DNA Casework Technical Procedures

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Section 1.1 General Considerations and Actions Related to DNA Extraction from Case Evidence

Section 1.1.1 Sampling Considerations

General

Before altering the evidence by collecting a sample for testing, the examiner will document its condition and, as needed, examine it using microscopy. This may be accomplished by description, sketching or photography.

Samples are typically chosen for DNA processing using *Sample Selection*. This is the practice of selecting items to test, or portions of items to test, based upon training, experience, and competence. This approach makes no assumptions of homogeneity.

Samples not described herein should be treated as deemed appropriate by the analyst. Additional guidance may be found in the Biology Technical Procedures.

Note

The manufacturer and lot number of a swab used to collect biological material from an item should be recorded in the bench notes.

Sampling evidence items

- If an item is swabbed in its entirety for **trace DNA** and permission to consume the evidence has not been given, a portion of the swab(s) should be retained for potential future testing.
- **Fingernail clippings** containing possible foreign material may be swabbed or processed by soaking. Stereomicroscopy may be helpful prior to soaking or swabbing. For soaking, see the suggested steps below.

Step	Action
1	Place the clipping(s) in a tube(s).
2	Soak in ~400 µL of PBS for ~1 hour at ~4°C or room temperature.
3	Vortex, then centrifuge ~5 minutes.
4	The clipping is then removed and set aside, and all but ~50 µL of the supernatant is removed to a separate tube.
5	The remaining cellular pellet is extracted.

- When typing the **actual nail material** from fingernails or toenails, cut the nail into smaller pieces.
- **Larger, diffuse stains** on cloth substrates should be cut into smaller pieces.
- Biological material deposited on a **hard or bulky substrate** should be scraped or

swabbed as appropriate.

- Stains on **leather** should be scraped or swabbed as appropriate.

Hairs

Hairs with roots should be cut approximately 0.5 cm or less from the root end and placed into a tube. A similar sized portion of the adjacent shaft should also be extracted as a control, unless the hair was submitted as a known reference standard and there is no expectation of foreign exogenous DNA.

Before sample collection, hairs should be examined for any adhering biological fluids (unnecessary if a known reference standard). If the biological fluid is of a probative nature, it can be swabbed with a sterile swab moistened with either purified water, or PBS, or TE^{-4}. Alternatively, the adhering material can be washed off and the liquid collected for extraction. For example, place the hair in a microfuge tube and add ~50 µL of PBS, then vortex and collect the liquid. Repeat the wash as necessary.

If the hair is to be mtDNA typed, refer to the appropriate mtDNA technical procedures for more detailed instructions.

Bones & teeth

Teeth may be crushed and placed directly into a microfuge tube, or alternatively, pulp from the tooth can be scraped out.

Bone may be pulverized, shaved or drilled. In instances where there is soft tissue or bone marrow present, DNA may be extracted using the organic DNA extraction protocol.

- DNA from bones and teeth may alternatively be extracted using a Demineralization Extraction Procedure in DNA Technical Procedures TP-18 (Volume II).
- If available in the laboratory, the Freezer/Mill, as described in Section 4.1 of DNA Technical Procedure TP-18 (Volume II), may be used for preparing bone samples and teeth.

Evidence on a slide

When biological material, including hair, is mounted on a microscope slide, the slide should first be cleaned on the outside surfaces and then the entire slide should be soaked in xylene until the cover slip detaches. Alternatively, a pipette may be used to stream the xylene over the slide until the cover slip detaches. Subsequently transferring the slide or hair to fresh xylene should remove any residual mounting medium, and this can be followed by an ethanol rinse. In the case of mounted cellular material, the air-dried sample can be collected by hydration and swabbing.

Consumption

Whenever possible, the examiner will ensure that sufficient sample (or representative sample) is left for possible reanalysis. If the entire sample must be consumed in order to obtain an interpretable result, the examiner should confer with the submitter and/or prosecutor to ensure that the legal implications of consuming the sample have been properly considered. In suspectless cases, this may be the detective or other law enforcement official. Typically, written permission to consume a sample is obtained from the agency.

For single hairs, the root may be collected without consulting the agency as long as shaft material remains for possible mitochondrial DNA testing.

Additional considerations

Batching

- Reasonable care should be taken to ensure that the risk of inadvertent transfer between high and low DNA concentration samples is minimized.
- Manually extracted evidence samples should be extracted at a different time than reference samples.
- Similarly, manually extracted penile swabs should be extracted separately from vaginal swabs.

Sample preparation and DNA extraction are <u>never</u> to take place in the same room where PCR amplification is conducted.

Special considerations apply when extracting samples for mtDNA analysis.

Section 1.1.2 Controls for DNA Extraction

Analytical controls

Analytical Controls for DNA Extraction

Controls are required to assess the quality of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results.

The following controls are described below:
- Quality control sample
- Reagent blank
- Substrate control

QC sample

The purpose of the quality control (QC) sample (also known as a positive extraction control) is to demonstrate that the analytical process worked properly. This sample is from a previously characterized source that is extracted and typed concurrently with the case samples. It serves as both an extraction control and a typing control for the process. It also serves as an internal blind control as the correct typing results are unknown to the analyst until the analysis is complete.

At least one QC sample should be included with each case or batch analysis. That is, if cases are batched, a single QC sample may serve as the QC sample for all the cases within the batch.

For multiple extraction sets within a given case, a new QC sample should be included for an additional extraction set if more than 60 days have passed or a reagent (based on lot number) has changed from the previous extraction.

For cases in which different extraction methods are employed, *e.g.,* organic extraction for evidence samples and Maxwell 16 robotic extraction for reference samples, a QC sample is included with each method. This may be a split of the same QC sample.

When extracting a non-semen QC sample with a set of differentially-extracted samples, the QC sample is typically added during the differential extraction steps such that SEB/ProK will be added to the sample.

In evaluating the QC sample as an extraction control, acceptable results include sufficient DNA yield for routine amplification and typing. The typing results should generate a DNA profile that is concordant with expected results. If the QC sample does not yield acceptable results, the DNA Technical Leader or designee should be consulted and any associated questioned or reference samples should be evaluated to determine if a re-extraction is necessary.

Reagent blank

A reagent blank is carried through the entire analytical process as part of each extraction set. The purpose of this sample is to detect DNA contamination that might occur from the reagents, the environment or between the samples being processed. It contains all of the reagents used during extraction, amplification and typing for each set of samples. Each separate extraction set (*e.g.,* evidence set, reference set) should have its own reagent blank(s). The reagent blank is carried through the amplification and typing process for each PCR system in which samples from a given set are typed. For samples extracted prior to July 2009, the reagent blank must be run in at least one PCR system.

In order to ensure that a sufficient quantity of reagent blank is available for each typing system and for any future tests, two separate reagent blanks should be used for any extraction set containing low-level evidentiary samples that may be consumed in such testing. While all reagent blanks should be quantitated, it is not necessary to type each reagent blank until/unless it is needed for this purpose. However, any reagent blank that indicates the presence of DNA above the qPCR limit of quantification in one or more detectors shall be amplified and typed in order to evaluate possible contamination.

Negative control results (*e.g.,* reagent blanks) that show background signal below the qPCR limit of quantification in one or more detectors do not necessarily require further characterization when there are multiple reagent blanks included for an extraction set. For sample sets in which multiple reagent blanks have been created, the reagent blank demonstrating the greatest signal, if any, should be the reagent blank that is amplified for STRs and typed using the most sensitive volume of the extraction set.

Substrate control

When appropriate, a similarly sized and apparently unstained portion of the substrate adjacent to the questioned stain should be collected and run through the typing process. A substrate control sample will not necessarily produce negative typing results. The possibility of other human biological material being present and contributing to the DNA content of a particular sample will be considered in the final interpretation. The knowledge, experience and judgment of the analyst are paramount when assessing the need for a substrate control sample, choosing the appropriate sample and evaluating the results.

Section 1.1.3 Making a slide

Overview

At analyst discretion, a slide(s) may be made to examine an evidence item for the presence of sperm and/or nucleated epithelial cells. When making slides prior to lysis and DNA extraction, whether it be for a differential extraction or another extraction method (*e.g.,* Organic or PrepFiler), this is the slide procedure to be used.

Note: Reagent volumes may be altered in order to accommodate the size or nature of a particular sample.

Slide

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Cover the sample in ~200-400 µL of PBS and incubate for ~1 hour at ~4°C or room temperature.</td></tr>
<tr><td>2</td><td>• Vortex the substrate for 15-30 seconds.
<table>
<tr><th>Then either</th><th>OR</th></tr>
<tr><td>Squeeze out as much liquid as possible</td><td>“Piggyback” the substrate
• Transfer it to a spin basket
• Place the spin basket back inside the microfuge tube and close the lid
• Spin for ~5 minutes in the microfuge</td></tr>
</table>
• Remove the substrate (and spin basket) from the tube.
• Save the substrate for DNA extraction.

Note: The Machery-Nagel Forensic Filter spin baskets should not be used when pelleting intact cells.</td></tr>
<tr><td>3</td><td>Carefully remove all except ~30 µL of the supernatant (aqueous extract) and place in freezer storage at approximately -20°C.</td></tr>
<tr><td>4</td><td>• Resuspend the cellular pellet in the ~30 µL of liquid remaining and
• transfer 3 µL to a microscope slide.
• Heat-dry the slide.</td></tr>
<tr><td>5</td><td>Stain slides as follows and examine microscopically (as per Biology Technical Procedures):
– Stain sample area with nuclear fast red for at least 1 minute. Do not let sample area dry out.
– Rinse gently with water.
– Stain with picroindigocarmine solution for up to 1 minute.
– Rinse with EtOH.</td></tr>
</table>

	– Air-dry the slide. – Examine the slide, recording the type of microscopy, the magnification, and any observations in the case notes. ***Note:*** Staining times depend on the concentration of the stain solution.
6	When performing the second slide of a differential extraction (*i.e.,* post-epithelial cell digestion), perform the following if nucleated epithelial cells remain: • Add an additional 400 µL of SEB and 10 µL of ProK • Incubate for ~30 minutes at ~56°C • Carefully remove and discard all except ~30 µL of the supernatant • 1-2 additional *manual* washes may be performed (see Steps 2-3 of the Differential Extraction/Manual Separation and Wash). ***Notes:*** *It is not necessary to make another microscope slide.* *A reagent blank from the set should be treated the same.*

Section 1.1.4 Post-extraction sample handling considerations

Concentrating a sample

A sample may be concentrated by using a variety of techniques. For example, a Speed-Vac System (*e.g.,* MiVac), heat block evaporation, or another method for concentrating DNA extracts (such as an approved ultrafiltration device) may be used. Note that evaporative methods are generally best for maximizing DNA yield while filtration devices are the better approach for minimizing PCR inhibition.

Important

- Related controls should be treated in the same manner as the sample extracts being concentrated.
- Techniques to avoid contamination shall be employed.

Reconstituting a sample

Sterile dH_2O may be used to reconstitute evaporated samples. Document appropriately.

Section 1.1.5 Appendix I - Reagents Used for DNA Extraction

Notes

- *These reagents may alternatively be purchased (molecular biology grade or equivalent).*
- *Formulations may be proportionately increased or decreased to obtain the desired volume.*
- *See the corresponding QC Worksheet for more information.*

0.5M EDTA

To prepare 0.5M EDTA, perform the following steps:

Step	Action
1	Dissolve 186.1 g of EDTA·$2H_2O$ (molecular biology grade &/or ≥99%) in 800 mL of dH_2O.
2	Adjust pH to 8.0 with 10-30 g of NaOH pellets.
3	Adjust the volume to 1 L
4	Autoclave the newly made solution.
5	Readjust the volume back to 1 L using sterile dH_2O.

1M DTT

To prepare 1M DTT, perform the following steps:

Step	Action
1	Dissolve 1.54 g Dithiothreitol (DTT; molecular biology grade &/or ≥98%) in sterile dH_2O for a total volume of 10 mL.
2	Aliquot into convenient size volumes.
3	Store frozen.

5M NaCl

To prepare 5M NaCl, perform the following steps:

Step	Action
1	Add 292.2 g of NaC1 (ACS reagent grade &/or ≥98%) to ~800 mL of dH_2O.
2	Adjust volume to 1 L with dH_2O.
3	Autoclave the newly made solution.
4	Readjust the volume back to 1 L using sterile dH_2O.

Christmas tree stain

See Biology Technical Procedures for Nuclear Fast Red Stain and Picroindigocarmine Stain.

PBS, pH 7.4

To prepare PBS (pH 7.4), perform the following steps:

Step	Action
1	Combine in ~800 mL of dH_2O: 8 g of NaCl (ACS reagent grade &/or ≥98%) 0.2 g of KCl (molecular biology grade &/or ≥99%) 1.44 g of Na_2HPO_4, anhydrous (reagent grade &/or ≥98%) 0.24 g of KH_2PO_4, anhydrous (reagent grade &/or ≥98%)
2	Adjust pH to 7.4 with HCl.
3	Autoclave the newly made solution.
4	Adjust volume to 1 L with sterile dH_2O.

PCIA

If not already at pH 8.0, adjust 100 mL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1; molecular biology grade) to pH 8.0 with ~ 6 mL of Tris base (pH 8.0).

ProK (20 mg/mL)

To prepare 20 mg/mL of Proteinase K, perform the following steps:

Step	Action
1	Dissolve 500 mg of proteinase K (nuclease free) in 25 mL of dH_2O.
2	Aliquot into convenient size volumes.
3	Store frozen.

SEB

To prepare Stain Extraction Buffer (10mM Tris; 10mM EDTA; 100mM NaC1; 2% SDS), perform the following steps:

Step	Action
1	Combine in ~855 mL of dH_2O: • 5 mL of 2M Tris, pH 8.0 • 20 mL of 0.5M EDTA, pH 8.0 • 20 mL of 5M NaC1
2	Autoclave the newly made solution.
3	Adjust volume to 900 mL with sterile dH_2O.
4	Add 100 mL of 20% SDS.

TE^{-4}

To prepare TE^{-4} (10mM Tris, pH 7.5; 0.1mM EDTA), perform the following steps:

Step	Action
1	Combine 5 mL of 2M Tris (pH 7.5) and 200 µL of 0.5M EDTA (pH 8.0).
2	Adjust volume to 1 L with dH_2O.
3	Autoclave the newly made solution.
4	Readjust the volume back to 1 L using sterile dH_2O.

2M Tris, pH 7.5

To prepare 2M Tris (pH 7.5), perform the following steps:

Step	Action
1	Dissolve 242.2 g of Tris base (ACS reagent grade &/or ≥99.8%) in 800 mL of dH_2O.
2	Adjust to pH 7.5 with concentrated HCl and adjust the volume to 1 L with dH_2O.
3	Autoclave the newly made solution.
4	Readjust the volume back to 1 L using sterile dH_2O.

2M Tris, pH 8.0

To prepare 2M Tris (pH 8.0), perform the following steps:

Step	Action
1	Dissolve 242.2 g of Tris base (ACS reagent grade &/or ≥99.8%) in 800 mL of dH_2O.
2	Adjust to pH 8.0 with concentrated HCl and the volume to 1 L with dH_2O.
3	Autoclave the newly made solution.
4	Readjust the volume back to 1 L using sterile dH_2O.

20% SDS

To prepare 20% SDS, perform the following steps:

Step	Action
1	Add 200 g of sodium dodecyl sulfate (SDS; molecular biology grade &/or ≥98.5%) to 700 mL of dH_2O.
2	Heat to ~65°C to dissolve.
3	Adjust volume to 1 L.

Section 1.2 Organic Extraction of DNA from Case Evidence

Section 1.2.1 Overview

Introduction

The following procedures describe the extraction of DNA from various biological materials. DNA is isolated through an organic extraction procedure that separates DNA from other cellular components. Following cell lysis and protein digestion, the cellular debris is removed with organic solvents. Ultra-filtration is then used to purify the recovered DNA.

In cases of sexual assault, the evidence may contain a mixture of spermatozoa and epithelial cells. Prior to organic extraction, the cellular components of such a mixture can be differentially separated so that the sperm and non-sperm cell fractions can be isolated and analyzed independently. Although this approach may not result in an absolute separation, it can generally provide a sperm fraction that is relatively free of non-sperm DNA.

Contents

The Organic DNA Extraction procedure includes the following topics:

Hazard warning

Phenol Chloroform is corrosive, highly toxic and a suspected teratogen. Use in a fume hood. Avoid skin contact and inhalation. Wear lab coat, gloves and protective eyewear when handling.

Note *Record reagent lot numbers on the appropriate checksheets.*

Section 1.2.2 Materials, Reagents, and Equipment for DNA Extraction

Materials & reagents The reagents for DNA extraction and their formulations are listed in Appendix I.

Equipment

- Pipettes
- Pipet tips – DNA/DNase/RNase free
- Centrifuges
- Incubator
- Dry-heat bath (for NucleoSpin devices)
- Compound microscope
- Microfuge 1.5 mL tubes – DNA/DNase/RNase free
- Spin baskets – DNA/DNase/RNase free
- Slide preparation materials—microscope slides, cover slips, mounting medium
- NucleoSpin XS filtration or Amicon Ultra-4 (Ultracel-30k) filtration devices
- For QIAcube workstation:
  - Qiagen 1.5 mL flip-cap tubes, or equivalent
  - Qiagen 2 mL flip-cap tubes
  - Qiagen sterile 1000 µL, wide-bore filter tips
  - Qiagen rotor adapters
  - Qiagen rotor adapter holder
  - Qiagen 30 mL reagent bottle
- Miscellaneous laboratory supplies

Section 1.2.3 Organic Extraction of DNA from Biological Materials

Overview This is the basic procedure for organic, non-differential extraction of DNA.

Notes Reagent volumes may be altered in order to accommodate the size or nature of a particular sample.

There are three different worksheets that describe the different approaches based on

the nature of the evidence sample:
- Organic Extraction of DNA from Biological Materials
- Organic Extraction of DNA from Biological Materials (with Microscopy)
- Organic Extraction of DNA from Hair or Nail Material

Each of these worksheets has two versions, depending on the filtration device used for clean-up: NucleoSpin XS or Amicon Ultra-4 (Ultracel-30k).

Slides

The extraction of a particular stain may begin with a PBS soak and microscopic evaluation, and then proceed directly to SEB/ProK without differential extraction. The steps for making a slide prior to DNA extraction are described in Section 1.1.3 Making a Slide.

Organic extraction

Perform the following steps for organic extraction of DNA from biological materials.

Step	Action
1	Add 400 µL of stain extraction buffer (SEB) to the sample in a microfuge tube.
2	Add 10 µL of 20 mg/mL Proteinase K (ProK). For hairs and nail fragments (when typing the actual nail material, not scrapings), add 20 µL of 1M DTT.
3	Ensure that the sample is completely covered by the liquid.
4	Incubate at ~56°C for ~2 hours. ***Notes:*** • Alternatively, the first digestion may be overnight, followed by the addition of more ProK and a ~2 hour incubation the following day. • If validated by the laboratory, the digestion steps for reference samples may be shortened to two half-hour digests with ProK.
5	Add an additional 10 µL of ProK to the tube.
6	Incubate at ~56°C overnight. ***Note:*** This second incubation may be shortened to ~2 hours.
7	Vortex the substrate for 15-30 seconds, squeeze out as much liquid as possible, and remove it. – Alternatively, the substrate may be left in the tube. If "piggybacking" the substrate, • transfer it to a spin basket.

	• Place the spin basket back inside the microfuge tube and close the lid. • Spin for ~5 minutes in the microfuge. • Remove the substrate and spin basket from the tube. ***Note:*** *These steps are simplified when using the Machery-Nagel Forensic Filter spin baskets.*
8	If saving the substrate, transfer it to a new microfuge tube for freezer storage.
9	• Add an equal volume (typically 400 µL) of phenol/chloroform/ isoamyl alcohol (25:24:1). – Alternatively, the extract may be added to a tube containing phenol/chloroform/isoamyl alcohol. • Shake the tube to achieve a milky emulsion.
10	Spin in microfuge for ~2 to 5 minutes to achieve phase separation and compression of the interface.
11	Transfer aqueous phase (top layer) to a new tube without disturbing the interface.
12	Do additional organic extractions (Steps 9-11) until the interface is clean and the aqueous phase is clear; usually 2 extractions are sufficient.
13	Proceed to clean-up using the NucleoSpin XS device. Alternatively, the Amicon Ultra-4 (Ultracel-30k) device may be used. ***Note:*** *The same type of filtration device is used for each sample in an extraction set.*

Section 1.2.4 Differential Extraction of DNA from Body Fluid/Semen Mixtures

Overview

A differential extraction is performed on potential body fluid/semen mixtures. The process separates sperm cells from non-sperm cellular lysate. The resulting sperm fraction is generally relatively free of non-sperm fraction DNA and vice versa, although many factors may affect the purity of the separation.

Notes

Reagent volumes may be altered in order to accommodate the size or nature of a particular sample.

This extraction process may be performed at the choice of the analyst in a number

of different ways:
- Manual separation/washes, organic extraction, and NucleoSpin purification
- QIAcube separation/washes, organic extraction, and NucleoSpin purification
- Manual separation/washes and PrepFiler extraction
- QIAcube separation/washes and PrepFiler extraction

See the appropriate Differential Extraction for Body Fluid/Semen Mixtures Worksheet.

First slide

Optional: Create a slide following Section 1.1.3 Making a Slide.

First digestion

Perform the following steps for the first digestion of the differential extraction.

Step	Action
1	Add the following to the sample substrate in the tube. • 400 µL of stain extraction buffer (SEB). • 10 µL of 20 mg/mL Proteinase K (ProK). ***Note:*** If using QIAcube for separation and wash, use the Qiagen 1.5 mL or equivalent tubes for these steps.
2	Incubate ~1 hour at ~56°C.
3	• Remove sample from incubation and vortex 15-30 seconds in order to loosen sperm cells. • Spin the tube briefly to remove any liquid in the lid.
4	• Remove the substrate, squeezing out as much liquid as possible. – Alternatively, piggyback the substrate. ***Note:*** *The Machery-Nagel Forensic Filter spin baskets should not be used when pelleting intact cells.*
5	If saving the substrate, transfer it to a new microfuge tube for freezer storage.

Separation and wash

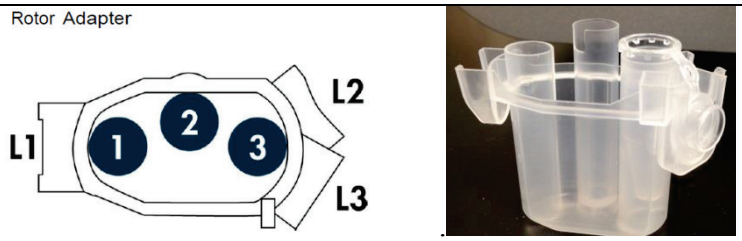
Perform the following steps to separate the non-sperm fraction from the sperm pellet and wash the sperm fraction. This may be performed manually or using the QIAcube robotic platform.

MANUAL:

Step	Action
1	Carefully transfer all except ~30 µL of the supernatant (non-sperm cell fraction) to a separately labeled tube and either: • return to ~56°C incubation or • set the tube aside until Step 2 of block **Organic processing of fractions** or • continue with PrepFiler (see Section 1.5) ***Note**: The post-epithelial cell digest slide may be prepared prior to the PBS washing step below.*
2	• Add 400-1000 µL of PBS, • gently resuspend sperm pellet, and • microfuge for ~5 minutes. • Carefully remove all except ~30 µL of the supernatant and discard.
3	Repeat Step 2, leaving ~30 µL each time. The number of wash steps may be varied at the analyst's discretion depending on the condition of the sample. A total of 3 washes is usually adequate; however, more washes are generally desirable in instances where high epithelial cell DNA and low sperm cell DNA is anticipated. ***Note:** If batching cases and some of the samples are washed more times than other samples, then the reagent blank should be washed corresponding to the sample that was washed the greatest number of times.*

QIACUBE:

Step	Action
1	Carefully insert the 1.5 mL tubes containing lysate and cellular pellet into the L3 slot of the rotor adapters. Be sure the lids are pushed all the way down in the slots. For convenience, the rotor adapter holder can be used.



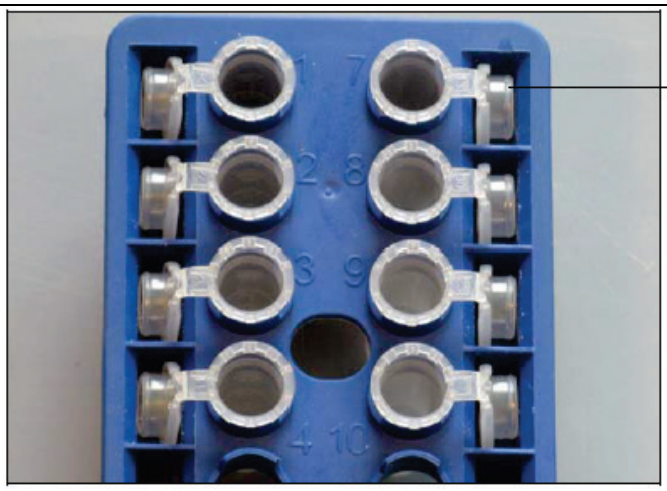
Important
Use either Qiagen 1.5 ml Elution Tubes or an equivalent tube.

2 Make the following selections on the QIAcube touchscreen:
- For 7 or fewer samples, run the 6 Protocol.
- For 8-12 samples, run the 12A and 12B protocols.

Step	Action
1	Choose "DNA"
2	Select "Pipetting" from the list of choices
3	Select "Epithelial and Sperm Cell" from the new list of choices
4	Select the appropriate protocol for the list: • "Separate Wash 6 FlipCap" • "Separate Wash 12A FlipCap" • "Separate Wash 12B FlipCap"
5	Press "Start."

3 Follow the prompts to set up the deck.

Important
- See Tables 1-3 in Appendix I to determine the volume of PBS and number of tips required for the run. It is generally recommended to start with 30 mL PBS (fill to demarcation on provided bottle).
- See the QIAcube loading chart (Figure 1) in Appendix I for the appropriate loading positions of the rotor adapters in the centrifuge and the tubes in the shaker rack.
  – To run 1 or 11 samples, a balance sample must be included (bringing the sample number up to 2 or 12).
- Non-sperm cell fraction tubes:
  – Use only Qiagen 2.0 mL round-bottom, flip-cap tubes in the shaker rack
  – Label the tubes prior to loading
  – Secure the lids into the slots as shown below

	
4	If running 12A/12B Protocols, follow the prompts to finish 12A and repeat Steps 2 and 3 above to start 12B. Replenish PBS and tips as needed. Note that the non-sperm cell lysates may be removed prior to starting the 12B Protocol.
5	At the completion of the QIAcube protocol, press "OK" and follow the prompts to: • Remove the 1.5 mL sperm fraction tubes and discard the rotor adapters • Remove the 2.0 mL non-sperm fraction tubes from the shaker rack • Remove reagents and labware • Discard used tips from waste drawer

Slide

Optional: Following Section 1.1.3 Making a Slide, create the second/post e-cell digest slide.

Next step

Choose either Organic processing or PrepFiler to proceed with lysis and purification.

Organic:	Proceed to Organic processing of fractions immediately below.
PrepFiler:	Proceed to Section 1.5

Organic processing of fractions

Perform the following steps to lyse the sperm fractions and organically extract the sperm and non-sperm lysates.

Step	Action
1	• Prepare a master-mix containing the following for each sperm fraction plus one, (or add the reagents individually): – 400 µL of SEB – 10 µL of ProK – 20 µL of liquid 1M DTT • Add 430 µL of this master-mix to each tube.
2	Incubate both fractions at ~56°C for ~2 hours. ***Notes:*** • *Alternatively, perform the first digestion overnight, followed by the addition of more ProK and a ~2 hour incubation the following day.* • *If the non-sperm cell fraction is not already in the ~56°C incubator, return it to ~56°C at this time.* • *The extraction of other sample types may also be started at this time.*
3	• Add another 10 µL of ProK to all of the samples and • continue incubating at ~56°C overnight. – This second incubation may be shortened to ~2 hours.
4	• Add an equal volume (typically 400 µL) of Phenol/Chloroform/ Isoamyl Alcohol (25:24:1). – Alternatively, the extract may be added to a tube containing phenol/chloroform/isoamyl alcohol. • Shake the tube by hand to achieve a milky emulsion in the tube. • Spin the tube for ~2 to 5 minutes to achieve phase separation and compression of the interface.
5	Transfer the aqueous phase (top layer) to a new tube without disturbing the interface.
6	Do additional organic extractions (Steps 13-14) until the interface is clean and the aqueous phase is clear; usually 2 extractions are sufficient.
7	Proceed to clean-up using the NucleoSpin XS device. Alternatively, the Amicon Ultra-4 (Ultracel-30k) device may be used. ***Note:*** *The same type of filtration device is used for each sample in an extraction set.*

Section 1.2.5 NucleoSpin XS Filtration Purification

Hazard warning

NT binding buffer contains guanidine thiocyanate which is harmful by inhalation, in contact with skin, and if swallowed. Upon contact with acids or bleach, guanidine thiocyanate will liberate toxic gas.

NucleoSpins

To purify a phenol extract using the NucleoSpin XS filtration device, perform the following procedure.

***Note**: Be sure ethanol has been added to the B5 Buffer prior to initial use of the kit.*

Step	Action
1	• Transfer aqueous layer from the organic extraction to a 1.5 mL tube containing 420 µL of TE^{-4} and 210 µL of binding buffer (NT). • Vortex and spin briefly.
2	• Load ~ ½ of the above solution to a NucleoSpin XS filtration device. (Do not add more than ~520 µL at one time). • Spin 30 seconds at ~11,000 x g. – Note orientation in rotor (*e.g.,* hinge up). • Discard waste tube and • replace with a new 2 mL capless tube.
3	• Add the remainder of the solution to the same NucleoSpin XS filtration device. • Spin 30 seconds at ~11,000 x g. • Discard waste tube and • replace with a new 2 mL capless tube. Repeat as needed until all of solution has been loaded into the NucleoSpin XS filtration device.
4	• Add 100 µL of wash buffer (B5). • Spin 2 minutes at ~11,000 x g. – The NucleoSpin XS filtration devices should be rotated ~180° from their orientation in the previous spin (*e.g.,* hinge down instead of hinge up). • Discard each waste tube and • replace with a labeled 1.5 mL capless tube.
5	• Add 6-15 µL of elution buffer (BE) to each tube. • Spin 1 minute at ~11,000 x g. ***Note:*** Volumes greater than 10 µL may be used but will not necessarily result in increased DNA yields.

6	• Add an additional 6-15 µL of elution buffer (BE) to each tube. • Spin 1 minute at ~11,000 x g.
7	• Remove NucleoSpin XS columns, and • heat tubes in 90°C dry-heat bath for ~5-8 minutes, depending on volumes of elution buffer used (*e.g.,* 5 minutes for two 6 µL elutions and 8 minutes for two 10 µL elutions).
8	• Transfer retentate to a storage tube, approximating the volume recovered. • Record the volumes. ***Note:*** *Once recovered, additional* TE^{-4} *may be added to extracts to obtain approximately equal volumes for the retentates. This is documented in the case notes. Although this may facilitate proportionality requirements for later testing, bear in mind that in general, the goal is to obtain a concentrated DNA extract.*

Next step Proceed to DNA quantitation.

Section 1.2.6 References

Reference documents

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Section 1.2.7 Appendix I – QIAcube Background and Reference Information

Background

Following centrifugation, the QIAcube will automatically transfer supernatants (non-sperm cell fractions) to the appropriate 2 mL tubes and then perform four 750 µl PBS washes of the sperm pellets. At the end of the protocol, sperm pellets in ~30 µl of PBS remain in the 1.5 mL tubes and ~380 µl of non-sperm fraction lysates are located in the 2 mL tubes in the shaker rack.

The protocol scripts were custom created by the Qiagen Group and are IDCR1079v1.

12 sample protocols

There are two 12 sample protocols, referred to as 12A Protocol and 12B Protocol. They are run sequentially such that 12A Protocol performs the separation and the first two of four washes and 12B Protocol performs the last two washes. The 12 sample protocols are run when there are 8-12 samples but can also be run for fewer than 8 samples. The 12B Protocol is manually initiated by the analyst upon completion of the 12A Protocol. Note that there is only one script for the 6 sample protocol, which performs the separation and all four washes when running 2-7 samples.

PBS/tip reference tables

Refer to the following tables to determine the volume of PBS and the number of tips required for a run.

6 Protocol

Number of Samples	Volume of PBS required (ml)	Number of tips required
2	11	18
3	14	27
4	17	36
5	20	45
6	23	54
7	26	63

Table 1. Volume of PBS and number of wide-bore filter tips required for the indicated number of samples using the 6 Sample Protocol.

12A Protocol

Number of Samples	Volume of PBS required (ml)	Number of tips required
2	8	10
3	9.5	15
4	11	20

5	12.5	25
6	14	30
7	15.5	35
8	17	40
9	18.5	45
10	20	50
12	23	60

Table 2. Volume of PBS and number of wide-bore filter tips required for the indicated number of samples using the 12A Protocol. Note that the full volume of 30 mL PBS may be used for all sample numbers when starting 12A.

12B Protocol

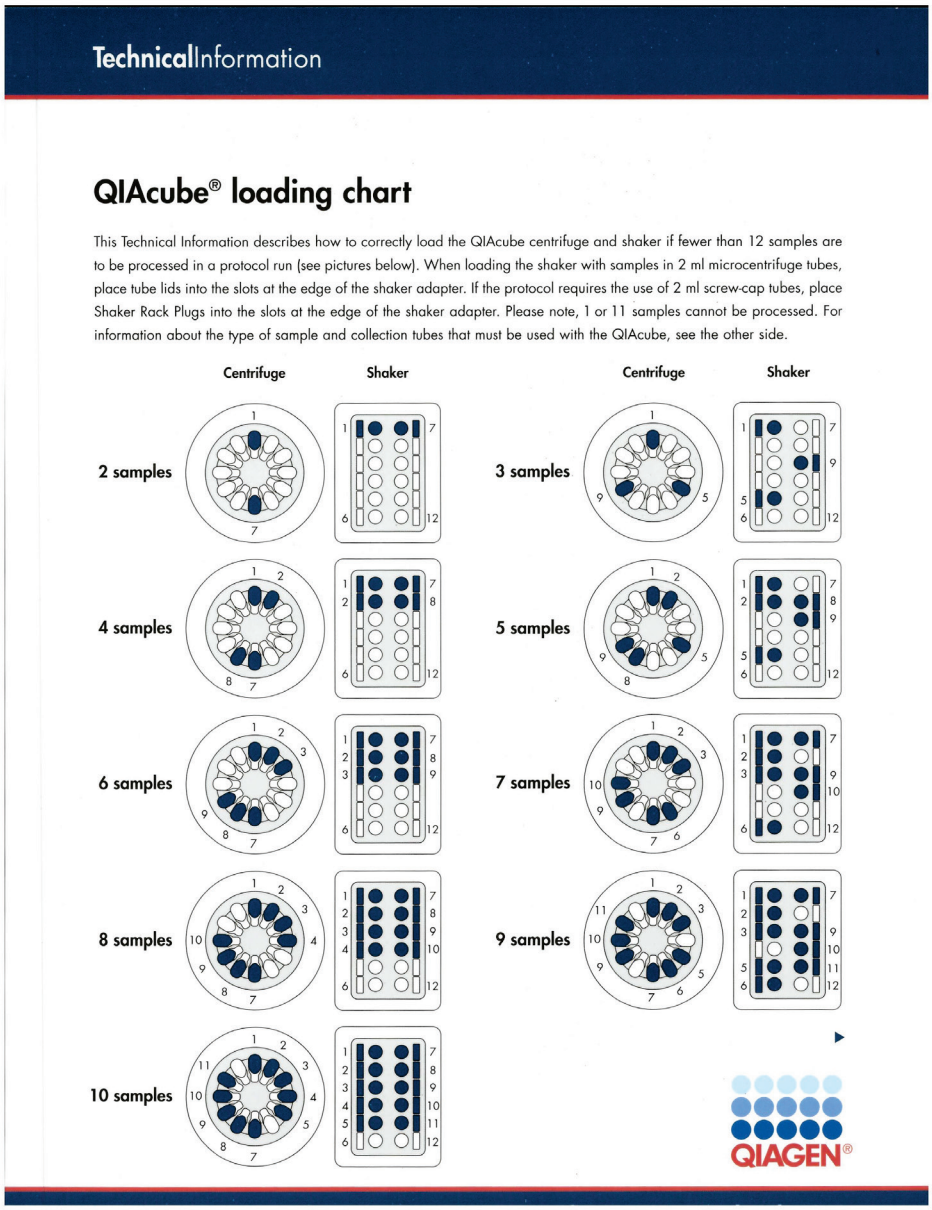
Number of Samples	Volume of PBS required (ml)	Minimum volume (ml) of PBS *to add* following 12A Protocol[¥]	Number of tips required
2	8		8
3	9.5		12
4	11		16
5	12.5		20
6	14		24
7	15.5		28
8	17		32
9	18.5	2	36
10	20	5	40
12	23	11	48

Table 3. Minimum volume of PBS and number of wide-bore filter tips to add following the 12A Protocol for the indicated number of samples.
[¥] This is the minimum volume of PBS to add if 12A starting volume was 30 mL.

Notes:

- If 12A is started with 64 tips, additional tips only need to be added when running 8-12 samples.
- If 12A is started with 30 mL PBS, additional PBS only needs to be added when running 9-12 samples.
- The formulation to calculate PBS volume is number of samples times 2 washes times 750 µL per wash; added to that total is a 5 mL overflow excess.
- The formulation to calculate tips is number of samples times 9 tips per sample; note that 9 tips total are used between 12A and 12B Protocols for a given sample.

Loading chart Refer to the loading chart below to properly load the QIAcube centrifuge.



TechnicalInformation

QIAcube® loading chart

This Technical Information describes how to correctly load the QIAcube centrifuge and shaker if fewer than 12 samples are to be processed in a protocol run (see pictures below). When loading the shaker with samples in 2 ml microcentrifuge tubes, place tube lids into the slots at the edge of the shaker adapter. If the protocol requires the use of 2 ml screw-cap tubes, place Shaker Rack Plugs into the slots at the edge of the shaker adapter. Please note, 1 or 11 samples cannot be processed. For information about the type of sample and collection tubes that must be used with the QIAcube, see the other side.

Figure 1. QIAcube loading chart for appropriate placement of <12 samples. Note that to run 1 or 11 samples, a balance sample must be included (bringing the sample number up to 2 or 12).

Expected run times

Approximate QIAcube run times (actual robot run time) are listed below.

6 Protocol

No. of samples	Time (min)
2	41
3	47
4	53
5	58
6	64
7	70

12A/12B Protocol

No. of samples	12A Time (min)	12B Time (min)	Total time (min)
2	24	18	42
7	40	31	71
8	43	34	77
9	46	37	83
10	50	39	89
12	56	45	101

Section 1.2.8 Appendix II - Clean-up Using the Amicon Ultra-4 (Ultracel-30k) Filtration Device

Ultra-4

To clean-up a phenol extract with an Amicon Ultra-4 device, perform the steps below.

Step	Action
1	Transfer the aqueous layer from the organic extraction to the Amicon Ultra-4 (Ultracel-30k) filtration device containing ~1.7 mL of TE^{-4}.
2	Ensure proper positioning in the rotor (membrane panel facing up) and spin for ~20 minutes at ~2000 x g. Discard effluent.
3	Reassemble the device, add ~2.0 mL of TE^{-4}, and spin for ~20 minutes at ~2000 x g. Discard effluent.
4	Reassemble the device, add ~2.0 mL of TE^{-4}, and spin for ~40-60 minutes at ~2000 x g. Discard effluent.
5	Transfer the retentate from the deadstop to a storage tube, approximating the volume recovered. Record the volumes. ***Note:*** *Once recovered, additional TE^{-4} may be added to extracts to*

	obtain approximately equal volumes for the retentates. This is documented in the case notes. Although this may facilitate proportionality requirements for later testing, bear in mind that in general, the goal is to obtain a concentrated DNA extract.

Section 1.2.9 Appendix III – Purification of Previously Extracted DNA Using a Nucleospin XS Filtration Device

Further purification

Previously extracted DNA may be further purified using a NucleoSpin XS filtration device, especially in the case of an extract containing a PCR inhibitor. See the following procedure:

Step	Action
1	Dilute previously extracted DNA with TE^{-4} for a final volume of 400 µL.
2	Add 100 µL of binding buffer (NT) to the diluted DNA. Vortex and spin briefly.
3	Load entire combined volume onto NucleoSpin XS filtration device.
4	Note orientation in rotor (*e.g.,* hinge up). Spin 30 seconds at ~11,000 x g. Discard waste tube and replace with a new 2 mL capless tube.
5	Add 100 µL of wash buffer (B5). The NucleoSpin XS filtration devices should be rotated ~180° from their orientation in the previous spin (*e.g.,* hinge down instead of hinge up). Spin 2 minutes at ~11,000 x g. Discard waste tubes and replace with labeled 1.5 mL capless tubes.
6	Add 6-15 µL of elution buffer (BE) to each tube. Spin 1 minute at ~11,000 x g.
7	Add an additional 6-15 µL of elution buffer (BE) to each tube. Spin 1 minute at ~11,000 x g.
8	Remove NucleoSpin XS columns and heat tubes in 90°C dry-heat bath for ~5-8 minutes, depending on volumes of elution buffer used (*e.g.,* 5 minutes for two 6 µL elutions and 8 minutes for two 10 µL elutions).
9	Transfer retentate to a storage tube, approximating the volume recovered. Record the volume.

Section 1.3 Extraction of Reference Samples Using the Maxwell 16

Section 1.3.1 Overview

Introduction

The following procedure describes the extraction of DNA from various biological materials that serve as reference samples. DNA is isolated from cellular material using the DNA IQ™ Casework Pro Kit for Maxwell® 16 with the Maxwell 16 LEV Instrument. A Maxwell 16 Instrument that has been reconfigured using the LEV conversion kit may also be used. Following cell lysis and protein digestion, the DNA IQ Casework Pro Kit for Maxwell 16 is used with the Maxwell 16 LEV Instrument to purify DNA from forensic casework samples using DNA IQ Resin magnetically transferred through a series of washes. The Maxwell 16 LEV Instrument has preprogrammed purification procedures and uses pre-dispensed reagent cartridges, eluting DNA samples into small, concentrated volumes.

Contents

The Maxwell 16 procedure includes the following topics:

Topic
Section 1.3.2 Materials, Reagents, Equipment, and Analytical Controls
Section 1.3.3 Maxwell 16 DNA Extraction Procedure for Reference Samples
Section 1.3.4 References
Section 1.3.5 Appendix I for *Operational Mode Setup*

Hazard warnings

DNA IQ extraction chemistry contains guanidinium thiocyanate,which is harmful by inhalation, in contact with skin, and if swallowed. Upon contact with acids or bleach, guanidinium thiocyanate will liberate toxic gas.

Section 1.3.2 Materials, Reagents, Equipment, and Analytical Controls

Materials & Reagents

- DNA IQ™ Casework Pro Kit for Maxwell® 16, includes:
  - Lysis Buffer
  - Elution Buffer
  - Sample cartridges
  - Elution tubes
- 1M Dithiothreitol (DTT)

Equipment

- Maxwell® 16 LEV Instrument (or a reconfigured Maxwell 16)
- Pipettes
- Pipet tips – DNA/DNase/RNase free
- Centrifuges
- Incubator
- Microfuge tubes – DNA/DNase/RNase free
- Spin baskets – DNA/DNase/RNase free
- Miscellaneous laboratory supplies

Analytical controls

Analytical Controls for DNA Extraction
Controls are required to assess the quality of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results.

Quality Control (QC) sample
In addition to the QC sample requirements described in Section 1.1, a QC sample must be included in each run.

Reagent blank
Each separate instrument run must contain at least one reagent blank. If an instrument run contains both liquid and stain samples, the reagent blank should be treated in the same manner as a stain.

Section 1.3.3 Maxwell 16 DNA Extraction Procedure for Reference Samples

Section 1.3.3.1 Overview

Contents The following topics are included in this section:

Section 1.3.3.2 Preliminary Considerations

Reference samples may include, but are not limited to, bloodstains, saliva swabs, liquid blood, and other tissues as well as blood samples collected at autopsy. The DNA IQ Casework Pro Kit for Maxwell 16 is acceptable for extraction of dried samples on solid substrates or for liquid blood samples.

Note: *The following worksheet is used for reference sample extraction: Maxwell 16® Robotic Extraction for Reference Samples*

Section 1.3.3.3 Preparation of Dried Reference Samples for Robotic Extraction

Dried sample preparation

Follow the steps below to prepare dried reference samples for extraction using the Maxwell 16.

Step	Action
1	Prepare Lysis Buffer based on the number of samples in a run (plus one): • Add 5 µL 1M DTT for every 500 µL of Lysis Buffer and • mix thoroughly. ***Note:*** *If a precipitate forms, warm Lysis Buffer to 37-60°C until it clears (no more than 5 minutes).*
2	Add 500 µL of Prepared Lysis Buffer to each sample in a labeled 1.5 mL tube. ***Note:*** *In this procedure, reagent volumes may be altered in order to accommodate the size or nature of a particular sample. However, the maximum volume that can be processed by the DNA IQ Casework Sample Cartridge is 750 µL.*
3	Incubate at ~70°C for ~30 minutes.
4	Briefly vortex and spin down tubes.
5	Remove the substrate and either discard or store frozen. If "piggybacking" the substrate, • transfer it to a spin basket. • Place the spin basket back inside the microfuge tube and close the lid. • Spin for ~5 minutes in the microfuge. • Remove the substrate and spin basket from the tube. • If saving the substrate, transfer it to a new microfuge tube for freezer storage. ***Note:*** *These steps are simplified when using the Machery-Nagel Forensic Filter spin baskets.*
6	Samples are now ready for processing with the Maxwell 16 LEV Instrument. ***Note:*** *At this point, do not refrigerate or freeze samples. Leave samples at room temperature overnight, if necessary.*

Section 1.3.3.4 Preparation of Liquid Blood Reference Samples for Robotic Extraction

Liquid blood preparation

Add 100 µL of Prepared Lysis Buffer to 50 µL of liquid blood in a labeled 1.5 mL tube.

Prepared Lysis Buffer
Based on the number of samples in a run (plus one): • Add 5 µL 1M DTT for every 500 µL of Lysis Buffer and • mix thoroughly. ***Note:*** *If a precipitate forms, warm Lysis Buffer to 37-60°C until it clears (no more than 5 minutes).*

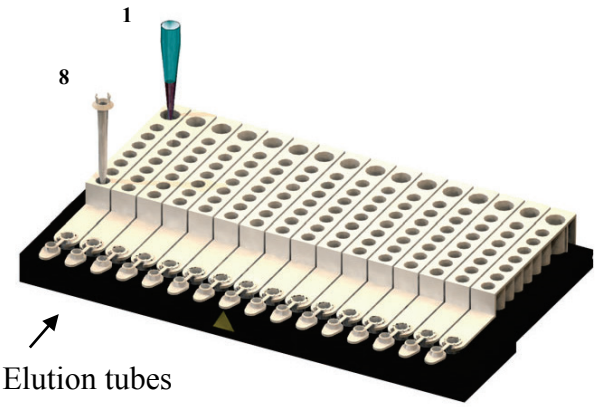
Section 1.3.3.5 Robotic Extraction of DNA from Reference Samples

Cartridge contents

Well #	Well Contents	User Adds
1	Lysis Buffer	Sample
2	DNA IQ Resin	
3	Lysis Buffer	
4	Wash Buffer	
5	Wash Buffer	
6	Wash Buffer	
7	Empty	
8	Empty	Plunger

Robotic extraction

Follow the steps below to extract DNA from reference samples using the Maxwell 16.

Step	Action
1	• Load the cartridges into the LEV cartridge rack with the tube holder facing toward the numbered side of the rack (Figure 1). – If fewer than 16 samples are being processed, load the cartridges from the center of the rack first and leave empty slots on the ends of the rack. • Remove seals. • For each sample cartridge, load a plunger into well #8 and transfer samples into well #1.  **Figure 1**
2	• Place labeled 0.5 mL Elution Tubes into the elution tube holder at the front of each cartridge. • Add 25 µL of Elution Buffer to each elution tube.
3	Turn on the Maxwell 16 LEV Instrument. – The instrument will home itself and display the firmware version number and the current operational mode setting. – The operational mode should be “LEV” (Low Elution Volume) and “Fnsc” (Forensic). – See Appendix I for instructions on how to change the operational mode settings.
4	• Scroll to “Run” on the Menu screen and press the Run/Stop button. • Open the door. • Press the Run/Stop button again to present the cartridge platform.
5	• Place the LEV cartridge rack containing the samples onto the Maxwell 16 platform. • Ensure that the desired volume of Elution Buffer has been added to

	the Elution Tubes prior to starting the automated method. • Press the Run/Stop button to retract the platform. • Close the door. Extraction begins automatically.
6	Following purification, • open the door, and • verify all plungers have been removed from the rod assembly (manually remove if necessary). • Press the Run/Stop button to present the cartridge platform.
7	Remove each Elution Tube and close.
8	Remove cartridges and plungers from instrument and discard.
9	Select "Yes" for an additional run or "No" to retract the platform. If no further runs are to be performed, power off the instrument.
10	Store extracted DNA at ~ 4°C or ~ -20°C.

Next step

Proceed to DNA quantitation.

Concentrating a sample

A sample may be concentrated by using a variety of techniques. For example, a Speed-Vac System (*e.g.,* MiVac), heat block evaporation, or another method for concentrating DNA extracts (such as an approved ultrafiltration device) may be used. Note that evaporative methods are generally best for maximizing DNA yield while filtration devices are the better approach for minimizing PCR inhibition.

Important

- Related controls should be treated in the same manner as the sample extracts being concentrated.
- Techniques to avoid contamination shall be employed, such as covering the sample during evaporation and/or using a bio hood or other dedicated space.

Reconstituting a sample

Sterile dH_2O may be used to reconstitute evaporated samples.

Section 1.3.4 References

Reference documents

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Section 1.3.5 APPENDIX I - Operational Mode Setup

Mode

Follow the steps below to change the operational mode.

Step	Action
1	Press the "Menu" button to get to the Menu screen.
2	Scroll down to move the cursor to "Setup." Press "Run/Stop" to select "Setup."
3	Scroll to "Forensic Mode" and press "Run/Stop" to select.
4	Scroll to "LEV Hardware" and press "Run/Stop" to select.
5	Turn the instrument off and then on to cycle the instrument power. Verify the instrument's operational mode setting on the display screen.

Section 1.4 Alkaline Differential Extraction of DNA from Body Fluid/Semen Mixtures

Section 1.4.1 Overview

Introduction

The Alkaline Differential Extraction procedure selectively lyses non-sperm cells using a 0.1N NaOH solution prior to lysis of sperm cells with a 1N NaOH solution. This procedure utilizes DNase I digestion steps between the lysis steps to remove residual non-sperm DNA. See Figure 1 in Appendix III for a color-coordinated diagram of the alkaline differential extraction.

Use of this procedure is intended for the Rapid DNA Service (RADS) program and other similar programs.

Contents

The Alkaline Differential Extraction procedure includes the following topics:

Topic
Section 1.4.2 Materials, Reagents, Equipment, and Analytical Controls
Section 1.4.3 Preparation
Section 1.4.4 Differential Extraction using NaOH
Section 1.4.5 NucleoSpin 96 Tissue Plate Clean-up of NaOH Sperm Fraction Lysates
Section 1.4.6 References
Section 1.4.7 Appendix I for *NucleoSpin XS Concentration of NaOH Non-sperm Fraction Lysates*
Section 1.4.8 Appendix II for *Diagram of Alkaline Differential Extraction*

Hazard warnings

- Sodium hydroxide (NaOH) is a corrosive and can cause respiratory tract, eye and skin burns.
- Glacial acetic acid is a corrosive that can burn all tissue, is harmful if inhaled, and is flammable.
- NTC Binding Buffer contains potassium thiocyanate, which is harmful by inhalation, in contact with skin, and if swallowed.

Important
Upon contact with acids or bleach, potassium thiocyanate will liberate toxic gas.

Warning

DNase I use should be separated from other processes by space and/or time to minimize the potential for DNase contamination.

Section 1.4.2 Materials, Reagents, Equipment, and Analytical Controls

Materials & Reagents

- NucleoSpin Alkaline Differential Extraction kit (Macherey Nagel)
- NucleoSpin XS kit (Macherey Nagel)

Equipment

- Four heat blocks and one 96-well block inset
- Three custom 2.2 mL heat transfer bases (V&P Scientific Inc)
- Incubator
- Slicprep device (Promega Corporation)
- Greiner, or similar, 96-well plates - DNase/RNase free
- Clear, white, purple, blue, green, and yellow 2.2 mL square-bottom 96-well plates – DNA/DNase/RNase free
- 96-well plate septa mats
- 96-well 0.2mL semi-skirt PCR plates – DNA/DNase/RNase free, PCR compatible
- 4 mL strip plates (or equivalent)
- Polyolefin sealing tape
- Alumaseal II foil tape plate covers (or other appropriate seals) – DNase/RNase free
- Two centrifuges with plate rotors
- miVac DNA Concentrator (or equivalent)
- NucleoSpin-96 Tissue plates
- 1.1 mL half-height 96-well plates
- 8-channel (L1000 or L1200, L200 and L20) pipettors
- Liquidator 96 pipettor, if desired
- Pipet tips – DNA/DNase/RNase free
- Miscellaneous laboratory supplies

Abbreviation key

For ease of use, the following MACHERY-NAGEL reagent abbreviations are noted below:
NSLB, Non-Sperm Lysis Buffer
SFLB, Sperm Lysis Buffer
NSNB, Non-Sperm Neutralization Buffer
SFNB, Sperm Neutralization Buffer

PBS, Wash Buffer
B5, Wash Buffer
DNB, DNase Buffer
SDB, Sample Dilution Buffer
NTC, Binding Buffer
DEB, DNA Elution Buffer
H2O, Sterile Water

Note *Record reagent lot numbers on the appropriate checksheet.*

Analytical controls

Analytical Controls for DNA Extraction

Controls are required to assess the quality of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results.

Semen Extraction Control Swab
A semen extraction control swab is run with every extraction set in place of the QC sample described in Section 1.1. However, the same requirements described in that section apply to this control swab.

Reagent Blank
Each extraction set should contain at least two reagent blanks. See Section 1.1 for further details on reagent blanks.

Before getting started Mark the left front corner of the Slicprep 96 spin basket and each 96-well plate to ensure the plates are not inadvertently rotated 180°.

Section 1.4.3 Preparation

Contents Preparation for the Alkaline Differential Extraction procedure includes the following topics:

Topic
Section 1.4.3.1 *Sample Preparation*
Section 1.4.3.2 *Reagent and Equipment Preparation*

Section 1.4.3.1 Sample Preparation

Note *For simplicity, samples are typically loaded continuously in columns (i.e. well A1, B1, C1…etc.), without skipping wells or columns.*

Preparing the samples

To prepare samples for differential extraction, perform the following steps:

Step	Action
1	Remove a new Slicprep device from the sealed package and place the 96 spin basket in a new 96-well Greiner plate (or similar 96-well plate). Save the bottom plate from the Slicprep device for use in Step 5 of Reagents & Equipment Preparation below (NSLB).
2	Place sample in the appropriate basket of the Slicprep 96 spin basket. • Total length of 1.5 cm or less for swab/shaft • For fabric (*e.g.,* proficiency testing), place cutting in well followed by a sterile, clean swab on top of it to keep the cutting submerged.
3	Push down the sample with a *NEW* P200 pipette tip (or other appropriate device) to ensure the sample is at the bottom of the corresponding well.
4	Record the pertinent sample information.
5	Repeat Steps 2 through 4 for each sample, including the semen extraction control swab. Be sure to include at least two reagent blanks.
6	Cover the spin basket assembly with a 96-well plate septa mat.
7	Continue to Section 1.4.3.2 *Reagent and Equipment Preparation*.

Section 1.4.3.2 Reagent and Equipment Preparation

Preparing reagents & equipment

Perform the steps below to prepare the reagents in the appropriate vessels:

Step	Action
1	Turn on all heat blocks and allow to warm to set temperature.
2	Confirm the 70°C incubator is on and contains DEB (1:6 TE^{-4}).
3	Remove 1x DNase (DNase I) from freezer and allow to thaw in a beaker containing cool tap water. ***Note:*** *DNase I should be kept at a temperature below room*

	temperature to maximize enzyme activity. **Caution** Mix reconstituted DNase I by gently inverting the tube. Do not vortex as the enzyme is particularly sensitive to physical denaturation.
4	PBS and H_2O Plate Preparation: The following preparatory steps are performed for the wash step which is intended to remove potential inhibitors and potentially buffering compounds (*i.e.,* blood, urine, feces…etc.) that may interfere with the Alkaline Differential Extraction procedure and/or downstream processes. Step / Action: 1 — Transfer 700 µL of Phosphate Buffered Saline (PBS) to the appropriate wells of a WHITE 2.2 mL square bottom 96-well deep well plate, label (*e.g.,* "PBS Soak"), and cover with polyolefin sealing tape. 2 — Transfer 650 µL of sterile DI H_2O to the appropriate wells of a PURPLE 2.2 mL square bottom 96-well deep well plate, label (*e.g* "H_2O Rinse"), and cover with polyolefin sealing tape.
5	Transfer 650 µL of NSLB (0.1N NaOH) to the appropriate wells of the Slicprep (CLEAR) deep well plate, label (*e.g.,* "NSF"), and cover with polyolefin sealing tape.
6	Transfer 650 µL of DNB (DNase Buffer) to the appropriate wells of a GREEN 2.2 mL square bottom 96-well plate, label (*e.g.,* "Pre DNase Buffer"), and cover with polyolefin sealing tape.
7	Transfer 650 µL of DNB (DNase Buffer) to the appropriate wells of a second GREEN 2.2 mL square bottom 96-well plate, label (*e.g.,* "Post DNase Buffer"), and cover with polyolefin sealing tape.
8	Transfer 650 µL of SFLB (1N NaOH) to the appropriate wells of a BLUE 2.2 mL square bottom 96-well plate, label (*e.g.,* "SF"), and cover with polyolefin sealing tape.
9	Continue to Section 1.4.4 *Alkaline Differential Extraction.*

Note: The plate color scheme described throughout this procedure may be altered as needed.

Section 1.4.4 Alkaline Differential Extraction

Section 1.4.4.1 Overview

Contents This section contains the following topics:

Notes: *The non-sperm and sperm lysis steps as well as the DNase I digestion steps are conducted using custom heat transfer bases from V&P Scientific, Inc. These are manufactured to fit the Slicprep/Abgene 2.2 mL plates.*

It is best to prepare centrifuge balances before beginning continuing with this procedure.

For each centrifugal spin of "~3000 x g up to ~30 seconds," the actual time while at ~ 3000 x g should be 10-20 seconds.

Section 1.4.4.2 Performing PBS Soak and H_2O Wash

The following steps are performed with the intent to remove potential inhibitors and potentially buffering compounds (*i.e.* blood, urine, feces, etc.) that may interfere with the Alkaline Differential Extraction procedure and/or downstream processes.

Note: *The plates prepared in Step 9 of Section 1.4.3.2 are utilized in this section.*

Step	Action
1	• ***SLOWLY*** (~5-10 seconds) transfer the Slicprep 96 spin basket into the plate containing the PBS and • incubate at room temperature for ~ 5 minutes.
2	• Gently lift Slicprep spin basket ~1/2", • insert U-shaped collar between the lip of the spin basket and the top of the plate and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds. ***Note:*** *Ensure the collar buttons are inserted into the spin basket divots.*
3	• Gently remove the spin basket assembly and • ***SLOWLY*** transfer it to the PURPLE 96-well plate containing the sterile DI H_20.
4	Incubate at room temperature for ~5 minutes.
5	• Gently lift Slicprep spin basket ~1/2", • insert U-shaped collar between the lip of the spin basket and the top of the plate and • quick spin the entire assembly at ~ 3000 x g up to ~30 seconds.
6	Continue to Section 1.4.4.3 *Performing Non-Sperm Cell Lysis*

Section 1.4.4.3 Performing Non-Sperm Cell Lysis

Non-sperm cell lysis

Perform non-sperm cell lysis as follows:

Step	Action
1	Pre-heat the CLEAR plate containing the NSLB (0.1N NaOH) on a 95°C heat block for 10 minutes.
2	***SLOWLY*** (~5-10 seconds) transfer the Slicprep 96 spin basket into the CLEAR plate containing the NSLB (0.1N NaOH) *without removing the plate from the heat bath*.
3	Heat for 1 minute on the 95 °C heat block. ***Note:*** *Do not remove plate from heat block until after the 1 minute lysis step is complete.*
4	Continue to Section 1.4.4.4 *Preparing Substrates for DNase I Digestion.*

Section 1.4.4.4 Preparing Substrates for DNase I Digestion

Preparing for DNase digestion

Prepare the substrates for DNase I digestion by neutralizing the residual NSLB (0.1N NaOH) as indicated below:

Step	Action
1	• Remove Slicprep spin basket/plate from heat block, • gently lift Slicprep spin basket ~1/2", • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds. ***Note:*** *Ensure the collar buttons are inserted into the spin basket divets.*
2	• Gently remove the spin basket assembly and • ***SLOWLY*** transfer it to the GREEN 96-well plate containing the DNB (DNase Buffer). ***Note:*** *The CLEAR plate contains the non-sperm fraction lysates which will be further processed in Section 1.4.4.6. Performing the Final Processing of the Non-sperm Fractions.*
3	Incubate at room temperature for ~5 minutes.
4	Continue to Section 1.4.4.5 *Performing the First DNase I Digestion.*

Section 1.4.4.5 Performing the First DNase I Digestion

First DNase digestion

Perform the first DNase I digestion as follows:

Step	Action
1	Transfer 650 µL of DNase I to the appropriate wells of a YELLOW 2.2 mL square bottom 96-well plate. **Caution** Mix reconstituted DNase I by gently inverting the tube. Do not vortex as the enzyme is particularly sensitive to physical denaturation.

2	• Gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds.
3	• Gently remove the spin basket assembly and • ***SLOWLY*** transfer it to the YELLOW 96-well plate containing the DNase I.
4	Incubate at 37 °C (***block set @ 44 °C***) for ~90 minutes.
5	Cover green Pre DNase Buffer plate with foil tape (or other appropriate seal) and discard into biohazard waste.
6	Continue to Section 1.4.4.6 *Performing the Final Processing of the Non-Sperm Fractions.*

Section 1.4.4.6 Performing the Final Processing of the Non-Sperm Fractions

Finishing the non-sperm fractions

Perform the final processing of the non-sperm fractions as described below:

Step	Action
1	Neutralize the non-sperm fractions/0.1N NaOH lysate by: • Adding 59 µL of NSNB (2 M Tris, pH 7.5) to each well containing the 0.1 N NaOH/DNA (CLEAR plate) and pipetting up and down repeatedly to mix. • Then, diluting the neutralized solutions in SDB (TE^{-4}) in a PCR plate. A 1/10 dilution is generally appropriate (*e.g.,* 10 µL of the neutralized solutions in 90 µL of SDB (TE^{-4})) to minimize PCR inhibition due to NaOH. The dilution is used for downstream processing. • *Optional*: Proceed to Appendix I for *NucleoSpin XS Concentration of NaOH Non-sperm Fraction Lysates*
2	Cover the neutralized DNA solutions and dilutions with Alumaseal II foil tape (or other appropriate seal).
3	Place plate in storage or proceed to quantitation step.
4	Continue to Section 1.4.4.7 *Performing the Second DNase I Digestion.*

Section 1.4.4.7 Performing the Second DNase I Digestion

Second DNase digestion

Perform the second DNase I digestion as described below:

Step	Action
1	Remove DNase I from the freezer and allow to thaw in a beaker of cool tap water approximately 15 minutes prior to starting the next step.
2	Transfer 650 µL of DNase I to the appropriate wells of a second YELLOW 2.2 mL square bottom 96-well plate.
3	• Gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds.
4	• Gently remove the spin basket assembly and • ***SLOWLY*** transfer it to the second YELLOW 96-well plate containing DNase I.
5	Incubate at 37 °C (***block set @ 44°C***) for ~60 minutes.
6	Continue to Section 1.4.4.8 *Preparing for Sperm-Cell Lysis*.

Section 1.4.4.8 Preparing for Sperm Cell Lysis

Preparing for sperm-cell lysis

To prepare for sperm cell lysis, perform the steps below:

Step	Action
1	• Remove Slicprep spin basket/plate from heat block, • gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds.
2	• Gently remove the spin basket assembly and • **SLOWLY** transfer it to the SECOND GREEN 96-well plate (Post DNase Buffer) containing 650 µL DNB (DNase Buffer).
3	Incubate at room temperature for ~5 minutes.
4	Pre-heat the BLUE plate containing SFLB (1N NaOH) on a 75°C heat block for 10 minutes (in preparation for sperm cell lysis).
5	Continue to Section 1.4.4.9 *Performing Sperm-Cell Lysis*.

Section 1.4.4.9 Performing Sperm-Cell Lysis

Sperm-cell lysis Perform sperm-cell lysis as described below:

Step	Action
1	• Gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g for a TOTAL of ~30 seconds (*i.e.,* ~10 sec actual time at ~ 3000 x g).
2	• Gently remove the spin basket assembly and • **SLOWLY** transfer it to the BLUE 96-well plate containing 650 µL of SFLB (1N NaOH) *without removing the plate from the heat bath*.
3	Incubate at 75 °C for 1 minute. ***Note:*** *Do not remove plate from heat block until after the 1 minute lysis step is complete.*
4	• Remove Slicprep spin basket/plate from heat block, • gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds.
5	Place the spin basket assembly back into the 96-well Greiner plate used in Step 1 of Sample Preparation (Section 1.4.3.1).
6	Continue to Section 1.4.4.10 *Neutralization of Sperm Fraction Lysates*.

Section 1.4.4.10 Neutralization of Sperm Fraction Lysates

Caution Once the glacial acetic acid has been added to each sample, proceed immediately to the remaining rinse/purification/concentration steps for the sperm fraction lysates. Prolonged incubation in glacial acetic acid may result in reduced DNA yields.

Neutralize lysates

Prepare the NaOH sperm fraction lysates for NucleoSpin 96-well tissue plate clean-up as indicated below:

Step	Action
1	Add 41 µL of SFNB (glacial acetic acid) to each original well containing the 1N NaOH/DNA, pipetting up and down carefully to mix.
2	Transfer the neutralized NaOH/DNA samples to a 4 mL strip plate (or equivalent).
3	Continue to Section 1.4.4.11 *Rinsing the Residual DNA from the Substrates*.

Section 1.4.4.11 Rinsing the Residual DNA from the Substrates

Rinse DNA

Rinse residual DNA from the substrates as indicated below:

Step	Action
1	Transfer 430 µL SDB (TE^{-4}) to the appropriate wells of the previously used BLUE 2.2 mL square bottom 96-well plate.
2	• Gently remove the spin basket assembly and • **SLOWLY** transfer it to the BLUE plate containing 430 µL SDB (TE^{-4}). • Incubate at room temperature for ~ 5 minutes.
3	• Gently lift Slicprep spin basket ~1/2”, • insert U-shaped collar between the lip of the spin basket and the top of the plate, and • quick-spin the entire assembly at ~ 3000 x g up to ~30 seconds.
4	Place the spin basket assembly back into the 96-well Greiner plate used in Step 1 of Sample Preparation (Section 1.4.3.1).
5	Transfer the residual DNA/ SDB (TE^{-4}) to the appropriate wells of the 4 mL strip plate (or equivalent) used in Step 2 of Neutralization of Sperm Fraction Lysates (Section 1.4.4.10).
6	Repeat Steps 1-5 once.
7	Continue to Section 1.4.4.12 *Completing the Extraction Process*.

Section 1.4.4.12 Completing the Extraction Process

Complete extraction

Complete the extraction process as described below:

Step	Action
1	Cover the yellow plates containing DNase I with Alumaseal II foil tape (or other appropriate seal) and discard into biohazard waste.
2	Cover green Post DNase Buffer plate with Alumaseal II foil tape (or other appropriate seal) and discard into biohazard waste.
3	Wrap the Slicprep/Greiner plate assembly in aluminum foil (or other appropriate seal), label appropriately, and store frozen. ***Note:*** *Substrate plate may be discarded after acceptable results have been obtained.*

Section 1.4.5 NucleoSpin 96 Tissue Plate Clean-up of NaOH Sperm Fraction Lysates

Section 1.4.5.1 Overview

Contents

This part contains the following topics:

Topic
Section 1.4.5.2 *Preparation of Sperm Fraction Lysates*
Section 1.4.5.3 *Loading the Neutralized NaOH Sperm Fraction Lysates in a NucleoSpin 96-well Tissue Plate*
Section 1.4.5.4 *Washing the Samples in the NucleoSpin 96 Tissue Plate*
Section 1.4.5.5 *Elution, Concentration, and Reconstitution of the Sperm Fraction DNA*

Section 1.4.5.2 Preparation of Sperm Fraction Lysates

Prepare lysates

Prepare the NaOH sperm fraction lysates for NucleoSpin 96-well tissue plate clean-up as indicated below:

Step	Action
1	Transfer 1000 µL of NTC Binding Buffer to the neutralized NaOH/DNA/TE^{-4} solutions, carefully pipetting up and down to mix.
2	Continue to Section 1.4.5.3 *Loading the Neutralized NaOH Sperm Fraction Lysates in a NucleoSpin 96-well Tissue Plate*.

Section 1.4.5.3 Loading the Neutralized NaOH Sperm Fraction Lysates in a NucleoSpin 96 Tissue Plate

Note *The NucleoSpin 96 plates are spun **un**covered during all centrifugation steps.*

Load the samples

Load the neutralized NaOH sperm fraction lysates in a NucleoSpin 96-well tissue plate according to the steps below.

Note: *Steps 1 through 4 are conducted with the NucleoSpin plate in a new or rotated 1.1 mL half-height 96-well plate for each spin. If rotated, the 1.1 mL half-height 96-well plate should be rotated 180° from its previous orientation in the centrifuge.*

Step	Action
1	• Transfer 700 µL of the neutralized lysates to the NucleoSpin 96-well plate. • Quick-spin ~30 seconds up to ~3000 x g.
2	• Transfer an additional 700 µL of the neutralized lysates to the NucleoSpin 96 plate. • Quick-spin ~30 seconds up to ~3000 x g.
3	• Transfer an additional 700 µL of the neutralized lysates to the NucleoSpin 96 plate. • Quick-spin ~30 seconds up to ~3000 x g.
4	• Transfer the remainder of the neutralized lysates to the NucleoSpin 96 plate. • Quick-spin ~30 seconds up to ~3000 x g.

5	Discard the 1.1 mL and strip plates into hazardous waste and the blue plate into biohazardous waste.
6	Continue to Section 1.4.5.4 *Washing the Samples in the NucleoSpin 96-well Tissue Plate.*

Section 1.4.5.4 Washing the Samples in the NucleoSpin 96 Tissue Plate

Washing the bound DNA

To wash the samples in the NucleoSpin 96 Tissue plate, follow the steps described below.

Note: *Steps 1 and 2 are conducted with the NucleoSpin plate in a new or rotated 1.1 mL half-height 96-well plate for each spin.*

Step	Action
1	• Wash with 500 µL of B5 Wash Buffer, • rotate the NucleoSpin 96 plate 180° from the final load orientation (Step 4 of Section 1.4.5.4 above) and • spin for 2 minutes at ~3000 x g.
2	• Wash with an additional 700 µL of Wash Buffer, • rotate both the NucleoSpin 96 and 1.1mL plates 180° from the previous step orientation, and • spin for 2 minutes at ~3000 x g.
3	Discard 1.1 mL plate(s) into hazardous waste.
4	Continue to Section 1.4.5.5 *Elution, Concentration, and Reconstitution of the Sperm Fraction DNA.*

Section 1.4.5.5 Elution, Concentration, and Reconstitution of the Sperm Fraction DNA

Elute, concentrate, & reconstitute sperm DNA

Elute, concentrate ,and reconstitute the sperm fraction DNA as described below:

Step	Action
1	• Transfer NucleoSpin 96 plate to a 96-well PCR plate in a plate holder, • transfer 30 µL of DEB (warmed to 70°C; 1:6 TE^{-4}) to each well, and • spin for 2 minutes at ~3000 x g.
2	• Transfer an additional 30 µL of DEB (warmed to 70°C; 1:6 TE^{-4}) to each well, • rotate both the NucleoSpin 96 and PCR plates 180° from the previous step orientation, and • spin for 2 minutes at ~3000 x g.
3	Remove and discard NucleoSpin 96 plate into hazardous waste.
4	Concentrate sperm fraction retentates to dryness in the miVac DNA Concentrator set at ambient temperature for roughly 2 hours. *In the absence of a miVac DNA Concentrator (or equivalent), sperm fractions may be dried as follows:* • In a 90°C dry-heat bath, heat the sperm fraction retentates in a 96-well plate for approximately 50 minutes, or as needed to completely dry the samples. • Remove the plate from the heat bath and allowed to cool approximately 5 minutes. ***Note:*** *Plate may be covered with Alumaseal II foil tape (or other appropriate seal) and stored frozen before or following (next step) reconstitution if quantitation will not immediately follow.*
5	• Reconstitute the samples in the desired volume of sterile DI H_2O and record the volume. (This may be done at a later time.) • Cover the plate with Alumaseal II foil tape (or other appropriate seal). • Vortex and quick-spin the plate. • Proceed to quantitation, or place plate in an appropriate storage location.

Section 1.4.6 References

Reference documents

Dissing, J., Rudbeck, L., Marcher, H., (1996) "A Five Minute Procedure for Extraction of Genomic DNA from Whole Blood, Semen and Forensic Stains for PCR" Advances in Forensic Haemogenetics 6:269-271.

Klinschar, M., Neuhuber, F., (2000) "Evaluation of an Alkaline Lysis Method for the Extraction of DNA from Whole Blood and Forensic Stains for STR Analysis" J. Forensic Sci. 45:669-673.

Garvin, A., Bottinelli, M., Gola, M., Conti, Conti, A, Soldati, G., (2009) "DNA Preparation from Sexual Assault Cases by Selective Degradation of Contaminating DNA from the Victim" J. Forensic Sci 6:1297-1303.

Hudlow, W., Buoncristiani, M. (2011) "Development of a Rapid, 96-Well Alkaline Based Differential DNA Extraction Method for Sexual Assault Evidence" Forensic Science International:Genetics In Press.

Section 1.4.7 Appendix I - NucleoSpin XS Concentration of NaOH Non-sperm Fraction Lysates

Introduction

This is an optional procedure used to concentrate non-sperm lysates containing low quantities of DNA. This procedure uses individual an NucleoSpin XS Kit with NTC Binding Buffer, replacing the NT Binding Buffer from the kit to enhance recovery.

Note: *Record reagent lot numbers on the appropriate checksheet.*

Warning

This procedure should not be used on bloody lysates as the lysate will generally clog the column.

Non-sperm fraction clean-up

To clean up NaOH non-sperm fraction lysates using NucleoSpin XS columns, perform the following steps:

Step	Action
1	Adjust the pH of the non-sperm fraction(s) by adding 5 µL of glacial acetic acid (SFNB) to each original well containing the neutralized 0.1N NaOH/DNA, carefully pipetting up and down to mix.
2	Transfer the neutralized NaOH/DNA samples to 2 mL TUBES containing 700 µL TE^{-4} (SDB) and mix.
3	Transfer 350 µL of **NTC** Binding Buffer to each of the neutralized NaOH/DNA/ TE^{-4} samples and mix.
4	First loading step: **Step** / **Action** 1 — Transfer 500 µL of the neutralized lysate to a NucleoSpin XS column. Repeat for each sample. 2 — Spin ~30 seconds up to ~11,000 x g. 3 — Discard the collection tubes. 4 — Transfer each NucleoSpin XS column to a new 2 mL collection tube.
5	Second loading step: **Step** / **Action** 1 — Transfer an additional 500 µL of the neutralized lysate to the NucleoSpin XS column for each sample. 2 — Spin ~30 seconds up to ~11,000 x g. 3 — Discard the collection tubes. 4 — Transfer the NucleoSpin XS columns to new 2 mL collection tubes.
6	Third loading step: **Step** / **Action** 1 — Transfer an additional 500 µL of the neutralized lysate to the NucleoSpin XS column for each sample. 2 — Spin ~30 seconds up to ~11,000 x g. 3 — Discard the collection tubes. 4 — Transfer the NucleoSpin XS columns to new 2 mL collection tubes.

7	Final loading step:

Step	Action
1	Transfer the remainder of the neutralized lysate to the NucleoSpin XS column for each sample.
2	Spin ~30 seconds up to ~11,000 x g.
3	Discard the collection tubes.
4	Transfer he NucleoSpin XS columns to new 2 mL collection tubes.

8	B5 Wash Buffer:

Step	Action
1	Wash each sample with 100 µL of B5 Wash Buffer.
2	Spin for 2 minutes at ~11,000 x g. ***Note:*** *NucleoSpin XS columns should be rotated 180° from their previous orientation in the centrifuge.*

9	BE elution:

Step	Action
1	Transfer the NucleoSpin XS columns to capless 1.5 mL tubes and transfer 6-15 µL of BE Buffer to each NucleoSpin XS column. ***Note:*** Volumes greater than 10 µL may be used but will not necessarily result in increased DNA yields.
2	Spin for 1 minute at ~11,000 x g.
3	Transfer an additional 6-15 µL of BE Buffer to each NucleoSpin XS column.
4	Spin for 1 minute at ~11,000 x g.

10	Removal of residual ethanol and sample concentration:

Step	Action
1	Remove NucleoSpin XS columns and heat tubes in 90°C dry-heat bath, generally for ~5-8 minutes.
2	Transfer retentate to a storage tube and record approximate amount recovered.

Section 1.4.8 Appendix II – Diagram of Alkaline Differential Extraction

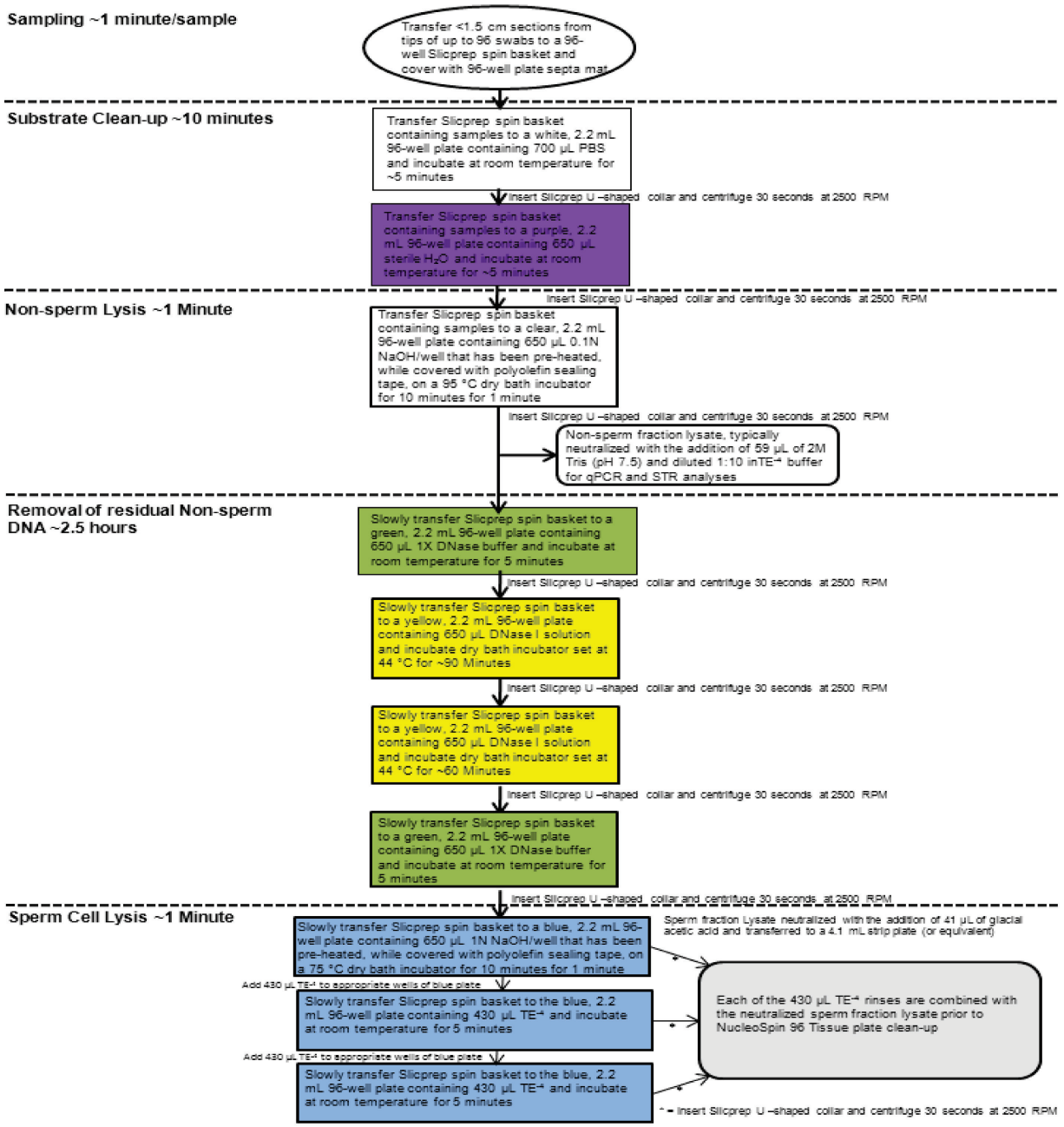


Figure 1

Section 1.5 PrepFiler™ DNA Extraction from Case Evidence

Section 1.5.1 Overview

Introduction

The PrepFiler™ Forensic DNA Extraction Kit is an alternative DNA extraction method to the other validated extraction methods available to casework analysts (*e.g.,* standard organic procedures and the Maxwell DNA IQ process). This procedure may be performed manually or in an automated format using the Tecan HIDEVO150 robotic platform.

The PrepFiler chemistry is based on reversible binding of DNA from a cellular lysate to magnetic particles. The particles with adhering DNA are washed to remove proteins and other cellular debris. The DNA is then released from the particles and collected in a buffer during the elution steps. The final elution volume is approximately 50 µL. Samples may be concentrated to lower volumes using a variety of approaches referenced herein.

Sample types and substrates

The PrepFiler kit is appropriate for the extraction of DNA from stains, swabs, and body-fluid samples (typically < ~40 µL). The kit will extract small amounts of tissue adhering to hair shafts, but will not fully digest the shafts.

A variety of sample types and substrates were tested in the PrepFiler DNA extraction validation. While most worked well, those substrates that are frequently challenging with other chemistries also produced suboptimal yields with PrepFiler and sometimes contained PCR inhibitors. These substrates included denim, soil, adhesives (envelopes), and leather. Thus direct lysis from such substrates is generally avoided if possible (*i.e.,* instead, swab the item for an indirect lysis).

See Section 1.5.3.4 for procedural modifications for samples containing soil and non-sperm fraction lysates from the organic differential extraction procedure.

The validated PrepFiler kit is not optimized for extraction from bones or samples with adhesives. Other available methods may prove to be better choices to maximize yield and purity (*e.g.,* extraction with demineralization for bones, organic for adhesives, *etc.*). PrepFiler is also not optimized for the clean-up of alkaline differential extraction lysates; these lysates are instead processed using NucleoSpin columns.

When a substrate is encountered which may result in suboptimal extraction with PrepFiler, it may be prudent to test a similar, non-evidentiary substrate to determine if any co-extracted component will interfere with the binding of the magnetic

particles to the magnet. This test may be accomplished by performing a "partial" PrepFiler extraction consisting of direct lysis, followed by the first particle-washing step (*i.e.,* addition of isopropanol, addition of Magnetic Particles, and binding of the particles to the magnet). If the particles flocculate or do not bind to the magnet at this step, then direct lysis will not be an efficient approach for PrepFiler extraction. In this case, either indirect sampling methods or alternative extraction methods should be considered.

Contents

The PrepFiler DNA Extraction procedure includes the following topics:

Hazard warnings!

- **PrepFiler Lysis Buffer** contains guanidine thiocyanate, which is harmful by inhalation, in contact with skin, and if swallowed. Causes eye, skin, and respiratory tract irritation. Upon contact with acids or bleach, guanidinium will liberate toxic gases.
- **PrepFiler Magnetic Particles** contain guanidine thiocyanate, which is harmful by inhalation, in contact with skin, and if swallowed. Causes eye, skin, and respiratory tract irritation. Upon contact with acids or bleach, waste containing this product will liberate toxic gases.
- **PrepFiler Wash Buffer A Concentrate** contains deoxycholate detergent, which is harmful by inhalation, in contact with skin, and if swallowed. Causes eye, skin, and respiratory tract irritation.

Section 1.5.2 Materials, Reagents, Equipment, and Analytical Controls

Materials & Reagents

- PrepFiler Automated Forensic DNA Extraction Kit (960 tests)
- 1M DTT
- TE^{-4}
- Isopropanol (100%)
- Ethanol (95%, molecular biology grade)

Equipment

- VorTemp Shaking Incubator (or other appropriate shaker/heater)
- Heat block or oven
- 1.5 mL Microfuge tubes – DNA/DNase/RNase free
- Minicentrifuge
- Vortex
- Pipettors
- Pipet tips – DNA/DNase/RNase free
- Miscellaneous laboratory supplies

- Manual:
  – Life Technologies/Applied Biosystems (or other appropriate) magnet rack for 1.5 mL tubes

- Automated:
  – Tecan Freedom HIDEVO150 Combination System, including TeShake and Magnetic Ring Stand
  – 96 deep, square-well processing plate (*e.g.,* Phenix P/N M-1810S or Life Tech P/N 4392904) – DNase/RNase free
  – 96-well reaction plates – DNA/DNase/RNase free, PCR compatible
  – Adhesive plate cover
  – Alumaseal II foil tape plate covers (or other appropriate seals) – DNase/RNase free
  – Tecan 100 mL troughs – DNA/DNase/RNase free
  – Conductive, filtered 200 and 1000 µL tips – DNA/DNase/RNase free (Tecan Pure)
  – Weak detergent (*e.g.,* Liquinox or RoboScrub)
  – Double-sided approximately ¾" foam tape (*e.g.,* 3M)
  – Approximately ¾" paper tape (*e.g.,* blue painter's or masking tape)

Note *Record reagent lot numbers on the appropriate checksheets.*

Analytical controls

Controls are required to assess the quality of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results. An extraction QC sample and a reagent blank are run with every extraction set. The same requirements described in Section 1.1 apply to the controls extracted with Prepfiler.

Section 1.5.3 PrepFiler Lysis

Section 1.5.3.1 Overview

Contents

Preparation for PrepFiler Lysis includes the following topics:

Topic
Section 1.5.3.2 *Reagent Preparation*
Section 1.5.3.3 *Sample Lysis*
Section 1.5.3.4 *Procedural Modifications for Specific Sample Types*

Section 1.5.3.2 Reagent Preparation

Preparing the lysis cocktail

To prepare the reagents used for PrepFiler lysis, perform the following steps:

Step	Action
1	Determine the number of samples to be extracted, including controls.
2	Determine the volume of PrepFiler Lysis Buffer needed as follows: Volume of buffer (in µL) = # of samples * 1.1 * 300 µL
3	Determine the volume of DTT needed as follows: **If…** / **Then…** The samples may contain semen, or are hair or nail material, / Volume of DTT (in µL) = # of samples * 1.1 * **5 µL.** Otherwise, / Volume of DTT (in µL) = # of samples * 1.1 * **3 µL.** ***Note:*** If the sample of interest is biological material *on the surface* of

	a hair or nail, a swab or scraping of the item should be extracted.
4	Prepare the Lysis Buffer cocktail by mixing the calculated quantities of PrepFiler Lysis Buffer and DTT. **Important** Lysis Buffer cocktail should be prepared freshly for each extraction. Any unused remaining cocktail should be appropriately discarded.

Section 1.5.3.3 Sample Lysis

Sample lysis Perform the steps below for sample lysis:

Step	Action
1	Add 300 µL of Lysis Buffer *cocktail* to each sample for extraction. The Lysis Buffer should cover each substrate.
2	Briefly vortex for 5 seconds and spin down tubes.
3	Place the sample tubes in the heater-shaker (*e.g.,* VorTemp Thermal Shaker). Incubate at ~ 900 RPM and ~ 70˚C for 60 minutes.
4	Remove the tubes and briefly spin down.
5	For each sample containing a substrate, remove the substrate (*e.g.,* by spin-basket centrifugation) from the lysate. ***Note:*** If particulates/solids remain after removing the substrate, spin down the particulates and carefully transfer the supernatant liquid to a new tube for extraction.
6	Inspect the tubes to ensure that each sample contains 180 - ~300 µL of lysate. If the volume is below 180 µL, add Lysis Buffer to increase the volume to ~300 µL. ***Note:*** Any volume in excess of 300 µL will not be processed unless transferred to an additional tube for processing.
7	Proceed to Manual (Section 1.5.4) or Automated (Section 1.5.5) PrepFiler DNA Extraction. • Allow the lysates to come to room temperature first. • The unprocessed lysate is stable at room temperature for approximately 24 hours. • To avoid precipitation, do NOT freeze, chill, or refrigerate the lysates.

Section 1.5.3.4 Procedural Modifications for Specific Sample Types

Soil samples Follow the steps below for a sample containing soil.

Step	Action
1	Prepare the sample: • Place up to 50 mg of soil sample into a 1.5 mL tube. • Add 100 µL of PBS. • Vortex for 10 seconds, then briefly centrifuge (up to 30 seconds, not longer). • Transfer ~70 µL of clear supernatant (free of residual soil) to a new 1.5 mL tube.
2	Prepare lysis cocktail by combining the following quantities of PrepFiler Lysis Buffer and DTT: PrepFiler Lysis Buffer = # of samples * 1.1 * 500 µL DTT = # of samples * 1.1 * 3 µL
3	Perform lysis: • Add 500 µL of PrepFiler Lysis *cocktail* to each sample supernatant. • Vortex for 5 seconds, then centrifuge briefly. • Incubate on a thermal shaker at ~70˚C and ~900 RPM for 30 minutes.
4	If there is soil residue in the tube, perform the following. Otherwise, continue to Step 5. • Centrifuge the tube at maximum speed (~16,000xg) for 5 minutes. • Transfer the clear supernatant (free of residual soil) to a new 1.5 mL tube.
5	For Automated PrepFiler DNA Extraction, split the ~570 µL of lysate into two tubes and continue to Section 1.5.5. For Manual PrepFiler DNA Extraction, continue with Step 6 below.
6	Bind genomic DNA to magnetic particles: • Add **20 µL** of magnetic particles into the tube containing the lysate. • Vortex at low speed (~500-1200 RPM) for 10 seconds to promote DNA binding, then centrifuge briefly. • Add **300 µL** of isopropanol to the lysate tube. • Vortex at low speed for 5 seconds, then centrifuge briefly. • Place the sample lysate tubes in a shaker or on a vortex (with adaptor), then mix at room temperature at ~900-1,000 RPM for 10 minutes.
7	Continue with the Manual PrepFiler DNA Extraction procedure, beginning with Step 1 of Section 1.5.4.4 Wash the Bound DNA.

Non-sperm fraction lysates

For non-sperm fraction lysates from the organic differential extraction procedure, the lysate is typically in a volume of ~400 µL. Therefore, follow the steps below.

Step	Action
1	Prepare samples: In a 1.5 mL tube, add PrepFiler Lysis Buffer and DTT to non-sperm fraction lysates in either formulation shown below. **Option** / **Non-sperm fraction lysate volume** / **PrepFiler Lysis Buffer** / **DTT** **A** / 50 µL / 300 µL / 3 µL **B** / 150 µL / 150 µL / 1.5 µL **A**: Use for routine non-sperm fraction processing. **B**: Use for non-sperm fractions in which it may be desirable to process a greater portion of the lysate (*e.g.,* to maximize an anticipated low quantity of DNA). **Important** Any volume in excess of 300 µL will not be processed unless transferred to an additional tube for processing. If needed, split the lysate volume among multiple 1.5 mL tubes. The multiple extracts may be recombined upon completion of PrepFiler extraction.
2	Freeze the remaining non-sperm cell fraction volumes in appropriately labeled tubes.
3	Continue with Step 2 of Section 1.5.3.3 Sample Lysis.

Section 1.5.4 Manual Prepfiler DNA Extraction

Section 1.5.4.1 Overview

Contents

This section contains the following topics:

Topic
Section 1.5.4.2 *Preparation for Extraction*
Section 1.5.4.3 *Bind DNA to Magnetic Particles*
Section 1.5.4.4 *Wash the Bound DNA*
Section 1.5.4.5 *Elute the DNA*

Section 1.5.4.2 Preparation for Extraction

Magnetic particles

Gently invert tube(s) repeatedly to fully suspend particles, then spin down very briefly to remove material from the cap.

If any light-tan/off-white precipitate is visually present in the tube of Magnetic Particles, incubate the tube for ~30 minutes at 37°C.

Reagent inventory

Assess the volume of reagents needed to ensure adequate quantities before starting.

Reagent	Volume needed
PrepFiler Magnetic Particles	15 µL x ___ samples = _____ total µL Magnetic Particles needed
Isopropanol	180 µL x ___ samples = _____ total µL isopropanol needed
Prepared PrepFiler Wash Buffer A (WBA)	900 µL x ___ samples = _____ total µL WBA needed WBA must have been previously prepared to include ethanol (see Appendix I).
Prepared PrepFiler Wash Buffer B (WBB)	300 µL x ___ samples = _____ total µL WBB needed WBB must have been previously prepared to include ethanol (see Appendix I).
PrepFiler Elution Buffer, or TE^{-4}	50 µL x ___ samples = _____ total µL Elution Buffer needed ***Note:*** TE^{-4} may be substituted for the Elution Buffer.

Section 1.5.4.3 Bind DNA to Magnetic Particles

Binding DNA Perform the steps below to bind the DNA to the magnetic particles.

Step	Action
1	• To prepare the magnetic particles for binding, repeatedly and gently invert the tube to confirm that no visible pellet of particles remains in the bottom of the tube. • Then, centrifuge briefly just to remove any material from the cap. ***Note:*** If transferring magnetic particles for many samples, repeat this step every ~5 minutes.
2	Verify lysates are at room temperature and the volumes are >180 µL. If not, add enough lysis buffer to bring the volume to approximately 300 µL.
3	Pipette 15 µL of magnetic particles into each tube containing sample lysate. ***Note:*** When not in use, keep the Magnetic Particle tubes tightly capped to avoid evaporation, which could cause precipitates to form.
4	• Vortex each sample at ***low*** speed (approximately 500 to 1,200 RPM) for ~10 seconds to promote DNA binding, • then centrifuge briefly.
5	Add 180 µL of isopropanol to each sample lysate tube. Isopropanol enhances the binding of DNA to the magnetic particles.
6	• Vortex at ***low*** speed (approximately 500 to 1,200 RPM) for ~5 seconds, • then centrifuge briefly.
7	• Place the sample lysate tubes in a shaker or on a vortex (with adaptor), • then mix at room temperature at 900-1,000 RPM for 10 minutes.

Section 1.5.4.4 Wash the Bound DNA

Washing bound DNA

Perform the steps below to wash the bound DNA.

Step	Action
1	• Vortex the sample tube at maximum speed (approximately 10,000 RPM) for 10 seconds, • then centrifuge briefly.
2	Place the sample tube in the magnetic stand and observe that the magnetic particles form a pellet against the back of the tube. Wait until the size of the pellet stops increasing (approximately 1 to 2 minutes). ***Notes:*** • Samples containing high levels of proteins or other impurities may require more time. • For some sample types such as blood, the solution may remain colored after the magnetic particles are separated.
3	With the sample tube remaining in the magnetic stand, use a pipette to carefully remove and discard all visible liquid phase into an appropriate waste container. ***Note:*** When removing the liquid phase, do not aspirate magnetic particles or disturb the magnetic particle pellet. **Important** In this first wash step, the waste should NOT be disposed down the sink or treated with bleach or acid.
4	Perform the following wash steps **THREE** times. **Step** / **Action** 1 — Add the appropriate volume of Wash Buffer to the sample tube: 1st wash = 600 µL of WBA 2nd wash = 300 µL of WBA 3rd wash = 300 µL of WBB 2 — Cap the sample tube and remove the tube from the magnetic stand. 3 — • Vortex the sample tube at maximum speed (approximately 10,000 RPM) until there is no visible magnetic particle pellet on the side of the tube

		(approximately 5 seconds), • then *very briefly* centrifuge. ***Note:*** It is acceptable to have magnetic particle aggregates suspended in the solution and adhering to the sides.
	4	Place the sample tube in the magnetic stand for 30 to 60 seconds until the beads form a pellet against the magnet.
	5	With the sample tube remaining in the magnetic stand, use a pipette to carefully remove and discard all visible liquid phase. ***Note:*** The particles are first washed with Wash Buffer A (WBA), which contains a detergent that can inhibit PCR when not washed away thoroughly. After two washes with WBA, the particles are washed with Wash Bufffer B (WBB) to remove residual WBA.
	6	Repeat Steps 1-5 twice, then continue to Step 5 below.
5	With the sample tube remaining in the magnetic stand, open the tube, then allow it to air-dry for ~7 to 10 minutes. This promotes evaporation of residual ethanol from the wash buffers. **Important** • Air-drying for more than 10 minutes may reduce DNA yield due to drying of the beads. • If room temperature is >25°C (>77°F), reduce the drying time to 5 minutes.	

Section 1.5.4.5 Elute the DNA

Elute Perform the steps below to elute the DNA.

Step	Action
1	Preheat the thermal shaker to 70°C for ~10 minutes. ***Note:*** When using the VorTemp thermal shaker, the RPM speed must not be set at "0." Some shaker motion is necessary to cause the fan to spin, enabling proper temperature control.
2	Add 50 µL of PrepFiler™ Elution Buffer or TE^{-4} to the sample tube.

	Note: Pure water should NOT be used for this elution step.
3	• Vortex the sample tube at maximum speed (approximately 10,000 RPM) until there is no visible magnetic particle pellet on the side of the tube (approximately 5 seconds), • then centrifuge briefly.
4	• Place the sample tube in a thermal shaker, • then incubate at 70°C and 900 RPM for 5 minutes.
5	• Vortex the sample tube at maximum speed (approximately 10,000 RPM) until there is no visible magnetic particle pellet on the side of the tube (approximately 2 seconds), • then centrifuge briefly.
6	• Place the sample tube in the magnetic stand, • then wait until the size of the pellet at the side of the tube stops increasing (at least 1 minute).
7	Being careful not to disturb the magnetic particle pellet, transfer the liquid (which contains the isolated genomic DNA) from the tube to a new, labeled tube for storage. Retentate volumes should be approximately 50 µL. ***Note:*** If the eluted DNA extract is turbid (for example, this may occur in tissue samples with a high fat content), centrifuge the tube for 5 to 7 minutes at maximum speed (approximately 10,000 RPM), then transfer the clear supernatant to a new 1.5-mL microcentrifuge tube.

Concentrating a sample

A sample may be concentrated by using a variety of techniques. For example, a Speed-Vac System (*e.g.,* MiVac), heat block evaporation, or another method for concentrating DNA extracts (such as an approved ultrafiltration device) may be used. Note that evaporative methods are generally best for maximizing DNA yield while filtration devices are the better approach for minimizing PCR inhibition.

Important

- Related controls should be treated in the same manner as the sample extracts being concentrated.
- Techniques to avoid contamination shall be employed, such as covering the sample during evaporation and/or using a bio hood or other dedicated space.

Reconstituting a sample

Sterile DI H_2O may be used to reconstitute evaporated samples.

Section 1.5.5 Automated Prepfiler DNA Extraction

Section 1.5.5.1 Overview

Overview

This section describes the procedures for setting up an HIDEVO150 PrepFiler DNA Extraction run. Up to 80 samples may be run at a time in either a "tube-to-tube" or "tube-to-plate" format. In the former, DNA extracts are deposited into 1.5 mL tubes and in the latter, extracts are deposited into a 96-well plate. In both instances, lysates are presented to the Tecan in 1.5 mL tubes, hence "tube-to-tube" and "tube-to-plate."

Typically, DNA quantification using the Quadruplex qPCR assay follows and then normalization and STR amplification setup are subsequently performed, followed by capillary electrophoresis and detection of STR loci using either a 3130, 3130*xl*, or 3500/3500xL Genetic Analyzer. The data from these instruments are then analyzed through GeneMapper ID or ID-X.

Contents

This section contains the following topics:

Hazard

To avoid potential crushing and piercing injuries from moving parts, do NOT reach into the Tecan work space when a script is running. If it is necessary to access the deck or work space during a run, either pause the script (wait for the green indicator light to begin flashing), stop the script (the indicator light turns off), or power down the Tecan.

Hazard

The barcode reader (*i.e.,* PosID) uses laser technology for scanning. Do not stare into the laser beam or its reflection.

Warning

Electromagnetic waves from a cellular phone may affect the function of liquid detection. Faulty detection of the liquid surface may cause the system to produce incorrect results. It is recommended to keep a distance of at least 6.5 feet from the instrument when using a cellular phone.

Section 1.5.5.2 Pre-Run Processing

Preparation for Extraction

Magnetic particles

Gently invert tube(s) repeatedly to fully suspend particles, then spin down very briefly to remove material from the cap.

If any light-tan/off-white precipitate is visually present in the tube of Magnetic Particles, incubate the tube for ~30 minutes at 37°C.

Notes on magnetic particles

- Difficulty achieving re-suspension:
  - Depending upon the last usage of the tubes, the re-suspension step (above) may require many inversions.
  - *Only if necessary*, vortex at medium speed until the particles are suspended, then centrifuge briefly. Vortexing will typically result in the formation of significant quantities of foam. Although the foam will reduce on standing, it may be necessary at a later stage in the procedure to either remove the foam using a pipettor or to transfer the non-foamed suspended particles to a clean 2 mL tube. The foam can interfere with liquid level detection on the Tecan.
- The automated procedure requires two tubes to be used, even if sufficient volume is present in a single tube for the run.
- When not in use, keep the Magnetic Particle tubes tightly capped to avoid evaporation, which could cause precipitates to form.

Reagent inventory

Assess the volume of reagents needed:

Reagent	Volume needed
PrepFiler Magnetic Particles	15 µL x ___ samples = _____ total µL Magnetic Particles needed
Isopropanol	207 µL x ___ samples + 5000 µL overfill = _____ total µL isopropanol needed
Prepared PrepFiler Wash Buffer A (WBA)	1035 µL x ___ samples + 5000 µL overfill = _____ total µL WBA needed WBA must have been previously prepared to include ethanol (see Appendix I).
Prepared PrepFiler Wash Buffer B (WBB)	345 µL x ___ samples + 5000 µL overfill = _____ total µL WBB needed WBB must have been previously prepared to include ethanol (see Appendix I).
PrepFiler Elution Buffer, or TE^{-4}	57.5 µL x ___ samples + 5000 µL overfill = _____ total µL Elution Buffer needed ***Note:*** Elution Buffer is simply TE^{-4}, thus TE^{-4} may alternatively be used.

Note: These formulations include a volume overfill where needed for robotic processing.

Generate Input File

Generate input file

Perform the following steps to generate the HIDEVO PrepFiler input file:

Step	Action
1	Open the Excel spreadsheet "*CADOJ_HIDEVO_TubesInput_v2_1_2.xltm*" and enable macros. ***Note:*** Higher version Excel template(s) may be used if performanced-checked.
2	Enter sample names. See details below for format specifics.

	General notes:
	• Include the case number with each sample. The format *LLYY#### sample name* is suggested, where LLYY#### represents a BFS laboratory case number.
	• No samples in the same run can have identical names.
	• A sample name *cannot* include spaces or particular characters, such as colons, commas, asterisks, and slashes.
	  o Permissible characters include -_(){}#.+
	  o Even a space *following* the sample name will *not* be accepted
	• A sample name *cannot* be a single, numeric 0 (zero).
	• All samples must be loaded contiguously; sequential tube and rack positions cannot be skipped.
	• Position 1 is Rack S1, P1 in the Excel template.

3	Follow the Excel spreadsheet instructions to perform the steps below. **Step** / **Action** 1 — Print the load map for inclusion in the case file: • Run **PrintLoadMap** to print the sample input sheet 2 — For the tube-to-plate format, also print the plate map which will indicate sample extract locations: • Run Print96well Extraction Plate Map 3 — Run **ExportPFiler** to generate the PrepFiler sample input file. Save this file as *PF_<instrument name>_<MMDDYY> _<operator initials>*. 4 — If desired for qPCR, • Run the appropriate **ExportCSV** to generate the qPCR sample tube or plate input file. Save this file as *qPCR_<instrument name>_ <MMDDYY>_<operator initials>*. • Run **ExportTXT** to generate the 7500 input file. Save this file as *7500PlateMap_<instrument name>_<MMDDYY> _<operator initials>*.
4	Record the PrepFiler file name on the Tecan HIDEVO150 PrepFiler checksheet and transfer the file to the appropriate instrument computer.
5	*Optional:* Save and re-name the Excel template as a macro-enabled file.

Rack Preparation

Tecan racks

To prepare the racks on the Tecan for processing, perform the following steps.

Step	Action
1	Obtain the PrepFiler lysate sample tubes. The lysates should be in 1.5 mL tubes at volumes in the range of 180-300 µL. ***Note:*** The lysate tubes should be at room temperature. Higher temperatures could reduce DNA binding to the magnetic particles.
2	Inspect the tubes to ensure that each contains at least 180 µL of lysate and to ensure that there are no bubbles at the bottoms of the tubes. If necessary, briefly centrifuge the tubes.
3	Arrange the tubes in Tecan 16-position Lysate ("L") tube racks according to the PrepFiler sample sheet.
4	For tube-to-tube format only, arrange a set of empty, labeled tubes in Tecan 16-position Sample ("S") tube racks so that the empty tubes are identically situated to the tubes in the "L" racks. These "S" tubes will eventually contain the extracted DNA.

Pre-run HIDEVO150 Checks and Maintenance

Frequency

These checks and maintenance procedures should be run prior to each sample run.

Pre-run check/ maintenance

Perform the following procedure for pre-run checks and maintenance on the HIDEVO150.

Step	Action
1	Boot up the computer connected to the HIDEVO150 system.
2	Load the appropriate sample input file for the run onto the computer.
3	As needed, perform the following maintenance tasks: • Empty and replace the DiTi waste bag. – *Note:* It is important to empty this before running a full plate to prevent obstructions during tip ejection due to the number of DiTis used. • Remove any plasticware (troughs, plates, etc.) that might have been left on the EVO deck. • Empty the Liquid Waste container. • Replenish the System Liquid with DI H_20.

	– ***Note:*** Ideally, this is done on the day preceding the automated run, so that the liquid has had an opportunity to de-gas.
4	Turn the Tecan power on by pressing in the green triangular button to the lower, right side of the deck.
5	Start up the EVOware software on the instrument computer and login to the software.
6	• Select the **Run maintenance** button • According to the table below, run the appropriate script(s) from EVOware:

If before starting the run...	**Then run the script...**
there are one or more DiTis on the liquid handling arm (LiHa)	*Combo_Drop_DiTis*
it is the first run of the day	*Combo_DailyStartUp*
it is not the first run of the day	*Combo_Flush* (enter **50 mL**)
when you run *Combo_DailyStartUp* or *Combo_Flush*, you see: • air bubbles in the syringe barrels or any supply lines and/or • intermittent flow from a DiTi cone	*Combo_Flush* one or more times until: • there are no visible air bubbles and • flow from the DiTi cones is constant

Notes:
- To exit the maintenance script, click the Cancel button in the software.
- The daily start-up script steps the user through a series of checks and system priming, including checking system liquid and waste, checking syringe and valve fittings, checking DiTi cones, and lastly checking placement of carriers and appropriate racks and labware for the run.
- The flush script simply primes and flushes the liquid system.

Setting Up the HIDEVO150 Deck

Deck Setup

In this section, the user will confirm that the deck is set up with the proper carriers and labware for the automated PrepFiler extraction procedure. Carriers and labware items will be changed out as needed. Figures 1a and 1b illustrate the PrepFiler Tube-to-Tube and Tube-to-Plate deck setups.

Note: It is not necessary to put all of the reagent troughs and plasticware onto the Tecan deck at this time.

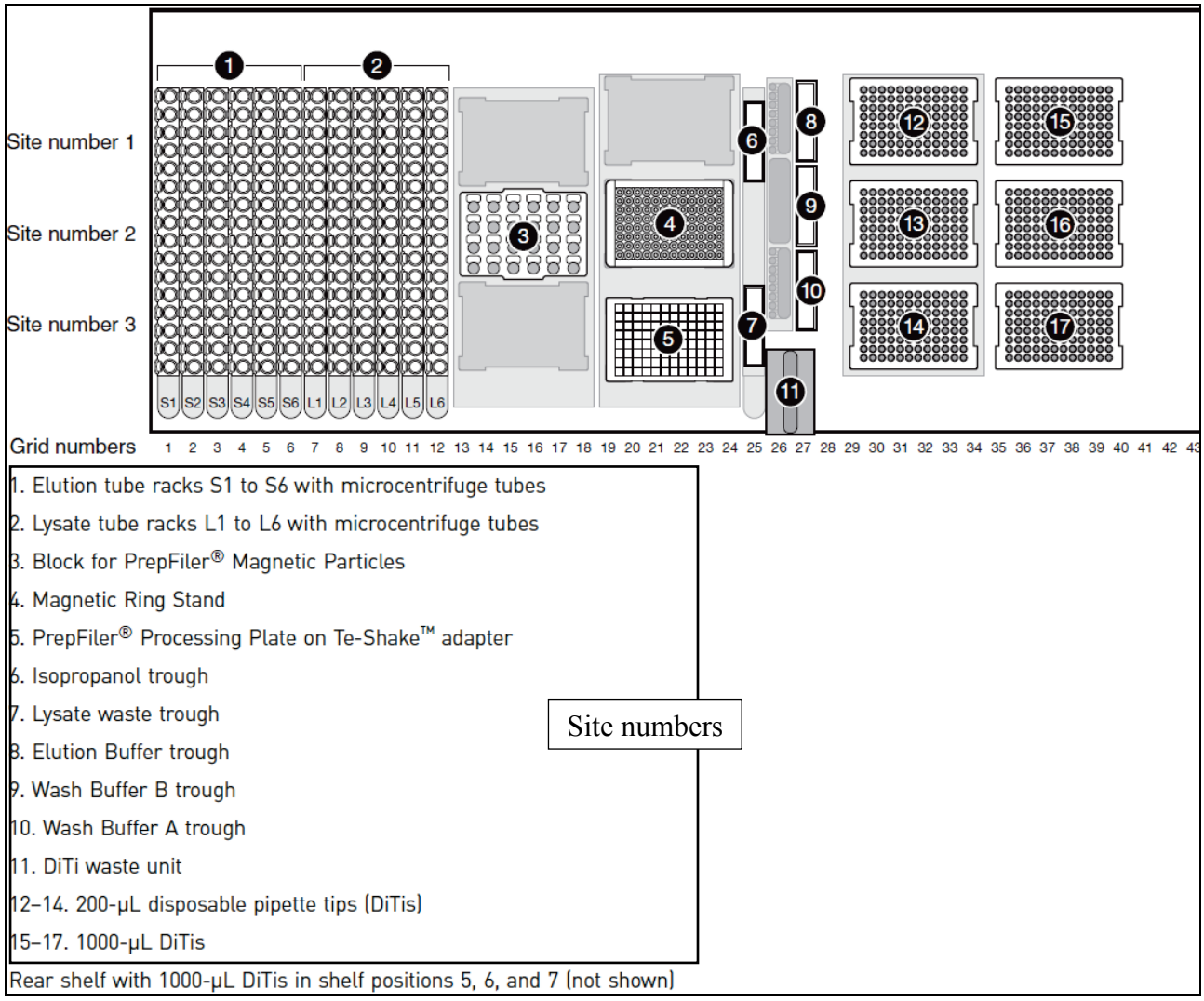
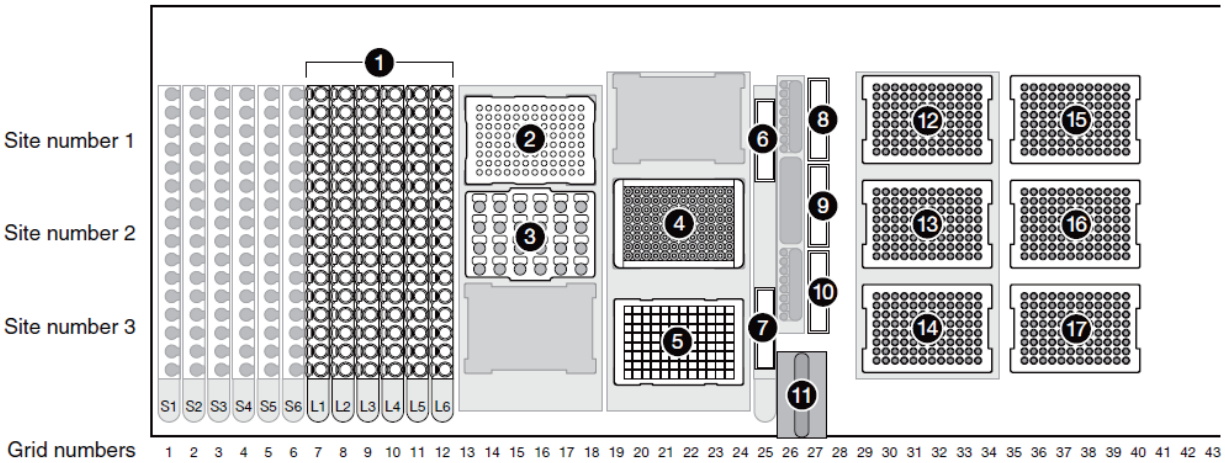


Figure 1a PrepFiler Tube-to-Tube deck setup.

Figure 7 Tubes-to-plate workstation layout.



1. Lysate tube racks L1 to L6 with microcentrifuge tubes
2. 96-Well Elution Plate
3. Block for PrepFiler™ Magnetic Particles
4. Magnetic Ring Stand
5. PrepFiler™ Processing Plate on Te-Shake™ adapter
6. Isopropanol trough
7. Lysate waste trough
8. Elution Buffer trough A
9. Wash Buffer B trough
10. Wash Buffer trough
11. DiTi waste unit

12–14. 200-µL disposable pipette tips (DiTis)

15–17. 1000-µL DiTis

Rear shelf with 1000-µL DiTis in shelf positions 5, 6, and 7 (not shown)

Figure 1b PrepFiler Tube-to-Plate deck setup.

Set up DiTi trays

To set up the DiTi trays, perform the steps below.

Step	Action
1	Obtain conductive, filter-tipped DiTis: • Three full trays of 200 µL • Six full trays of 1000 µL ***Note:*** A full extraction run of 80 samples will require ~2.5 trays of 200 µL and ~5 trays of 1000 µL DiTis. **Important** In contrast to the HIDEVO qPCR/STR modules, the PrepFiler module

	resets the 200 µL and 1000 µL DiTi positions to the first position in the first tray for each run. Consequently, it is *necessary* to start each PrepFiler run with a full set of DiTis.
2	Load each of the 1000 µL DiTi trays into an aluminum DiTi box/rack, taking care to orient the notch in the tray to the pin (Figure 2a) or nose (Figure 2b) in the box. • See Figure 2a or 2b, depending upon the type of DiTi box that is used on your Tecan system.
3	Place three of these DiTi boxes into the three positions at grid 35 (see Figures 1 and 2a/b).
4	Place the other three DiTi boxes into positions 5, 6, and 7 of the DiTi shelf. • See Figures 2a/b for correct orientation of the boxes. • The boxes should be placed flush against the rear of the shelf. **Important** All other positions in the DiTi shelf should be empty. The Tecan will "crash" if anything is in position 4 at the beginning of a run.
5	Place three full trays of 200 µL DiTis in the carrier at grid 29, taking care to orient the notch in each tray as shown in Figure 2a/b.

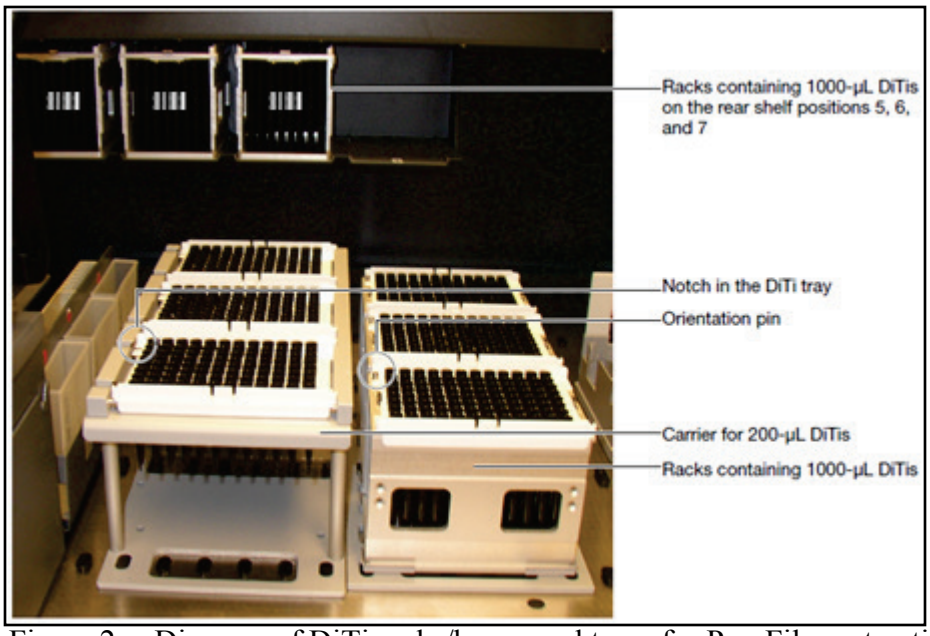


Figure 2a. Diagram of DiTi racks/boxes and trays for PrepFiler extraction. The barcodes on the DiTi racks on the rear shelf should be visible, facing the operator.

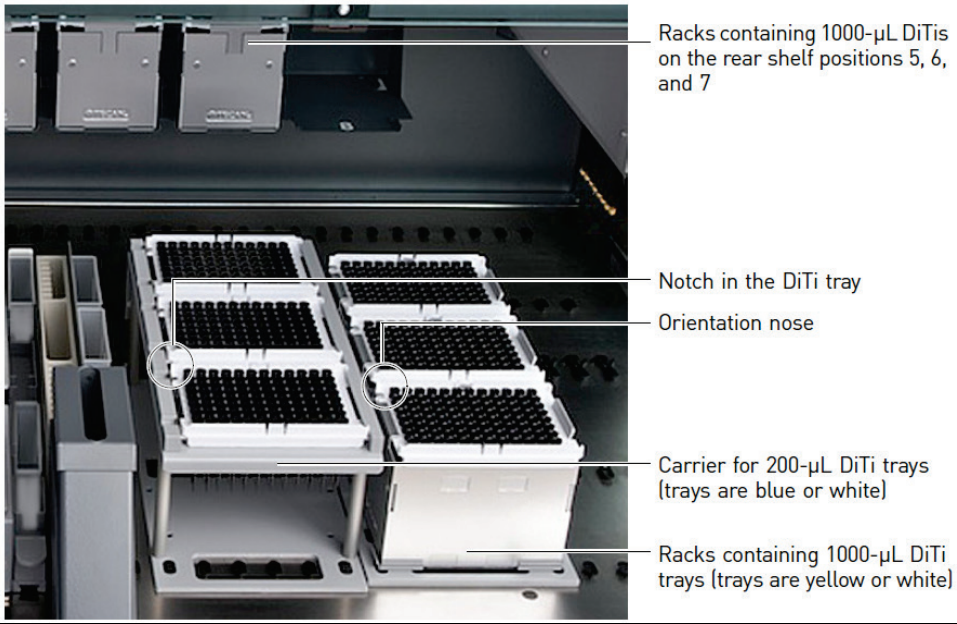
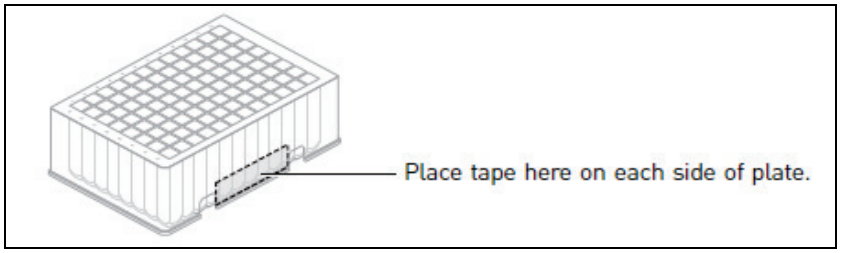


Figure 2b. Diagram of alternative DiTi racks/boxes and trays for PrepFiler extraction.

Load processing plate

To set up the HIDEVO150 deck, follow the steps below.

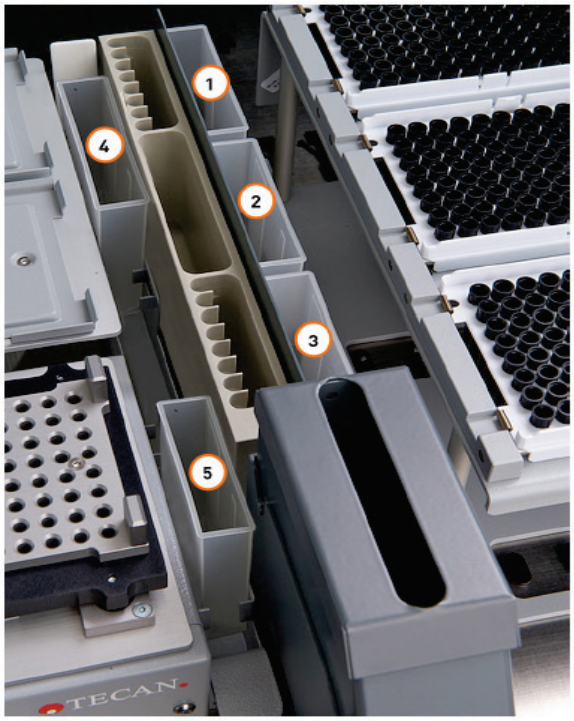
Step	Action
1	Obtain a 96 square-well processing plate and place two layers of tape on each side of the plate between the two notches in the plate (see Figure 3 below). • Use "spongy" double-sided tape for the first layer on each side, • then use painter's or masking tape for the second layer on each side.  Figure 3 ***Note:*** The tape layers provide a gripping surface for the RoMa fingers.
2	• Place the plate onto the TeShake carrier with position A1 oriented in the standard "far-left" position. • Manually remove and replace the plate to ensure that the plate is able to smoothly slide on and off of the TeShake support posts. If not, discard and replace with a newly taped plate.

3	Check position 2 of the TeShake carrier to confirm that the AB magnet ring plate is present.
4	*Optional:* The operator may check that the RoMa is properly configured to transfer the Processing Plate (to and from the TeShake and the Magnet Ring) and/or the DiTi boxes (to and from the DiTi shelves and the carrier at grid position 35). To test RoMa vectors prior to the extraction run, perform the following:

Step	Action
1	From the *Runtime Controller* window, select **Edit an existing script**. [Do not select **Run an existing script**.] ***Note:*** If the *Runtime Controller* window is not open, select File, then Open, and choose the script; skip to Step 4.
2	**Start your selection** and choose the script: • *Combo_Test_Only_RoMa_ProcessingPlate* to test only the Processing Plate transfers • *Combo_Test_All_RoMa_Vectors_Only* to test both the Processing Plate and DiTi box transfers
3	Click the green arrow to continue.
4	Choose **Run** from the drop-down menu, NOT **Run direct**.
5	Verify the **Run full script?** box is checked and click **Run** again.
6	After performing the script(s), confirm that the deck is configured properly before continuing with the procedure.

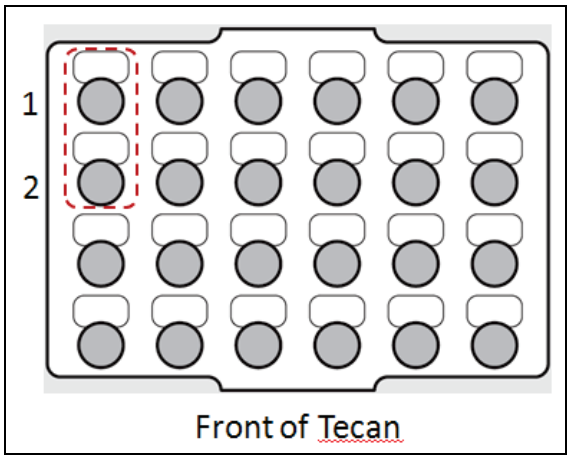
Load PrepFiler reagents

Perform the following steps to load the PrepFiler reagents on the Tecan deck.

Step	Action
1	Obtain the inventoried PrepFiler extraction reagents (see Preparation for Extraction in Section 1.5.5.2).
2	Obtain five clean 100 mL troughs and label as follows: • "I" for Isopropanol • "WBA" for prepared Wash Buffer A • "WBB" for prepared Wash Buffer B • "EB" for Elution Buffer, or "TE^{-4}" for TE^{-4} • "L" for lysate waste
3	Using the calibration marks on the reagent troughs, pour the approximate calculated volumes into each appropriate trough. ***Notes:*** • Use buffer solutions poured freshly from their storage containers. • The waste trough ("L") is empty. • Do not re-use buffer solutions remaining in troughs from previous runs. • Properly dispose of remaining solutions.
4	Place troughs on the Tecan deck according to Figure 4.  **Trough Layout** 1. Elution Buffer trough (grid 27, position 1) 2. Prepared Wash Buffer B trough (grid 27, position 2) 3. Prepared Wash Buffer A trough (grid 27, position 3) 4. Isopropanol trough (grid 25, position 1) 5. Lysate waste trough (grid 25, position 3) Figure 4. Trough Layout for HIDEVO PrepFiler Extraction Module

Load Particles

Perform the following steps to load the PrepFiler Magnetic Particles.

Step	Action
1	Obtain two tubes of PrepFiler Magnetic Particles and gently invert the tubes to mix the contents.
2	*Very briefly* centrifuge the particle tubes to remove liquid from the caps.
3	Carefully open the tubes and inspect for the presence of any bubbles above the meniscus level of the liquid or for the presence of a thin film stretching across the opening of the tube. • Use the corner of a clean Kimwipe or the end of a clean wooden applicator stick to pop any such bubbles, which could otherwise interfere with Tecan's liquid-level detection system. • If there is foam on the surface of the Magnetic Particles, remove the foam with a pipet.
4	Place the un-capped tubes in block positions 1 and 2 of the Magnetic Particles carrier that is located at deck position 2 of grid 13 (labeled as #3 in Figures 1a/1b). Make sure that the Magnetic Particles carrier is oriented exactly as shown in Figure 5 (and not rotated 180˚).  Figure 5. PrepFiler Magnetic Particle Carrier showing Block Positions 1 and 2.

Load the Lysate Racks and Sample Tubes/Plate

Load samples Perform the following steps to load the lysate and sample collection tubes.

Step	Action
1	Re-inspect the lysate tubes in the "L" racks to ensure that tubes are properly positioned and that the liquid is at the bottom of each tube. If necessary, centrifuge briefly.
2	Uncap each sample tube, one-by-one, and replace in the tube rack.
3	Load the lysate "L" tube racks onto the Tecan workspace starting at grid 7 (cross-reference Figures 1a/1b).
4	**Tube-to-Tube format:** Load the "S" tube racks containing the uncapped, empty 1.5 mL sample tubes onto the Tecan workspace starting at grid 1 (cross-reference Figure 1a). ***Note***: Make sure that the racks are properly loaded. In particular, the "S" and "L" tube racks should be identically arranged, with the "S" racks starting at grid 1 and the "L" racks starting at grid 7. **Tube-to-Plate format:** Place an empty, labeled 96-well plate in position 1 of grid 13 (see #2 in Figure 1b). The DNA extracts will be eluted into this plate. Be sure to *center* the plate on the plate rack.

Section 1.5.5.3 During Run Processing

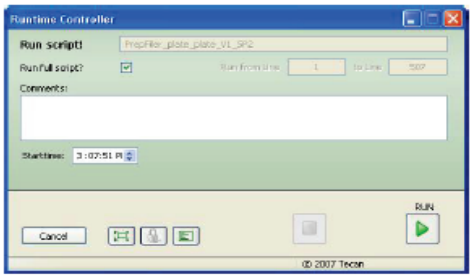
Start the Extraction Script

Cautionary note

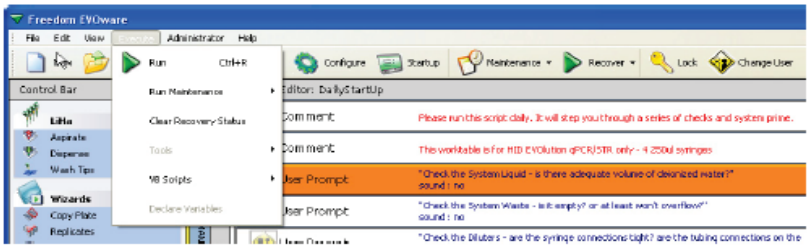
IMPORTANT! Do not click in the Runtime Controller or Freedom EVOware script dialog, or the run stops and cannot be restarted.

If you intentionally or accidentally stop the run, take the following steps to clear the script recovery status before you start a new run:

- Click **Cancel** in the Runtime Controller.



- In the Freedom EVOware script dialog, note the highlighted orange line; this is the step where the run stopped.
- In the Freedom EVOware script dialog, select **Execute ▸ Clear Recovery Status** from the menu. You can now start a new run.



Opening the script

Follow the procedure below to open the appropriate HIDEVO150 PrepFiler DNA extraction script.

Note: The script runs for approximately 1.5-4 hours, depending on the number of samples to be processed.

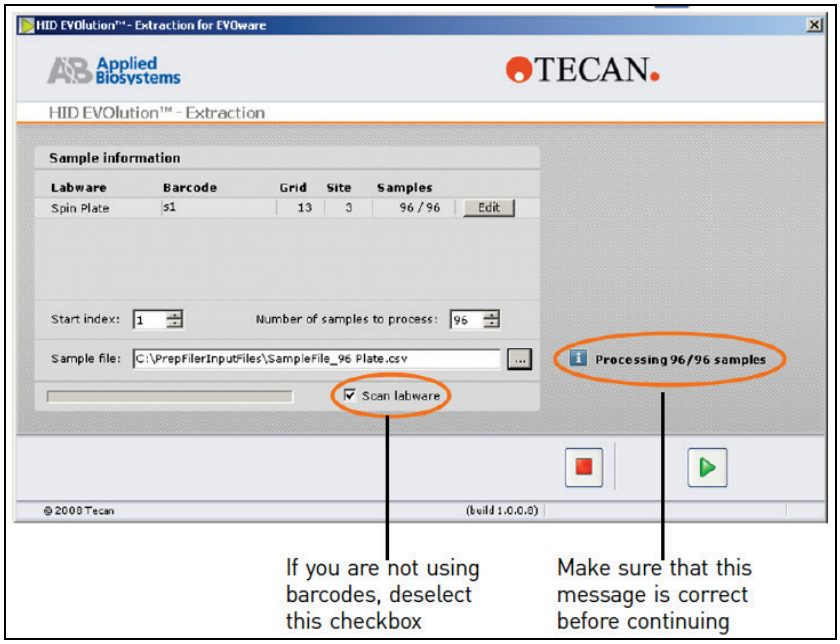
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Perform the following steps to start the appropriate script:
<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>From the Runtime Controller window, select Edit an existing script. [Do not select Run an existing script.]</td></tr>
<tr><td>2</td><td>Start your selection and choose the script (see table below).</td></tr>
<tr><td>3</td><td>Click the green arrow to continue.</td></tr>
</table>
If the Runtime Controller window is not open, select File, then Open and choose the script.
<table>
<tr><th>Scripts</th><th>EVOware v. 2.1</th><th>EVOware v. 2.4</th></tr>
<tr><td>Tube-to-Tube format</td><td>modPrepFiler_tubes_tubes Combo_V1_SP1</td><td>mod_PrepFiler_tubes_tubes Combo_V2</td></tr>
<tr><td>Tube-to-Plate format</td><td>modPrepFiler_tubes_plate Combo_V1_SP1</td><td>mod_PrepFiler_tubes_plate Combo_V2</td></tr>
</table></td></tr>
<tr><td>2</td><td>Choose Run from the drop-down menu, NOT Run direct.</td></tr>
<tr><td>3</td><td>Verify the Run full script? box is checked and click Run again.</td></tr>
</table>

Start the Run

Start the run

Perform the following steps to start the extraction run:

Step	Action
1	The *HID Evolution EVOware* window should now be open behind the *Runtime Controller*. Move the *Runtime Controller* aside to proceed with the prompts in the *HID Evolution EVOware* window.
2	In the Sample Information screen (see below), click the […] box to select the

	sample input file for the run.
3	• Upon importing the input file, the wizard will automatically determine the number of samples to process. Inspect this value to ensure that it is correct for your run. • Deselect the **Scan labware** checkbox and click OK. • Click the green arrow to continue.  **Important** Because we do not currently use bar-coded samples or plates, the "Scan labware" checkbox in the Sample Information screen of the "HID EVOlution™ – Extraction" wizard has to be deselected (unchecked). If this box remains selected (*i.e.,* if a "checkmark" is showing), then any sample information that you have manually entered or imported may be overwritten.
4	Bypass the request for lot number entry by clicking the green arrow to continue. Record lot numbers and all other pertinent information on the checksheet for the run.
5	At the Load Worktable window, verify **Liquid Level Detection** is checked, and • click the loaded box for each piece of labware or rack on the deck, *or* • click loaded all if all pieces are known to be loaded. • Click the green arrow to continue. ***Note:*** WBA was also known as Wash Buffer and WBB was also known as Wash 3.
6	Respond appropriately to the DiTi user prompt to execute the run.
7	The script will proceed first by checking the reagent trough volumes and

then by transferring 15 µL of magnetic particles to each sample well. After the magnetic particles are transferred, the HIDEVO script will pause to provide a User Prompt window, which will allow the operator to visually inspect the lysate tubes for the presence of magnetic particles prior to proceeding with the automated extraction procedure.

At the pause window:

Step	Action
1	Remove the lysate rack(s) from the Tecan deck.
2	Visually inspect each tube for the brown tint indicating the presence of transferred particles,
3	Document any tubes that do not contain particles.
4	To such tubes, • manually add 15 µL of PrepFiler magnetic particles, • then "tip mix" the well contents.
5	Return rack(s) to correct original position(s).
6	*Optional:* Remove and tightly cap the PrepFiler Magnetic Bead tubes to avoid solvent evaporation.

8	Continue running the automated extraction script.

Section 1.5.5.4 Post-Run Processing

Post-run Sample Handling

Post-run sample handling

Process samples following completion of the script as described below.

Step	Action
1	At the end of the run, visually inspect the extract "S" tubes or the extract 96-well plate, the lysate "L" tubes, and the processing plate, recording any noteworthy observations in the bench notes.
2	Also note any unusual results in the User/Maintenance log for the HIDEVO system.
3	Cap the extract tubes or seal the extract plate and move to appropriate storage, or proceed to further processing steps (*e.g.,* qPCR). ***Note:*** The extracts are eluted in a final volume of ~45 µL TE^{-4}.

Post-run Tecan Clean-Up and Maintenance

Clean-up/ maintenance

Perform the following clean-up and maintenance steps after the run.

Step	Action
1	Run the *Combo_Flush.esc* maintenance script: • Choose the script • Click **Run** • Respond with **10** in the user interface to wash with 10 mL of dH2O
2	If it is the last run of the day: • exit EVOware, • respond **Yes** to moving all arms to their home positions, and • turn off the HIDEVO150 by depressing the **ON** button.
3	Remove the Lysate Waste trough ("L") from the Tecan deck, and pour the lysate waste into an appropriately labeled waste container. **Important** This waste should NOT be disposed of down the sink or treated with bleach or acid.
4	Remove and discard the other consumables and plasticware on the Tecan deck.
5	If needed: • re-fill the System Liquid carboy with de-ionized water (*e.g.,* Millipore), • empty the Waste Liquid carboy, • empty/replace the DiTi waste bag, and • wipe down the deck. ***Note:*** When cleaning the HIDEVO150, **never pour or squirt liquids (not even water) on the deck**. Liquids could leak below the deck and short out electronic circuit boards. Instead, wipe the deck with towels dampened with appropriate liquids (*e.g.,* weak detergent, then water, then alcohol, as needed). Tecan carriers and reagent blocks should be treated similarly, in order to avoid exposing them unduly to corrosive agents. **Important** Never clean the instrument while it is powered on.
6	As needed, remove any carriers from the deck and clean with weak detergent, water and/or alcohol. Allow to dry.

Post-Run Electronic File Handling

The output files generated by the HIDEVO150 run are generated automatically on the Tecan control PC, in the folders or respective subfolders of either:

- *C:\HIDEvolutionExtractionFiles\Export\HID_...run name* or
- *C:\ HIDEvolutionExtractionFiles\DNAExtraction_...run name*

Typically, these files are not utilized or retained.

Section 1.5.6 References

Reference documents

PrepFiler™ Automated Forensic DNA Extraction Kit: Automated Extraction on the HID EVOlution™ – Extraction System – User Guide, Applied Biosystems, Part Number 4393917 Rev C 12/2010.

PrepFiler and PrepFiler BTA Forensic DNA Extraction Kits – User Guide, Applied Biosystems, Part Number 4463348 Rev. C 1/2012.

Brevnov, M., Pawar, H., Mundt, J., Calandro, L., Furtado, M., Shewale, J. (2009) "Developmental Validation of the PrepFiler Forensic DNA Extraction Kit for Extraction of Genomic DNA from Biological Samples" J Forensic Sci 54(3)

Strangegaard, M., Hjort, B., Hansen, T., Hoflund, A., Mogensen, H., Hansen, A., Morling, N. (2013) "Automated extraction of DNA from biological stains on fabric from crime cases. A comparison of a manual and three automated methods" FSIG 7(3).

Section 1.5.7 Appendix I – Preparation of Wash Buffers

WBA

Perform the following to prepare Wash Buffer A (WBA):

Step	Action
1	Combine 26 mL of PrepFiler Wash Buffer A Concentrate with 74 mL of 95% ethanol (molecular biology grade). ***Note:*** The volumes may be proportionately adjusted as needed.
2	Store at room temperature for *up to six months* in a tightly capped container(s) to avoid ethanol evaporation.

WBB

Perform the following to prepare Wash Buffer B (WBB):

Step	Action
1	Combine 200 mL of PrepFiler Wash Buffer B Concentrate with 300 mL of 95% ethanol (molecular biology grade). ***Note:*** The volumes may be proportionately adjusted as needed.
2	Store at room temperature for *up to six months* in a tightly capped container(s) to avoid ethanol evaporation.

Section 1.5.8 Appendix II – Preparation of 95% Ethanol

95% Ethanol

Ninety-five percent ethanol (molecular biology grade) may be purchased for use in the WBA and WBB. However, it may also be prepared in-house using 200 proof molecular biology grade ethanol, as described below.

Step	Action
1	Combine the volume of 200 proof (100%) ethanol with the corresponding volume of DI H_2O shown below: **Total volume (mL)** / **Ethanol (mL)** / **DI H_2O (mL)** 500 / 482.5 / 17.5 ***Notes:*** • The volumes may be proportionally adjusted as needed. • Due to the partial molar change of water, the total volume will shrink to less than 500 mL, but the mass and molar percentages will be the same, *i.e.* 95% Ethanol.
2	Store at room temperature for up to six months in a tightly capped container(s) to avoid evaporation.

Section 2.1 Quadruplex qPCR Procedure

Section 2.1.1 INTRODUCTION

An estimation of the DNA content of each sample should be conducted before proceeding with STR (short tandem repeat) amplification and analysis. This can be accomplished by quantitative PCR (qPCR), which specifically amplifies and detects primate DNA to assess the quantity of amplifiable DNA in a sample. Successful STR amplification and analysis can benefit not only from an accurate quantification of extracted DNA, but also from an estimation of the quality of that DNA. DNA extracts from forensic samples can be of reduced quality because of degradation due to prolonged environmental exposure or because of the presence of co-extracted PCR inhibitors, both of which can lead to lowered success rates for STR genotyping. The Quadruplex qPCR assay described here assesses DNA fragmentation by simultaneously quantifying two primate-specific nuclear DNA target sequences of different lengths. PCR inhibition is detected by including amplification of an internal PCR control (IPC) sequence. The quantity of male DNA is detected by quantifying a Y-chromosome-specific target sequence. Knowledge of DNA quantity and quality at the outset enables appropriate downstream decisions for improved analytical results.

The Polymerase Chain Reaction (PCR) can be utilized for real-time qPCR quantitation. Real-time quantitative PCR is defined as the detection of products generated at each cycle of PCR so that the accumulation of PCR products for an unknown sample can be compared to the corresponding accumulation for a series of standard samples containing known quantities of template DNA. Through this comparison, real-time qPCR is used to estimate the initial quantity of specific template DNA in the unknown sample. For the protocol described here, this analysis is performed using the TaqMan® Universal PCR Master Mix (No AmpErase® UNG) and AmpliTaq® Gold DNA Polymerase manufactured by Life Technologies/Applied Biosystems and a nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix. The nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix contains a Cy5-labeled nuTH01 nuclear probe, a VIC-labeled nuSRY nuclear probe, a FAM-labeled nuCSF nuclear probe, a NED-labeled IPC probe, the synthetic oligonucleotide target sequence of the IPC probe, BSA, and forward and reverse primers for each target sequence of interest. The Primer/Probe Mix is prepared and tested for quality control along with the Universal Master Mix by the Richmond QC group.

After preparation, the nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix is stored at approximately –20°C. Upon receipt, the ABI TaqMan® Universal PCR Master Mix (No AmpErase® UNG) is stored at approximately –20°C. When the ABI Master Mix is subsequently separated into ~1 mL aliquots for distribution, it is stored at approximately +4°C. Once thawed, both the Universal Master Mix and Primer/Probe Mix are stored at approximately +4°C until depleted.

Appendices

Appendix I (Section 2.1.7) - *Using HID Real-Time PCR Analysis Software v1.2 (HIDv1.2) to Collect and Analyze Quadruplex qPCR Data on the 7500 Real-Time Instrument*
Appendix II (Section 2.1.8) - *Troubleshooting*
Appendix III (Section 2.1.9) - *Guidelines for Truncated qPCR Runs*
Appendix IV (Section 2.1.10) - *An Approach to Preparing a Dilution Series for Unknown Samples Using Option B (2 µL samples)*

Notes

- *Collect Quadruplex data directly onto the local harddrive of the 7500 control computer, rather than over a network or USB drive. If the network goes down or is even just briefly interrupted, then it is likely all data will be lost.*
- *Clicking around too much with the mouse during a run may result in the data collection software freezing, in which case data loss is likely.*

Section 2.1.2 EQUIPMENT AND REAGENTS

2.1.2.1 Equipment

- 7500 Real Time PCR System instrument, Life Technologies/Applied Biosystems
- MicroAmp Optical 96-well half skirt reaction plate (10 plates/box), Life Technologies/Applied Biosystems P/N N801-0560 (or equivalent) - DNA/DNase/RNase free, PCR compatible
- Optical Adhesive Covers (100 covers/box), Life Technologies/Applied Biosystems P/N 4311971 (or equivalent)
- Adhesive Seal Applicator Kit (5 spatulas/kit), Life Technologies/Applied Biosystems P/N 4333183
- Microfuge tubes - DNA/DNase/RNase free
- Pipettors
- Pipet tips - DNA/DNase/RNase free
- Mini centrifuge
- Vortex
- Centrifuge with plate rotar or alternative plate spinner

2.1.2.2 Reagents

- TE^{-4}
- Primer probe mix
- Human Genomic DNA: Male (100 µg), Promega P/N G1471
- Water, Molecular Biology Grade (10 x 50 mL), VWR P/N 62111-410 or equivalent
- 7500 Real Time PCR System Spectral Calibration Kit, Life Technologies/Applied Biosystems P/N 4349180
- TaqMan® Universal PCR Master Mix, No AmpErase® UNG (5 mL), Life Technologies/Applied Biosystems P/N 4324018 (May also be purchased as 10 x 5 mL, Life Technologies/Applied Biosystems P/N 4324020 or 50 mL, Life Technologies/Applied Biosystems P/N 4326614.)
- AmpliTaq® Gold DNA Polymerase (5 U/µL) 5 x 1,000 units with Buffer II and $MgCl_2$ solution, Life Technologies/Applied Biosystems P/N N8080249 (May be purchased with either Buffer I or Buffer II, with or without $MgCl_2$ and in any quantity.)
- Custom Oligonucleotides from Eurofins MWG Operon (Huntsville, Alabama):

Oligo Name	Sequence
nuTH01-F	5' - AGG GTA TCT GGG CTC TGG - 3'
nuTH01-R	5' - GGC TGA AAA GCT CCC GAT TAT - 3'
nuSRY-F	5' - TGG CGA TTA AGT CAA ATT CGC - 3'

nuSRY-R 5' - CCC CCT AGT ACC CTG ACA ATG TAT T - 3'
IPC-F 5' - AAG CGT GAT ATT GCT CTT TCG TAT AG - 3'
IPC-R 5' - ACA TAG CGA CAG ATT ACA ACA TTA GTA TTG - 3'
Note: *These are typically custom synthesized on the 1 µmole scale and purified as salt-free.*

- Primers from Life Technologies/Applied Biosystems (South San Francisco, CA):
  Oligo Name Sequence
  nuCSF-F 5' - GGG CAG TGT TCC AAC CTG AG - 3'
  nuCSF-R 5' - GAA AAC TGA GAC ACA GGG TGG TTA - 3'
  Note: *These primers are ordered as "Sequence Detection Primers." They are typically custom synthesized on the 80,000 pmol scale, lyophilized and desalted.*
- IPC assay target from Eurofins MWG Operon (Huntsville, Alabama):
  Oligo Name Sequence
  IPC-oligo 5' - AAG CGT GAT ATT GCT CTT TCG TAT AGT TAC CAT GGC AAT GCT TAG AAC AAT ACT AAT GTT GTA ATC TGT CGC TAT GT - 3'
  Note: *This is a 77 bp synthetic single-stranded oligonucleotide custom synthesized on the 50 nmol (or 1 µmol) scale and purified as salt-free.*
- Cy5-labeled nuTH01 TaqMan probe from Eurofins MWG Operon (Huntsville, Alabama):
  Oligo Name Sequence
  nuTH01-probe 5' – *Cy5*- ATT CCC ATT GGC CTG TTC CTC CCT T –*BHQ 3* - 3'
  Note: *This TaqMan probe is typically custom synthesized on the 1 µmole scale and purified by HPLC.*
- FAM-labeled nuCSF TaqManMGB probe, VIC labeled nuSRY TaqManMGB probe and the NED-labeled IPC TaqManMGB probe from Life Technologies/Applied Biosystems (South San Francisco, CA):
  Oligo Name Sequence
  nuSRY-probe 5'- *VIC*-CCC TGC TGA TCT GCC T-*MGB-NFQ* - 3'
  nuCSF-probe 5' - *FAM*- CAA CCT GCT AGT CCT T-*MGB-NFQ* - 3'
  IPC-probe 5' - *NED*- TAC CAT GGC AAT GCT-*MGB-NFQ* - 3'
  Note: *These probes are typically custom synthesized on the 20 nmole scale and HPLC purified.*

Section 2.1.3 BACKGROUND INFORMATION ON THE Primers and Probes for the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay

The primers used for the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay are listed below. The primers are named for the genome amplified (nu = nuclear), the specific gene amplified (TH01, SRY, CSF) and the direction of the primer (F = forward, R = reverse).

nuTH01-F	5' - AGG GTA TCT GGG CTC TGG - 3'
nuTH01-R	5' - GGC TGA AAA GCT CCC GAT TAT - 3'
nuSRY-F	5'- TGG CGA TTA AGT CAA ATT CGC – 3’
nuSRY-R	5'- CCC CCT AGT ACC CTG ACA ATG TAT T – 3’
nuCSF-F	5' - GGG CAG TGT TCC AAC CTG AG - 3'
nuCSF-R	5' - GAA AAC TGA GAC ACA GGG TGG TTA - 3'
IPC-F	5' - AAG CGT GAT ATT GCT CTT TCG TAT AG - 3'
IPC-R	5' - ACA TAG CGA CAG ATT ACA ACA TTA GTA TTG - 3'

The probes used for the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay are listed below. Again, the probes are named for the genome amplified, the specific gene amplified, and the word “probe.” FAM, Cy5, VIC and NED indicate the fluorescent dye attached to the 5' end of the oligonucleotide probe. The quenchers are indicated by BHQ (Black Hole Quencher) or NFQ (Non-Fluorescent Quencher) and are located at the 3' end of each probe. MGB indicates that the probe contains a Minor Groove Binder moiety to increase the bonding interaction between the probe and the target DNA.

nuTH01-probe	5' - *Cy5*-ATT CCC ATT GGC CTG TTC CTC CCT T-*BHQ 3* - 3'
nuSRY-probe	5' - *VIC*-CCC TGC TGA TCT GCC T-*MGB-NFQ* - 3'
nuCSF-probe	5' - *FAM*-CAA CCT GCT AGT CCT T-*MGB-NFQ* - 3'
IPC-probe	5' - *NED*-TAC CAT GGC AAT GCT-*MGB-NFQ* - 3'

The TH01, SRY and CSF target sequences are co-amplified using the nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix. The nuTH01 amplicon length varies between 170-192 bp, depending upon the number of tetra-nucleotide repeats (n= 5-11) contained in the template DNA. The nuSRY amplicon is 137 bp in length. The nuCSF amplicon is 67 bp in length. The IPC assay targets a 77 bp synthetic single-stranded oligonucleotide (IPC-oligo, sequence shown below) that is spiked into the Primer/Probe Mix. This target sequence is also co-amplified using the nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix.

IPC-oligo 5' - AAG CGT GAT ATT GCT CTT TCG TAT AGT TAC CAT
GGC AAT GCT TAG AAC AAT ACT AAT GTT GTA ATC
TGT CGC TAT GT - 3'

Section 2.1.4 QUANTITATION USING THE nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay

2.1.4.1 Analytical Controls and Standards for Quantitation

Controls and standards are required to assess the quantity of the extracted DNA as well as the effectiveness, accuracy and precision of the analytical procedures in a particular case. Evaluation of the controls is essential to the proper interpretation of test results.

In addition to any extraction reagent blanks, extraction controls (e.g., QC samples), and substrate controls, the following standard and control samples are included in each nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR run:

2.1.4.1.1 Quantitation Standards

Dilutions of Promega "Human Genomic DNA: Male" are used as standards for relative quantitations. Typically, these standards range from 32 ng down to 0.044 ng. The concentration of the standard DNA stock is 32 ng/uL. This stock is prepared by the QC group. All standards are prepared from this stock of DNA.

2.1.4.1.2 Negative control

The negative amplification control contains the reagents used to prepare the qPCR amplification Master Mix in addition to TE^{-4}, which replaces the DNA sample. The purpose of this sample is to detect DNA contamination that might occur from the PCR reagents, the PCR set-up environment or between the samples being prepared.

2.1.4.2 Preparation of the Plate Record for the Run and Warming Up the ABI 7500 Instrument

Note: *Go to Section 2.1.7, Appendix I for using HID Real-time PCR Analysis Software v.1.2 (HIDv1.2) to collect and analyze Quadruplex Assay data on the 7500 instrument.*

1. Turn on the 7500 Real Time PCR System instrument and allow it to warm up for ~15 minutes before beginning the qPCR run.

2. While the instrument is warming up and prior to physically preparing the sample plate for the run, it may be convenient to use the ABI 7500 software to set up the plate record for the run and to printout a hard copy of this record to aid in preparation of the plate, as described here in steps 2 through 6. Alternatively, steps 2-6 may be performed after preparation of the sample plate and immediately prior to the qPCR run.

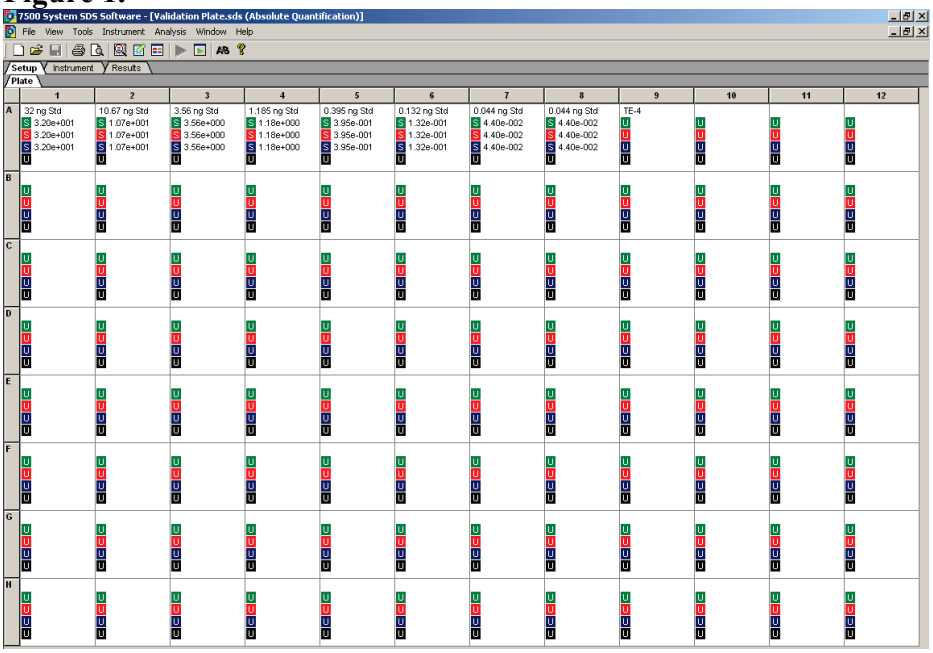
Log onto the computer attached to the instrument and open the Sequence Detection Software version 1.3 7500 System SDS Software by clicking on the *7500 System Software* shortcut found on the desktop. When the software is open, select *Open…* from the *File* menu.

3. In the browser window that appears, select *All SDS Files*, or select *SDS Templates (*.sdt)* to limit the list to template files. Browse to find the SDS template *Quadruplex assay*.

4. Open the SDS template file; a 96-well format will appear on the screen. Because any changes can be saved over the template accidentally, save the file with a new name *immediately* after opening the template file. Choose *Save As…* from the *File* menu, assign the file name as *Quadruplex assay <MMDDYY> <INITS-#>*, where MMDDYY is the date of the run, INITS are the initials of the instrument operator, and -# represents the run number for that date (e.g., -1 for the first run, -2 for the second, etc.), and save as an SDS document (*.sds).

The start-up screen shows that all wells in the plate have been assigned four detectors (Quad nuSRY is the top green symbol, Quad nuTH01 is the red symbol second from the top, Quad nuCSF the blue symbol second from the button, IPC the lower black symbol) so that qPCR data will be obtained for the three nuclear reactions as well as for the IPC reaction. The top row of the plate contains the appropriate sample names and quantities for the standard curve and the TE^{-4} negative control. Figure 1 shows this initial screen.

Figure 1.



5. Record the run in the log for the 7500 Real Time PCR System instrument.

6. Determine the well positions for the samples to be quantified, noting that wells A1-A9 are used for the quantitation standards and the negative amplification control. (Any wells that are not used for these standard and control samples may be used for unknown samples.)

Use the 7500 software to populate all sample wells with a sample indicator [sample name, six-digit number (e.g., for case LLYY-######, use YY-####), dilution, etc.] by entering the indicator in the

Sample Name field in the *Well Inspector*. The Well Inspector can be opened in several ways: by double clicking on the well of interest; by choosing *Well Inspector* from the *View* menu; or by clicking on the *Well Inspector* button on the toolbar. To keep the file size small, empty wells may be omitted by highlighting all empty wells and choosing *Omit Well* in the *Well Inspector*. Alternatively, empty wells may be omitted after the run is completed during data analysis.

Note: *Make sure that you do not omit any wells that contain sample. Data are* ***not*** *collected for omitted wells.*

Ensure that the Passive Reference field in the lower right corner of the Well Inspector has ROX chosen for all samples. To aid in sample loading, the plate map may be printed using the *Print* option under the *File* menu.

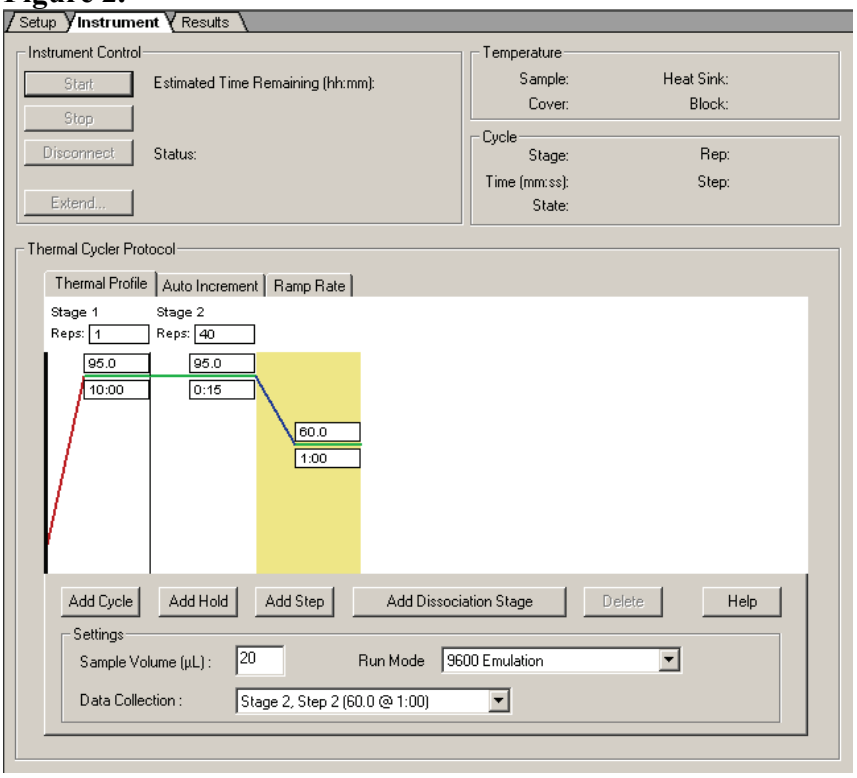
Note: *If the sample names are too long, they may be 'cut-off' in the printed plate map. This can be avoided by reducing the size of the printed text, as follows. Choose File > Page Setup > Properties > Advanced (under Paper/Quality) > Scaling, then entering an appropriate reduction percent (75 is usually adequate) using the keyboard, followed by OK.*

7. Select the *Instrument* tab at the top of the window. Figure 2 shows the Instrument window. The Sample Volume field should indicate *20 µL* reactions and *9600 Emulation sh*ould be chosen from the drop-down menu in the *Run Mode* field. *Stage 2, Step 2 (60.0 @ 1:00)* should be indicated in the drop-down menu in the *Data Collection* field. The Thermal Profile should indicate the thermal cycler parameters listed below.

Pre-heat: (1 cycle)	95°C, 10 minutes
40 cycles:	95°C, 15 seconds
	60°C, 1 minute

8. The *Start* button at the top of the screen will not be active. This is because when any changes are made to the plate record, the software will not allow the instrument to be run until the plate record has been saved. Choose *Save* from the *File* menu or click the *Save Document* button on the toolbar. This action will activate the *Start* button. Do not start the run at this point; instead proceed to the next section, which describes the preparation of standard DNA samples, unknown/test samples, and Master Mix.

Figure 2.



2.1.4.3 Preparation of DNA Quantitation Standards, Test Samples and Master Mix

There are two options for preparing a run using the Quadruplex qPCR assay, Option A and Option B. Option A allows the analyst to use up to 4 µL of sample per assay; Option B allows for up to 2 µL of sample. These options are provided for convenience in setting up the run, and do not result in any differences in the final composition of the qPCR reaction mix. Instructions for each option are described below. Notice that there is a corresponding check-sheet for each option. Be sure to use the appropriate check-sheet for the option you are using.

Note: *Do not mix the procedures for Options A and B. For example, do not use standard DNA dilutions prepared using Option A if you are using Master Mix prepared using Option B.*

2.1.4.3.1 Option A: Using up to 4 µL of Template DNA per Assay

1. Mix the contents of the Human Genomic DNA: Male standard DNA tube thoroughly and spin briefly in a microfuge to remove any liquid from the cap. Record the QC lot #'s for the standard DNA and the TE^{-4}.

2. Dilute the DNA quantitation standards as described below. Mix well between each dilution. Dilutions should be used on the same day on which they are prepared.

8 ng/µL dilution:	3 µL of 32 ng/µL Human Genomic DNA: Male + 9 µL TE^{-4}
2.67 ng/µL dilution:	4 µL of 8 ng/µL dilution + 8 µL TE^{-4}

0.889 ng/µL dilution:	4 µL of 2.67 ng/µL dilution + 8 µL TE^{-4}
0.296 ng/µL dilution:	4 µL of 0.889 ng/µL dilution + 8 µL TE^{-4}
0.099 ng/µL dilution:	4 µL of 0.296 ng/µL dilution + 8 µL TE^{-4}
0.033 ng/µL dilution:	4 µL of 0.099 ng/µL dilution + 8 µL TE^{-4}
0.011 ng/µL dilution:	4 µL of 0.033 ng/µL dilution + 8 µL TE^{-4}

3. As appropriate, dilute each test/unknown sample to the desired extent in TE^{-4} buffer.

For any sample that is suspected to contain significant amounts of co-extracted PCR inhibitors (e.g., an extract from a soil sample), it may be advantageous to run a dilution series (1:1, 1:5, 1:10, ...) of that sample in order to obtain a more accurate qPCR quantitation (see Appendix IV and discussion in section 2.1.5.5).

Note: *The nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay generates more accurate results for samples whose quantitation values fall within the standard curve. This should be considered when diluting unknown samples.*

4. Determine the number of control, standard and test samples to be analyzed.

Note: *The nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay standard curve is constructed with nine wells of the following nuclear template quantities (all in ng): 32, 10.67, 3.56, 1.19, 0.395, 0.132, 0.044 (duplicate samples) and TE^{-4}.*

5. Determine the desired location of each sample in a 96-well format, noting that wells A1-A9 are used for standard and control samples (see Figure 1). If you have previously printed a plate map for your run, you may wish to refer to that printout as you prepare the plate.

6. Mix the nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix thoroughly and spin briefly in a microfuge to remove any liquid from the cap. Mix the TaqMan® Universal PCR Master Mix by gently shaking/swirling the reagent container, then spin down.

Note: *Both of these mixes contain photo-sensitive dyes. Insofar as possible, minimize the exposure of these solutions to light.*

Obtain a tube of QC'ed AmpliTaq® Gold DNA Polymerase (5U/µL) from the freezer. Record the lot or QC #'s for both mixes and the polymerase.

7. The qPCR Master Mix should be prepared fresh for each run. To account for volume lost during pipetting, the following calculations include an extra 12% of each component of the mix. Prepare the Master Mix by adding the following volumes of reagents to a microfuge tube:

# reactions x 11.2 µL	TaqMan® Universal PCR Master Mix (No AmpErase® UNG)
# reactions x 6.16 µL	nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix
# reactions x 0.56 µL	5 U/µL AmpliTaq® Gold DNA Polymerase

8. Mix thoroughly (e.g., a brief pulse on a vortex unit) and spin the tube briefly in a microfuge to remove any liquid from the cap.

9. Dispense 16 µL of the Master Mix into the correct wells in an ABI MicroAmp® Optical 96-well Reaction Plate, an E&K Semi-Skirt 96 PCR Plate, or equivalent.

10. Opening one tube at a time, add 4 µL of test sample, quantitation standard or TE^{-4} buffer to each appropriate well. After the addition of template to the appropriate wells, cover the prepared plate with an Optical Adhesive Cover. The final reaction volume in each well is 20 µL.

Note: *With Option A, if less than 4 µL of an unknown sample is used, the volume must be "made up" with TE^{-4} buffer so that the total volume in the well is 20 µL. For example, if you want to use only 1 µL of an unknown sample, this can be accomplished by adding 3 µL of TE^{-4} buffer and 1 µL of the unknown sample to the well.*

Note: *Sterile deionized water may be used to reconstitute evaporated samples.*

11. With a spatula, press the optical cover onto the surface of the plate being careful not to introduce wrinkles in the cover. With the edge of the spatula, press the optical cover firmly horizontally and vertically at all intersections across the plate as well as around the outer edge of the plate to secure the adhesive seal. Bring the plate into the PCR product room.

Skip past the next section ("Option B") to the section titled "Quantitation with the 7500 Real Time PCR System."

2.1.4.3.2 Option B: Using up to 2 µL of Template DNA per Assay

1. Mix the contents of the Human Genomic DNA: Male standard DNA tube thoroughly and spin briefly in a microfuge to remove any liquid from the cap. Record the QC lot #'s for the standard DNA and the TE^{-4}.

2. Dilute the DNA quantitation standards as described below. Mix well between each dilution. Dilutions should be used on the same day on which they are prepared.

16 ng/µL dilution:	3 µL of 32 ng/µL Human Genomic DNA: Male + 3 µL TE^{-4}
5.33 ng/µL dilution:	2 µL of 16 ng/µL dilution + 4 µL TE^{-4}
1.78 ng/µL dilution:	2 µL of 5.33 ng/µL dilution + 4 µL TE^{-4}
0.59 ng/µL dilution:	2 µL of 1.78 ng/µL dilution + 4 µL TE^{-4}
0.20 ng/µL dilution:	2 µL of 0.59 ng/µL dilution + 4 µL TE^{-4}
0.066 ng/µL dilution:	2 µL of 0.20 ng/µL dilution + 4 µL TE^{-4}
0.022 ng/µL dilution:	2 µL of 0.066 ng/µL dilution + 4 µL TE^{-4}

3. As appropriate, dilute each test/unknown sample to the desired extent in TE^{-4} buffer.

For any sample that is suspected to contain significant amounts of co-extracted PCR inhibitors (e.g., an extract from a soil sample), it may be advantageous to run a dilution series (1:1, 1:5, 1:10, ...) of that sample in order to obtain a more accurate qPCR quantitation (see Appendix IV and discussion in section 2.1.5.5).

Note: *The nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay generates more accurate results for samples whose quantitation values fall within the standard curve. This should be considered when diluting unknown samples.*

4. Determine the number of control, standard and test samples to be analyzed.

Note: *The nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay standard curve is constructed with nine wells of the following nuclear template quantities (all in ng): 32, 10.67, 3.56, 1.19, 0.395, 0.132, 0.044 (duplicate samples) and* TE^{-4}.

5. Determine the desired location of each sample in a 96-well format, noting that wells A1-A9 are used for standard and control samples (see Figure 1). If appropriate, refer to a previously printed plate map for your qPCR run.

6. Mix the nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix thoroughly and spin briefly in a microfuge to remove any liquid from the cap. Mix the TaqMan® Universal PCR Master Mix by gently shaking/swirling the reagent container, then spin down.

Note: *Both of these mixes contain photo-sensitive dyes. Insofar as possible, minimize the exposure of these solutions to light.*

Obtain a tube of QC'ed AmpliTaq® Gold DNA Polymerase (5U/µL) from the freezer. Record the lot or QC #'s for both mixes and the polymerase.

7. The qPCR Master Mix should be prepared fresh for each run. To account for volume lost during pipetting, the following calculations include an extra 12% of each component of the mix. Prepare the Master Mix by adding the following volumes of reagents to a microfuge tube:

# reactions x 11.2 µL	TaqMan® Universal PCR Master Mix (No AmpErase® UNG)
# reactions x 6.16 µL	nuTH01-nuSRY-nuCSF-IPC Quadruplex Primer/Probe Mix
# reactions x 0.56 µL	5 U/µL AmpliTaq® Gold DNA Polymerase
# reactions x 2.24 µL	TE^{-4}

8. Mix thoroughly (e.g., a brief pulse on a vortex unit) and spin the tube briefly in a microfuge to remove any liquid from the cap.

9. Dispense 18 µL of the Master Mix into the correct reaction wells in an ABI MicroAmp® Optical 96-well Reaction Plate, an E&K Semi-Skirt 96 PCR Plate, or equivalent.

10. Opening one tube at a time, add 2 µL of test sample, quantitation standard or TE^{-4} buffer to each appropriate plate well. After the addition of template to all wells, cover the prepared plate with an Optical Adhesive Cover. The final reaction volume in each well is 20 µL.

Note: *With Option B, if less than 2 µL of an unknown sample is used, the volume must be "made up" with TE^{-4} buffer so that the total volume in the well is 20 µL. For example, if you want to use 1 µL of an unknown sample, this can be accomplished by adding 1 µL of TE^{-4} buffer and 1 µL of the unknown sample to the well.*

Note: *Sterile deionized water may be used to reconstitute evaporated samples.*

11. With a spatula, press the optical cover onto the surface of the plate being careful not to introduce wrinkles in the cover. With the edge of the spatula, press the optical cover firmly horizontally and vertically at all intersections across the plate as well as around the outer edge of the plate to secure the adhesive seal. Bring the plate into the PCR product room.

2.1.4.4 Quantitation with the 7500 Real Time PCR System

The following operations assume that the ABI 7500 Real Time PCR system is powered on and warmed up, that a plate record has been previously created and saved for the qPCR run, and that a qPCR reaction plate has been prepared following the protocol for either Option A or B. If these steps have not been performed, follow the instructions provided earlier in this protocol.

Note: *Go to Section 2.1.7, Appendix I for using HID Real-time PCR Analysis Software v.1.2 (HIDv1.2) to collect and analyze Quadruplex Assay data on the 7500 instrument.*

1. Inspect the plate to make sure that liquid is at the bottom of each well. Be careful to avoid air bubbles at the bottom of each well, which can interfere with fluorescence detection. If necessary, spin down the plate to avoid such bubbles. Bubbles at the tops of the wells are not a problem.

2. Open the 7500 instrument tray. To do this, first apply pressure to the depressed mark on the right corner of the tray and then release. The tray will move out automatically. If the tray stops before the entire 96-hole surface is accessible, gently assist the forward movement of the tray.

3. Place the plate in the exposed instrument plate holder *making sure that well A1 is in the upper left corner*.

4. Close the instrument tray. To do this, apply pressure to the depressed mark on the right corner of the tray. Push the tray back until the tray latches into place.

5. Use the 7500 SDS system software to confirm that the run information is accurate (*i.e.,* that the plate record accurately reflects the sample placement and that the run parameters are appropriate). If necessary, make any changes to the sample information or run parameters, then re-save the plate

record. To start the run, select the *Instrument* tab (the *Start* button should be active), then click *Start*. Status information regarding the run will be displayed, including the estimated time remaining, temperature status, and cycle status. The run will finish in ~2 hours.

Note: *If the Start button under the Instrument tab is inactive, first make sure that you have re-saved the plate record as described in step 5. If the Start button is still inactive, then the connection between the 7500 and the computer may need to be re-initialized. To do this, close the file and re-open it.*

6. While the instrument is running, qPCR amplification curves and related data can be monitored in real-time. To do this, select the *Results* tab at the top of the window, then select the *Amplification Plot* tab. Amp curves will be displayed for highlighted wells.

7. The instrument automatically saves data to the output .sds file as the run progresses. When the run is finished, the estimated time remaining will be blank, as indicated under the *Instrument* tab. At run completion, the data need not be re-saved to the file. The file will be ready for analysis.

Note: *At the discretion of the analyst, it is possible to end a run earlier than 40 cycles. The guidelines for such a "truncated" run can be found in Appendix III. The potential disadvantage for truncating a run includes omitting the lower points in the standard DNA dilution curve, thus raising the lower limit of detected DNA in the control and unknown samples. This disadvantage, however, may be offset by the need to end a qPCR run early. In addition, if a run ends prematurely due to a power failure, the data collected up to the point of the power failure can be analyzed according to the guidelines in Appendix III.*

8. Exit the 7500 System SDS software. Move the qPCR data file to the designated network drive.

Note: *The file on the network drive will be purged after 30 days (based on the date of creation of the file).*

9. Turn off the 7500 Real Time PCR System instrument, remove the sample plate from the instrument block and discard it in the PCR product trash bin.

Note: *The lamp on the 7500 Real Time PCR System instrument is rated to have a lifetime of ~2000 hours. The instrument should be turned off soon after run completion; avoid leaving the instrument on overnight unnecessarily.*

2.1.4.5 7500 System SDS Software (version 1.3) Analysis

The data can be analyzed on the computer connected to the instrument, or the file can be transferred to a different computer for analysis as long as the 7500 System SDS software (v1.3) has been loaded onto that computer. For routine qPCR runs, the Absolute Quantification assay is used to analyze data. (Other assays, e.g., the Background and Pure Spectra assays, are used for periodic instrument calibration and are not performed for each run.)

Note: *Go to Section 2.1.7, Appendix I for using HID Real-time PCR Analysis Software v.1.2 (HIDv1.2) to collect and analyze Quadruplex Assay data on the 7500 instrument.*

The Absolute Quantification assay uses data collected for a standard DNA dilution series to develop a standard curve for each run, from which quantifications of the unknown samples can be interpolated (or extrapolated). The presence of a passive reference dye (ROX) in each sample is used to normalize the fluorescence intensity across the 96-well plate. The amplification curves for each sample are brought to equivalent initial baseline values of zero by an automatic baselining routine. The cycle threshold (Ct) for each curve is determined automatically by using an analyst-defined fluorescence threshold.

The specific steps for using the Absolute Quantification assay to analyze data from a nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR run are as follows:

1. Transfer the .sds output file to the SDS Documents folder of the computer that will be used for data analysis. This transfer can be accomplished by using a networked drive or a portable memory device.

To open the .sds file for analysis:

- Open the 7500 System SDS software.
- If a “Product Registration” dialog box opens, click the *Cancel* button.
- From the *File* menu choose *Open…*
- Browse to find the correct .sds file and click *Open*.

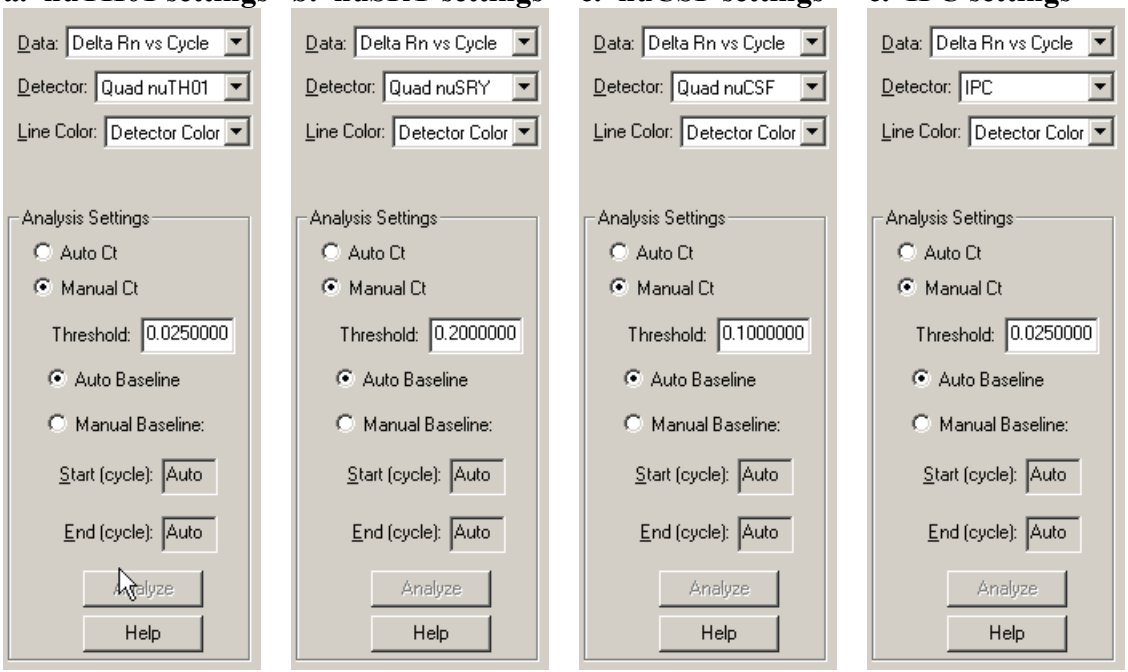
2. The *Plate* tab within the *Setup* tab will be shown when the document opens. Before proceeding with data analysis, examine the ROX signal intensities for several samples as part of a per-run quality evaluation of the instrument. Under the *Results* tab, open the *Components* tab. A window displaying the fluorescence data for each detector (FAM, VIC, NED, Cy5, and ROX) is displayed. The ROX signal should be visible, typically as a magenta line (make sure the ROX “box” is checked “on”). Visually examine the intensity level of the ROX signal for wells A3, A6 and A9 by clicking sequentially on the appropriate boxes in the plate map below the fluorescence plots. Make sure that the ROX fluorescence is ≥14,000 units for each of the three wells. If the ROX signal is <14,000 fluorescence units for any of the three wells, indicate the value(s) and the well position(s) in the 7500 logbook. Data from the qPCR run may still be analyzed and interpreted to give DNA quantitations, but consistently low ROX signals could indicate that the tungsten-halogen excitation lamp needs to be replaced.

3. Analysis occurs automatically upon opening the .sds file. Review the quantification results by selecting the *Plate* tab within the *Results* tab. Make sure that the appropriate default analysis settings were used for the automatic data analysis:

- Select the *Amplification Plot* tab within the *Results* tab.
- In the window at the right of the plot, check that *Delta Rn vs Cycle* is chosen from the *Data* drop-down menu.
- Either *Well Color* or *Detector Color* may be selected in the *Line Color* drop-down menu. This will be a preference left up to the analyst. ***Note:*** *Line color indicates in which color the amplification curve lines will be displayed on the screen. Selecting Well Color will cause all amp curves for a particular well to be displayed in the same color regardless of detector, while selecting Detector Color will cause all amp curves for a particular detector to be displayed in the same color regardless of well/sample.*
- Select the *Quad nuTH01* detector from the *Detector* drop-down menu and check the *Analysis Settings*. See Figure 3a (below). Ensure the following selections:
- Manual Ct with a *0.025* Threshold setting.
- Auto Baseline feature.
- If necessary, modify the settings to match these default values.
- Select the Quad nuSRY detector from the Detector drop-down menu and check the Analysis Settings. See Figure 3b (below). Ensure the following selections:
- Manual Ct with a *0.20* Threshold setting.
- Auto Baseline feature.
- Select the Quad nuCSF detector from the Detector drop-down menu and check the Analysis Settings. See Figure 3c (below). Ensure the following selections:
- Manual Ct with a *0.10* Threshold setting.
- Auto Baseline feature.
- If necessary, modify the settings to match these default values.
- Select the IPC detector from the Detector drop-down menu and check the Analysis Settings. See Figure 3d (below). Ensure the following selections:
- Manual Ct with a *0.025* Threshold setting.
- Auto Baseline feature.
- If necessary, modify the settings to match these default values.

Figure 3

a. nuTH01 settings **b. nuSRY settings** **c. nuCSF settings** **c. IPC settings**



4. The data were automatically analyzed with the default settings present when the document was opened. If no settings were changed in step 3, then re-analysis is not needed. Proceed to step 5.

If any settings were changed in step 3, the data will need to be re-analyzed, as indicated by the color change in the threshold plot line (green to red). To re-analyze:

- Click *Analyze* in the window to the right of the plot, or
- From the *Analysis* menu choose *Analyze*, or
- Click the green arrow in the toolbar at the top of the screen.

5. Return to the *Results/Amplification Plot* tab. Select the *Standard Curve* tab at the top of the screen and evaluate the quality of both the nuTH01, nuSRY and nuCSF standard curves by choosing first one then the other in the *Detector* drop-down menu.

- For all detectors, the R^2 value should be ≥ 0.98.
- For all detectors, the slope of the standard curve should be between -2.9 and -4.0.

6. While evaluating the quality of the standard curve, also evaluate the quality of the amplification plots, analysis settings and negative control results according to the interpretation guidelines (next section). If necessary, make any changes to the analysis settings or standards as allowable by the interpretation guidelines and re-analyze the data as described in step 4 above.

Use the Well Inspector to omit appropriate wells (e.g., empty wells that were not omitted prior to data collection, or, for a batched plate, sample-containing wells that will be analyzed by someone else). Results for omitted wells will not be included in the printed report. Re-analyze if you omit any wells.

Note: *If wells are omitted after data collection, the data will need to be re-analyzed before it can be exported as the Data.csv file.*

7. Select *Save* from the *File* menu to save the data under the same name. (If the data was not re-analyzed, there is no need to re-save the file. In this case, the *Save* option in the *File* menu will not be active.)

8. Print the absolute quantification results.

9. Print the nuTH01 and nuCSF standard curves (printing the nuSRY curve is optional).
   - Choose *Quad nuTH01* from the *Detector* drop-down menu.
   - Select *Print…* from the *File* menu and click *OK*.
   - Choose *Quad nuCSF* from the *Detector* drop-down menu.
   - Select *Print…* from the *File* menu and click *OK*.
   - If desired, choose *Quad nuSRY* from the *Detector* drop-down menu.
   - Select *Print…* from the *File* menu and click *OK*.
   - A copy of each standard curve should be placed in all related case folders.

10. The quantitation results for the run can be summarized, viewed and printed by exporting the data from the 7500 System SDS Software so that it can be analyzed using the Excel spreadsheet template *qPCR Casework-Degradation Quadruplex.xlt*.

To use this spreadsheet, first export the qPCR results table from the 7500 System SDS Software by using the *File* menu (*Export > Results...*), naming the table *Data.csv* at the prompt and saving it into the folder C:\Documents and Settings\All Users\Desktop. This is the only file that needs to be in a specific location on the hard drive. ***Note:*** *If your Desktop is found on the D: drive, save the file to D:\Documents and Settings\All Users\Desktop.*

11. After exporting the Data.csv file, open *Quadruplex qPCR Casework-Degradation.xlt* by double-clicking on the desktop icon. At the prompt, enable the spreadsheet to run macros.

Note: *Do not save the spreadsheet prior to running the macros. Saving it will cause the macro "Data Entry" to halt.*

12. Click on the following three macro-buttons in the sequence given:
   - "Case Info Entry" button – to enter the case number(s), analyst initials and date.
   - "Data Entry: C Drive" or "Data Entry: D Drive" button, as appropriate for the desktop
   - drive on your analysis computer, to enter the relevant data from the "Data.csv"
   - results table into the spreadsheet.
   - "Print Page" button – to print the results table for inclusion in the case file.

Note: *Do not simply use the standard Excel "print" function, which will result in multiple blank pages printed out along with the table page.*

The print out, which should be included in the lab notes for any relevant case folders, will contain the following results for each sample:

- *Sample Name*
- *ΔIPC* - the IPC Ct value of the sample minus the average IPC Ct value for the standard DNA and control samples in wells A2-A9
- *ng nuTH01* - the quantitation value measured by the nuTH01 portion of the Quadruplex assay
- *ng nuCSF* - the quantitation value measured by the nuCSF portion of the Quadruplex assay
- *Ratio* - the quotient from the operation (ng nuCSF) ÷ (ng nuTH01)
- *Male/Human Ratio* – the quotient from the operation (ng nuSRY) ÷ (ng nuTH01)

Quantitation values are rounded to two significant figures. The quantities are reported as ng of nuclear DNA per well. To convert the concentration to units of ng/µL, you will need to divide the reported values by the volume of sample you added to each well. The lower limit of quantification (LQ) for each assay has been set at 0.0125 ng of nuclear DNA. The limit of quantification is defined here as the level above which signal from DNA is reproducibly distinguished above noise; below this limit, these are considered indistinguishable. Any result below this limit is listed as "<LQ" on the printout from the Quadruplex qPCR Nuclear Degradation Excel spreadsheet.

Section 2.1.5 QUANTITATION INTERPRETATION GUIDELINES

2.1.5.1 Introduction

The interpretation of results in casework is necessarily a matter of professional judgment and expertise. Not every situation can or should be covered by a pre-set rule, nor is it expected that competent analysts will always be in full agreement in a particular situation. However, it is important that the laboratory develops and adheres to minimum criteria for interpretation of analytical results. These criteria are based on validation studies, literature references, and casework experience, and are developed with maximum input from analysts. It is expected that these interpretation guidelines will continue to evolve as the collective experience of the laboratory grows.

The purpose of these guidelines is to establish a general framework and to outline minimum standards to ensure that:

- conclusions in casework reports are scientifically supported by the analytical data, including that obtained from appropriate standards and controls.
- interpretations are made as objectively and consistently as possible from analyst to analyst.

2.1.5.2 Evaluation of the Analysis Settings

2.1.5.2.1 Ct Threshold and Baseline Settings

The Analysis Settings for qPCR assays analyzed on the 7500 Real Time PCR System instrument are comprised of two separate settings: the Ct threshold and the baseline. Although the 7500 System SDS Software version 1.3 has the capability to automatically assign both Ct and baseline values for each amplification curve, the validation was completed using the automatic feature only for baseline analyses. For Ct analyses, analyst-determined ("manual") values were used. Specifically, the nuTH01 data were analyzed with a Ct threshold of 0.025, the nuSRY data were analyzed with a Ct threshold of 0.20, the nuCSF data with a Ct threshold of 0.10, and the IPC data with a Ct threshold of 0.025. These pre-determined analysis settings should be suitable for analyzing data for most qPCR runs. However, during analysis of each nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay plate, the efficacy of the settings should be reviewed using the following criteria:

1. Correct baseline settings should lead to a flat early portion of each amplification curve (when viewing the amplification curve in linear mode on the Y-axis). By enabling the automatic baseline feature, the 7500 software should automatically identify and apply appropriate settings to give flat baselines.

2. The Ct threshold should be set within the exponential (geometric) phase of the logarithmic scale amplification plot. In addition, the threshold should be set high enough to avoid noise in the background signal.

2.1.5.2.2 Examining Amplification Curves

Amplification curves should be examined to make sure that the auto-baselining feature of the 7500 software led to flat baselines (~zero slope) in the pre-exponential-growth portions for each sample.

1. Select the *Amplification Plot* tab (under the *Results* tab).
2. Select *Quad nuTH01* from the *Detector* drop-down menu.
3. To look at the amplification curve for a particular well, single click on the well of interest in the 96-well plate grid at the bottom of the screen. To overlay multiple amplification curves, hold down the *Ctrl* key or the *Shift* key while clicking on wells to highlight individual or multiple samples respectively. All wells in the plate may be selected by clicking the square in the upper-left corner of the grid above the "A" indicating row A of the plate.
4. It is easier to determine if the baseline is flat in the desired range if the plots are viewed with a linear Y-axis scale. To change the Y-axis scale, double-click on the Y-axis to open the *Graph Settings* window. Under *Post Run Settings* change the Y-axis scale from *Log* to *Linear* and click *OK*.
5. Select *Quad nuSRY* from the *Detector* drop-down menu and highlight all samples. As needed, use the instructions in steps 1-4 to determine if the baseline is flat in the appropriate portion of each amplification curve.
6. Select *Quad nuCSF* from the *Detector* drop-down menu and highlight all samples. As needed, use the instructions in steps 1-4 to determine if the baseline is flat in the appropriate portion of each amplification curve.

7. Select *IPC* from the *Detector* drop-down menu and highlight all samples. As needed, use the instructions in steps 1-4 to determine if the baseline is flat in the appropriate portion of each amplification curve.
8. If any amplification curves show sloping baselines that would be expected to significantly alter the Ct for that curve, the sample(s), well position(s), and detector(s) should be documented in the bench notes. A sloping baseline would likely be due to a fluorescence artifact (e.g., a noise spike or a shift in the background) in an early cycle of the qPCR run. Such an artifact would typically be apparent by viewing the "raw" fluorescence for the sample under the *Results/Component* tab.

2.1.5.2.3 Reviewing Pre-determined Ct Threshold Settings

1. Select the *Amplification Plot* tab.
2. Select *Quad nuTH01* from the *Detector* drop-down menu and highlight the standards to be used in the nuTH01 standard curve (wells A1 to A8; 32 – 0.044 ng).
3. With the Y-axis in logarithmic scale, the threshold should appear as a green horizontal line across the standards within the exponential (geometric) phase of the amplification curves and should avoid instances of noisy signal. Noise appears on the plot as either jagged lines or the disappearance of the lower region of the exponential phase of the curve.

Note: *Sometimes the software does not display the threshold line on the plot correctly, but instead displays it near the bottom of the plot. If this occurs, place the cursor in the box indicating the threshold value and press Enter on the keyboard. The software will then move the threshold line to the correct location on the plot.*

4. The logarithmic amplification curve should be a smooth line that increases linearly and then typically levels off at a plateau. Non-smooth areas do occur occasionally within the exponential phase of amplification curves, but the Ct threshold should avoid instances of these non-smoothed areas if possible.
5. Select *Quad nuSRY* from the *Detector* drop-down menu and highlight the standards to be used in the nuSRY standard curve (wells A1 to A8; 32 – 0.044 ng).
6. With the same criteria used above to evaluate the nuTH01 Ct Threshold, ensure that the nuCSF Ct Threshold is set appropriately.
7. Select *Quad nuCSF* from the *Detector* drop-down menu and highlight the standards to be used in the nuCSF standard curve (wells A1 to A8; 32 – 0.044 ng).
8. With the same criteria used above to evaluate the nuTH01 Ct Threshold, ensure that the nuCSF Ct Threshold is set appropriately.
9. Select IPC from the Detector drop-down menu and highlight wells A1-A8.
10. With the same criteria used above to evaluate the nuTH01 Ct Threshold, ensure that the IPC Ct Threshold is set properly.
11. If any changes are made to the *Analysis Settings*, re-analyze the data and document the change and its rationale.

2.1.5.3 Evaluation of the Standard Curve

2.1.5.3.1 nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay Standard Curve Construction

The nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay standard curve is constructed with the following nuclear template quantities (all in ng): 32, 10.67, 3.56, 1.19, 0.395, 0.132, and 0.044 (duplicate samples). The 0.044 ng sample is run in duplicate because the precision of the nuTH01, nuSRY and nuCSF assays is reduced as the amount of template decreases (0.044 ng corresponds to ~15 initial copies of target sequence for the nuTH01 and nuCSF assays and ~7 initial copies of target sequence for the nuSRY assay).

2.1.5.3.2 Evaluation of the nuTH01 Standard Curve

1. Select the *Standard Curve* tab.
2. Select *Quad nuTH01* from the *Detector* drop-down menu and examine the Slope and R^2 values. The R^2 value should be at least 0.98, and the slope should be between -2.9 and -4.0.
3. If the R^2 value is less than 0.98 or the slope condition is not met, one or both of the 0.044 ng data points may be removed from the standard curve to improve the standard curve. (To remove a sample from the standard curve, follow the steps described below in section 2.1.5.3.5.) The low number of nuclear copies at this level of template can lead to poor precision in the Ct values.
4. If any point(s) higher than 0.044 ng in the standard curve appears to be an outlier, that point may be removed from construction of the standard curve.

2.1.5.3.3 Evaluation of the nuSRY Standard Curve (Optional)

1. Select the *Standard Curve* tab.
2. Select *Quad nuSRY* from the *Detector* drop-down menu and read the Slope and R^2 values. The R^2 value should be at least 0.98 and the slope should be between -2.9 and -4.0.
3. If the R^2 value is less than 0.98 or the slope condition is not met, one or both of the 0.044 ng data points may be removed from the standard curve to improve the standard curve. (To remove a sample from the standard curve, follow the steps described below in section 2.1.5.3.5 "Removal of Samples from the Standard Curve.") The low number of nuclear copies at this level of template can lead to poor precision in the Ct values.
4. If any point(s) higher than 0.044 ng in the standard curve appears to be an outlier, that point may be removed from construction of the standard curve.

2.1.5.3.4 Evaluation of the nuCSF Standard Curve

1. Select the *Standard Curve* tab.
2. Select *Quad nuCSF* from the *Detector* drop-down menu and read the Slope and R^2 values. The R^2 value should be at least 0.98 and the slope should be between -2.9 and -4.0.
3. If the R^2 value is less than 0.98 or the slope condition is not met, one or both of the 0.044 ng data points may be removed from the standard curve to improve the standard curve. (To

remove a sample from the standard curve, follow the steps described below in section 2.1.5.3.5.) The low number of nuclear copies at this level of template can lead to poor precision in the Ct values.

4. If any point(s) higher than 0.044 ng in the standard curve appears to be an outlier, that point may be removed from construction of the standard curve.

2.1.5.3.5 Removal of Samples from the Standard Curve

1. To remove a sample from the standard curve, select the *Plate* tab and double-click the sample to be removed.
2. In the row corresponding to the appropriate detector in the *Well Inspector*, choose *Unknown* from the *Task* drop-down menu.
3. The symbol for that detector in the *Plate* window will immediately change from an *S* (S) to a *U* (U).
4. Close the *Well Inspector* and re-analyze the data. Document the change and its rationale.

Note: *Alternatively, an outlier data point may be removed from construction of the standard curve by either deselecting the detector from the Use column in the Well Inspector or simply omitting the well.*

2.1.5.3.6 Addition of Samples to the Standard Curve

1. To add a sample back into the standard curve, select the *Plate* tab and double-click the sample to be added.
2. In the row corresponding to the appropriate detector in the *Well Inspector*, choose *Standard* from the *Task* drop-down menu.
3. The symbol for that detector in the *Plate* window will immediately change from a *U* (U) to an *S* (S).
4. In the *Quantity* column enter the number of known nanograms in that sample in the row corresponding to the appropriate detector.
5. Close the *Well Inspector* and re-analyze the data. Document the change and its rationale.

2.1.5.4 Evaluation of the Negative Control

The protocol requires the inclusion of at least one negative control. This control consists of all reagents used in the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay except that it includes either 2 (Option B) or 4 (Option A) µL of TE^{-4} in place of the DNA template. Although only one negative control per run is required, multiple negative controls may be included if desired. A negative control sample should show negative amplifications ("<LQ" as reported by the *Quadruplex qPCR Casework-Degradation.xlt* analysis spreadsheet) for the nuclear assays, but should show a positive amplification for the IPC assay, indicating that qPCR amplification and detection are working properly.

Note: *"<LQ" corresponds to the lower limit of quantification (0.0125 ng) for each nuclear assay.*

If nuTH01, nuSRY and/or nuCSF amplifications in the negative control sample are detected *above* the lower limits of quantification, the sample shall be amplified and typed in order to be evaluated for potential contamination and how it may have occurred. Generally, if the level of nuclear DNA

detected by qPCR would not be expected to result in detectable signals in downstream analyses (e.g., in detected STR alleles), then the detected qPCR signal would not indicate contamination of significance.

Negative control results (*e.g.,* reagent blanks) that show background signal <LQ in one or more detectors do not necessarily require further characterization when there are multiple reagent blanks included for an extraction set. In this situation, the reagent blank demonstrating the greatest signal, if any, should be the reagent blank that is amplified for STRs and typed using the most sensitive volume of the extraction set.

Note: *If samples have been diluted in a different lot of TE^{-4} than the standard curve, analysts may include a negative control for the different TE^{-4} lot.*

An early cycle baseline artifact ("ECBA") may be periodically observed, typically resulting in a false positive quantitation value in one detector of a negative control. This type of qPCR result can be readily identified by a large quantitation value in one detector and no signal in the other two (for "unknowns"). The resulting inauthentic amplification curve can be verified by the shape of the curve and by early baseline elevation in the raw component data.

2.1.5.5 PCR Inhibition and Interpretation of the IPC Amplification Curves

The purpose of an IPC (internal PCR control) assay is to provide an internal indication of the success of each PCR amplification. This assay (i) indicates that the qPCR master mix components were successfully prepared and that the qPCR instrument is working properly and (ii) detects for the presence of co-extracted PCR inhibitors in the unknown samples. The IPC assay amplifies a synthetic oligonucleotide ("IPC-oligo") that is spiked into the Primer/Probe mix at a relatively fixed concentration (~90,000 oligo copies per assay). Since every sample in a run will contain roughly the same quantity of synthetic oligonucleotide target sequences, the IPC Ct values for all samples in the run should be nearly identical (assuming that no samples contain co-extracted PCR inhibitors). For the Quadruplex assay, these IPC Ct values generally fall between 25 and 27 cycles. There is some run-to-run variability in this Ct range, presumably due to differences in preparations of the qPCR master mix. As discussed in more detail below, the possibility for PCR inhibition is indicated in those samples that show delays in Ct (> ~0.75 to 1 cycles) of the IPC assay.

A positive IPC amplification is defined to be one in which the IPC Ct value falls within the range expected for samples that are known to be non-inhibited. Since each qPCR run includes a set of non-inhibited, control samples (the DNA standards and TE^{-4}), the IPC Ct values for these samples can be used to determine an "average control IPC Ct" value for the run. This average IPC Ct for the control samples, which is calculated automatically by the Excel worksheet template *qPCR Casework-Degradation Quadruplex.xlt* as part of routine data analysis, is compared to the IPC Ct for each unknown by taking the following difference:

$$\Delta IPC = (\text{IPC Ct of sample}) - (\text{average control IPC Ct})$$

Note: *The calculation of the "average control IPC Ct" value does not use results from the 32*

ng standard DNA sample (well A1) because co-amplification of the relatively large quantity of nuclear DNA in this sample reduces the efficiency of the IPC amplification, resulting in a delay in the IPC Ct that is unrelated to the presence of co-extracted PCR inhibitors.

Detection of PCR inhibition is important primarily for two reasons. First, PCR inhibition in the qPCR can lead to an underestimation of the quantity of nuclear DNA in the sample, a result that would likely cause the analyst to inadequately dilute the sample for downstream STR amplification. If this underestimation were significant, the STR amplification could be adversely affected due to the combined effects of using excess template DNA in the presence of excess PCR inhibitor. Second, if PCR inhibitors are known to be present, then an analyst can decide how to avoid possible downstream implications (*e.g.,* sub-optimal STR amplifications), for example, by diluting the sample or by re-purifying the extract to reduce the inhibitor content (*e.g.,* NucleoSpin filtration).

2.1.5.5.1 Guidelines for ΔIPC Interpretation

Some guidelines are listed below for using the ΔIPC value to assess the accuracy of qPCR quantification. Keep in mind that these guidelines are based on validation studies using hematin, which cannot be expected to reproduce the effects of the entire array of inhibitors encountered in genuine forensic samples.

1) Identify those samples that show a ΔIPC greater than 0.75 cycles; the printout from the Excel worksheet (*qPCR Casework-Degradation Quadruplex.xlt*) will flag such samples. These samples *possibly* contain PCR inhibitors and should be evaluated further. For samples that show a ΔIPC less than ~0.75 cycles, the nuTH01 assay should reliably quantify the DNA concentration in the original extract. (***Note**: For STR amplifications using the ABI kits, the nuTH01 quantification result should be used for dilution calculations, because the nuTH01 target length (~180bp) is intermediate in the range of STR allele lengths.*)

2) If ΔIPC is greater than 0.75 cycles AND the quantity of template is large (≥ ~20 ng by either the nuTH01 or nuCSF assays), then the delay in IPC Ct may NOT be due to PCR inhibition, but instead due to competition among the four qPCR reactions in the Quadruplex assay. This possibility can be confirmed by comparing the slope of the nuCSF amplification curve for the sample in question to the slope of the nuCSF curve for one of the DNA standards in the run. If the two slopes are visually similar, then the presence of significant PCR inhibition is unlikely. If the delay in IPC Ct is due to a large quantity of DNA, then the qPCR quantification should be suitable for use in downstream analysis (e.g., for calculating dilutions for STR amplifications).

3) If ΔIPC is greater than 0.75 cycles AND the quantity of template is not large (≤ ~20 ng), then the effects of PCR inhibition should be considered before proceeding to downstream applications. As indicated by the validation studies with hematin, a ΔIPC of ~0.75 cycles was not associated with a significant underestimation of DNA quantity, and in this case, it might be appropriate to use the qPCR results to go directly to downstream analysis. However, a ΔIPC of ~1 cycle likely means that the qPCR quantification is inaccurate (e.g., underestimating the quantity of DNA by ~30% or more) and due caution should be exercised in using the qPCR result to calculate dilutions for downstream analyses. Moreover, if ΔIPC is ~1 (or greater), the risk for carry-over of PCR inhibitors into downstream PCR amplifications is increased. For such samples, the best approach is to obtain qPCR

data on a dilution series (1:5, 1:10, 1:20 ...) of the extract, as described in Appendix IV. In this approach, *the least diluted sample that does not show effects of PCR inhibition can be expected to provide the most accurate qPCR quantification.* Once the DNA quantity in the extract is accurately determined, consideration of the extent of dilution needed for downstream analysis (e.g., the dilution ratio to achieve 0.1 ng/µL for an STR amplification) in combination with the ΔIPC value may provide enough information to assess the likelihood for inhibitor carry-over into the downstream application. If these considerations predict significant PCR inhibition, the analyst may choose to avoid downstream effects by taking appropriate measures, such as sample dilution or re-purifying the extract to remove inhibitors (*e.g.,* NucleoSpin filtration).

4) If ΔIPC indicates any qPCR inhibition, then the qPCR degradation ratio (see next section) should be interpreted carefully. The nuTH01, nuSRY and nuCSF assays do not respond identically to the presence of PCR inhibitors. Consequently, even with relatively low levels of inhibitors, the qPCR degradation ratio can be expected to deviate from the "actual" ratio of target sequence copies present in the sample. For instance, in a genuinely high-molecular-weight sample, it is possible that PCR inhibition could cause the nuCSF amplification to be more delayed than the nuTH01 amplification, leading to a qPCR degradation ratio less than unity, *i.e.,* to the unrealistic result that the sample contains more DNA fragments that are longer than ~180 bp than there are fragments longer than 67 bp.

5) It should be noted that the ΔIPC value should not be used by itself to predict the success or failure of the subsequent STR amplification, as it does not take into consideration the quantity of DNA and/or PCR inhibitor(s) in the STR reaction relative to the quantity in the qPCR reaction. As an example, consider two hypothetical samples, A and B. For sample A, qPCR reveals a "high" nuTH01 DNA concentration (8 ng/µL) and "mild" PCR inhibition (ΔIPC = 0.7), whereas for sample B, qPCR reveals a "low" DNA concentration (0.1 ng/µL) and the same "mild" level of inhibition (ΔIPC = 0.7). Even though qPCR indicates the same level of PCR inhibition for samples A and B, the effects of this inhibition on the subsequent STR amplifications are unlikely to be equivalent. That is, sample A will be *diluted* to achieve the desired quantity of template in the STR amplification (with a concomitant reduction in the concentration of PCR inhibitor), while sample B is unlikely to be diluted and much more likely to show inhibition effects in the STR amplification. Therefore, before predicting the effects of PCR inhibition in downstream STR amplifications, the relative quantity of DNA and PCR inhibitor(s), as estimated from qPCR, should be taken into consideration. We suggest here a simple and general approach to address this issue. For each sample quantified by qPCR, a "normalized inhibition factor" (NIF) is calculated to estimate the degree of inhibition per nanogram of sample:

$$\text{NIF} = (\Delta\text{IPC (cycles)}) \div (\text{quantity of DNA (ng)})$$

The larger the NIF, the more likely the sample is to exhibit PCR inhibition effects in downstream STR amplifications

For the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR assay, it is possible to calculate three independent NIF values for each sample: a nuTH01 autosomal NIF (ΔIPC/ng nuTH01), a nuCSF autosomal NIF (ΔIPC/ng nuCSF) and a nuSRY Y-chromosomal NIF (ΔIPC/ng nuSRY). As shown in Table I, the nuTH01 autosomal NIF is useful for assessing potential inhibition effects in downstream Identifiler STR amplifications. In particular, samples with nuTH01 autosomal NIF values greater than ~1 are seen to produce incomplete Identifiler profiles or null amplifications. These results effectively

establish a threshold of 1 for the nuTH01 autosomal NIF, which, if reached, is indicative of potential PCR inhibition in downstream Identifiler STR amplifications.

Table I. I-NIF: Quadruplex qPCR and Identifiler STR Results (run 061406)

Sample µM Hematin	ΔIPC	nuTH01(ng)	Normalized Inhibition Factor (I-NIF) (ΔIPC/ nuTH01(ng))	% Identifiler Peaks Detected
0.00	-0.18	2.30	-0.08	100
10.00	-0.10	2.65	-0.04	100
20.00	0.50	2.15	0.23	100
30.00	1.10	1.40	0.79	100
40.00	1.50	1.20	1.25	39
50.00	1.90	0.70	2.73	0
60.00	2.70	0.24	11.25	0
70.00	3.10	0.08	39.24	0
80.00	4.00	0.00	-	0

Table II shows that samples with nuSRY Y-chromosomal NIF values greater than 2 are likely to produce incomplete Yfiler profiles or null amplifications, effectively establishing a Y-chromosomal NIF threshold of ~2 for predicting downstream amplification effects. It is possible that the increase in the NIF threshold value from ~1 for the autosomal nuTH01 value to ~2 for the Y-chromosomal nuSRY value is a reflection of the different template amounts used in the two STR assays; twice as much DNA (and PCR inhibitor) is typically utilized in the 1 ng Identifiler reactions than in 0.5 ng Yfiler reactions, and thus the ratio of PCR inhibitor to DNA can be twice as high before it has an equivalent effect. When the I-NIF is greater than 1, or the Y-NIF is greater than 2, reduced template amplification and/or sample clean-up should be considered. Although we do not currently use nuCSF autosomal NIF values, these might be helpful for predicting downstream inhibition effects in MiniSTR amplifications. Finally, although our results indicate that NIF values are potentially useful for identifying samples that may show inhibition in downstream PCR amplifications, the NIF approach does not cover every situation. For example, some samples that contain low quantities of DNA *and* PCR inhibitors may yield insignificantly small ΔIPC values, especially when a small volume (1-4 µL) of sample is amplified with the nuTH01-nuSRY-nuCSF-IPC Quadruplex assay. For such samples, the larger quantities and volumes utilized in the STR amplification may contain sufficient PCR inhibitor(s) to prevent amplification.

Table II. Y-NIF: Quadruplex qPCR and Yfiler STR Results (run 061406BH)

Sample µM Hematin	ΔIPC IPC	nuSRY(ng)	Normalized Inhibition Factor (Y-NIF) (ΔIPC/ nuSRY(ng))	% Yfiler Peaks Detected
0.00	-0.18	1.88	-0.09	100
10.00	-0.10	1.68	-0.06	100
20.00	0.50	1.36	0.37	100
30.00	1.10	0.92	1.20	100
40.00	1.50	0.68	2.21	76
50.00	1.90	0.40	4.75	0
60.00	2.70	0.20	13.50	0
70.00	3.10	0.08	38.75	0
80.00	4.00	0.01	500.00	0

Note: *When a Y-NIF is calculated the presence of excessive quantities of female DNA should be considered, as the ΔIPC may be a result of excessive female DNA and not due to the presence of PCR inhibitor(s).*

2.1.5.6 Interpretation of the qPCR "Degradation Ratio"

2.1.5.6.1 Introduction

The Quadruplex qPCR assay amplifies two independent autosomal target sequences with different lengths:

target sequence	target length (~ limit of detectable DNA fragment size)
nuTH01	~180 bp (depending upon TH01 genotype of template)
nuCSF	67 bp

This difference in target sequence length has implications for quantifying DNA in degraded samples. In effect, the nuCSF portion of the Quadruplex assay quantifies those DNA fragments in the extract that are at least 67 bp in length, while the nuTH01 portion quantifies those fragments that are at least ~180 bp in length. For samples containing non-degraded DNA, the quantity of nuCSF fragments will be nearly identical to the quantity of nuTH01 fragments because nearly all of the fragments will be longer than 180 bp. However, if the DNA in a sample is highly degraded (e.g., average fragment length ~150 bp), then the sample will contain more nuCSF targets than nuTH01 targets. In this case, the nuCSF portion of the Quadruplex assay will amplify and quantify more "DNA" than the nuTH01 portion of the assay. (***Note***: *Ideally, the nuTH01 assay should never quantify more "DNA" than the nuCSF assay.*) This effect is analogous to what is commonly seen for STR profiles of degraded DNA, where peaks for the smaller amplicons (e.g., AMEL, D3S1358) are progressively more intense than peaks for the larger amplicons (e.g., CSF1PO, D7S820). This type of STR profile for degraded DNA is often called the "wedge" or "ski slope" effect, and STR profiles for increasingly degraded DNA will exhibit increasingly pronounced wedge effects. In the same way, the nuCSF and nuTH01 qPCR

quantifications will increasingly diverge as the DNA is increasingly fragmented, and this divergence in qPCR quantification can be used to estimate the extent of DNA degradation.

Using the Quadruplex qPCR assay, the extent of DNA degradation is represented by the "qPCR Degradation Ratio:"

qPCR Degradation Ratio = (nuCSF quantity) ÷ (nuTH01 quantity)

For high-molecular-weight DNA, this ratio will be approximately 1. As the DNA becomes increasingly fragmented, the ratio will increase to values of 2-3 for moderately degraded samples and to values >5-10 for highly degraded samples.

If the nuTH01 and nuCSF assays are giving different quantitations, which value should be used in making dilutions for STR amplifications? As discussed in more detail below, it is better to use the nuTH01 quantity, because the nuTH01 target length falls within the range of sizes of the STR alleles in the ABI amplification kits. In general, for degraded DNA samples, it is important to consider the sizes of the target sequences in the downstream application (e.g., 100-375 bp for Identifiler) and to use the qPCR assay that best approximates these sizes. For downstream typing systems that rely on the amplification of short amplicons (e.g., mini-STR systems), it might be appropriate to use the quantity supplied by the shorter nuCSF target.

2.1.5.6.2 Guidelines for Interpretation of the qPCR Degradation Ratio

Unless otherwise indicated, template calculations for Identifiler Plus amplifications should use results from the nuTH01 assay because the nuTH01 target is similar in length to the STR alleles and template calculations for Yfiler amplifications should use the nuSRY target as the nuSRY target is a better representation of male DNA present in mixed gender samples.

1) The qPCR Degradation Ratio is listed for each sample on the printout from the Excel worksheet (*qPCR Casework-Degradation Quadruplex.xlt*). For samples with Degradation Ratios less than 3, the nuTH01 quantity can be used for downstream STR analysis, and the effects of DNA degradation on STR genotyping are expected to be minimal. Samples with Degradation Ratios greater than 3 will be flagged by the Excel worksheet for further examination.

There are two circumstances in which the Degradation Ratio is likely to be inaccurate and should be interpreted with due caution. First, if the sample shows evidence for inhibition in the ΔIPC, then the Degradation Ratio is likely to be inaccurate due to the different responses of the nuCSF and nuTH01 assays to the PCR inhibition. In particular, relatively low levels of PCR inhibitor may lead to degradation ratios <1. Second, if either (or both) of the qPCR quantifications is low (e.g., < ~50-100 pg per assay), then imprecision in the quantification(s) can be expected to reduce the accuracy of the Degradation Ratio.

2) If the Degradation Ratio for a sample is between 3 and 5, the STR profile can be expected to exhibit a pronounced wedge effect, but relatively few artifacts (e.g., cross-dye pull-up) can be expected, most of which may be remedied by re-injecting the PCR product at shorter time(s).

3) As the Degradation Ratio increases above 5, more pronounced effects on the STR profile can be expected, including increased incidences of pull-up, off-scale peaks (at the smaller loci), incomplete non-template nucleotide addition ("-A" shouldering), called stutter peaks, and allelic drop-outs (at the larger loci). Potential remedies for some of these effects include re-injecting at shorter times or repeating amplification with less template.

If qPCR data for an extract indicate that it contains highly degraded DNA, but that the DNA yield is fairly high, it is worth considering performing more than one STR amplification at the outset, for example, a "1 ng" amplification based on the nuTH01 results and another (more dilute) "1 ng" amplification based on the nuCSF results (or some result in between the nuTH01 and nuCSF quantitation values). This approach, to use different template quantities in different amplifications, acknowledges the reality that there is no single quantification value that can represent the "true" DNA concentration in a degraded sample.

2.1.5.7 Interpretation of the qPCR nuSRY:nuTH01 ratio

The Quadruplex qPCR assay amplifies a total human nuclear target (nuTH01) and a human male sequence (nuSRY) with different lengths:

target sequence	target length (~ limit of detectable DNA fragment size)
nuTH01	~180 bp (depending upon TH01 genotype of template)
nuSRY	137 bp

These targets can be compared by the ratio of the quantity of male DNA detected by the nuSRY target to the ratio of total human DNA detected by the nuTH01 target.

Male DNA:Total Human DNA Ratio = (nuSRY quantity) ÷ (nuTH01 quantity)

For a single-source male sample this value is expected to be approximately 1. This expectation was tested in the validation of the Quadruplex assay by quantifying 225 single-source samples from known male donors in three self-identified populations (US Black, US Caucasian, and US Hispanic). Male DNA:Total human DNA ratios in the population study ranged from 0.3 to 2.13. Ratios less than 1 may be attributed to inhibition and/or primer or probe binding site mutations, and ratios greater than 1 generally correlated with degradation ratios greater than 1. Therefore, the level of degradation should be considered when interpreting the male DNA:total human DNA ratio. As ratios less than 1 have been observed in known single-source male samples, ratios less than 1 should not necessarily be assumed to be a mixed gender sample. However, ratios less than 0.3 are indicative of a male:female mixture and when the ratio is equal to or less than 0.1 Y-STR typing should be considered.

Section 2.1.6 REFERENCES

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Section 2.1.7 APPENDIX I - Using HID Real-Time PCR Analysis Software v1.2 (HIDv1.2) to Collect and Analyze Quadruplex Assay Data on the 7500 Real-Time Instrument

Overview

The Quadruplex Assay was originally developed and validated using 7500 System Detection Software (SDS) developed by Applied Biosystems. This manufacturer has developed new, updated software called HID Real-Time PCR Analysis Software. While the newer software has not fundamentally changed, the graphical user interface (GUI) has changed as well as the input and output file formats. As a result, the procedural steps are slightly different. Thus when using the HID Real-Time PCR Analysis Software for Quadruplex Assay runs, the step-wise procedure described in this appendix should be followed for data collection and analysis of results. Note that there are no changes to the chemistry or run conditions of the Quadruplex Assay itself nor the analysis parameters or interpretation of Quadruplex data.

Contents

This appendix includes the following topics.

Topic
Set-up
Adding samples
Manually adding samples
Importing samples
Omit and save
Verify and start
Data analysis
Data.csv

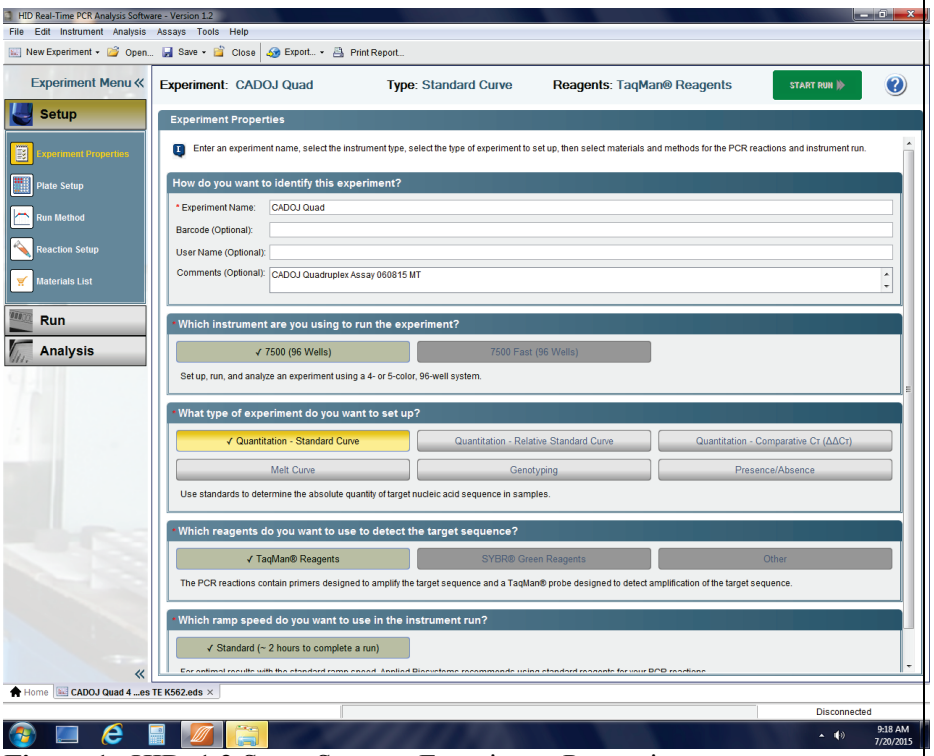
Note

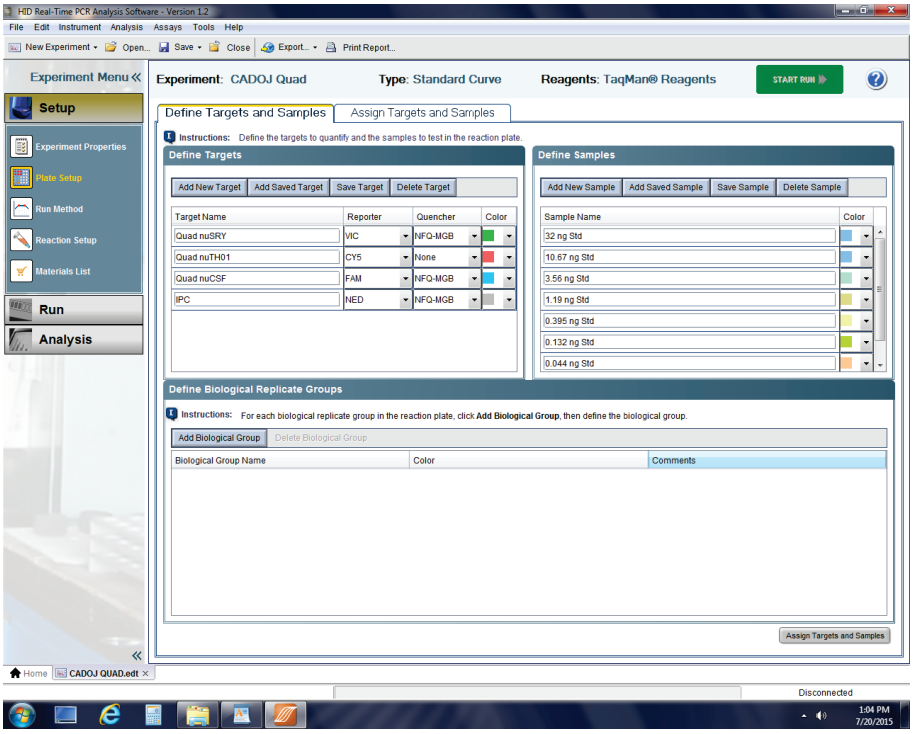
This procedure assumes that the default PCR volume setting in the HIDv1.2 software has previously been set at 20 µL.

Set-up

Perform the following steps to set up a run using HID Real-Time PCR Analysis Software version 1.2 (or higher, if performance-checked).

Step	Action
1	Power up the 7500 instrument and allow it to warm up (~15 min).
2	If needed, power up and login to the computer that controls the 7500.
3	Double-click the “HID Real-Time PCR Analysis Software v1.2” icon to run the software.

4	At the resulting login screen, select a user name from the dropdown menu. ***Notes:*** • Do not login using "GUEST." • No password is required. • **IMPORTANT**: If a *new* user name is entered at this step in the procedure, the default volume *must be changed* from 50 µL to 20 µL after login by using the top menu "Tools/Performance" dropdown, then by choosing the "Defaults" tab.
5	At the Home screen, click the "Custom Assays" icon.
6	Use "File/Open" in the top menu bar to open the Quadruplex template file (Quadruplex Assay.edt). This template file, which is typically located in the C:/Applied Biosystems/7500/experiments/ directory, contains all of the run and analysis settings that are appropriate for the Quadruplex assay (with the exception of the PCR volume – see Step 4 Note).
7	After opening the template file, select "Experiment Properties" under the "Experiment Menu/Setup" tab (left panel) to confirm the properties shown in Figure 1 (7500; Quantitation – Standard Curve; TaqMan® Reagents; Standard).  **Figure 1**. HIDv1.2 Setup Screen: Experiment Properties

8	Under "Experiment Menu/Setup/Plate Setup," select the "Define Targets and Samples" tab to confirm the four targets of the Quadruplex assay displayed in the "Define Targets" tab.  **Figure 2**. HIDv1.2 Setup Screen: Plate Setup – Define Targets and Samples

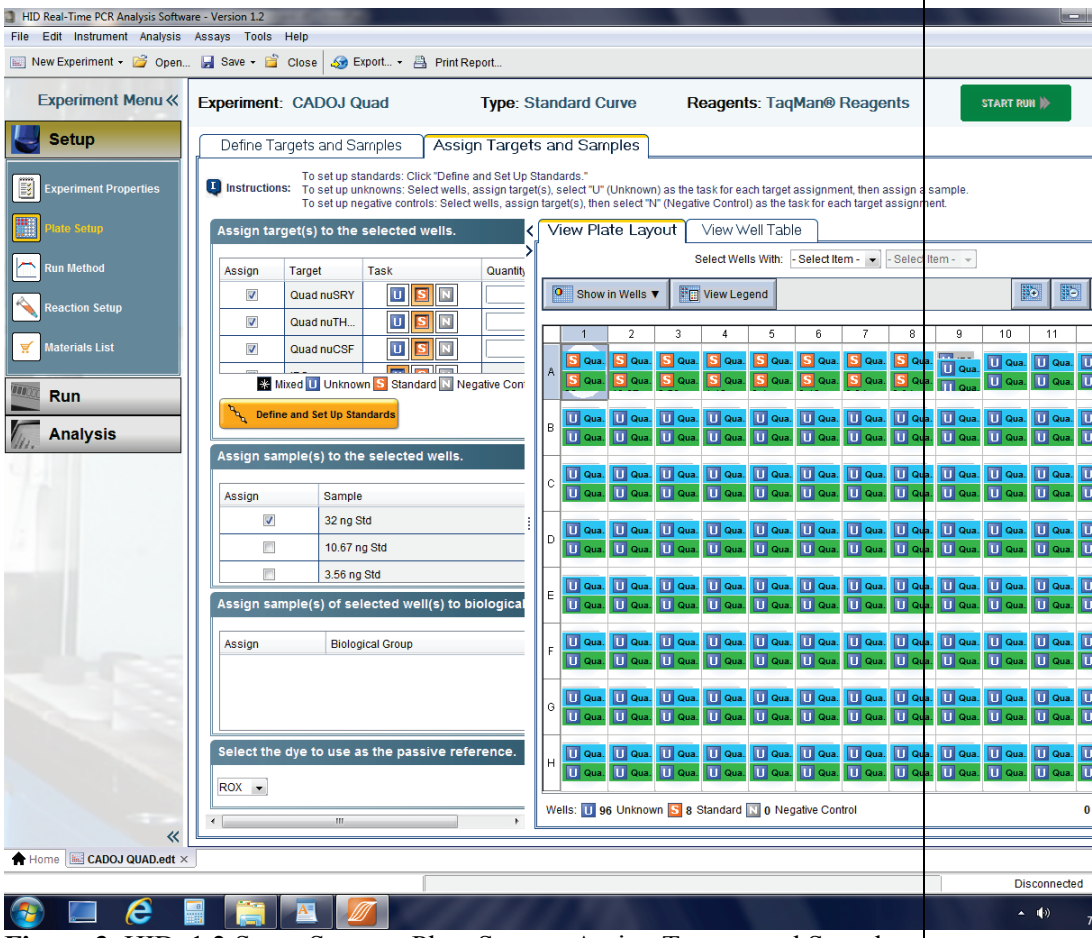
Adding samples

As indicated in the "Define Samples" tab (Figure 2), the template file only defines the DNA Standard dilutions and TE^{-4} as samples. As described in the next section, additional samples may be incorporated in the run plate using either a manual entry method or by importing a properly formatted text (.txt) file. See the appropriate procedure below.

Manually added samples

The manual addition of a sample to a run plate consists defining the sample by adding its name to a sample list and assigning that sample name to a well position in the run plate. To manually add samples, follow the steps below.

Step	Action
1	In the "Experiment Menu/Setup/Plate Setup/Define Targets and Samples" tab (Figure 2): • Click the "Add New Sample" button in the "Define Samples" panel • Enter the name of the new sample
2	Repeat Step 1 for each new sample to be added.

	Note: *If you want to run replicate samples, you only need to define a single name for the entire set of replicates.*
3	Go to the "Experiment Menu/Setup/Plate Setup/Define Targets and Samples" tab and click the "Assign Targets and Samples" tab (Figure 3).  **Figure 3.** HIDv1.2 Setup Screen: Plate Setup – Assign Targets and Samples
4	Specify the well position of the new sample: • Click the appropriate sample well in the "View Plate Layout" panel • "Check" the appropriate sample name in the "Assign sample(s) to the selected wells" panel to assign the "checked" sample to the highlighted well
5	Repeat Step 4 for each newly added sample.
6	Verify the assignments have been accurately made by either • holding the cursor over each appropriate well in the "View Plate Layout" panel, or • by examining the list of samples in the "View Well Table" panel
7	Continue with **Save run file**.

Importing samples

To import samples using a .txt-formatted file, follow the steps below.

Step	Action
1	Verify the Quadruplex assay template file is open; this template file specifies the parameters for the Quadruplex qPCR run.
2	Use "File/Import" in the top dropdown menu to browse to the .txt-formatted file to import for the run. For example, this file may have been generated for Tecan/HIDEvo sample processing using a version of the CADOJ_HIDEVO_ TubesInput Excel spreadsheet.
3	Import the .txt file, which will overwrite all previous *sample* information that is in the Quadruplex Assay.edt template file.
4	After importing the file, check the sample information for accuracy: • Select the "Experiment Menu/Setup/Plate Setup" tab • Select the "View Well Table" tab to view the sample information for each well position
5	To add or delete a sample, or make other changes, see **Manually added samples** above.
6	Continue with **Save run file**.

Save run file

Save the ".eds" qPCR run file as Quadruplex Assay <*MMDDYY*> <*INITS*-#>, where MMDDYY is the date of the run, INITS are the initials of the analyst/operator, and -# represents the run number for that date (*e.g.,* -1 for the first run, -2 for the second, *etc.*).

Verify and start Verify the parameters and start the run as described below.

Step	Action
1	Select the "Experiment Menu/Setup/Run Method" tab to ensure proper qPCR and collection settings (see Figure 4):

Holding Stage	Step 1: 64% ramp to 95.0ºC; hold for 10:00 min ***Note:*** *64% heating ramp rate is equivalent to "9600 emulation"*
Cycling Stage	Number of Cycles = 40; Enable AutoData (NOT selected); Starting Cycle: 2 • Step 1: 64% ramp to 95.0ºC (00:15 sec) • Step 2: 100% ramp to 60.0ºC (1:00 min); "Data Collection On" for this step

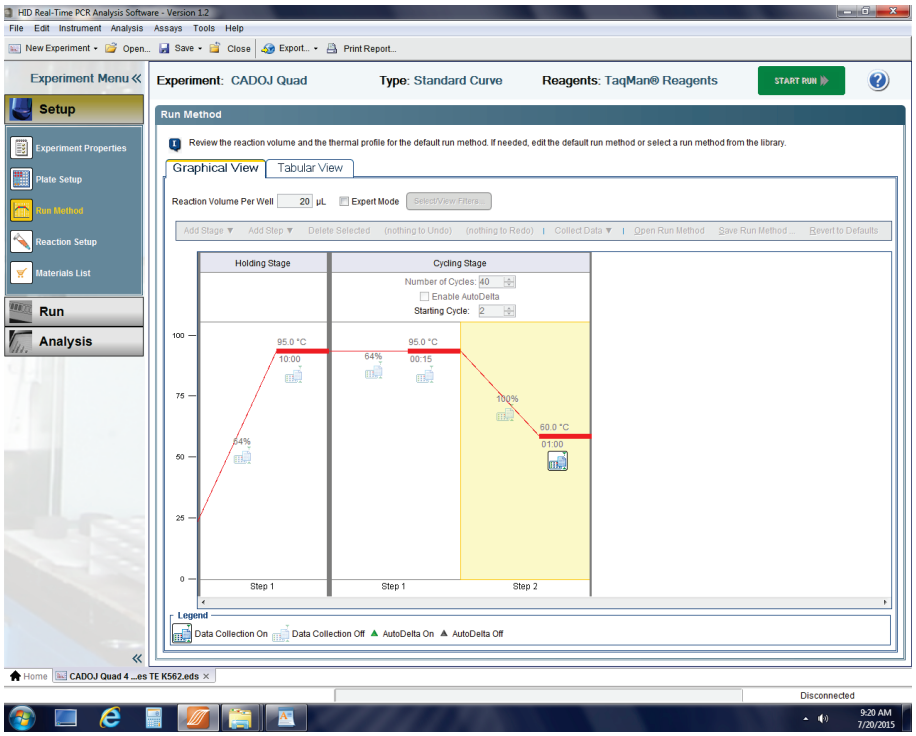


Figure 4. HIDv1.2 Setup Screen: Run Method

2	Use the Tools/Preferences drop-down in the top menu to confirm that the reaction volume per well is 20 µL. If it is not set to 20 µL, make the change and apply it to the run. ***Note:*** *"GUEST" login does not allow you to make this change*

3	If any changes are made, re-save your .eds file.
4	Place the sealed qPCR run plate in the 7500, making sure to orient well A1 correctly. Close the 7500 tray.
5	Select the "Experiment Menu/Run/" window, then use the "Start Run" button to start the run. ***Note:*** *Unlike with the previous SDS Collection software, if the 7500 lid has not reached 105ºC, there is no indication in the HID software that the 7500 is waiting for the lid to heat up before it begins temperature cycling; the HID software will just indicate that the instrument is "Running," and the "Temperature Plot" window will show a flat temperature of ~25ºC. However, once the lid has heated, the temperature cycling will begin, which can confirmed in the "Temperature Plot" window.*
6	When the run is completed, turn off the 7500 and continue to data analysis.

Data analysis

Perform the following steps for data analysis using HID Real-Time PCR Analysis Software version 1.2 (or higher, if performance-checked).

Step	Action
1	Data analysis is performed by first selecting the "Experiment Menu/Analysis" window.
2	From the "View Plate Layout" tab, omit empty wells by selecting (highlighting) the empty wells, then by using right-click "Omit." ***Note:*** *All empty wells must be deleted for correct scaling of amplification plots and so that no "empty" results will be exported for later data processing.*
3	Use the "Analysis Settings" button (upper right corner) to confirm the "Ct Settings" for analyzing each qPCR target in the Quadruplex assay. As shown in Figure 5, the settings should be: **Target** / **Threshold** / **Baseline Start/End** IPC / 0.025 / AUTO nuCSF / 0.1 / AUTO nuSRY / 0.2 / AUTO nuTH01 / 0.025 / AUTO

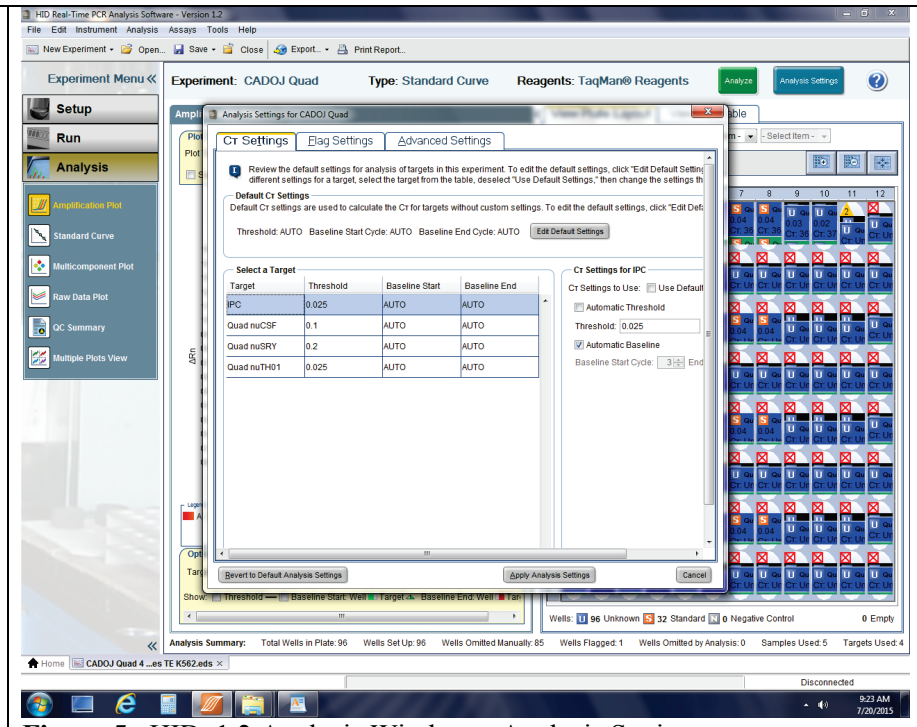


Figure 5. HIDv1.2 Analysis Window – Analysis Settings

4	Click the "Analyze" button to re-analyze the data and save the changes to the .eds file.
5	Select the "Multicomponent Plot" window in the Experiment Menu panel to check that the ROX reference signals for wells A3, A6 and A9 are each ≥14,000 fluorescence units (or use wells B1, E1, and G1 for Tecan HIDEvo150 formatted qPCR runs). If any signals are low, briefly note in the comments section of the User Log for the run.
6	Use the "Amplification Plot" window to evaluate the quality of amplification plots, analysis settings, and negative control results according to Section 2.1.5 Quantification Interpretation Guidelines. Use the drop-down settings to customize plot views (*e.g.,* delta Rn vs. cycle, linear). ***Note:*** *Any sorting of the data in the HID software will potentially affect the downstream processing through Excel programs.*
7	Evaluate the quality of the nuTH01 and nuCSF standard curves, and, if desired, the quality of the nuSRY standard curve. This can be done by: • using the "View Plate Layout" to select ONLY the standard sample wells, • then select "Standard Curve" to view the standard curve window. The standard curves settings may be changed at the top of this window, and the standard curve quality parameters can be viewed at

	the bottom of the window. The R^2 values should be ≥0.98, and the slopes of the standard curves should be between -2.9 and -4.0. If it is necessary to omit any standard wells to improve the standard curve quality parameters, note which wells were removed. ***Note:*** *An entire well must be omitted in this software, rather than just the problematic detector(s).*
8	If necessary, re-analyze the data, then save the .eds file.
9	Print the nuTH01 and nuCSF standard curves using the printer icon in the "Standard Curves" window, making sure to have the standard wells highlighted so that the data points will be displayed in the printouts. If desired, similarly print the nuSRY standard curve.
10	Export the qPCR results in Excel (.xls) format: – Select (highlight) only the non-omitted samples in the well plate. Note that only results for highlighted samples will be exported. – From the main toolbar (top), select "Export." – In the "Export Properties" tab of the "Export Data" window, edit the "Export File Name," if necessary. – Confirm (change, if needed) the following settings: ▪ Select data to export = Results ONLY ▪ Select one file or separate files = One File ▪ File Type = .xls – Browse to the desired directory, then "Start Export." – Close the export tool when complete.
11	Exit the HIDv1.2 software, saving any changes to the .eds file during analysis, and shut down the 7500.

Data.csv

To create a "Data.csv"-formatted file from the exported .xls results file, follow the steps below.

Step	Action
1	Open the Excel spreadsheet "HIDOutput_to_Data_csv_Converter_ v1_1.xlt" file (or higher, if performance-checked).
2	Select "Clear Input Sheet."
3	Select "Import HIDv1.2 Results File" and browse to the .xls file that was previously exported in the HIDv1.2 software.
4	Select "Export Data.csv File" to export the converted file; save as "Data.csv" to C:\\Macros.
5	Select "Print Report Sheet" to print the raw data.
6	Inspect the printed Report Sheet to confirm that all expected samples and results are included, and that no results for any empty wells are included. If necessary, re-open the HIDv1.2 software, and re-export your data so that results are shown only for expected samples.
7	Exit the spreadsheet without saving.

Section 2.1.8 APPENDIX II – Troubleshooting

Identifying potential evaporation

Under the *Component tab*, evaporation of a well during a qPCR run is typically indicated by a creep of the ROX signal in that well over the course of the run as well as the baseline of the other dyes creeping before the exponential portion of the curve. Note that component data is spectrally deconvolved but the baselines have not been normalized. The occurance of evaporation can additionally be verified by the total volume in the well after the run and examination of the seal.

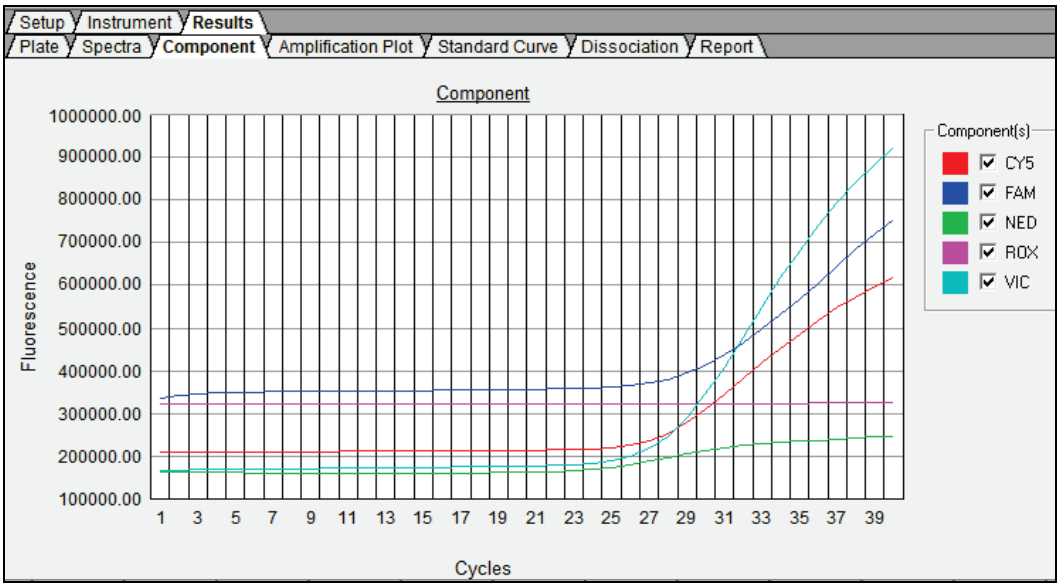


Figure 1. The component data from a successful run.

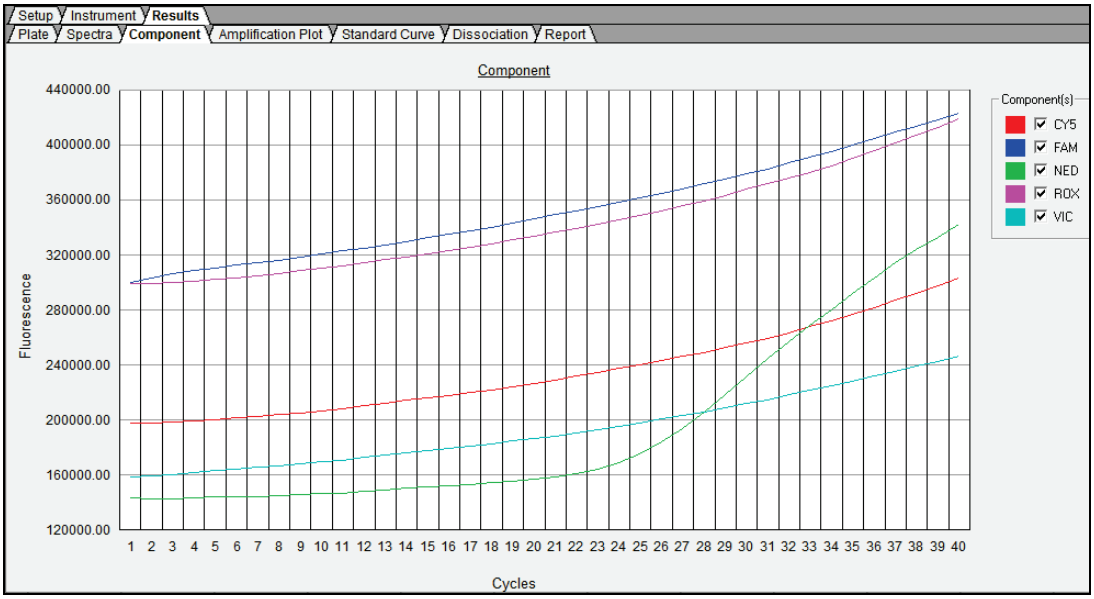


Figure 2. The component data from a well which evaporated during a qPCR run. Note the characteristic creep of the ROX signal.

Unexpected male signal

Quantitation of relatively large quantities of female-only DNA may result in the apparent detection of very low quantities of male (nuSRY) DNA. If small quantities of male DNA are detected in the presence of relatively large quantities of female DNA, the authentic presence of male DNA in the sample should be confirmed by inspecting the nuSRY amplification curve. In particular, look for an increased (~exponential) slope in the curve, which would indicate an authentic nuSRY amplification.

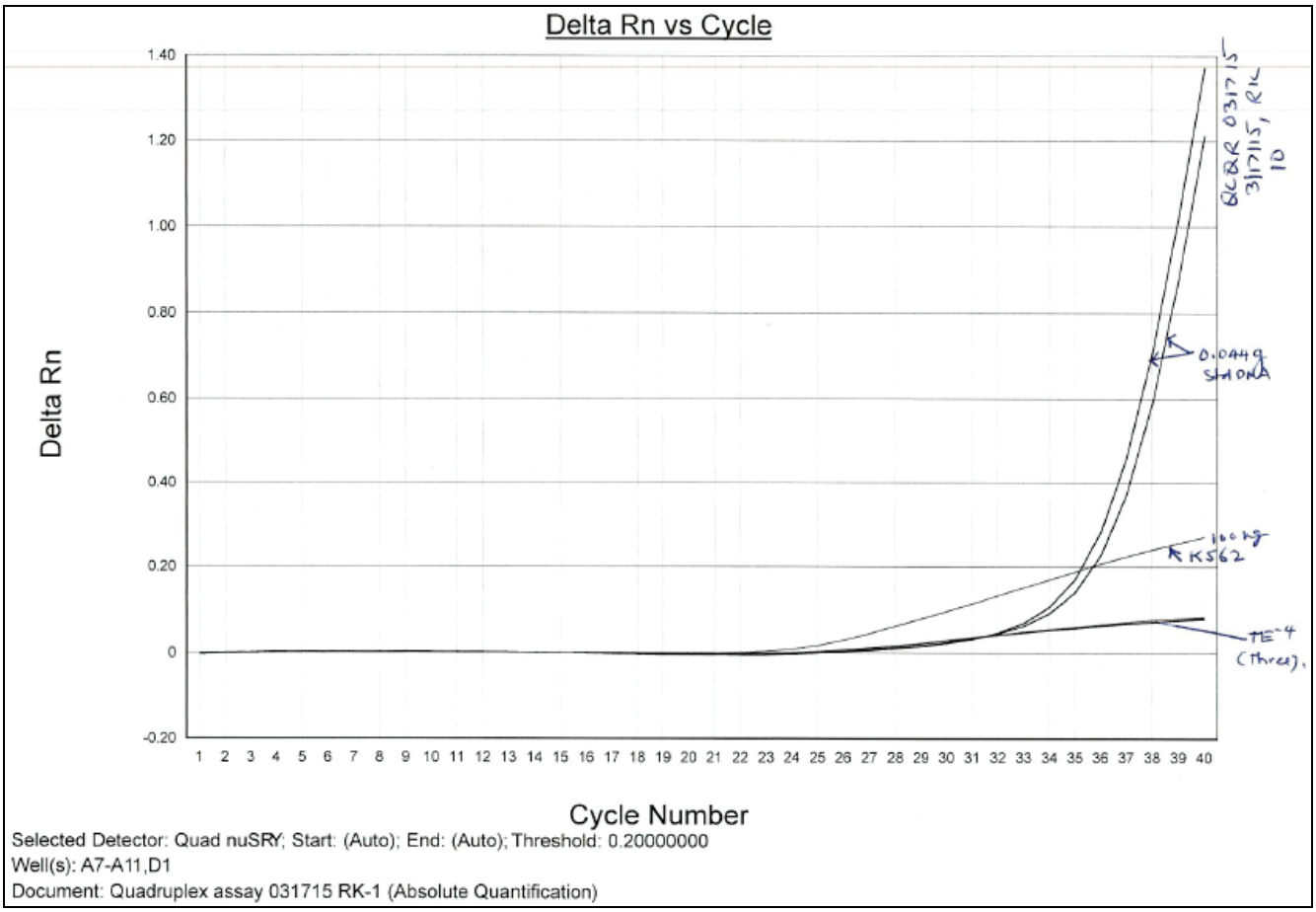


Figure 3. An example of nuSRY target results of a large amount of female DNA (100 ng K562). The inauthentic male signal shown is due to spectral cross-talk into the VIC/nuSRY filter.

Section 2.1.9 APPENDIX III - Guidelines for Truncated qPCR Runs

2.1.7.1 Introduction

Runs will typically end after 40 qPCR cycles. A run, however, may be truncated at less than 40 cycles at the discretion of the analyst, but only if adequate qPCR data are collected to deduce DNA quantities for the desired samples. (***Note**: It is also possible that a run may be truncated due to a power failure, under which circumstances the qPCR data may be interpreted using these same guidelines.*)

In order to use data from a truncated run, the amplification curves for *at least three* of the standard DNA dilutions need to cross the qPCR threshold. (That is, at least three points must be used to construct the nuTH01, nuSRY and nuCSF standard curves.) For such truncated runs, the data will need to be evaluated to determine a lower limit of detection, which should be recorded in the analyst's bench notes. This evaluation uses the standard curve parameters (listed by the ABI 7500 analysis software on the printout of each curve) for that run. An example is shown below for calculating the lower limit of detection for the nuTH01 qPCR portion for a hypothetical run that was stopped at 32 cycles and that gave a nuTH01 standard curve with a slope of -3.45 and an intercept of 31.33:

y = mx + b
Ct = m(log Co) + b, which rearranges to
Co = 10^((Ct-b)/m), or
Co = 10^((32 - 31.33)/(-3.45)) = 0.64 ng

For this example, the lower limit of detection for the nuTH01 portion of the Quadruplex assay would be 0.64 ng. Lower quantities than this would not be detectable in standard, control, or unknown samples. The lower limit of detection for the nuCSF portion of the run can be calculated similarly from the slope and intercept values of the nuCSF standard curve.

2.1.7.2 Truncating a run on the ABI 7500

(1) In real-time, use the Results/Amplification Plot tab of the 7500 collection software to examine the amplification curves of the desired samples to ensure that these curves cross threshold for each of the nuTH01, nuSRY and nuCSF portions of the Quadruplex assay.
(2) Similarly, use the collection software to examine the amplification curves of the DNA standard samples to ensure that at least three of the amplification curves cross threshold for each of the nuTH01, nuSRY and nuCSF portions of the Quadruplex assay.
(3) If the preceding conditions are met, use the Stop button under the Instrument tab of the 7500 collection software to truncate the run.
(4) Data analysis is performed as usual, except to calculate and annotate the lower limit of detection, as described above. In calculating the DNA standard curve, the 7500 software will automatically ignore any amplification curves that do not cross threshold.

Section 2.1.10 APPENDIX IV - An Approach to Preparing a Dilution Series for Unknown Samples Using Option B (2 µL samples)

Co-extracted PCR inhibitors can lead to a significant underestimation of the amount of DNA by qPCR. It may be possible to avoid such an underestimation by collecting qPCR data from a dilution series (1:5, 1:10, 1:20, etc.) of the extract so that the inhibition effects are diluted out. If a sample is suspected of containing significant quantities of PCR inhibitors (e.g., colored extracts, or extracts from soil or bone samples), it may be advantageous to prepare a dilution series from the outset. An example of a dilution series, which uses Option B (2 µL sample volumes) and requires a total of 4 µL of extract, is shown below:

Volume of Extract or Diluted Extract (ratio)	Volume of TE^{-4}	Dilution Ratio in 2 µL of qPCR Sample
2 µL of neat	0 µL	1:1 (neat)
2 µL of neat	8 µL	1:5
2 µL of 1:5	2 µL	1:10
2 µL of 1:5	6 µL	1:20
2 µL of 1:5	18 µL	1:50

Notes:

(1) Although the effects of PCR inhibition are expected to be diluted out by achieving a dilution ratio of ~1:20, some samples may require higher levels of dilution (e.g., 1:50 or 1:100). If the inhibitors are not soluble, dilutions may not be effective in their removal.

(2) Keep in mind that higher levels of extract dilution will not only remove the effects of PCR inhibition but will also contain smaller quantities of DNA, possibly reducing quantitation precision or giving null results.

Section 3.2 AMPFℓSTR Identifiler Plus Amplification

Section 3.2.1 Overview

Introduction

The Polymerase Chain Reaction (PCR) is utilized for Short Tandem Repeat (STR) typing. This analysis is performed using the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit manufactured by Life Technologies/Applied Biosystems. Each kit provides sufficient reagents for 200 tests.

Contents of the Amplification Kit

Each kit includes:
- AmpFℓSTR® Identifiler® Plus Master Mix,
- AmpFℓSTR® Identifiler® Plus Primer Set
- AmpFℓSTR® Control DNA 9947A, and
- AmpFℓSTR® Identifiler® Plus Allelic Ladder.

Contents

Section 3.2 contains the following topics:

Section 3.2.2 Materials, Reagents, and Equipment

Materials and reagents

- TE^{-4}
- AmpFℓSTR® Identifiler® Plus PCR Amplification Kit

Equipment

- 9700 Thermal cycler, Life Technologies/Applied Biosystems
- 0.5 mL and 0.2 mL GeneAmp® thin-walled reaction tubes – DNA/DNase/RNase free, PCR compatible
- 96-well amplification plates – DNA/DNase/RNase free, PCR compatible
- Amplification plate covers –DNase/RNase free, PCR compatible
- MicroAmp® strip caps – DNA/DNase/RNase free, PCR compatible
- Speed-Vac System, or other concentrator

- Pipettors
- Pipet tips – DNA/DNase/RNase free
- Mini centrifuge
- Vortex
- Miscellaneous laboratory supplies

Section 3.2.3 AMPFℓSTR Identifiler Plus Amplification

Contents This section contains the following topics:

Section 3.2.3.1 Analytical Controls and Standard for Amplification

Purpose of the controls and standards

Controls and standards are required to assess
- the quantity and quality of the extracted DNA as well as,
- the effectiveness, accuracy and precision of the analytical procedures.

Important
Evaluation of the controls is essential to the proper interpretation of the test results.

Quantitation

An estimation of the DNA content of the sample (*e.g.,* qPCR) will be made prior to STR analysis. In general, 0.5-1.25 ng of DNA is recommended by ABI for STR amplification, although correct results may be obtained outside of this range. Samples containing less than 500 pg and more than 1.25 ng have been reproducibly amplified and correctly typed for STR markers.

Positive control

This sample ensures that the amplification and typing process is working properly. This control is included in the AmpFℓSTR Identifiler Plus typing kit.

The profile of control DNA 9947A is:
D8S1179 (13,13), D21S11 (30,30), D7S820 (10,11), CSF1PO (10,12), D3S1358 (14,15), TH01 (8,9.3), D13S317 (11,11), D16S539 (11,12), D2S1338 (19,23),D19S433 (14,15), VWA (17,18), TPOX (8,8), D18S51 (15,19), Amelogenin (X,X), D5S818 (11,11), FGA (23,24).

Negative controls

The negative controls used in the amplification procedure are described below.

Negative amplification control

Purpose	To detect contamination that might occur from the • PCR reagents • PCR set-up environment, or • between the samples being prepared.
What it contains	Only the reagents used to prepare the PCR amplification mixture for each batch of samples, including sample buffer (TE^{-4}).
When it's used	It is created during amplification set-up and carried through the typing process.

Reagent blank

Purpose	To detect DNA contamination that might occur from the • reagents, • the environment, or • between the samples being processed.
What it contains	All of the reagents used during extraction, amplification and typing for each set of samples.
When it's used	It is carried through the entire analytical process as part of each extraction set and through the amplification and typing process for *each* PCR system in which the evidence is typed. For samples extracted prior to July 2009, the reagent blank must be run in at least one PCR system. The reagent blank is amplified using the same concentration conditions as required by the samples containing the least amount of DNA in the extraction set and it should be typed using the most sensitive conditions for the samples of the extraction set.

Substrate control sample

When appropriate, a similarly sized and apparently unstained portion of the substrate adjacent to the questioned stain should be collected and run through the typing process. A substrate control sample will not necessarily produce negative typing results. The possibility of other human biological material being present and contributing to the DNA content of a particular sample will be considered in the final interpretation.

The knowledge, experience and judgment of the analyst are paramount when assessing the

need for a substrate control sample, choosing the appropriate sample, and evaluating the results.

Quality control (QC) sample

The purpose of this control is to demonstrate that the analytical process worked properly.

This is a sample from a previously characterized source that is extracted and typed concurrently with the case samples. It serves as:

- an extraction control and a typing control for the process, and
- an internal blind control as the correct typing results are unknown to the analyst until the analysis is complete.

Section 3.2.3.2 Amplification Procedure

Procedure

Follow these steps to complete the amplification.

Step	Action
1	Turn on the 9700 thermal cycler (TC) and confirm the Identifiler Plus amplification parameters listed below. <u>DNA Thermal Cycler 9700 (TC 9700)</u> Pre-denaturation and enzyme activation: 95°C, 11 minutes Cycle (28 cycles): 94°C, 20 seconds 59°C, 3 minutes Final extension: 60°C, 10 minutes Hold temperature: 4°C
2	• Label the required number of 0.5-mL or 0.2-mL GeneAmp® Thin-Walled reaction tubes. • Alternatively, a 96-well amplification plate may be used. • Fill out the AmpFlSTR Identifiler Plus Amplification Sample Checksheet with all pertinent information, including well number if using a 96-well amplification plate.
3	For each sample to be amplified, does the sample volume exceed 10µL? • ***If no,*** go to Step 4. • ***If yes,*** the sample may be concentrated by using a technique for concentrating extract – *e.g.,* a Speed-Vac System, *or* – simple evaporation, *or* – another method for concentrating DNA such as an approved ultrafiltration device, – and go to Step 4.

	Note: Appropriate reagent controls should be treated in the same manner as the sample extracts being concentrated. Techniques to avoid contamination shall be employed, such as covering the sample during evaporation and/or using a bio hood.
4	For each sample, combine in the following order: • Quantity of TE^{-4} needed for a total volume of 10 µL • Sample: – Extracted DNA: typically target 0.5 to 1.25 ng of DNA in a total volume of 10 µL – Positive amplification control: target 0.5 to 1 ng of Control DNA 9947A in a total volume of 10 µL – Negative amplification control: 10 µL of TE^{-4} ***Notes:*** • Sterile deionized water may be used to reconstitute evaporated samples. • Positive and negative amplification controls associated with samples being typed shall be amplified concurrently in the same instrument with the samples at all loci and with the same primers as the forensic samples. All samples typed shall also have the corresponding amplification controls typed.
5	Prepare a "master mix" by adding the following volumes of reagents: # samples x 11 µL AmpFℓSTR Identifiler Plus Master Mix # samples x 5.5 µL AmpFℓSTR Identifiler Plus Primer Set ***Note:*** The formulation above provides slight excess. A 1.5-mL microfuge tube provides enough volume for a maximum of 88 samples.
6	Mix thoroughly and spin the tube briefly in a mini centrifuge to remove any liquid from the cap.
7	Dispense 15 µL of master mix into each GeneAmp Thin-Walled reaction tube, *or* a 96-well amplification plate.
8	• Add the prepared sample (10 µL) to each reaction tube, or well. • After the addition of sample, cap each tube before proceeding to the next sample. ***Notes:*** • Mixing and vortexing are unnecessary. • The final reaction volume in each tube is 25 µL. ***If using a 96-well amplification plate*** • Place a MicroAmp 96-well full plate cover (or another appropriate seal) over the plate once the plate is ready for amplification. • If needed, briefly spin the covered plate in a centrifuge.

	Alternative sample preparation • The 10 µL sample may be prepared directly in the thin-wall amplification tubes, or wells of a 96-well plate. • Add the 15 µL of reaction master mix into each GeneAmp Thin-Walled reaction tube, closing each tube before proceeding to the next sample.
9	• Place the tubes or plate into the TC 9700 and start the thermal cycler program. • Complete the AmpFℓSTR Identifiler Plus Amplification using TC9700 checksheet.
10	• After amplification is complete and the thermal cycler has reached 4°C, remove the tubes or 96-well amplification plate from the instrument block. • Either proceed to capillary electrophoresis, *or* • place the tubes in storage, as follows: – The amplified products should be protected from light. – If using a 96-well amplification plate, remove the plate cover and seal the wells with strip caps (or another appropriate seal) prior to storage. – The products can **either** be stored in a refrigerator for short periods of time *or* in a freezer for longer periods.

Section 3.2.4 References

See Section 3.10.

Section 3.3 Identifiler Plus 3130/3130*xl* Procedure

Section 3.3.1 INTRODUCTION

This procedure module describes the use of the Life Technologies/Applied Biosystems 3130 four-capillary and 3130*xl* 16-capillary electrophoresis instruments in conjunction with the AmpFℓSTR® Identifiler Plus® PCR Amplification Kit and GeneMapper software. It does not contain sections on amplification and genotyping using the analysis software. Amplification and genotyping are performed as described in the relevant technical procedures. This module provides details on capillary electrophoresis and detection with the 3130 and 3130*xl* Genetic Analyzers. References that are specific to the 3130 and 3130*xl* Genetic Analyzers are also included.

Note: *See Appendix I (Section 3.3.5) for Creating New Results Groups and Instrument Procedures*
See Appendix II (Section 3.3.6) for Electronic File Naming Conventions for 3130/3130xl STR Analysis
See Appendix III (Section 3.3.7) for 3130/3130xl Genetic Analyzer Computer Maintenance

Section 3.3.2 EQUIPMENT AND REAGENTS

3.3.2.1 Equipment

- 3130 or 3130*xl* Genetic Analyzer, Life Technologies/Applied Biosystems
- 4-capillary and 16-capillary arrays
- Microfuge tubes
- Mini centrifuge
- Vortex
- Centrifuge with plate rotor or alternative plate spinner
- 96-well reaction plates
- MicroAmp strip caps
- Genetic Analyzer septa plate covers
- Heat blocks or thermal cyclers
- Ice blocks or ice
- Polypropylene 50 mL tubes
- Luer lock syringe
- Pipettors
- Pipet tips – DNA/DNase/RNase free

3.3.2.2 Reagents

- GeneScan-600 [LIZ] internal size standard
- Hi Di formamide
- Identifiler Plus allelic ladder (AmpFℓSTR® Identifiler Plus® PCR Amplification Kit)
- Genetic Analyzer POP-4 polymer
- Genetic Analyzer 10X buffer with EDTA
- Matrix standard DS-33
- Deionized water

Section 3.3.3 CAPILLARY ELECTROPHORESIS AND DETECTION WITH THE 3130/3130XL GENETIC ANALYZERS

Capillary electrophoresis is performed only in the PCR Product Room. All pipettors and other equipment in the PCR Product Room are dedicated for post-PCR use only.

3.3.3.1 Analytical Controls and Standards for Capillary Electrophoresis

- **Internal Size Standard (GeneScan®-600 [LIZ] version 2)**
  GeneScan®-600 [LIZ] version 2 (GS600v2 [LIZ]) contains dye-labeled plasmid DNA fragments of known size that are co-injected with the sample to allow estimation of STR allele sizes.

- **AmpFℓSTR Identifiler Plus Allelic Ladder**
  The allelic ladder provided in the AmpFℓSTR Identifiler Plus Amplification Kit is used to determine the genotypes of samples. While the ladder includes primarily the common alleles, additional alleles exist and may be detected.

3.3.3.2 Setting up the 3130/3130*xl* Genetic Analyzers

***Note**: When replacing or replenishing polymer, if the entire volume of 3130/3130xl Genetic Analyzer POP-4™ is not needed, pour the desired volume of polymer into a clean 3130/3130xl polymer bottle. A minimum of 2 mL of polymer is needed to prepare the instrument when replacing polymer. A minimum of 1mL of polymer is needed to prepare the instrument when replenishing polymer.*

1. If needed, turn on the 3130/3130*xl* Genetic Analyzer computer. The computer must be powered on before the instrument. Log onto the computer.

2. Turn on the 3130/3130*xl* Genetic Analyzer. Wait for the status light to turn solid (not flashing) green.

3. Launch the 3130/3130*xl* Genetic Analyzer Data Collection software version 3.0 (or higher, if performance checked) by double-clicking on the desktop shortcut icon. Alternatively, launch Data Collection software directly from the **Start** menu, point to **All Programs**, then **Applied Biosystems**, **Data Collection,** and select **Run 3130/3130xl Data Collection v3.0**.

4. The **Service Console** will be displayed. By default, all applications are off, indicated by red circles. They launch automatically with the 3130/3130*xl* Data Collection Software. As each application activates, the red circles (off) change to yellow triangles (activating), and then to green squares (on) when they are fully functional. When all four applications are running (displaying green squares), the **Foundation Data Collection** window will be displayed. This may take several minutes to complete.

5. In the **Foundation Data Collection** window, click on the + next to each folder as desired to expand subfolders in the left tree pane. Select items from this tree pane to open manager, viewer, and other program windows.

6. Record the pertinent information in the instrument log.

7. If necessary, make 1X Genetic Analyzer buffer with EDTA:
   - Dilute, e.g., 3 mL of 10X Genetic Analyzer buffer with EDTA to 1X concentration with 27 mL of deionized water (or 4 mL with 36 mL water) in a 50-mL polypropylene tube.

8. The polymer delivery pump water trap should be flushed with deionized water approximately monthly to keep the pump clean and to clear bubbles. If the water trap does not need to be flushed, proceed to step 9.

   Flush the water seal trap:
   - Fill a 20-mL Luer lock syringe with deionized water and expel any bubbles from the syringe.
   - Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.
   - Open the Luer fitting by grasping the body of the fitting and turning it (and the attached syringe) approximately one-half turn counterclockwise.
   - Open the exit fitting at the top left side of the pump block by turning it approximately one-half turn counterclockwise.
   - Hold an empty tube or beaker under the exit fitting to receive approximately 5 mL of waste.
   - Flush the trap by pushing slowly and steadily on the syringe plunger. It is VERY important to flush slowly; it should take approximately 30 seconds to flush 5 mL of deionized water through the trap.
   - Close the Luer fitting by turning it clockwise until it seals against the pump block. Do NOT over tighten.

- Close the exit fitting by turning it clockwise until it seals against the pump block. Do NOT over tighten.
- Remove the syringe from the Luer fitting. Hold the fitting with one hand while turning the syringe counterclockwise with the other hand.

9. **Install Array Wizard.**
   - Determine if the capillary array must be installed or replaced. The array should yield acceptable data up to at least 150 runs; a run is one set of 4 (3130) or 16 (3130*xl*) samples. In practice, acceptable data has been obtained from significantly more than 150 runs on a single array. If the capillary array does not need to be replaced, continue to step 10.
   - Remove the 3130/3130*xl* Genetic Analyzer POP-4™ polymer from the refrigerator, loosen the cap and allow it to warm to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature by gentle mixing.
   - Click <***instrument name***> in the tree pane. Select **Install Array Wizard** from the **Wizards** menu at the top of the screen. Follow all prompts in the wizard to install or replace the array.
   - If necessary, select **Update Cap Array Info** wizard from the **Wizards** menu at the top of the screen to correct any entries or to update capillary array and serial number information.
   - When the Install Array Wizard is finished proceed to step 13.

10. **Water Wash Wizard**.
    - The polymer delivery pump chamber, lower polymer block, channels and tubing should be washed with deionized water approximately monthly to keep the blocks clean and in good working order. If the instrument does not need to be washed, proceed to step 11.
    - Remove the 3130/3130*xl* Genetic Analyzer POP-4™ polymer from the refrigerator, loosen the cap and allow it to warm to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature by gentle mixing.
    - Click <***instrument name***> in the tree pane. Select **Water Wash Wizard** from the **Wizards** menu at the top of the screen. Follow all prompts in the wizard to wash the polymer delivery pump chamber, lower polymer block, channels and tubing with water.
    - When the Water Wash Wizard is finished, proceed to step 13.

11. **Replenish Polymer Wizard**.
    - Determine whether the polymer and buffer need to be changed or supplemented. Approximately 25-40 µL of polymer is used per run of 4 samples (3130) and 50-80 µL of polymer for each run of 16 samples (3130*xl*). If the polymer and buffer do not need to be changed, proceed to step 12.
    - Remove the 3130/3130*xl* Genetic Analyzer POP-4™ polymer from the refrigerator, loosen the cap and allow it to warm to room temperature. If precipitate is present in the bottle when removed from cold storage, it should go back into solution at room temperature by gentle mixing.

- Click <***instrument name***> in the tree pane. Select **Replenish Polymer Wizard** from the **Wizards** menu at the top of the screen. Follow all prompts in the wizard to replenish or replace the polymer. When the Replenish Polymer Wizard is finished, proceed to step 13.

12. **Bubble Remove Wizard.**
    - Check the polymer delivery pump, channels and tubing for the presence of bubbles. Proceed to step 13 if no bubbles are present.
    - If bubbles are present, click <***instrument name***> in the tree pane. Select **Bubble Remove Wizard** from the **Wizards** menu at the top of the screen. Follow all prompts in the wizard to remove bubbles.
    - If persistent bubbles remain, perform a water wash (see step 10).

13. **Replace buffer and water:**
    - With the instrument doors closed, press the **Tray** button on the outside of the instrument to bring the autosampler to the forward position. Once the autosampler has stopped moving, open the doors.
    - Remove cathode buffer and water reservoirs from the instrument.
    - Dispose of remaining fluid and rinse reservoirs and septa strips with deionized water.
    - Dry all parts.
    - Fill the cathode buffer reservoir to the line with 1X Genetic Analyzer buffer with EDTA (~17 mL), seal with a septa strip, and replace in position 1.
    - Fill the water reservoirs to the line with deionized water, seal with septa strips, and replace in positions 2 and 4.
    - Position 3 remains empty.
    - If needed replace the buffer in the anode buffer reservoir:
        - Remove the anode buffer reservoir from the instrument.
        - Dispose of remaining fluid and rinse with deionized water.
        - Fill the anode buffer reservoir to the line with 1X Genetic Analyzer buffer and place under the lower pump block.
    - Close the instrument doors and press the **Tray** button on the outside of the instrument to return the autosampler to its previous position.

14. If necessary, select **Update Cap Array Info** wizard from the **Wizards** menu at the top of the screen to correct any entries or to update capillary array and serial number information.

15. Close the 3130/3130*xl* doors. Autosampler should return to the previous position and submerge the ends of the capillaries. If this does not occur automatically, press the **Tray** button on the outside of the instrument to bring the autosampler back to the previous position.

3.3.3.3 Spatial Calibration

A spatial calibration creates a spatial map of the array for the CCD (Charged-Coupled Device). This map defines the pixel number at the center of fluorescence in each capillary.

***Note**: A spatial calibration must be performed each time a capillary array is installed, replaced, or temporarily removed from the detection block. A spatial calibration is unnecessary if the instrument is already set-up, whether or not additional polymer has been added or the buffer has been replaced.*

1. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl >** <***instrument name***> **> Spatial Run Scheduler**.

2. In the Protocol drop-down menu in the Spatial Protocols section, select either **3130SpatialNoFill_1** (if the capillaries contain fresh polymer) or **3130SpatialFill_1** (if the capillaries do not contain fresh polymer).

   ***Note**: If the spatial calibration is performed more than once, there is no need to fill the capillaries with polymer the second time.*

3. Click **Start**. The spatial profile window will turn black when the spatial calibration begins.

4. Once the spatial calibration is complete, evaluate the spatial calibration. There should be 4 (3130) or 16 (3130*xl*) single sharp peaks, all of similar peak height and at least approximately 6,000 RFU. The presence of small shoulders is acceptable. Each peak should have an orange cross symbol at the top of the peak and the peaks should be separated by increments of approximately 13-16 position values.

5. If the data meet these passing criteria, press **Ctrl-Alt-PrintScreen** to copy the image. Open **WordPad** (or another comparable program), and **Paste** under **Edit**. Then choose **Save As** under **File** and name as *Spatial <date> <analyst's initials>*. Save this to a Spatial Calibrations folder on the C drive. It will be retained there for five years, after which time it may be deleted. Choose **Quit** under **File** to exit **WordPad**.

6. In 3130/3130*xl* Data Collection, click **Accept** to write the calibration data to the database.

   If the spatial calibration data do not meet the criteria for passing, do not print the image. Click **Reject** and repeat steps 2-5 above. It may be helpful to fill the capillaries again.

3.3.3.4 Spectral Calibration

A spectral calibration creates a calibration file to correct for the overlap of fluorescence emission spectra from each of the dyes. The Identifiler Plus dyes (6-FAM, VIC, NED, PET, LIZ) are referred to as the G5 dye set.

A spectral calibration should be performed if the laser, optics, or CCD camera have been realigned or replaced by a service engineer, or when pull-up/pull-down peaks become excessive (>>6%). It is not necessary to perform a spectral calibration if installing an array. If the instrument has been giving acceptable data and a service engineer has not replaced or realigned the laser, optics, or CCD camera, the analyst may omit this calibration procedure.

If the 3130/3130*xl* is new or has had the Data Collection software reinstalled, the condition number bounds of the Spect36_POP4_1 run module should be verified prior to performing a spectral calibration. This is done by choosing this module under **Protocol Editor** and clicking **Edit Param**. The condition number bounds for the G5 dye set should be set at 7-12. Edit the parameters if they are different and save the changes.

1. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl > Protocol Manager** to open the **Protocol Manager** window.

2. In the **Instrument Protocols** pane, click **New** and the **Protocol Editor** dialog box will open.

3. Complete the **Protocol Editor** dialog box:
   - Type G5Spectral for the procedure.
   - If desired, type a description for the procedure.
   - Select **Spectral** in the Type drop-down list.
   - Select **G5** in the Dye Set drop-down list.
   - Select **POP4** in the Polymer drop-down list.
   - Select **36** in the Array Length drop-down list.
   - Select **Matrix Standard** in the Chemistry drop-down list.
   - Select **Spect36_POP4_1** in the Run Module drop-down list.
   - Click **OK**.

4. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl > Plate Manager** and click **New** to open the **New Plate Dialog** box.

5. Complete the **New Plate Dialog** box:
   - Type a name for the plate.
   - If desired, type a description for the plate record.
   - Select **Spectral Calibration** in the Application drop-down list.
   - Select **96-well** in the Plate Type drop-down list.
   - Enter a name for each the owner and the operator.
   - Click **OK**.

6. Complete the **Spectral Calibration Plate Editor** dialog box:
   - In the Sample Name column, enter a sample name then click the next cell. The value 100 automatically displays in the Priority column. ***Note:*** See Section 3.3.3.6 Plate Records for sample name restrictions.
   - If desired, enter any additional comments or notations for the sample in the Comments column.
   - Select **G5Spectral** in the Instrument Protocol 1 drop-down list
   - Highlight the entire row and select **Edit > Fill Down Special**. The software will automatically fill in the appropriate well numbers for a single run.
   - Click **OK** to complete the plate record.

7. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl >** <***instrument name***> **> Run Scheduler > Plate View**.

8. Search for the plate record:
   - If there are a limited number of plates in the database, choose **Barcode** in the Type of Search drop-down menu and click **Find All**. All plates in the database will display in the plate record section.
   - Perform an advanced search by selecting **Advanced** in the Type of Search drop-down menu. Use the drop-down condition list and values next to the category (or multiple categories) to define search conditions. Click **Search** to display all plates in the database that match the search criteria in the plate record section.

WARNING! CHEMICAL HAZARD! Formamide is an irritant and a teratogen. Avoid skin contact and inhalation. Use in a well-ventilated area. Wear lab coat, gloves, and protective eyewear when handling.

Note*: Formamide should be aliquoted in a fume hood. Minimize light exposure to the matrix standard.*

9. Prepare standards for spectral calibration by mixing 1.5 µL (3130) or 5 µL (3130*xl*) of Matrix Standard Set DS-33 with 58.5 µL (3130) or 195 µL (3130*xl*) of HiDi Formamide. Vortex cocktail and spin briefly. Dispense 10 µL of matrix standard cocktail into wells A1-D1 (3130) or A1-H2 (3130*xl*) of a 96-well plate. Cover the plate with a septa mat. Heat-denature the matrix standards for 3 minutes at 95°C. Snap-cool the plate for 3 minutes.

   Note: *The matrix standard volume may be adjusted as needed.*

10. Ensure that each sample is positioned correctly at the bottom of each well. Assemble the 96-well plate on a plate base and snap a plate retainer over the plate and base. Press **Tray** to bring the autosampler forward, open the 3130/3130*xl* doors, and place the sample assembly on the autosampler. The plate position indicator in the **Plate View** should now show a yellow grid. Close the doors. The autosampler should automatically return to its previous position. If the autosampler does not respond upon door closing, either press the **Tray** button or open and close the doors again. Ensure that all the capillaries are submerged in buffer at site 1 (or water at site 2 or 3).

11. Check and refill fluids on the instrument as necessary.

12. Link the plate record to the samples by highlighting the plate record and then clicking on the plate position indicator. When linked, the plate position indicator will change from yellow to green.

13. Click the **Run Instrument** button on the toolbar (green triangle) to begin the run.

14. The **Processing Plates** dialog box opens. Click **OK** to begin the run.

15. When the run has concluded, evaluate the spectral calibration profile for each capillary via the **Spectral Viewer**. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl >** <***instrument name***> **> Spectral Viewer**.

16. In the Dye Set drop-down list, select **G5**.

17. In the plate diagram, green wells indicate passed capillaries while tan wells indicate failed capillaries. Select each well on the plate diagram in turn to view the spectral results for each capillary.

18. Evaluate the spectral profile (top display) and raw data (lower display) for each capillary:
    - Verify that the order of the peaks in the spectral profile from left to right is blue-green-yellow-red-orange.
    - Verify that the order of the peaks in the raw data profile from left to right is orange-red-yellow-green-blue.
    - Verify that the peaks in the spectral profile do not contain gross overlaps, dips, or other irregularities.
    - Re-run the spectral calibration if more than 1 capillary failed on the 3130 or more than 3 capillaries failed on the 3130*xl*.

    Note: *It is generally useful to review the spectral calibration run log file to determine the cause of failure for any non-passing matrices. This can be found at E:\AppliedBiosystems\UDC\DataCollection|data|ga3130|<3130>\SpectralCalTmpFiles\G5*.txt*

19. If desired, rename the spectral calibration run. (The spectral file default name is the day, date, and time of the run.)
    - Click **Rename**.
    - In the **Rename Calibration** dialog box, enter the run number, dye set, and date performed using underscores instead of spaces (<*instrument name/run number*> _G5_<*date* >). This file is electronically maintained until a suitable replacement is created. Record the name of the spectral calibration in the instrument log.
    - Click **OK**.

20. The results from a new spectral calibration automatically become the active spectral calibration. To change the active spectral calibration:
    - In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl > <*instrument name*> > Spectral Viewer**.
    - Select **G5** in the Dye Set drop-down menu.
    - In the List of Calibrations for Dye Set G5 drop-down list, select the desired spectral calibration. The spectral profiles and raw data will be displayed.
    - If the spectral calibration is acceptable, click **Set**. This becomes the active spectral calibration.

3.3.3.5 Preparing Identifiler Plus Samples for Capillary Electrophoresis

Hazard warning

Formamide is an irritant and a teratogen.

Precautions:
- Avoid skin contact and inhalation.
- Use in a well-ventilated area.
- Wear lab coat, gloves and protective eyewear when handling.

Reagent notes

- *The formamide/GS600v2 [LIZ] master mix should be made and aliquoted in a fume hood.*
- *Minimize light exposure to GS600v2 [LIZ] and allelic ladder.*

Sample note

Sterile deionized water may be used to reconstitute evaporated samples.

Run setup notes

- *Typically, allelic ladder is included in more than one well of a run, depending on the number of samples to be injected.*
- *Every set of 4 (3130) or 16 (3130xl) wells to be injected should contain at least formamide and GS600v2 [LIZ]; in other words, there should be no empty wells such that every capillary is injecting at minimum formamide and GS600v2 [LIZ].*
- *Alternatively, formamide alone may be used to fill the excess wells in a run.*

Prepare samples

Perform the steps below to prepare samples for capillary electrophoresis.

Step	Action
1	Combine 8.5 µL HiDi formamide and 0.5 µL GeneScan-600 [LIZ] v2 size standard per sample well. For ease, volumes shown in the table below may be used. Note that multiples of 16 are shown in bold for the 3130*xl*. ***Note:*** *If reduced GS600v2 [LIZ] peak heights are needed (e.g., to reduce the peak height of the TPOX artifact), the amount of size standard may be reduced (e.g., to 0.3 µL). The formamide volume should increase by the amount the size standard has decreased (e.g., to 8.7 µL), so that the total volume per well remains at 9 µL. See Step 2 for more information.*

8.5 µL /0.5 µL formulation:

Number of injections (*i.e.,* wells)	HiDi formamide* (~ microliters)	GS600v2 [LIZ] * (~ microliters)
4	37.4	2.2
8	74.8	4.4
12	112.2	6.6
16	**149.6**	**8.8**
20	187.0	11.0
24	224.4	13.2
28	261.8	15.4
32	**299.2**	**17.6**
36	336.6	19.8
40	374.0	22.0
44	411.4	24.2
48	**448.8**	**26.4**
52	486.2	28.6
56	523.6	30.8
60	561.0	33.0
64	**598.4**	**35.2**
68	635.8	37.4
72	673.2	39.6
76	710.6	41.8
80	**748.0**	**44.0**
84	785.4	46.2
88	822.8	48.4
92	860.2	50.6
96 (full plate)	**897.6**	**52.8**

*Calculations are based on the following:
(X + Y) x **8.5** µL formamide
(X + Y) x **0.5** µL GS600v2 [LIZ] size standard
X = (# amplified samples) + (# ladder wells) + (# GS600v2 [LIZ] wells)
Y = 10% of X to provide slight excess for pipetting

2 If a 8.7 µL /0.3 µL formulation is desired, the volumes of HiDi formamide and GeneScan-600 [LIZ] v2 size standard shown in the table below may be used. Note that multiples of 16 are shown in bold for the 3130*xl*.

Otherwise, continue to Step 3.

8.7 µL /0.3 µL formulation:

Number of injections (*i.e.,* wells)	HiDi formamide* (~ microliters)	GS600v2 [LIZ] * (~ microliters)
4	38.3	1.3
8	76.6	2.6
12	114.8	4.0
16	**153.1**	**5.3**
20	191.4	6.6
24	229.7	7.9
28	268.0	9.2
32	**306.2**	**10.6**
36	344.5	11.9
40	382.8	13.2
44	421.1	14.5
48	**459.4**	**15.8**
52	497.6	17.2
56	535.9	18.5
60	574.2	19.8
64	**612.5**	**21.1**
68	650.8	22.4
72	689.0	23.8
76	727.3	25.1
80	**765.6**	**26.4**
84	803.9	27.7
88	842.2	29.0
92	880.4	30.4
96 (full plate)	**918.7**	**31.7**

*Calculations are based on the following:
(X + Y) x **8.7** µL formamide
(X + Y) x **0.3** µL GS600v2 [LIZ] size standard
X = (# amplified samples) + (# ladder wells) + (# GS600v2 [LIZ] wells)
Y = 10% of X to provide slight excess for pipetting

3	Mix the tube and spin briefly in a mini centrifuge.
4	Label a clean 96-well plate and aliquot 9 µL of the formamide/ GS600v2 [LIZ] master mix into each well to be used. Any unused wells in a run of four (or 16 for 3130*xl*) should also contain 9 µL of the formamide/GS600v2 [LIZ] master mix.
5	Add 1.0 µL of PCR product or allelic ladder to each well and cover the plate with a 96-well plate septa mat. **Important** The standard input volume of 1.0 µL may be reduced but not increased.
6	If needed, briefly spin the 3130/3130*xl* 96-well plate.
7	Heat-denature the samples for three minutes at 95◦C.
8	Snap-cool samples immediately for a minimum of three minutes.

9	Prepare the plate assembly: • Place the sample plate into a 3130/3130*xl* plate base. • Snap the plate retainer over the plate and plate base.
10	Load the plate assembly: • With the instrument doors closed, press the **Tray** button to bring the autosampler to the forward position. • Place the plate assembly on the autosampler. • The corresponding position in the Plate View window (**GA Instruments > /ga3130xl >** <***instrument name***> **> Run Scheduler > Plate View**) of 3130/3130*xl* Data Collection should change color (gray to yellow). • Close the instrument doors. • The autosampler should automatically return to the back position and submerge the capillary/electrode ends into one of the reservoirs. • If the autosampler does not respond upon door closing, either press the **Tray** button or open and close the doors again.
11	Repeat steps 1-10 if a second plate is to be run on a 3130*xl* Genetic Analyzer.

Note: *The 3130/3130xl septa mats may be re-used several times before discarding. See the procedure described below:*

1. *Soak the previously used septa mat in 5% bleach for approximately 5-15 minutes.*
2. *Rinse the septa mat thoroughly with running tap water, followed by dH_20.*
3. *Allow the septa mat to air dry.*
4. *Once dry, store the septa mat in a clean location.*

3.3.3.6 Plate Records

Plate records are data tables in the instrument database that store information about the plates and samples such as plate name, type, owner, sample well numbers, dye set information, run modules, etc. Sample name restrictions limit the use of non-letter and non-number characters to -_(){}#.+. In addition, spaces are NOT allowed.

Each plate of samples must have a plate record. If two plates are to be run on a 3130*xl*, then two plate records must be created—one for each plate.

1. Before creating a new plate record, check that the desired Results Group and Instrument Procedures already exist on the instrument.
   - Go to **GA Instruments > Results Group** and see if the desired Results Group is listed.
   - Go to **GA Instruments > /ga3130xl > Protocol Manager** and see if the desired Instrument Protocol is listed.
   - If either is not yet created on the instrument, create and save the desired files. (See Appendix I, "Creating New Results Groups and Instrument Procedures.")
   - Alternatively, a new plate record can be started and if the desired Results Group or Instrument Protocol is not listed, select **New** in the appropriate drop-down menu and follow instructions in the Appendix I under "Creating New Results Groups and Instrument Procedures."

2. A new plate record can be created directly in the Data Collection Software (go to step 3) or in Excel on a separate computer and imported into the Data Collection software (skip to step 7). To create a new plate record, go to **GA Instruments > /ga3130xl > Plate Manager** in the tree pane of the Data Collection software and click **New** to open the **New Plate Dialog** box.

3. Complete the **New Plate Dialog** box:
   - Type a name for the plate (e.g., 16MCE5_mmddyy).
   - Select **GeneMapper-Generic** in the Application drop-down list.
   - Select **96-well** in the Plate Type drop-down list.
   - Enter a name for the owner (the analyst) and the operator (the analyst).
   - Click **OK**.

   Note: *If setting up two plates to be run on a 3130xl, an "A" may be included in the name of the plate record for the plate in the left deck position (e.g., 16MCE5A_mmddyy). Similarly, a "B" may be included for the right-hand plate (e.g., 16MCE5B_mmddyy).*

4. Fill in the plate record with the appropriate information.
   - Enter sample names in the Sample Names column.
   - As samples are added to the plate record, the Priority column automatically populates with a default value of 100. Changing this value to a smaller number will cause that set of 4 (3130) or 16 (3130*xl*) samples to be injected before others on the plate record. In other words,

regardless of location on the plate, runs proceed in the order of their priority number (smallest to largest). If no priority numbers are entered (other than the default), runs will proceed in the order in which they are listed in the plate record.

- As an option, the following fields may be filled out in the plate record: Sample Type, Size Standard, Panel, Analysis Method. In the Sample Type column, enter ladder, positive control, and negative control for allelic ladder, positive amplification control, and negative amplification control, respectively. This information will be used to autopopulate the Sample Type field when importing sample files into a GeneMapper project. The SnpSet field and User Defined columns are not generally utilized.
- In the Results Group 1 column, choose **Identifiler_Generic** from the drop-down menu.
- In the Instrument Protocol 1 column, choose **Identifiler5sec** from the drop-down menu.

***Note**: All samples and controls, including the allelic ladder, should be injected at least twice per run. For case samples, the quality of the two injections must be sufficient to ensure reproducibility of typing results. One good injection is sufficient for positive and negative controls.*

Note:** Modified modules may additionally or alternatively be chosen. Modifications may include shorter injection times, lower injection voltages, and longer run times. Refer to Appendix I Creating New Results Groups and Instrument Protocols. Modified modules should reflect the changes made in the title of the Instrument Protocol (e.g.,* **Identifiler1.5kV).*

***Note:** The standard injection conditions of 5 seconds at 3kV may be reduced, but not increased. The standard run time of 1500 seconds (25 minutes) may be increased. Two injections with the same injection conditions are needed for interpretation of evidence samples.*

- Add additional Results Group and Instrument Protocol columns by choosing **Add Sample Run** from the **Edit** menu at the top of the screen. Complete these columns as above.

- Highlight the Results Groups and Instrument Protocol columns and select **Edit > Fill Down** to complete these columns for all samples.

***Note:** Duplicate injections can be identified in the Extracted Data folder by the file suffix ".2" (or ".3" if a triplicate injection, etc.).*

5. When finished, click **OK**. The changes will be automatically saved and the plate record will appear in the plate record list.

6. Export the plate record so that a copy can be saved with the sample files when the run is complete.
   - Go to **GA Instruments > /ga3130xl > Plate Manager** in the tree pane of the Data Collection software and locate the plate record (see Spectral Calibration section for details on searching for plate records).
   - Highlight the plate record in the list and click the **Export** button.
   - Save as a tab delimited text file (".txt") to the desired location.

7. Repeat steps 2-6 if a second plate is to be run on a 3130*xl* Genetic Analyzer.

3.3.3.7 Capillary Electrophoresis

1. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl >** <***instrument name***> **> Run Scheduler > Plate View**.

2. Search for the plate record:
   - If there are a limited number of plates in the database, choose **Barcode** in the Type of Search drop-down menu and click **Find All**. All plates in the database will display in the plate record section.
   - Perform an advanced search by selecting **Advanced** in the Type of Search drop-down menu. Use the drop-down condition list and values next to the category (or multiple categories) to define search conditions. Click **Search** to display all plates in the database that match the search criteria in the plate record section.

3. Link the plate record to the plate on the autosampler by highlighting the plate record and then clicking on the plate position indicator. When linked, the plate position indicator will change from yellow to green.

4. Repeat Steps 2 and 3 if a second plate is to be run on a 3130*xl* Genetic Analyzer.

5. For networked computers, disconnect the computer from the network by unplugging the cord from the ethernet wall or computer port or by using another appropriate procedure.

6. Click the **Run Instrument** button on the toolbar (green triangle). When the **Processing Plates** dialog box opens, click **OK** to begin the run.

7. When running the 3130/3130*xl* Genetic Analyzer, leave the data collection software open to the Instrument Status window (**GA Instruments > /ga3130xl >** <***instrument name***> **> Instrument Status**.).

   Data for a set of injections can be viewed as it is being collected by going to **GA Instruments > /ga3130xl >** <***instrument name***> **>Capillaries Viewer.**

Data for completed injections can be viewed using analysis software (GeneMapper, GeneScan) while a run is in progress. Sample files are added to the run folder on the E drive (E:\Applied Biosystems\udc\data) at the completion of each set of injections.

If it is necessary to add injections to a run in progress, go to **GA Instruments > /ga3130xl > Plate Manager** in the tree pane of the Data Collection software. Select the plate record from the list and click **Edit.** Add additional Results Group and Instrument Protocol columns by choosing **Add Sample Run** from the **Edit** menu at the top of the screen. Fill in the Results Group and Instrument Protocol fields for the samples to be injected. Click **OK**. The plate record will be updated and the additional runs will appear in the Run View window (**GA Instruments > /ga3130xl > <*instrument name*> > Run Scheduler > Run View**).

8. When the run has completed, review the data to ensure that it is of sufficient quality for each sample. If additional injections are needed for the samples or a subset of the samples from a run, edit the original plate record to perform a new run to minimally include those samples requiring another injection, the positive and negative amplification controls, and two injections of allelic ladder. *This becomes a new run.*

Note: *Any corresponding reagent blank(s) should be included if the samples re-injected are run on an instrument different from the original and/or if the run conditions have potentially increased in sensitivity.*

To edit a previous plate record:
- Go to **GA Instruments > /ga3130xl > Plate Manager** in the tree pane of the Data Collection software.
- Search for the plate record using either **Barcode** or **Advanced** Search.
- Highlight the plate record name and click **Duplicate**.
- Edit the plate name accordingly in the **Plate Name** field of the **Plate Dialog Box.**
- Click **OK.**
- Delete the all of the Results Group and Instrument Protocol information for the sets of samples that will not be re-injected.
- In the Sample Name column, delete the sample names for all sets of samples that will not be re-injected. (The plate record cannot be saved if Results Group and Instrument Protocol information is not provided for every sample listed in the Sample Name column).
- If necessary, add additional Results Group and Instrument Protocol columns for the desired number of injections.

3.3.3.8 Data Transfer

1. For networked computers, reconnect the computer to the network by plugging the cord into the ethernet wall or computer port or by using another appropriate procedure.

2. Locate data in the **Data** or **Extracted Data** folder on the E drive and perform the following:
   - Rename the run folder as *RF <instrument name/run number> <date >* (See Section 3.3.6 Appendix II).
   - Export the plate record into the run folder and print the sample names, well numbers, and modules used for inclusion in the case file(s) (*e.g.,* open in Excel, delete extraneous columns, print in landscape).
   - Transfer the run folder to the desired workstation for analysis.

3.3.3.9 3130/3130xl Genetic Analyzer Shutdown

Note: *It is not necessary to shutdown the 3130/3130xlGenetic Analyzer and the associated computer following a run.*

1. Estimate the length of time before the instrument will be run again. Depending on the time frame, either a short-term (continue to step 3) or long-term (continue to step 2) shut down may be performed if desired.

2. (Long-term shutdown) From the toolbar at the top of the Data Collection software, select **Instrument Shutdown Wizard** and follow the prompts, then skip to Step 4.

3. (Short-term shutdown) Go to **GA Instruments > /ga3130xl > *<instrument name>* > Manual Control**. In the **Send Defined Command** drop-down menu, select **Autosampler**. In the **Command Name** drop-down menu, select **Move autosampler to site.** In the **Value** menu, select **Waste** and click **Send Command**. Wait for the autosampler to stop moving and **Send Command** becomes active, before proceeding. In the **Send Defined Command** drop-down menu, select **Polymer Delivery Pump**. In the **Command Name** drop-down menu, select **Fill 36 cm capillary array** and click **Send Command**. The main objective of a short-term shutdown is to preserve the array by filling it with polymer and keeping the capillary ends submerged in buffer or water. Steps 4-6 may optionally be performed.

4. **Exit** Data Collection software application.

5. Depress the 3130/3130*xl* Genetic Analyzer power button to shut down the instrument.

6. Turn off the computer by selecting **Shutdown** from the **Start** menu.

Section 3.3.4 REFERENCES FOR THE 3130/3130*xl* GENETIC ANALYZERS

Applied Biosystems (2004), "3130/3130*xl* Genetic Analyzers Getting Started Guide," Applied Biosystems P/N 4352715.

Applied Biosystems (2004), "3130/3130*xl* Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide," Applied Biosystems P/N 4352716, Rev. B.

Applied Biosystems (2004), "3130/3130*xl* Genetic Analyzers Quick Reference Card," Applied Biosystems P/N 4362825.

Applied Biosystems (2005), "3130/3130*xl* Genetic Analyzers Using Data Collection Software v3.0," User Bulletin, Applied Biosystems P/N 4363787, Rev. A.

Section 3.3.5 APPENDIX I - Creating New Results Groups and Instrument Procedures

This procedure describes the creation of plate records using the Results Group **Identifiler_Generic** and the Instrument Protocol **Identifiler5sec**. If these features are not yet created on the 3130/3130*xl* instrument to be utilized, create them by following the steps below.

3.3.5.1 Creating New Results Groups

1. In the tree pane of the Data Collection software, go to **GA Instruments > Results Group**.

2. Click **New…** to open the **Results Group Editor**.

3. On the **General** tab, type in “Identifiler_Generic” for the name of the Results Group.

4. On the **Analysis** tab, select “GeneMapper-Generic” as the Analysis Type.

5. On the **Destination** tab, check the box next to **Use Custom Location** and type in “E:\Extracted Data” as the custom location.

6. On the **Naming** tab, type "RF" as the Prefix in the Run Folder Name Format section and ensure that "_" is the selected Name Delimiter. "Plate Name" should be chosen from the drop-down menu in the first field under Format and the other two fields should remain "none".

   Also while on the **Naming tab**, choose the desired Sample File Name Format (*e.g.,* well position, sample name, capillary number) using the format drop-down menu.

7. Click **OK** to save the Results Group.

3.3.5.2 Creating New Instrument Procedures

Because the **Identifiler5sec** procedure utilizes the Run Module **Identifiler5sec**, this Run Module must be created before establishing the **Identifiler5sec** procedure.

1. To create the **Identifiler5sec** Run Module, in the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl > <*instrument name*> > Module Manager** and click **New** to open the **Run Module Editor** dialog box.

2. Complete the **Run Module Editor** dialog box:
   - Type "Identifiler5sec" as the name for the new module.
   - Select **Regular** in the Type drop-down list.
   - Select **HIDFragmentAnalysis36_POP4** in the Template drop-down list.
   - If desired, type a description for the procedure.
   - The **Identifiler5sec** parameters are identical to those of the HID default module on the 3130 Genetic Analyzer. Therefore, no changes need to be made to the Run Module Settings for the 3130. However, for the 3130*xl* Genetic Analyzer, the **Identifiler5sec** injection time parameter is different from that of the HID default module. Therefore, change the **Injection_Time** parameter from 10 seconds to 5 seconds *for the 3130xl*. The remaining parameters are identical.
   - Click **OK**.

3. Next, create the Instrument Protocol. In the tree pane of the Data Collection software, go to **GA Instruments > /ga3130xl > Protocol Manager** and click **New** to open the **Protocol Editor**.

4. Complete the **Protocol Editor** dialog box:
   - Type "Identifiler5sec" as the name for the procedure.
   - If desired, type a description for the procedure.
   - Select **Regular** in the Type drop-down list.
   - Select **Identifiler5sec** in the Run Module drop-down list.
   - Select **G5** in the Dye Set drop-down list.
   - Click **OK** to save the Instrument Protocol.

Section 3.3.6 APPENDIX II - Electronic File Naming Conventions for 3130/3130*xl* STR Analysis

The instrument run number and the case number are used to facilitate electronic data tracking. Each casework 3130 or 3130*xl* instrument is assigned an identifier, and each run is assigned a consecutive number. For purposes of illustration, this is simply referred to as "#MCE#" in the examples below. In the circumstance where two or more of the same file/folder types are generated on the same day, they will be distinguished by the addition of -2, -3, etc. (e.g., CaseNumber GM-2 *mmddyy* Analyst's Initials).

Note: The software will automatically add the appropriate extensions to file names. The following is a list of extensions used by Data Collection and GeneMapper ID/ID-X:

Sample file	.fsa/.hid
Plate record	.plt/.txt
Project	.ser
Size standard	.szs/.xml
Analysis parameters	.xml
Plot settings file	.xml
Panels and bins	.txt

3.3.6.1 Run set-up documents

Sample name (case sample):	CaseNumber_Sample Name…(up to 14 characters)[2]
Sample name (control):	#MCE# _Sample Name… (up to 24 characters total)[2,3]
Plate record name:	#MCE#_*mmddyy* [4,5,6]

[2]The 3130/3130*xl* Genetic Analyzer Data Collection software will automatically add the well position as a prefix and the capillary number as a suffix to the sample name (*e.g.*,B01_ CaseNumber_Sample Name_002, E04_#MCE# _Sample Name _001). Duplicate injections of the same sample will be appended with .2, .3 etc. (e.g.,B01_ CaseNumber_Sample Name_002.2).

[3]Control samples are identified with the instrument run number (e.g., #MCE# ladder, #MCE# FAM). When cases are batched, this includes the batched control samples (*e.g.,* quality control sample, reagent blanks, positive and negative amplification controls). When needed for clarity, the case number may alternatively be used. For plates set up on the Tecan HIDEVO, an instrument run number is not generally used as part of the sample file name since that is determined later in the process flow. Instead, Plate ID (amplification plate name) is automatically appended to all sample files.

[4]This date ("*mmddyy"*) is the date of the 3130/3130*xl* run.

[5]The plate names may include an "A" or "B" if two plates were run together on a 3130*xl*. This distinguishes the run folders from one another (e.g., 16MCE5A_mmddyy and 16MCE5B_mmddyy).

[6]Other unique identifiers, such as the amplification plate name (*e.g.,* date run and analyst initials), may be used to name the plate record and run folder.

3.3.6.2 Post run documents

Run Folder: RF_ #MCE#_ *mmddyy*[4]
Case Folder: CaseNumber CF Analyst's Initials
Batch Folder: RunFolderUniqueIdentifier BF Analyst's Initials

The 3130/3130*xl* Collection software automatically creates run folders. The plate record name will be appended automatically as a suffix to the run folder name.

If multiple runs are performed for a case, the additional run folders are added to the Case or Batch Folder.

3.3.6.3 GeneMapper ID documents

GeneMapper Project: CaseNumberIP GM *mmddyy* Analyst's Initials[1,5]
2nd Reader Project (optional): CaseNumberIP GM 2nd reader *mmddyy* Reviewer's Initials[1,5, 6]

[1]Use "IP" for samples amplified with the Identifiler Plus kit.
[5]The project date is the date the project was created.
[6]Analysis files generated by the second reader are saved to the Case/Batch Folder.

Note that a Run Folder unique identifier may be used for a batch project, as opposed to Case Number for a case-specific project.

See Section 3.9 for GeneMapper ID-X electronic file naming conventions.

3.3.6.4 Case/Batch Folder organization

The following example demonstrates typical Case Folder organization:

CaseNumber CF Analyst's Initials
RF_ #MCE#_ *mmddyy*
#MCE#_*mmddyy* (plate record)
(Sample files)
CaseNumberIP GM *mmddyy* Analyst's Initials

The following example demonstrates typical Batch Folder organization:

#MCE# BF Analyst's Initials
RF_ #MCE#_ *mmddyy*
#MCE#_*mmddyy* (plate record)
(Sample files)
#MCE#IP GM *mmddyy* Analyst's Initials

Section 3.3.7 APPENDIX III - 3130/3130*x*/ Genetic Analyzer Computer Maintenance

Before a run will start, the Data Collection software automatically checks the space on drives C, D, E, and F to ensure that there is sufficient space to store the newly created sample file data. All runs are stored to both D and E drives. The Data Collection software will send a warning message if a drive is getting full. For the D drive, which houses the Data Collection Oracle database, this message occurs when the drive is 70-75% full.

To check drive space:

- In the tree pane of the Data Collection software, go to **GA Instruments > Database Manager** to open the **Database Manager** window.
- Check the space available on each drive under the **Free Disk Space Status** section.
- If there is insufficient space in the database for additional runs, the Data Collection software will prompt you in the **Database Status** section to "**Cleanup Processed Plates**." This should be performed promptly (see instructions below). At 78% full, the software will not start a run.

If a warning message is generated when a run is started, the system status light in the bottom left-hand corner of the data collection window will flash red. View the message by going to **GA Instruments > /ga3130xl > <*instrument name*> > Instrument Status > Event Log**. The log will describe the reason for the warning message. To delete runs from the D and E drives, see the procedures described below. Once completing the message request, click the **Clear Errors** button in the bottom right corner of the **Event Log** to clear the errors and stop the system status light from blinking.

The **Cleanup Processed Plates** utility will erase all runs in the Oracle database. It will additionally delete all plate records and processed frame data in the database. Do not run this utility more than once a day as previously extracted sample files may be overwritten. Alternatively, plate records and runs may be manually deleted from the database one at a time.

To perform the **Cleanup Processed Plates** utility on the **D drive**:

- In the tree pane of the Data Collection software, go to **GA Instruments > Database Manager** to open the **Database Manager** window.
- Click **Cleanup Processed Plates** button.
- Click **OK** in the dialog box that opens. It may take several minutes to cleanup the database if it contains a lot of data or is full.

Note: *Spatial and Spectral Calibrations are not deleted by the Cleanup Processed Plates utility.*

The Cleanup Processed Plates utility does not erase the extracted runs from the E drive. Runs on the E drive should be manually deleted periodically. It may be most convenient to do this when Cleanup Processed Plates is run on the D drive.

To clean-up the **E drive** space:

- Open the **E drive** and locate the **Extracted Data** folder.
- Verify that the run folders have been transferred for analysis and archiving.
- Transfer the run folders to be deleted to the **Recycle Bin** and **Delete.**

Section 3.7 Identifiler Plus Mixture Interpretation Procedures and Guidelines

Section 3.7.1 Overview

Introduction

This section contains information, guidance, and procedures on the deconvolution and documentation of DNA mixtures. A mixture is a DNA typing result originating from two or more individuals. Because an individual's contribution to a mixed biological sample is generally proportional to their yield in PCR product, DNA results of mixed samples may be further refined through qualitative and quantitative assessments. Examples of such assessments include identification of foreign alleles, minimum peak height ratio expectations, and mixture proportion evaluations. The deconvolution of a mixed DNA sample is the separation of contributors based on such assessments and underlying assumptions.

Discernment of typing results through deconvolution may effectively constitute a single-source profile, such as the major contributor in a two-person mixture. In other instances, discernment of genotypes may be limited to only some loci, yielding mixed or partial profiles. Mixed or partial profiles may be used for comparison to other profiles when an appropriate statistical calculation can be made, should an inclusion result from such comparison.

Genetic Analyzer Model

This procedure was originally written for Genetic Analyzer 3130/3130*xl* data. It has since been updated to incorporate Genetic Analyzer 3500/3500xL data. The concepts between the two platforms are the same despite the difference in the scale of the signal. However, note that many of the examples given throughout this procedure use the 3130/3130*xl* scale.

For simplicity, where noted, "3130" refers to both the 3130 and 3130*xl* models and "3500" refers to both the 3500 and 3500xL models.

Contents The following topics are covered:

Section 3.7.2 Manual Deconvolution of Mixed DNA Profiles

Section 3.7.2.1 Overview

Purpose This section contains guidance and procedures on the deconvolution of DNA mixtures without the use of MixMaster. As part of mixture deconvolution, assumptions made are reviewed throughout the process to ensure an appropriate and scientifically sound interpretation.

Note that MixMaster mimics the manual mixture deconvolution approach.

Contents The following outlines the steps used in the recognition and deconvolution of DNA mixtures.

Procedural Steps
1. Identify Alleles from Artifactual Peaks
2. Determine Whether the Sample is a Mixture
3. Determine Whether There are Known Contributors that can be Assumed to be Present
4. Determine How Many Contributors are Present in the Mixture
5. Determine if All Alleles can be Assumed to be Present
6. Determine the Mx to be Used for Deconvolution
7. Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes
8. Apply the Calculated Mx Range to Determine Possible Unknown Donor Genotypes

The above steps are illustrated for 4-allele, 3-allele, and 2-allele locus interpretation of two-person mixtures in Appendix II.

Section 3.7.2.2 Identify Alleles from Artifactual Peaks

Allele or artifact?

Perform the following steps to determine which peaks will be called alleles as opposed to artifacts. Note that Section 3.10.5 contains more information on artifactual peaks and this step may have already been accomplished as part of GeneMapper analysis.

Step	Action
1.	Edit non-allelic artifactual peaks as appropriate. This includes: • Pull-up/pull-down • Non-reproducible artifacts (*e.g.,* spikes) • Reproducible artifacts (*e.g.,* dye blobs, non-specific amplification artifacts)
2	Determine which DNA peaks are callable peaks and edit as appropriate. This is framed by the following criteria: • Analytical threshold (AT) – 50 RFU (3130), 150 RFU (3500) • Stochastic threshold (ST) – 365 RFU (3130), 1,075 RFU (3500) • Presence of a possible DNA peak in at least two injections • N-4 stutter – see Section 3.10.5 for locus filters • N+4 stutter – see Section 3.10.5 for tiered global filters • Incomplete non-templated nucleotide addition
3.	Go to Section 2. *Determine Whether the Sample is a Mixture*

Section 3.7.2.3 Determine Whether the Sample is a Mixture

Is it a mixture? Perform the following steps to determine if the sample may be a mixture of DNA sources. See Section 3.10.6 for additional information.

Step	Action
1.	Assess the following to determine whether the sample is a mixture:

If ...	Then ...
Only one locus shows three alleles while the remaining loci are consistent with a single source sample,	The sample may be a mixture, however the possibility of a triallelic genotype in a single-source sample should be considered.
More than two alleles are observed at more than one locus,	The sample is likely a mixture.
Heterozygous peak height ratios show imbalance below the minimum expectations but there are no more than two alleles at each locus,	The sample may not be a mixture. The sample may be exhibiting stochastic effects due to low template.
Only one locus shows two imbalanced alleles while the remaining loci are consistent with a single source sample,	The possibility of a primer binding site mutation or triallelic genotype (three genetic copies with two alleles being the same) should be considered.

2.	If the sample *is* considered to be a mixture, continue to *Determine Whether There are Known Contributors that can be Assumed to be Present*.
3.	If the sample *is not* considered to be a mixture, return to Section 3.10 to complete the interpretation.

Section 3.7.2.4 Determine Whether There are Known Contributors that can be Assumed to be Present

Known donor?

Apply the following criteria to determine whether a known contributor can be assumed to be present in the data. See Sections 3.7.4.2 for more information on assumed donors.

Step	Action
1	<table><tr><th>If ...</th><th>Then ...</th></tr><tr><td>You *can* reasonably assume the presence of a known contributor,</td><td>The conditional known individual's profile may be subtracted from the mixture.</td></tr><tr><td>You *cannot* reasonably assume the presence of a known contributor,</td><td>The mixture should be interpreted blindly.</td></tr></table>
2	Continue to *Determine How Many Contributors are Present in the Mixture.*

Section 3.7.2.5 Determine How Many Contributors are Present in the Mixture

How many contributors?

Perform the following steps to determine how many contributors are present in a mixture.

Step	Action
1	Determine how many contributors are present in the mixture. <table><tr><th>If ...</th><th>Then ...</th></tr><tr><td>There are *no more than 4 alleles* at each locus,</td><td>The sample is a probable 2-person mixture.</td></tr><tr><td>There are loci with *more than 4 alleles* **but** *no more than 6 alleles,*</td><td>The sample is a probable 3-person mixture.</td></tr><tr><td>There are loci with *more than 6 alleles,*</td><td>The sample contains DNA from at least 4 individuals.</td></tr></table> ***Note:*** Peak height ratio imbalance may indicate an additional donor(s).

2	If there is an assumed donor, verify the number of contributors. **If ...** / **Then ...** There are *no more than 2 foreign alleles* at each locus, — The sample is a probable 2-person mixture. There are loci with *more than 2 foreign alleles* **but** *no more than 4 foreign alleles,* — The sample is a probable 3-person mixture. There are loci with *more than 4 foreign alleles,* — The sample contains DNA from at least 4 individuals.
3	If it is not possible to clearly determine an exact number of contributors to be assumed, this should be clearly reflected in the bench notes and suitability for comparisons may be curtailed. ***For example,*** a mixture may have a clear major donor but possibly more than one minor donor. In this situation, it should be made clear that there are *at least* two donors. The determined profile for the major donor would be suitable for comparison. A determined profile(s) for the minor(s) would be suitable for comparison if an assumption of one or two minor contributors could be made. Furthermore, it may be fitting to interpret the minor donor under two scenarios for two different results (assuming one versus two minor donors).
4	Continue to *Determine if All Alleles can be Assumed to be Present*.

Section 3.7.2.6 Determine if All Alleles can be Assumed to be Present

All alleles detected?

Apply the following criteria to each locus to determine if all alleles can be assumed to be present in the data. In general, it is easier to determine whether all alleles have been detected with increasing peak height and for those loci with a greater number of alleles present (*i.e.,* 2N or 2N-1 where N is the number of assumed individuals).

If ...	Then ...
All alleles assumed to be unshared are above the stochastic threshold, taking stutter into account,	It may be possible to assume that all alleles have been detected for each contributor. ***Note:*** This may not be the case if there is significant differential degradation or allele overlap.
• The mixture is a major/minor mixture • The major donor is the profile to be deconvoluted • The unshared major donor peaks are all above the stochastic threshold,	It can be assumed that all alleles have been detected for the major donor.
• The mixture is a major/minor mixture • The minor donor is the profile to be deconvoluted,	It may be possible to assume all alleles have been detected for the minor contributor if: • There is one minor donor • If single minor peaks are above the stochastic threshold, after taking stutter into account. • For the loci with two minor peaks, those peaks appear to pair together and both are at or above the analytical threshold.
The data is the result of a three-person mixture,	It may be possible to assume all alleles have been detected for one or more contributors depending on peak heights and allele stacking.

Notes: It is common practice to assume the detection of all alleles for a major donor while not detecting all alleles for the minor donor. It is also possible to assume the detection of all alleles for a donor(s) at some loci but not others.

Assumed or not?

Once it has been determined whether it is reasonable to assume that all alleles were detected, proceed as follows:

If ...	Then ...
All alleles *are* assumed to be present at a locus,	The possible genotype scenarios using only the detected alleles are considered, including potential alleles in artifactual positions.
All alleles *are not* assumed to be present at a locus,	The mixture should be interpreted with the assumption that there may be one or more alleles undetected. This typically results in an allele call(s) at the relevant locus.

Continue to *Determine the Mx to be Used for Deconvolution.*

Section 3.7.2.7 Determine the Mixture Proportion (Mx) to be Used for Deconvolution

Mx use

A mixture proportion (Mx) is utilized to assist in the determination of donor genotypes for mixtures of varying donor proportions, either with or without an assumed donor. This includes both clear major/minor donor situations as well as those where the donor proportions are more similar (*i.e.,* "indistinguishable").

A mixture proportion can be calculated to distinguish between not only two donors but various combinations of donors when there are more than two individuals present.

See Section 3.7.4.3 for additional information.

Criteria for determining Mx

Mx values are determined for a mixture interpretation by assessing the Mx from individual loci with both:

- Non-overlapping donor alleles
- All alleles assumed to be detected for all donors

This is most obvious at loci where there are two alleles observed for each contributor (*e.g.,* four alleles in a two-person mixture).

Calculating Mx; Assumed Donor

Follow the steps below for Mx determinations from a *two-person mixture* in which **one donor is assumed to be present**.

For 4-allele loci:

Step	Action
1	Identify all loci with 4 alleles.
2	Determine which donor appears to be "minor" (at overall lower RFU). The peak height total for this donor will need to *always* be in the numerator for the following calculations.
3	Add the peak heights of the alleles for the minor donor.
4	Add the peak heights of all four alleles.
5	Divide the total for the minor donor alleles by the sum of the peak heights from all four alleles. This is the Mx, or Mixture proportion, for this locus.
6	Repeat this process at as many 4-allele loci as possible.

For 3-allele loci:

Step	Action
1	Identify all loci with 3 alleles where the *assumed donor is homozygous* and the unknown donor appears to be heterozygous.
2	Determine which donor appears to be the "minor" donor. The peak height total for this donor will always be in the numerator for the following calculations. ***Note:*** The *same* donor used in the numerator for the 4-allele locus calculations has to be in the numerator for the 3-allele locus calculations.
3	If the minor donor is heterozygous, add the peak heights for this donor.
4	Add the peak heights of all three alleles.
5	Divide the total for the minor donor alleles by the sum of the peak heights from all three alleles. This is the Mx, or Mixture proportion, for this locus.
6	Repeat this process at as many 3-allele loci as possible where the *assumed donor is homozygous*.
7	Proceed to "**Average Mx & Mx range**."

Calculating Mx; No Assumed Donor

When **a donor cannot be assumed** to be present and mixture proportions are needed for interpretation, Mx values may be determined for a *two-person mixture* using 4-allele loci as described below:

Step	Action
1	Identify a locus with 4 alleles.
2	Add the peak heights of the two shortest alleles ("minor donor").
3	Add the peak heights of all alleles.
4	Divide the total for the minor donor by the total for all alleles. This is the Mx, or Mixture proportion, for this locus.
5	Repeat Steps 1- 4 until all 4-allele loci have an Mx.
6	Proceed to "**Average Mx & Mx range**."

Three allele loci may be used when **a donor cannot be assumed** for a *two-person mixture* only if the minor peaks pair exclusively with one another (*i.e.,* a clear major homozygote and minor heterozygote).

Step	Action
1	Identify the 3-allele locus in which there is a clear major homozygote and minor heterozygote.
2	Add the peak heights of the two shortest alleles ("minor donor").
3	Add the peak heights of all three alleles.
4	Divide the total for the minor donor by the total for all alleles. This is the Mx, or Mixture proportion, for this locus.
5	Average this Mx in with the Mx values above from the 4-allele loci.

Average Mx & Mx range

After determining the Mx's for each locus, determine the average Mx and range of acceptable Mx values as follows:

Step	Action
1	Average together all Mx values calculated from 4-allele and 3-allele loci. ***Note:*** All individual Mx's should be calculated with the minor donor in the numerator. Similarly, for 1:1 mixtures with an assumed contributor, the same donor should always be in the numerator.
2	If the Mx is needed for the major donor, subtract the average from 1: average major Mx = 1 – average minor Mx
3	Add 0.3 to the average Mx. This is the upper value for the Mx range.
4	Subtract 0.3 from the average Mx. This is the lower value for the Mx range.
5	Continue to *Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes*.

Note: The Mx range may be adjusted as appropriate within 0.2 – 0.5.

Section 3.7.2.8 Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes

PHR use

Heterozygous peak height ratios (PHRs) are utilized to assist in the determination of donor genotypes for mixtures of varying donor proportions. This includes both clear major/minor donor situations as well as those where the donor proportions are more similar ("indistinguishable").

Acceptable PHR thresholds

The minimum acceptable PHR used for a mixture interpretation is dependent upon the peak height of the taller allele for the particular two peaks in question, as follows:

For 3130:

If the taller peak is...	Then ...
The taller peak is at least 1200 RFU	The minimum acceptable PHR is 50%
The taller peak is 750-1199 RFU	The minimum acceptable PHR is 40%
The taller peak is 365-749 RFU	The minimum acceptable PHR is 30%
The taller peak is <365 RFU	Any PHR is acceptable

For 3500:

If the taller 3500 peak is...	Then ...
The taller peak is at least 5000 RFU	The minimum acceptable PHR is 50%
The taller peak is 2700-4999 RFU	The minimum acceptable PHR is 40%
The taller peak is 1075-2699 RFU	The minimum acceptable PHR is 25%
The taller peak is <1075 RFU	Any PHR is acceptable

Typically, when a PHR is below the minimum expectation in a mixed DNA sample, the data is best explained by there being more than one donor present. Mixture interpretation assumptions should be re-evaluated throughout the deconvolution process, particularly the number of contributors assumed to be present.

Calculate mixture PHRs

Perform the following steps to apply the PHR thresholds to the mixture data.

Step	Action
1	At each locus, calculate the PHRs for potential heterozygote allele pairings. This is typically performed using a 100% PHR as a first pass and testing whether each peak may be shared. Some peaks will obviously not be shared based on peak height (and assumed donor genotype, if relevant) while others will require calculations. **Example:** A two-person mixture with 3 peaks at the locus being evaluated: 600 RFU (unshared, donor A), 1300 RFU (testing if shared), 800 RFU (unshared, donor B). Subtract the height of one unshared peak from the height of the shared allele. The PHR for the other donor is the remainder paired with the unshared allele from the second donor. Therefore, 1300–600 = 700; 700/800 = 87.5% PHR; or 1300-800 = 500; 500/600 = 83%.
2	Apply the appropriate minimum expected PHR thresholds based on the peak height of the taller peak for each pairing to determine acceptable versus rejected pairings. **Example:** The PHR of 87.5% (or 83%) exceeds the minimum expectation and therefore the genotype would be accepted as a possibility based on the PHR.
3	If a PHR does not meet or exceed the minimum expectation, it is appropriate in most instances to check whether the genotype(s) would be included when calculating PHRs based on a minimum contribution. See the Steps below for *"Minimum contribution."* **Example:** A two-person mixture with 3 peaks at the locus being evaluated: 400 RFU (unshared, donor A), 900 RFU (shared), 1300 RFU (unshared, donor B). Using a 100% PHR, 900–400 = 500; 500/1300 = 38.5% PHR. This is below the 50% minimum. ***Note:*** If the maximum contribution needs to be calculated, go to that section below.
4	Continue to *Apply the Calculated Mx Range to Determine Possible Unknown Donor Genotypes.*

Minimum contribution

To calculate a minimum contribution, perform the following steps:

Step	Action
1	Identify a peak of one donor to be tested as *unshared* and use its height to determine the minimum acceptable PHR. **Example:** Unshared peak is 400 RFU so 30% will be used.
2	Multiply the RFUs of the unshared peak by the minimum acceptable PHR. **Example:** 400 RFU X 0.30 = 120 RFU So, 120 RFU is used as the minimum peak height contribution to the shared peak.
3	Subtract the minimum peak height contribution from the shared allele. **Example:** 900-120 = 780 RFU
4	The PHR for the other donor is the remainder paired with the unshared allele from the second donor. **Example:** 780/1300 = 60%
5	Apply the appropriate minimum expected PHR threshold to the adjusted PHR for the pairing (Step 4 above) to determine whether the genotype is now acceptable.

Maximum contribution

To calculate the maximum contribution of an allele and the corresponding PHR, perform the following steps:

Step	Action
1	Using the *unshared* peak, identify the minimum acceptable PHR. **Example:** Peak is 120 RFU so 30% will be used.
2	Divide the RFUs of the unshared peak by the minimum acceptable PHR. **Example:** 120 RFU / 0.30 = 400 RFU

3	Evaluate within which minimum PHR the newly calculated peak height falls. **Example:** For 400 RFU, the minimum expected PHR is 30%.
4	If the minimum remains the same, determine the PHR for the second donor using the maximum contribution for the first donor and assess whether the PHR is accepted and the genotype is therefore possible. Otherwise, continue to Steps 5 and 6.
5	Repeat Step 2 using the different minimum acceptable PHR. **Example:** See Example B below.
6	If the minimum PHR expectation is straddling two ranges, the maximum RFU value for the lower range is typically used. **Example:** See Example C below.

Examples

The following are examples of how the maximum contribution is used.

- Example A: Unshared peak = 120 RFU
  1.) 120/0.30 = 400 RFU; stop here and use 400 RFUs as maximum. contribution to shared peak
- Example B: Unshared peak = 350 RFU
  1.) 350/0.30 = 1167 RFU; outside 30% range so go to # 2.
  2.) 350/0.40 = 875 RFU; stop here and use 875 RFUs as maximum contribution to shared peak
- Example C: Unshared peak = 250 RFU
  1.) 250/0.30 = 833 RFU; outside 30% range so go to # 2.
  2.) 250/0.40 = 625 RFU; outside 40% range so go to # 3.
  3.) Use a value of 749 RFU for the maximum contribution because it is the maximum value for the 30% range (*i.e.,* 250/749 = 33%).

Section 3.7.2.9 Apply the Calculated Mx Range to Determine Possible Unknown Donor Genotypes

Applying Mx range

Perform the following steps to apply the Mx range to mixture data.

Step	Action
1	Using the potential allele pairings not excluded based on PHRs, calculate Mx values at each locus.
2	Compare each Mx value to the Mx range to determine which pairings are possible based on peak height ratios and mixture proportions.
3	It may be necessary to evaluate whether a genotype possibility excluded based on Mx might actually be included when the PHRs are adjusted using the *Minimum or Maximum contribution* described in *Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes*.

Section 3.7.3 MixMaster

Section 3.7.3.1 Overview

Introduction

MixMaster IDP is a two-person mixture deconvolution tool that has been validated for use with *Identifiler Plus* results. MixMaster is not an Expert System. It is not meant to replace the criminalist's assessment and judgment. MixMaster is a calculation tool that mimics the manual deconvolution approach.

The purpose of this calculation tool is to assist the analyst in determining the allowable genotypes given the Bureau-defined thresholds. However, the ultimate interpretation is dependent upon the analyst's review of all available data within the context of the Bureau's procedures. The analyst may add or redact genotypes based on the evaluation of the data and the reasoning shall be documented in the notes.

Contents

This section contains the following topics:

Section 3.7.3.2 MixMaster Functionality

How it works

Mixture deconvolution is based upon the concepts of relative peak heights and the additive nature of overlapping alleles between two contributor's genotypes. Foundational discussions of 2-person mixture interpretation and deconvolution can be found in Clayton *et al*. (FSI 41 1998 55-70) and Gill *et al*. (FSI 41 1998 41-53).

MixMaster does not use the concept of "residuals" as discussed in Gill *et al*. and applied in the mixture application of Life Technologies/Applied Biosystems GeneMapper ID-X. For a combination of genotypes to be allowed, the individual PHRs and the minor donor mixture proportion should fall within the accepted, Bureau-defined ranges. The MixMaster approach is similar, but not identical, to that described in Gill *et al.* (FSI 148 2005 181–189) and applied in FSS-i^3.

MixMaster adjusts the observed data, cycling through randomized locus mixture proportions and stutter levels. With each cycle, it assesses which pairs of major and minor contributor genotypes would be allowed given predetermined peak height ratios, randomized stutter (N+4 and N-4) subtraction, and mixture proportion thresholds. Possible genotypes take into account allelic drop out ("F") as well as minor contributor alleles overlapping major contributor stutter and incomplete nucleotide addition (INA) peaks.

The following flow chart is a graphical representation of the MixMaster process.

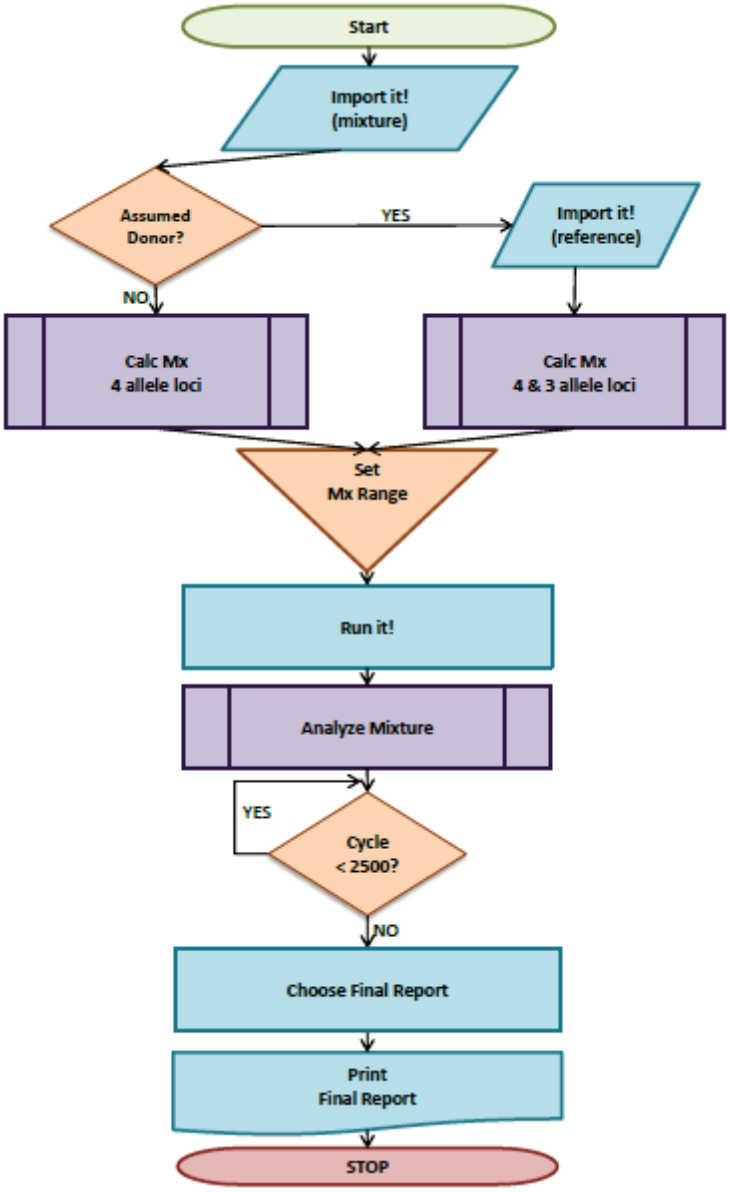


Figure 1

Initial mixture assessment

Prior to using MixMaster, the candidate mixture should be assessed to establish whether it is appropriate for deconvolution with MixMaster. It should meet the following criteria:

- Two-person mixture
- Reasonable to assume bi-allelic donors with no null alleles
- Inter-locus mixture proportions are not excessively affected by inhibition or differential degradation (one template DNA source degraded to a different degree than the second template DNA source)
- Intra-locus balance not excessively affected by degradation (especially for wide-spread alleles)

Important!

Prior to proceeding to deconvolution, *STR results imported into MixMaster should always be checked for* correct allele and artifactual peak assignments. Verify that peaks in stutter positions belong in those positions rather than artifact slots. If needed, manual edits should be made by the analyst.

MixMaster should not be used for mixtures that appear to have significant inhibition or differential degradation (a greater degree of degradation in one contributor's DNA than in the other's) as the donor proportions will potentially vary considerably across the entire profile.

MixMaster assumes bi-allelic donors with no imbalances caused by primer binding site mutations. Both of these genetic anomalies may negatively affect the observed Mx.

Assumed donor *partial* profiles in MixMaster

When an assumed contributor is used, MixMaster will calculate Mx at all loci meeting the stated criteria. If the assumed profile is incomplete, Mx will only be calculated at those loci with assumed donor genotypes.

For example, if D21S11, D2S1338, and FGA are all amenable to calculating Mx, and the assumed contributor's genotypes are entered at all three loci, then all three individual locus Mx values will be averaged for the overall Mx. However, if the assumed contributor's profile is incomplete and lacks D2S1338 and FGA, then the Mx will be based solely upon D21S11.

If the assumed contributor's profile lacks results for all three loci, MixMaster will revert to the Mx calculated when there is no assumed contributor.

MixMaster Override Mx

The user may use an override Mx value in the following situations:

- If the user determines that the average Mx is skewed
  - For example, artifacts affecting the peak height(s) of one contributor,
- When there are no four-allele loci or three-allele loci with an assumed homozygote

An override Mx may be set using the approach described in Appendix I.

Thresholds in MixMaster

The *analytical threshold* is the peak detection threshold set during data analysis. The *stochastic threshold* is the RFU value entered in the Peak Height Ratio section "Start Range 2."

F alleles in MixMaster

When three or fewer alleles are detected at a locus, MixMaster will consider the presence of drop-out alleles. Possible drop-out alleles are given an F designation. Because F alleles fall below the analytical threshold, their peak heights are unknown and can range from 0 RFU up to one RFU below the analytical threshold. For example, with a 3130 analytical threshold of 50 RFU, an F allele could range from 0 to 49 RFU. Similarly, an F allele could range from 0 to 149 RFU on the 3500. For each cycle, MixMaster will randomly assign an RFU level within the relevant range when evaluating possible genotypes that include F alleles.

PHRs in MixMaster

MixMaster allows for a total of four PHR ranges, mirroring those ranges used for single-source sample genotyping and manual mixture deconvolutions (see *Use Heterozygous Peak Height Ratios for Determining Possible Unknown Donor Genotypes* and Section 3.7.4.3 of this procedure for more information). Specifically, the RFU value entered is the starting point of the range, and the PHR entered will be applied to all genotypes where the taller allele is at or above the starting point RFU but below the starting point RFU for the next range.

Range 1 is for heterozygous genotypes where the taller allele falls within the stochastic range. In Range 1, the predefined stochastic PHR setting is 0%, meaning that allelic drop-out can occur.

The starting point of Range 2 defines the RFU at which genotypes are no longer in the stochastic range. In other words, this is when the taller allele is at or above the stochastic threshold and the second allele is expected to be detectable above the analytical threshold.

For example, a possible genotype pairing of alleles with heights of 520 and 910 RFU would be assessed using a 40% PHR threshold, because the taller allele was above the starting point for the 40% PHR range (750 to 1199 RFU) but less than the starting point for the 50% PHR range (1200 and higher).

Filtered Stutter in MixMaster

The stutter thresholds in MixMaster are preset and based upon values established by the Bureau (see Section 3.10.5 for more information). These thresholds correspond to those used for single-source sample genotyping and manual mixture deconvolutions.

Because minor contributors can have alleles that are filtered in GeneMapper due to overlap in stutter positions, stutter peaks are also imported and considered in MixMaster deconvolutions at each cycle. The deconvolution calculations will allow for the filtered stutter peak to be anywhere from 0 RFU up to its full height.

Alleles in Stutter Positions

The MixMaster deconvolution calculations will allow for the stutter contribution from a neighboring allele to be anywhere from 0% up to the maximum stutter percentage.

For example, if a locus with a 10% N-4 threshold and 9.2% N+4 threshold has three successive alleles, 7, 8, and 9, MixMaster will…

- Subtract anywhere in the range 0-10% the full height of allele 8 from the height of allele 7;
- Subtract anywhere in the range 0-10% the full height of allele 9 <u>and</u> anywhere in the range 0-9.2% the full height of allele 7 from the height of allele 8; and
- Subtract anywhere in the range 0-9.2% the full height of allele 8 from allele 9

Notes:

- The stutter-subtracted heights of the alleles are never used to determine the possible amount of stutter that will be subtracted from a neighboring peak.
- Peaks that have been filtered as possible stutter are also used to subtract for possible stutter.

INA in MixMaster

Because minor contributors can have alleles that are filtered or deselected in GeneMapper due to overlap in incomplete nucleotide addition (INA) positions, INA peaks are also imported and considered in MixMaster deconvolutions.

For each cycle, the height of the "allelic" portion of the INA peak will be anywhere from 0 RFU (*i.e.,* the allele actually dropped out and the peak is 100% INA) to the full height of the peak (*i.e.,* the peak is 100% allele with no actual INA).

Note: Peaks that have been designated as possible INA are also used to subtract for possible stutter, when relevant.

Shared alleles in MixMaster

When considering possible genotype pairings, MixMaster calculates the relative contribution to a shared allele from each donor based on the randomized Mx at each cycle. This process allows for one or both contributors to have a low but acceptable PHR.

For example, for three alleles (A, B, and C) where the tested pairing is A,B and B,C, MixMaster performs the following:

1. A randomized Mx is determined that falls within the allowed Mx range.
2. The minor contributor's contribution to the shared allele would be calculated as their total proportion of the locus RFU (*i.e.,* the randomized Mx multiplied by the total RFU for A, B, and C) minus the RFU of their unshared allele. If this results in a negative number, MixMaster will instead proceed with 0 RFU for the peak height.
3. The resultant peak heights for the two genotypes would then be compared to the PHR thresholds and the allowable Mx range for compliance.

Randomization

The randomization of cycling in MixMaster applies what has been described as a binary approach to interpretation. MixMaster does not take a probablistic approach to mixture interpretation. All variables are allowed to vary equally across their range instead of being varied through the application of probability curves.

For example, if the stutter threshold is 15%, then all possible levels of stutter from 0% up to 15% stutter are treated as equally possible. This binary approach is well suited to the application of a Random Match Probability statistic.

MixMaster caveat

A manual mixture interpretation might identify a pair of genotypes that would be deemed possible, but only through the combination of numerous rare events. Analyses using MixMaster may not include those combinations when the combined rarity of the event is much lower than the inverse of the number of cycles selected. In other words, for any given mixture, genotype combinations may exist that are mathematically possible when taking several of the variables to their limits. MixMaster may not necessarily test such combinations due to the randomization of cycling. An analyst considering such situations in a result will likely need to supplement MixMaster results with manual calculations.

For example, when a genotype combination would require multiple instances of maximum N-4 stutter and maximum N+4 stutter subtraction and the Mx to be on the edge of the range, the combination may be included in a manual deconvolution but not necessarily for a MixMaster deconvolution. The combined chance of such random, extreme boundary assignments occurring concurrently is unlikely to be included by 2,500 cycles.

Section 3.7.3.3 Preparing GeneMapper results

Exporting GeneMapper txt files

To prepare import files for MixMaster, perform the following steps in GeneMapper ID/ID-X to create export tables.

Step	Action
1	Open a GeneMapper project.
2	In the "Samples" tab, select one injection of the sample you wish to export. ***Note:*** The mixture and assumed contributor have to be exported separately.
3	Display plots with a table. *Casework Plot Settings* may be used. The table should include columns with the following headers: • Dye/Sample Peak • Sample File Name • Marker • Allele • Size • Height ***Note:*** The order of the columns is not important.
4	If not done previously, electronically edit the profile, removing labels from any stutter, INA peaks, or other artifacticious peaks. ***Note:*** MixMaster will import peaks labeled as alleles and all unlabeled peaks, filtered and/or deselected, in N-4, N+4, and INA positions.
5	Under the File menu, click "Export Table."
6	Save the exported results as a ".txt" file. ***File name notes:*** • The file does not need to be retained. • There are no limitations on the file name. – The import function provides an opportunity to adjust the sample name prior to inserting the profile into the worksheet. • For convenience, MixMaster will attempt to reduce the file name to just the item number. – It looks for two underscores ("_") before the item number, the second being directly before the item number, and one underscore after the

	item number. – For example, a file name of "B02_BKYY####_2-5sp_002.2" would be shortened to "2-5sp."

Section 3.7.3.4 Importing GeneMapper results

Peak import facts

"OL" labels for microvariant alleles need to be converted to the determined microvariant calls.

Alleles designated "<A" or ">B" should be modified to a standard allele designation based on its basepair size for deconvolution in MixMaster. This designation is simply for MixMaster as it will not accept the "<" and ">" symbols; it does not represent the true allele designation. The final report should reflect the conventional "<A" or ">B" designation from GeneMapper.

Filtered peaks are entered in blocks associated with the respective allele. For example:
- For 9.3 and 10 alleles, the 8.3 stutter would be entered into the 9.3 allele block and the 9 stutter into the 10 allele block.
- For 8 and 8.3 alleles, where a 7.3 was detected above the stutter filter but was interpreted as INA from the 8, it would be entered as 7.3 INA in the 8 block. Similarly, 8.2 INA would be entered in the 8.3 allele block.

Peaks that are possible INA of stutter peaks, N+4 stutter peaks, or other artifacts that might also contain low RFU alleles can instead be entered in the two peak entry positions labeled "Artifact."
- When utilizing these entry slots for INA or N+4, select the adjacent cell containing the word "Artifact" to activate a drop-down window, and modify the entry to the appropriate designation ("Stutter" or "INA") .

Note: Once MixMaster has run, an alert will be given if there are loci that contain more stutter/INA/artifact peaks than MixMaster can interpret as possible alleles. For those loci, deconvolution will not be performed.

Opening the workbook

Perform the following to open and prepare MixMaster.

Step	Action
1	Open MixMaster (version 1.1, or higher if performance checked) and enable macros.
2	Verify the interpretation thresholds are correctly set, according to the validated procedure: • Mx range will default to 0.3 (see Mx range in Section 3.7.4.3) • Analytical threshold (RFU level to call an allele) should be 50 RFU for 3130 and 150 RFU for 3500 • Peak height ratio thresholds should be as indicated in the manual deconvolution section *Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes* • Cycle number should be at least 2500
3	If applicable, enter the assumed reference profile. **If…** / **Then…** Entering electronically: Click on the "Import it!" macro button and import the genotypes from an exported GeneMapper txt file. ***Note:*** When using "Import it!" it is important to review the entries prior to mixture deconvolution. Entering the genotypes manually: Type in the values ***Note:*** For homozygotes, enter the allele twice; for single alleles in the stochastic range, enter the allele once. **Important** When importing an assumed donor from GeneMapper, loci with single allele values will be modified to be homozygotes with two allele calls (*e.g.,* a 12 will be turned into a 12,12.) If a locus has only one allele and it falls within the stochastic range, the imported profile *should be manually edited* to remove the second allele.

4	Enter the mixed DNA sample, including not only obvious alleles but also associated filtered peaks (*e.g.,* possible stutter and/or INA). **If…** / **Then…** Entering electronically, — Click on the "Import it!" macro button and import the genotypes from an exported GeneMapper txt file. ***Note:*** When using "Import it!" it is important to review the entries prior to mixture deconvolution. Entering the genotypes manually, — Type in the values

Section 3.7.3.5 Running MixMaster

Run It!

Run It! To run the MixMaster deconvolution, perform the following steps:

Step	Action
1	If applicable, enter an override Mx and an override assumed donor major or minor call. See Appendix I *Setting Override Mx* for more information.
2	Select the number of randomization cycles. The minimum cycle number is 2500 and is suggested for routine use. There is no maximum. More cycles equates to more randomized combinations of stutter contributions/INA levels/Mx that MixMaster can evaluate. This means that rare combinations from the true donors will more likely be included. Note that repeating a run with the same cycle number won't necessarily give the same results due to the randomization of the cycling process. ***Note:*** Excel 2007 runs macros more slowly than Excel 2003 or earlier. For example, a full 15-locus profile run for 2,500 cycles might take minutes to complete with Excel 2000 or 2003 but an hour using Excel 2007. To minimize the run time, set the "Show Analysis?" cell to NO.

3	Click on the "Run it!" macro button.
4	Review the allowed pairs of minor:major genotypes. ***Note:*** On the MixMaster worksheet, the allowed pairs are *not* limited by the assumed donor's genotypes.

Report It!

Report It!

To create a report of MixMaster results, perform the following steps:

Step	Action
1	Select your report settings. • Reduced: The results for each contributor (minor and major treated separately) are reduced to a list of non-overlapping genotypes. – For example, a minor donor who could be 12,12 or 12,13 or 12,F is reduced to just 12,F. – This setting is optimal for reporting an RMP statistic. – This is the default setting. • Not Reduced: All genotype pairs will be included in the report, even when they overlap – For example, the first pair is 12,13 minor with 14,14 major and the second pair is 12,F minor with 14,14 major. Both are listed even though the second pair includes the genotypes contained within the first pair. – This setting is required when reporting an LR statistic to preserve the genotype pairs. • Assumed: The list is filtered to include only pairs that contain the assumed donor's requisite genotype. – This is the default setting. • Not Assumed: The list is not filtered using the assumed donor's requisite genotype.
2	Click on the "Report it!" macro button.
3	Enter the case number, analyst, and date.
4	Click on the "Print it!" macro button.

5	Review the results and edit the printout as needed.
6	After data analysis, the run parameter and profile cells are locked to prevent discordance between these values and the interpreted genotypes. Any modifications will clear the results of the current interpretation. To enter new run parameters or manually change the profiles, click on the "Modify The Search" macro button. To import new profiles, click on the "Import it!" macro button.

Clear It!

Clear It! To start a new interpretation, click on the "Clear it!" macro button, clear both overrides, and begin again.

Section 3.7.4 Mixture Interpretation Guidelines

Section 3.7.4.1 Overview

Introduction These criteria for the deconvolution and interpretation of DNA mixtures are based on validation studies, literature references, and casework experience. They were developed with maximum input from analysts. It is expected that these criteria will continue to evolve as the collective experience of the BFS Laboratories grows.

Purpose The purpose of these guidelines is:

- To bring uniformity to mixture interpretation within the Bureau
- To ensure that conclusions in casework reports are scientifically supported by the analytical data
- To enable interpretations to be made as objectively as possible

These guidelines are used in conjunction with those described in Section 3.5.

Documentation (SWGDAM 3.5.2; 3.6.5)

Documentation of assumptions used for a mixture deconvolution should be included in the bench notes. This minimally includes:

- assumptions made as to the number of contributors,
- individuals/profiles assumed to be present,
- whether all alleles have been assumed to be detected for a particular contributor, particular locus (loci), or the overall results,
- any calculated mixture proportions and the corresponding range(s), and
- any other relevant assumptions made which are not stated in the Technical Procedure.

The optional Mixture Evaluation Worksheet may be used to facilitate such documentation.

Notes:

- Unless modified from the Technical Procedures, heterozygous PHR assumptions, stutter filters, and the applied analytical and stochastic thresholds do not need to be restated in the notes.
- Unless noted otherwise, biallelic donors are assumed with no null or partial null alleles.

Contents

The following topics are covered:

Section 3.7.4.2 Characterizing a Mixture

Single-source or mixture (SWGDAM 3.4; 3.4.1; 3.4.2)

Generally, a sample is considered to have originated from a single individual if one or two alleles are present at all loci for which typing results were obtained, and the peak height ratios for all heterozygous loci are within the empirically determined values. However, a single-source sample may occasionally be observed to have greater than two alleles at a locus due to a genetic anomaly (*e.g.,* trisomy) or peak height imbalance due to a primer binding site mutation. Peak height imbalance is more common when low quantities of DNA are amplified due to stochastic effects.

A sample is generally considered to have originated from more than one individual if three or more alleles are present at one or more loci (excepting genetic anomalies). Thus the minimum number of loci for determination of whether a sample is a mixture is one. Additionally, a sample may be considered to be a mixture if the peak height ratios for heterozygous pairs are below the empirically determined minimum heterozygous peak height ratio expectations.

Minimum number of contributors (SWGDAM 3.4.1; 3.4.2)

The minimum number of contributors to a mixed sample can be determined based upon a combination of relative peak heights and the loci that exhibit the greatest number of allelic peaks.

As an example, if at most four alleles are detected per locus, then the DNA typing results might appear consistent with having arisen from two individuals. However, if a locus with four alleles exhibits peaks that cannot pair exclusively with one another based on peak height ratios, then there may be a third contributor.

Peaks that do not meet the analytical and stochastic thresholds may be used in this assessment.

Amelogenin (SWGDAM 3.2.1.2; 4.6.3.1)

Results at the gender locus Amelogenin may be used in establishing the presence of a mixture and/or male DNA (*e.g.,* presence of major X and low-level Y alleles).

Major/minor genotyping (SWGDAM 3.5; 3.5.1; 3.5.2.2; 3.5.4; 3.5.4.2; 3.5.4.3)

If a sample contains a predominance of an individual's DNA, this results in what is categorized as a "distinguishable" mixture, whereby there is a distinct contrast in the peak heights among the different contributors' alleles. In such instances, major and/or minor contributors may be determined. The deduced profiles typically constitute single-source profiles. Discernment of genotypes for the major or minor contributors may be determined at some, but not necessarily all, loci.

Mixture proportions and PHRs may be used to quantitatively distinguish major and minor contributor genotypes from one another. The application of these quantitative characteristics is particularly useful in determining whether an allele may be shared among the contributors. Genotypes attributed to the major and minor contributors should meet the established PHR expectations and the calculated Mx proportion range (see Section 3.7.4.3 below for more information).

Indistinguishable mixtures (SWGDAM 3.5; 3.5.1; 3.5.6)

When major or minor contributors cannot be distinguished because of similarity in signal intensities, the sample is categorized as an "indistinguishable" mixture. The classification as indistinguishable may be limited to some, not all, of the loci for which DNA typing results are obtained and does not imply that the profile is uninterpretable. Individuals may still be included or excluded as possible contributors to an indistinguishable mixture. With an assumed donor, a deduced single-source profile may be possible.

Mixture proportions and PHRs may be used to quantitatively distinguish genotypes from each contributor. The application of these quantitative characteristics is particularly useful in determining whether an allele may be shared among the contributors. Genotypes attributed to each contributor should meet the established PHR expectations and the calculated Mx range (see Section 3.7.4.3 below for more information).

Assumed contributors (SWGDAM 3.5; 3.5.7; 3.5.7.1; 3.5.7.2)

Intimate
Evidence collected directly from a person's body is considered intimate and is generally expected to yield DNA from the individual from whom the sample was taken. If another source of DNA is present in sufficient quantity in such a sample, a mixture of DNA is likely to be detected. Based on this expectation, a conditional known sample (*e.g.,* the victim) may be subtracted from the mixture results to facilitate identification of the foreign alleles/genotypes. The obligate foreign alleles/genotypes may effectively constitute a single-source profile or a mixture profile (*i.e.,* if there are multiple additional DNA contributors) for comparison. Examples of intimate samples include oral, vaginal, rectal, penile, and other body swabs.

Personal
Personal items are generally expected to yield DNA from the owner/routine user of the item. If another source of DNA is present in sufficient quantity in such a sample, a mixture of DNA is likely to be detected. If the item appears to have had limited sources of DNA based on the results and/or other case-specific information warranting a contributor assumption, a conditional known sample (*e.g.,* the victim) may be subtracted from the mixture results to facilitate identification of the foreign alleles/genotypes. Examples of personal items include clothing, bed linens, and personal equipment (*e.g.,* toothbrush, computer).

Portable items that may easily come into contact with multiple individuals (*e.g.,* ski mask, gang-related evidence) should be treated cautiously with regard to assumed contributors.

A conditional sample may additionally include results obtained from a substrate sample or other sub-item result expected to have had close contact and/or mixing with the biological material of interest.

Multiple items/ same source (SWGDAM 3.6.4)

A conclusionary statement can be made that all DNA typing results from a mixed sample may be accounted for by a particular combination of profiles when the following conditions are met:
1.) Deconvolution of a DNA mixture is unnecessary (see below), *and*
2.) Two or more individuals cannot be excluded as potential contributors, *and*
3.) All of the DNA typing results in question (*e.g.,* all alleles detected, majors of a category II three-person mixture, *etc.*) are accounted for by previously determined profiles/sources.

For example, "All of the detected alleles may be accounted for by a mixture of DNA from the victim and a consensual partner."

Interpretation of a DNA mixture may be unnecessary due to the existence of other evidence providing greater probative value. Some examples include:

- When there is a single-source male in sperm fraction and the non-sperm fraction is a mixture consistent with the sperm donor and the victim, the non-sperm fraction is not typically deconvoluted.
- When all alleles of a mixture can be accounted for by the victim and a consensual partner, and there was no foreign DNA detected that can be attributed to the assailant, the mixture may not need to be deconvoluted.
- When a foreign, male profile has been determined from a vaginal swab result, a DNA mixture from the victim's jeans consistent with DNA from that individual and the victim may not need to be deconvoluted.

As suggested by the examples above, this approach is case context-dependent. For example, deconvolution and statistical calculations *are* typically appropriate is when it is *not* reasonable to assume the probative results from the various items are from the same individual (*e.g.,* evidentiary items collected from a burglary).

Section 3.7.4.3 Quantitative Assessment of a Mixture

Peak Height Ratio

PHR Thresholds (SWGDAM 3.3; 3.3.1; 3.3.1.1; 3.3.2; 3.5.4.1; 3.5.4.2)

Peak height ratio expectations differ with changes to the overall peak heights and therefore a tiered approach to applying peak height ratios is used, as outlined below. Note that there is no minimum expected peak height ratio when the taller peak falls below the stochastic threshold (*i.e.,* 0%).

Based on Identifiler Plus empirical data generated by the Bureau Laboratories, the following minimum PHRs were determined for use at all STR loci:

For 3130:

When the taller peak is between…	Then the minimum acceptable peak height ratio is…
0 - 364 RFU	0%
365 - 749 RFU	30%
750 – 1199 RFU	40%
1200 RFU and up	50%

For 3500:

When the taller 3500 peak is between…	Then the minimum acceptable peak height ratio is…
0 – 1074 RFU	0%
1075 – 2699 RFU	25%
2700 – 4999 RFU	40%
5000 RFU and up	50%

Note that per analyst discretion, a minimum expected PHR may be reduced, but not increased when performing a manual deconvolution; these thresholds are “fixed” in MixMaster.

PHR Minimum & Maximum

To deconvolute loci with potentially shared alleles, the minimum and maximum possible contributions to a shared peak may need to be calculated. In a two-person mixture, this involves loci with less than four alleles present. In a three-person mixture, the loci with less than six alleles present are frequently involved.

The purpose of looking at minimum and maximum contributions to a shared allele is to ensure that all possible genotype pairings are included given the Mx range and the minimum expected peak height ratios (PHRs). The various ways to divide a shared

allele to include the most genotypes possible can also be determined by trial and error. Thus minimum and maximum contributions account for worst case scenarios that may be less likely but still possible given the interpretation assumptions.

Minimum and maximum contributions are described in detail in *Use Heterozygous Peak Height Ratios (PHRs) for Determining Possible Unknown Donor Genotypes*.

Mixture Proportion

Mixture proportion (SWGDAM 3.5.3; 3.5.4.1)

A mixture proportion (Mx) is a quantitative characteristic used to assess the potential contribution of a donor at each locus.

For two-person mixture deconvolution, the Mx is defined as the RFU sum of the minor contributor alleles divided by the total RFU sum of all alleles.

$$Mx = \frac{\sum RFU_{Minor}}{\sum RFU_{Total}}$$

When there is no contributor assumed to be present, Mx is calculated at loci with four alleles, and the minor contributor is assigned the two lower RFU alleles, even though that may not be the true genotype pairing.

When an assumed contributor is present in a two-person mixture, Mx is calculated for loci where there is no allelic overlap. This includes four-allele loci and three-allele loci where the assumed contributor is a homozygote. The formula then becomes:

$$Mx = \frac{\sum RFU_{Assumed}}{\sum RFU_{Total}}$$

When the assumed contributor is the major donor to the mixture (averaged across the entire profile), the Mx is calculated as:

$$Mx = 1 - \frac{\sum RFU_{Assumed}}{\sum RFU_{Total}}$$

When using an assumed contributor, the same donor should always be in the numerator.

Mx with >2 donors (SWGDAM 3.5.3)

A mixture proportion can be calculated to distinguish between not only two donors but various combinations of donors when there are more than two individuals' DNA present. For example, it may be used to distinguish an individual(s) in a three-person mixture.

Average Mx (SWGDAM 3.5.3)

The average Mx represents the overall Mx for the profile and is calculated from the individual locus Mx values. MixMaster also uses an average Mx to assess the results at the individual loci.

Mx Range (SWGDAM 3.5.3)

A range around the average Mx is created to compensate for locus-to-locus Mx variation. The range is typically set as $Mx_{avg} \pm 0.3$ (MixMaster default). The range may be adjusted as appropriate within 0.2 – 0.5.

Validation at the BFS Jan Bashinski DNA Laboratory showed that although most 2-person mixtures with an assumed donor are within ± 0.2, nearly all data was within ± 0.3; this included case data not conditioned on a contributor and instances of mild differential degradation.

When the average Mx is 0.3 (equivalent to a minor:major donor ratio of ~1:3), the lower bound for the Mx range is 0. Therefore, an Mx of 0.3 or less allows for the complete loss of the minor contributor genotype.

Differential degradation (SWGDAM 3.5.3.1)

Differential degradation is varying levels of DNA degradation amongst the contributors to a DNA mixture. This may impact mixture proportions and expected PHRs across the entire profile. Therefore, great caution should be exercised in applying such quantitative assessments to the data.

Differential degradation example

Figure 2 shows the blue panel of a two-person mixture with the alleles of an assumed donor highlighted. The foreign contributor DNA was degraded while that of the assumed was not. The overall average Mx for the profile was 0.35. The Mx was re-calculated with the profile divided between high and low molecular weight loci. The average Mx for the low molecular weight (LMW) loci was 0.43 and the average Mx for the high molecular weight (HMW) loci was less than 0.20. Although the LMW and HMW Mx ranges (±0.3) overlap, they span a broader range collectively than the overall profile range. The effect is that fewer genotypes would be considered possible and thus the true genotype at any one locus may not actually be included. Furthermore, with an Mx of 0.2, drop-out is clearly possible, something that would not necessarily be considered with an Mx of 0.35.

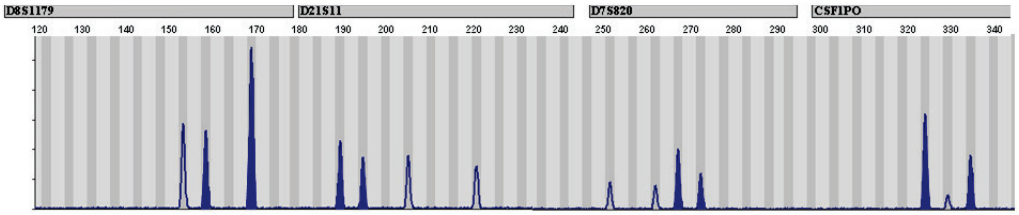


Figure 2

Stutter *versus* minor peaks (SWGDAM 3.5.8; 3.5.8.1; 3.5.8.2; 3.5.8.3)

For mixtures in which minor contributor genotype(s) are being considered, stutter may be indistinguishable from potential minor alleles. This is considered in deconvolution when deriving potential genotypes from applied minimum PHR expectations and the calculated Mx range. As a result, a filtered peak in a stutter position may be included as a potential minor allele.

Generally, when the height of a peak in a stutter position exceeds the stutter threshold(s), that peak is designated as an allele.

Stutter *v.* minor Example 1

In the example below, the peak in the position of a 6 allele would be filtered as stutter by GeneMapper but should be considered as a possible minor allele in the deconvolution. The 6 (118 RFU) could pair with the 12 (380 RFU), resulting in a 31% PHR. Assuming a three-person mixture (Category II – see next section) and all alleles have been detected, the possible minor genotypes are: 6,12 7,12 8,12 10,12 12,12

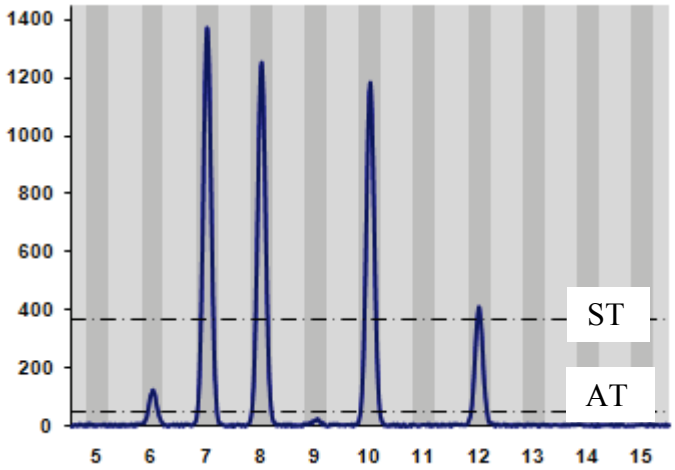


Figure 3

Stutter *v.* minor Example 2

In this example, taking N+4 at 9.2% stutter into account leads to the inclusion of an additional possible genotype (*i.e.,* 7,10).

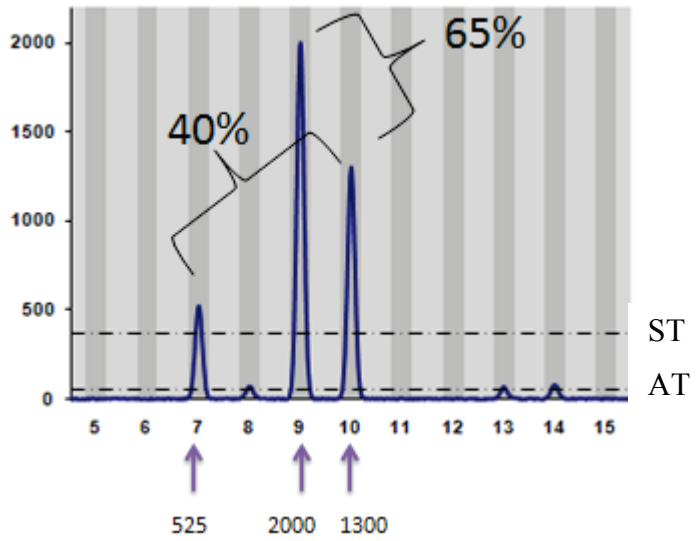


Figure 4

With N+4 considered (9.2%):
2000 x 0.092 = 184 RFU
1300 - 184 = 1116 RFU
PHR for 7,10 becomes 525/1116 = 47%

Section 3.7.4.4 Three-person Mixtures

Categorized

3-person mixtures (SWGDAM 3.5.5; 3.5.6)

Using criteria outlined below and the tiered PHR thresholds, three person mixtures may be categorized as follows:

- Category I: one major and two minors
- Category II: two majors and one minor
- Category III: three indistinguishable contributors
- Category IV: one major, one intermediate, one minor

Notes:
- Some mixtures may not fit neatly into only one category (see Figures 2 and 23).
- Any three-person mixture that does not fit into category I, II, or IV, should be treated as a category III mixture.

Mx for > 2-person mixtures (SWGDAM 3.5.3)

Mixture proportion may be applied to a donor or combination of donors in mixtures with more than two contributors. It can be used to assess the proportion of major, minor, or intermediate donor(s) to the donor pool.

Category I

To determine whether a three-person mixture falls into Category I (one major/two minors), it should meet the following criteria.

Stage	Description
At 6-allele loci	• The two tallest peaks cannot pair with any of the other 4 peaks using PHRs. • The Mx for the major contributor (sum of the two tallest peaks divided by the sum of all six peaks) is at least 0.62.
At 5-allele loci with an apparent major homozygote	• The tallest peak cannot pair with any of the other four peaks. • The apparent homozygote cannot be fully accounted for by being shared with the next two tallest peaks. • The Mx for the major contributor (sum of the tallest peak divided by the sum of all five peaks) is at least 0.62.

Cat I, Mx ≥0.62

In a 3-person mixture in which a Category I classification is being considered, the Mx for the major contributor should be at least 0.62. This basement threshold is derived from the minimum peak height ratio expectations, as described below.

Considering the worst case 3130 scenario (the lowest acceptable peak height ratio of 30%), the taller peaks should be at least 365 RFU and have a peak height ratio less than 30% when evaluated for pairing with any of the minor alleles. Given that, the minor alleles detected would each be < 110 RFU. At that level, the Mx would be less than 0.624, as illustrated below.

$$Mx = \frac{365 + 365}{110 + 110 + 110 + 110 + 365 + 365} = 0.624$$

Similarly, for the worst case 3500 scenario (the lowest acceptable peak height ratio of 25%), the taller peaks should be at least 1075 RFU and have a peak height ratio less than 25% when evaluated for pairing with any of the minor alleles. As follows, the minor

alleles detected would each be < 269 RFU. At that level, the Mx would be less than 0.67, as illustrated below.

$$Mx = \frac{1075 + 1075}{269 + 269 + 269 + 269 + 1075 + 1075} = 0.67$$

Cat I, Example 1

See Figure 5 below for an example of a six-allele locus in a Category I mixture. All major/minor PHRs are less than the lowest minimum PHR.

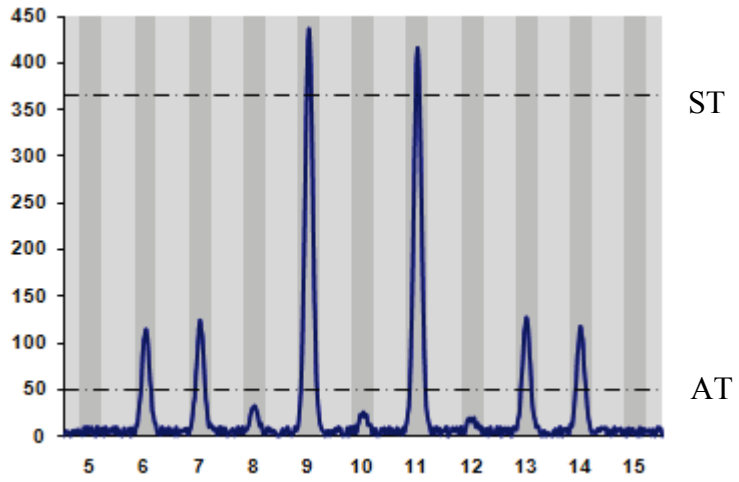
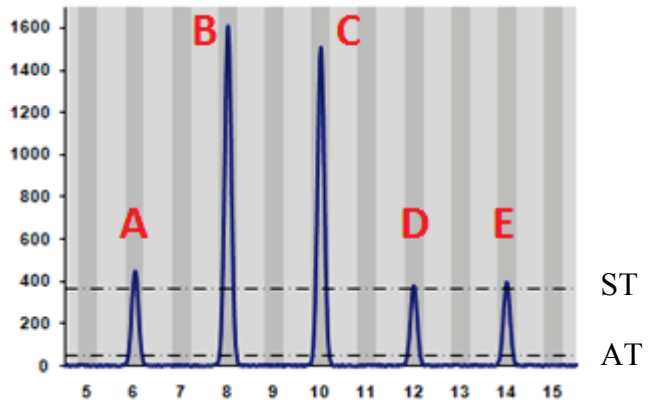


Figure 5

Cat I, Example 2

See Figure 6 below for an example of a five-allele locus in a Category I mixture with an assumed donor.



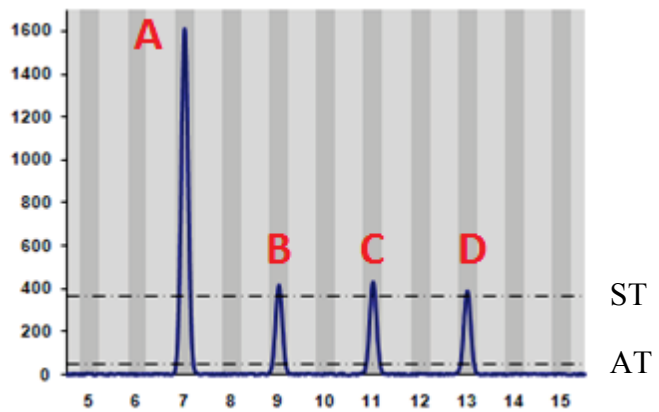
$$\text{Major Mx} = \frac{B + C}{A + B + C + D + E}$$

Assumed donor = A,A
B & C cannot pair with A, D, or E based on PHR

Figure 6

Cat I, Example 3

See Figure 7 below for an example of a four-allele locus in a Category I mixture with an assumed donor.



$$\text{Major Mx} = \frac{A}{A + B + C + D}$$

Assumed donor = B,B
A cannot pair with B, C, or D based on PHR

Figure 7

Category I – Suitable for Comparison (Minor) (SWGDAM 3.6.2.1)

For loci in which the minor contributors are present at a low level, only those loci in which there are at least three minor alleles may be suitable for comparison and statistical evaluation for the minor contributors.

Category II

To determine whether a three-person mixture falls into Category II (two majors/one minor), it should meet the following criteria:

Stage	Description
At 6-allele loci	• None of the four tallest peaks could pair with either of the two shortest peaks.
At 5-allele loci	• If there are four tall peaks and one short peak, none of the four tall peaks could pair exclusively with the short peak. • If there are three tall peaks and two short peaks, the shortest of the three taller peaks cannot be fully explained by being shared with the two shorter peaks.

Note: Once major and minor alleles are determined, it may be possible over at least part of the profile to treat the two major contributors as a two-person mixture.

Cat II, Example 1

See Figure 8 below for an example of a six-allele locus in a Category II mixture. All major/minor PHRs are less than 40%.

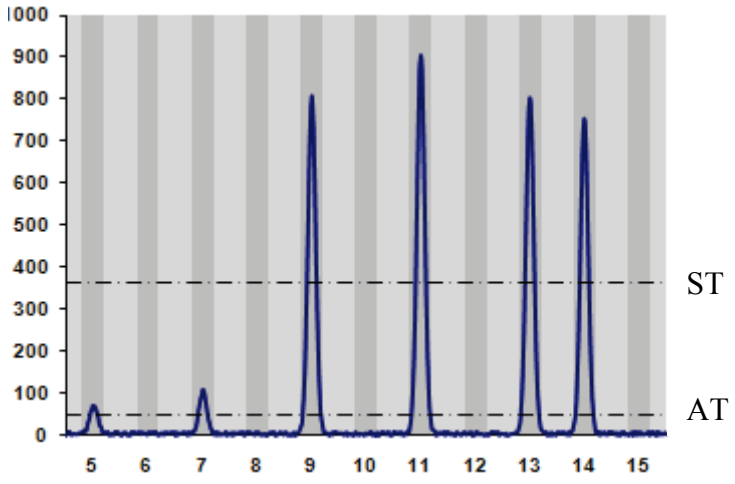


Figure 8

Cat II, Example 2

See Figure 9 below for an example of a five-allele locus in a Category II mixture. All major/minor PHRs are less than 40%.

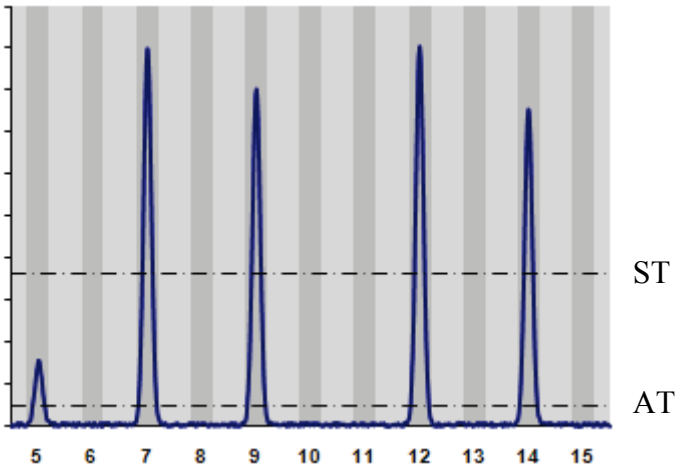


Figure 9

Cat II, Example 3

See Figure 10 below for an example of a five-allele locus in a Category II mixture. In this example, a combination of 6,10 and 8,10 do not fully explain the height of the 10 allele.

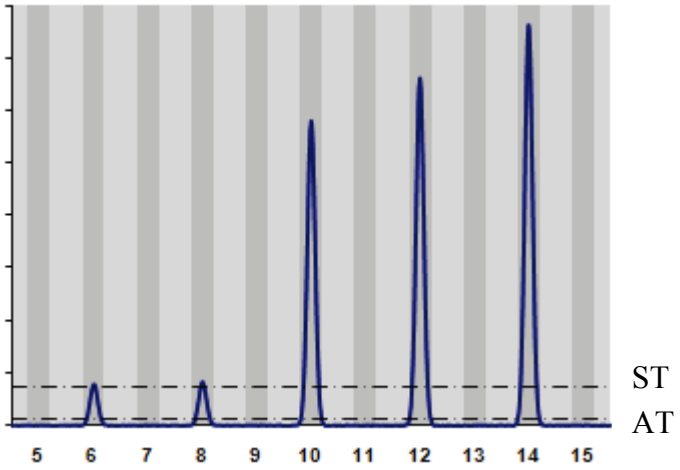


Figure 10

Category III

To determine whether a three-person mixture falls into Category III (indistinguishable), it should meet the following criteria:

Stage	Description
At 6-allele loci	• There are no peaks that can pair exclusively with only one other peak.

At 5-allele loci	• There are no peaks that can pair exclusively with only one other peak, AND
	  • One peak can be fully explained by being shared with 2 other peaks, *or*
	  • One peak could be paired with an undetected peak

Cat III, Example 1

See Figure 11 below for an example of a six-allele locus in a Category III mixture.

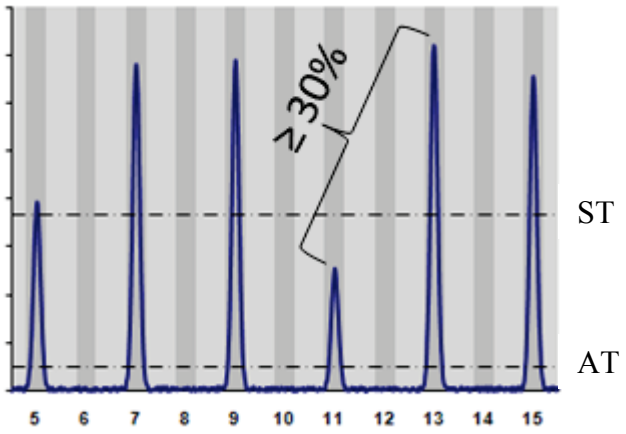


Figure 11

Cat III, Example 2

See Figure 12 below for an example of a five-allele (potential sixth allele), low-level locus in a Category III mixture.

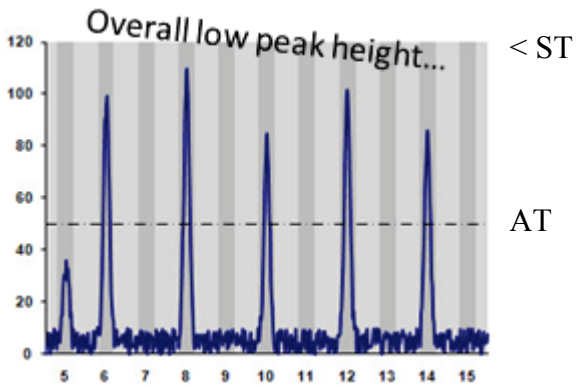


Figure 12

Cat III, Example 3

See Figure 13 below for an example of a five-allele locus in a Category III mixture which highlights allele stacking.

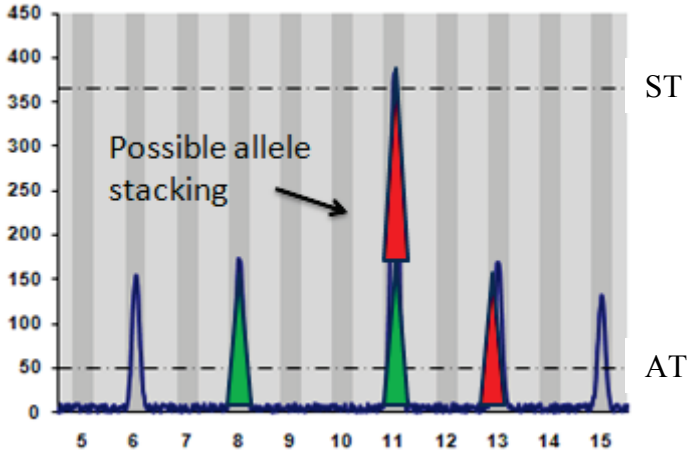


Figure 13

Category III - Suitable for Comparison (SWGDAM 3.6.2.1)

- If it can be demonstrated that alleles are not dropping out, all possible genotype combinations for any contributor should be determined and should be suitable for comparison and statistical evaluation.
- If alleles could be dropping out, a locus should only be used for comparison and statistical evaluation if 5 or 6 alleles are detected.

Cat III, Example 4

See Figure 14 below for an example of a five-allele locus in a Category III mixture in which the following assumptions are made:

- One assumed contributor
- All alleles have been detected (no drop-out)
- No significant separation between the two foreign contributors

Assumed Contributor 10,12

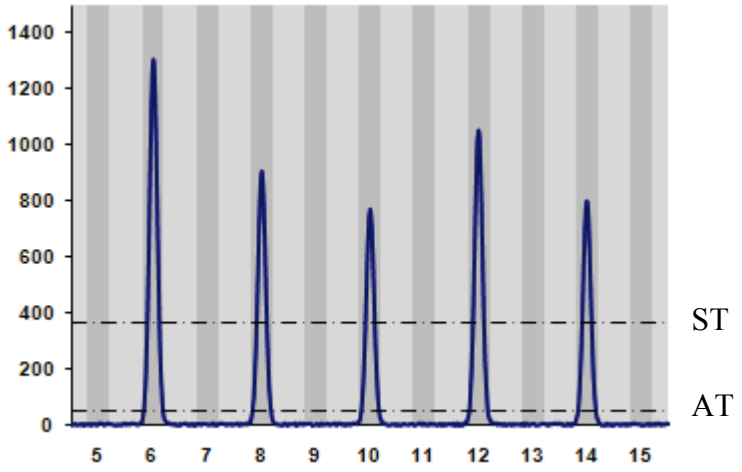


Figure 14

Given the assumptions and considering PHRs (10 allele cannot be shared),

- The genotypes to include for the foreign contributors are: 6,6 8,14 8,8 6,14 14,14 6,8 6,12 8,12 12,14.
- The genotypes to exclude for the foreign contributors are: 10,10 10,12 12,12 6,10 8,10 10,14.

Cat III, Example 5

See Figure 15 below for an example of a three-allele locus in a Category III mixture in which the following assumptions are made:

- One assumed contributor
- All alleles have been detected (no drop-out)
- No significant separation between the two foreign contributors

Assumed Contributor 7,10

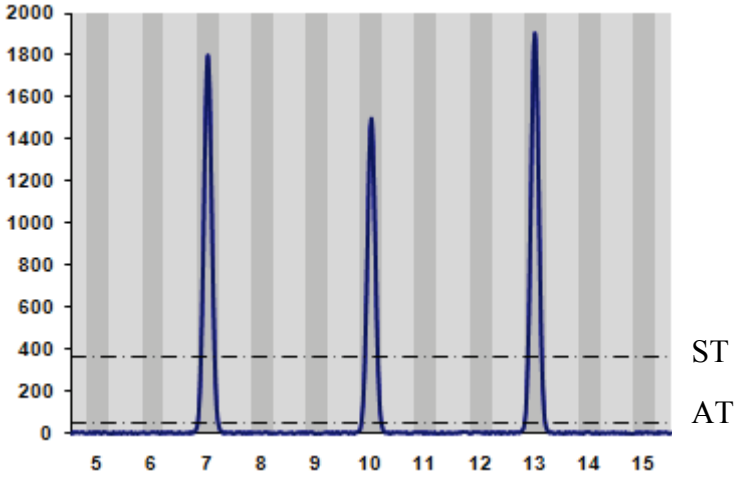


Figure 15

Given the assumptions and considering PHRs:

- The genotypes to include for the foreign contributors are: 7,7 7,13 10,13 7,10 10,10 13,13

Cat III, Example 6

See Figure 16 below for an example of a five-allele locus in a Category III mixture in which the following assumptions are made:

- One assumed donor
- Drop-out is possible
- No significant separation between the two foreign contributors

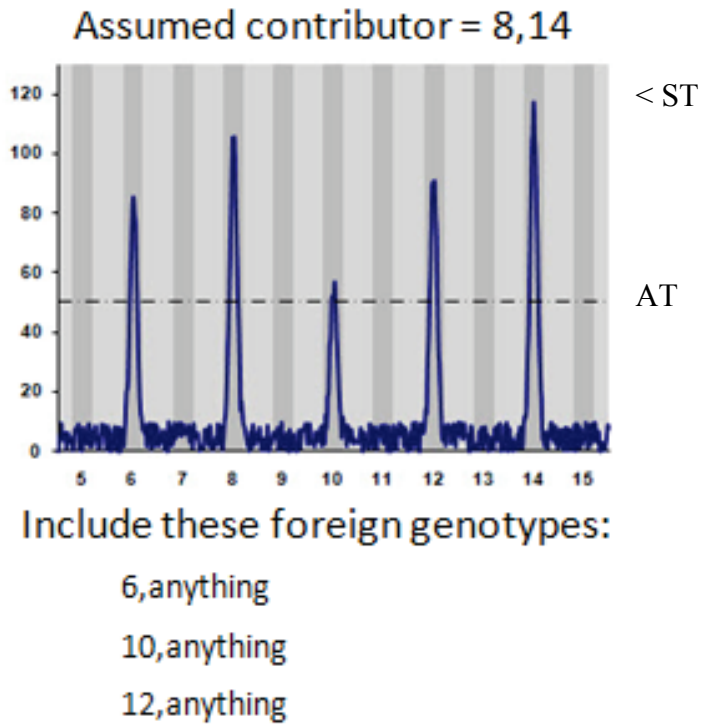


Figure 16

Cat III, Example 7

See Figure 17 below for an example of a four-allele locus in a Category III mixture in which the following assumptions are made:

- One assumed donor
- Drop-out is possible
- No significant separation between the two foreign contributors

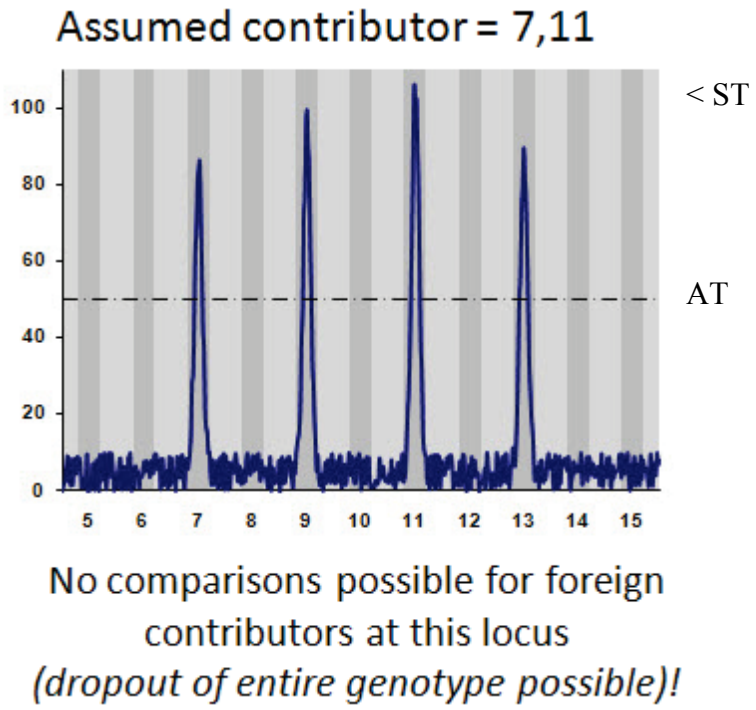


Figure 17

Category IV

A three-person mixture falls into Category IV (major/intermediate/minor) if:

- At least one six-allele locus is present AND
- At <u>all</u> 6 allele loci, 3 pairs of alleles exist that cannot pair with any other alleles (based on PHRs).

Cat IV, Example

See Figure 18 below for an example of a six-allele locus in a Category IV mixture in which unambiguous pairings can be made based upon minimum PHR expectations: 5,7 and 9,11 and 13,15.

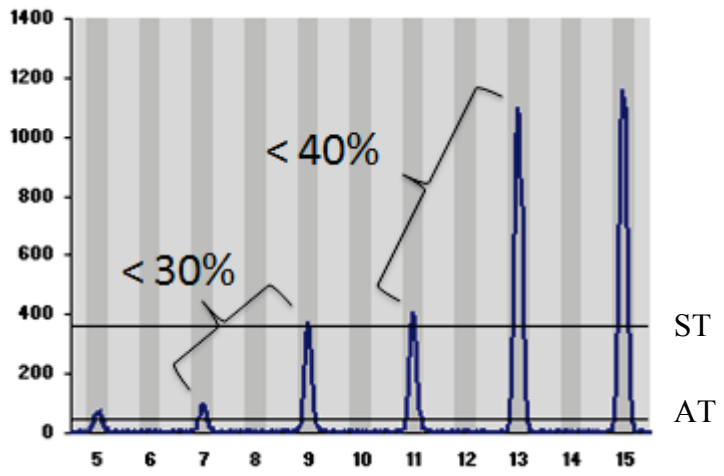


Figure 18

Category IV Limitations

- At some loci, it may not be possible to interpret for individual contributors.
- At all loci, the combination of all possible genotypes from non-minor peaks should typically include the major and intermediate contributors.
- At loci where allele sharing is a concern, you may not be able to determine separate genotypes for the major and intermediate contributors.

Category IV Alternatives

If a three-person mixture has at least one six-allele locus present but does not fall into Category IV above, consider the following scenarios:

If ...	Then ...
Significant separation does not exist between the minor and intermediate contributors, but does exist between the intermediate and major,	The mixture may be treated as a Category I mixture (one major and two minors).
Significant separation does not exist between the intermediate and major contributors, but does exist between the minor and intermediate,	The mixture may be treated as a Category II mixture (two majors and one minor).

Assumed Contributors to Three-person Mixtures

One assumed contributor

For three-person mixtures in which one donor is assumed, see the following table for guidance on further interpretation.

If ...	Then ...
Significant separation (based on PHRs) exists between the two foreign contributors,	Further interpretation of one or both foreign contributors should be possible. For example, • a Category I mixture with an assumed minor, or • a Category II mixture with an assumed major.
Significant separation (based on PHRs) does NOT exist between the two foreign contributors AND it can be assumed that the foreign contributor alleles are not dropping out or masked by the assumed contributor,	All possible genotype combinations for the foreign contributors should be determined at all loci where it is appropriate. For example, • a Category II mixture with an assumed minor, or • a high-RFU Category III mixture.
Significant separation (based on PHRs) does NOT exist between the two foreign contributors AND the assumed contributor alleles could be masking the foreign contributor alleles and/or the foreign contributor alleles could be dropping out,	Only loci with 3 or 4 foreign alleles should be used for comparison and statistical evaluation. For example, • a Category I mixture with an assumed major, or • a low-RFU Category III mixture.
Both foreign contributors are low-level,	Only those loci with at least three foreign alleles are suitable for comparison and statistical evaluation.

Two assumed contributors

For three-person mixtures in which two donors are assumed, see the following table for guidance on further interpretation.

If ...	Then ...
Two donors are assumed to be present in a three-person mixture,	Foreign allele calls are at least possible.
There are two foreign alleles at a locus,	Foreign genotypes are possible.
Significant separation exists between the foreign contributor and both of the assumed contributors (*e.g.,* a Category I mixture with both minors assumed),	Foreign genotypes are possible.
It can be demonstrated that the foreign contributor and at least one of the assumed contributors have not dropped out (*e.g.,* a Category II mixture with the minor and one major assumed or a high-RFU Category III with two assumed contributors),	Foreign genotypes are possible.
The foreign contributor is minor and/or dropout is possible for the foreign contributor,	Only loci with at least one foreign allele are suitable for comparison.

Additional considerations in three-person mixtures

Missing alleles

There are typically only three situations that account for missing alleles:

1. Allelic dropout
2. Stacking
3. Stutter

These are described in more detail below.

Allelic dropout

After considering worst-case scenario PHRs (*e.g.,* the maximum contribution of an unshared allele relative to another peak), dropout should be considered if the remaining peak height is less than the stochastic threshold, which results in an allele call only.

As the number of detected alleles decreases, the minimum peak height for assigning genotypes should increase. See the following examples for further elaboration.

Allelic dropout examples

- At a 5-allele locus, one allele below the stochastic threshold *could* be paired with one allele less than the analytical threshold.

- At a 4-allele locus, one allele below 730 RFU *could* be paired with one allele greater than the analytical threshold of 50 RFU and one allele less than 50 RFU, as illustrated in Figure 19 below. In this 3-person mixture situation, the 10 allele is less than 730 RFU and the two low-level peaks could account for the entire height of the 10 allele.

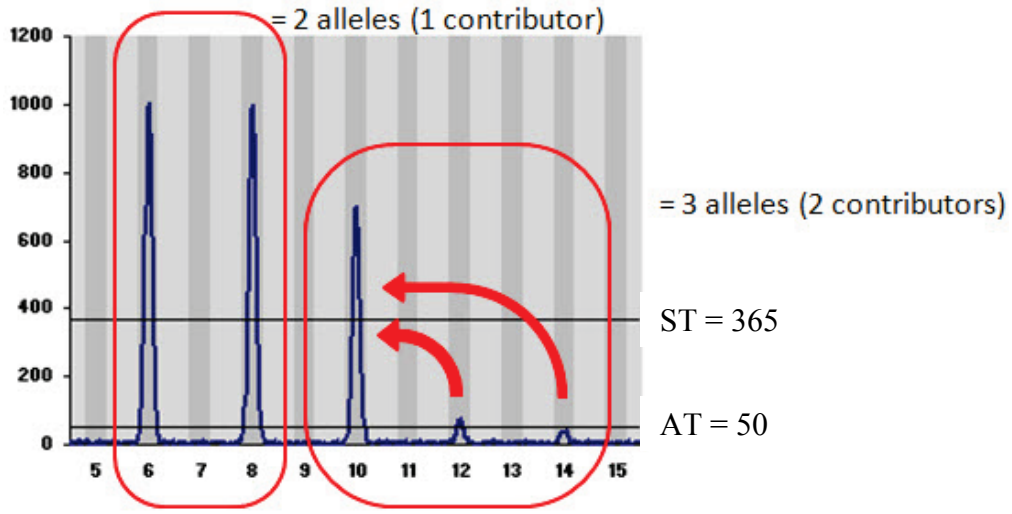


Figure 19

- At a 3-allele locus, one allele below 730 RFU *could* be paired with two alleles less than 50 RFU, as illustrated below in Figure 20. In this 3-person mixture situation, the 10 allele is less than 730 RFU and the two peaks below the analytical threshold could account for the entire height of the 10 allele.

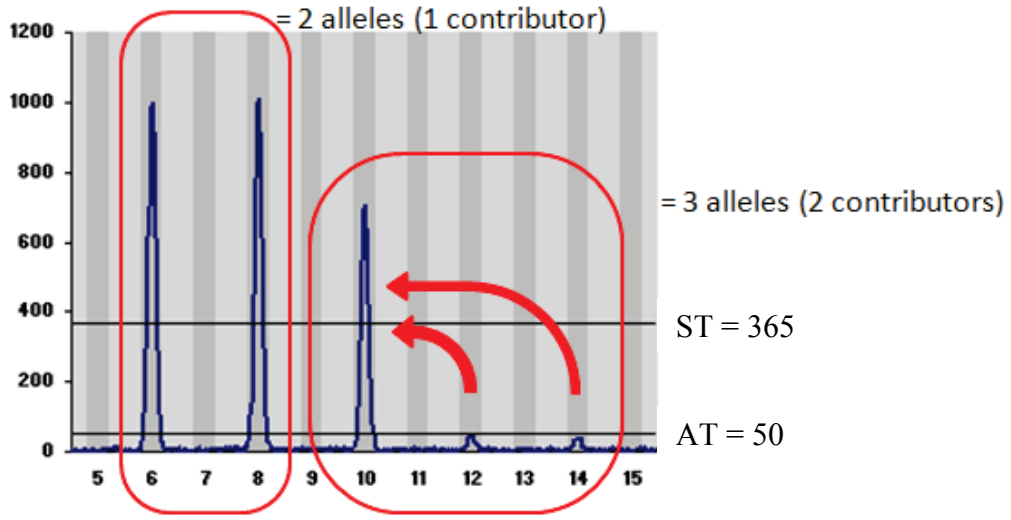


Figure 20

- At a 2-allele locus, one allele below 730 RFU *could* be paired with two alleles less than 50 RFU. In this scenario, the one allele above 730 RFU would be a homozygote (similar to Figure 20 above).

- At a locus with a single allele, one allele less than 1095 RFU *could* be paired with three alleles less than 50 RFU, as illustrated in Figure 21 below.

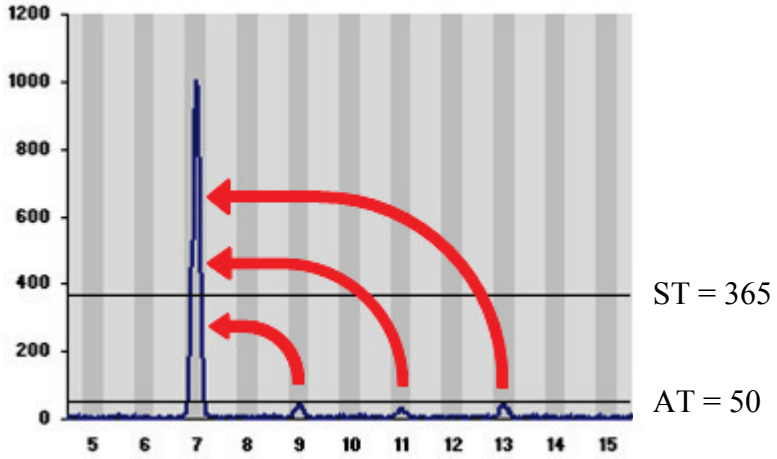


Figure 21

F alleles in 3-person mixtures

Given the preceding illustrated points, the maximum peak height of a *potentially shared* allele that may be paired with an F sister allele(s) can be summarized below. In general, as the number of detected alleles decreases, the maximum number of potential F alleles increases and therefore the maximum peak height of the potentially shared allele may increase.

# of Detected Alleles	Max # of Missing Alleles	Max # of Potential F Alleles Paired with the Same Detected Allele	3130 Max Peak Height of *Potentially Shared* Allele	3500 Max Peak Height of *Potentially Shared* Allele
6	0	n/a	n/a	n/a
5	1	n/a	<365	<1075
4	2	n/a	<365	<1075
3	3	2	<730	<2150
2	4	2	<730	<2150
1	5	3	<1095	<3225

Stacking

When peaks in an apparently high-level 3-person mixture exceed the stochastic threshold, the effects of possible stacking of minor alleles should be considered. Two or more low-level peaks whose partner peaks are less than the analytical threshold could be stacked on top of each other to create the appearance of one taller peak above the stochastic threshold. See Figures 19, 20, and 21 for examples.

Stacking should also be considered at loci where the possibility of dropout has been ruled out and where it appears there is balance between 2 pairs of peaks. For example, at a 4-allele locus, a taller peak could be paired with two smaller peaks leaving a taller, fourth peak to be a homozygote. For example, as illustrated below in Figure 22, one possible genotype scenario would include: 6,6 and 8,10 and 8,12.

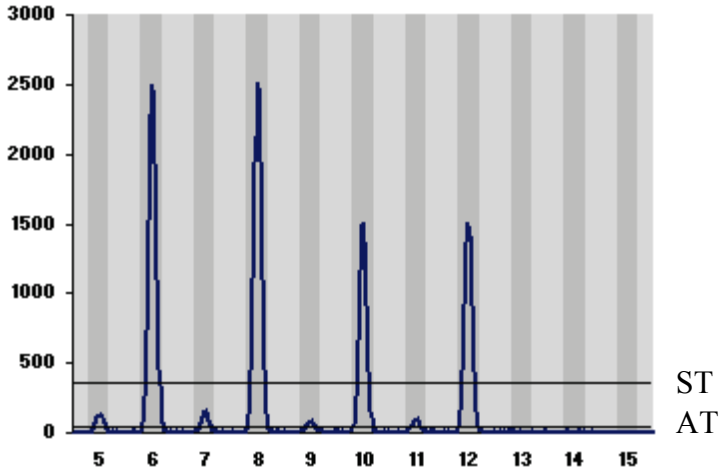


Figure 22

Stutter

When 5 or fewer alleles are detected, alleles in stutter positions (filtered out by the software or deselected by the analyst) should be considered. For example, a peak greater than the stochastic threshold may be paired with a filtered stutter peak and have an acceptable PHR (see Stutter *v.* Minor Example 1 – Figure 3).

When unacceptable PHRs indicate that two alleles could not be paired, the possibility that stutter could be increasing one of the peak heights should be considered. If one of the peaks falls in the stutter position of another peak in the mixture, subtracting the maximum possible stutter (N-4 and/or N+4) may bring the allele pair into an acceptable PHR range (see Stutter *v.* Minor Example 2 – Figure 4).

Degradation

Caution should be taken in evaluating a degraded mixture as being truly from no more than three contributors. If significant separation exists between contributors, interpretation of some loci may still be possible. Furthermore, if five or six alleles are detected at a locus, that locus (loci) may still be used for comparison.

If degradation affects all contributors to a mixture equally, the ratio of contributors will remain relatively constant with the overall RFU level decreasing at the larger loci. In such situations, it may be possible to assume all alleles have been detected at the LMW loci but not at the HMW loci.

If differential degradation occurs, significant separation of the contributors may exist at the LMW loci but not at the HMW loci. When this occurs, tools such as PHR, mixture proportion, and mixture categories can become unreliable as well as assumptions regarding the numbers of contributors. In cases of extreme differential degradation, interpretation may not be possible at loci with fewer than five alleles.

Differential degradation example

See Figure 23 below for an example of differential degradation transitioned between Categories I and II.

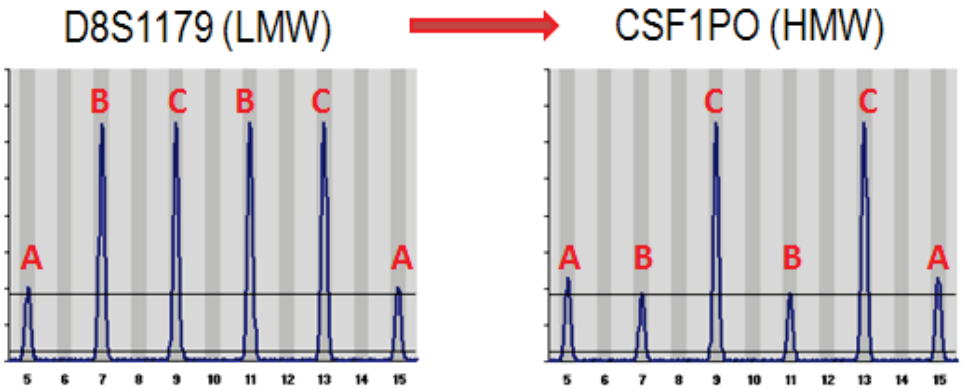


Figure 23

> 3-person mixtures

In the case of a mixed DNA sample containing DNA from at least four individuals, the usefulness of PHRs and mixture proportions is greatly diminished due to allele stacking. Because of potential allele stacking, the number of detected alleles becomes a poor estimator of the number of contributors to the DNA mixture. Consequently, with increasing contributors, estimates of the numbers of donors become less accurate.

If there is a donor(s) that can be assumed to be present, stacking and stochastic uncertainties may be reduced.

For example, in a four-person mixture, it may only be possible to determine genotypes at loci with 7-8 alleles, despite the assumption of four contributors across the entire profile. With the subtraction of a known contributor, genotypes may be determined at additional loci.

Section 3.7.5 References

The following references are supplemental to those included in Section 3.10:

Bill, M., Gill, P., Curran, J., Clayton, T., Pinchin, R., Healy, M., Buckleton, J. (2005) "Pendulum – a guideline-based approach to the interpretation of STR mixtures." Forensic Science International 148:181-191.

Clayton, T.M., Whitaker, J.P., Sparkes, R., Gill, P. (1998) "Analysis and interpretation of mixed forensic stains using DNA STR profiling." Forensic Science International 91:55-70.

Gill, P., Sparkes, R., Pinchin, R., Clayton, T., Whitaker, J., Buckleton, J. (1998) "Interpreting simple STR mixtures using allele peak areas." Forensic Science International 91:41-53.

Gill, P., Brenner, C.H., Buckleton, J.S., Carracedo, A., Krawczak, M., Mayr, W.R., Morling, N., Prinz, M., Schneider, P.M., Weir, B.S. (2006) "DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures." Forensic Science International 160:90-101.

Gill, P., Brown, R.M., Fairley, M., Lee, L., Smyth, M., Simpson, N., Irwin, B., Dunlop, J. Greenhalgh, M., Way, K., Westacott, E.J., Ferguson, S.J., Ford, L.V., Clayton, T., Guiness, J. (2008) Letter to the Editor: "National recommendations of the Technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes." Forensic Science International: Genetics 2:76-82.

Section 3.7.6 Appendix I – Setting Override Mx in MixMaster

Mx estimates from 3-allele loci

For two-person mixtures that lack loci with four detected alleles (or three alleles with a homozygous assumed contributor), Mx can be estimated using three allele loci. This can be done manually, as described in this appendix, or using Mx CALculator; either way, appropriate documentation should be included in the case file.

Consideration is given to the possibilities that the mixture may represent:
- a heterozygous genotype combined with a homozygous genotype, or
- two heterozygous genotypes that overlap at one allele

For the formulae below, A, B, and C represent the RFUs of the three detected alleles.

Notes:

- While PHRs below 60% are observed, applying a higher threshold generally yields a more accurate estimate of the Mx when using three-allele loci.
- When there is an assumed contributor to a two-person mixture, care should be taken to ensure that the assumed person's alleles are consistently used for the major or minor contributor at each locus, regardless of whether they appear to have flipped from major to minor (or vice versa) at any one locus.

Heterozygote-homozygote pairing

For a potential heterozygote-homozygote pairing, the Mx may be estimated as follows.

Step	Action
1	Calculate peak height ratios for each pair of alleles.
2	For each allele pair with a PHR greater than 60% (see notes below), calculate a minor contributor Mx:

If…	Then…
The third allele's RFU (representing the contribution of the homozygous genotype) is lower than the allele pair's combined RFU (representing the contribution of the heterozygous genotype),	$Mx = \frac{A}{A+B+C}$
The third allele's RFU is higher than the allele pair's combined RFU,	$Mx = \frac{A+B}{A+B+C}$

Heterozygote-heterozygote pairing

For a potential heterozygote-heterozygote pairing, the Mx may be estimated as follows.

Step	Action
1	For each pair of alleles, calculate a PHR using the combined RFU of the allele pair (representing the unshared alleles) and the RFU of the third allele (representing the shared allele). **If…** / **Then…** The third allele's RFU is lower than the allele pair's combined RFU, → $PHR = \frac{C}{A+B}$ The third allele's RFU is higher than the allele pair's combined RFU, → $PHR = \frac{A+B}{C}$
2	For each combination with a PHR greater than 60%, calculate a minor contributor Mx based upon the lower RFU of the unshared alleles. This assumes proportional contributions for the shared allele. $$Mx = \frac{A}{A+B}$$

Locus Mx

Average the Mx values from the potential heterozygote-homozygote pairings and heterozygote-heterozygote pairings.

For example, if two combinations were possible for heterozygote-homozygote pairings and one combination was possible for a heterozygote-heterozygote pairing, the average Mx would be the sum of the three individual Mx values divided by 3.

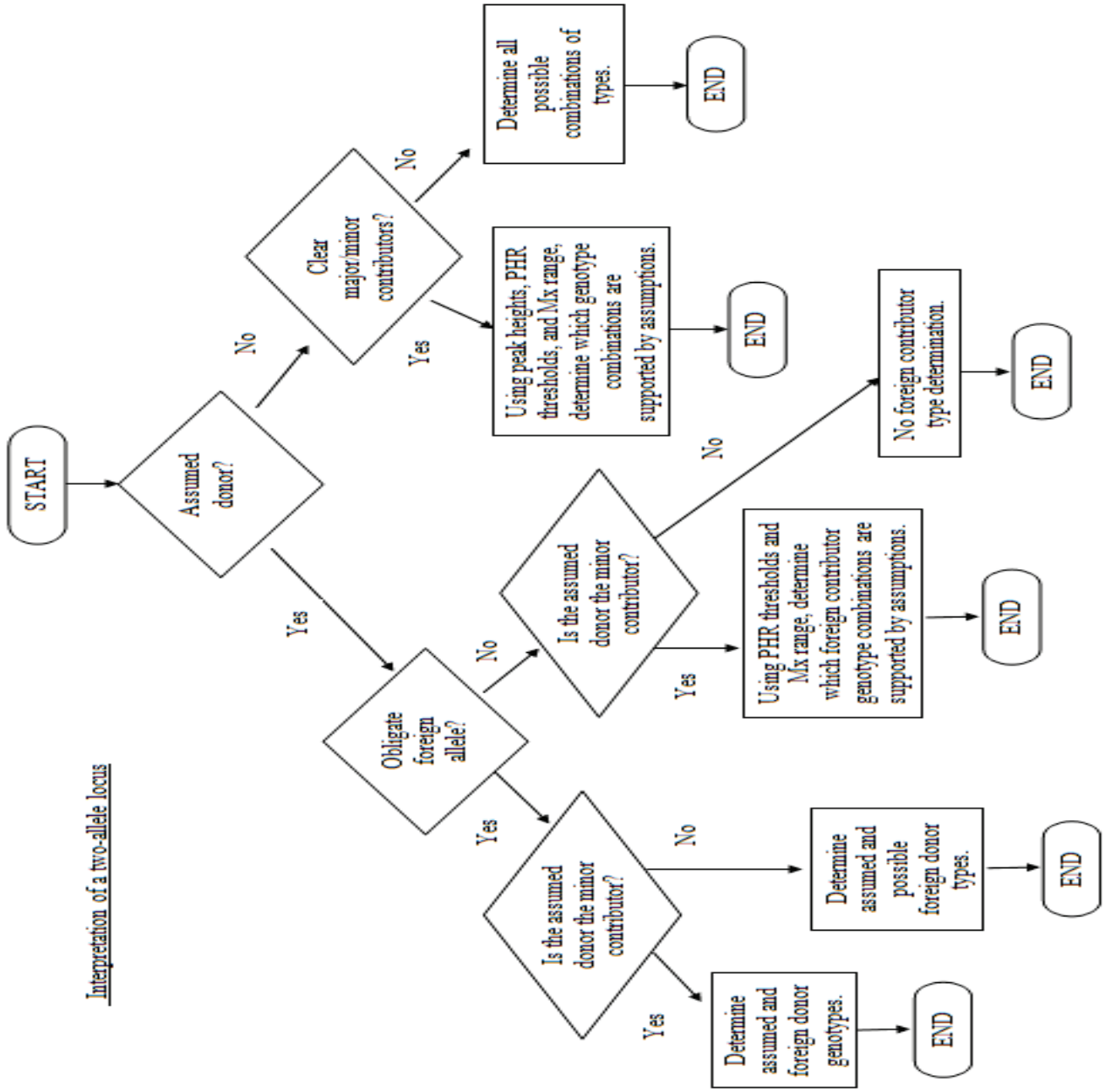
Average Mx

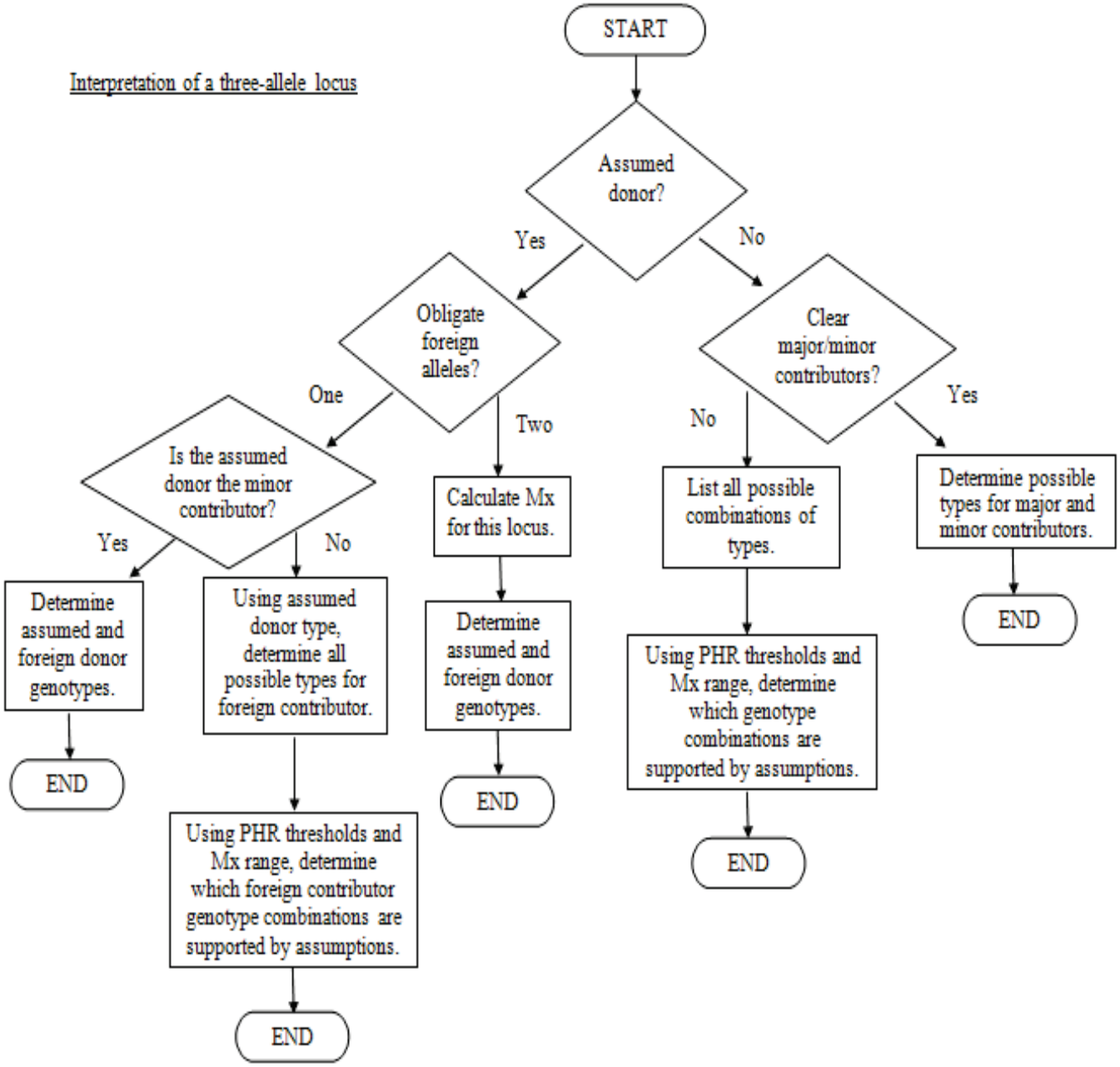
Average the Mx values from all three-allele loci for which an Mx was estimated.

Section 3.7.7 Appendix II – Two-Person Mixture Flow Charts

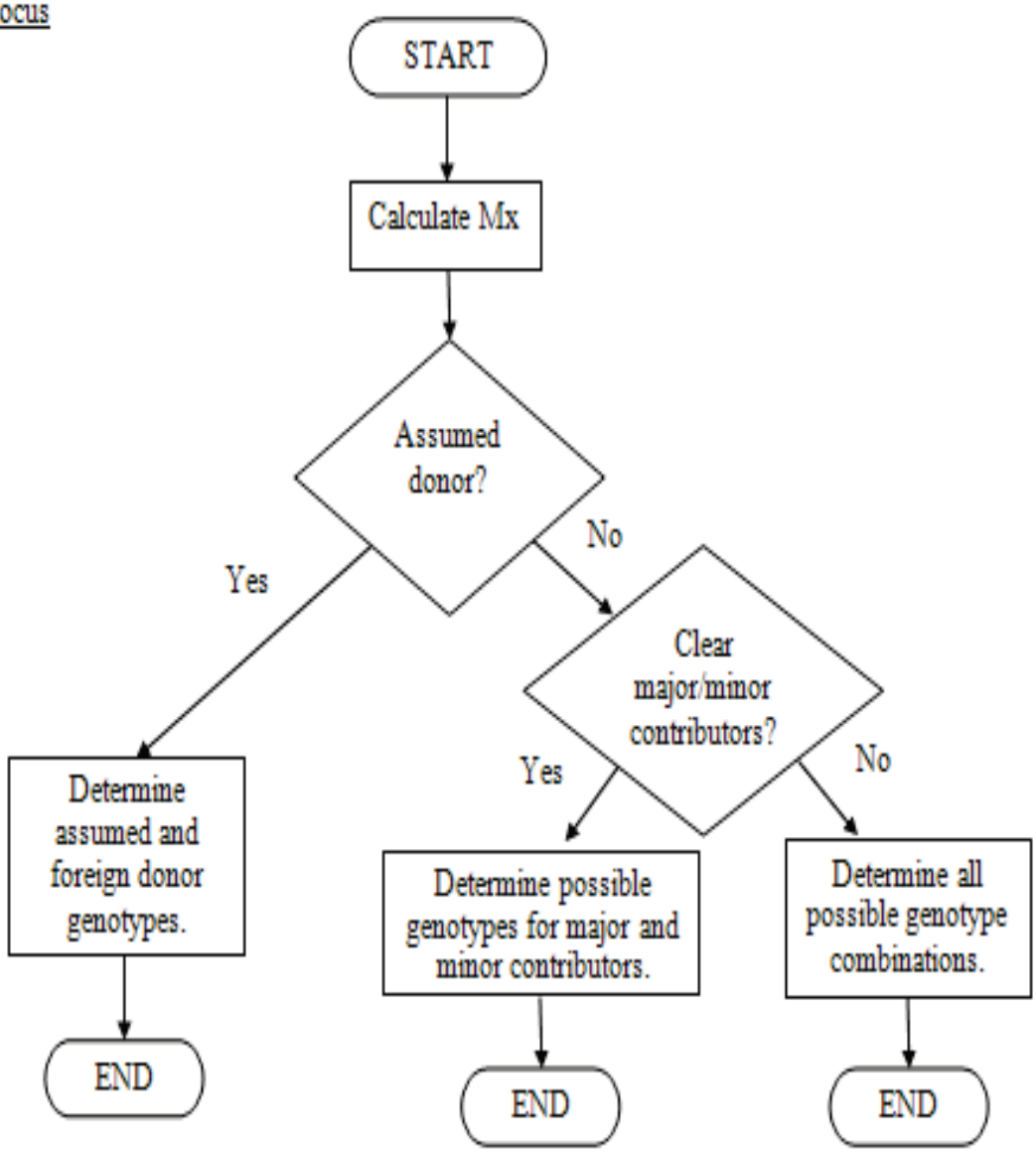
Flow charts

The following flow charts diagram the interpretation process for 2-allele, 3-allele, and 4-allele loci in two-person DNA mixtures.




Interpretation of a three-allele locus
START
Assumed donor?
Yes
No
Obligate foreign alleles?
Clear major/minor contributors?
One
Two
No
Yes
Is the assumed donor the minor contributor?
Calculate Mx for this locus.
List all possible combinations of types.
Determine possible types for major and minor contributors.
Yes
No
Determine assumed and foreign donor genotypes.
Using assumed donor type, determine all possible types for foreign contributor.
Determine assumed and foreign donor genotypes.
Using PHR thresholds and Mx range, determine which genotype combinations are supported by assumptions.
END
END
END
END
Using PHR thresholds and Mx range, determine which foreign contributor genotype combinations are supported by assumptions.
END

Interpretation of a four-allele locus



Section 3.8 3500/3500xL Genetic Analyzer Procedure

Section 3.8.1 Introduction

Introduction

This procedure describes the use of the Life Technologies/Applied Biosystems 3500 (array of 8) and 3500xL (array of 24) capillary electrophoresis instruments for STR typing purposes using the Identifiler Plus, MiniFiler, and Yfiler PCR amplification kits. It does not contain sections on amplification and genotyping of STRs. Amplification and genotyping are performed as described in the relevant technical procedures.

Contents

This section contains the following topics:

Hazard warning

Formamide is an irritant and a teratogen. Avoid skin contact and inhalation. Use in a well-ventilated area (*e.g.,* fume hood). Wear lab coat, gloves, and protective eyewear when handling.

Caution

Minimize light exposure to the matrix standard, the internal size standard GeneScan-600 [LIZ] version 2, and the allelic ladder.

Section 3.8.2 Materials, Reagents, Equipment, and Analytical Controls

Materials & reagents

- Anode Buffer Container (ABC) 3500 Series
- Cathode Buffer Container (CBC) 3500 Series
- 3500/3500xL POP-4® Polymer, 384 or 960 samples
- Conditioning Reagent 3500 Series
- GeneScan™ 600 LIZ® Size Standard v2.0 Size Standard 3500 Series
- Hi Di formamide
- Identifiler Plus allelic ladder (AmpFlSTR Identifiler Plus PCR Amplification Kit)
- Matrix standard DS-33
- Deionized water

Equipment

- 3500/3500xL Genetic Analyzer, Life Technologies/Applied Biosystems
- 3500 8-capillary array and/or 24-capillary array
- 96-Well Septa for 3500 Series Genetic Analyzers
- 3500 series Cathode Buffer Container Septa
- 96-well retainer & base set (Standard) 3500 Series Genetic Analyzers
- Microfuge tubes
- Mini centrifuge
- Vortex
- Centrifuge with plate rotor or alternative plate spinner
- 96-well reaction plates
- MicroAmp strip caps
- Genetic Analyzer septa plate covers
- Heat blocks or thermal cyclers
- Ice blocks or ice
- Polypropylene 50 mL tubes
- Luer-lock syringe
- Pipettors
- Pipet tips

Analytical controls

The analytical controls and standards for capillary electrophoresis include:

Internal Size Standard GeneScan®-600 [LIZ] version 2.0
GeneScan®-600 [LIZ] v2.0 (GS600v2 [LIZ]) contains dye-labeled plasmid DNA fragments of known size that are co-injected with the sample to allow estimation of STR allele sizes.

Allelic Ladder
The allelic ladder provided in each STR kit is used to determine the genotypes of samples amplified from the same kit (*e.g.,* Identifiler Plus, MiniFiler, and Yfiler). While the ladders primarily include the common alleles, additional alleles exist and may be detected.

Important

Capillary electrophoresis is performed only in the PCR Product Room. All pipettors and other equipment in the PCR Product Room are dedicated for post-PCR use only.

The 3500/3500xL license must be renewed annually. Upon expiration, the instrument will not operate.

Section 3.8.3 Setting up the 3500/3500xL Genetic Analyzer

Section 3.8.3.1 Initial Set-Up

3500/3500xL Set-up

Perform the following steps to set up the 3500/3500xL Genetic Analyzer.

Step	Action
1	If not already on, turn on the 3500/3500xL Genetic Analyzer. **Important** Wait for the status light to turn solid (not flashing) green.
2	Turn on the computer attached to the instrument and logon. **Important** *Before* launching the Data Collection software, wait for the green checkmark to appear in the 3500 Server Monitor icon (lower, right-hand corner of the Windows taskbar).
3	Launch the 3500 Series Data Collection software version 1.0 (or higher, if performance checked) by double-clicking on the desktop shortcut icon.
4	After the Data Collection Software has finished launching, the Login dialog box is displayed. Enter the User Name and Password in the 3500 Log In dialog box.
5	The 3500 Series Data Collection **Dashboard** screen appears after the software successfully launches. If needed, click the Dashboard button to bring up this screen.
6	Refresh the dashboard by clicking on the **Refresh** button in the Consumables Information Section.

Preferences

Data Collection preferences may be set and adjusted by opening the window labeled **Preferences**. In particular:

- **System/Instrument Settings/Instrument Name** may be set to the name of the instrument.
- **User/Plate Setup** may be set to **HID, POP4,** and **36** for **Application, Polymer Type,** and **Capillary Length,** respectively; and to **Plate** or **Table** for **Assign Plate Contents View.**
- **User/Run Setup** is generally set to match the folder location designated in the Results Group (*e.g.,* D:\Applied Biosystems\3500\Data); data will go to this folder in the absence of an assigned Results Group and File Name Convention.

Section 3.8.3.2 Maintenance

Before starting

Note that the Maintenance Wizards are accessed via the left navigation pane. After choosing **Maintenance** from the top right-hand side of the screen, click on **Maintenance Wizards** in the left navigation pane.

Data Collection will tick down the dials in the Dashboard every 10 minutes but it does not refresh the Consumables Information window unless prompted.

Monthly Maintenance

The following should be performed approximately monthly or at least within a month prior to a run:

- Flush the water seal trap
- Perform the *Wash Pump and Channels* wizard

The other tasks are performed as needed.

Water Trap

To flush the water seal trap, perform the following steps.

Step	Action
1	Fill a 20-mL Luer lock syringe with deionized water and expel any bubbles from the syringe.
2	Attach the syringe to the forward-facing Luer fitting at the top of the pump block. Hold the fitting with one hand while threading the syringe onto the fitting with the other hand.
3	Open the Luer fitting by grasping the body of the fitting and turning it (and the attached syringe) approximately one-half turn

	counterclockwise.
4	Flush the trap by pushing slowly and steadily on the syringe plunger. It is VERY important to flush slowly; do not use excessive force. It should take approximately 30 seconds to flush 5 mL of deionized water through the trap.
5	Retighten the fitting by turning it back approximately one-half turn clockwise. Remove the syringe by holding the fitting with one hand while reverse threading the syringe out of the fitting with the other hand. Make sure the fitting is closed.
6	Record the pertinent information in the instrument log.

Wash

To run the *Wash Pump and Channels* wizard, perform the following steps.

Step	Action
1	Choose **Maintenance**, and then **Maintenance Wizards**.
2	Click on the **Wash Pump and Channels** wizard button.
3	Follow the prompts to complete the task.
4	Record the pertinent information in the instrument log.

Fill Array

The array may be filled with polymer periodically to extend the life of the array.

To fill an array, perform the steps below.

Step	Action
1	Choose **Maintenance**, and then **Maintenance Wizards**.
2	Start the **Fill Array with Polymer** wizard.
3	Follow the prompts to complete the task.

Shutdown

The 3500/3500xL may be shutdown when desired (*e.g.,* during an extended period of no use). It is not necessary to shut down the Genetic Analyzer and associated computer following a run.

To shut down the 3500/3500xL, perform the steps below.

Step	Action
1	Start the **Shutdown the Instrument** wizard from the **Maintenance Wizards** screen. ***Note:*** *This wizard takes 60 minutes to complete.*
2	Place a conditioning reagent pouch onto the instrument after

	removing the polymer bag.
3	If the Polymer Pouch will be stored for later use, place a pouch cap securely on the polymer bag after removing it from the instrument, indicate the date of removal, and store the polymer in a refrigerator. Otherwise, discard the Polymer Pouch.
4	Exit Data Collection software application.
5	Turn off the computer by selecting **Shutdown** from the **Start** menu.
6	Depress the 3500/3500xL Genetic Analyzer power button to shut down the instrument.

Restart

It is beneficial to perform a periodic restart of the computer and the 3500/3500xL, as described below:

Step	Action
1	Exit Data Collection software application.
2	Restart the computer by selecting **Shutdown** from the **Start** menu.
3	Depress the 3500/3500xL Genetic Analyzer power button to shut down the instrument.
4	Turn on the 3500/3500xL and wait for a solid green light to illuminate.
5	When there is a solid green light, turn on the computer and login.
6	Launch Data Collection and login.

Septa mat re-use

The 3500 septa mats may be re-used several times before discarding. See the cleaning procedure described below:

Step	Action
1	Soak the previously used septa mat in 5% bleach for approximately 5-15 minutes.
2	Rinse the septa mat thoroughly with running tap water, followed by dH20.
3	Allow the septa mat to air dry.
4	Once dry, store the septa mat in a clean location.

Section 3.8.3.3 3500/3500xL Reagent Preparation

Notes on POP4

- Both the 384 and 960 sample (total injections) polymer pouches have enough polymer to accommodate one priming event and four bubble removes per pouch in addition to the sample count.
- Upon attempting to start a run, Data Collection will present an alert when there is insufficient polymer remaining for the number of injections requested to be performed. Either the polymer must be replaced or injections removed in order to start the run.
- Polymer should not be on the Genetic Analyzer for more than approximately one month total time (*i.e.,* not including intervening days stored in a refrigerator). Once this time has been reached, the polymer should be discarded or marked for training/research purposes.

Notes on Buffer

- The ABC and CBC each contain 1x Genetic Analyzer buffer.
- When installing the ABC, before removing the adhesive seal, tip the container sideways to transfer any buffer in the small chamber to the larger chamber. There should be enough buffer in the large chamber to reach the fill line on the container.
- When installing the CBC, after removing the adhesive seal, pat the container with Kimwipes to remove the excess liquid on the lid. Be sure septa covers are fully in contact with the container lid.
- As prompted by the software, this buffer is replaced if it has been on the instrument for more than seven days.
- As long as a run is started prior to the seventh day expiration time, the buffer may be used and Data Collection will not prevent the completion of the run. However, once started, Data Collection will not allow additional injections to be added to the run.

Notes on Arrays

- The 8-capillary and 24-capillary arrays should yield acceptable data up to at least 160 injections. In practice, acceptable data has been obtained from significantly more.
- When the array is replaced, new spatial and spectral calibrations both need to be performed.

Set-up Wizards

Run the appropriate wizard for the task(s) needed.

Step	Action
1	**Refresh** the Consumables Information.
2	Choose **Maintenance**, and then **Maintenance Wizards**.

3	Click on the appropriate wizard button for the task needed as outlined below. **If ...** / **Then ...** A new array needs to be installed, / Start the **Install Capillary Array** wizard. Fresh polymer is needed, / Start the **Replenish Polymer** wizard. There are bubbles in the channels, / Start the **Remove Bubbles** wizard. Fresh anode buffer is needed, / Open the door and replace the anode buffer container (ABC). Fresh cathode buffer is needed, / Bring the tray forward, open the door, and replace the cathode buffer container (CBC).
4	Follow the prompts to complete the task.
5	Click **Refresh** from the Dashboard to update the screen.
6	Record the pertinent information in the instrument log.

Section 3.8.3.4 Spatial Calibration

Defined

A spatial calibration creates a spatial map of the array for the CCD (Charged-Coupled Device). This map defines the pixel number at the center of fluorescence in each capillary.

When to perform

A spatial calibration must be performed each time the detection cell door is opened (*e.g.,* array installation or removal). A spatial calibration is unnecessary if the instrument is already set-up, whether or not a new polymer pouch has been added or the buffer has been replaced.

Spatial calibration

Perform the following steps for a 3500/3500xL Genetic Analyzer spatial calibration.

Step	Action
1	Access the Spatial Calibration screen by selecting **Maintenance**, then **Spatial** under **Calibrate**.
2	Select **No Fill**, or **Fill** to fill the array with polymer before starting the calibration.

	***Note**: If the spatial calibration is performed more than once, there is no need to fill the capillaries with polymer the second time.*
3	Select **Perform QC Checks** to have Data Collection check each capillary against a specified range for spacing and intensity. Data Collection will calculate the following: **Attribute** / **Calculation** / **Threshold** Average peak height / Sum of all peaks divided by number of peaks / Must be >6,400 RFU for 8-capillary instruments or >3,000 for 24-capillary instruments Uniformity (*i.e.,* peak height similarity) / Standard deviation divided by average peak height / Must be 0.2 or less Capillary spacing / Max spacing minus min spacing / Must be 2 pixels or less
4	Click **Start Calibration.**
5	Evaluate the calibration once complete. • There should be 8 single sharp peaks, all of similar peak height and at least approximately 6,400 RFU. • The presence of small shoulders is acceptable. • Each peak should have a marker at the top of the peak. • The peaks should be separated by increments of 13-16 position values.
6	Does the data meet the passing criteria? **If ...** / **Then ...** Yes / Click **Accept Results** and proceed to Step 7. No / Click **Reject Results** and repeat Steps 1-5. • It may be helpful to fill the capillaries again. • The array detection cell may need to be cleaned with methanol.
7	**Export** and **Save** the file. Click **View Spatial Calibration Report**. Click **Print**. In the Printer dialog box, select **CutePDF Writer** as the printer to save the report as a pdf file. Name the report as *Spatial Report <Instrument name> <date> <analysts initials>* and Save this to a Spatial Calibrations folder on the computer. It will be retained

	for five years, after which time it may be deleted.

Section 3.8.3.5 Spectral Calibration

Defined

A spectral calibration creates a calibration file to correct for the overlap of fluorescence emission spectra from each of the dyes. The Identifiler Plus dyes (6-FAM, VIC, NED, PET, LIZ) are referred to as the G5 dye set.

When to perform

Data Collection requires a new spectral calibration for each new array installed. Similar to a spatial calibration, only one spectral calibration is maintained by the software. Each new spectral calibration overwrites the previous calibration.

A spectral calibration should also be performed if the laser, optics, or CCD camera have been realigned or replaced by a service engineer, or when pull-up/pull-down peaks become excessive (>>6%).

It is not necessary to perform a spectral calibration following a servicing if a service engineer has not replaced or realigned the laser, optics, or CCD camera, the array will not be changed and the instrument is giving acceptable data.

Notes

- *Formamide should be aliquoted in a fume hood.*
- *Minimize light exposure to the matrix standard.*

Spectral calibration

Perform the following steps for a 3500/3500xL Genetic Analyzer spectral calibration.

Step	Action
1	Check and replace fluids on the instrument as necessary.
2	Optional: Set the oven temperature by clicking **Start Pre-heat**.
3	Prepare standards for spectral calibration by mixing 3 µL of Matrix Standard Set DS-33 with 297 µL of HiDi Formamide. Vortex cocktail and spin briefly.
4	Dispense 10 µL of matrix standard cocktail into wells A1-H1 (3500) or A1-H3 (3500xL) of a 96-well plate. Cover the plate with a septa mat. ***Note:*** *The matrix standard volume may be adjusted as needed.*
5	Heat-denature the matrix standards for 3 minutes at 95ºC. Snap-cool

	the plate for 3 minutes.
6	Select **Maintenance**, then **Spectral** under **Calibrate.** ***Note:*** *You do not create a Plate for the calibration. The software uses the predetermined positions A1-H1 or A1-H3 for calibration.*
7	Select the number of wells in the spectral calibration plate and specify the plate location in the instrument.
8	Select **Matrix Standard** as the Chemistry Standard and **G5** as the Dye Set.
9	(Optional) Select **Allow Borrowing** if you want an adjacent passing capillary to replace a failed capillary. Otherwise, no failed capillaries will be allowed in a passing spectral calibration. If borrowing is allowed, Data Collection will allow: • For 8-capillary 3500's, one adjacent-capillary borrowing event (*i.e.,* one capillary to fail) • For 24-capillary 3500xL's, up to three adjacent-capillary borrowing events (*i.e.,* 3 capillaries to fail)
10	Load the plate onto the 3500/3500xL: • Ensure that each sample is positioned correctly at the bottom of each well. • Assemble the 96-well plate on a plate base and snap a plate retainer over the plate and base. • Press **Tray** to bring the autosampler forward. • Open the door and place the sample assembly on the autosampler. • Close the door.
11	Click **Start Run**. The system sets up three injections. If the first injection does not pass the set parameters, injection 2 is performed. If injection 2 doesn't pass, then injection 3 is performed. Data Collection uses the following criteria for passing a capillary matrix: • The five peaks must be detected. • Each peak must be at or above the minimum required amplitude threshold (*i.e.,* 750 RFU). • Spikes are absent or not interfering with peak recognition. • The baseline is not elevated. • The Condition Number must be below the upper bound (*e.g.,* 1.0 - 13.5 range for G5). • The Quality Value must be at or above the minimum Quality Value (*e.g.,* 0.95 for G5). • The software evaluates all the spectral runs (1, 2, or 3) performed collectively.

12	Assess the results when the run has concluded: • Passing capillaries are shown in green. • Failing capillaries are shown in red. • Borrowed capillaries are shown in yellow with an arrow indicating the adjacent capillary from which results were borrowed. ***Note:*** *To determine the cause of failure for any non-passing capillaries, it is generally useful to review the Spectral Calibration Report and/or the xml log files (D:\Applied Biosystems\3500\datastore\SpectralCalibrations*.xml.*
13	For each capillary, evaluate the spectral and raw data by clicking on a capillary in the table. • Verify that the order of the peaks in the spectral profile from left to right is blue-green-yellow-red-orange. • Verify that the order of the peaks in the raw data profile from left to right is orange-red-yellow-green-blue. • Verify that the peaks in the spectral profile do not contain gross overlaps, dips, or other irregularities.
14	To accept the spectral calibration, click **Accept.** Otherwise, re-run the spectral calibration (repeat Steps 1-13). ***Note:*** *Once accepted, the results from a passing new spectral calibration automatically become the active spectral calibration for that particular dye set.*
15	To archive the accepted spectral calibration results: Click **View Spectral Calibration Report**. Click **Print**. In the Printer dialog box, select **CutePDF Writer** as the printer to save the report as a pdf file. Name the report as *Spectral Report <Instrument name> <date> <analyst's initials>* and Save this to a Spectral Calibrations folder on the computer. It will be retained for five years, after which time it may be deleted.

Section 3.8.3.6 Preparing Samples for Capillary Electrophoresis

Before starting

- Typically, allelic ladder is included in more than one well of an entire run, depending on the number of samples to be injected.
- Every set of 8 or 24 wells to be injected should contain at least formamide and GS600v2 [LIZ]; in other words, there should be no empty wells such that every capillary is injecting at minimum formamide and GS600v2 [LIZ]. Alternatively,

formamide alone may be used to fill the excess wells in a run. Note that data from excess wells need not be collected (*i.e.,* included in the Plate).

- The plate record on the 3500/3500xL is referred to as the Plate.

Prepare Samples

Perform the following steps to prepare samples for capillary electrophoresis.

Step	Action
1	Optional: Preheat the oven by setting the temperature on the **Dashboard** to **60ºC** and clicking **Start Pre-Heat**. The pre-heat step takes approximately 30 minutes and will automatically stop after 2 hours.
2	Combine the necessary amount of HiDi formamide and GS600v2 [LIZ] size standard as shown below.

Number of injections (*i.e.,* wells)	HiDi formamide* (~ microliters)	GS600v2 [LIZ] * (~ microliters)
8	74.8	4.4
16	149.6	8.8
24	**224.4**	**13.2**
32	299.2	17.6
40	374.0	22.0
48	**448.8**	**26.4**
56	523.6	30.8
64	598.4	35.2
72	**673.2**	**39.6**
80	748.0	44.0
88	822.8	48.4
96 (full plate)	**897.6**	**52.8**

* Calculations are based on the following:
(X + Y) x 8.5 µL formamide
(X + Y) x 0.5 µL GS600v2 [LIZ] size standard

X = number of amplified samples + number of ladder wells + number of GS600v2 [LIZ] wells
Y = 10% of X to provide slight excess for pipetting

3	Mix the tube and spin briefly in a mini centrifuge.
4	Label a clean 96-well plate and aliquot 9 µL of the formamide/GS600v2 [LIZ] master mix into each well to be used. Any unused wells in a run of eight should also contain 9 µL of the formamide/GS600v2 [LIZ] master mix.
5	Add 1.0 µL of PCR product or allelic ladder to each well and cover the plate with a 96-well septa plate cover. The standard input volume of 1.0 µL may be reduced but not increased.

	Notes: • *Sterile deionized water may be used to reconstitute evaporated samples.* • *3500 series 96-well septa plate covers may be used interchangeably with those from the 3130 series.*
6	If needed, briefly spin the 96-well plate.
7	Heat-denature the samples for three minutes at 95ºC.
8	Snap-cool samples immediately for a minimum of three minutes.
9	Prepare the plate assembly: • Place the sample plate into a 3500 Series plate base. • Snap the plate retainer over the plate and plate base.
10	With the instrument doors closed, press the **Tray** button to bring the autosampler to the forward position.
11	Place the plate assembly on the autosampler and close the instrument door.
12	Repeat steps 2-11 if a second plate is to be run on the 3500/3500xL Genetic Analyzer.

Section 3.8.3.7 Create/Import a Plate

Defined

Plates are data tables in the instrument database that store information about a run, such as sample names, plate name, sample well numbers, dye set information, assays, *etc.,* similar to a Plate Record on the ABI Prism 3130 instrument. Each plate of samples will have an assigned Plate in the Data Collection software. If two plates of samples are to be run on a 3500/3500xL, then two Plates must be created—one for each.

Before starting

All samples and controls, including the allelic ladder, should be injected at least twice per run. For case samples, the quality of the two injections must be sufficient to ensure reproducibility of typing results. One good injection is sufficient for positive and negative controls.

Two injections with the *same injection conditions* are needed for interpretation of evidence samples.

The standard run time of 1210 seconds (both 3500 and 3500xL) may be increased. The standard injection conditions may be reduced, but not increased. These are:
• 15 seconds at 1.2kV on the 3500

• 24 seconds at 1.2kV on the 3500xL

Modified assays may additionally or alternatively be chosen. Modifications may include shorter injection times, lower injection voltages, and longer run times. Refer to Appendix I Creating New Assays, File Name Conventions, and Results Groups. Modified assays should reflect the changes made in the assay name (*e.g.,* Casework_POP4_3500_8sec).

More than one protocol can be assigned to an assay. For example, if samples are to be injected twice with two different injection durations, then a single assay with the two protocols is chosen. Alternatively, to use a different protocol for a replicate injection, specify a re-injection in the Monitor Run screen after you start the run.

Duplicate injections are identified by the file suffix "(2)" or, "(3)" if a triplicate injection, *etc.*

Create a Plate

Perform the following steps to create a Plate. A plate may alternatively be imported, as described in the next block.

Step	Action
1	In the Dashboard, click the **Create Plate From Template** button to display the Open Plate Template from the Library dialog box. Alternatively, a plate may be created from **Create New Plate**.
2	Filter the templates by selecting **HID** in the Filter.
3	• Select either: – A previously created template (*e.g.,* **Casework_template**), or – **HID_36_POP4** (3500) or **HID_36_POP4_xl** (3500xL) • And click **Open**.
4	Enter the Plate name. Verify the following are selected: **96 wells, HID, 36,** and **POP4**. ***Note:*** *If setting up two plates with the same name, an "A" may be included in the name of the plate record for the plate in the left deck position (e.g., 16MCE5A_mmddyy). Similarly, a "B" may be included for the right-hand plate (e.g., 16MCE5B_mmddyy).*
5	Click **Assign Plate Contents.**
6	From either **Plate View** or **Table View**: • Enter sample names in the appropriate wells • Choose an Assay to assign to each sample (*e.g.,* **Casework _POP4_3500**) • Choose a File Name Convention for each sample (*e.g.,* **Casework_3500**)

	• Choose a Results Group for each sample (*e.g.,* **3500**) • Specify Sample Type at least for Allelic Ladder wells. ***Notes:*** *Spaces may be used in sample names*.
7	Click **Save Plate** to save the Plate; see Section 3.8.7 Appendix III for plate naming conventions.
8	From **Table View**, click on **Export** to export a .txt file of the Plate. Save the exported Plate to the hard drive.
9	Repeat Steps 1-8 if a second plate is to be created and run.

Import a Plate

A plate may alternatively be imported. Perform the following steps to import a Plate.

Step	Action
1	Go to the Library and click **Import**. ***Note:*** *The Plate Name is not retained if import occurs through* ***Assign Plate Contents****.*
2	Navigate to the Plate for import and click **Import**. Address the prompt that indicates whether the import was successful.
3	Under **Maintenance**, choose **Main Workflow** in the left navigation pane.
4	Under **Setup**, choose **Assign Plate Contents.** Click **Open Plate** and choose **Edit Existing Plate…** from the drop-down menu.
5	Filter the templates by selecting **HID** in the Filter and choose the appropriate Plate to open.
6	From either **Plate View** or **Table View**, verify the following have been entered: • Sample names in the appropriate wells o An Assay assigned to each sample (*e.g.,* **Casework_POP4_3500**) • A File Name Convention for each sample (*e.g.,* **Casework_3500**) • A Results Group for each sample (*e.g.,* **3500**) • Sample Type designated at least for Allelic Ladder wells. ***Notes:*** *Spaces may be used in sample names*.
7	If any changes were made: • Click **Save Plate** to save the Plate. • From **Table View**, click on **Export** to create a .txt file of the Plate. • Save the exported Plate to the hard drive.
8	Repeat Steps 1-7 if a second plate is to be imported and run.

Section 3.8.3.8 Start a Run

Start Perform the following steps to start a run.

Step	Action
1	**Link Plate for Run.** Either: • With the Plate open in **Assign Plate Contents**, click **Link Plate for Run**. Be sure the plate is linked to the correct position (*i.e.,* A or B), or • Go to Main Workflow/Run Instrument/Load Plates for Run and click **Link Plate**.
2	If there is a second plate to be linked, click **Link Plate** for that second plate.
3	**Refresh** the Consumables Information and verify the correct spectral calibration is indicated.
4	Set up the Injection List. Either: • Click **Create Injection List** to open the virtual injection list, or • Go to Main Workflow/Run Instrument/ Preview Run.
5	In the Injection List view: • Select any columns by highlighting the row(s) from the list on the left that need to be injected more than once and click on **Duplicate** as many times as additional injections are needed. • Injection order may be modified by selecting a column(s) and then using the **Move Up** or **Move Down** arrows. • If insufficient polymer remains for the desired number of injections, an error message will appear preventing the start of the run. • The list includes all injections, whether running one plate or two. • To view Plate A versus Plate B injections, toggle between the **Plate A** and **Plate B** tabs (top, right side of the window).
6	Click on **Start Run.**

Section 3.8.3.9 Monitor and Modify a Run

Start Perform the following steps to monitor and modify a run.

Step	Action
1	After the run has started, the **Monitor Run** screen is automatically displayed.
2	To monitor injection status and sample quality while the run is in progress: • Injection status is indicated under Injection List Details (top,left) • The data from completed samples may be viewed by selecting the desired well in the plate view (top, right) and then selecting the Sample Tab under Instrument Run Views and Flags (bottom, left). • Both raw data and data analyzed using the assigned QC Protocol may be viewed by clicking **Review Results**. – Click the **HID Samples** button to update the sample file list. – Scroll through the sample list to view each injection. – This view may also be accessed from Main Workflow/Review Results/ View Fragment/HID Results.
3	Additional injections may be added before the run completes, as follows: **Steps** / **Action** 1 — If needed, return to the injection list view (Main Workflow/ Run Instrument/Monitor Run). 2 — Either select the relevant column(s) or a particular well(s) from the plate view (top, right). 3 — Click **Re-Injection**. 4 — In the Re-injection dialog box, make the appropriate selections and click **OK**. **Result** The additional injections should appear in the injection list.
4	When the run has completed, review the data to ensure that it is of sufficient quality for each sample.
5	Locate the Plate txt file and transfer it to the Run Folder (in the Data Folder of the D drive).
6	Transfer the Run Folder to the desired workstation for analysis.

7	Print sample names, well numbers, and assays from the Plate for inclusion in the case file(s). Indicate the number of injections for each sample. If injections were added during the run using a different assay, that assay name &/or those different conditions should be documented.

Section 3.8.3.10 Additional Run

A follow-up run

If the initial run has completed and additional injections are needed for the samples or a subset of the samples from a run, either:

- Create a new Plate (see previous Section 3.8.3.7 Create/Import a Plate) or,
- Edit the original Plate (see below).

This becomes a new run; record run information in pertinent instrument log. The new run should *minimally* include:

- Those samples requiring another injection,
- The positive and negative amplification controls,
- Two injections of allelic ladder, and
- Any corresponding reagent blank(s) *if* the samples re-injected are run on an instrument different from the original and/or if the run conditions have potentially increased in sensitivity.

Edit original Plate

To edit the original Plate:

Step	Action
1	Click on **Open Plate** from the **Assign Plate Contents** screen. When prompted, choose yes to unlink the plate.
2	Select the desired plate and **Save As** a new Plate with the new run number in the plate name. The plate name should be updated in order to have a new run folder created.
3	Assign plate contents to the sample wells being re-injected following Steps 6-10 in previous Section 3.8.3.7 Create/Import a Plate. ***Note:*** *All samples in an array (i.e., one or three columns of eight wells) will be re-injected but only the data for those samples in the column(s) with an assigned assay will be saved to sample files. In other words, even though all 8 or 24 samples in the set will be re-injected, not all the data needs to be collected.*

4	Continue with Sections 3.8.3.8 Start a Run and 3.8.3.9 Monitor and Modify a Run.

Section 3.8.4 References

Applied Biosystems 3500/3500xL Genetic Analyzer, 3500 Series Software 2 User Guide (PN 4476988c) July 2013

Applied Biosystems 3500/3500xL Genetic Analyzer User Bulletin (PN 4469192A) June 2011

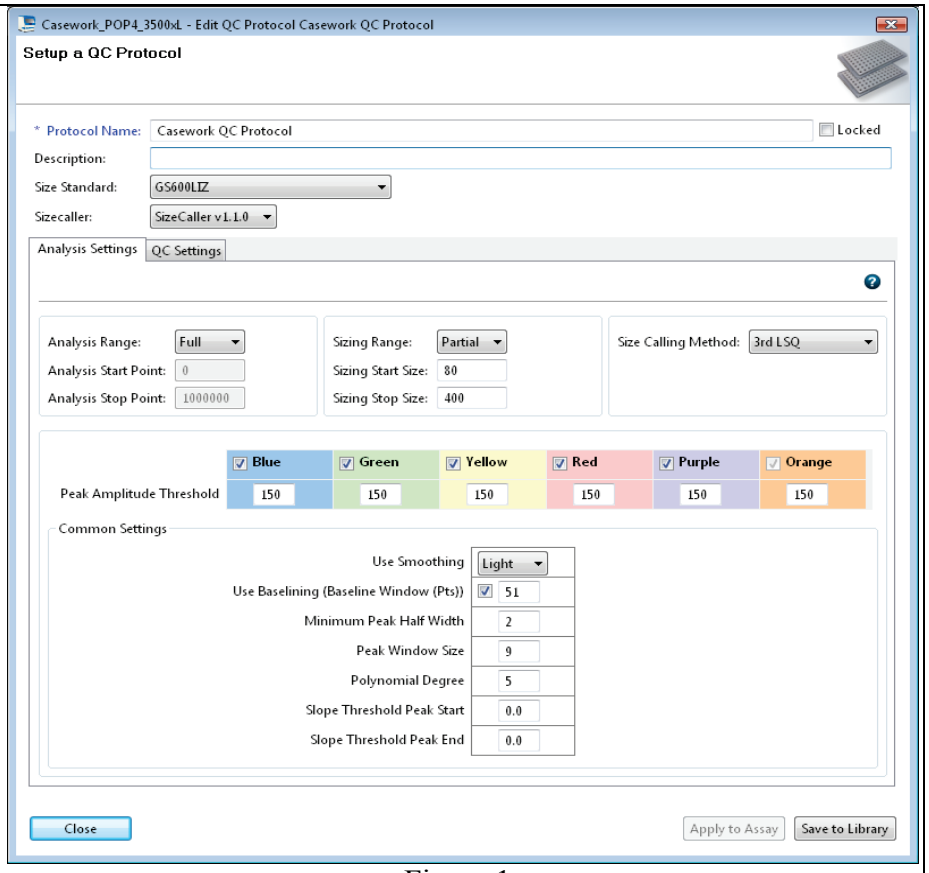
Section 3.8.5 Appendix I - Creating New QC Protocols

Casework QC Protocol

The Casework QC protocol can be used to assist the analyst in evaluating data for re-injections while the instrument is running. For example, the software will flag sample wells that fail SQ (sizing quality).

To create the Casework QC Protocol, perform the following steps.

Step	Action
1	Under **Maintenance**, choose **Main Workflow** in the left navigation pane.
2	Under **Setup**, choose **Assign Plate Contents**, go to **Assays** box and select **New** under **Action**.
3	Select **Create New** across from **QC Protocol**.
4	Name the protocol as **Casework QC Protocol**.
5	The Analysis Settings should be as shown below. The QC Settings may remain at the default values.

	 Figure 1
6	Click **Save to Library**.

Section 3.8.6 Appendix II - Creating New Assays, File Name Conventions, Results Groups, and Plate Templates

New Assays To create a new Assay, perform the following steps.

Step	Action
1	Under **Maintenance**, choose **Main Workflow** in the left navigation pane.
2	Under **Setup**, choose **Assign Plate Contents**, go to **Assays** box and select **New** under **Action**.
3	Name your assay in the **Setup an Assay** dialog box and select **HID** for the Application Type. • Routine assays run under standard conditions are named as: – "Casework_POP4_3500" for Identifiler Plus, MiniFiler, and Yfiler on

	the 3500 – "Casework_POP4_3500xL" for Identifiler Plus, MiniFiler, and Yfiler on the 3500xL • The standard injection conditions are: – 3500: 15 seconds at 1.2kV – 3500xL: 24 seconds at 1.2kV • For assays modified from the standard conditions, be sure to include information reflecting the change in the assay name (*e.g.,* Casework_POP4_ 3500_8sec). – The injection conditions (voltage/time) may be reduced but not increased. – The default run time of 1210 seconds (3500 and 3500xL) may be increased.
4	If more than one Instrument Protocol will routinely be used (*i.e.,* different injection or run parameters for the same wells), then click the **Yes** circle after "Do you wish to assign multiple instrument protocols to this assay?" Otherwise, select **No**.
5	Select the Instrument Protocol(s) to apply to the assay, or click on **Create New**. The routine Instrument Protocol used for Identifiler Plus, MiniFiler, and Yfiler is the default "HID36_POP4_G5_NT3200" for 3500 and "HID36_POP4xl_G5_NT3200" for 3500xL. ***Note:*** *Although normalization parameters are built into these Instrument Protocols, the data is not collected with normalization applied.*
6	Select the QC Protocol (*i.e.,* Casework QC Protocol).
7	Click **Save to Library**.

New File Name Conventions

To create a new File Name Conventions, perform the following steps.

Step	Action
1	Under **Maintenance**, choose **Main Workflow** in the left navigation pane.
2	Under **Setup**, choose **Assign Plate Contents.** Go to the **File Name Conventions** box and select **New** under **Actions**.
3	Enter a new name for the File Name Convention. For routine use, name as "Casework_3500" or "Casework_3500xL."
4	Choose the attributes to be used in sample file naming. For routine use, the following are recommended in the listed order, using a delimiter underscore:

	• Well position • Sample name • Plate name • Capillary number
5	Select File Location as **Default File Location "D:\Applied Biosystems\3500\Data**." ***Note:*** *The file location specified in File Name Convention is used only if a Results Group is not specified for a well.*

New Results Group

To create a new Results Group, perform the following steps.

Step	Action
1	Under **Maintenance**, choose **Main Workflow** in the left navigation pane.
2	Under **Setup**, choose **Assign Plate Contents.** Go to **Results Groups** box and select **New** under **Actions**.
3	Enter a new name for the Results Group. For routine use, name as "3500" or "3500xL."
4	Select attributes and delimeters in the Select Results Group Attributes dialog box. • Select Prefix and Plate Name by selecting each attribute and clicking on "Add." • For Prefix, enter "RF." • Select "Underscore_" as the delimeter for each attribute., check "Add between attributes," highlight the list of selected attributes, and click Add. • Order the attributes as Prefix_Plate Name_Results Group Name.
5	For **Reinjection Folder Option**, select "Store reinjection sample files with original sample files (same level)."
6	For **Folder Option**, select: • Default file location "D:\Applied Biosystems\3500\Data\RF_<Plate Name>_3500\" • Include a Results Group Name folder **Run Folder Name** These settings result in the Run Folder name as either "RF_<Plate Name>_3500" or "RF_<Plate Name>_3500xL."
7	Click **Save to Library**.

Plate template

A run template may be set up to have a Plate auto-populate with an assay, file name convention, and results group routinely run.

To set up the Casework_template, perform the following steps:

Step	Action
1	From the **Dashboard**, click the **Create New Plate** button.
2	Name the Plate as "Casework_template."
3	Verify the other fields on the left have **96, HID, 36,** and **POP4** chosen and click **Assign Plate Contents**.
4	Choose the assay, file name convention, and results group to add from the library: • "Casework_POP4_3500," "Casework_3500," and "3500" for routine 3500 runs • "Casework_POP4_3500xL," "Casework_3500xL," and "3500xL" for routine 3500xL runs
5	Under **Save Plate**, choose Save As Template. This template will now reside in the Library and may be used to create Plates for future runs under standard conditions with Identifiler Plus, MiniFiler, and Yfiler (**Create Plate from Template** button on the **Dashboard**).

Section 3.8.7 Appendix III - Electronic File Naming Conventions

Naming

The instrument run number, case number, and plate name are used to facilitate electronic data tracking. Each casework 3500/3500xL instrument is assigned an identifier, and each run is assigned a consecutive number. For purposes of illustration, this is simply referred to as "#MCE#" in the examples below.

In the circumstance where two or more of the same file/folder types are generated on the same day, they will be distinguished by the addition of -2, -3, *etc.* (*e.g.,* CaseNumber IDX-2 *mmddyy* Analyst's Initials).

File extensions

The software will automatically add the appropriate extensions to file names. The following is a list of extensions used by Data Collection and GeneMapperID-X:

Sample file .hid
Project .ser
Size standard .xml
Analysis parameters .xml

Plot settings file .xml
Table settings file .xml
Panels and bins .txt

Run set-up documents

Sample name (case sample): CaseNumber_Sample Name_Plate Name…[1]
Sample name (control): Sample Name_Plate Name…[1,2]
Plate name: #MCE#_*mmddyy*[3,4]

[1]The Casework_3500 File Name Convention will automatically add the well position as a prefix and the plate name and capillary number as suffixes to the sample name (*e.g.*, B01_ CaseNumber_SampleName_ PlateName_02, E04_#MCE# _Sample Name _Plate Name_01). Duplicate injections of the same sample will be appended with (2), (3), etc. (*e.g.,* B01_ CaseNumber_SampleName_PlateName_02(2)).

[2]Control samples are uniquely identified with the plate name, whether batched or not. The case number is generally also included with case samples. The run number may additionally be used for clarity.

[3]This date ("*mmddyy"*) is the date of the 3500/3500xL run.

[4]Other unique identifiers, such as the amplification plate name (*e.g.,* date run and analyst initials, STRPlate_<date>_<time>, *etc.*), may be used to name the Plate.

Post-run documents

Run Folder: RF_Plate Name_3500/3500xL
Case Folder: CaseNumber CF Analyst's Initials

The 3500/3500xL Data Collection software automatically creates run folders. The 3500/3500xL Results Group will result in the Run Folder named as either RF_PlateName_3500 or RF_PlateName_3500xL. If two plates are run together, the software will create separate Run Folders for each plate. Additional injections run as a new run (set up following the completion of the first run) will be added to a new run folder as long as the Plate Name has been updated. If multiple runs are performed for a case, the additional Run Folders are added to the Case Folder.

ID-X files

GeneMapper Project: CaseNumberIP IDX *mmddyy* Analyst's Initials[1,2]
2nd Reader Project (optional): CaseNumberIP IDX 2nd reader *mmddyy* Reviewer's Initials[1,2, 3]

[1]Use "IP" for Identifiler Plus projects, "MF" for MiniFiler projects, and "YF" for Yfiler projects.

[2]The project date is the date the project was created.

[3]Analysis files generated by the second reader are saved to the case folder.

Case Folder organization

The following example demonstrates typical case folder organization:

CaseNumber CF Analyst's Initials (Case Folder)

- RF_PlateName_3500/3500xL (Run Folder)
  - Sample files
  - Plate.txt
- CaseNumberIP IDX *mmddyy* Analyst's Initials (Identifiler Plus Project)
- CaseNumberMF IDX *mmddyy* Analyst's Initials (MiniFiler Project)
- CaseNumberYF IDX *mmddyy* Analyst's Initials (Yfiler Project)

Section 3.8.8 Appendix IV - 3500/3500xL Genetic Analyzer Computer Maintenance

Drive space

Before a run will start, the Data Collection software automatically checks the space on drives C, D, E, and F to ensure that there is sufficient space to store the newly created sample file data. Runs are stored to the D drive.

The Data Collection software will send a warning message if a drive is getting full (70-75% full). At 78% full, the software will not start a run.

If there is insufficient room to store data from a new run, the following message will appear:
"Remove data: the drive is getting full"

Check Drives

To check drive space:

Step	Action
1	Open Computer.
2	Right-mouse click on C drive.
3	Select Properties.
4	Click **General** tab.

Insufficient drive space

If there is insufficient space:

Step	Action
1	Archive run folders and sample files.
2	Delete sample file data from the drive D.
3	Empty the contents of the Recycle Bin.

Library clean-up

User-created items in the Library may be either manually delete or purged using Purge as described below.

Step	Action
1	Under the **Manage** window, choose **Purge**.
2	Click **Yes** to the warning message that this action will be permanent and cannot be undone.
3	Specify the date category and range for those files to be deleted, then click **OK**. **Important** All non-factory default files in that range within the Library will be permanently deleted.
4	Click **Yes** to the purge warning message. ***Notes:*** • *Factory provided items will not be purged.* • *Purge will not delete the spatial and spectral calibrations. This information is not stored in the Library.*

Note: *Items in the library may be archived to a .dsz file and subsequently restored to the library via the* ***Archive*** *and* ***Restore*** *functions under the* ***Manage*** *drop-down window. This may be useful if the Data Collection is being upgraded or the computer hard drive must be rebuilt or replaced.*

Defragment

Defragment the hard drive space:

Step	Action
1	Go to Start/All Programs/Accessories/System Tools/Disk Defragmenter.
2	If desired, click **Analyze disk** to determine if defragmentation is needed.
3	To proceed with defragmentation, click **Defragment disk** and follow prompts.

Section 3.8.9 Appendix V - About the Data Collection Library and the Files It Stores

Contents

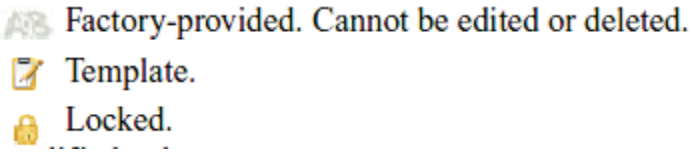
The Library is not a true database (*e.g.,* Oracle) but is managed as such (*e.g.,* access and maintenance). Thus use and modifications to files within the Library occur through the Data Collection software.

The library contains:

- Items selected when setting up a run
  - Plates
  - Assays
  - Filename conventions
  - Results groups
- Items selected when creating an assay
  - Instrument protocols
  - Primary analysis protocols (*e.g.,* QC)
  - Optional secondary analysis protocols (*e.g.,* HID analysis)
- Items selected when creating instrument, sizecalling, and QC protocols
  - Dye sets
  - Size standards

Library symbols

Entries in the library are flagged with the following symbols:



Plate

A Plate contains sample information and any assigned Assays, File Name Conventions, and Results Groups. The Plates Library contains all Plates that have been saved in the software (those already run and those to be run).

Assay

An Assay contains the Instrument Protocol (dye set and run configuration). It also contains a primary Analysis Protocol that is needed to collect data (*e.g.,* Casework QC protocol) and an optional secondary Analysis Protocol.

An Assay must be assigned to all named sample wells on a Plate before it can be linked and run.

File Name Convention

A File Name Convention specifies the naming conventions for sample data files. If one is not assigned to a sample injection, the default format is <sample name>_<well>.

Results Group

A Results Group is used to name, sort, and customize the folders in which sample data files are stored. The file location specified in a Results Group overrides the file location in the File Name Convention specified for a well. It also overrides the data location specified under Preferences.

Instrument protocol

An Instrument Protocol contains the parameters that control the instrument during data acquisition. More than one Instrument Protocol may be added to a single assay.

Dye set

When an Instrument Protocol is created, a dye set is added to the protocol. A dye set defines the following for an Instrument Protocol:

- Dye colors
- Order of the dye peaks in the matrix standard
- Spectral analysis parameters

Size standard

When a QC Protocol is created, a size standard is added to the protocol. A size standard defines the sizes of known fragments. It is used to generate a standard curve, which is used to determine the sizes of fragments in unknown samples.

QC protocol

A QC Protocol is used as the primary analysis protocol. It defines peak definition, sizing, and quality values. This is added to an Assay when the Assay is created. The QC Protocol defines the basic analysis parameters under which data may be viewed as “analyzed” using Review Results/View Fragment/HID Results.

HID analysis protocol

An HID analysis protocol is used as a secondary, optionally defined analysis protocol. It is used for auto-analysis with GeneMapper ID-X. An Assay does not require an HID analysis protocol.

Library templates

When files are used from the Library as templates for a new run, the template is copied by the software and modifications made are independent of the original item in the Library. The changes may be saved to only the assigned Plate and/or the Library copy as well.

Section 3.8.10 Appendix VI - Troubleshooting

Red light

If the indicator light becomes red, the following are possible causes and remediations:

Cause	Remediation
Loss of communication between the computer and instrument.	Restart both – see Section 3.8.3.2 Maintenance
Reservoir septa were pulled off the reservoir by the array.	Securely replace the septa and restart the run.
Smashed array due to lack of use of full plate septa covers.	1.) Use a full plate septa cover with each plate of samples to be run, even if the plate is not full. A partial plate covering does not keep the clip cover perpendicular to the array. 2.) Replace array (see Section 3.8.3.3 3500/3500xL Reagent Preparation) and restart run.

RFID tag

If the instrument isn't reading an RFID tag properly, be sure the tag is correctly facing the instrument. For example, the POP4 pouch may be installed facing either direction but the RFID reader may have difficulty reading the tag if it is facing toward the installer.

Run log

It may be useful to review the run log for a particular run. This is access through **View Logs**, under the **Tools** drop-down menu. Once open, choose the appropriate run to view.

Add from library

If the desired Assay, File Name Convention, and/or Results Group does not appear as a choice in the respective box when setting up a Plate, go to that box, click on the drop-down window, choose **Add From Library**, make the relevant selection from the list (filter for HID for ease), and choose **Add to Plate**.

Section 3.9 Identifiler Plus Genotyping Using GeneMapper® ID-X Version 1.4

Section 3.9.1 Overview

Purpose of the software

GeneMapper® ID-X software (ID-X) is used to:

- Determine base pair sizes for alleles,
- Assign labels to alleles,
- Assign quality values to genotypes, and
- Build tables containing allele information.

About ID-X

There are two forms of GeneMapper ID-X. The basic form is a full copy, which is a fully functioning version. A full copy of the software can have different user accounts, all on the same workstation computer. Alternatively, the user accounts can be set up to access the full copy database over a server. This form of GeneMapper ID-X is a client copy.

One (or more) full copy resides permanently in each BFS laboratory with client workstation copies accessing the full copy for routine analysis. An additional (or more) full copy is available for use that does not interact with a server; this standalone workstation may be used remotely if it has been installed on a laptop computer.

The setup of GeneMapper ID-X described in Appendix I must occur for each full copy. (Note that the setup can be simplified by importing exports of the relevant files.) As needed, user accounts must be setup on each full copy as well, either directly on the same workstation (as described in Appendix II) or through a client copy.

How ID-X analysis works

ID-X determines base pair sizes by comparing alleles to an internal size standard. Allele designations are assigned by comparing the averaged sizes obtained for the alleles in the allelic ladder with the sizes obtained for the unknown sample alleles. Process Quality Value (PQV) flags may be used to evaluate the data.

Overview of procedure

The ID-X procedure involves:
- Creating a New Project with Analyzed Data
- Evaluating and Editing Data
- Printing and Archiving Data

Technical review

In performing a technical review, the reviewer assesses the criteria outlined on technical review forms PF-0022 and PF-0017. As part of the review, the technical reviewer performs a second reading of the STR typing data by reviewing the printed ID-X electropherograms and tabular data. The reviewer also checks the electronic or printed size standard and allelic ladder data. The reviewer may review and/or reanalyze additional electronic data if he/she feels it is advantageous in a particular case.

Contents

This section contains the following topics:

Section 3.9.2 Analytical Controls and Standards for Capillary Electrophoresis and Genotyping

Purpose/use

The allelic ladder provided in the AmpFℓSTR Identifiler Plus Amplification Kit is used to determine the allele designations of samples. While the ladder primarily includes the common alleles, additional alleles exist and may be detected in samples.

GeneMapper ID-X software assigns allele designations to peaks by comparison to allelic ladder(s) injected in the same run. The specific size assigned to each ladder allele may vary between instruments *and* between injections on one instrument.

Allelic Ladder

The STR alleles included in the ladder, and the size ranges they span, are listed in Table 1.

Table 1

Locus	Dye Color	Ladder Alleles	Size Range (bp)	Dye Label
D8S1179	Blue	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	128-172	6-FAM
D21S11	Blue	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	187-243	6-FAM
D7S820	Blue	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	258-294	6-FAM
CSF1PO	Blue	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	303-349*	6-FAM
D3S1358	Green	12, 13, 14, 15, 16, 17, 18, 19	114-142	VIC
TH01	Green	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3	165-204	VIC
D13S317	Green	8, 9, 10, 11, 12, 13, 14, 15	206-250*	VIC
D16S539	Green	5, 8, 9, 10, 11, 12, 13, 14, 15	257-301*	VIC
D2S1338	Green	15, 16, 17, 18, 19, 20, 21, 22, 23, 24,25, 26, 27, 28	301-366*	VIC
D19S433	Yellow	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	106-140	NED
vWA	Yellow	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	157-209	NED
TPOX	Yellow	6, 7, 8, 9, 10, 11, 12, 13	217-261*	NED
D18S51	Yellow	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27	265-345	NED
Amelogenin	Red	X, Y	107 (X), 113 (Y)	PET
D5S818	Red	7, 8, 9, 10, 11, 12, 13, 14, 15, 16	135-171	PET
FGA	Red	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	215-353	PET

Note about sizing information

- Sizes listed for each range are based on sequence data and include the additional nucleotide resulting from non-template nucleotide addition.
- Loci marked with asterisks contain non-nucleotide linkers to modify inter-locus spacing, and thus the size ranges listed for these markers are approximations.

Internal Size Standard

The internal size standard GeneScan®-600 [LIZ] version 2 (GS600v2 [LIZ]) contains dye-labeled DNA fragments of known size that are co-injected with the sample to allow estimation of STR allele sizes.

Section 3.9.3 Creating a New Project with Analyzed Data

Before you begin

If ID-X has not been previously set up, proceed to Appendix I *Setting Up GeneMapper ID-X v. 1.4 for Identifiler Plus*. Once ID-X set-up is complete, return to this section to analyze data.

Case versus batch projects

Below are approaches that may be taken in creating and saving a project:

- Import only the sample files relevant to a particular case from a Run Folder, perform data analysis, and save the case-specific project to a Case Folder for archiving. All data is printed from this case-specific project.
- Import all (or relevant) sample files from a Run Folder and perform data analysis. Data may be printed directly from the batch project and/or from an edited and renamed project (*i.e.,* a specific case number). Projects from which data are printed for case files should be electronically archived (*e.g.,* to the Case Folder).

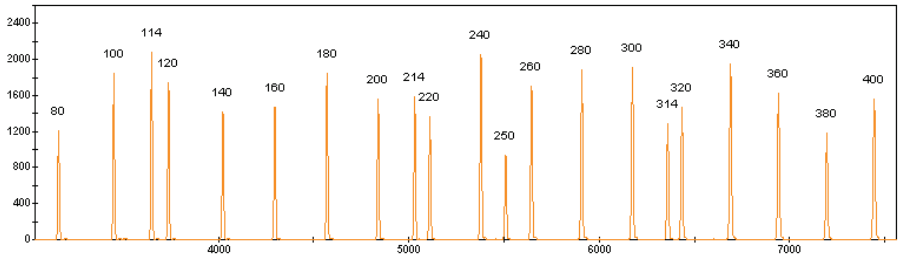
***Note*:** If the Sample Origin Path is not preserved, it may be re-established in ID-X by selecting *Define new Sample File path…* under *Edit*.

Create a new project

Follow these steps to create a new project.

Step	Action
1	• Create a Case Folder or Batch Folder in ID-X Case Data on the desired workstation computer. • Copy the run folder into the Case Folder or Batch Folder.
2	Open ID-X version 1.4 (or higher, if performance evaluated) and log into the software.
3	Create a new project by: – Selecting *Add Samples to Project* under the *Edit* menu.

	– Locate the correct run folder(s), – Click *Add to List*, – And then click the *Add* button. ***Note:*** *If sample files from more than one run folder are to be analyzed, all run folders may be imported into one project because ladder injections from each run folder are averaged separately.*
4	To examine the raw data of each sample: – Click the sample folder in the navigation pane once to view the sample files in the folder. – Click on a sample file and then choose the *Raw Data* tab. – Scroll through the list of sample files to examine each injection. – When finished, click the run folder or project node to return to the main window.
5	To remove a sample from the project (*e.g.,* GS600v2 only, failed, or extraneous injections), highlight the sample and select *Delete from project* under the *Edit* menu. Alternatively, samples may be deleted while viewing electropherograms by selecting *Mark Sample for Deletion*. When the plot window is closed, the sample will be deleted from the project. ***Note****: All injections from a run must be maintained in the run folder.*
6	From the *Table Setting* pull down menu, choose Casework Table Settings.
7	Adjust "Sample Types" to the correct settings. • Allelic ladder injections used for genotyping need to be set to *Allelic Ladder*. • 9947A may be set to *Positive Control.* ID-X will check 9947A genotypes. • Negative controls (*e.g.,* PCR and reagent blanks) may be set to *Negative Control.* ID-X will also check the negative controls for labeled peaks. • All others should be set to *Sample.*
8	Under Panel, *Identifiler_DOJ_Panel_v1.2* should be selected.
9	Under Analysis Method, select either: • Casework_IDP_3500 • Casework_IDP_3130 Open the analysis method and check to be sure that the default settings match those shown in Appendix I (Figure 1 for 3500 or Figure 2 for 3130).

	***Notes**:* • The analysis range may need to be modified to include the GS600v2 80-bp and 400-bp peaks. • The size calling method is 3rd Order Least Squares.
10	Under Size Standard, *Casework_GS600_LIZ(80-400)* should be selected. – The default settings should match those shown in Figure 1.  **Figure 1**
11	Analyze sample files by clicking the menu bar *Green Arrow*. When prompted, name and save the project, either as: • <Case Number>IP IDX <date of ID-X analysis> <analyst's initials> for a case-specific project or, • <Run Folder name>IP IDX <date of ID-X analysis> <analyst's initials> for a batch project. ***Note:*** The Run Folder unique identifier can be the run number (*e.g.* #MCE#) &/or run date (*e.g.* STRplate_mmddyy) &/or another suitable tracking identifier.

Next steps Continue with the steps for Evaluating and Editing Data.

Section 3.9.4 Evaluating and Editing Data

Note Customized table and plot setting profiles may be generated to accommodate analyst-specific data viewing preferences.

Evaluate and edit data

Follow the steps below to evaluate and edit the data.

Step	Action
1	Confirm that the size standard peaks have been correctly assigned. • This can be aided by use of the Sizing Quality (SQ) PQV. • It may also be helpful to view the raw data. • See Figure 1 for the correct GS600v2 peak sizes. Were the peaks labeled correctly? • ***If yes,*** continue to Step 2. • ***If no,*** complete the following actions. To edit a size standard peak assignment (*i.e.,* when a sample has a red flag for SQ), – click once on the sample in the *Samples* view, – then on the *Size Match Editor* toolbar icon. – Edit the size standard peak accordingly. **If GS600v2 peaks were labeled incorrectly due to ...** / **Then ...** An artifactual peak(s) / Left-click once on the peak and right-click to make the necessary edit. The 400bp peak not collected during the run / The sample will need to be re-run. It may be beneficial to increase the run time.
2	Re-analyze any edited sample files. If an allelic ladder injection was edited: – Re-analyze all injections in the project. This is necessary to create new average values (offsets) for allele assignments. – To re-analyze all injections, change an analysis criterion to "activate" the unedited injections for re- analysis (*e.g.,* change the Analysis Method from *Casework_IDP_3500* to *Default* and then back to *Casework_IDP_3500*).

3	Review sample and allelic ladder results. Evaluate the following parameters: – Peak shape and height. – Quality of spectral separation (baselines should be relatively flat, and any pattern of pronounced peaks or dips below true DNA peaks should not be present). – Peak profile (examine for artifactual peaks). If there are extraneous peaks in an electropherogram, it may be useful to examine the raw data. – Identification of off-scale peaks may be aided by the OS Process Component-based Quality Value (PQV) in the *Samples* view. – Ensure that the alleles in the allelic ladder results have been correctly assigned by ID-X. – Allelic ladder peaks should be labeled as shown in Figure 2 (at the end of this section). ***Additional help in evaluating data*** Data evaluation may be assisted by use of additional PQV values. These are viewed in the table produced under the *Genotypes* tab. The *Casework Table Settings* profile will display the following PQVs: BIN, PHR, LPH, AN, CC. The *Analysis Summary* tab has further sample evaluation information based on other PQVs. See Appendix I for guidance on creating custom Table Settings profiles.
4	Electronically edit extraneous peaks between 95 and 375 bp, including extraneous peaks between locus ranges (unlabeled peaks). ***Extraneous peaks include:*** – Pull-up, – Pull-down, – Spikes, – Dye-labeled kit artifacts (“dye blobs”), – Incomplete non-template nucleotide addition peaks, – Labeled stutter peaks, and – Non-specific amplification artifacts. ***Note:*** It is not necessary to edit the 6FAM-labeled kit artifact (blue dye blob) that is frequently observed at approximately 96 bp. ***To make electronic edits:***

Step	Action
1	Left-click once on the peak label, right-click once on the same label, and choose *Delete Label*.
2	Enter the edit in the *Reason for Change* textbox. The abbreviations below may be used for edits.

Edit	Abbreviation
Pull-up	PU
Pull-down	PD
Incomplete Nucleotide Addition	INA
Non-reproducible artifact	NRA
Reproducible artifact	RA
Low-level inconclusive	LLI, *
N-4 Stutter	N-4
N+4 Stutter	N+4
Size standard artifact	SSA

3	***Result:*** The label box will be modified to display the comment and a diagonal line ("slash") will be drawn through the label box.

Note*:* Alternatively, edits may be made manually on print-outs.

5 Is there a microvariant allele or other callable allele requiring an edit?

- ***If no,*** continue to Section 3.9.5 Printing and Archiving Data.
- ***If yes,*** determine the allele call and complete the following actions.
- The allele call determination may be performed manually or following Appendix I of Section 3.10. See Section 3.10.4 for more information.

To edit a microvariant allele or other callable allele:

Step	Action
1	Left-click once on the peak label and right-click once on the same label.
2	Choose *Rename Allele* and *Custom* from the pop-up menus instead of Delete Allele.
3	Enter the allele designation in the *Allele Name* textbox and click *OK*.
4	In the *Edit Allele Comment* textbox, enter the original ID-X designation in parentheses (*e.g.,* "(OL Allele)") and click *OK*.
5	***Result:*** The label box will be modified to display the new information.

Note*:* Alternatively, edits may be made manually on print-outs.

AmpFlSTR Identifiler Plus allelic ladder peaks are shown here in Figure 2.

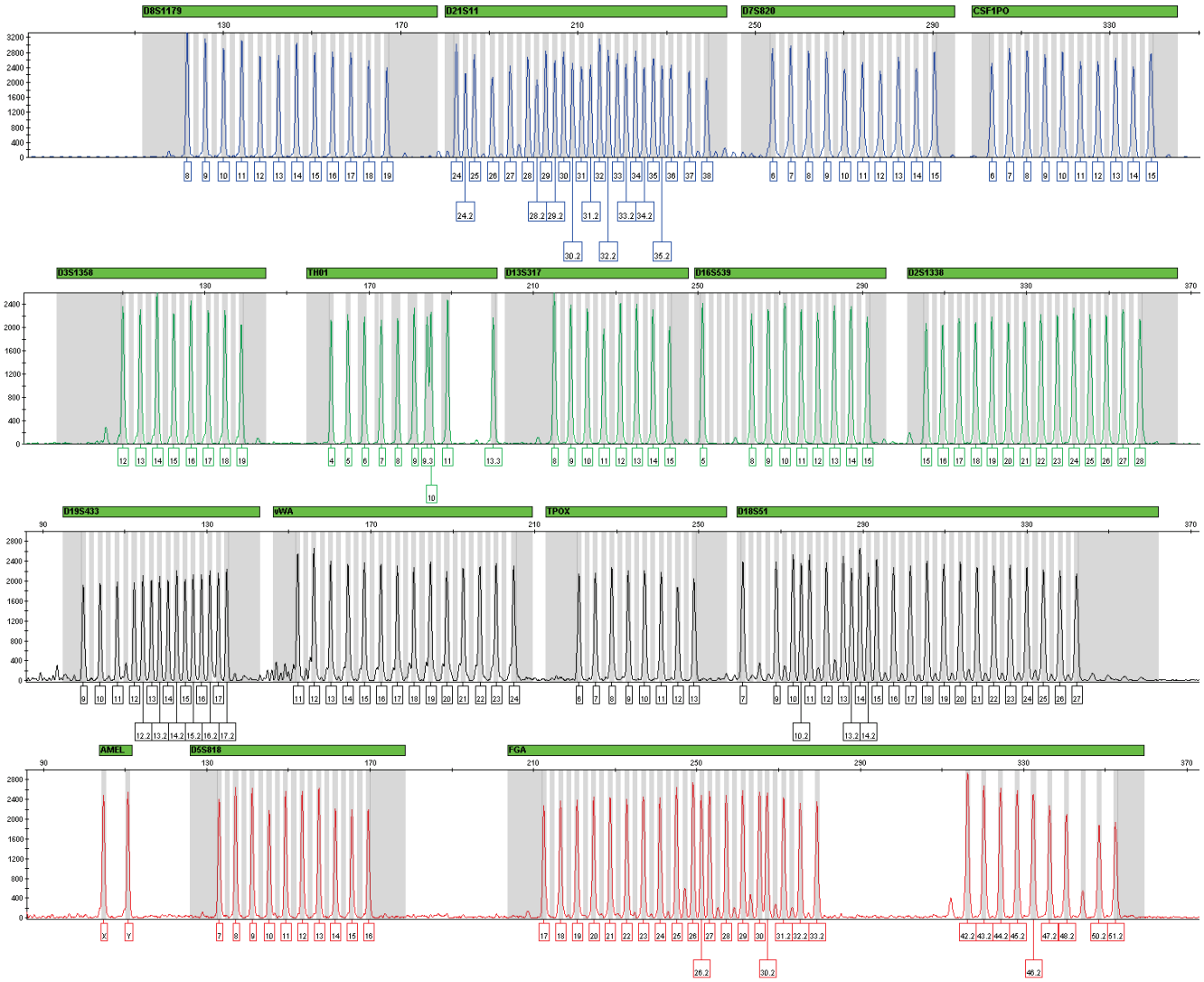


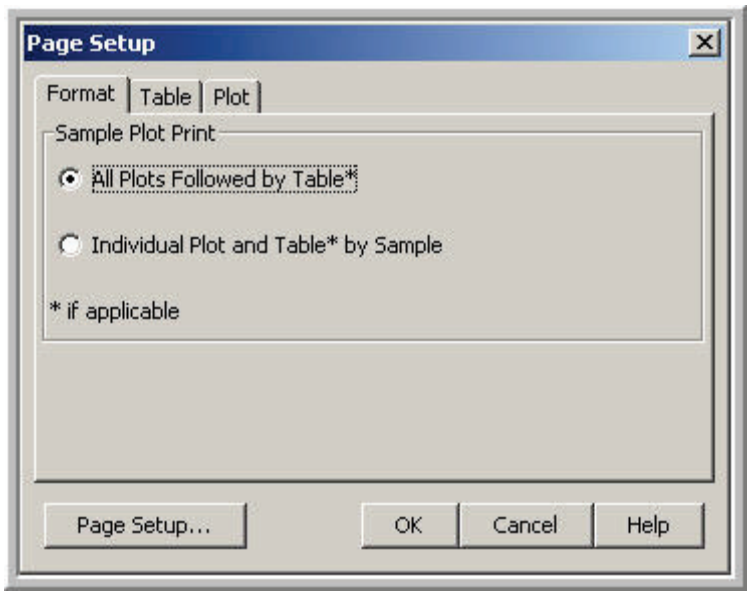
Figure 2

Next steps Continue with the steps for Printing and Archiving Data.

Section 3.9.5 Printing and Archiving Data

Print and archive data

Follow the steps below to print and archive the data.

Step	Action
1	Before printing: • Make sure that the table settings are showing the sizing table with the electropherograms. • Verify *Page Set-up* from the plots view appears as shown in Figures 3, 4, and 5 (parameters may be modified with different printing conditions). • Adjust margins as needed from *Print*, also from the plots view.  **Figure 3**

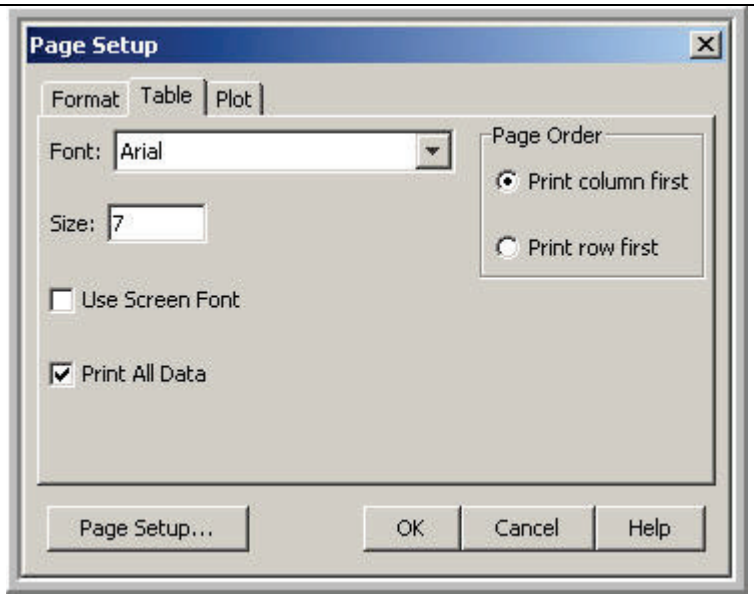


Figure 4

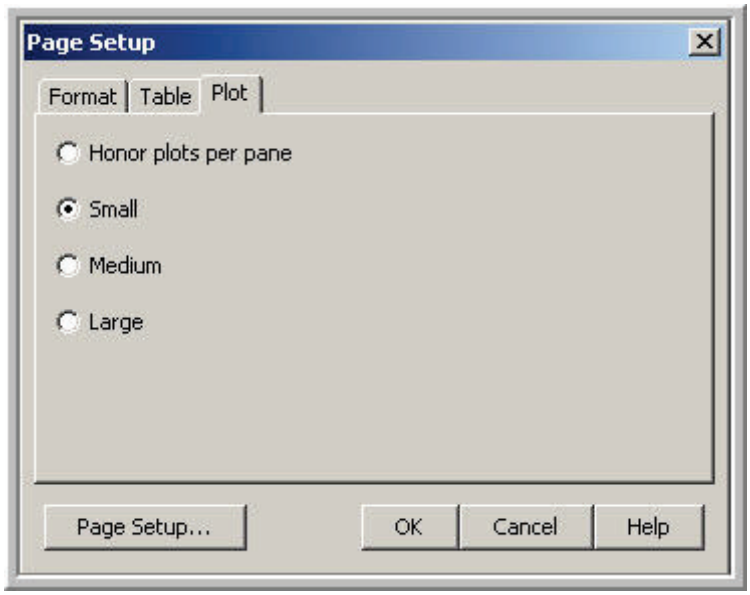


Figure 5

2	After reviewing data and completing electronic edits, select samples to print from the *Samples* tab. **<u>See sections below</u>**: *Printing specifications* and *Notes about printing samples.* ***Note:*** If creating case-specific projects from a temporary batch project, this is generally completed prior to printing as the appropriate case number will be on each printout (*i.e.,* project name).
3	Make any other edits manually that were not addressed electronically (*e.g.,* spikes in orange, artifacts between the locus ranges).

4	*Save* and *export* the project. • Under *Tools*, open *GeneMapper ID-X Manager.* • Highlight the desired project under the *Projects* tab and click *Export*. • At the prompt, enter the project name and navigate to the desired location. • Click *OK*.
5	• Copy the electronic Case Folder or Batch Folder onto one CD for the case file and verify that the folder has been recorded. • Move the folder to a secure network.

Printing specifications

Full view is defined as visualization of the highest peak apexes in the upper half of the printed electropherogram.

Casework Evidence/ Reference Samples (if single-source): Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection. At the analyst's discretion, a close-up view may also be printed.

Casework Evidence Samples (if a mixture): For one injection, print the blue, green, yellow, and red tabular data and electropherograms at full view and close-up view. For at least one other injection, print the blue, green, yellow and red tabular data and electropherograms in close-up view.

Casework Evidence Samples (if a partial profile): For one injection, print the blue, green, yellow, and red tabular data and electropherograms at full view and close-up view. For at least one other injection, print the blue, green, yellow and red tabular data and electropherograms in close-up view.

Negative Amplification Controls, Reagent Blanks, and Other Blanks: Print blue, green, yellow, and red tabular data and electropherograms at 300 (3500)/ 100 (3130) RFU from one injection.

Positive Amplification Control and Quality Control Sample: Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection.

Notes about printing samples

When to print in close-up view
It may be necessary to print duplicate sample injections in a close-up view (for example, if an apparent single-source sample has a low-level, third possible allele present due to mutation).

Allelic ladders
Because the genotyping bins are based on an average of all the allelic ladders, it is

not necessary to print the allelic ladders nor the GS600v2 (orange) electropherograms and tabular data for the allelic ladders.

Batch projects
A unique laboratory identifier must be included on each printed ID-X page. For example, the batch name as the project name or the case number in the sample name field.

Section 3.9.6 References

Applied Biosystems (2012), "GeneMapper® ID-X Software Version 1.4," Applied Biosystems User Bulletin P/N 4477684, Rev.A.

Applied Biosystems (2009), "GeneMapper® ID-X Software Version 1.2," Applied Biosystems Reference Guide P/N 4426481, Rev.A.

Applied Biosystems (2007), "GeneMapper® ID-X Software Version 1.0," Applied Biosystems Administrator's Guide P/N4376327, Rev.A.

See also Section 3.10.

Section 3.9.7 Appendix I – Setting Up GeneMapper ID-X v. 1.4 for Identifiler Plus

Contents

This appendix describes the steps used to set-up GeneMapper ID-X v. 1.4 (or higher if performance checked) for analysis of Identifiler Plus data from both the 3500/3500xL and 3130/3130*xl* Genetic Analyzers. Some of these steps must be completed while logged into the administrator/gmidx account, thus it is recommended to complete the set up using this login.

Setting up GeneMapper ID-X includes the topics listed below.

Topic
Panels and bins
Analysis method parameter file
Table settings file
Casework plot settings file
Other plot settings files
Size standard table

Important
The files created in this appendix are seen and potentially used by all user accounts.

Note: *Customized table and plot setting profiles may be generated to accommodate analyst-specific data viewing preferences. Be cognizant that the commonly used files are used by all user accounts.*

About Administrator/ gmidx

The Administrator/gmidx user account allows the user full access to all functions of the GeneMapper ID-X software. This includes access to analyze, view, edit, and print data (as with the Casework Analyst – see Appendix II Creating User Accounts in GeneMapper ID-X) as well as all administrative function access (such as Security Manager) and creating analysis method parameters.

This account has access to all projects, including those of the Casework, GeneMapper ID-X, and Admin Security Groups.

This account can reset Casework Analyst passwords. However, if the password of this Administrator account is lost, it cannot be retrieved by another user nor Life Technologies/Applied Biosystems.

Panels & bins Perform the following to install and verify the panels and bins.

Step	Action
1	Open GeneMapper ID-X v. 1.4 (ID-X) and log into the software as the Administrator/gmidx user account.
2	Under the *Tools* tab, select *Panel Manager*. Click once in the left pane on the *Panel Manager* node and once on the *AmpFLSTR_DOJ_Panels_v1.2* node to open the folder. Continue to Step 4. If *AmpFLSTR_DOJ_Panels_v1.2* is *not* present, go to Step 3.
3	If *AmpFLSTR_DOJ_Panels_v1.2* is *not* present, perform the following steps.

Step	Action
1	Obtain a copy of *Identifiler_DOJ_Panel_v1.2.txt* and *Identifiler_DOJ_Bins_v1.2*.
2	Copy each to *C:/AppliedBiosystems/GeneMapperID-X/Panels* on the workstation computer.
3	Open ID-X and, under the *Tools* tab, *Panel Manager*.
4	Click once on *Panel Manager* in the left navigation pane.
5	Under the *File* menu, choose *Import Panels*.
6	Select the *Identifiler_DOJ_Panel_v1.2* panel and choose "Casework Security Group". Then, click *OK*. ***Note:*** *The stutter ratio data will not be imported.*
7	Click once on the *AmpFlSTR_DOJ_Panels_v1.2* folder and, under the *File* menu, choose *Import Bin Set.*
8	Select the *Identifiler_DOJ_Bins_v1.2* bin set.
9	Enter the marker specific stutter information: • Click once on the *Identifiler_DOJ_Panel_v1.2* node to open the folder and see each locus in the panel. • Repeat for each locus: – Click once on the *D8S1179* node to "open" that locus. – Click once on *Stutter Ratio & Distance* – Choose *New* to enter the *Minus Stutter* information: ▪ Type of Stutter: *Minus stutter* ▪ Ratio (between 0-1): *0.11* (for D8S1179; see Table 1in this section below for the other STR loci; enter 0 for Amelogenin) ▪ From Distance (bp): 3.25 ▪ To Distance (bp): 4.75 ▪ Click *Apply,* then *OK*.
10	Return to Step 2 of **Panels & bins**.

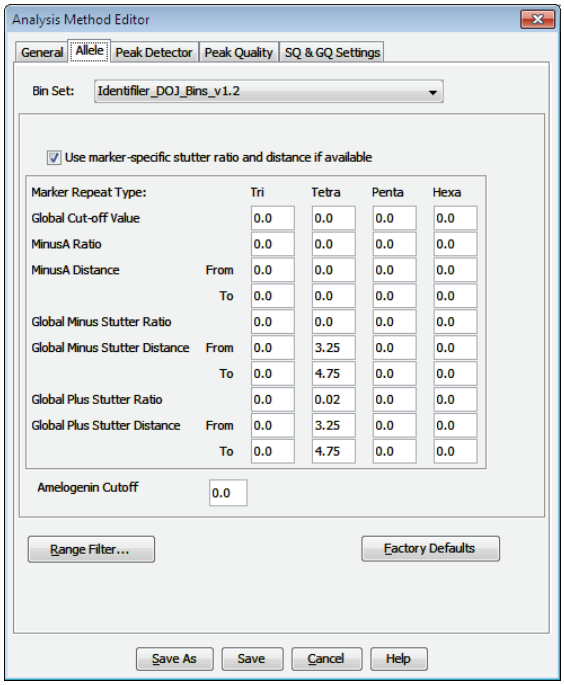
4	Verify *Identifiler_DOJ_Bins_v1.2* is the selected bin set from the drop-down menu in the *Panel Manager* tool bar.
5	Verify the Marker Specific Stutter Ratio is set correctly for each locus. • Click once on the *Identifiler_DOJ_Panel_v1.2* node to open the folder and see each locus in the panel. • Repeat for each locus: – Click once on the *D8S1179* node to "open" that locus. – Click once on *Stutter Ratio & Distance* node to view the marker specific stutter ratio and verify the distance settings (3.25 to 4.75 bp).
6	Click *Apply* if changes were made. Click *OK*.

D8S1179	11%
D21S11	12%
D7S820	10%
CSF1PO	10%
D3S1358	14%
TH01	7%
D13S317	11%
D16S539	11%
D2S1338	14%
D19S433	16%
vWA	16%
TPOX	8%
D18S51	20%
D5S818	11%
FGA	16%

Table 1

3500 Analysis Method

To create the *Casework_IDP_3500* Analysis Method, perform the following steps. Note that this analysis method will apply a global 2% N+4 filter.

Step	Action
1	Under the *Tools* tab: • Select *GeneMapper ID-X Manager* • Click on the *Analysis Methods* tab and on *New*
2	Under the *General* tab: • Analysis type should indicate *HID* • Enter "Casework_IDP_3500" for Name • Choose "Casework Security Group" for Security Group • Enter "Identifiler Plus" for Description
3	Under *Allele*: • Choose *Identifiler_DOJ_Bins_v1.2* for Bin Set • Select "Use marker-specific stutter ratio and distance if available." • Make the necessary edits to the "Tetra" colum to match Figure 1 below, including the "Global Plus Stutter Ratio" at 2%.  **Figure 1** • The remaining values are used at the factory default.
4	Under *Peak Detector*: • Peak Detection Algorithm should indicate *Advanced.* • Carefully fill in the values and match the selections shown in Figure 2.

Analysis Method Editor

General | Allele | Peak Detector | Peak Quality | SQ & GQ Settings

Bin Set: Identifiler_DOJ_Bins_v1.2

☑ Use marker-specific stutter ratio and distance if available

Marker Repeat Type:		Tri	Tetra	Penta	Hexa
Global Cut-off Value		0.0	0.0	0.0	0.0
MinusA Ratio		0.0	0.0	0.0	0.0
MinusA Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Global Minus Stutter Ratio		0.0	0.0	0.0	0.0
Global Minus Stutter Distance	From	0.0	3.25	0.0	0.0
	To	0.0	4.75	0.0	0.0
Global Plus Stutter Ratio		0.0	0.02	0.0	0.0
Global Plus Stutter Distance	From	0.0	3.25	0.0	0.0
	To	0.0	4.75	0.0	0.0

Amelogenin Cutoff 0.0

Range Filter... | Factory Defaults

Save As | Save | Cancel | Help

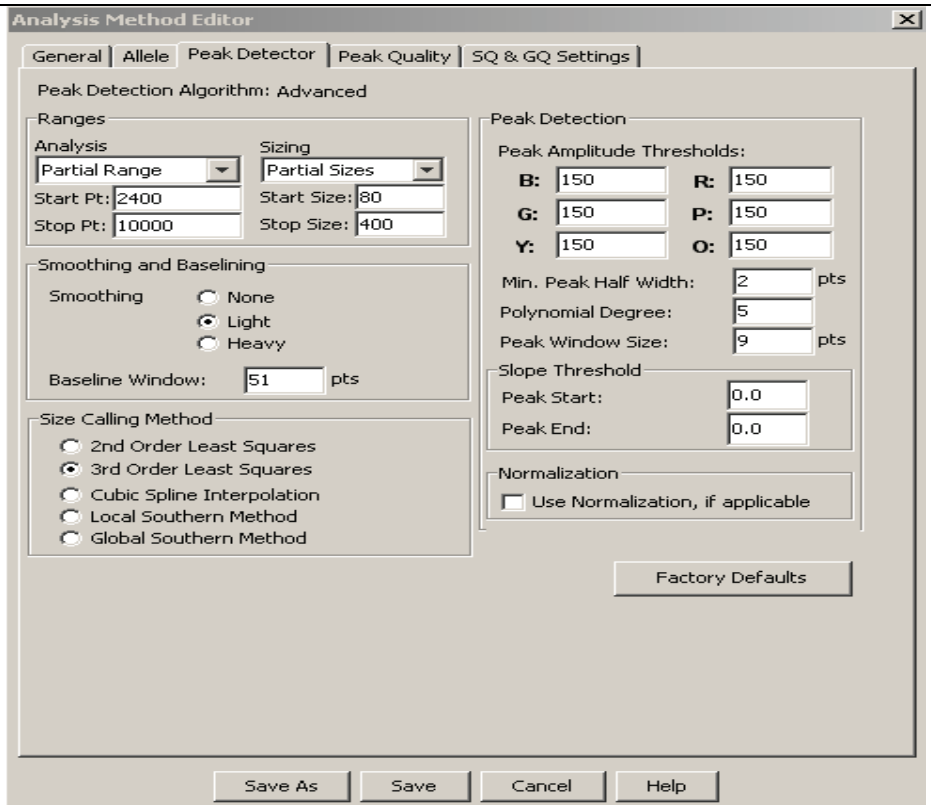


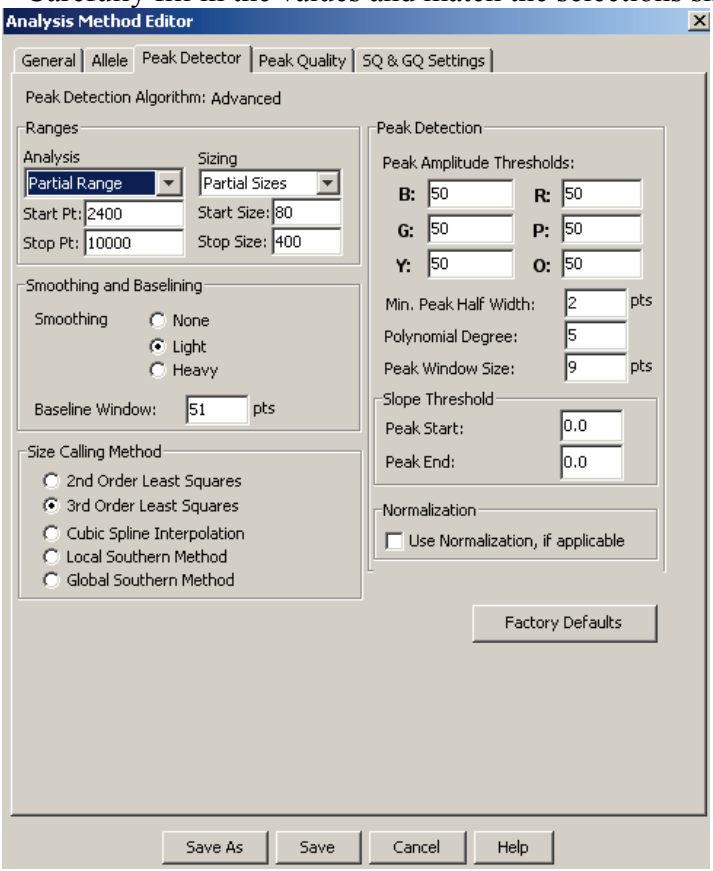
Figure 2

Important
Be careful to NOT select normalization.

5	Under *Peak Quality,* the values should be set as follows: • Signal Level, Homozygous min peak height: *1,075* • Signal Level, Heterozygous min peak height: *150* • Max Peak Height: *32,000* • Heterozygote balance, Min peak height ratio: *0.60* • Peak morphology, Max peak width (basepairs): 1.5 (default) • Allele number, Max expected alleles: – For autosomal markers and Amel 2 (default) – For Y markers 1 (default) • Allelic Ladder Spike: Spike Detection: *Enable*; Cut-off value: *0.2* • Sample Spike Detection: Spike Detection: *Disable*
6	Under *SQ & GQ Settings,* the values should remain at the factory defaults.
7	Save As *Casework_IDP_3500* with "Casework Security Group."

3130 Analysis Method

To create the *Casework_IDP_3130* Analysis Method, perform the following:

Step	Action
1	Under the *Tools* tab: • Select *GeneMapper ID-X Manager* • Click on the *Analysis Methods* tab and on *New*
2	Under the *General* tab: • Analysis type should indicate *HID* • Enter "Casework_IDP_3130" for Name • Choose "Casework Security Group" for Security Group • Enter "Identifiler Plus" for Description
3	Under *Allele*: • Choose *Identifiler_DOJ_Bins_v1.2* for Bin Set • The remaining values are used at the factory default.
4	Under *Peak Detector*: • Peak Detection Algorithm should indicate *Advanced* • Carefully fill in the values and match the selections shown in Figure 2.  **Figure 2**

	Important Be careful to NOT select normalization.
5	Under *Peak Quality,* the values should be set as follows: • Signal Level, Homozygous min peak height: *365* • Signal Level, Heterozygous min peak height: *50* • Max Peak Height: *8,000* • Heterozygote balance, Min peak height ratio: *0.60* • Peak morphology, Max peak width (basepairs): 1.5 (default) • Allele number, Max expected alleles: – For autosomal markers and Amel 2 (default) – For Y markers 1 (default) • Allelic Ladder Spike: Spike Detection: *Enable*; Cut-off value: *0.2* • Sample Spike Detection: Spike Detection: *Disable*
6	Under *SQ & GQ Settings,* the values should remain at the factory defaults.
7	Save As *Casework_IDP_3130* with "Casework Security Group."

Table settings file

To create the *Casework Table Settings*, perform the following:

Step	Action
1	In *GeneMapper Manager ID-X*, click the *Table Settings* tab and click *New*. The *Table Settings Editor* window will open.
2	Under the *General* tab, name the file "*Casework Table Settings*" with "Casework Security Group."
3	Under *Samples*: • Set the font to Arial and size 9. • Sort by Sample Type (Ascending), then Sample File (Ascending), and then None. • Check the boxes shown below. The remaining boxes should be unchecked. – #1 Status – #2 Sample File – #6 Sample Type – #9 Analysis Method – #10 Panel – #11 Size Standard – #14 Run Name – #28 Off-scale (SOS) – #29 Size Quality (SQ) – #30 Sample Spike (SSPK)

4	Under *Genotypes*: • Set the font to Arial and size 9. • Set Allele Settings: Number of Alleles to 4. • Sort by Sample File (Ascending), then Dye (Ascending), and then None. • Check the boxes shown below. The remaining boxes should be unchecked. – #1 Sample File – #6 Marker – #8 Allele – #17 Marker Edit (ME) – #19 Out of Bin Allele (BIN) – #20 Peak Height Ratio (PHR) – #21 Low Peak Height (LPH) – #24 Allele Number (AN) – #27 Control Concordance (CC)
5	Click *OK*.

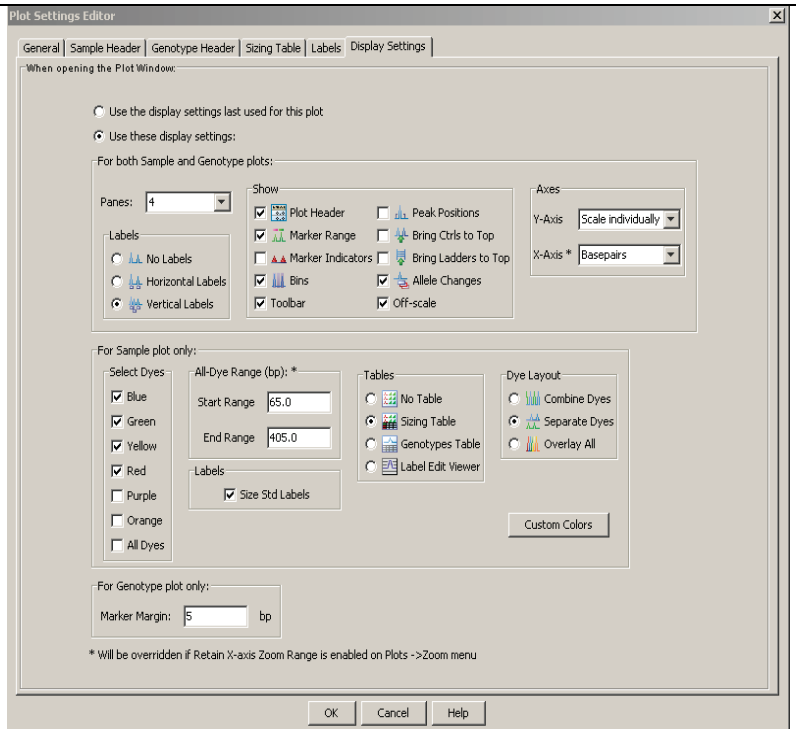
Casework plot settings file

To create *Casework Plot Settings*, perform the following steps.

Note: *See Section 3.9.5 Printing and Archiving Data to set printing preferences for electropherograms and tabular data.*

Step	Action
1	In *GeneMapper ID-X Manager,* click on the *Plot Settings* tab and click on *New.* The *Plot Settings Editor* window will open.
2	Name the file as "Casework Plot Settings" and select "Casework Security Group."
3	Under the *Sample Header* tab, check the boxes shown below. The remaining boxes should be unchecked. • #1 Sample File • #5 Sample Off-scale (SOS) • #6 Sizing Quality (SQ)
4	Under *Genotype Header,* check the boxes shown below. The remaining boxes should be unchecked. • #1 Sample File • #4 Marker • #5 Off-scale (OS) • #6 Out of Bin Allele (BIN) • #7 Peak Height Ratio (PHR) • #8 Low Peak Height (LPH) • #11 Allele Number (AN) • #14 Control Concordance

5	Under *Sizing Table*, • Set the font to Arial and size 9. • Check the boxes shown below. The remaining boxes should be unchecked. – #1 Dye/Sample Peak – #2 Sample File Name – #3 Marker – #4 Allele – #5 Size – #6 Height – #8 Data Point
6	Under *Labels,* • Set the font to Arial and size 9. • Select the labels shown below for Assigned Allele, Custom Allele, Allelic Ladder, and Artifact. – Label 1: Allele Call – Label 2: Height – Label 3: AE Reason for Change (***Note:*** For Allelic Ladder, can be None) – Label 4: None (default) • Under *When opening the Plot Window*: – Check Display virtual allele label in black – Set Label color: Dye Color - Border
7	Under *Display Settings,* adjust the settings to those shown in Figure 3.

	 **Figure 3**
8	Click *OK*.

Other plot settings files

Modified plot settings files may be created for routine use. Some examples are described below.

Casework GS600v2 3500:

Step	Action
1	Click once on *Casework Plot Settings* and **Save As** *Casework GS600v2 3500*.
2	Open *Casework GS600v2 3500.*
3	Under Display Settings, change: • Y-Axis to "Scale to _____" and enter a value that will result in the orange peaks displayed in the approximate upper half of the electropherogram. This will result in the same Y scale for each orange electropherogram, facilitating comparison between injections. • Select Dyes to deselect Blue, Green, Yellow, and Red (only Orange will be chosen)
4	Click *OK*.
5	Repeat Steps 1-4 for a *Casework GS600v2 3130* plot settings file.

Casework Zoom 3500:

Step	Action
1	Click once on *Casework Plot Settings* and **Save As** *Casework Zoom 3500*.
2	Open *Casework Zoom 3500.*
3	Under Display Settings, change the Y-Axis to "Scale to 300" and deselect Orange.
4	Click *OK*.
5	Repeat Steps 1-4 for a *Casework Zoom 3130* plot settings file, entering 100 RFU for the Y-Axis scale.

Casework Compare:

Step	Action
1	Click once on *Casework Plot Settings* and **Save As** *Casework Compare*.
2	Open *Casework Compare.*
3	Under Display Settings, change: • Select Dyes to deselect Green, Yellow, Red and Orange (only Blue will be chosen). This will result in a by-color comparison, which is convenient when comparing multiple injections of the same sample. Once Blue has been reviewed, it can be deselected in the tool bar and another color can be chosen. • Table to No Table
4	Click *OK*.

Size standard table

To create the *Casework_GS600_LIZ_(80-400)* Size Standard Table, perform the following steps.

Step	Action
1	In *GeneMapper ID-X Manager,* click on the *Sizing Standard* tab and once on *GS600_LIZ_(80-400).* Choose Save As and name the file *Casework_GS600_LIZ_(80-400).* Save the new file with "Casework Security Group."
2	Open the new file and verify the sizes have been correctly entered: 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400
3	Verify *Orange* is selected from the Size Standard Dye menu.
4	Click *OK,* then *Done.*

Section 3.9.8 Appendix II – Creating User Accounts in GeneMapper ID-X v. 1.4

Contents

This appendix describes the steps taken to create a Casework Analyst user account. It also includes the additional user-specific steps for setting up the account and workstation.

Setting up GeneMapper ID-X includes the topics listed below.

Topic
Create account
Project Options
Data folder

About Casework Analyst

The Casework Analyst user account allows the user access to projects through the Casework Security Group. Projects saved under the GeneMapper ID-X generic group are also accessible.

The default Profile associated with this user group is Analyst. This profile has the following access associated with the Casework and GeneMapper ID-X Security Groups:

- Create, import, view, edit, and delete projects
- Create, import, view, edit, and delete plotting settings
- Create, import, view, edit, and delete table settings
- Create, import, view, edit, and delete size standard files
- Import, view, edit, and delete panels
- Edit and save modifications to the size range and analysis range of analysis method parameters
- Analyze, edit, and export data

Create account

Follow the steps below to create a Casework Analyst user account.

Step	Action
1	Start the *Security Manager*: • Under *Admin*, choose *Security Manager*. • Enter the administrator account user identification and password to login.
2	Select *Casework Analyst* as the user account type from the left

	navigation pane (under the Users folder).
3	Select *Edit*, then *Duplicate* in the Access Control Administration window.
4	In the Name field of the General section, replace "Clone of Casework Analyst(1)" with the caseworker's network user identification. ***Notes:*** • This login name is not case-sensitive and must be ≤ 30 characters. • A user account cannot be created with the same name that has been previously deleted.
5	In the User Details section, enter the user's first and last name in the Full Name field.
6	Deselect *Show EULA*. This will prevent the display of the End User License Agreement every time a user logs in.
7	Leave Status set to *Active*.
8	Optional: Select *Pre-Expire* to prompt a user to change their password the first time logging into their account.
9	Click *Set Password* to create the password for this user account. In the Change Password dialog box, enter the new password twice. When finished, click *OK*. ***Note:*** *Passwords for casework user accounts may be reset through the administrator/gmidx account.*
10	Verify Profile under Control Properties is set to *Analyst*.
11	Verify "Associate 'Clone of Casework Analyst(1)' with …" is set to *Casework User Group*.
12	Select *File*, then *Save* in the Access Control Administration window to save the new user account.
13	Close the Access Control Administration window and, if desired, restart ID-X to login using the new user account. ***Notes:*** • *The new user account cannot be accessed without restarting ID-X.* • *The first time the new user logs in, the caseworker's network user identification will have to be manually entered. Thereafter, the caseworker's network user identification will appear in the drop-down menu.*

Project Options

The project option preferences are specific to each user account. To set these preferences, follow the steps described below.

Step	Action
1	Login to GeneMapper ID-X v. 1.4 (ID-X) using the Casework Analyst user account.
2	Under *File,* select *Project Options.*
3	Under the *General* tab, select: • *Open Blank Project* from the project sub-section • *Casework Security Group* from the data access control menu
4	Under *Add Samples*, select the following from the pull-down menus: • Set Analysis Method to *Casework_IDP_3500* OR *Casework_IDP_3130*. • Set Size Standard to *Casework_GS600_LIZ(80-400)*. • Set Panel to *Identifiler_DOJ_Panel_v1.2*. • The sample type may be set to the "Read from Data collection Info field" radio button.
5	Choose the *Analysis* tab and select the following: • Select *Stop analysis and display Analysis Requirements Summary***.** • Select *Stop analysis and display Allelic Ladder Analysis Summary* if one or more allelic ladders do not meet sizing and/or genotyping requirements. • After Analysis, select *View Sample Table*. • Do NOT select the option to bring low quality samples to the top. This button has a bug that prevents analysis. • Select the "Symbols" radio button under Quality Metrics Display. • Leave the option to duplicate homozygous alleles unselected.
6	Click *OK.*

Note: *These preferences are set at the user's discretion and may be modified as preferred.*

Data folder

Create a folder called ID-X Case Data on the user's workstation desktop. *For example,* C:\Documents and Settings\All Users\Desktop. Case and run folders should be stored here when analyzing data in ID-X. A standard location for case and run folder placement when creating projects facilitates preservation of the virtual path of origin (the link between sample files and a project).

Section 3.9.9 Appendix III - Viewing and Re-analysis of ID-X Data on Another Workstation Computer

Analyzed data

Users have access to view all projects within the same full copy of GeneMapper ID-X (ID-X).

To view projects on a different full copy of ID-X (as may be done for a technical review), the project must be imported to the ID-X database. To import a project, perform the following steps.

Step	Action
1	Open *GeneMapper ID-X Manager* under the *Tools* window
2	Select *Import* under the *Projects* tab.
3	Navigate to the project file, click *Import* and *Done*.
4	Open the project by choosing *Open Project* under the *File* window.

Raw data

To access and view raw data, the run folder for the project should also be present on the workstation calling up the project and in the original sample path (location). When a project is created, ID-X creates a link between the project and sample files (*i.e.,* raw data). Raw data from sample files is not stored in the project. Thus, if sample files are removed from a workstation, the raw data cannot be accessed for the particular project as the link has been broken.

The link may be re-established by one of two approaches:

Either	Approach
1	Place a copy of the sample files in the correct path of origin on the workstation computer. This path is recorded in *Info* of each sample file, as *Sample Origin Path*. To access *Info:* • Open the project of interest. • Click once on the run folder node in the left *Navigation Pane* to open the run folder. • Click once on a sample file. • Select the *Info* tab for that sample to view the *Sample Origin Path.*
2	Reestablish the Sample Origin Path by directing the project to the new folder location for the run folder, as follows: • Open the project of interest. • Select all the samples within the project that need the path reestablished.

	• Under *Edit*, choose *Define new Sample File path*. • At the prompt, navigate to the run folder containing the sample files and click *Select*.

Reanalysis

To re-analyze data within an existing project, access to the sample files is required and the run folder must be in the correct path of origin.

Section 3.9.10 Appendix IV - Cleaning the GeneMapper ID-X Database

ID-X clean-up

GeneMapper ID-X database clean-up is recommended to keep the stored information manageable and maximize efficient software and computer functioning.

To delete a project from ID-X:

Step	Action
1	Open *GeneMapper ID-X Manager* from under the *Tools* tab.
2	Select the project(s) to delete.
3	Click on *Delete* and click *Yes* to remove the project(s) permanently.

Important
Prior to deleting a project in GeneMapper ID-X, ensure that the project has been exported for permanent storage.

Section 3.9.11 Appendix V – Audit Record Settings and Management in GeneMapper ID-X

Overview

GMID-X has an administrative auditing capability to audit specific actions desired. This includes various actions while using GMID-X (*e.g.,* allele edits) and various files applied (*e.g.,* panels and bins). BFS DNA casework sections do not utilize this tracking system, other than to directly electronically edit peak labels when needed. Rather, key information is technically and administratively reviewed as described elsewhere and all relevant analysis parameters are addressed in the DNA Technical Procedures.

For peak label changes, BFS DNA caseworkers already "track" peak label changes indicating why a peak has been edited by either 1.) electronically entering the "reason for change" in the dialog box, or 2.) manually documenting the

change/reasoning on the printed electropherogram. Either way, such changes are indicated as LLI, PU, NRA, RA, *etc.* for a deleted allele or with the custom allele name for a microvariant, per the DNA Technical Procedures. The audit tracking system cannot be completely deactivated because it will cause the "reason for change" dialog box to no longer be available. The preferences described below enable this one feature to remain functional.

Set audit record preferences

To turn off all but allele editing audit records, perform the following steps.

Step	Action
1	Open ID-X.
2	Login with the administrator account.
3	Under **Admin**, open **Audit Manager** and choose **Setting.**
4	Enter the administrator account login name and password.
5	Under **Auditing**, verify **On** is selected.
6	• Click on **Allele** under *Name* in *Audit Map Objects.* • For each attribute listed (e.g., deleted, modified, and created), choose **On** under *State.*
7	• Click on **Sample** under *Name* in *Audit Map Objects.* • For **ALLELE**, choose **On** under *State*. • For all other attributes listed, choose **Off**.
8	• Click on **Run** under Name in Audit Map Objects. • For **SAMPLE**, choose **On** under *State*. • For all other attributes listed, choose **Off**.
9	• Click on **Project** under *Name* in *Audit Map Objects.* • For **RUN**, choose **On** under *State*. • For all other attributes listed, choose **Off**.
10	The state can be **Off** for all listed attributes under each of the following: **Kit, Size Standard, Marker, Panel, Analysis Method, Bin Set, Bin, and Matrix**.
11	Exit Audit Manager and GMID-X. The changes are automatically saved when you close the Audit Map Configuration window. However, settings are not applied until you close and *restart the GMID-X software*.

Managing the audit records

This audit tracking system in GMID-X automatically compiles audit records for designated actions set up as preferences, such as the allele editing feature described above. The compiled audit records occupy disk space and therefore periodically need to be deleted, especially as the disk space approaches its maximum limit defined in the software (*i.e.,* 40,000 records).

Check & delete audit record counts

To check the Audit Record Count and delete the audit records, perform the following steps.

Step	Action
1	Open GMID-X.
2	Login with the administrator account
3	Under **Admin**, open **Audit Manager** and choose **Show Audit Record Count.**
4	It is strongly recommended that the count not exceed 40,000. Click **OK** to close the window.
5	To delete all audit records: • Under **Admin**, open **Audit Manager** • Choose **Delete All Audit Records** • Select **Yes** to delete the records • Select **OK** in the prompt "All audit records were deleted successfully"
6	Exit GMID-X.

Section 3.10 Identifiler Plus STR Interpretation Guidelines

Section 3.10.1 Overview

Introduction

The interpretation of results in casework is necessarily a matter of professional judgment and expertise. Not every situation can or should be covered by a pre-set rule; nor is it expected that competent analysts will always be in full agreement in a particular case.

However, it is important that the laboratory develops and adheres to minimum criteria for interpretation of analytical results. These criteria are based on validation studies, literature references, and casework experience, and were developed with maximum input from analysts. It is expected that these interpretation guidelines will continue to evolve as the collective experience of the laboratory grows.

Purpose of the guidelines

The purpose of these guidelines is to establish a general framework and to outline minimum standards to ensure that:

- Conclusions in casework reports are scientifically supported by the analytical data, including those obtained from appropriate standards and controls.
- Interpretations are made as objectively as possible, and consistently from analyst to analyst.

Contents

This section contains the following topics:

Section 3.10.2 Preliminary Evaluation of Data

Heterozygous/ homozygous genotypes

Heterozygous alleles appear as a two-peak pattern. The genotype is reported as each allele call value separated by a comma. On average, heterozygous alleles have an allelic peak height ratio of greater than 60%. Peak height ratios of heterozygous alleles are defined as the ratio of the lower peak's height to the higher peak's height, expressed as a percentage. Peak height ratios less than 60% may be observed in single source samples, especially when the overall peak heights are low.

Homozygous alleles appear as single peaks and are reported as genotypes (the allele call value duplicated and separated by a comma). Results indicating a homozygous type with low peak heights should be interpreted with greater caution, especially when obtained from samples suspected to contain degraded DNA or PCR inhibition.

Analytical threshold (SWGDAM 1.1)

The analytical threshold is defined as the minimum height requirement at and above which peaks can be reliably distinguished from background noise. Analysis of sample files for each of the dye channels is routinely performed at an analytical threshold of:

- 50 relative fluorescence units (RFU) for data from the 3130/3130*xl*
- 150 RFU for data from the 3500/3500xL

This serves as the minimum peak amplitude for peaks to be assigned allele designations. This threshold was determined empirically based on internal validation data generated by the BFS Jan Bashinski DNA Laboratory.

Peak Reporting (SWGDAM 3.2.1.2; 3.4.3)

Peaks greater than or equal to the analytical threshold in at least two injections may be assigned allele designations and reported. In instances where a peak is above the analytical threshold in one injection and below the analytical threshold in the duplicate injection, the peak will be considered inconclusive and not reported as an allele.

Similarly, in instances where a possible minor allele is co-migrating with a stutter peak, the peak must exceed the locus stutter threshold in at least two injections to be reported as a minor allele. Otherwise, the peak will be considered inconclusive and not reported as an allele. Note that, regardless of the stutter threshold, a peak in a stutter position may be reported as a *possible* minor allele when considering the possible minor component genotypes from a mixture.

While fluorescent signal lower than the analytical threshold may be useful in troubleshooting or to provide guidance in mixture interpretation, peaks below the analytical threshold will not be interpreted for reporting purposes.

Multiple results / one sample (SWGDAM 3.4.3; 3.4.3.1)

For case sample extracts, a minimum of two injections are needed to ensure the reproducibility of typing results. Additional injections and/or amplifications of a given sample may be relied upon for interpretation.

Deconvolution of DNA mixture typing results is typically performed on one of at least two injections. The injection chosen is at the analyst's discretion. However, low-level alleles not detected in at least two injections, may not be used for comparisons, statistics, and reporting (excluding F alleles). See the preceding section on *Peak Reporting* for more information.

Any comparisons and statistics are based upon the interpreted results.
Examples include:
- a single-source profile
- a determined profile from a DNA mixture deconvolution
- a composite profile (see the following section on *Composite profile*)

Composite profile (SWGDAM 3.4.3.1)

In considering multiple injections or amplifications of a sample, a composite profile may be reported if an allele is detected at at least the analytical threshold in a minimum of two injections. This includes composite results of an extract from two typing kits, such as Identifiler Plus and MiniFiler.

However, in the case of allelic data from separate extractions of different locations on a given evidentiary item, unless there is a reasonable expectation of the DNA to have come from the same individual(s), a composite profile may not be reported for this situation.

Case circumstances suggesting a common source, sample splitting from an original common item (*e.g.,* sperm and non-sperm cell fractions of a vaginal swab), different areas of the same item in close proximity (*e.g.,* cuttings of underwear), and spatter patterns may provide a reasonable expectation of the DNA having come from the same individual(s). Given analyst judgment, a composite profile may be generated under such circumstances. Moreover, indications of the same contributor should exist between the results at several loci to be combined and the reasoning for combining the results should be clearly stated in the bench notes.

Offscale peaks defined

By default, the maximum peak amplitude that can be obtained for raw data is approximately:
- 8000 RFU on the 3130/3130*xl* Genetic Analyzers;
- 32,000 RFU on the 3500/3500xL Genetic Analyzers

Peaks exceeding this limit are determined to be "off-scale" by the software. Specifically, Data Collection assigns a tag to off-scale peaks for recognition by GeneMapper ID and GeneMapper ID-X (ID-X).

Use of off-scale data (SWGDAM 3.1; 3.1.1.3)

Off-scale peaks may be assigned allele designations if pull-up, raised baselines, artificially elevated stutter peaks, or artifacts caused by an excess of input DNA (*e.g.*, peaks caused by incomplete 3´ terminal nucleotide addition) have been ruled out. These artifacts should be noted on the GeneMapper ID /ID-X printouts.

Peak height values for off-scale peaks should not be used in quantitative aspects of interpretation.

A sample that exhibits one or more unacceptable off-scale peaks may be either re-injected using a shorter injection time and/or lower injection voltage, re-run using less PCR product, or re-amplified using less input DNA.

Section 3.10.3 Controls Required to Assess Analytical Procedures

Extraction & Amplification Controls

Purpose of the controls (SWGDAM 1.3)

The following controls and standards are used to assess the effectiveness, accuracy and precision of the analytical procedures:

- Positive amplification control 9947A
- Quality Control (QC) sample
- Negative controls (including negative amplification controls and reagent blanks)

Evaluation of the controls is essential to the proper interpretation of the test results.

Positive & QC (SWGDAM 1.3; 1.3.1)

In order for the analytical results to be reported, the positive amplification control and QC sample should yield typing results that are consistent with the known profiles for these samples.

However, if it can be demonstrated that low or no signal was seen in one of these controls as the result of insufficient template DNA or product, the results from the initial amplification may be reported as long as at least one of these controls (positive amplification control or QC sample) gives an accurate typing result.

The presence of artifactual peaks does not invalidate a positive control or QC sample.

Positive & QC *continued* (SWGDAM 1.3; 1.3.2)

Failure to give the expected type
In the event that the positive amplification control or a QC sample fails to give the expected types, the analyst and technical reviewer will evaluate the results in an attempt to determine the cause and seriousness of the discrepancy.

For the positive amplification control and the quality control sample, where the correct types are known, contamination is defined as the presence of foreign allelic peaks greater than or equal to the analytical threshold.

Consideration will be given to the extent to which the results from other samples in the chain of analysis may have been affected. The extent to which a failed QC sample affects the other samples in an analytical set will be evaluated on a case-by-case basis.

For example, if a sample mix-up occurred for the QC sample of a particular case during the extraction process, this would not necessarily affect the typing results for a second case that was extracted separately but amplified and typed with the first case.

Negative controls (SWGDAM 1.3.1; 1.3.2)

Negative control samples (negative amplification control and reagent blank) should show no allelic peaks at or above the analytical/interpretation threshold. If any such peaks are detected in a negative control sample, that sample must be evaluated for potential contamination and how it may have occurred.

Contamination (SWGDAM 1.3)

Due to the potentially complex nature of some evidence samples, possible contamination will be assessed on a sample-by-sample basis. To assist in troubleshooting possible contamination, samples may be re-analyzed at less than the analytical threshold. However, peaks less than the analytical threshold may not be reported.

If contamination is determined to be present in an evidence or reference sample or any of the controls governing the analysis of that sample, the contamination may be classified as either *not serious* or *serious.*

Not Serious
If the level or nature of the contamination does not account for or interfere with the genotype calls of the sample(s) in question, it is considered not serious.

In this situation, the analyst
- May report the results for the case sample(s).
- Will evaluate the contamination and document it in the bench notes.
- Will prepare an Instance of Contamination Report for inclusion in the case file and the Contamination Log.
- Will note the occurrence of contamination in the laboratory report.

Serious
If the level or nature of the contamination does account for or interfere with the genotype calls of the sample(s) in question, it is considered serious.

In this situation, the analyst
- Will repeat the procedures to the appropriate extent for the affected samples.
- Will evaluate the contamination and document it in the bench notes.
- Will prepare an Instance of Contamination Report for inclusion in the case file and the Contamination Log.
- Will note the occurrence of contamination in the laboratory report.

Review
The Technical Leader will review all contamination reports.

Other considerations

Potential issue with reference samples
Although reference samples are typically single-source, transfusion should be considered as a possible source of additional alleles. When a reference sample contains a secondary source that is determined not to be the result of contamination by the laboratory, this will be documented.

Sizing & Genotyping Controls

Size standards & allelic ladders (SWGDAM 1.2)

How GeneMapper works

GeneScan-600 [LIZ] version 2 is used as the Internal Size Standard. This size standard is run with every sample to normalize injection-to-injection peak migration differences. Using GeneMapper ID/ID-X , sizes are assigned to the 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, and 400 base-pair GeneScan-600 [LIZ] peaks.

Size windows based on the allelic ladder are used to assign allele designations. It is therefore necessary to ensure that each allele in the AmpFlSTR Identifiler Plus Allelic Ladder has been sized for genotyping.

Sizing precision (SWGDAM 1.2)

The method used for genotyping employs a ± 0.5-bp "window" around the size obtained for each allele in the AmpFℓSTR Identifiler Plus Allelic Ladder injection(s) from the given run. These sizes may be averages of each allele if more than one allelic ladder injection is used for allele assignments.

Off-ladder sample alleles
A ± 0.5-bp window allows for the detection and correct assignment of potential off-ladder sample alleles whose true size is only one base different from an allelic ladder allele.

- A sample allele that sizes outside a window could be an off-ladder allele (an allele of a size that is not represented in the allelic ladder), or
- an allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement imprecision.

Effect of temperature on sizing precision

Fluctuation in ambient temperature during the course of a set of capillary injections may affect sizing precision.

Recognizing the effects of temperature on a run
An indicator that room temperature fluctuation may have affected a run is the occurrence of shifts in the migration of peaks from one injection to the next.

- Variations in peak migration may become apparent while determining the optimal analysis range for a run.
- It may result in size variation between injections such that allelic ladder peaks differ by more than 0.5 bp from allelic peaks in other injections.
- *Result:* This will cause GeneMapper ID/ID-X to assign these alleles as off-ladder alleles.

Section 3.10.4 Allele Designation

About genotypes

Genotypes are determined from the diagnostic peaks of the appropriate color (fluorescent dye label) and size range for a particular locus. Allele calls are assigned by comparing the sizes obtained for the unknown sample alleles with the sizes obtained for the alleles in the allelic ladder(s). Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories.

Locus designation (SWGDAM 2.1.1)

The following locus range approximations were determined by Life Technologies/ Applied Biosystems for Human Identification analysis.

The allelic bin definitions are stored within GeneMapper ID/ID-X using the bin set "Identifiler_DOJ_Bins_v1.2." Typically, multiple ladders are used in an analysis and allelic bins are determined by averaging the designated ladders in the project. Ladders within a single run folder are used for calculating allelic bin offsets and subsequent genotyping.

6-FAM-labeled loci:

- D8S1179 118.0 to 183.5 bp
- D21S11 184.5 to 247.5 bp
- D7S820 251.0 to 298.5 bp
- CSF1PO 302.12 to 348.63 bp

VIC-labeled loci:

- D3S1358 98.0 to 148.0 bp
- TH01 159.0 to 205.0 bp
- D13S317 205.64 to 250.16 bp
- D16S539 255.3 to 301.81 bp
- D2S1338 304.8 to 370.31 bp

NED-labeled loci:

- D19S433 101.0 to 148.0 bp
- VWA 151.0 to 213.5 bp
- TPOX 216.98 to 260.99 bp
- D18S51 263.49 to 365.0 bp

PET-labeled loci:

- Amelogenin 106.0 to 114.0 bp
- D5S818 128.0 to 180.0 bp
- FGA 206.25 to 360.0 bp

Allele designation (SWGDAM 2.1.2; 2.1.2.1; 2.1.2.2)

The allelic ladder provided in the AmpFℓSTR Identifiler Plus PCR Amplification Kit contains the majority of alleles for each locus.

The laboratory designates alleles as numerical values in accordance with recommendations of the International Society of Forensic Genetics.

The number of 4-bp repeat units observed in an allele that is present in the ladder is designated by an integer.

Alleles that contain an incomplete repeat motif are designated by an integer representing the number of complete repeats, followed by a decimal point and the number of bases in the incomplete repeat (*e.g.*, the 26.2 allele in FGA).

Alleles not aligning (SWGDAM 2.1.2; 2.1.2.2; 2.1.2.3)

The GeneMapper software is used to automatically convert allele sizes into allele designations. Alleles not aligning with those in the allelic ladder have been detected both within and outside the range of the allelic ladder for each locus.

Allele designations for such alleles are assigned as follows:

When the allele at a locus is ...	Then the allele designation will be ...
Smaller than the lowest molecular weight allele (A) in the allelic ladder	"<A."
Larger than the largest molecular weight allele (B) in the allelic ladder	">B."
An off-ladder allele occurring within the ladder region	Determined by interpolation. Either calculate the allele call manually or refer to Section 3.10.10 Appendix I *Designation of Microvariant Alleles.* ***Note:*** The off-ladder allele designation should be confirmed using at least two sample injections. Print the electropherograms and tabular data for both sample injections.
An FGA allele that falls between the 33.2 and 42.2 ladder alleles	">33.2"

Comparison of <A & >B alleles (SWGDAM 2.1.2.3)

Comparison of two < or > alleles among samples is typically performed by comparing the base pair size of each off-ladder allele. To be considered the same allele, the sizes should be within ± 0.5 bp of one another. The other loci should additionally support a common DNA source to declare an inclusion.

Stochastic threshold (SWGDAM 3.2; 3.2.1)

The stochastic threshold is the peak height value at and above which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele has not occurred. The stochastic threshold may also be referred to as the homozygote threshold as it is the threshold above which a single peak may be genotyped as a homozygote. This threshold has been determined through validation studies to be as follows when using the Identifiler Plus kit:

- 365 RFU on the 3130/3130*xl* Genetic Analyzers
- 1,075 RFU on the 3500/3500xL Genetic Analyzers

A single peak must be at least the height of the stochastic threshold in at least one injection to be genotyped as a homozygote.

Concordance (SWGDAM 1.4)

When a sample is analyzed using STR multiplexes that contain redundant loci (*e.g.,* MiniFiler and Identifiler Plus), the results from the redundant loci should be consistent with one another. However, if amplification of one of the redundant loci is successful with one amplification kit but not the other, the successful amplification may be reported. In cases where low-level alleles are detected at or above the analytical threshold with one kit but not the other, the low-level alleles may be reported. On occasion, results may vary between loci due to primer sequence variation between the typing kits that can lead to a partial or full null allele.

Section 3.10.5 Non-Allelic Peaks

Artifact designations

Artifacts can occur and should be noted where appropriate for editing purposes. Several types of artifacts are discussed in detail below.

Pull-up / pull-down (SWGDAM 3.1; 3.1.1)

Recognizing pull-up

Smaller artifactual peaks can appear in other colors under allelic peaks. This phenomenon is termed "pull-up" and is the result of spectral overlap between the fluorescent dyes. If a pull-up peak is above the peak amplitude threshold, it will be sized at a similar size as the allelic peak. The shape of the peak may appear similar to a true DNA peak or sigmoidal, as a doublet, or otherwise irregularly-shaped.

Recognizing pull-down

Pull-down is also the result of spectral overlap between the fluorescent dyes. When an oversubtraction occurs, the result is a dip into the baseline. The baseline can only be positive values so this dip causes a raised baseline, or pull-down, in the other colors of the proximal size/data point range of the allelic peak(s).

Pull-up and pull-down can occur as a result of the following:

- Application of a less than optimal spectral calibration. The run may need to be repeated with a different spectral calibration. However, even when the spectral calibration is satisfactory, pull-up and pull-down may still be seen.
- Amplification using excess input DNA can lead to off-scale peaks. If necessary, samples can be re-amplified using less input DNA, or re-run using shorter injection times or less product.

Stutter (SWGDAM 3.1; 3.1.1)

Recognizing stutter

In addition to an allele's primary peak, artifactual stutter peaks can occur at four-base intervals. The stutter peak most commonly observed is four bases smaller than the primary peak ("N-4"). However, it is also possible to see a peak that is four bases larger ("N+4") than the primary peak.

Stutter peaks may be due to repeat slippage during amplification. Sequence variation can affect the amount of stutter; a lower amount of stutter is produced from alleles with increased sequence variation between repeats.

N-4 Stutter thresholds (SWGDAM 3.1; 3.1.1)

Stutter peaks are evaluated by examining the ratio of the stutter peak height to the primary peak height, expressed as a percentage. This percentage can vary by locus. Moreover, longer alleles within a locus generally have a higher N-4 stutter percentage than the shorter alleles.

The ***GeneMapper thresholds for N-4 stutter*** are set at the values shown below in Table 2. Typically, N-4 stutter is expected to be less than these percentages.

Table 2

D8S1179	11%
D21S11	12%
D7S820	10%
CSF1PO	10%
D3S1358	14%
TH01	7%
D13S317	11%
D16S539	11%
D2S1338	14%
D19S433	16%
vWA	16%
TPOX	8%
D18S51	20%
D5S818	11%
FGA	16%

Reference: These thresholds are based on validation studies performed at the BFS Jan Bashinski DNA Laboratory and represent the overall locus mean plus four standard deviations.

N+4 Stutter threshold (SWGDAM 3.1; 3.1.1)

Although uncommon, **N+4 stutter** has been observed as high as 9.2% in Identifiler Plus validation studies performed at the BFS Jan Bashinski DNA Laboratory. While the presence of N+4 stutter does not complicate the interpretation of single-source samples, the possible presence of this PCR artifact should be considered when interpreting mixture results.

Though N+4 stutter does not exhibit the predictability of N-4 stutter, the percentage relative to the parent allele does reproducibly increase with decreasing parent peak height. Therefore, the following tiered stutter thresholds are applied to **Identifiler Plus 3500/3500xl** results:

Allele RFU	Maximum N+4 Stutter Percentage
≤ 2499	9.2%
2500 - 5199	5.0%
≥ 5200	2.0%

Important

- GMID-X will apply a global 2.0% filter when using the Casework_IDP_3500 analysis parameters. As relevant, the analyst *manually* applies the 5.0% and 9.2% filters.
- The analyst also *manually* applies the combination of N+4 and N-4 filters for a peak in an N-4/N+4 overlap position because GMID-X applies the filters separately, rather than additively.
- MixMaster IDP 3500 is programmed to apply all three filters and the N-4/N+4 overlap additively when a peak is in an N-4/N+4 position.

Causes of elevated stutter peak heights (SWGDAM 3.1; 3.1.1)

The presence of peaks in stutter positions exhibiting percentages greater than the threshold values may indicate a DNA mixture.

However, stutter peak heights may also be elevated above the expected thresholds by the following:

- The measurement of percent stutter may be unnaturally high for **main peaks that are off-scale**, *i.e.*, have exceeded the linear dynamic range for detection. This is approximately 8,000 RFU for the 3130/3130*xl* instruments and approximately 32,000 RFU for the 3500/3500xl instruments. Because “raw data” from both instrument models has already had the spectral calibration applied, viewing the raw data is not a means for determining whether a peak was detected off-scale. Rather, the Genotypes Display in GeneMapper has a PQV designated as "OS” that is set to flag off-scale peaks automatically. If a stutter peak is greater than the maximum expected and the primary peak is off-scale, the analyst should interpret the results with caution. The sample may be subsequently re-injected with less product or for a shorter time/less

voltage, or the sample may need to be re-amplified using less input DNA.

- For **alleles differing by two repeat units**, the stutter peak from the larger allele may overlap the trailing shoulder of the smaller allele and therefore exhibit an increased stutter percentage for the larger allele. This will not occur if the smaller allele peak drops to baseline before reaching the stutter peak.

- Stutter may exceed a threshold simply as a result of **normal statistical variation**. Specifically, the thresholds are based on an overall mean plus four standard deviations calculation at each locus.

- Apparent elevated stutter may be the result of a **somatic mutation** (*i.e.,* Type I mutations; Clayton *et al* 2004 JFS, Rolf *et al* 2002 FSI, Gill, P. 2002 BioTechniques). For example, such mutations may be seen in rapidly dividing buccal cells or spermatozoa. These situations are generally the result of a minor proportion of the otherwise wildtype cell population containing the mutated genotype (*i.e.,* mosaicism). "IPA," or indeterminate possible allele, may be used in the benchnotes to annotate such occurrences in apparent single-source samples.

- In some situations, **an allelic peak in a mixture may co-migrate with the stutter peak** of another allelic peak, resulting in an apparent stutter peak height greater than that typically observed for stutter in a single-source sample.

Incomplete non-template nucleotide addition (SWGDAM 3.1; 3.1.1)

Amplification conditions have been set to maximize the non-template addition of a 3′ terminal nucleotide by AmpliTaq Gold DNA polymerase. Failure to attain complete terminal nucleotide addition may result in "split peaks," visualized as two peaks that are one base apart. Except for microvariant alleles, the presence of peaks differing by one base pair is diagnostic of this phenomenon.

Other artifacts (SWGDAM 3.1; 3.1.1)

In addition to the artifacts described above, the following anomalies can arise:

Dye blobs

Artifacts which are distinguished from labeled DNA alleles because the peak morphology is not consistent with labeled DNA may be reproducibly detected. These artifacts are dye-labeled primer by-products from the Identifiler Plus kit.

For example:

- a blue dye artifact is frequently observed at 96 bp and other artifacts may be present within the D8S1179 locus range,
- a green dye artifact may be present in the locus range for D3S1358, and
- a red dye artifact may be present in the Amelogenin locus range.

Spikes
Peaks of the approximately same size, frequently present in all five colors, are not the result of dye-labeled DNA and do not indicate a spectral calibration problem. The shape of these peak-like artifacts often differs from the shape of dye-labeled DNA peaks and these artifacts are generally not reproducible.

TPOX Artifact
There is frequently an artifactual peak(s) observed at the TPOX locus when the Identifiler Plus kit is run in combination with the GS600v2 LIZ size standard. This artifactual peak typically follows each TPOX allele at approximately 2.5-3.1 bases larger in size. The internal validation studies performed at the BFS Jan Bashinski DNA Laboratory showed the artifact to increase in height over time, to as high as approximately 30% of the corresponding TPOX allele 48 hours after preparation of the plate for capillary electrophoresis. Because the size of this artifact does not correspond with allele bins for TPOX, it is readily identified and may be noted as "SSA" (size standard artifact).

Section 3.10.6 Single-Source, Partial, and Mixture Profiles

Purpose of this section

This section describes conditions in which the data would lead to the conclusion that the source of the DNA is either from a single person or may be from more than one person. If the data indicate the presence of DNA from more than one person, the analyst should proceed to the Identifiler Plus Mixture Interpretation Procedures and Guidelines (Section 3.7) for more information.

Considerations (SWGDAM 3.4)

It is generally possible to estimate a minimum number of contributors to a DNA sample by considering the number of alleles present at a locus, the appearance of allelic patterns within and between loci, peak heights, and/or peak height ratios. Peaks that do not meet the analytical and stochastic thresholds may be used in this assessment.

The data may indicate a mixture if there are more than two alleles per locus and/or a peak present in a stutter position shows significantly greater peak height than that typically observed for stutter in a single-source sample.

However, some data may be best explained by:

- The possible presence of a "null" or "partial null" allele – an allele that is not detected or has significantly reduced peak height due to a mutation in the primer annealing region or a deletion
- A possible genetic anomaly, such as trisomy, mosaicism, and chimerism.

Single-source (SWGDAM 3.4; 3.4.2)

A single-source profile is a DNA typing result determined to have originated from one individual based on peak height ratios and the number of alleles at each locus. A sample may be considered to be from a single contributor when the observed number of alleles at each locus does not exceed two (assuming no genetic anomalies) and intra- and inter-locus peak heights observed are appropriately balanced. Single-source peak height ratios may be less balanced when low level results are observed.

Peak height ratios (SWGDAM 3.3; 3.3.1; 3.4.2)

Peak height ratios lower than 60% may indicate a mixture, especially when seen at more than one locus. However, a single-source sample may also exhibit peak height ratios below 60%, particularly when the overall peak heights are low.

Analysts should consider results at all loci when interpreting samples that exhibit peak height ratios of less than 60%. Depending upon the sample source, the loci in question, the number of loci affected and the percent disparity between allele peak heights, the sample may need to be re-amplified. This determination is made by the analyst at their discretion and should include an evaluation of all loci for that sample.

Partial profiles (SWGDAM 3.6.2; 3.6.2.2; 3.6.3; 3.5.4.3)

Partial profiles may be obtained when the DNA template is degraded, in low quantity, or when PCR inhibitors are present. A negative or inconclusive result at some loci may not impact allele designations at the remaining loci.

At low levels of DNA template, stochastic effects may cause loss of an allele or a substantial imbalance of alleles at a locus. Imbalanced amplification of heterozygous allele pairs at low template levels is not necessarily an indication of a mixture, and the absence of an allele may not be a valid basis to exclude a potential contributor.

Mixed profiles (SWGDAM 3.4; 3.6.3)

Refer to the Identifiler Plus Mixture Interpretation Procedures and Guidelines (Section 3.7).

Section 3.10.7 Comparison of DNA Typing Results

Purpose of this section (SWGDAM 3.6)

This section provides guidelines that may be used in formulating conclusions resulting from comparisons of single-source profiles and the results of mixed DNA samples (*e.g.,* determined "single-source" and mixed profiles).

General categories of conclusions include:

- Inclusion
- Exclusion
- Inconclusive
- No result

Comparisons (SWGDAM 3.6.1)

Comparisons with any known samples should only be performed following the interpretation of evidentiary samples, other than those of assumed contributors.

Inclusion (SWGDAM 3.6.3)

For single-source samples, if each homozygous allelic peak greater than or equal to the stochastic threshold and each heterozygous peak greater than or equal to the analytical threshold in the evidence sample is observed in the reference standard, then the STR profile from the evidence sample matches that of the reference.

An individual may also be included when a partial profile is observed in an evidence sample (due to low template DNA, degradation and/or inhibition) if the evidence alleles detected correspond with those in the reference sample.

If an individual's alleles are present or can be accounted for in a deconvolved mixture result, the individual may be included (or cannot be excluded) as a possible source of that component of the mixed biological material.

Inclusive findings may be reported for any profile determined to be suitable for comparison, regardless of the number of alleles/genotypes compared and the resulting discriminating power of the statistical calculation. It may be appropriate to convey the significance, or insignificance, of the reported statistics through additional language. For example, weak statistics may be reported as possible instances of inclusion due to chance alone; "strong evidence" is reported for instances of at least 1 in 10 million across all reported populations (see Section 3.10.8.1, Genotype Frequencies).

Exclusion (SWGDAM 3.6.3)

If allelic peaks in the profile from a reference standard are not found in the profile of the evidence sample, and their absence cannot be attributed to insufficient template, degradation, inhibition, masking at a stutter position, or a genetic anomaly, then the individual is excluded as a possible source of the biological material present in the sample.

As a matter of policy and at analyst discretion, a sample that is determined to be unsuitable for the calculation of a statistic (*e.g.,* due to an indeterminate number of contributors), and therefore unsuitable for an inclusion statement, may be compared to reference standards for exclusionary purposes only. Such samples are those which do not support the inclusion of an individual based on a lack of alleles in common with the evidence result and/or a lack of "expected" alleles when considering locus zygosity, DNA fragment length, allele frequency, etc. The exclusion should be clearly documented and discussed with the Technical Leader. Such results may be worded as follows: "John Doe appears to be excluded as a DNA contributor to item X. However, due to the nature of the data, the results for the mixed sample are unsuitable for inclusionary statements." For such data, the analyst may inform the client that submitting additional references may be inappropriate.

Inconclusive (SWGDAM 3.6.3; 3.6.6)

When it is not possible to conclude with a reasonable level of confidence whether an individual is excluded or included as a potential contributor, the results are inconclusive. Specifically, when a potential inclusion cannot be paired with an appropriate statistic, the DNA typing results obtained for that sample are unsuitable for comparison and considered inconclusive. Inconclusive results may apply to a particular locus or loci in a profile or the overall profile.

For example, an appropriate statistic cannot be calculated if there is a possibility of allele drop-out *and* a fixed number of contributors to a mixture cannot be reasonably assumed for the component in question. These conditions are problematic as they lead to uncertainty about the possibility of entire genotypes being undetected in a mixture. Under such circumstances, comparisons are not typically made as no appropriate statistic can be given.

A determination of inconclusive may apply to one component of a mixture (*e.g.,* minor contributor results in a mixture with an interpretable major component) or to the overall results (*e.g.,* low-level *possible* mixture with peaks below the analytical threshold). For example, in a mixture of one major DNA contributor and at least one minor contributor, comparisons may be made to the determined major contributor and a statistic would be calculated for any inclusion. However, the minor DNA contributor(s) may be unsuitable for comparison if a fixed number of contributors cannot be reasonably assumed.

Inconclusive DNA results are typically due to:

- An inability to determine whether entire genotypes at one or more loci may have dropped out from a DNA mixture (*e.g.,* low-level mixture);
- An inability to reasonably assume a fixed number of contributors to a mixture (*e.g.,* at least two, maybe three DNA contributors);
- The presence of excessive background DNA, as indicated by the results for a corresponding substrate sample; and/or
- The presence of excessive DNA template confounding the background of DNA typing results.

It may be beneficial to re-amplify a sample with inconclusive results, if possible, or to seek additional probative evidence.

No result

A finding of "no results" is reported when no non-artifactual fluorescent signal greater than or equal to the analytical threshold is observed. A conclusion of "no results" may be obtained for some or all loci of a particular sample.

Section 3.10.8 Statistical Analysis of DNA Typing Results and Corresponding Formulae

Section 3.10.8.1 Overview

Introduction (SWGDAM 4)

In forensic DNA testing, statistical calculations are performed to aid in the assessment of the significance of an inclusion. These calculations are performed on evidentiary DNA profiles that are established as relevant in the context of the case. These calculations include the random match probability (RMP), the combined probability of exclusion/inclusion (CPE/CPI), or the likelihood ratio (LR).

The genetic loci used and the assumptions made for statistical calculations performed should be clearly stated in the bench notes.

Important! (SWGDAM 5.1)

If there is a reasonable possibility that dropout could have led to the loss of an entire genotype, then a statistical calculation *should not* be performed at that locus for the mixture component of interest (*e.g.,* probative minor partial profile). This extends to even a single allele missing for a CPI calculation.

Similarly, the product rule should not be applied when the resultant set of combined profiles would not include all individuals who would be included as possible contributors to the mixture.

Choosing a statistic (SWGDAM 4.6.1)

The following table is provided to give guidance in determining which statistical approach(s) is appropriate for a given result.

Profile(s) for comparison	RMP	CPI [3]	LR [1]
Single-source	X		X
Single major contributor	X		X
Multiple major contributors	X [2]	X [2]	X
Single minor contributor	X	X	X
Multiple minor contributors	X [2]	X	X
Indistinguishable mixture	X [1]	X	X

[1]Restricted or unrestricted
[2]Restricted
[3]All potential alleles identified during interpretation are included in the statistical calculation. Assumes all alleles detected (*i.e.,* no drop-out).

Statistics on evidence only (SWGDAM 4.2)

For calculating the CPI or RMP, any DNA typing results used for statistical analysis must be derived from evidentiary items and *not* known samples.

In instances where a known sample yields a partial profile, statistics calculated on the evidentiary profile are restricted to those loci successfully typed in the known sample being compared.

Inclusions (SWGDAM 4.1; 4.4)

A statistic must be provided in support of any inclusion that is determined to be relevant in the context of a case, irrespective of the number of alleles detected and the quantitative value of the statistical analysis.

See also *Multiple stains* below and *Documentation continued* in Section 3.7.4.1.

Exclusionary conclusions do not require statistical analysis.

Multiple samples/ one statistic (SWGDAM 4.1.1)

Generally, statistics are calculated for each probative inclusion associated with a case. However, when multiple samples from the same, or in some instances separate, items have provided genetic information that is consistent with originating from a common source(s) but having various levels of discrimination, the statistics for the probative typing results that provide the most genetic information and/or the highest discrimination potential are often calculated and reported. The justification for the item/profile chosen from which to calculate the statistics should be documented in the notes.

Examples of such situations include:

- Results from a cervical swab and a vaginal swab, collected from the same individual
- Multiple blood stains on the same item
-
- ***Notes:***
- When the same single-source, full profile is obtained from multiple probative items, the statistics are typically calculated and reported once for the profile.
- Statistics for a more informative profile should not be confused with representing the statistics for the less informative results.
- See also *Multiple items/same source* in Section 3.7.4.2

Multiple statistics (SWGDAM 4.2; 4.6.2)

Different calculations may be performed for the same mixture component if different assumptions as to the number of contributors are made and clearly stated in the bench notes.

However, it is not appropriate to calculate a composite statistic using multiple statistical approaches and/or varied assumptions across a multi-locus profile. For example, a mixture statistic cannot be calculated using CPI at some loci and RMP at others because

they rely upon different fundamental assumptions about the number of contributors to the mixture.

Inconclusive loci (SWGDAM 4.3; 4.3.1; 3.6.2.1); 3.6.6

Inconclusive loci must not be used in a statistical analysis. See *Inconclusive* in Section 3.10.7 for more information on inconclusive loci.

Statistical analysis may, however, be performed for a mixture component in question in the presence of another inconclusive component. For example, the major contributor to a mixture may be used for statistical analysis despite inconclusive minor component results.

Restricted versus unrestricted (SWGDAM 4)

Quantitative peak height information and mixture proportion assessments may or may not be used in the interpretation of an evidentiary profile. Calculations performed using interpretations which incorporate this information are termed "restricted." When this quantitative peak height information is not utilized, the resultant calculation is termed "unrestricted."

Genotype frequencies (SWGDAM 4.5)

Frequency estimates

Genotype frequencies for STR loci will be calculated using Caucasian, Hispanic, and African American population data from:

- "Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians," Budowle *et al*. (1999), Journal of Forensic Sciences 44(6): 1277-1286
- "Genotype profiles for six population groups at the 13 CODIS short tandem repeat core loci and other PCR-based loci," Budowle *et al*. (1999), Forensic Science Communications 1(2).
- "Population data on the STR loci D2S1338 and D19S433," Budowle, B. (2001), Forensic Science Communications 3(3).
- Erratum. Moretti, T. R., Budowle, B. and Buckleton, J. S. (2015), Journal of Forensic Sciences 60(4): 1114-1116.

BFS laboratories have developed Excel worksheets to automatically perform the calculations.

A five-event minimum allele frequency will be used for rare alleles. For each individual allele, an observed allele count of less than five is raised to five. This modified allele count is converted to a frequency and used for all subsequent genotype calculations.

Reporting estimates

- Additional racial/ethnic population data known to be relevant to the case may also be used when available.
- Frequency estimates will be reported to two significant figures.
- A threshold value of 1 in 10 million across all reported populations should be obtained in order to use the phrase "strong evidence" in reports.
- Subject to analyst discretion, frequencies are not usually calculated on non-probative samples, such as the victim's type on a vaginal swab.

Contents

This section contains the following topics:

Section 3.10.8.2 RMP

RMP (SWGDAM 4)

The Random Match Probability (RMP) is the probability of randomly selecting an unrelated individual from the population who could be a potential contributor to an evidentiary profile.

The application of the RMP inherently includes an assumption of the number of contributors to the DNA result. If an exact number of contributors cannot be assumed, this statistic cannot be applied appropriately to an inclusive result.

The application of the RMP to mixture calculations is sometimes refered to as a modified RMP. This procedure applies the term RMP to both single-source profiles and mixture calculations.

Single-source (SWGDAM 4.6; 5.2)

The formulae used in calculating the frequency of a single-source DNA profile are shown in the table below.

Genotype	Frequency Formula	NRC Formula Reference	Notes/Variations
Heterozygote	Probability (PQ) = 2pq	4.1b	
Homozygote	Probability (PP) = $p^2 + p(1-p)\theta$, where $\theta = 0.01$ ***Note:*** This is the National Research Council 1996 (NRC II) formula.	4.4a	When a small, isolated population (*e.g.*, Native Americans) is relevant, θ is set to 0.03.
Single peak present below the stochastic threshold	Probability (PF) = $p^2 + p(1-p)\theta + 2p(1-p)$, where $\theta = 0.01$	SWGDAM 5.2.1.3.2	This formula accounts for instances where the second peak of a heterozygote may have not been detected. ***Note:*** This formula is algebraically identical to $2p-p^2+ p(1-p)\theta$.
Multi-locus STR profile	Multiply genotype frequencies from each locus.	N/A	This is known as the Product Rule.

Conditional subpopulation calculations

Refer to the NRC formulae 4.10a and 4.10b. These formulae determine the probability of selecting a random person with the same genotype as the evidence donor, given that the two individuals are from the same subpoulation.

Formulae used with results from mixtures (SWGDAM 4.6)

The following possible situations may exist from mixture results:

If ...	Then ...
Genotypes are determined at all loci typed,	The frequency for the determined profile is calculated in the same way as a single-source sample (using formulas 4.1b and 4.4a above). Alternatively, LR may be used.
A contributor(s) is known or reasonably assumed to be present allowing the profile of the unknown contributor(s) to be inferred,	The frequency for the determined profile is calculated in the same way as a single-source sample (using formulas 4.1b and 4.4a above). Alternatively, LR may be used.
Contributor genotypes can be determined at some loci but not others	The statistical significance may be calculated using formulas 4.1b and 4.4a, and adjusting for single peaks when needed. ***Note***: CPI or LR may alternatively be calculated when appropriate.
Multiple single peaks at a locus are below the stochastic threshold and drop-out is a consideration	It may be appropriate to use the following for each allele below the stochastic threshold: Probability (PF) = $p^2 + p(1-p)\theta + 2p(1-p)$, where $\theta = 0.01$ ***Note:*** Double-counted heterozygotes should be subtracted.
The mixture is complex and/or low-level, such that the following assumptions cannot be reasonably made: • a fixed number of contributors *and* • at least one allele has been detected for each contributor	Statistics are not calculated and the data is reported as inconclusive.

Multiple genotypes at a locus (SWGDAM 5.2.2)

When more than one genotype is possible at a locus, the RMP is calculated by summing the individual genotype frequencies at the locus and then multiplying the composite locus frequency by the other locus frequencies (Product Rule).

RMP Caution (SWGDAM 5.2.2.4; 5.2.2.3.1)

Care should be taken to not report a calculated RMP greater than 1.0. This can occur due to:

- The application of theta in the standard homozygote formula but not in the heterozygote formula
- The double-counting of heterozygote genotype frequencies if not subtracted when using multiple single allele calculations at a locus

Unrestricted RMP (SWGDAM 5.2.2.6)

An unrestricted RMP includes an assumption of the number of contributors, but peak height and quantitative assessment information is not utilized.

For two-person mixtures with no drop-out assumed, the formulae for loci displaying one, two, or three alleles are identical to a CPI calculation. For loci displaying four alleles, homozygous genotypes would not typically be included. The unrestricted RMP in this case requires the subtraction of homozygote genotype frequencies. In other words, for alleles P, Q, R, and S, the formula is
$(p + q + r + s)^2 - p^2 - q^2 - r^2 - s^2$.

Untested Relatives (SWGDAM 4.6; 5.2.3)

Probability of Untested Relatives Matching the Evidence Profile

The possibility that a *close relative of a suspect* is a potential contributor to an evidence profile should be considered on a case-specific basis.

For those situations where there is reason to believe that a close relative is involved, a reference sample should be collected from that individual, if possible, for comparison to the evidence profile to avoid the need to rely on a probability-based estimate of a coincidental match.

If the putative relative cannot be typed, the analyst may calculate the probability of finding the evidence profile in such an individual using the NRC II Formula 4.8 or Formula 4.9, as appropriate. The Bureau has validated Excel spreadsheets for such calculations.

Relative Formulae (SWGDAM 4.6; 5.2.3.1; 5.2.3.2)

For *noninbred unilineal relatives* (relatives who have at most one gene identical by descent at a locus), the formulae can be expressed in terms of the kinship coefficient, F:

Genotype of suspect	Probability of same genotype in a relative
PP (4.8a Homozygote)	$p^2+4p(1-p)F$
PQ (4.8b Heterozygote)	$2pq+2(p+q-4pq)F$

where the value for F is:

Kinship coefficient F	Familial relationship
1/4	parent and offspring
1/8	half-siblings
1/8	uncle and nephew
1/8	grandparent and grandchild
1/16	first cousins

Full siblings, being bilineal rather than unilineal, require different formulae:

Genotype of suspect	Probability of same genotype in a full sibling
PP (4.9a Homozygote)	PP $(1+2p+p_2)/4$
PQ (4.9b Heterozygote)	PQ $(1+p+q+2pq)/4$

Conditional subpopulation calculations (SWGDAM 5.2.3.3)

Conditional subpopulation corrections could also be applied to these formulae if needed.

Section 3.10.8.3 CPI

CPI (SWGDAM 4; 4.6)

The combined probability of inclusion is produced by multiplying each locus probability of inclusion (PI), where PI represents the percentage of the population that can be included as potential contributors to a DNA mixture. CPI is also known as Random Man Not Excluded (RMNE). Unlike the RMP, the CPI makes no assumption as to the number of contributors.

The combined probability of exclusion (CPE) provides an estimate of the frequency of individuals who are excluded as possible contributors to the mixture. CPE is equivalent to 1-CPI.

CPI limitations (SWGDAM 4.6.3)

The CPI is typically applied to all alleles detected in a mixture, subject to the limitations of:

- No assumptions are made with regard to the number of donors
- All alleles are assumed to be detected and above the stochastic threshold

As the number of contributors increases, the minimum peak height for assigning genotypes should similarly increase. In other words, because of the potential for allele stacking, the stochastic threshold becomes less reliable as the point at which it is reasonable to assume a sister allele has not dropped out.

CPI caution (SWGDAM 4.6.3; 4.6.3.1)

When using CPI to calculate the probability that a randomly selected person would be included as a contributor to the mixture, loci with alleles below the stochastic threshold may not be used for statistical purposes to support an inclusion. In these instances, the potential for allelic dropout raises the possibility of contributors having genotypes not encompassed by the interpreted alleles.

Restricted CPI (SWGDAM 5.3.5.3; 5.3.5.3.1; 3.5.2.1)

A restricted CPI may be applied to multiple major contributors despite the presence of minor contributor(s) alleles below the stochastic threshold. Low-level peaks may not be included if those of higher RFUs are clearly in a distinct group, separate from the low-level contributor(s). In other words, a restricted CPI can be calculated for the major proportion of a clear major/ minor mixture.

However, caution should be exercised in applying a restricted CPI. As the number of contributors to a DNA mixture increases, it becomes increasingly unreliable to assign major *versus* minor peaks due to potential allele stacking.

PI formulae (SWGDAM 5.3.1; 5.3.3)

The PI is calculated as the square of the sum of the allele frequencies for each locus. The PE is 1 – PI.

Combined PI (SWGDAM 5.3.2; 5.3.4)

The CPI is the product of the individual locus PIs (*i.e.,* CPI = $PI_1 * PI_2 * \ldots PI_N$). Once the allele frequencies are summed within each locus and the sum is squared, the PIs are multiplied to obtain a combined probability of inclusion. This calculation provides an estimate of the frequency of individuals who cannot be excluded as possible contributors to the mixture.

The CPE is 1 – CPI, or $1 - [(1 - PE_1) * [(1 - PE_2) * \ldots * (1 - PE_N)]$.

The Bureau has validated an Excel spreadsheet for such calculations.

Section 3.10.8.4 LR

LR

The likelihood ratio compares the probabilities of detecting the evidence types given two competing hypotheses which are mutually exclusive. The identity and actual number of contributors to the mixture are considered in calculating this ratio.

Typically, the numerator contains the inclusionary ("prosecution") hypothesis and the denominator the exclusionary ("defense") hypothesis.

LR v. RMP (SWGDAM 5.4.1)

When the evidence profile is determined to be single source, and the reference and evidence profiles are identical at all loci, the likelihood ratio is simply 1/RMP.

Specifically, the numerator hypothesis would assume the person in question's contribution so the probability of observing the results matching his/her profile would be 1.0. The hypothesis for the denominator would assume the person in question is not the contributor so the denominator is the RMP.

LR factors (SWGDAM 5.4.2)

The calculation of the LR in a mixture is dependent upon the evidence profile, the comparison reference profile(s), and the individual hypotheses.

Unrestricted LR (SWGDAM 5.4.2.1)

An unrestricted LR is the LR calculated without taking peak heights into consideration.

Restricted LR (SWGDAM 5.4.2.2)

A restricted LR is the LR calculated once relative peak heights are taken into consideration.

Note: Within an STR profile, some loci may have results that give identical restricted and unrestricted LRs.

Section 3.10.8.5 Kinship Testing

Kinship index

Relationship Testing (including Parentage) using the Kinship Index

The laboratory performs kinship analysis in certain situations. These include:

- Identification of remains
- Cases where an evidentiary stain is compared to relatives of a victim for whom no reference sample is available
- Criminal paternity cases

In these instances, the analyst should use the likelihood ratio approach and calculate a kinship (*e.g.,* paternity, maternity, parentage, sibship) index. The Bureau has developed Excel worksheets to automatically perform the kinship calculations. A kinship index of at least 10,000 across all reported populations should be obtained in order to use the phrase *"strong evidence"* in reports.

While the LR is the preferred approach for kinship testing calculations, on occasion the analyst may also use the Random Man Not Excluded/ Probability of Inclusion/ Probability of Exclusion in parentage cases.

Parentage index example

In a paternity case, the likelihood ratio compares the probability a child would inherit the observed profile if the known mother and alleged father were the parents versus if the known mother and a random man were the parents. In cases where a man and woman are thought to be the biological parents of an individual for whom there is no reference sample, the parentage index (sometimes referred to as a reverse parentage index) compares the probability of a child having the observed profile if the tested couple were the parents versus if a random couple were the parents.

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Section 3.10.10 Appendix I – Designation of Microvariant Alleles

Overview

When an off-ladder allele occurs within the ladder region, the allele designation will be determined by interpolation. This appendix describes an interpolation process for determining the microvariant allele designation.

Capturing size information

Follow these steps to capture the size information in GeneMapper ID/ID-X.

Step	Action
1	In GeneMapper ID/ID-X, display the electropherograms and sizing table for – all ladder injections used in the project for sample analysis, and – at least two injections of the sample containing the microvariant allele.
2	• Click on the **All** button in the menu bar to hide all the colors. • Then click once on the color for the allele in question. – For example, if the microvariant allele is at D8S1179, click on **Blue** to display only the 6FAM data for all the injections chosen. • Click on the **Sizing Table** button in the menu bar if the table is not already displayed. (This button is in the center of the three to the far right.)
3	Click once on the **Overlay All** button to overlay the particular color of data (*e.g.,* blue) from each injection displayed. (This button is at the far right of the button in the center of the toolbar.)
4	Zoom in on the locus in question (*e.g.,* D8S1179).
5	Click once under the area of the ladder peak that is to the left of the microvariant allele. This will select all displayed injections of the desired ladder peak. Be sure to click below the peak area of the shortest peak to ensure inclusion of all overlayed peaks.
6	Holding the Ctrl key down, click once under the area of the ladder peak to the right of the microvariant allele. Similarly, this will select all displayed injections of this second desired ladder peak. Again, be sure to click below the peak area of the shortest peak to ensure inclusion of all overlayed peaks.
7	Still holding the Ctrl key down, click once under the area of the microvariant allele peaks to select all displayed injections of this allele.
8	Under **View/Plots**, choose **Table Filter**>**Show Selected Rows**.
9	Under **File**, choose **Export Table**. Name the file as <CaseNumberIP Microvariant *mmddyy*> where IP represents Identifiler Plus and *mmddyy* represents the date the file was created. Save the table as a ***.txt** and ***.csv** file (the default tab- and comma-delimited file choice) to the Case Folder.

10	*If not already done,* print the electropherograms and tabular data as usual (see Printing and Archiving Data in Section 3.9.5 of this procedure) from at least two injections of the sample containing the microvariant allele for inclusion in the case file. *If desired,* additional print-outs may be included in the file (*e.g.*, a zoomed-in comparison of the sample to the ladder alleles).

Calculating the microvariant allele designation

Follow these steps to calculate a microvariant allele designation.

Step	Action
1	• Open Excel. • Choose **File**>**Open**. • Change the dropdown file option to **All files (*.*)** • Navigate to the ***.txt** table file created above (see *Capturing the size information)*. • Select **Open** and **Finish**.
2	If there were sample alleles overlapping with ladder alleles from the sample containing the microvariant allele, delete these other alleles so that they are not inadvertently included in the following calculations. For example, if the genotype of the sample in question is D8S1179 12, 12.2, the 12 allele data from the sample will likely be exported from GeneMapper ID/ID-X with the D8S1179 12 ladder allele data. To delete these rows of data in the Excel table, – Highlight the row by clicking on the number to the far left that corresponds with the row – Right-click and choose delete – Repeat as needed
3	Highlight all columns (A-G) and under **Data**, choose **Sort**. In the window that opens, choose to sort by **Size**.
4	In an unused cell, type **=average(**. Then click once in the top cell containing the size for the smaller Allelic Ladder allele and keeping the mouse clicked down, drag the cursor down to include all the sizes (*i.e.*, injections) for that allele. Let the mouse up when you are in the last cell to be included. Type **)** and hit **Enter** on the keyboard. The cell should now show the average size for the allele. ***Note:*** This average is also the size used by GeneMapper ID/ID-X in assigning allele calls for that particular allele throughout the project.
5	Repeat Step 3 in another unused cell for the larger Allelic Ladder allele.
6	Determine the microvariant allele call for each individual injection of the sample in question using the ladder averages and the microvariant allele

formula given below. This may be done in Excel or by hand.

Note: The off-ladder allele designation should be confirmed using at least two sample injections.

Formula	$$S_{a,x} = \left[S_a + X\left(\frac{S_b - S_a}{n} \right) \right] \pm 0.50$$
Definitions	• $S_{a.x}$ = the bin size range for the microvariant • S = the base pair size for the alleles • Note these are average values for the Allelic Ladder alleles. • a = the smaller ladder allele • b = the larger ladder allele • X = the microvariant number (1, 2, or 3; this is estimated by the analyst) • n = the number of base pairs between S_a and S_b in the allelic ladder (*e.g.,* a whole number such as 2 or 4).
Example	A sample has an off-ladder allele (OLA) at 250.85 bp. The flanking alleles (*e.g.,* 20 and 21) in the Allelic Ladder are 4 bases apart and the average sizes from the Allelic Ladder injections for each are 248.81 and 252.91 bp, respectively. Because the microvariant lies about halfway between the allelic ladder peaks, the OLA is presumed to be a 2-bp variant. Using the formula above: $$S_{a,x} = \left[248.81 + 2\frac{(252.91 - 248.81)}{4} \right] \pm 0.50$$ $$S_{a,x} = [248.81 + 2.05] \pm 0.50 = 250.86 \pm 0.50$$ Therefore, the range for the 2-bp variant is 250.36 to 251.36 bp. Since the OLA was measured at 250.85 bp, it is reported as a 20.2 allele.

7	**Save** the ***.txt, *.csv** file as an Excel file (***.xls**) to the Case Folder.
8	**Print** the Excel file to be included in the case file. The calculations showing the microvariant allele determination should also be included in the case file.

Section 3.10.11 Appendix II – Glossary of Terms

This glossary is supplemental to the terms defined within the body of this procedure as well as Section 3.7.

- **Allelic dropout**: Failure to detect an allele at or above the analytical threshold or failure to amplify an allele during PCR. These alleles are represented by an "F" and are assumed to have a peak height from 0 to 49 RFU (3130) or 0 to 149 RFU (3500).
- **Baseline**: The reference value (anchored at zero) set by the software; the software calculates this value for each data point using a moving window size (typically set at 51 data points).
- **Consistent with:** Agreeing or concordant with.
- **Determined profile:** A single-source or mixture profile that is derived from DNA mixture results using deconvolution. A determined profile may also be called a foreign or deduced profile.
- **F allele:** See allelic dropout definition.
- **Likely:** Reasonably believed or expected; probable.
- **Major profile/component/proportion**: The profile from the predominant contributor(s) to a mixture.
- **Minor profile/component/proportion**: The profile from the lesser, secondary contributor(s) to a mixture.
- **Mixture consistent with X contributors:** Based on the number of alleles, peak height ratios, and mixture proportion, the mixture appears to have no more and no less than X contributors.
- **Mixture Deconvolution**: The separation of contributors to a DNA mixture that is based on quantitative assessments and assumptions.
- **Mixture of *at least* X contributors**: Based on the number of alleles, peak height ratios, and the possibility that not all alleles have been detected, there are at least X contributors, but it is possible that there are more than X.
- **Mixture profile**: The determined profile from a DNA mixture which contains alleles and/or genotypes representing more than one individual.
- **Mutually exclusive:** Of or pertaining to a situation involving two or more events, possibilities, etc., in which the occurrence of one precludes the occurrence of the other.
- **Noise**: The background signal detected by a data collection instrument.
- **Partial profile**: The profile of one or more individuals which lacks allelic information at one or more tested loci.
- **Peak:** A distinct, triangular section of an electropherogram that projects above the baseline.
- **Peak height, or peak amplitude**: The point at which the signal intensity of the peak is highest.
- **Possible:** That which may or can be, exist, or happen.
- **Probable:** Likely to occur or prove true; having more evidence for than against.
- **Shared Allele:** A detected allele is the result of DNA contributed from more than one donor.
- **Signal-to-noise ratio:** An assessment used to establish an analytical threshold to distinguish allelic peaks (signal) from background/instrumental noise.

- **Stacking**: An additive effect due to the co-amplification and co-migration of the same allele from more than one donor. Such overlap is expected given STR allele frequencies.
- **Stochastic effects:** The observation of intra-locus peak height imbalance and/or allele drop-out resulting from random, disproportionate amplification of alleles in low-quantity template samples.
- **Suitable for comparison:** The result may be compared to reference standard results and an appropriate statistic can be calculated for an inclusive finding.
- **Unshared allele**: A detected allele that is the result of DNA from only one donor.

Section 4.1 AmpFℓSTR Yfiler Amplification Procedure

Section 4.1.1 INTRODUCTION

The Polymerase Chain Reaction (PCR) is utilized for Short Tandem Repeat (STR) typing of loci on the Y chromosome (Y-STRs). This analysis is performed using the AmpFℓSTR® Yfiler® PCR Amplification Kit manufactured by Life Technologies/Applied Biosystems. Each kit provides sufficient reagents for 100 tests. Included in each kit are AmpFℓSTR PCR Reaction Mix, AmpFℓSTR Yfiler Primer Set, AmpFℓSTR Control Female DNA 9947A, AmpFℓSTR Positive Male Control DNA 007, AmpliTaq Gold™ DNA Polymerase, and AmpFℓSTR Yfiler Allelic Ladder.

Following amplification, electrophoresis and detection of AmpFℓSTR Yfiler™ STR loci are performed using a 3500/3500xL Genetic Analyzer (Section 3.8), followed by GeneMapper ID-X version 1.4 analysis (Section 4.3).

All Yfiler data is interpreted according to Section 4.4.

Section 4.1.2 EQUIPMENT AND REAGENTS

4.1.2.1 Equipment

- 9700 Thermal cycler, Life Technologies/Applied Biosystems
- 0.5 mL and 0.2 mL GeneAmp thin-walled reaction tubes – DNA/DNase/RNase free, PCR compatible
- 96-well amplification plates – DNA/DNase/RNase free, PCR compatible
- Amplification plate covers – DNase/RNase free, PCR compatible
- MicroAmp strip caps – DNA/DNase/RNase free, PCR compatible
- Speed-Vac System, or other concentrator
- Micellaneous laboratory supplies

4.1.2.2 Reagents

- TE^{-4}
- AmpFℓSTR® Yfiler® PCR Amplification Kit

Section 4.1.3 AMPFℓSTR YFILER AMPLIFICATION

1.1.3.1 Analytical Controls and Standards for Amplification

Controls and standards are required to assess the quantity and quality of the extracted DNA as well as the effectiveness, accuracy and precision of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results.

4.1.3.1.1 Quantitation

An estimation of the total human DNA and male DNA content of the sample, preferably using Quadruplex qPCR (refer to the Procedure for the nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay Using the 7500 Real Time PCR System), should be made prior to STR analysis. If the sample is a single-source male reference sample or there is other information (*e.g.,* Identifiler Plus typing results) about the sample, then the sample may not need to be re-quantitated. In general, 0.5-1.0 ng of male DNA is recommended by ABI for STR amplification, although correct results may be obtained outside of this range. For this protocol, a narrower range, 0.5 to 0.8 ng, should be targeted for amplification if sufficient male DNA is available. However, samples less than 125 pg have been reproducibly amplified and correctly typed for Y-STR markers.

4.1.3.1.2 Positive amplification control (007)

This sample ensures that the amplification and typing process is working properly. This control is included in the AmpFℓSTR Yfiler typing kit. The haplotype of control DNA 007 is:
DYS456 (15), DYS389I (13), DYS390 (24), DYS389II (29),
DYS458 (17), DYS19 (15), DYS385a/b (11,14),
DYS393 (13), DYS391 (11), DYS439 (12), DYS635 (24), DYS392 (13),
Y GATA H4 (13), DYS437 (15), DYS438 (12), and DYS448 (19).

4.1.3.1.3 Negative controls

- The **female DNA 9947A control** (10ng/µl) is used to determine specificity of the Yfiler kit and should be run with each set of amplifications. No amplification products should be observed when female DNA is amplified.

- The **negative amplification control** contains only the reagents used to prepare the PCR amplification mixture for each batch of samples, including sample buffer (TE^{-4}). The purpose of this control is to detect contamination that might occur from the PCR reagents, the PCR set-up environment or between the samples being prepared.

- A **reagent blank** is carried through the entire analytical process as part of each extraction set and through the amplification and typing process for *each* PCR system in which the evidence

is typed. For samples extracted prior to July 2009, the reagent blank must be run in at least one PCR system.

The purpose of this control is to detect DNA contamination that might occur from the reagents, the environment or between the samples being processed. It contains all of the reagents used during extraction, amplification and typing for each set of samples. The reagent blank is amplified using the same concentration conditions as required by the samples containing the least amount of DNA in the extraction set and it should be typed using the most sensitive conditions for the samples of the extraction set.

4.1.3.1.4 Substrate control sample

When appropriate, a similarly sized and apparently unstained portion of the substrate adjacent to the questioned stain should be collected and run through the typing process. A substrate control sample will not necessarily produce negative typing results. The possibility of other human biological material being present and contributing to the DNA content of a particular sample will be considered in the final interpretation. The knowledge, experience and judgment of the analyst are paramount when assessing the need for a substrate control sample, choosing the appropriate sample and evaluating the results.

4.1.3.1.5 Quality Control (QC) sample

The purpose of this control is to demonstrate that the analytical process worked properly. This is a sample from a previously characterized source (male or female) that is extracted and typed concurrently with the case samples. It serves as both an extraction control and, if from a male source, a typing control for the process. It also serves as an internal blind control as the correct typing results are unknown to the analyst until the analysis is complete.

If the QC sample is indicated to be from a female source, the QC should be amplified using the same amount of DNA as the male amplification control, 007 (*e.g.,* 0.5 ng).

4.1.3.2 Amplification

1. Turn on the 9700 thermal cycler (TC) and confirm the Yfiler amplification parameters listed below.

<u>DNA Thermal Cycler 9700 (TC 9700)</u>

Pre-denaturation and enzyme activation:	95°C, 11 minutes
Cycle (30 cycles):	94°C, 1 minute
	61°C, 1 minute
	72°C, 1 minute
Final extension:	60°C, 80 minutes
Hold temperature:	4°C

2. Label the required number of 0.5-mL or 0.2-mL GeneAmp® Thin-Walled reaction tubes. Alternatively, a 96-well amplification plate may be used. Fill out the AmpFℓSTR Yfiler Amplification Sample Checksheet with all pertinent information, including well number if using a 96-well amplification plate.

3. For each sample, combine in the following order:
   - Quantity of TE^{-4} needed for a total volume of 10 µL
   - Sample:
     - Extracted DNA: typically target 0.5 to 0.8 ng of male DNA in a total volume of 10 µL
     - Positive amplification control: 5 µL (0.5 ng) of Control DNA 007
     - Control female DNA: 1 µL (10 ng) of Control DNA 9947A
     - Negative amplification control: 10 µL of TE^{-4}

   Note: *Less than 0.5 ng positive amplification control may be amplified.*

   Note: *If a sample volume exceeds 10 µL the sample may be concentrated to approximately 10 µL by using a technique for concentrating the extract. For example, a Speed-Vac System, simple evaporation, or another method for concentrating DNA extracts such as an approved ultrafiltration device may be used. Appropriate reagent controls should be treated in the same manner as the sample extracts being concentrated. Techniques to avoid contamination shall be employed, such as covering the sample during evaporation, and using a bio hood.*

   Note: *Sterile deionized water may be used to reconstitute evaporated samples.*

   Note: *Positive and negative amplification controls associated with samples being typed shall be amplified concurrently in the same instrument with the samples at all loci and with the same primers as the forensic samples. All samples typed shall also have the corresponding amplification controls typed.*

4. Prepare a "master mix" by adding the following volumes of reagents:

   # samples x 10.12 µL PCR Reaction Mix
   # samples x 5.5 µL AmpFℓSTR Yfiler Primer Set
   # samples x 0.88 µL AmpliTaq Gold DNA Polymerase

   Note*: The formulation above provides slight excess. A 1.5-mL microfuge tube provides enough volume for a maximum of 88 samples.*

5. Mix thoroughly and spin the tube briefly in a microfuge to remove any liquid from the cap.

6. Dispense 15 µL of the master mix into each GeneAmp® Thin-Walled reaction tube, or a 96-well amplification plate.

7. Add the prepared sample (10 µL) to each reaction tube. After the addition of sample, cap each tube before proceeding to the next sample. Mixing and vortexing are unnecessary. The final reaction volume in each tube is 25 µL.

   Note: *Alternatively, the 10 µL sample may be prepared directly in the thin-wall amplification tubes. Add the 15 µL of the reaction master mix into each GeneAmp Thin-Walled reaction tube. After the addition of reaction mix, cap each tube before proceeding to the next sample. The final reaction volume in each tube is 25 µL.*

   Note*: If using a 96-well amplification plate, place a MicroAmp 96-well full plate cover (or another appropriate seal) over the plate once the plate is ready for amplification. If needed, briefly spin the covered plate in a centrifuge.*

8. Place the tubes or plate into the TC 9700 and start the thermal cycler program. Complete the documentation of the amplification.

9. After amplification is complete and the thermal cycler has reached 4°C, remove the tubes from the instrument block and proceed to capillary electrophoresis. Alternatively, place the tubes in storage. The amplified products should be protected from light and can be stored in a refrigerator for short periods of time. For longer periods, the products should be stored in a freezer.

   Note: *If using a 96-well amplification plate, remove the plate cover and seal the wells with strip caps (or another appropriate seal) prior to storage..*

Section 4.1.4 References

See Section 4.4.

Section 4.3 Yfiler Genotyping Using GeneMapper® ID-X, Version 1.4

Section 4.3.1 Overview

Purpose of the software

GeneMapper® ID-X software (ID-X) is used to:

- Determine base pair sizes for alleles,
- Assign labels to alleles,
- Assign quality values to genotypes, and
- Build tables containing allele information.

About ID-X

There are two forms of GeneMapper ID-X. The basic form is a full copy, which is a fully functioning version. A full copy of the software can have different user accounts, all on the same workstation computer. Alternatively, the user accounts can be set up to access the full copy database over a server. This form of GeneMapper ID-X is a client copy.

One (or more) full copy resides permanently in each BFS laboratory with client workstation copies accessing the full copy for routine analysis. An additional (or more) full copy is available for use that does not interact with a server; this standalone workstation may be used remotely if it has been installed on a laptop computer.

The setup of GeneMapper ID-X described in Appendix I must occur for each full copy. (Note that the setup can be simplified by importing exports of the relevant files.) As needed, user accounts must be setup on each full copy as well, either directly on the same workstation (as described in Appendix II) or through a client copy.

How ID-X analysis works

ID-X determines base pair sizes by comparing alleles to an internal size standard. Allele designations are assigned by comparing the averaged sizes obtained for the alleles in the allelic ladder with the sizes obtained for the unknown sample alleles. Process Quality Value (PQV) flags may be used to evaluate the data.

Overview of ID-X analysis procedure

The ID-X procedure involves:

- Creating a New Project with Analyzed Data
- Evaluating and Editing Data
- Printing and Archiving Data

Technical review

In performing a technical review, the reviewer assesses the criteria outlined on technical review forms PF-0022 and PF-0017. As part of the review, the technical reviewer performs a second reading of the STR typing data by reviewing the printed ID-X electropherograms and tabular data. The reviewer also checks the electronic or printed size standard and allelic ladder data. The reviewer may review and/or reanalyze additional electronic data if he/she feels it is advantageous in a particular case.

Contents

This section contains the following topics:

Section 4.3.2 Analytical Controls and Standards for Capillary Electrophoresis and Genotyping

Purpose/use

The allelic ladder provided in the AmpFℓSTR Yfiler Amplification Kit is used to determine the allele designations of samples. While the ladder primarily includes the common alleles, additional alleles exist and may be detected in samples.

GeneMapper ID-X software assigns allele designations to peaks by comparison to allelic ladder(s) injected in the same run. The specific size assigned to each ladder allele may vary between instruments *and* between injections on one instrument.

Allelic Ladder

The STR alleles included in the ladder, and the size ranges they span, are listed in Table 1.

Table 1

Locus	Dye Color	Ladder Alleles	Number of Bases in Repeat	Approximate Size Range (bp)	Dye Label
DYS456	Blue	13,14,15,16,17,18	4	103-123	6-FAM
DYS389I	Blue	10,11,12,13,14,15	4	142-170	6-FAM
DYS390	Blue	18,19,20,21,22,23, 24, 25,26,27	4	193-237	6-FAM
DYS389II	Blue	24,25,26,27,28,29, 30, 31,32,33,34	4	254-294	6-FAM
DYS458	Green	14,15,16,17,18,19, 20	4	137-161	VIC
DYS19	Green	10,11,12,13,14,15, 16, 17,18,19	4	175-211	VIC
DYS385	Green	7,8,9,10,11,12,13, 14,15,16,17,18,19, 20,21, 22,23,24,25	4	243-315	VIC
DYS393	Yellow	8,9,10,11,12,13,14, 15,16	4	107-143	NED
DYS391	Yellow	7,8,9,10,11,12,13	4	148-180	NED
DYS439	Yellow	8,9,10,11,12,13,14, 15	4	200-228	NED
DYS635	Yellow	20,21,22,23,24,25, 26	4	242-270	NED
DYS392	Yellow	7,8,9,10,11,12,13, 14,15, 16,17,18	3	291-327	NED
Y GATA H4	Red	8,9,10,11,12,13	4	122-142	PET
DYS437	Red	13,14,15,16,17	4	182-202	PET
DYS438	Red	8,9,10,11,12,13	5	223.5-248.5	PET
DYS448	Red	17,18,19,20,21,22, 23,24	6	276-324	PET

Internal Size Standard

The internal size standard GeneScan®-600 [LIZ] version 2 (GS600v2 [LIZ]) contains dye-labeled DNA fragments of known size that are co-injected with the sample to allow estimation of STR allele sizes.

Section 4.3.3 Creating a New Project with Analyzed Data

Before you begin

If ID-X has not been previously set up, proceed to Appendix I *Setting Up GeneMapper ID-X v. 1.4 for Yfiler*. Once ID-X set-up is complete, return to this section to analyze data.

Case versus batch projects

Below are approaches that may be taken in creating and saving a project:

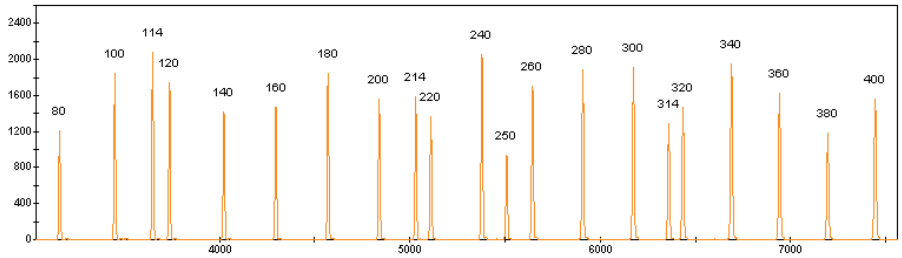
- Import only the sample files relevant to a particular case from a Run Folder, perform data analysis, and save the case-specific project to a Case Folder for archiving. All data is printed from this case-specific project.
- Import all (or relevant portion) sample files from a Run Folder and perform data analysis. Data may be printed directly from the batch project and/or from an edited and renamed project (*i.e.,* a specific case number). Projects from which data are printed for case files should be electronically archived (*e.g.,* to the Case Folder).

***Note*:** If the Sample Origin Path is not preserved, it may be re-established in ID-X by selecting *Define new Sample File path…* under *Edit*.

Create a new project

Follow these steps to create a new project.

Step	Action
1	• Create a Case Folder or Batch Folder in ID-X Case Data on the desired workstation computer. • Copy the run folder into the Case Folder or Batch Folder.
2	Open ID-X version 1.4 (or higher, if performance evaluated) and log into the software.
3	Create a new project by: – Selecting *Add Samples to Project* under the *Edit* menu. – Locate the correct run folder(s), – Click *Add to List*, – And then click the *Add* button. ***Note:*** *If sample files from more than one run folder are to be analyzed, all run folders may be imported into one project because ladder injections from each run folder are averaged separately.*
4	To examine the raw data of each sample: – Click the sample folder in the navigation pane once to view the sample files in the folder. – Click on a sample file and then choose the *Raw Data* tab. – Scroll through the list of sample files to examine each injection. – When finished, click the run folder or project node to return to the main

	window.
5	To remove a sample from the project (*e.g.,* GS600v2 only, failed, or extraneous injections), highlight the sample and select *Delete from project* under the *Edit* menu. Alternatively, samples may be deleted while viewing electropherograms by selecting *Mark Sample for Deletion*. When the plot window is closed, the sample will be deleted from the project. ***Note**: All injections from a run must be maintained in the run folder.*
6	Choose *Casework Table Settings* from the *Table Setting* pull down menu.
7	Adjust "Sample Types" to the correct settings. • Allelic ladder injections used for genotyping need to be set to *Allelic Ladder*. • 007 may be set to *Positive Control.* ID-X will check the 007 genotypes. • Negative controls (*e.g.,* PCR and reagent blanks) may be set to *Negative Control.* ID-X will also check the negative controls for labeled peaks. • All others should be set to *Sample.*
8	Under Panel, *YFiler_v1.2X* of *AmpFlSTR_Panels_v3X* should be selected.
9	Under Analysis Method, *Casework_ YF_3500* should be selected. – Open the analysis method and check to be sure that the default settings match those shown in Figure 1 of Appendix I. ***Notes**:* • The analysis range may need to be modified to include the GS600v2 80-bp and 400-bp peaks and all sample peaks. • The size calling method is 3rd Order Least Squares.
10	Under Size Standard, *Casework_GS600_LIZ(80-400)* should be selected. – The default settings should match those shown in Figure 1.  **Figure 1**
11	Analyze sample files by clicking the menu bar *Green Arrow*. When prompted, name and save the project, either as: • <Case Number>YF IDX <date of ID-X analysis> <analyst's initials> for a case-specific project or, • <Run Folder name>YF IDX <date of ID-X analysis> <analyst's initials> for a batch project.

Next steps Continue with the steps for Evaluating and Editing Data.

Section 4.3.4 Evaluating and Editing Data

Note Customized table and plot setting profiles may be generated to accommodate analyst-specific data viewing preferences.

Evaluate and edit data Follow the steps below to evaluate and edit the data.

Step	Action
1	Confirm that the size standard peaks have been correctly assigned. • This can be aided by use of the Sizing Quality (SQ) PQV. • It may also be helpful to view the raw data. • See Figure 1 for the correct GS600v2 peak sizes. Were the peaks labeled correctly? • ***If yes,*** continue to Step 2. • ***If no,*** complete the following actions. To edit a size standard peak assignment (*i.e.,* when a sample has a red flag for SQ), – click once on the sample in the *Samples* view, – then on the *Size Match Editor* toolbar icon. – Edit the size standard peak accordingly. **If GS600v2 peaks were labeled incorrectly due to ...** / **Then ...** An artifactual peak(s) / Left-click once on the peak and right-click to make the necessary edit. The 400bp peak not collected during the run / The sample will need to be re-run. It may be beneficial to increase the run time.
2	Re-analyze any edited sample files. If an allelic ladder injection was edited: – Re-analyze all injections in the project. This is necessary to create new average values (offsets) for allele assignments. – To re-analyze all injections, change an analysis criterion to "activate" the

	unedited injections for re- analysis (*e.g.,* change the Analysis Method from *Casework_YF_3500* to *Default* and then back to *Casework_ YF_3500*).
3	Review sample and allelic ladder results. Evaluate the following parameters: – Peak shape and height. – Quality of spectral separation (baselines should be relatively flat, and any pattern of pronounced peaks or dips below true DNA peaks should not be present). – Peak profile (examine for artifactual peaks). If there are extraneous peaks in an electropherogram, it may be useful to examine the raw data. – Identification of off-scale peaks may be aided by the OS Process Component-based Quality Value (PQV) in the *Samples* view. – Ensure that the alleles in the allelic ladder results have been correctly assigned by ID-X. – Allelic ladder peaks should be labeled as shown in Figure 2 (at the end of this section). ***Additional help in evaluating data*** Data evaluation may be assisted by use of additional PQV values. These are viewed in the table produced under the *Genotypes* tab. The *Casework Table Settings* profile will display the following PQVs: BIN, PHR, LPH, AN, CC. The *Analysis Summary* tab has further sample evaluation information based on other PQVs. See Appendix I of Section 3.9 for guidance on creating custom Table Settings profiles.
4	Electronically edit extraneous peaks between 95 and 340 bp, including extraneous peaks between locus ranges (unlabeled peaks). ***Extraneous peaks include:*** – Pull-up, – Pull-down, – Spikes, – Dye-labeled kit artifacts (“dye blobs”), – Incomplete non-template nucleotide addition peaks, and – Non-specific amplification artifacts. ***To make electronic edits:***

Step	Action
1	Left-click once on the peak label, right-click once on the same label, and choose *Delete Label*.
2	Enter the edit in the *Reason for Change* textbox. The abbreviations below may be used for edits.

Edit	Abbreviation
Pull-up	PU

Pull-down	PD
Incomplete Nucleotide Addition	INA
Non-reproducible artifact	NRA
Reproducible artifact	RA
Low-level inconclusive	LLI, *
N-2 Stutter	N-2
N+2 Stutter	N+2
N-3 Stutter	N-3
N+3 Stutter	N+3
N-4 Stutter	N-4
N+4 Stutter	N+4
N-5 Stutter	N-5
N-8 Stutter	N-8
N-9 Stutter	N-9

3	***Result:*** The label box will be modified to display the comment and a diagonal line ("slash") will be drawn through the label box.

Note*:* Alternatively, edits may be made manually on print-outs.

5 Is there a microvariant allele or other callable allele requiring an edit?

- ***If no,*** continue to Section 4.3.5 Printing and Archiving Data.
- ***If yes,*** determine the allele call and complete the following actions.
- The allele call determination may be performed manually or as described in Section 3.10.10, Appendix I. See Section 4.4.4.2 for more information.

To edit a microvariant allele or other callable allele:

Step	Action
1	Left-click once on the peak label and right-click once on the same label.
2	Choose *Rename Allele* and *Custom* from the pop-up menus instead of Delete Allele.
3	Enter the allele designation in the *Allele Name* textbox and click *OK*.
4	In the *Edit Allele Comment* textbox, enter the original ID-X designation in parentheses (*e.g.,* "(OL Allele)") and click *OK*.
5	***Result:*** The label box will be modified to display the new information.

	***Note**:* Alternatively, edits may be made manually on print-outs.

AmpFlSTR Yfiler Allelic Ladder peaks are shown here in Figure 2.

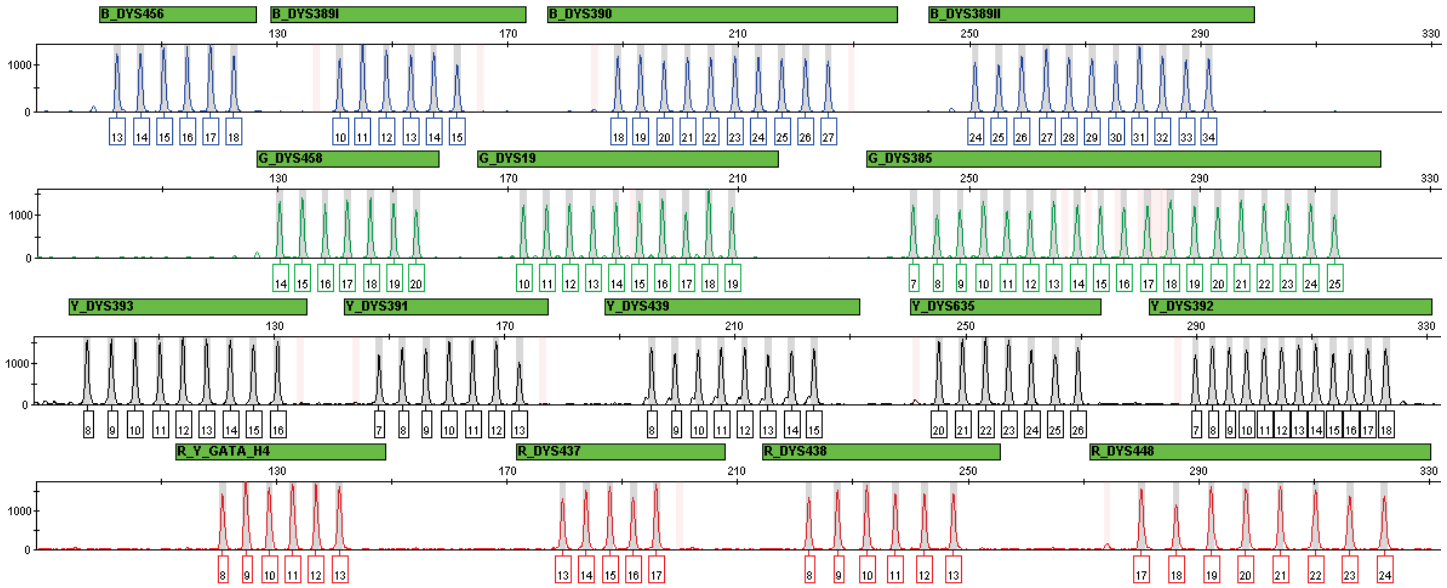


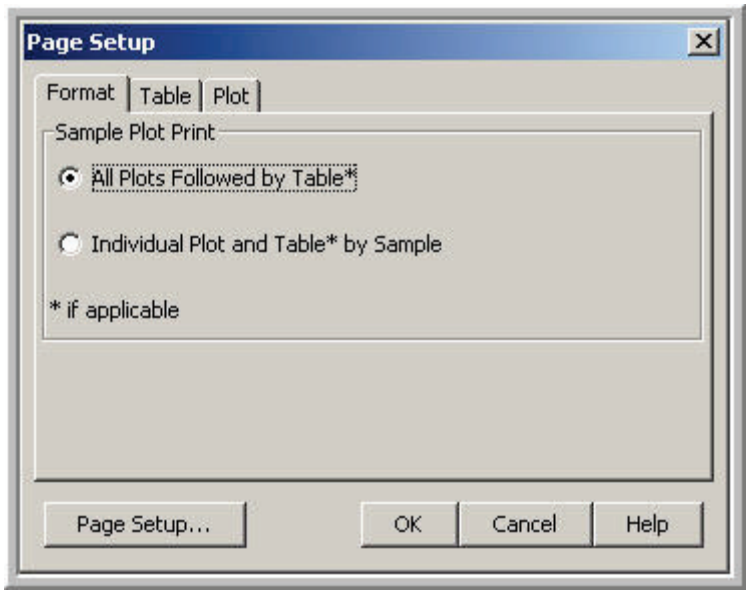
Figure 2

Next steps Continue with the steps for Printing and Archiving Data.

Section 4.3.5 Printing and Archiving Data

Print and archive data

Follow the steps below to print and archive the data.

Step	Action
1	Before printing: • Make sure that the table settings are showing the sizing table with the electropherograms. • Verify *Page Set-up* from the plots view appears as shown in Figures 3, 4, and 5 (parameters may be modified with different printing conditions). • Adjust margins as needed from *Print*, also from the plots view.  **Figure 3**

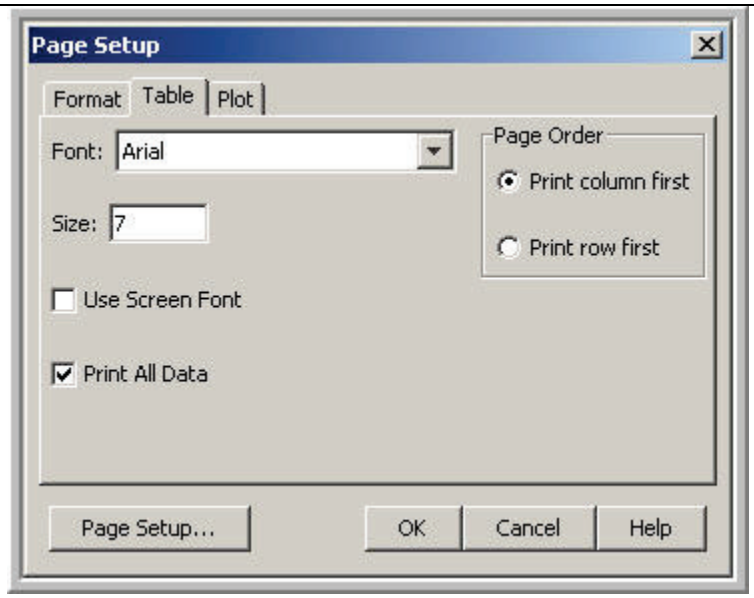


Figure 4

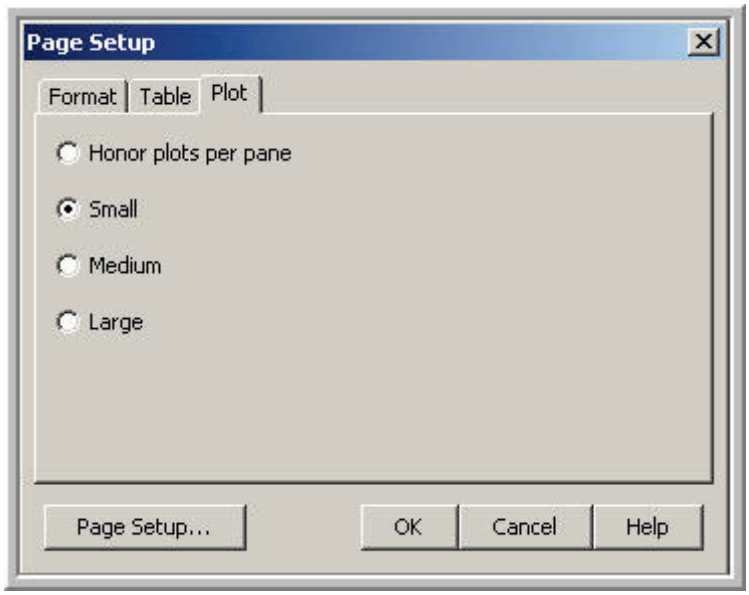


Figure 5

2	After reviewing data and completing electronic edits, select samples to print from the *Samples* tab. **See sections below**: *Printing specifications* and *Notes about printing samples.* ***Note:*** If creating case-specific projects from a temporary batch project, this is generally completed prior to printing as the appropriate case number will be on each printout (*i.e.,* project name).
3	Make any other edits manually that were not addressed electronically (*e.g.,* spikes in orange, artifacts between the locus ranges).

4	*Save* and *export* the project. • Under *Tools*, open *GeneMapper ID-X Manager.* • Highlight the desired project under the *Projects* tab and click *Export*. • At the prompt, enter the project name and navigate to the desired location. • Click *OK*.
5	• Copy the electronic Case Folder or Batch Folder onto one CD for the case file and verify that the folder has been recorded. • Move the folder to a secure network.

Printing specifications

Full view is defined as visualization of the highest peak apexes in the upper half of the printed electropherogram.

Casework Evidence/ Reference Samples (if single-source): Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection. At the analyst's discretion, a close-up view (*e.g.*, 300 RFU) may also be printed.

Casework Evidence Samples (if a mixture or a partial haplotype): For one injection, print the blue, green, yellow, and red tabular data and electropherograms at full view and close-up view (*e.g.,* 300 RFU). For at least one other injection, print the blue, green, yellow and red tabular data and electropherograms in close-up view.

Negative Amplification Controls, Reagent Blanks, and Other Blanks: blue, green, yellow, and red tabular data and electropherograms at 300 RFU from one injection.

Positive Amplification Control and Quality Control Sample: Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection. If the Quality Control sample is indicated to be from a female, print sample according to the Negative Amplification Control instructions.

Notes about printing samples

When to print in close-up view
It may be necessary to print duplicate sample injections in a close-up view (for example, if an apparent single-source sample has a low-level, second possible allele present due to mutation).

Allelic ladders
Because the genotyping bins are based on an average of all the allelic ladders, it is not necessary to print the allelic ladders nor the GS600v2 (orange) electropherograms and tabular data for the allelic ladders.

Batch projects
A unique laboratory identifier must be included on each printed ID-X page. For example, the batch name as the project name or the case number in the sample name field.

Section 4.3.6 References

Applied Biosystems (2012), "GeneMapper® ID-X Software Version 1.4," Applied Biosystems User Bulletin P/N 4477684, Rev.A.

Applied Biosystems (2009), "GeneMapper® ID-X Software Version 1.2," Applied Biosystems Reference Guide P/N 4426481, Rev.A.

Applied Biosystems (2007), "GeneMapper® ID-X Software Version 1.0," Applied Biosystems Administrator's Guide P/N4376327, Rev.A.

Section 4.3.7 Appendix I – Setting Up GeneMapper ID-X v. 1.4 for Yfiler

Contents

This appendix describes the steps used to set-up GeneMapper ID-X v. 1.4 (or higher if performance checked) for analysis of Yfiler data. Some of these steps must be completed while logged into the administrator/gmidx account, thus it is recommended to complete the set up using this login. The table settings file, plot settings files, and size standard table are the same as those used for Identifiler Plus; refer to Appendix I in Section 3.9.

Setting up GeneMapper ID-X includes the topics listed below.

Topic
Panels and bins
Analysis method parameter file

Important
The files created and used in this appendix are accessible to all ID-X user accounts.

About Administrator/ gmidx

The Administrator/gmidx user account allows the user full access to all functions of the GeneMapper ID-X software. This includes access to analyze, view, edit, and print data (as with the Casework Analyst – see Section 3.9 Appendix II Creating User Accounts in GeneMapper ID-X) as well as all administrative function access (such as Security Manager) and creating analysis method parameters.

This account has access to all projects, including those of the Casework, GeneMapper ID-X, and Admin Security Groups.

This account can reset Casework Analyst passwords. However, if the password of this Administrator account is lost, it cannot be retrieved by another user nor Life Technologies/ Applied Biosystems.

Panels & bins Perform the following to install and verify the YFiler panels and bins.

Step	Action
1	Open GeneMapper ID-X v. 1.4 (ID-X) and log into the software as the Administrator/gmidx user account.
2	Under the *Tools* tab, select *Panel Manager*. Click once in the left pane on the *Panel Manager* node and once on the *AmpFlSTR_Panels_v3X* node to open the folder. Continue to Step 4. If *AmpFlSTR_Panels_v3X* is *not* present, go to Step 3.
3	If *AmpFlSTR_Panels_v3X* is *not* present, perform the following steps.

Step	Action
1	Obtain a copy of *AmpFLSTR_Panels_v3X.txt* and *AmpFlSTR_Bins_v3X.txt.*
2	Copy each to *C:/AppliedBiosystems/GeneMapperID-X/Panels* on the workstation computer.
3	Open ID-X and, under the *Tools* tab, *Panel Manager*.
4	Click once on *Panel Manager* in the left navigation pane.
5	Under the *File* menu, choose *Import Panels*.
6	Select the *AmpFLSTR_Panels_v3X.txt* panel and choose "Casework Security Group". Then, click *OK*. ***Note:*** *The stutter ratio data will not be imported.*
7	Click once on the *AmpFlSTR_Panels_v3X* folder and, under the *File* menu, choose *Import Bin Set.*
8	Select the *AmpFlSTR_Bins_v3X.txt* bin set.
9	Enter the marker specific stutter information: • Click once on the *AmpFlSTR_Panels_v3X* node to open the folder and see each kit in the panel. • Click once on *YFiler_v1.2X* to open the folder and see each locus in the panel. • Repeat for each locus, according to Table 1 in Step 5: – Click once on the locus node to "open" that locus. – Click once on *Stutter Ratio & Distance* – Choose *New* to enter the *Minus or Plus Stutter* information: ▪ Type of Stutter (*e.g.,* Minus stutter) ▪ Ratio (between 0-1): (*e.g.,* 0.15 for DYS456) ▪ From Distance (bp): (*e.g.,* 3.25) ▪ To Distance (bp): (*e.g.,* 4.75) • Click *Apply,* then *OK*.

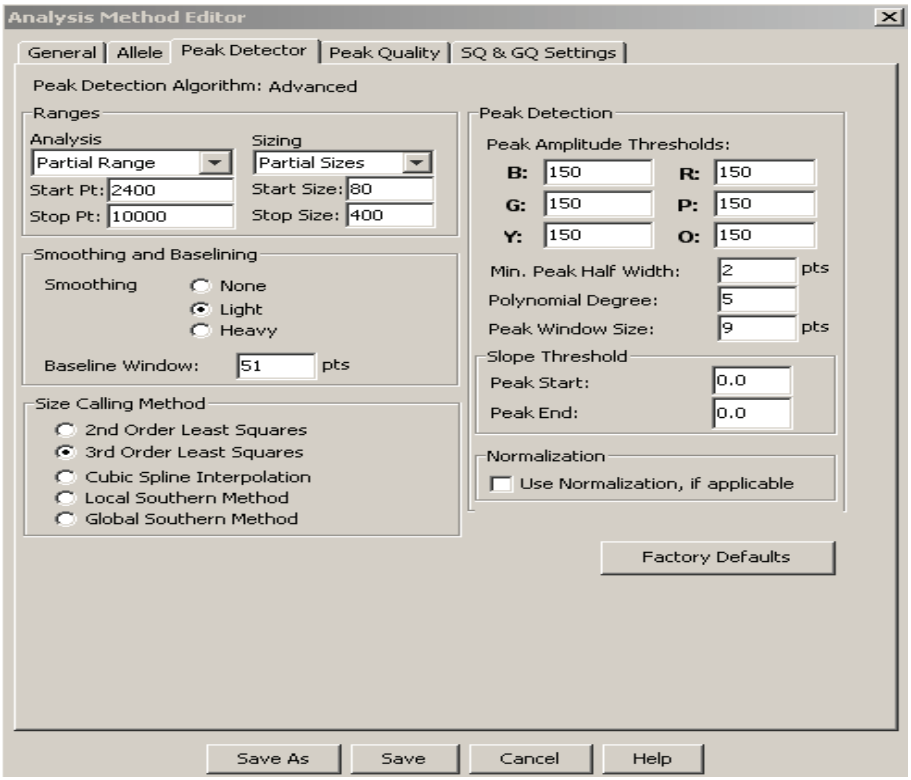
	10 | Return to Step 2 of **Panels & bins**.
4	Verify *AmpFlSTR_Bins_v3X* is the selected bin set from the drop-down menu in the *Panel Manager* tool bar.
5	Verify the Marker Specific Stutter Ratio is set correctly for each locus. • Click once on the *AmpFlSTR_Panels_v3X* node to open the folder and see each kit in the panel. • Click once on *YFiler_v1.2X* to open the folder and see each locus in the panel. • Repeat for each locus: – Click once on the locus node to "open" that locus. – Click once on *Stutter Ratio & Distance* node to view the marker specific stutter ratio and verify the direction and distance (bp) settings.
6	Click *Apply* if changes were made. Click *OK*.

Locus	**Stutter location**	**Stutter Threshold**	**Stutter Distance (bp)**
DYS456	n-4	15%	3.25 to 4.75
DYS389I	n-4	10%	3.25 to 4.75
DYS390	n-4	14%	3.25 to 4.75
DYS389II	n-4	17%	3.25 to 4.75
DYS458	n-4	14%	3.25 to 4.75
DYS19	n-4	13%	3.25 to 4.75
	n-2	11%	1.25 to 2.75
	n+2	4%	1.25 to 2.75
DYS385a/b	n-4	16%	3.25 to 4.75
DYS393	n-4	16%	3.25 to 4.75
	n-8	5%	7.25 to 8.5 (max value allowed)
DYS391	n-4	11%	3.25 to 4.75
DYS439	n-4	12%	3.25 to 4.75
DYS635	n-4	14%	3.25 to 4.75
DYS392	n-3	20%	2.25 to 3.75
	n+3	9%	2.25 to 3.75
GATA H4	n-4	11%	3.25 to 4.75
DYS437	n-4	10%	3.25 to 4.75
DYS438	n-5	6%	4.25 to 5.75
DYS448	n-6	4%	5.25 to 6.75

Table 1

Analysis Method

To create the *Casework_YF_3500* Analysis Method, perform the following:

Step	Action
1	Under the *Tools* tab: • Select *GeneMapper ID-X Manager* • Click on the *Analysis Methods* tab and on *New*
2	Under the *General* tab: • Analysis type should indicate *HID* • Enter "Casework_YF_3500" for Name • Choose "Casework Security Group" for Security Group • Enter "YFiler" for Description
3	Under *Allele*: • Choose *AmpFLSTR_ Bins_v3X* for Bin Set • The remaining values are used at the factory default.
4	Under *Peak Detector*: • Peak Detection Algorithm should indicate *Advanced* • Carefully fill in the values and match the selections shown in Figure 1  **Figure 1** **Important** Be careful to NOT select normalization.

5	Under *Peak Quality,* the values should be set as follows: • Signal Level, Homozygous min peak height: *150* • Signal Level, Heterozygous min peak height: *150* • Max Peak Height: *32,000* • Heterozygote balance, Min peak height ratio: *0* • Peak morphology, Max peak width (basepairs): 1.5 (default) • Allele number, Max expected alleles: – For autosomal markers and Amel 2 (default) – For Y markers 1 (default) • Allelic Ladder Spike: Spike Detection: *Enable*; Cut-off Value: *0.2* • Sample Spike Detection: Spike Detection: *Disable*
6	Under *SQ & GQ Settings,* the values should remain at the factory defaults.
7	Save As *Casework_YF_3500* with "Casework Security Group."

Section 4.4 Y-STR INTERPRETATION GUIDELINES

Section 4.4.1 Introduction

The interpretation of results in casework is necessarily a matter of professional judgment and expertise. Not every situation can or should be covered by a pre-set rule; nor is it expected that competent analysts will always be in full agreement in a particular case. However, it is important that the laboratory develops and adheres to minimum criteria for interpretation of analytical results. These criteria are based on validation studies, literature references, and casework experience, and were developed with maximum input from analysts. It is expected that these interpretation guidelines will continue to evolve as the collective experience of the laboratory grows.

The purpose of these guidelines is to establish a general framework and to outline minimum standards to ensure that:

- Conclusions in casework reports are scientifically supported by the analytical data, including that obtained from appropriate standards and controls.
- Interpretations are made as objectively as possible, and consistently from analyst to analyst.

Section 4.4.2 Preliminary Evaluation of Data

A peak is defined as a distinct, triangular section of an electropherogram that projects above the baseline. The peak height, or peak amplitude, is the point at which the signal intensity of the peak is highest.

4.4.2.1 Analytical and Stochastic Thresholds

The analytical thresholds are defined as the minimum and maximum peak amplitudes that are acceptable for peaks that will be assigned allele designations. Analysis of sample files is routinely performed at a peak detection (analytical) threshold of:

- 50 relative fluorescence units (RFU) for data from the 3130/3130*xl*
- 150 RFU for data from the 3500/3500xL

By default, the maximum peak amplitude that can be obtained for raw data is approximately:

- 8000 RFU on the 3130/3130*xl* Genetic Analyzers
- 32,000 RFU on the 3500/3500xL Genetic Analyzers

Peaks exceeding this limit are determined to be “off-scale” by the software. Specifically, Data Collection assigns a tag to off-scale peaks for recognition by GeneMapper ID/ID-X. Peaks derived from off-scale data may be assigned allele designations if pull-up, pull-down/raised baselines, artificially elevated stutter peaks, or artifacts caused by an excess of input DNA (*e.g.*, peaks caused by incomplete 3´ terminal nucleotide

addition) have been ruled out. These artifacts should be noted on the GeneMapper ID/ID-X printouts. Peak height values for off-scale peaks should not be used in quantitative aspects of interpretation. A sample that exhibits one or more unacceptable off-scale peaks may be either re-injected using a shorter injection time and/or lower injection voltage, re-run using less PCR product, or re-amplified using less input DNA.

The minimum signal intensity used to assign alleles was determined empirically based on data generated by the laboratory. Peaks greater than or equal to the analytical threshold in at least two injections may be assigned allele designations and reported. In instances where a peak is above the analytical threshold in one injection and below in the second injection, the peak will be considered inconclusive and not reported as an allele. Similarly, in instances where a possible minor allele is co-migrating with a stutter peak, the peak must exceed the locus stutter threshold in at least two injections to be reported as a minor allele; otherwise, the peak will be considered inconclusive.

In considering multiple injections or amplifications of a sample, a composite haplotype may be reported if an allele is detected at at least the analytical threshold in a minimum of two injections. However, in the case of allelic data from separate extractions of different locations on a given evidentiary item, unless there is a reasonable expectation of the DNA to have come from the same individual, a composite haplotype may not be reported for this situation.

Fluorescent signal lower than the analytical threshold will not be interpreted for reporting purposes.

4.4.2.2 Size Standards and Allelic Ladders

On the 3130/3130*xl* Genetic Analyzers, GeneScan-500 [LIZ] is used as the Internal Size Standard. This size standard is run with every sample to normalize injection-to-injection peak migration differences. Using GeneMapper ID, sizes are assigned to the 75, 100, 139, 150, 160, 200, 300, 350, 400, and 450 base-pair GS-500 [LIZ] peaks. No value is assigned to the 250- and 340-bp peaks. In instances in which the 450-bp peak was not collected by the Data Collection software, sample analysis may proceed as usual with an adjusted Size Standard Table.

On the 3500/3500xL Genetic Analyzers, GeneScan-600 [LIZ] version 2 is used as the Internal Size Standard. This size standard is run with every sample to normalize injection-to-injection peak migration differences. Using GeneMapper ID-X , sizes are assigned to the 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, and 400 base-pair GeneScan-600 [LIZ] peaks.

Size windows based on the allelic ladder are used to assign allele designations. It is therefore necessary to ensure that each allele in the allelic ladder has been sized for genotyping.

4.4.2.3 Sizing Precision

The method used for genotyping employs a ± 0.5-bp "window" around the size obtained for each allele in the `AmpFℓSTR` Yfiler Allelic Ladder injection(s) from the given run. These sizes may be averages of each allele if more than one allelic ladder injection is used for allele assignments. A ± 0.5-bp window allows for the detection and correct assignment of potential off-ladder sample alleles whose true size is only one base different from an allelic ladder allele. A sample allele that sizes outside a window could be an off-ladder

allele (an allele of a size that is not represented in the allelic ladder) or an allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement imprecision.

Fluctuation in ambient temperature during the course of a set of capillary runs may affect sizing precision. An indicator that room temperature fluctuation may have affected a run is the occurrence of shifts in the migration of peaks from one run to the next, which is most easily and more commonly observed in the GS500 peaks (especially the 250-bp peak). Variations in peak migration may become apparent while determining the optimal analysis range for a comparison set. Significant room temperature fluctuation may result in size variation between injections such that allelic ladder peaks differ by more than 0.5 bp from allelic peaks in other injections. This will cause GeneMapper ID/ID-X to assign these alleles as off-ladder alleles.

Section 4.4.3 Controls required to assess analytical procedures

The following controls and standards are used to assess the effectiveness, accuracy and precision of the analytical procedures:

- Positive amplification control 007
- Negative controls (including negative amplification controls and reagent blanks)
- Quality Control (QC) sample

Evaluation of the controls is essential to the proper interpretation of the test results.
In order for the analytical results to be reported, the positive amplification control and QC sample, if from a male source, should yield typing results that are consistent with the known haplotypes for these samples. However, if it can be demonstrated that low or no signal was seen in one of these controls as the result of insufficient template DNA or product, the results from the initial amplification may be reported as long as at least one of these controls (positive amplification control or male QC sample) gives an accurate typing result. The presence of artifactual peaks does not invalidate a positive control or QC sample.

In the event that the positive amplification control or a QC sample fails to give the expected types, the analyst and technical reviewer will evaluate the results in an attempt to determine the cause and seriousness of the discrepancy. Consideration will be given to the extent to which the results from other samples in the chain of analysis may have been affected. The extent to which a failed QC sample affects the other samples in an analytical set will be evaluated on a case-by-case basis. For example, if a sample mix-up occurred for the QC sample for a particular case during the extraction process, that would not necessarily affect the typing results for a second case that was extracted separately but amplified and typed with the first case.

Negative control samples (female DNA 9947A control, negative amplification control, system water control, and reagent blank), as well as female QC samples should show no allelic peaks at or above the analytical/interpretation threshold. If any such peaks are detected in a negative control sample, that sample must be evaluated for potential contamination and how it may have occurred.

For the positive amplification control and the quality control sample, where the correct types are known, contamination is defined as the presence of foreign allelic peaks greater than or equal to the analytical threshold.

Although reference samples are typically single-source, transfusion should be considered as a possible source of additional alleles. When a reference sample contains a secondary source that is determined not to be the result of contamination by the laboratory, this will be documented.

Due to the potentially complex nature of some evidence samples, possible contamination will be assessed on a sample-by-sample basis. To assist in troubleshooting possible contamination, samples may be re-analyzed at less than the analytical threshold. However, peaks less than the analytical threshold may not be reported.

If contamination is determined to be present in an evidence or reference sample or any of the controls governing the analysis of that sample, the contamination may be classified as not serious if the level or nature of the contamination does not account for or interfere with the typing calls of the sample(s) in question. In this situation, the results for the case sample(s) may be reported. The possible contamination will be evaluated and documented in the bench notes, and the analyst will prepare an Instance of Contamination Report for inclusion in the case file and the Contamination Log. If the contamination is determined to be serious, the procedures will have to be repeated to the appropriate extent for the affected samples. Evaluation and documentation in the case file and Contamination Log will also be required. The Technical Leader will review all contamination reports. The occurrence of contamination should be noted in the laboratory report.

Section 4.4.4 Designation

Types are determined from the diagnostic peaks of the appropriate color (fluorescent dye label) and size range for a particular locus. Allele calls are assigned by comparing the sizes obtained for the unknown sample alleles with the sizes obtained for the alleles in the allelic ladder(s). Types, not sizes, are used for comparison of data between runs, instruments, and laboratories.

4.4.4.1 Locus Designation

The following locus range approximations were determined by Life Technologies/Applied Biosystems for Human Identification analysis. The allelic bin definitions are stored within GeneMapper ID/ID-X using the corresponding bin set. Typically, multiple ladders are used in an analysis and allelic bins are determined by averaging the designated ladders in the project. Ladders within a single run folder are used for calculating allelic bin offsets and subsequent typing.

6-FAM-labeled loci:

- DYS456 100 to 127 bp
- DYS389I 134 to 178 bp
- DYS390 185 to 245 bp
- DYS389II 246 to 302 bp

VIC-labeled loci:

- DYS458 133 to 165 bp
- DYS19 167 to 219 bp
- DYS385 235 to 323 bp

NED-labeled loci:

- DYS393 104 to 144 bp
- DYS391 146 to 181 bp
- DYS439 192 to 236 bp
- DYS635 241 to 274 bp
- DYS392 286 to 335 bp

PET-labeled loci:

- Y GATA H4 114 to 150 bp
- DYS437 174 to 210 bp
- DYS438 215.5 to 256.5 bp
- DYS448 273 to 332 bp

4.4.4.2 Allele designation

The allelic ladder provided in the AmpFℓSTR Yfiler PCR Amplification Kit contains the majority of alleles for each locus. The alleles contained in the allelic ladder were named by the kit manufacturer.

The number of repeat units observed in an allele that is present in the ladder is designated by an integer. Alleles that contain an incomplete repeat motif are designated by an integer representing the number of complete repeats, followed by a decimal point and the number of bases in the incomplete repeat. The GeneMapper ID/ID-X software is used to automatically convert allele sizes into allele designations. Alleles not aligning with those in the allelic ladder have been detected both within and outside the range of the allelic ladder for each locus. Alleles smaller than the lowest molecular weight allele (A) in the allelic ladder for that locus will be designated as "<A." Alleles larger than the largest molecular weight allele (B) in the allelic ladder for that locus will be designated as ">B." When an off-ladder allele occurs within the ladder region, the allele designation will be determined by interpolation The off-ladder allele designation should be confirmed using at least two sample injections. Print both sample injection electropherograms with tabular data.

4.4.4.3 Artifacts

Artifacts can occur and should be noted where appropriate for editing purposes.

4.4.4.3.1 Pull-up / pull-down

Smaller artifactual peaks can appear in other colors under allelic peaks. This phenomenon is termed "pull-up" and is the result of spectral overlap between the fluorescent dyes. If a pull-up peak is above the peak amplitude threshold, it will be sized at a similar size as the allelic peak. The shape of the peak may appear similar to a true DNA peak or sigmoidal, as a doublet, or otherwise irregularly-shaped.

Similarly, pull-down is also the result of spectral overlap between the fluorescent dyes. When an oversubtraction occurs, the result is a dip into the baseline. The baseline can only be positive values so this dip causes a raised baseline, or pull-down, in the other colors of the proximal size/data point range of the allelic peak(s).

Pull-up and pull-down can occur as a result of the following:

- Application of a less than optimal spectral calibration or matrix. The run may need to be repeated with a different spectral calibration or re-analyzed with a different matrix.
- Amplification using excess input DNA can lead to off-scale peaks. If necessary, samples can be re-amplified using less input DNA, or re-run using shorter injection times or less product.

Even when the spectral calibration is satisfactory, pull-up and pull-down may be seen.

4.4.4.3.2 Stutter

In addition to an allele's primary peak, artifactual stutter peaks can occur at different base pair intervals depending on the number of bases in the repeating unit. In general, the stutter peak most commonly observed is one repeating unit smaller than the primary peak (*e.g.,* "n-4"). However, it is also possible to see a peak that is one or more repeating units larger or smaller (*e.g.,* "n+4" or "n-8") than the primary peak.

Note: *For DYS19 it is common to observe n-2 stutter and/or n+2 stutter.*

Stutter peaks may be due to repeat slippage during amplification. Sequence variation can affect the amount of stutter; a lower amount of stutter is produced from alleles with increased sequence variation between repeats.

Stutter peaks are evaluated by examining the ratio of the stutter peak height to the primary peak height, expressed as a percentage. This percentage can vary by locus. Moreover, longer alleles within a locus generally have a higher stutter percentage than the shorter alleles.

The GeneMapperID/ID-X thresholds for stutter are set at those values shown in Table 2:

Table 2

Locus	Stutter location	Stutter Threshold
DYS456	n-4	15%
DYS389I	n-4	10%
DYS390	n-4	14%
DYS389II	n-4	17%
DYS458	n-4	14%
DYS19	n-4	13%
DYS385a/b	n-4	16%
DYS393	n-4	16%
DYS391	n-4	11%
DYS439	n-4	12%
DYS635	n-4	14%
DYS392	n-3	20%
DYS392	n+3	9%
GATA H4	n-4	11%
DYS437	n-4	10%
DYS438	n-5	6%
DYS448	n-6	4%
DYS19*	n-2	11%
DYS19*	n+2	4%
DYS393*	n-8	5%

Note: Although GeneMapper ID thresholds for stutter cannot be set for the asterisked loci, the stutter percentage thresholds are set at those values shown. Electronic or manual edits will need to be made to the electropherograms for these three stutter percentage thresholds when using GeneMapper ID.

These stutter thresholds are based on validation studies performed at the BFS Sacramento Laboratory and represent the locus mean plus four standard deviations determined individually for each locus. Typically the stutter percentages for each locus are expected to be less than these percentages.

The presence of peaks in the stutter positions exhibiting percentages greater than these values may indicate a DNA mixture. However, stutter peak heights may also be elevated above the expected thresholds by the following:

- The measurement of percent stutter may be unnaturally high for main peaks that are off-scale, *i.e.*, have exceeded the linear dynamic range for detection (raw data greater than approximately 8,000 on the 3130 and 32,000 RFU on the 3500). The Genotypes Display in GeneMapper ID/ID-X has a PQV that can be set to flag off-scale peaks automatically, designated as "OS." If a stutter peak is greater than the maximum expected and the primary

peak is off-scale, the analyst should interpret the results with caution. The sample may be subsequently re-injected with less product or for a shorter time, or the sample may need to be re-amplified using less input DNA.

- For alleles differing by two repeat units, the stutter peak from the larger allele may overlap the trailing shoulder of the smaller allele and therefore exhibit an increased stutter percentage. This will not occur if the smaller allele peak drops to baseline before reaching the stutter peak.

- Stutter may exceed a threshold simply as a result of normal statistical variation. Specifically, the thresholds are based on a mean plus four standard deviations calculation.

- Apparent elevated stutter may be the result of a **somatic mutation** (*i.e.,* Type I mutations; Clayton *et al* 2004 JFS, Rolf *et al* 2002 FSI, Gill, P. 2002 BioTechniques). For example, such mutations may be seen in rapidly dividing buccal cells or spermatozoa. These situations are generally the result of a minor proportion of the otherwise wildtype cell population containing the mutated genotype (*i.e.,* mosaicism). "IPA," or indeterminate possible allele, may be used in the benchnotes to annotate such occurrences in apparent single-source samples.

- In some situations, an allelic peak in a mixture may co-migrate with the stutter peak of another allelic peak, resulting in an apparent stutter peak height greater than that typically observed for stutter in a single-source sample.

4.4.4.3.3 Incomplete non-template nucleotide addition

Amplification conditions have been set to maximize the non-template addition of a 3´ terminal nucleotide by AmpliTaq Gold DNA polymerase. Failure to attain complete terminal nucleotide addition may result in "split peaks," visualized as two peaks that are one base apart. Except for microvariant alleles, the presence of peaks differing by one base pair is diagnostic of this phenomenon.

4.4.4.3.4 Other artifacts

In addition to the artifacts described above, the following anomalies can arise:

- Artifacts which are distinguished from labeled DNA alleles because the peak morphology is not consistent with labeled DNA may be reproducibly detected. These artifacts are dye-labeled primer by-products from the Yfiler kit. For example, a green dye artifact is frequently observed at approximately 87-90 bp and a yellow dye artifact may be observed at approximately 90 bp. These artifacts are also referred to as "dye blobs."
- Peaks of the approximately same size, frequently present in all five colors, are not the result of dye-labeled DNA and do not indicate a spectral calibration problem. The shape of these peak-like artifacts ("spikes") often differs from the shape of dye-labeled DNA peaks and these artifacts are generally not reproducible.

- Low-level artifacts have been observed in the DYS437 and DYS385 loci in the n-5 position as well as the DYS385 locus in the n-9 position.

Section 4.4.5 Interpretation of Results

The following situations describe conditions in which the data would lead to the conclusion that the source of the male DNA is either from a single male or from more than one male. It is generally possible to estimate the number of male contributors by considering the number of alleles present at a locus and among all loci. When an assumption is made in the interpretation of the Y-STR data of a male DNA mixture, that assumption should be clearly stated in the laboratory report.

The following are considerations in interpreting sample haplotypes:

- The presence of more than one allele per locus (except DYS385 a/b which typically has two alleles, but may have just one), especially at more than one locus, may indicate a mixture.
- The presence of a peak at a stutter position that is significantly greater in peak height than that typically observed for stutter in a single-source sample may indicate a mixture.
- The possible presence of a “null” or “partial null” allele – an allele that is not detected or has significantly reduced peak height due to a mutation in the primer-annealing region or a deletion.

4.4.5.1 Single Male Contributor

A sample may be considered to be from a single male contributor when the observed number of alleles at each locus does not exceed one (assuming no genetic anomalies, see 4.4.5.6), except for DYS385 a/b which typically has two alleles, but may have just one.

4.4.5.2 Mixtures with major and minor male contributors

A sample may be considered to be a mixture of major and minor male contributors if there is a distinct contrast in peak heights among the alleles within loci. It may be possible to determine major and minor contributors in mixtures of two males if there are significant differences in peak height. However, it may be difficult to discern minor alleles from stutter and these mixtures should be interpreted with caution.

4.4.5.3 Mixtures with a known contributor(s)

In some situations, when the presence of one of the contributors (*e.g.*, the victim) can be assumed based on the nature of the sample, the genetic haplotype of the unknown contributor may be inferred. Depending on the haplotypes in the specific instance, this can be accomplished by subtracting the contribution of the known donor from the mixed haplotype.

4.4.5.4 Mixtures with indistinguishable contributors

When major or minor contributors cannot be distinguished, single donor haplotypes generally cannot be discerned. An exception would be in an intimate sample in which the foreign alleles are derived based on the haplotype of a known contributor (see Section 4.4.5.3 above).

4.4.5.5 Partial haplotypes

Partial haplotypes may be obtained when the DNA template is degraded, in low quantity, or when PCR inhibitors are present. A negative or inconclusive result at some loci may not impact allele designations at the remaining loci.

4.4.5.6 Genetic Anomalies

It is possible to observe a variety of variants that include, but are not limited to:

- deletion of portions of the Y chromosome that may affect one or more loci
- duplication or deletion of a locus (*e.g.,* DYS390, DYS392)
- deletion of a single base (*e.g.,* microvariant)
- transversion of a base that can affect mobility
- triplication or quadruplication

For current information regarding observed genetic anomalies see the Y Chromosome Haplotype Reference Database website at www.yhrd.org or the National Institute of Standards and Technology's website at www.cstl.nist.gov/biotech/strbase/.

See Appendix I for some examples of genetic anomalies that were observed by the BFS Sacramento Laboratory during its validation studies.

Section 4.4.6 Conclusions

The following guidelines may be used in formulating conclusions resulting from comparisons of single-source samples and some male mixtures with known reference samples.
General categories of conclusions include, but are not limited to:

4.4.6.1 Inclusion

For single-source samples, if each allelic peak is greater than or equal to the analytical threshold in the evidence sample and is observed in the reference standard, then the STR male haplotype from the evidence sample matches that of the reference. An individual may also be included when a partial haplotype is observed in an evidence sample (due to low template DNA, degradation and/or inhibition) if the evidence alleles detected are the same as those at the corresponding loci in the reference sample.

If a single-source haplotype can be deduced from a mixture, the haplotype may be used for comparison purposes and an individual may be included (or cannot be excluded) as a possible source of some of the biological material present in the sample. Such profiles include (a) those of major (and potentially minor) contributor(s) to a distinguishable mixture, and (b) for an intimate sample, those foreign alleles derived from separation of a conditional known sample (*e.g.,* from the victim) type.

In instances where an individual cannot be excluded as a source of the evidentiary haplotype, a haplotype frequency estimate must be provided in the report. In instances where a single-source haplotype cannot be deduced from a mixture, the results may only be used for exclusionary purposes.

4.4.6.2 Exclusion

If allelic peaks in the male haplotype from a reference standard are not found in the haplotype of the evidence sample, and their absence cannot be attributed to insufficient template, degradation, inhibition, masking at a stutter position, or a genetic anomaly, then the individual is excluded as a possible source of the biological material present in the sample.

4.4.6.3 Inconclusive or uninterpretable

Depending on the results of a particular sample, some alleles may not be detected at low levels, and it may not be possible to determine conclusively whether or not a particular individual is included or excluded.

The interpretation of results for an evidentiary sample may be confounded by the presence of excessive background male DNA, as indicated by the results for the corresponding substrate sample.

4.4.6.4 No results

A finding of “no results” is reported when no non-artifactual fluorescent signal greater than or equal to the analytical threshold is observed. A conclusion of “no results” may be obtained for some or all loci of a particular sample.

Section 4.4.7 Statistical Interpretation

4.4.7.1 Haplotype statistics

In instances where an individual cannot be excluded as a source of the evidentiary haplotype, a haplotype frequency estimate must be provided in the report. Since Y-STR loci are located on the non-recombining part of the Y-chromosome, they should be considered linked as a single locus.

A consolidated United States Y-STR population database (www.usystrdatabase.org) consisting of anonymous population profiles from various ethnic groups has been established and should be used for reporting the significance of a Y-STR match. The search of the database provides the number of times a specific haplotype is observed in the database. Haplotype searches of the population database should be conducted using all loci for which results were obtained from the evidentiary sample. In cases where less information is obtained from the known sample, only those loci for which results were obtained from both the known and evidentiary sample should be used in the population database search.

The basis for the haplotype frequency estimation is the counting method. The application of a confidence interval corrects for database size and sampling variation.

An upper bound confidence interval should be calculated from the results of a database search in order to provide a conservative estimate of the population frequency of a haplotype. An upper 95% confidence interval value (upper bound) should be presented as a percentage of individuals within each population who are estimated to be included as potential sources of the evidentiary haplotype. The upper bound confidence interval estimate is dependent on the size of the database, and the estimates are therefore likely to change as the database size changes.
The results of the upper bound confidence interval calculation are typically included in the report.

The following formulae are used to calculate the upper bound estimate:

In cases where the haplotype has been observed in the database:

The conservative Clopper and Pearson formula to calculate the upper 95 percent confidence limit in this case would be

$$\sum_{k=0}^{x} \binom{n}{k} p_0^k (1-p_0)^{n-k} = 0.05$$

where n = database size, x = the number of observations of the haplotype in the database, k = 0, 1, 2, 3 … x observations, and p = the haplotype frequency at which x or fewer observations are expected to occur 5% of the time.

This cumulative binomial distribution formula is solved for p through serial iterations and therefore requires the use of a computer program.

In cases where the haplotype has not been observed in the database:

$1-\alpha^{1/n}$
α is the confidence coefficient (0.05 for a 95% confidence interval), and n is the number of individuals in the database. This is equivalent to the Clopper and Pearson formula listed above where x = 0 (*i.e.* no matching haplotypes).

Example: 0 matches in 4,004
$1-(0.05)^{[1/4,004]} = 0.000748$
= 0.075% (~1 in 1,340)

By following the directions for entering haplotype data at the U.S. Y-STR Database, the formulae listed above are used to calculate frequency estimates. Typically, frequency estimates for African American, Caucasian, and Hispanics will be used for reporting purposes.

4.4.7.2 Relatives

Patrilineal male relatives will have the same male haplotype (barring genetic mutations). The possibility that a close relative of a suspect is a potential contributor to an evidence haplotype should be considered.

4.4.7.3 Paternity

Due to the inheritance of the male haplotype from father to son, it is possible to perform paternity analysis in criminal paternity cases. This may be particularly useful in analyzing products of conception where it may not be possible to separate the maternal DNA from the DNA of a male fetus. In the instance that the alleged father's haplotype is very similar to the male child's haplotype, mutation rates for the locus or loci should be taken into consideration. For current Y-STR mutation rates see the Y Chromosome Haplotype Reference Database website at www.yhrd.org.

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Section 4.4.9 APPENDIX I - Examples of observed Genetic Anomalies

Deletion of DYS456 and DYS393

A sample with a deletion at each of these loci was analyzed at the BFS Sacramento Laboratory (see Figure 1 below).

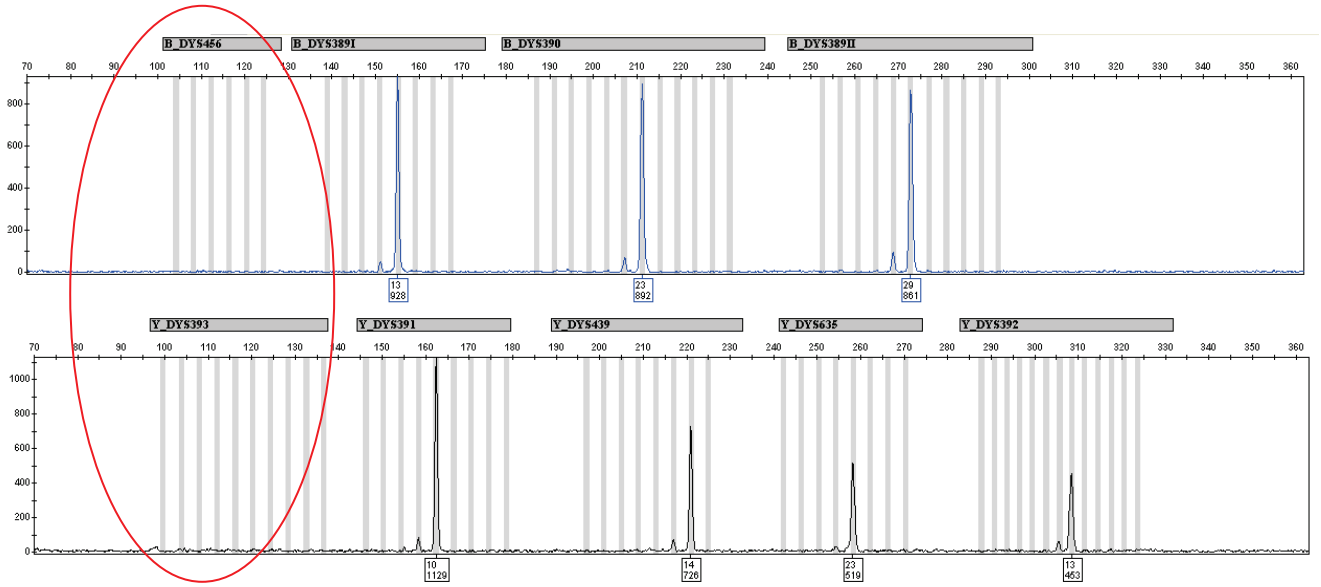


Figure 1

DYS392 "10.2" Variant

Variant allele = "10.2" (see Figure 2 below), from a sample that was analyzed at the BFS Sacramento Laboratory.

Report this variant allele as a 10.2 or an 11 with a footnote at the bottom of the table.

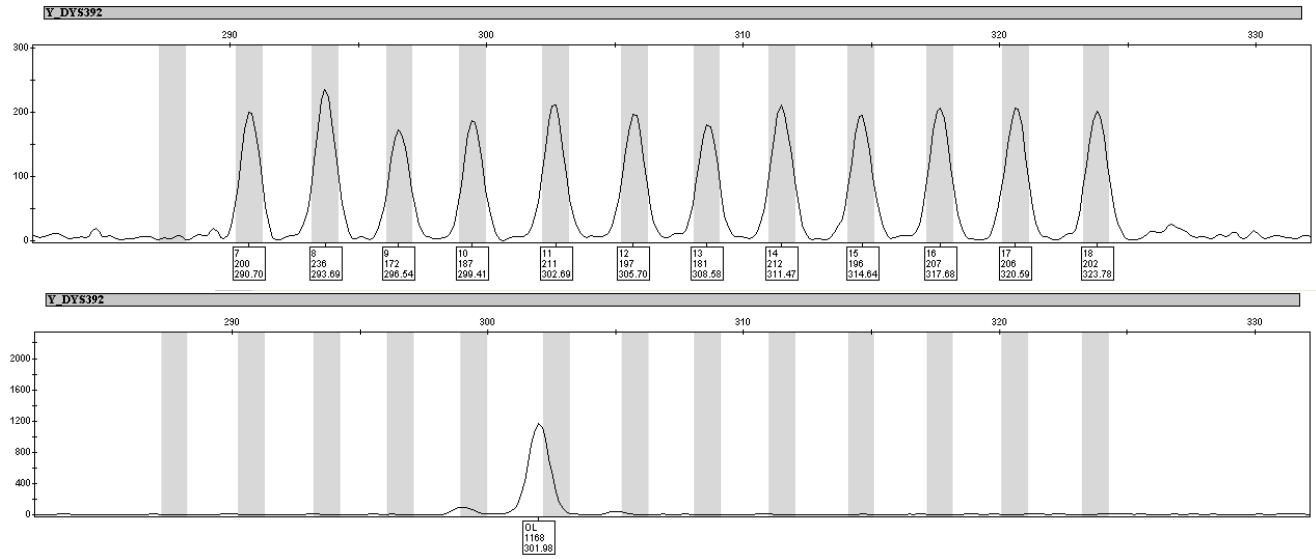


Figure 2

A "10.2" variant was sequenced by the National Institute of Standards and Technology (NIST). The variant is a "C" to "G" transversion 180bp upstream of the STR repeat region. The mutation causes an apparent mobility shift of approximately 0.75bp such that the allele falls outside of the +/- 0.5bp genotyping bin. See **www.cstl.nist.gov/biotech/strbase/STRseq.htm**.

Section 7 – Abbreviations

This list is sorted by description and is not all inclusive. Analysts may use additional abbreviations so long as they are defined in the case record.

AP	Acid phosphatase
add'l., addl., addtl	Additional
…	Additional marking(s) not recorded
AR	Administrative review
ALS	Alternate light source
amel	Amelogenin
ASCLD/LAB	American Society of Crime Laboratory Directors/Laboratory Accreditation Board
amp, amp'd, ampd	Amplification, amplified
Amy	Amylase
app	Apparent
~, ≈, approx	Approximately
aq	Aqueous
AE	Aqueous extract
assoc.	Associated
btw, btwn	Between
blk	Black
BF	Bright field
Brn	Brown
bpb, BPB	Brown paper bag
BFS	Bureau of Forensic Services
Cal-EMA	California Emergency Management Agency
CE	Capillary electrophoresis
CF	Case folder
CID, CID'd	Case number, initial(ed), date(d)
cm	Centimeter
cerv, cx	Cervical, cervix
Δ	Change
X-mas A	Christmas Tree Stain A (nuclear fast red)
X-mas B	Christmas Tree Stain B (picroindigocarmine)
CE	Coin envelope, capillary electrophoresis (context)
CODIS	Combined DNA Index System

Abbreviations, Continued

CD	Compact disc
conc	Concentrate(d), concentrating
Consis	Consistent
c (with a line over top), c/, c	Contains, containing
cont, con't, contd	Continued
Cont, cntrl, ctrl, ctl,	Control
Xref	Cross reference
dk	Dark
DBF	Date body found
DLC, DOLC	Date of last contact
↓	Decreased, low, little
°	Degree(s)
DI	Deionized
DI H2O, dH_2O	Deionized water
DNA	Deoxyribonucleic acid
DOJ	Department of Justice
Det	Detected
diam	Diameter
Dig.	Digital
dil, dil'n	Diluted, dilution
D	Direct
DLE	Division of Law Enforcement
dm	Dry mount
Docu	Documentation
DR	Donor ratio
EC, e. cell(s), ECF	Epithelial cell(s), epithelial cell fraction
ECBA	Early cycle baseline artifact (a qPCR phenomenom)
env	Envelope
=	Equal (to)
ETS, ets, et/s	Evidence tape seal(ed)
ETSID	Evidence tape seal(ed), initial(ed), date(d)
Evid, evid, evd	Evidence
Exclu, Excl	Excluded
ext, EXT	Extract
extn	Extraction
XL	Extra large
FRS	Family reference sample
FBI	Federal Bureau of Investigation
FL	Fluorescence

FD	Foreign donor
FR, frz, fzr	Freezer
GMID	GeneMapper ID
≥, ≤	Greater than or equal to, less than or equal to
>, <	Greater than, less than
HS, H/S	Heat sealed
HSID	Heat sealed, initialed, dated
HL	Hospital label
HV1, HVI	Hyper-variable region 1
HV2, HVII	Hyper-variable region 2
INA, -A, N-1	Incomplete nucleotide addition
inc	Inconclusive
↑	Increased, high, a lot
Indiv.	Individuals
inhib.	Inhibition
ID, ID'd	Initial(ed), date(d)
IL	Injection list
IPA	Indeterminate possible allele
ISO	International Organization of Standardization
Interp	Interpretation
JT	Justice Trax
KM, K-M	Kastle-Meyer
LIP	Labeled in part
Lab.	Labeled
lg(r)	Large(er)
circled “L”	Left
<LQ	Less than the limit of quantification (qPCR)
ltr	Letter
lt	Light
LTS	Long-term storage
LLI	Low-level inconclusive
mjr., Maj	Major
man	Manila
ME	Manila envelope
m (with a line over top)	Marked
max	Maximum
ME, MEO	Medical Examiner, Medical Examiner's Office
med	Medium
MT	Microcentrifuge (microfuge) tube
λ, µL	Microliter
micro	Microscope, microscopy

Abbreviations, Continued

mm	Millimeter
min	Minimum
Min., mnr	Minor
misc	Miscellaneous
mtDNA, mito	Mitochondrial DNA
N-4	N-4 stutter
N+4	N+4 stutter
N	Neat
(-), -, neg, Neg	Negative
NC	Negative control
≠	No match, excluded, eliminated
NR	No results
Φ , Θ	No, not, none
NDR	Non diagnostic region
NNEC	Non-nucleated epithelial cells
NRA	Non-reproducible artifact
NS, NSF	Non-sperm fraction
NA, N/A, n/a	Not applicable
ND	Not detected, no DNA detected
≠	Not equal (to)
n/e, N/E	Not examined
NFR	Nuclear fast red
NEC	Nucleated epithelial cell(s)
#, num	Number
Obs	Observed
Obv	Obvious
OW	Off white
OWC	Off white compact
OCJP	Office of Criminal Justice Planning
OES	Office of Emergency Services
OHS	Office of Homeland Security
orig	Original
OL	Overlay, off ladder
OMR	Out of marker range
O/N	Overnight
pkg	Package(d), packaging
pg(s)	Page(s)
PHI	Peak height imbalance

Abbreviations, Continued

PHR	Peak height ratio
pend	Pending
PC	Phase contrast
PCIA	Phenol/chloroform/isoamyl alcohol
PH, PHE, phe	Phenolphthalein
PIC	Picroindigocarmine
Pl	Plastic
PD	Police Department
PCR	Polymerase chain reaction
Pos, +, (+)	Positive
PC	Positive control, phase contrast (context)
poss.	Possible
prev	Previous, previously
prop	Property
∝	Proportional to
PD	Pull-down
PT	Primer tail outside allelic region
PU	Pull-up
QC	Quality control
Quant, qt	Quantification, quantity (context)
rxn	Reaction
RB	Reagent blank
RBE, ERB	Reagent blank (evidence)
RBR, RRB	Reagent blank (reference)
RG, RG H2O	Reagent grade, reagent grade water
rec, rec'd, recd, rcd, rcvd	Received
R/b	Red/brown
REF, ref	Reference, refrigerator (context)
rfu(s)	Relative fluorescence unit(s)
RA	Reproducible artifact
ret, rtn, retd, ret'd	Returned
(circled “R”)	Right
RT	Room temperature
RF	Run folder
SS	Sample sheet
SAE	Sexual assault evidence
SCO	Sheriff-Coroner's Office
SO	Sheriff's Office

Abbreviations, Continued

STR	short tandem repeat
slt	slight
Sm	small
SF, SP	sperm fraction
Stats	statistics
stut	stutter
sub, subm	submission
subst, Sub	substrate
SCO, SC	substrate control
(S), (s), susp	suspect
SB	swab blank
TS, T/S, t/s	tape seal(ed)
TSID, T/S/I/D, T/S/ID, t/s/I	tape seal(ed) initial(ed), date(d)
TR	technical review
temp	temperature, temporary (context)
∴	therefore
TC	thermal cycler
→	to, transferred to
TMTC	too many to count
TNTC	too numerous to count
UV	ultra violet
vag	vaginal, vagina
verf	verify, verified
VB	vial blank
(V), (v), vic	victim
Vis	visible
H2O	water
WB	water blank
wm	wet mount
wht, wt	white
WPB	white paper bag
w/, W/	with
w/I, W/I	within
w/o	without
YG, yg	yield gel

Section 11.1 AmpFℓSTR MiniFiler Amplification Procedure

Section 11.1.1 INTRODUCTION

The Polymerase Chain Reaction (PCR) is utilized for Short Tandem Repeat (STR) typing. This analysis is performed using the AmpFℓSTR® MiniFiler® PCR Amplification Kit manufactured by Life Technologies/Applied Biosystems. Each kit provides sufficient reagents for 100 tests. Included in each kit are AmpFℓSTR MiniFiler Master Mix, AmpFℓSTR MiniFiler Primer Set, AmpFℓSTR Control DNA 007, and AmpFℓSTR MiniFiler Allelic Ladder.

Following amplification, electrophoresis and detection of AmpFℓSTR® MiniFiler® STR loci are performed using a 3500/3500xL Genetic Analyzer (Section 3.8), followed by GeneMapper ID-X version 1.4 analysis (Section 11.3).

All MiniFiler data is interpreted according to Section 11.4.

Section 11.1.2 EQUIPMENT AND REAGENTS

11.1.2.1 Equipment

- 9700 Thermal cycler, Life Technologies/Applied Biosystems
- 0.5 mL and 0.2 mL GeneAmp thin-walled reaction tubes – DNA/DNase/RNase free, PCR compatible
- 96-well amplification plates – DNA/DNase/RNase free, PCR compatible
- Amplification plate covers – DNase/RNase free, PCR compatible
- MicroAmp strip caps – DNA/DNase/RNase free, PCR compatible
- Speed-Vac System, or other concentrator
- Pipettors
- Pipet tips – DNA/DNase/RNase free
- Mini centrifuge
- Vortex
- Miscellaneous laboratory supplies

11.1.2.2 Reagents

- TE^{-4}
- AmpFℓSTR® MiniFiler® PCR Amplification Kit

Section 11.1.3 AmpFℓSTR MINIFILER AMPLIFICATION

11.1.3.1 Analytical Controls and Standards for Amplification

Controls and standards are required to assess the quantity and quality of the extracted DNA as well as the effectiveness, accuracy and precision of the analytical procedures. Evaluation of the controls is essential to the proper interpretation of the test results.

11.1.3.1.1 Quantitation

An estimation of the DNA content of the sample (e.g., qPCR) will be made prior to STR analysis. In general, 0.25-0.75 ng of DNA is recommended by ABI for MiniFiler amplification, although correct results may be obtained outside of this range. Samples containing less than 0.25 ng and more than 0.75 ng have been reproducibly amplified and correctly typed for STR markers using the MiniFiler kit.

11.1.3.1.2 Positive amplification control (007)

This sample ensures that the amplification and typing process is working properly. This control is included in the AmpFℓSTR MiniFiler typing kit. The profile of control DNA 007 is:
D13S317 (11,11), D7S820 (7,12)
Amelogenin (X,Y), D2S1338 (20,23), D21S11 (28,31)
D16S539 (9,10), D18S51 (12,15)
CSF1PO (11,12), FGA (24,26).

11.1.3.1.3 Negative controls

- The **negative amplification control** contains only the reagents used to prepare the PCR amplification mixture for each batch of samples, including sample buffer (TE^{-4}). The purpose of this control is to detect contamination that might occur from the PCR reagents, the PCR set-up environment or between the samples being prepared.

- A **reagent blank** is carried through the entire analytical process as part of each extraction set and through the amplification and typing process for *each* PCR system in which the evidence is typed. For samples extracted prior to July 2009, the reagent blank must be run in at least one PCR system.

The purpose of this control is to detect DNA contamination that might occur from the reagents, the environment or between the samples being processed. It contains all of the reagents used during extraction, amplification and typing for each set of samples. The reagent blank is amplified using the same concentration conditions as required by the samples containing the least amount of DNA in the extraction set and it should be typed using the most sensitive conditions for the samples of the extraction set.

11.1.3.1.4 Substrate control sample

When appropriate, a similarly sized and apparently unstained portion of the substrate adjacent to the questioned stain should be collected and run through the typing process. A substrate control sample will not necessarily produce negative typing results. The possibility of other human biological material being present and contributing to the DNA content of a particular sample will be considered in the final interpretation. The knowledge, experience and judgment of the analyst are paramount when assessing the need for a substrate control sample, choosing the appropriate sample and evaluating the results.

11.1.3.1.5 Quality Control (QC) sample

The purpose of this control is to demonstrate that the analytical process worked properly. This is a sample from a previously characterized source that is extracted and typed concurrently with the case samples. It serves as both an extraction control and a typing control for the process. The QC sample should be typed with each PCR system in which the evidence is typed. It also serves as an internal blind control as the correct typing results are unknown to the analyst until the analysis is complete.

11.1.3.2 Amplification

1. Turn on the 9700 thermal cycler (TC) and confirm the MiniFiler amplification parameters listed below.

   DNA Thermal Cycler 9700 (TC 9700)

Pre-denaturation and enzyme activation:	95°C, 11 minutes
Cycle (30 cycles):	94°C, 20 seconds
	59°C, 2 minutes
	72°C, 1 minute
Final extension:	60°C, 90 minutes
Hold temperature:	4°C

2. Label the required number of 0.5-mL or 0.2-mL GeneAmp® Thin-Walled reaction tubes. Alternatively, a 96-well amplification plate may be used. Fill out the AmpFℓSTR MiniFiler Amplification Sample Checksheet with all pertinent information, including well number if using a 96-well amplification plate.

3. For each sample, combine in the following order:
   - Quantity of TE^{-4} needed for a total volume of 10 µL
   - Sample:
     - Extracted DNA: typically target ~0.5 ng of DNA in a total volume of 10 µL
     - Positive amplification control: 0.25 to 0.5 ng (2.5 to 5 µL) of Control DNA 007 in a total volume of 10 µL
     - Negative amplification control: 10 µL of TE^{-4}

   Note: *If a DNA sample is significantly degraded, the nuCSF result from the qPCR quantitation may be the most appropriate value for determining the amount of DNA to be amplified using the MiniFiler kit.*

   Note*: If a sample volume exceeds 10 µL the sample may be concentrated to approximately 10 µL, e.g., by using a technique for concentrating the extract. For example, a Speed-Vac System, simple evaporation, or another method for concentrating DNA extracts such as an approved ultrafiltration device may be used. Appropriate reagent controls should be treated in the same manner as the sample extracts being concentrated. Techniques to avoid contamination shall be employed, such as covering the sample during evaporation and/or using a bio hood.*

   Note: *Sterile deionized water may be used to reconstitute evaporated samples.*

   Note: *Positive and negative amplification controls associated with samples being typed shall be amplified concurrently in the same instrument with the samples at all loci and with the same primers as the forensic samples. All samples typed shall also have the corresponding amplification controls typed.*

4. Prepare a "master mix" by adding the following volumes of reagents to a 1.5-mL microfuge tube:

   # samples x 11 µL AmpFℓSTR MiniFiler Master Mix
   # samples x 5.5 µL AmpFℓSTR MiniFiler Primer Set

   Note*: The formulation above provides slight excess. A 1.5-mL microfuge tube provides enough volume for a maximum of 88 samples.*

5. Mix thoroughly and spin the tube briefly in a mini centrifuge to remove any liquid from the cap.

6. Dispense 15 µL of the *prepared* master mix (MiniFiler Master Mix plus Primer Set) into each GeneAmp® Thin-Walled reaction tube, or a 96-well amplification plate.

7. Add the prepared sample (10 µL) to each reaction tube. After the addition of sample, cap each tube before proceeding to the next sample. Mixing and vortexing are unnecessary. The final reaction volume in each tube is 25 µL.

   Note: *Alternatively, the 10 µL sample may be prepared directly in the thin-wall amplification tubes. Add the 15 µL of the prepared master mix (MiniFiler Master Mix plus Primer Set) into each GeneAmp Thin-Walled reaction tube, closing each tube before proceeding to the next sample. The final reaction volume in each tube is 25 µL.*

   Note*: If using a 96-well amplification plate, place a MicroAmp 96-well full plate cover (or another appropriate seal) over the plate once the plate is ready for amplification. If needed, briefly spin the covered plate in a centrifuge.*

8. Place the tubes or plate into the TC 9700 and start the thermal cycler program. Complete the AmpFℓSTR MiniFiler Amplification using TC9700 checksheet.

9. After amplification is complete and the thermal cycler has reached 4°C, remove the tubes from the instrument block and proceed to capillary electrophoresis. Alternatively, place the tubes in storage. The amplified products should be protected from light and can be stored in a refrigerator for short periods of time. For longer periods, the products should be stored in a freezer.

   Note: *If using a 96-well amplification plate, remove the plate cover and seal the wells with strip caps (or another appropriate seal) prior to storage.*

Section 11.1.4 References

See Section 11.4.

Section 11.3 MiniFiler Genotyping Using GeneMapper® ID-X, v.1.4

Section 11.3.1 Overview

Purpose of the software

GeneMapper® ID-X software (ID-X) is used to:

- Determine base pair sizes for alleles,
- Assign labels to alleles,
- Assign quality values to genotypes, and
- Build tables containing allele information.

About ID-X

There are two forms of GeneMapper ID-X. The basic form is a full copy, which is a fully functioning version. A full copy of the software can have different user accounts, all on the same workstation computer. Alternatively, the user accounts can be set up to access the full copy database over a server. This form of GeneMapper ID-X is a client copy.

One (or more) full copy resides permanently in each BFS laboratory with client workstation copies accessing the full copy for routine analysis. An additional (or more) full copy is available for use that does not interact with a server; this standalone workstation may be used remotely if it has been installed on a laptop computer.

The setup of GeneMapper ID-X described in Appendix I must occur for each full copy. (Note that the setup can be simplified by importing exports of the relevant files.) As needed, user accounts must be setup on each full copy as well, either directly on the same workstation (as described in Appendix II) or through a client copy.

How ID-X analysis works

ID-X determines base pair sizes by comparing alleles to an internal size standard. Allele designations are assigned by comparing the averaged sizes obtained for the alleles in the allelic ladder with the sizes obtained for the unknown sample alleles. Process Quality Value (PQV) flags may be used to evaluate the data.

Overview of procedure

The ID-X procedure involves:

- Creating a New Project with Analyzed Data
- Evaluating and Editing Data
- Printing and Archiving Data

Technical review

In performing a technical review, the reviewer assesses the criteria outlined on technical review forms PF-0022 and PF-0017. As part of the review, the technical reviewer performs a second reading of the STR typing data by reviewing the printed ID-X electropherograms and tabular data. The reviewer also checks the electronic or printed size standard and allelic ladder data. The reviewer may review and/or reanalyze additional electronic data if he/she feels it is advantageous in a particular case.

Contents

This section contains the following topics:

Section 11.3.2 Analytical Controls and Standards for Capillary Electrophoresis and Genotyping

Purpose/use

The allelic ladder provided in the AmpFℓSTR MiniFiler Amplification Kit is used to determine the allele designations of samples. While the ladder primarily includes the common alleles, additional alleles exist and may be detected in samples.

GeneMapper ID-X software assigns allele designations to peaks by comparison to allelic ladder(s) injected in the same run. The specific size assigned to each ladder allele may vary between instruments *and* between injections on one instrument.

Allelic Ladder

The STR alleles included in the ladder, and the size ranges they span, are listed in Table 1.

Table 1

Locus	Dye Color	Ladder Alleles	Approximate Size Range (bp)	Dye Label
D13S317	Blue	8, 9, 10, 11, 12, 13, 14, 15	90-139	6-FAM
D7S820	Blue	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	141.5-193.5	6-FAM
Amelogenin	Green	X, Y	99.3 (X), 109.3 (Y)	VIC
D2S1338	Green	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28	110.9-179.9	VIC
D21S11	Green	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	180.6-250.6	VIC
D16S539	Yellow	5, 8, 9, 10, 11, 12, 13, 14, 15	70-122	NED
D18S51	Yellow	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27	122.4-210.4	NED
CSF1PO	Red	6, 7, 8, 9, 10, 11, 12, 13, 14, 15	84.6-132.6	PET
FGA	Red	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	136.4-296.4	PET

Note about sizing information

- Sizes listed for each approximate range are those listed in the GeneMapper MiniFiler bins.
- Except for D13S317, the loci contain non-nucleotide linkers to modify inter-locus spacing.

Internal Size Standard

The internal size standard GeneScan®-600 [LIZ] version 2 (GS600v2 [LIZ]) contains dye-labeled DNA fragments of known size that are co-injected with the sample to allow estimation of STR allele sizes.

Section 11.3.3 Creating a New Project with Analyzed Data

Before you begin

If ID-X has not been previously set up, proceed to Appendix I *Setting Up GeneMapper ID-X v. 1.4 for MiniFiler*. Once ID-X set-up is complete, return to this section to analyze data.

Case versus batch projects

Below are approaches that may be taken in creating and saving a project:

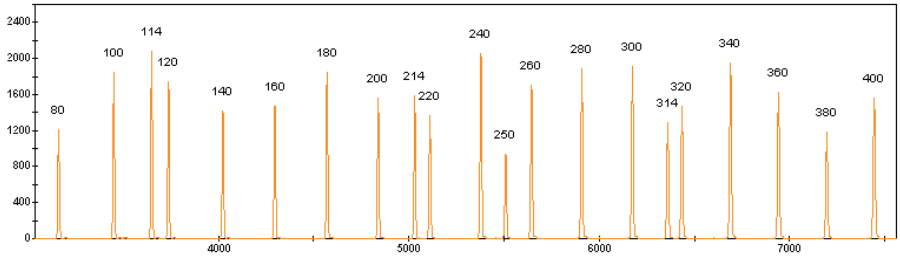
- Import only the sample files relevant to a particular case from a Run Folder, perform data analysis, and save the case-specific project to a Case Folder for archiving. All data is printed from this case-specific project.
- Import all (or relevant portion) sample files from a Run Folder and perform data analysis. Data may be printed directly from the batch project and/or from an edited and renamed project (*i.e.,* a specific case number). Projects from which data are printed for case files should be electronically archived (*e.g.,* to the Case Folder).

***Note*:** If the Sample Origin Path is not preserved, it may be re-established in ID-X by selecting *Define new Sample File path…* under *Edit*.

Create a new project

Follow these steps to create a new project.

Step	Action
1	• Create a Case Folder or Batch Folder in ID-X Case Data on the desired workstation computer. • Copy the run folder into the Case Folder or Batch Folder.
2	Open ID-X version 1.4 (or higher, if performance evaluated) and log into the software.
3	Create a new project by: – Selecting *Add Samples to Project* under the *Edit* menu. – Locate the correct run folder(s), – Click *Add to List*, – And then click the *Add* button. ***Note:*** *If sample files from more than one run folder are to be analyzed, all run folders may be imported into one project because ladder injections from each run folder are averaged separately.*
4	To examine the raw data of each sample: – Click the sample folder in the navigation pane once to view the sample files in the folder. – Click on a sample file and then choose the *Raw Data* tab. – Scroll through the list of sample files to examine each injection. – When finished, click the run folder or project node to return to the main window.
5	To remove a sample from the project (*e.g.,* GS600v2 only, failed, or extraneous injections), highlight the sample and select *Delete from project* under the *Edit* menu. Alternatively, samples may be deleted while viewing electropherograms by selecting *Mark Sample for Deletion*. When the

	plot window is closed, the sample will be deleted from the project. ***Note**: All injections from a run must be maintained in the run folder.*
6	Choose *Casework Table Settings* from the *Table Setting* pull down menu.
7	Adjust "Sample Types" to the correct settings. • Allelic ladder injections used for genotyping need to be set to *Allelic Ladder*. • 9947A may be set to *Positive Control.* ID-X will check 9947A genotypes. • Negative controls (*e.g.,* PCR and reagent blanks) may be set to *Negative Control.* ID-X will also check the negative controls for labeled peaks. • All others should be set to *Sample.*
8	Under Panel, *MiniFiler_v1.2X* of *AmpFlSTR_Panels_v3X* should be selected.
9	Under Analysis Method, *Casework_ MF_3500* should be selected. – Open the analysis method and check to be sure that the default settings match those shown in Figure 1 of Appendix I. ***Notes**:* • The analysis range may need to be modified to include the GS600v2 80-bp and 400-bp peaks and all sample peaks. • The size calling method is 3rd Order Least Squares.
10	Under Size Standard, *Casework_GS600_LIZ(80-400)* should be selected. – The default settings should match those shown in Figure 1.  **Figure 1**
11	Analyze sample files by clicking the menu bar *Green Arrow*. When prompted, name and save the project, either as: • <Case Number>MF IDX <date of ID-X analysis> <analyst's initials> for a case-specific project or, • <Run Folder name>MF IDX <date of ID-X analysis> <analyst's initials> for a batch project.

Next steps Continue with the steps for Evaluating and Editing Data.

Section 11.3.4 Evaluating and Editing Data

Note Customized table and plot setting profiles may be generated to accommodate analyst-specific data viewing preferences.

Evaluate and edit data Follow the steps below to evaluate and edit the data.

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Confirm that the size standard peaks have been correctly assigned.
• This can be aided by use of the Sizing Quality (SQ) PQV.
• It may also be helpful to view the raw data.
• See Figure 1 for the correct GS600v2 peak sizes.

Were the peaks labeled correctly?
• If yes, continue to Step 2.
• If no, complete the following actions.

To edit a size standard peak assignment (i.e., when a sample has a red flag for SQ),
– click once on the sample in the Samples view,
– then on the Size Match Editor toolbar icon.
– Edit the size standard peak accordingly.

<table>
<tr><th>If GS600v2 peaks were labeled incorrectly due to ...</th><th>Then ...</th></tr>
<tr><td>An artifactual peak(s)</td><td>Left-click once on the peak and right-click to make the necessary edit.</td></tr>
<tr><td>The 400bp peak not collected during the run</td><td>The sample will need to be re-run. It may be beneficial to increase the run time.</td></tr>
</table></td></tr>
<tr><td>2</td><td>Re-analyze any edited sample files.

If an allelic ladder injection was edited:
– Re-analyze all injections in the project. This is necessary to create new average values (offsets) for allele assignments.
– To re-analyze all injections, change an analysis criterion to “activate” the unedited injections for re- analysis (e.g., change the Analysis Method from Casework_MF_3500 to Default and then back to Casework_MF_3500).</td></tr>
<tr><td>3</td><td>Review sample and allelic ladder results. Evaluate the following</td></tr>
</table>

	parameters: – Peak shape and height. – Quality of spectral separation (baselines should be relatively flat, and any pattern of pronounced peaks or dips below true DNA peaks should not be present). – Peak profile (examine for artifactual peaks). If there are extraneous peaks in an electropherogram, it may be useful to examine the raw data. – Identification of off-scale peaks may be aided by the OS Process Component-based Quality Value (PQV) in the *Samples* view. – Ensure that the alleles in the allelic ladder results have been correctly assigned by ID-X. – Allelic ladder peaks should be labeled as shown in Figure 2 (at the end of this section). ***Additional help in evaluating data*** Data evaluation may be assisted by use of additional PQV values. These are viewed in the table produced under the *Genotypes* tab. The *Casework Table Settings* profile will display the following PQVs: BIN, PHR, LPH, AN, CC. The *Analysis Summary* tab has further sample evaluation information based on other PQVs. See Appendix I of Section 3.9 for guidance on creating custom Table Settings profiles.
4	Electronically edit extraneous peaks between ~65 - ~350bp, including extraneous peaks between locus ranges (unlabeled peaks). ***Extraneous peaks include:*** – Pull-up, – Pull-down, – Spikes, – Dye-labeled kit artifacts (“dye blobs”), – Incomplete non-template nucleotide addition peaks, and – Non-specific amplification artifacts. ***To make electronic edits:***

Step	Action
1	Left-click once on the peak label, right-click once on the same label, and choose *Delete Label*.
2	Enter the edit in the *Reason for Change* textbox. The abbreviations below may be used for edits.

Edit	Abbreviation
Primer Tail Outside Allelic Region	PT
Pull-up	PU
Pull-down	PD
Incomplete Nucleotide Addition	INA

	Non-reproducible artifact: NRA Reproducible artifact: RA Low-level inconclusive: LLI, * N-4 Stutter: N-4 N+4 Stutter: N+4
3	***Result:*** The label box will be modified to display the comment and a diagonal line ("slash") will be drawn through the label box.

***Note**:* Alternatively, edits may be made manually on print-outs.

5 Is there a microvariant allele or other callable allele requiring an edit?

- ***If no,*** continue to Section 11.3.5 Printing and Archiving Data.
- ***If yes,*** determine the allele call and complete the following actions.
- The allele call determination may be performed manually or as described in Section 3.10.10, Appendix I. See also Section 11.4.4.2 for more information.

To edit a microvariant allele or other callable allele:

Step	Action
1	Left-click once on the peak label and right-click once on the same label.
2	Choose *Rename Allele* and *Custom* from the pop-up menus instead of Delete Allele.
3	Enter the allele designation in the *Allele Name* textbox and click *OK*.
4	In the *Edit Allele Comment* textbox, enter the original ID-X designation in parentheses (*e.g.,* "(OL Allele)") and click *OK*.
5	***Result:*** The label box will be modified to display the new information.

***Note**:* Alternatively, edits may be made manually on print-outs.

AmpFlSTR MiniFiler Allelic Ladder peaks are shown here in Figure 2.

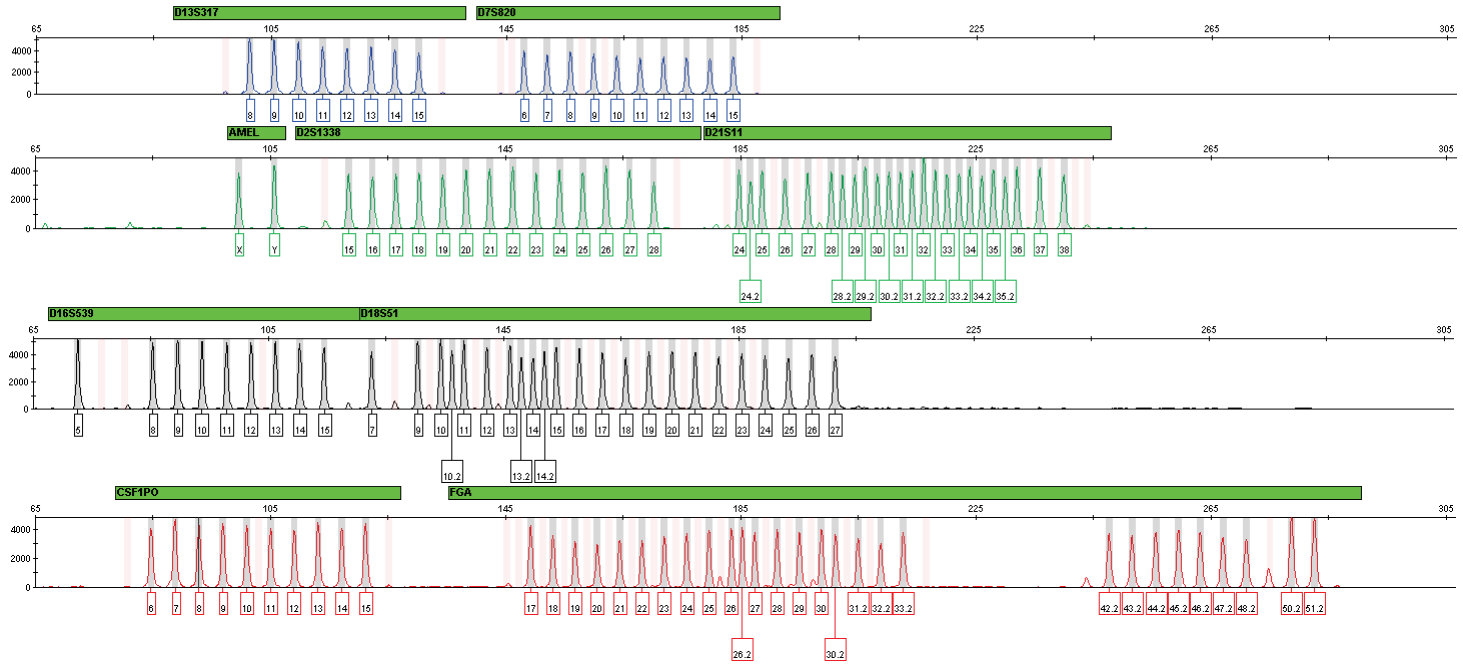


Figure 2

Next steps Continue with the steps for Printing and Archiving Data.

Section 11.3.5 Printing and Archiving Data

Print and archive data Follow the steps below to print and archive the data.

Step	Action
1	Before printing: • Make sure that the table settings are showing the sizing table with the electropherograms. • Verify *Page Set-up* <u>from the plots view</u> appears as shown in Figures 3, 4, and 5 (parameters may be modified with different printing conditions). • Adjust margins as needed from *Print*, also <u>from the plots view</u>.

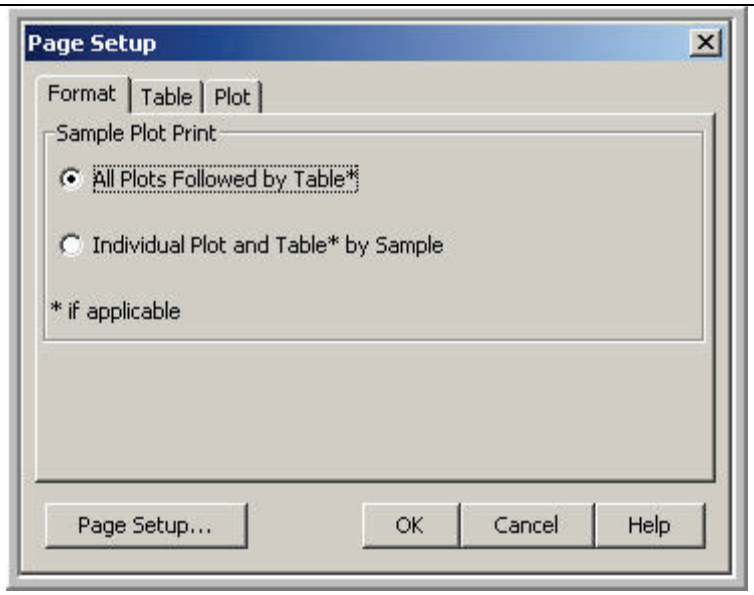


Figure 3

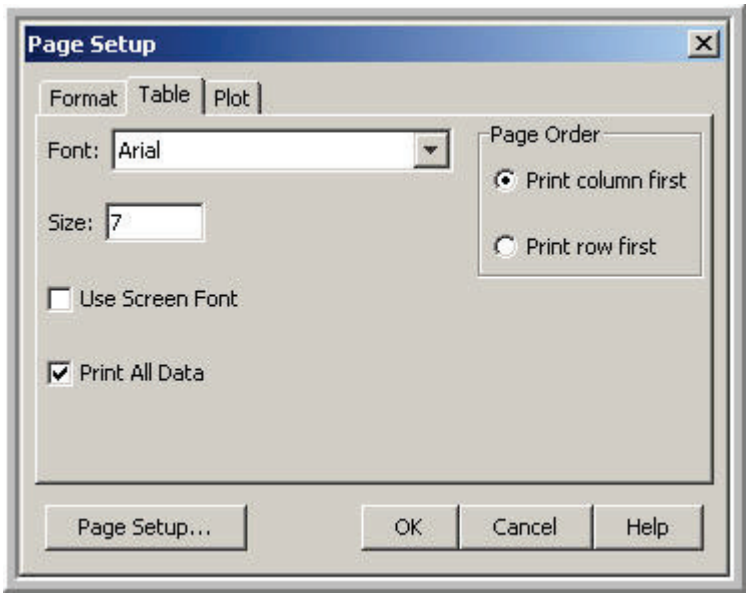
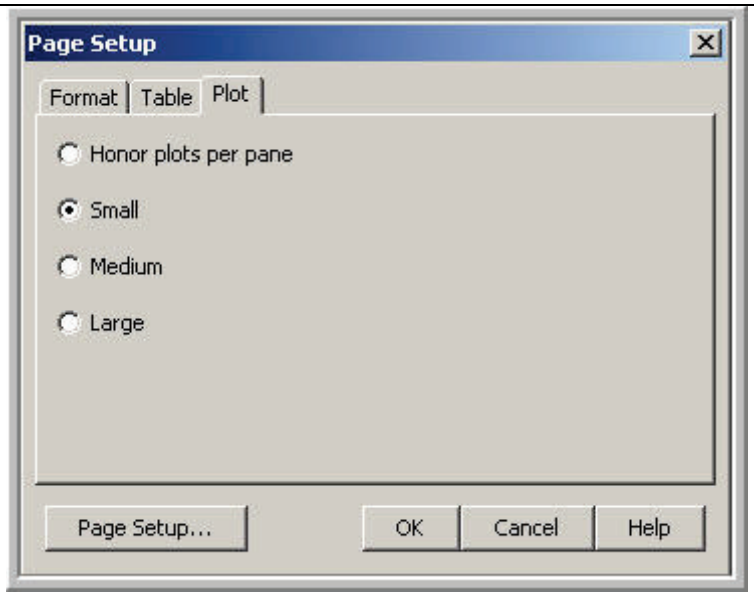


Figure 4

	 **Figure 5**
2	After reviewing data and completing electronic edits, select samples to print from the *Samples* tab. **See sections below**: *Printing specifications* and *Notes about printing samples.* ***Note:*** If creating case-specific projects from a temporary batch project, this is generally completed prior to printing as the appropriate case number will be on each printout (*i.e.,* project name).
3	Make any other edits manually that were not addressed electronically (*e.g.,* spikes in orange, artifacts between the locus ranges).
4	*Save* and *export* the project. • Under *Tools*, open *GeneMapper ID-X Manager.* • Highlight the desired project under the *Projects* tab and click *Export*. • At the prompt, enter the project name and navigate to the desired location. • Click *OK*.
5	• Copy the electronic Case Folder or Batch Folder onto one CD for the case file and verify that the folder has been recorded. • Move the folder to a secure network.

Printing specifications

Full view is defined as visualization of the highest peak apexes in the upper half of the printed electropherogram.

Casework Evidence/ Reference Samples (if single-source): Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection. At the analyst's discretion, a close-up view (*e.g.*, 300 RFU) may also be printed.

Casework Evidence Samples (if a mixture): For one injection, print the blue, green, yellow, and red tabular data and electropherograms at full view and in close-up view (*e.g.,* 300 RFU). For at least one other injection, print the blue, green, yellow and red tabular data and electropherograms in close-up view.

Casework Evidence Samples (if a partial profile): For one injection, print the blue, green, yellow, and red tabular data and electropherograms at full view and close-up view (*e.g.,* 300 RFU). For at least one other injection, print the blue, green, yellow and red tabular data and electropherograms in close-up view.

Negative Amplification Controls, Reagent Blanks, and Other Blanks: Print blue, green, yellow, and red tabular data and electropherograms at 300 RFU from one injection.

Positive Amplification Control and Quality Control Sample: Print blue, green, yellow, and red tabular data and electropherograms in full view from one injection.

Notes about printing samples

When to print in close-up view
It may be necessary to print duplicate sample injections in a close-up view (for example, if an apparent single-source sample has a low-level, third possible allele present due to mutation).

Allelic ladders
Because the genotyping bins are based on an average of all the allelic ladders, it is not necessary to print the allelic ladders nor the GS600v2 (orange) electropherograms and tabular data for the allelic ladders.

Batch projects
A unique laboratory identifier must be included on each printed ID-X page. For example, the batch name as the project name or the case number in the sample name field.

Section 11.3.6 References

Applied Biosystems (2012), "GeneMapper® ID-X Software Version 1.4," Applied Biosystems User Bulletin P/N 4477684, Rev.A.

Applied Biosystems (2009), "GeneMapper® ID-X Software Version 1.2," Applied Biosystems Reference Guide P/N 4426481, Rev.A.

Applied Biosystems (2007), "GeneMapper® ID-X Software Version 1.0," Applied Biosystems Administrator's Guide P/N4376327, Rev.A.

Section 11.3.7 Appendix I – Setting Up GeneMapper ID-X v. 1.4 for MiniFiler

Contents

This appendix describes the steps used to set-up GeneMapper ID-X v. 1.4 (or higher if performance checked) for analysis of MiniFiler data. Some of these steps must be completed while logged into the administrator/gmidx account, thus it is recommended to complete the set up using this login. The table settings file, plot settings files, and size standard table are the same as those used for Identifiler Plus; refer to Appendix I in Section 3.9.

Setting up GeneMapper ID-X includes the topics listed below.

Topic
Panels and bins
Analysis method parameter file

Important
The files created and used in this appendix are accessible to all ID-X user accounts.

About Administrator/ gmidx

The Administrator/gmidx user account allows the user full access to all functions of the GeneMapper ID-X software. This includes access to analyze, view, edit, and print data (as with the Casework Analyst – see Section 3.9 Appendix II Creating User Accounts in GeneMapper ID-X) as well as all administrative function access (such as Security Manager) and creating analysis method parameters.

This account has access to all projects, including those of the Casework, GeneMapper ID-X, and Admin Security Groups.

This account can reset Casework Analyst passwords. However, if the password of this Administrator account is lost, it cannot be retrieved by another user nor Life

Technologies/Applied Biosystems.

Panels & bins Perform the following to install and verify the MiniFiler panels and bins.

Step	Action
1	Open GeneMapper ID-X v. 1.4 (ID-X) and log into the software as the Administrator/gmidx user account.
2	Under the *Tools* tab, select *Panel Manager*. Click once in the left pane on the *Panel Manager* node and once on the *AmpFlSTR_Panels_v3X* node to open the folder. Continue to Step 4. If *AmpFlSTR_Panels_v3X* is *not* present, go to Step 3.
3	If *AmpFlSTR_Panels_v3X* is *not* present, perform the following steps. **Step** / **Action** 1 — Obtain a copy of *AmpFLSTR_Panels_v3X_Panels.txt* and *AmpFLSTR_Panels_v3X_AmpFlSTR_Bins_v3X_bins.txt.* 2 — Copy each to *C:/AppliedBiosystems/GeneMapperID-X/Panels* on the workstation computer. 3 — Open ID-X and, under the *Tools* tab, *Panel Manager*. 4 — Click once on *Panel Manager* in the left navigation pane. 5 — Under the *File* menu, choose *Import Panels*. 6 — Select the *AmpFLSTR_Panels_v3X_Panels.txt* panel and choose "Casework Security Group". Then, click *OK*. ***Note:*** *The stutter ratio data will not be imported.* 7 — Click once on the *AmpFlSTR_Panels_v3X* folder and, under the *File* menu, choose *Import Bin Set.* 8 — Select the *AmpFlSTR_Bins_v3X.txt* bin set. 9 — Enter the marker specific stutter information: • Click once on the *AmpFlSTR_Panels_v3X* node to open the folder and see each kit in the panel. • Click once on the *MiniFiler_v1.2X* to open the folder and see each locus in the panel. • Repeat for each locus: – Click once on the *D13S317* node to "open" that locus. – Click once on *Stutter Ratio & Distance* – Choose *New* to enter the *Minus Stutter* information: ▪ Type of Stutter: *Minus stutter* ▪ Ratio (between 0-1): *0.14* (for D13S317; see Table 1 in this section below for the other STR loci; enter 0 for Amelogenin)

	▪ From Distance (bp): 3.25 ▪ To Distance (bp): 4.75 ▪ Click *Apply,* then *OK*.
10	Return to Step 2 of **Panels & bins**.
4	Verify *AmpFlSTR_Bins_v3X* is the selected bin set from the drop-down menu in the *Panel Manager* tool bar.
5	Verify the Marker Specific Stutter Ratio is set correctly for each locus. • Click once on the *AmpFlSTR_Panels_v3X* node to open the folder and see each kit in the panel. • Click once on the *MiniFiler_v1.2X* to open the folder and see each locus in the panel. • Repeat for each locus: – Click once on the *D13S317* node to "open" that locus. – Click once on *Stutter Ratio & Distance* node to view the marker specific stutter ratio and verify the distance settings (3.25 to 4.75 bp).
6	Click *Apply* if changes were made. Click *OK*.

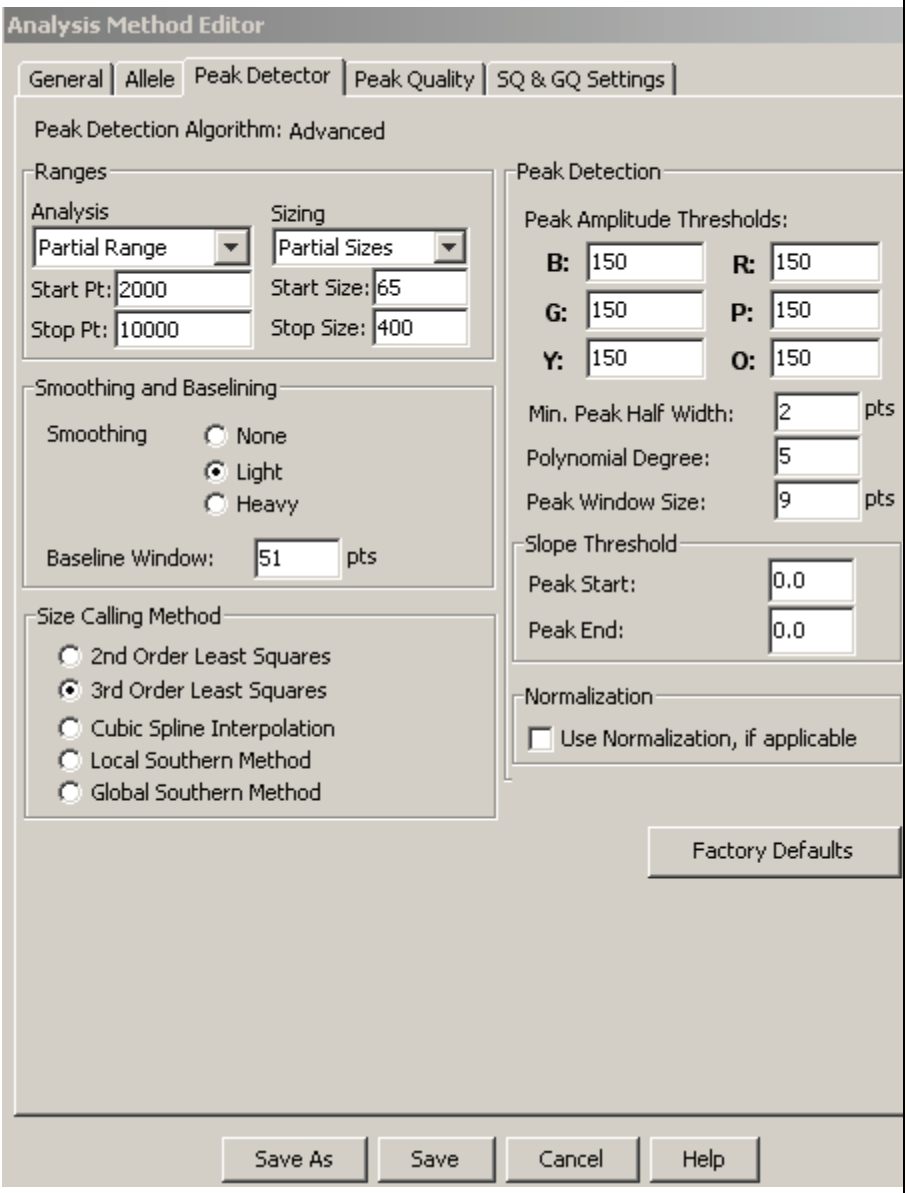
D13S317	14%
D7S820	11%
D2S1338	18%
D21S11	16%
D16S539	15%
D18S51	18%
CSF1PO	14%
FGA	15%

Table 1

Analysis Method

To create the *Casework_MF_3500* Analysis Method, perform the following:

Step	Action
1	Under the *Tools* tab: • Select *GeneMapper ID-X Manager* • Click on the *Analysis Methods* tab and on *New*
2	Under the *General* tab: • Analysis type should indicate *HID* • Enter "Casework_MF_3500" for Name • Choose "Casework Security Group" for Security Group • Enter "MiniFiler" for Description

3	Under *Allele*: • Choose *AmpFLSTR_ Bins_v3X* for Bin Set • The remaining values are used at the factory default.
4	Under *Peak Detector*: • Peak Detection Algorithm should indicate *Advanced*. • Carefully fill in the values and match the selections shown in Figure 1.  **Figure 1** **Important** Be careful to NOT select normalization. ***Note:*** The analysis range may be modified as needed.

5	Under *Peak Quality,* the values should be set as follows: • Signal Level, Homozygous min peak height: *2400* • Signal Level, Heterozygous min peak height: *150* • Max Peak Height: *32,000* • Heterozygote balance, Min peak height ratio: *0.60* • Peak morphology, Max peak width (basepairs): 1.5 (default) • Allele number, Max expected alleles: – For autosomal markers and Amel 2 (default) – For Y markers 1 (default) • Allelic Ladder Spike: Spike Detection: *Enable*; Cut-off value: *0.2* • Sample Spike Detection: Spike Detection: *Disable*
6	Under *SQ & GQ Settings,* the values should remain at the factory defaults.
7	Save As *Casework_MF_3500* with "Casework Security Group."

Section 11.4 Minifiler Interpretation Guidelines

Section 11.4.1 Introduction

The interpretation of results in casework is necessarily a matter of professional judgment and expertise. Not every situation can or should be covered by a pre-set rule; nor is it expected that competent analysts will always be in full agreement in a particular case. However, it is important that the laboratory develops and adheres to minimum criteria for interpretation of analytical results. These criteria are based on validation studies, literature references, and casework experience, and were developed with maximum input from analysts. It is expected that these interpretation guidelines will continue to evolve as the collective experience of the laboratory grows.

The purpose of these guidelines is to establish a general framework and to outline minimum standards to ensure that:

- Conclusions in casework reports are scientifically supported by the analytical data, including those obtained from appropriate standards and controls.
- Interpretations are made as objectively as possible, and consistently from analyst to analyst.

Section 11.4.2 Preliminary Evaluation of Data

A peak is defined as a distinct, triangular section of an electropherogram that projects above the baseline. The peak height, or peak amplitude, is the point at which the signal intensity of the peak is highest.

Heterozygous alleles appear as a two-peak pattern and, on average, have an allelic peak height ratio of greater than 65%. Peak height ratios of heterozygous alleles are defined as the ratio of the lower peak's height to the higher peak's height, expressed as a percentage. Peak height ratios less than 65% may be observed in single source samples, especially when the overall peak heights are low.

Homozygous alleles appear as single peaks. Results indicating a homozygous type with low peak heights should be interpreted with greater caution, especially when obtained from samples suspected to contain degraded DNA or PCR inhibition.

11.4.2.1 Analytical and Stochastic Thresholds

The analytical thresholds are defined as the minimum and maximum peak amplitudes that are acceptable for peaks that will be assigned allele designations. Analysis of sample files is routinely performed at a peak detection (analytical) threshold of:

- 50 relative fluorescence units (RFU) for data from the 3130/3130*xl*
- 150 RFU for data from the 3500/3500xL

By default, the maximum peak amplitude that can be obtained for raw data is approximately:

- 8000 RFU on the 3130/3130*xl* Genetic Analyzers
- 32,000 RFU on the 3500/3500xL Genetic Analyzers

Peaks exceeding this limit are determined to be "off-scale" by the software. Specifically, Data Collection assigns a tag to off-scale peaks for recognition by GeneMapper ID/ID-X. Peaks derived from off-scale data may be assigned allele designations if pull-up, pull-down/raised baselines, artificially elevated stutter peaks, or artifacts caused by an excess of input DNA (*e.g.*, peaks caused by incomplete 3´ terminal nucleotide addition) have been ruled out. These artifacts should be noted on the GeneMapper ID/ID-X printouts. Peak height values for off-scale peaks should not be used in quantitative aspects of interpretation. A sample that exhibits one or more unacceptable off-scale peaks may be either re-injected using a shorter injection time and/or lower injection voltage, re-run using less PCR product, or re-amplified using less input DNA.

The stochastic threshold is the peak height value at and above which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele has not occurred. The stochastic threshold may also be referred to as the homozygote threshold as it is the threshold above which a single peak may be genotyped as a homozygote. This threshold has been determined through validation studies to be as follows when using the MiniFiler kit:

- 600 RFU on the 3130/3130*xl* Genetic Analyzers
- 2400 RFU on the 3500/3500xLGenetic Analyzers

A single peak must be at least the height of the stochastic threshold in at least one injection to be genotyped as a homozygote.

The minimum signal intensities used to assign alleles or genotypes were determined empirically based on data generated by the laboratory. Peaks greater than or equal to the analytical threshold in at least two injections may be assigned allele designations and reported. Single peaks between the analytical threshold and the stochastic threshold should be reported as alleles rather than genotypes, since one allele of a heterozygote may not be detected at this level for stochastic reasons. In instances where a peak is above the analytical threshold in one injection and below in the duplicate injection, the peak will be considered inconclusive and not reported as an allele.

Similarly, in instances where a possible minor allele is co-migrating with a stutter peak, the peak must exceed the locus stutter threshold in at least two injections to be reported as a minor allele. Otherwise, the peak will be considered inconclusive and not reported as an allele. Note that, regardless of the stutter threshold, a peak in a stutter position may be reported as a *possible* minor allele when considering the possible minor component genotypes from a mixture.

In considering multiple injections or amplifications of a sample, a composite profile may be reported if an allele is detected at at least the analytical threshold in a minimum of two injections. However, in the case of allelic data from separate extractions of different locations

on a given evidentiary item, unless there is a reasonable expectation of the DNA to have come from the same individual, a composite profile may not be reported for this situation.

Fluorescent signal lower than the analytical threshold will not be interpreted for reporting purposes.

11.4.2.2 Size Standards and Allelic Ladders

On the 3130/3130*xl* Genetic Analyzers, GeneScan-500 [LIZ] is used as the Internal Size Standard. This size standard is run with every sample to normalize injection-to-injection peak migration differences. Using GeneMapper ID, sizes are assigned to the 75, 100, 139, 150, 160, 200, 300, 340, 350, 400, and 450 base-pair GS-500 [LIZ] peaks. No value is assigned to the 250-bp peak. In instances in which the 450-bp peak was not collected by Data Collection software, sample analysis may proceed as usual with an adjusted Size Standard Table.

On the 3500/3500xL Genetic Analyzers, GeneScan-600 [LIZ] version 2 is used as the Internal Size Standard. This size standard is run with every sample to normalize injection-to-injection peak migration differences. Using GeneMapper ID-X , sizes are assigned to the 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, and 400 base-pair GeneScan-600 [LIZ] peaks.

Size windows based on the allelic ladder are used to assign allele designations. It is therefore necessary to ensure that each allele in the allelic ladder has been sized for genotyping.

11.4.2.3 Sizing Precision

The method used for genotyping employs a ± 0.5-bp "window" around the size obtained for each allele in the AmpFℓSTR MiniFiler Allelic Ladder injection(s) from the given run. These sizes may be averages of each allele if more than one allelic ladder injection is used for allele assignments. A ± 0.5-bp window allows for the detection and correct assignment of potential off-ladder sample alleles whose true size is only one base different from an allelic ladder allele. A sample allele that sizes outside a window could be an off-ladder allele (an allele of a size that is not represented in the allelic ladder) or an allele that does correspond to an allelic ladder allele, but whose size is just outside a window because of measurement imprecision.

Fluctuation in ambient temperature during the course of a set of capillary runs may affect sizing precision. An indicator that room temperature fluctuation may have affected a run is the occurrence of shifts in the migration of peaks from one run to the next, which is most easily and more commonly observed in the GS500 peaks (especially the 250-bp peak). Variations in peak migration may become apparent while determining the optimal analysis range for a comparison set. Significant room temperature fluctuation may result in size variation between injections such that allelic ladder peaks differ by more than 0.5 bp from allelic peaks in other injections. This will cause GeneMapper ID/ID-X to assign these alleles as off-ladder alleles.

Section 11.4.3 Controls required to assess analytical procedures

The following controls and standards are used to assess the effectiveness, accuracy and precision of the analytical procedures:

- Positive amplification control 007
- Negative controls (including negative amplification controls and reagent blanks)
- Quality Control (QC) sample

Evaluation of the controls is essential to the proper interpretation of the test results. In order for the analytical results to be reported, the positive amplification control and QC sample should yield typing results that are consistent with the known profiles for these samples. However, if it can be demonstrated that low or no signal was seen in one of these controls as the result of insufficient template DNA or product, the results from the initial amplification may be reported as long as at least one of these controls (positive amplification control or QC sample) gives an accurate typing result. The presence of artifactual peaks does not invalidate a positive control or QC sample.

In the event that the positive amplification control or a QC sample fails to give the expected types, the analyst and technical reviewer will evaluate the results in an attempt to determine the cause and seriousness of the discrepancy. Consideration will be given to the extent to which the results from other samples in the chain of analysis may have been affected. The extent to which a failed QC sample affects the other samples in an analytical set will be evaluated on a case-by-case basis. For example, if a sample mix-up occurred for the QC sample for a particular case during the extraction process, that would not necessarily affect the typing results for a second case that was extracted separately but amplified and typed with the first case.

Negative control samples (negative amplification control, system water control, and reagent blank) should show no allelic peaks at or above the analytical/interpretation threshold. If any such peaks are detected in a negative control sample, that sample must be evaluated for potential contamination and how it may have occurred.

For the positive amplification control and the quality control sample, where the correct types are known, contamination is defined as the presence of foreign allelic peaks greater than or equal to the analytical threshold.

Although reference samples are typically single-source, transfusion should be considered as a possible source of additional alleles. When a reference sample contains a secondary source that is determined not to be the result of contamination by the laboratory, this will be documented.

Due to the potentially complex nature of some evidence samples, possible contamination will be assessed on a sample-by-sample basis. To assist in troubleshooting possible contamination, samples may be re-analyzed at less than the analytical threshold. However, peaks less than the analytical threshold may not be reported.

If contamination is determined to be present in an evidence or reference sample or any of the controls governing the analysis of that sample, the contamination may be classified as not serious if the level or nature of the contamination does not account for or interfere with the genotype calls of the sample(s) in question. In this situation, the results for the case sample(s) may be reported. The possible contamination will be evaluated and documented in the bench notes, and the analyst will prepare an Instance of Contamination Report for inclusion in the case file and the Contamination Log. If the contamination is determined to be serious, the procedures will have to be repeated to the appropriate extent for the affected samples. Evaluation and documentation in the case file and Contamination Log will also be required. The Technical Leader will review all contamination reports. The occurrence of contamination should be noted in the laboratory report.

Section 11.4.4 Designation

Genotypes are determined from the diagnostic peaks of the appropriate color (fluorescent dye label) and size range for a particular locus. Allele calls are assigned by comparing the sizes obtained for the unknown sample alleles with the sizes obtained for the alleles in the allelic ladder(s). Genotypes, not sizes, are used for comparison of data between runs, instruments, and laboratories.

11.4.4.1 Locus Designation

The following locus range approximations were determined by Life Technologies/Applied Biosystems for Human Identification analysis. The allelic bin definitions are stored within GeneMapper ID/ID-X using the corresponding bin set. Typically, multiple ladders are used in an analysis and allelic bins are determined by averaging the designated ladders in the project. Ladders within a single run folder are used for calculating allelic bin offsets and subsequent genotyping.

6-FAM-labeled loci:

- D13S317 90.0 to 139.0 bp
- D7S820 141.5 to 193.5 bp

VIC-labeled loci:

- Amelogenin 99.3 to 109.3 bp
- D2S1338 110.9 to 179.9 bp
- D21S11 180.6 to 250.6 bp

NED-labeled loci:

- D16S539 70.0 to 122.0 bp
- D18S51 122.4 to 210.4 bp

PET-labeled loci:

- CSF1PO 84.6 to 132.6 bp
- FGA 136.4 to 296.4 bp

11.4.4.2 Allele designation

The allelic ladder provided in the AmpFℓSTR MiniFiler PCR Amplification Kit contains the majority of alleles for each locus. The alleles contained in the allelic ladder were named by the kit manufacturer in accordance with the recommendations of the DNA Commission of the International Society for Forensic Haemogenetics.

The number of 4-bp repeat units observed in an allele that is present in the ladder is designated by an integer. Alleles that contain an incomplete repeat motif are designated by an integer representing the number of complete repeats, followed by a decimal point and the number of bases in the incomplete repeat (*e.g.*, the 26.2 allele in FGA).

The GeneMapper software is used to automatically convert allele sizes into allele designations. Alleles not aligning with those in the allelic ladder have been detected both within and outside the range of the allelic ladder for each locus. Alleles smaller than the lowest molecular weight allele (A) in the allelic ladder for that locus will be designated as "<A." Alleles larger than the largest molecular weight allele (B) in the allelic ladder for that locus will be designated as ">B." When an off-ladder allele occurs within the ladder region, the allele designation will be determined by interpolation. The off-ladder allele designation should be confirmed using at least two sample injections. Print both sample injection electropherograms with tabular data.

Note: *For locus FGA, alleles that fall between the 33.2 and 42.2 ladder alleles will be reported as >33.2.*

11.4.4.3 Artifacts

Artifacts can occur and should be noted where appropriate for editing purposes.

11.4.4.3.1 Pull-up/Pull-down

Smaller artifactual peaks can appear in other colors under allelic peaks. This phenomenon is termed "pull-up" and is the result of spectral overlap between the fluorescent dyes. If a pull-up peak is above the peak amplitude threshold, it will be sized at a similar size as the allelic peak. The shape of the peak may appear similar to a true DNA peak or sigmoidal, as a doublet, or otherwise irregularly-shaped.

Similarly, pull-down is also the result of spectral overlap between the fluorescent dyes. When an oversubtraction occurs, the result is a dip into the baseline. The baseline can only be positive values so this dip causes a raised baseline, or pull-down, in the other colors of the proximal size/data point range of the allelic peak(s).

Pull-up (or pull-down) can occur as a result of the following:

- Application of a less than optimal spectral calibration or matrix. The run may need to be repeated with a different spectral calibration.
- Amplification using excess input DNA can lead to off-scale peaks. If necessary, samples can be re-amplified using less input DNA, or re-run using shorter injection times or less product.

Even when the spectral calibration is satisfactory, pull-up and pull-down may still be seen.

11.4.4.3.2 Stutter

In addition to an allele's primary peak, artifactual stutter peaks can occur at four-base intervals. The stutter peak most commonly observed is four bases smaller than the primary peak ("n-4"). However, it is also possible to see a peak that is four bases larger ("n+4") than the primary peak.

Stutter peaks may be due to repeat slippage during amplification. Sequence variation can affect the amount of stutter; a lower amount of stutter is produced from alleles with increased sequence variation between repeats.

Stutter peaks are evaluated by examining the ratio of the stutter peak height to the primary peak height, expressed as a percentage. This percentage can vary by locus. Moreover, longer alleles within a locus generally have a higher stutter percentage than the shorter alleles.

The GeneMapper ID/ID-X thresholds for n-4 stutter are set at those values shown in Table 2.

Table 2

Locus	Marker Specific Stutter Ratio (filter threshold %)
D13S317	14
D7S820	11
D2S1338	18
D21S11	16
D16S539	15
D18S51	18
CSF1PO	14
FGA	15

These thresholds are the values determined by Life Technologies/Applied Biosystems and used in the kit panel. Typically, n-4 stutter is expected to be less than these percentages.

The presence of peaks in the stutter positions exhibiting percentages greater than these values may indicate a DNA mixture. However, stutter peak heights may also be elevated above the expected thresholds by the following:

- The measurement of percent stutter may be unnaturally high for main peaks that are off-scale, *i.e.*, have exceeded the linear dynamic range for detection (raw data greater than approximately 8,000 RFU on the 3130 and 32,000 RFU on the 3500). However, the "raw data" has already had the spectral calibration applied and therefore is not a means for confirming whether a peak was detected off-scale. The Genotypes Display in GeneMapper ID/ID-X has a PQV that can be set to flag off-scale peaks automatically, designated as "OS." If a stutter peak is greater than the maximum expected and the primary peak is off-scale, the analyst should interpret the results with caution. The sample may be subsequently re-injected with less product or for a shorter time, or the sample may need to be re-amplified using less input DNA.

- For alleles differing by two repeat units, the stutter peak from the larger allele may overlap the trailing shoulder of the smaller allele and therefore exhibit an increased stutter percentage. This will not occur if the smaller allele peak drops to baseline before reaching the stutter peak.

- Stutter may exceed a threshold simply as a result of normal statistical variation.

- Apparent elevated stutter may be the result of a somatic mutation (*i.e.,* Type I mutations; Clayton et al 2004 JFS, Rolf et al 2002 FSI, Gill, P 2002 BioTechniques). For example, such mutations may be seen in rapidly dividing buccal cells or spermatozoa. These situations are generally the result of a minor proportion of the otherwise wildtype cell population containing the mutated genotype (*i.e.,* mosaicism). "IPA," or indeterminate possible allele, may be used in the benchnotes to annotate such occurrences.

- In some situations, an allelic peak in a mixture may co-migrate with the stutter peak of another allelic peak, resulting in an apparent stutter peak height greater than that typically observed for stutter in a single-source sample.

- Although less common, n+4 stutter has been observed, typically no more than 4.5%. While the presence of n+4 stutter does not complicate the interpretation of single-source samples, the possible presence of this PCR artifact should be considered when interpreting mixture results.

11.4.4.3.3 Incomplete non-template nucleotide addition

Amplification conditions have been set to maximize the non-template addition of a 3´ terminal nucleotide by DNA polymerase. Failure to attain complete terminal nucleotide addition may result in "split peaks," visualized as two peaks that are one base apart. Except

for microvariant alleles, the presence of peaks differing by one base pair is diagnostic of this phenomenon.

11.4.4.3.4 Other artifacts

In addition to the artifacts described above, the following anomalies can arise:

- Artifacts in the non-calling region may appear in the 6-FAM (~70 bp) and VIC (~80 bp) dyes.
- Low level artifacts in the calling region may appear in the 6-FAM (~117 and ~127 bp), VIC (~118 bp), and NED (~166 bp) dyes.
- Amplification of quantities of DNA that exceed the optimal template level may lead to artifacts that have been characterized as secondary stutter products in the D13S317 and D21S11 loci. Their mobility varies with that of the main amplification products.
- Low level artifacts observed at ~7-9 bp less than the allelic peak for D13S317 and ~2.5 bp less than the allelic peak for D2S1338.
- Peaks of the same size, often present in all five colors, are not the result of dye-labeled DNA and do not indicate a spectral calibration problem. The shape of these peak-like artifacts ("spikes") often differs from the shape of dye-labeled DNA peaks and these artifacts are generally not reproducible.

Section 11.4.5 Interpretation of Results

The following situations describe conditions in which the data would lead to the conclusion that the source of the DNA is either from a single person or from more than one person. It is generally possible to estimate the number of contributors by considering the number of alleles present at a locus, the appearance of allelic patterns within and between loci, peak heights, and/or peak height ratios. Due to the increased sensitivity and stochastic effects observed with this kit, additional caution should be applied to the interpretation of MiniFiler STR typing results.

Peak height ratios lower than 65% may indicate a mixture, especially when seen at more than one locus. However, a single-source sample may also exhibit peak height ratios below 65%, especially when the overall peak heights are low. Analysts should consider results at all loci when interpreting samples that exhibit peak height ratios of less than 65%. Depending upon the sample source, the loci in question, the number of loci affected and the percent disparity between allele peak heights, the sample may need to be re-amplified and typed. This determination is made by the analyst at their discretion and should include an evaluation of all loci for that sample.

The following are also considerations in interpreting sample profiles:

- The presence of more than two alleles per locus, especially at more than one locus, may indicate a mixture. However, some individuals may exhibit more than two alleles at a locus due to genetic anomalies (*e.g.*, trisomy, mosaicism, and chimerism).
- The presence of a peak at a stutter position that is significantly greater in peak height than that typically observed for stutter in a single-source sample may indicate a mixture.
- The possible presence of a "null" or "partial null" allele – an allele that is not detected or has significantly reduced peak height due to a mutation in the primer-annealing region or a deletion.

11.4.5.1 Single Contributor

A sample may be considered to be from a single contributor when the observed number of alleles at each locus does not exceed two (assuming no genetic anomalies), and intra- and inter-locus peak heights observed are appropriately balanced. Single-source peak height ratios may be less balanced when low level results are observed.

11.4.5.2 Mixtures with major and minor contributors

A sample may be considered to be a mixture of major and minor contributors if there is a distinct contrast in peak heights among the alleles. In some situations, a major or minor contributor's profile may be determined at some, but not all, loci.

11.4.5.3 Mixtures with a known contributor(s)

In some situations, when the presence of one of the contributors (*e.g.*, the victim) can be assumed based on the nature of the sample, the genetic profile of the unknown contributor may be inferred. Depending on the profiles in the specific instance, this can be accomplished by subtracting the contribution of the known donor from the mixed profile. It may be possible to infer the foreign profile by examining peak height ratios.

11.4.5.4 Mixtures with indistinguishable contributors

When major or minor contributors cannot be distinguished because of similarity in peak heights or the presence of shared or masked alleles, individuals may still be included or excluded as possible contributors to the mixture.

When an assumption is made in the interpretation of the STR data of a complex DNA mixture, that assumption should be clearly stated in the laboratory report.

11.4.5.5 Partial profiles

Partial profiles may be obtained when the DNA template is degraded, in low quantity, or when PCR inhibitors are present. A negative or inconclusive result at some loci may not impact allele designations at the remaining loci.

At low levels of DNA template, stochastic effects (*i.e.*, unequal signal from alleles in a heterozygote due to chance sampling and/or unequal amplification) may cause loss of an allele or a substantial imbalance of alleles at a locus. Imbalanced amplification of heterozygous allele pairs at low template levels is not necessarily an indication of a mixture, and the absence of an allele may not be a valid basis to exclude a potential contributor.

Section 11.4.6 Conclusions

The following guidelines may be used in formulating conclusions resulting from comparisons of single-source samples and mixtures with known reference samples.

General categories of conclusions include, but are not limited to:

11.4.6.1 Inclusion

For single-source samples, if each homozygous allelic peak greater than or equal to the stochastic threshold and each heterozygous peak greater than or equal to the analytical threshold in the evidence sample is observed in the reference standard, then the STR profile from the evidence sample matches that of the reference. An individual may also be included when a partial profile is observed in an evidence sample (due to low template DNA, degradation and/or inhibition) if the evidence alleles detected are the same as those at the corresponding loci in the reference sample. Generally, if an individual's alleles are present or can be accounted for in a mixture, the individual may be included (or cannot be excluded) as a possible source of some of the biological material present in the sample.

11.4.6.2 Exclusion

If allelic peaks in the profile from a reference standard are not found in the profile of the evidence sample, and their absence cannot be attributed to insufficient template, degradation, inhibition, masking at a stutter position, or a genetic anomaly, then the individual is excluded as a possible source of the biological material present in the sample.

11.4.6.3 Inconclusive or uninterpretable

In situations where allelic peaks below the analytical threshold are observed in a sample, it may not be possible to determine a complete profile. Depending on the results of a particular sample, some alleles may not be detected at these low levels, and it may not be possible to determine conclusively whether or not a particular individual is included or excluded.

The interpretation of results for an evidentiary sample may be confounded by the presence of excessive background DNA, as indicated by the results for the corresponding substrate sample.

11.4.6.4 No results

A finding of "no results" is reported when no non-artifactual fluorescent signal greater than or equal to the analytical threshold is observed. A conclusion of "no results" may be obtained for some or all loci of a particular sample.

Section 11.4.7 Statistical Interpretation

See Section 3.10.8 of the Identifiler Plus STR Interpretation Guidelines.

Section 11.4.8 References

See also Identifiler Plus references.

Applied Biosystems (2007), "AmpFℓSTR® MiniFiler™ PCR Amplification Kit User Guide,", Rev. B.

Butler, J.M., Shen, Y., McCord, B.R. (2003) "The development of reduced size STR amplicons as tools for analysis of degraded DNA." Journal of Forensic Sciences 48(5):1054-1064.

Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) "A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets." Journal of Forensic Sciences 49(4):733-740

Drabek, J., Chung, D.T., Butler, J.M., McCord, B.R. (2004) "Concordance study between miniplex STR assays and a commercial STR typing kit." Journal of Forensic Sciences 49(4):859-860.

Hill, C.R., Kline, M.C., Mulero, J.J., Lagace, R.E, Chang, C-W., Hennessy, L.K., Butler, J.M. (2007) "Concordance study between the AmpFℓSTR MiniFiler PCR Amplification Kit and conventional STR Typing Kits." Journal of Forensic Sciences 52(4):870-873

Luce, C., Montpetit, S., Gangitano, D., O'Donnell, P. (2009) "Validation of the AmpFℓSTR MiniFiler PCR amplification kit for use in forensic casework." Journal of Forensic Sciences 54(5):1046-1054.

Mulero, J.J., Chang, C-W, Lagace, R.E., Wang, D.Y., Bas, J.L., McMahon, T.P., Hennessy, L.K. (2008) "Development and validation of the AmpFℓSTR MiniFiler PCR amplification kit: a miniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA." Journal of Forensic Sciences 53(4):838-852.

Section 13.1 Quadruplex qPCR Setup Using the Tecan Freedom HIDEVO150 Combo System

Section 13.1.1 Overview

Introduction

This section describes the use of the Tecan Freedom HIDEVO150 Combo System for Quadruplex qPCR setup using the *CA_Quad_tubesCombo_nomix* script and the *CA_Quad_plateCombo_nomix* script. The Quadruplex qPCR assay is used for the quantification of DNA samples. Typically, DNA normalization and STR amplification setup are subsequently performed on the HIDEVO150 with the AmpFℓSTR® Identifiler® Plus Amplification Kit, followed by capillary electrophoresis and detection of the STR loci using either a 3130, 3130*xl*, or 3500/3500xL Genetic Analyzer. The data from these instruments are then analyzed through GeneMapper ID/ID-X.

Contents

This procedure contains the following topics:

Hazard

To avoid potential crushing and piercing injuries from moving parts, do NOT reach into the Tecan work space when a script is running. If it is necessary to access the deck or work space during a script, either pause the script (wait for the green indicator light to begin flashing), stop the script (the indicator light turns off), or power down the Tecan.

Hazard

The barcode reader (*i.e.,* PosID) uses laser technology for scanning. Do not stare into the laser beam or its reflection.

Warning

Electromagnetic waves from a cellular phone may affect the function of liquid detection. Faulty detection of the liquid surface may cause the system to produce incorrect results. It is recommended to keep a distance of at least 6.5 feet from the instrument when using a cellular phone.

Section 13.1.2 Materials, Reagents, and Equipment

Materials & reagents

- Quadruplex qPCR assay reagents
- TE^{-4}
- Deionized water
- Weak detergent (*e.g.,* Liquinox or RoboScrub)

Equipment

- Tecan Freedom HIDEVO150 Combo System
- 1.5 mL Microfuge tubes – DNA/DNase/RNase free
- Mini centrifuge
- Vortex
- Centrifuge with plate rotor or alternative plate spinner
- Adhesive plate cover
- 96-well optical reaction plates – DNA/DNase/RNase free, PCR compatible
- VWR 5 mL screw-cap tubes
- Conductive, filtered 50 µL tips – DNA/DNase/RNase free
- Pipettors
- Pipet tips – DNA/DNase/RNase free
- Miscellaneous laboratory supplies

Section 13.1.3 Pre-run Processing

Section 13.1.3.1 Overview

Introduction

This section describes the procedures for setting up an HIDEVO150 Quadruplex qPCR Setup run. This is a procedure which specifically amplifies and detects primate DNA for the purpose of assessing the quantity of amplifiable DNA in a sample. For more information on the Quadruplex assay, refer to Section 2.1, procedure for nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay.

Contents

This part contains the following topics:

Section 13.1.3.2 About the Input Files

Required files

The HIDEVO150 Quadruplex qPCR setup procedure requires two input files:

1. a sample input file, which specifies the name and position of each sample tube in the 16-position tube racks or in the 96-well sample plate for the HID EVOware software.

2. a plate map file required by the AB7500 system software for the qPCR run.

Figure 1a

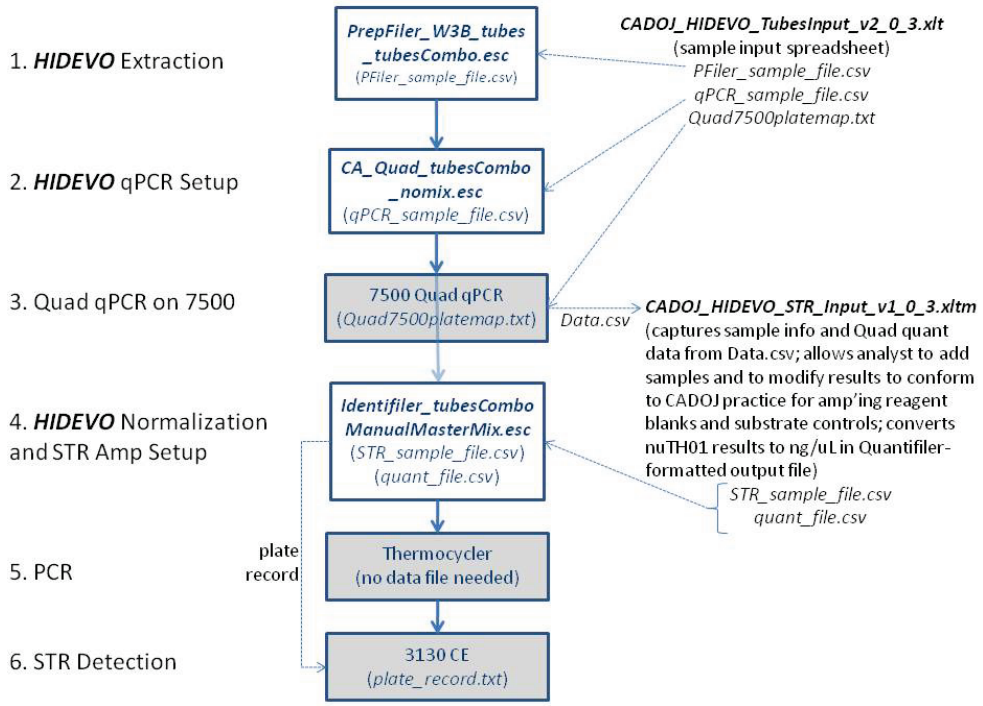


Figure 1a. An overview of HIDEVO150 sample tube processing at the CA DOJ.

- The bold arrows represent the rough flow of the actual samples through the processing.
- The thin, dotted arrows represent the flow of data files generated to connect the processes.
- Steps 1, 2, and 4 represent the three HIDEVO150 automation modules (extraction, quantification setup, normalization/amp setup), while steps 3, 5, and 6 represent operations performed on AB instrument platforms.

Figure 1b

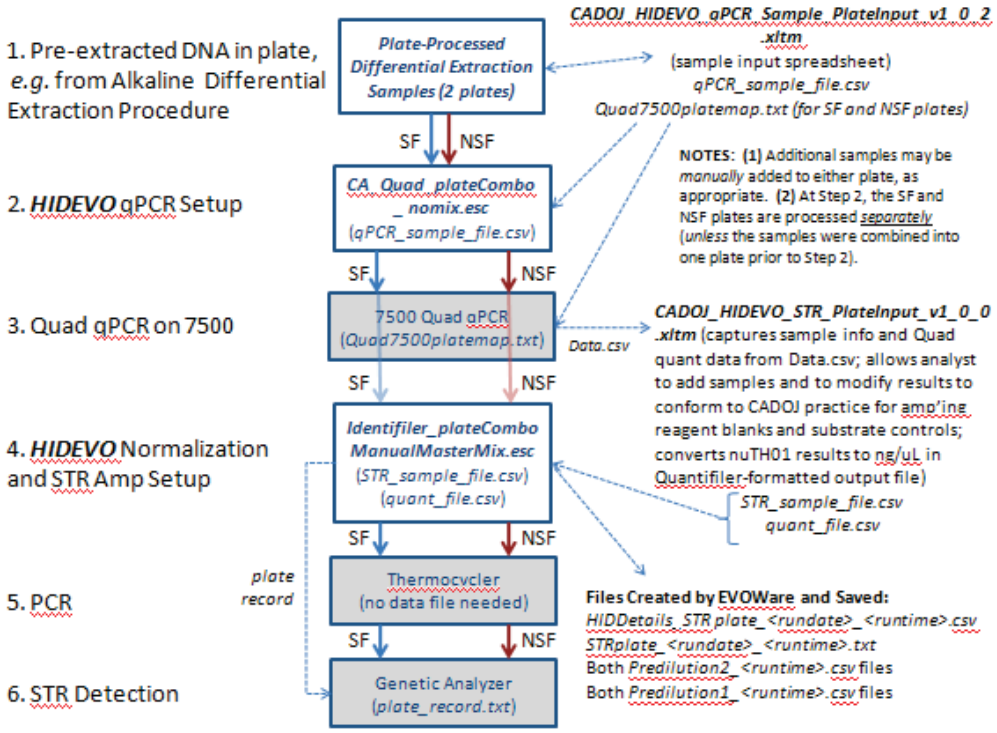


Figure 1b. An overview of HIDEVO150 sample plate processing at the CA DOJ. Differentially-extracted sample plates are illustrated as an example.

- The bold arrows represent the rough flow of the actual samples through the processing.
- The thin, dotted arrows represent the flow of data files generated to connect the processes.
- Steps 1, 2, and 4 represent the three HIDEVO150 automation modules (extraction, quantification setup, normalization/amp setup), while steps 3, 5, and 6 represent operations performed on AB instrument platforms.

Background

Each HIDEVO150 automation module is identified by a specific EVOware script file (.esc), which requires at least one input file (in parentheses in Figure 1) for processing.

Due to the custom nature of our quadruplex qPCR protocol, our data files are not created only by EVOware, but are also created or modified by locally-developed VBA-enabled Excel templates (right-hand side of Figures 1a and 1b).

Sample Volumes

The HIDEVO150 qPCR setup script will use 2 µL of extract for each full-volume 20 µL amplification reaction.

Section 13.1.3.3 Generate Input Files

Generating input files

Perform the following to generate the sample and 7500 input files.

***Note**: If proceeding from the Tecan automated PrepFiler procedure with no changes to the sample setup, continue to Section 13.1.3.4.*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Open the appropriate Excel template and enable macros.
<table>
<tr><th>If samples are in</th><th>Then use</th></tr>
<tr><td>1.5mL sample tubes</td><td>CADOJ_HIDEVO_TubesInput_v2_0_3.xlt</td></tr>
<tr><td>96-well PCR plate</td><td>CADOJ_HIDEVO_qPCR_Sample_PlateInput _v1_0_2.xltm</td></tr>
</table>
Note: Higher version Excel template(s) may be used if performanced-checked.</td></tr>
<tr><td>2</td><td>Enter sample names. See details below for format specifics.

General notes:
• Include the case number with each sample. The format LLYY####_sample name is suggested, where LLYY#### represents a BFS laboratory case number.
• No samples in the same run can have identical names.
• A sample name cannot include spaces or particular characters, such as colons, commas, asterisks, and slashes.
  o Permissible characters include -_(){}#.+
• A sample name cannot be a single, numeric 0 (zero).

Sample tube format:
• All samples must be loaded contiguously; sequential tube and rack positions cannot be skipped.
• Position 1 is Rack S1, P1 in the Excel template; see Build Sample Racks for the Run for tube placement in racks.

96-well format:
• All samples must be loaded contiguously; sequential wells and columns of the plate cannot be skipped.
• Position 1 is well A1, followed by B1, C1, and so forth.</td></tr>
<tr><td>3</td><td>For sample tube format:</td></tr>
</table>

Step	Action
1	Run **PrintLoadMap** to print the sample input sheet for inclusion in the case file.
2	Run **ExportCSV** to generate the sample input file. Save this file as *qPCR_<instrument name>_<MMDDYY> _<operator initials>*.
3	Run **ExportTXT** to generate the 7500 input file. Save this file as *7500PlateMap_<instrument name>_<MMDDYY> _<operator initials>*.

4 For 96-well format:

Step	Action
1	• Use the macro buttons (on opened 1. Input worksheet) to clear worksheets 1_SF. Input and 1_NSF. Input. • Then, to copy the sample info into these worksheets, click Copy Sample Names to SF and NSF Worksheets.
2	Select the 1_SF. Input worksheet to enter sample information for any additional samples (*e.g.,* reference and body swab extracts) that were added to the sperm-fraction (SF) extraction plate.
3	For the SF plate: • Print the load map. • Generate the Sample and 7500 Input files for the plate using the export csv and txt macro buttons. • Name as SF*_qPCR_<instrument name>_<MMDDYY> _<operator initials>* and SF*_7500PlateMap_<instrument name>_<MMDDYY> _<operator initials.* • Transfer the files to the Tecan computer.
4	Select the 1_NSF. Input worksheet to enter sample information for any additional samples (*e.g.,* reference and body swab extracts) that were added to the non-sperm-fraction (NSF) extraction plate. ***Note:*** This step is not relevant if only one plate is being processed.
5	For the NSF plate: • Print the load map. • Generate the Sample and 7500 Input files for the plate using the export csv and txt macro buttons. • Name as NSF*_qPCR_<instrument name> <MMDDYY> <operator initials>* and

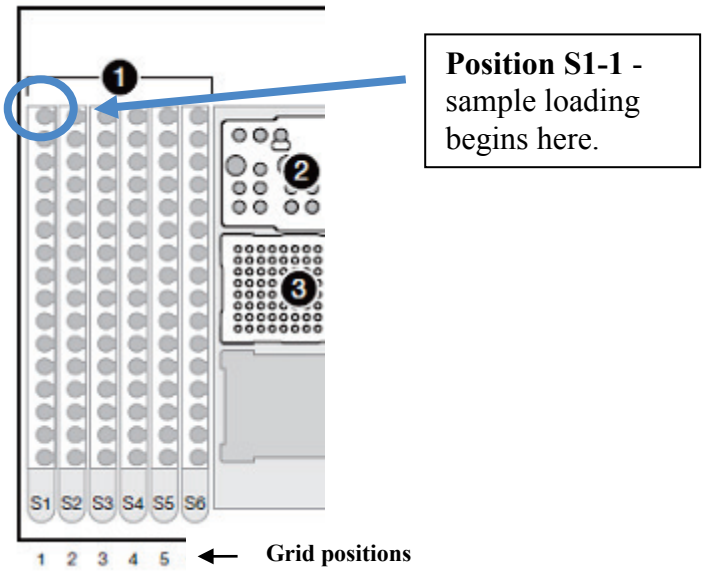
	NSF_*7500PlateMap_<instrument name>_<MMDDYY> _<operator initials.* • Transfer the files to the Tecan computer. ***Note:*** This step is not relevant if only one plate is being processed. ***Note:*** *For non-differentially extracted samples*, skip the first worksheet (1.Input) and instead enter samples directly into the 1_SF.Input worksheet and generate the export files. In bypassing the first worksheet, "SF" (or "NSF") will not be appended to the sample names.
5	Record the file names on the appropriate Tecan HIDEVO150 qPCR Plate Setup checksheet and transfer the files to the appropriate instrument computer.
6	*Optional:* Save and re-name the Excel template as a macro-enabled file.

Note When using the plate format, note that the remainder of the procedure is performed *separately* for each plate (*e.g.,* SF and NSF plates).

Section 13.1.3.4 Build Sample Racks or Sample Plate(s) for the Run

Sample preparation Perform the following to begin sample setup.

Step	Action
1	Verify that each sample has sufficient volume for the qPCR setup and downstream DNA normalization and STR setup procedures. **For sample tubes:** Because at least 15 µL of sample volume is generally required for reliable aspiration from the 1.5 mL tubes, sample volumes for the qPCR setup procedure should typically be at least **17 µL** to ensure sufficient remaining volume for downstream automation procedures. **For 96-well format:** For 96-well plate format, typically at least **14 µL** is needed to ensure sufficient remaining volume for downstream automation procedures. These volumes may vary, however, based on a laboratory's site validation. **Important** If insufficient volume is present, the Tecan may be unable to detect or

	transfer *any* sample volume. In other words, it will not necessarily add whatever volume is present. ***Note:*** *Sterile deionized water may be used to reconstitute evaporated samples.*
2	Ensure that the sample volumes are spun down to the bottoms of the 1.5 mL sample tubes or plate wells. **Important** There should be no air bubbles at or near the bottom of any sample.
3	For sample tubes, see below. For plate format, continue to Section 13.1.3.5. For sample tubes, assemble the *capped* sample tubes for the run into their proper positions in the 16-position tube racks (S1, S2, S3, S4, S5). **Rack Facts** • The tubes must be arranged in the racks sequentially, typically starting at position S1-1. • S1-1 is the furthest back position in the most left-hand rack, as shown in Figure 2.  *Figure 2.* Tecan loading rack positions. • A maximum run contains 80 samples (5 filled racks), excluding the size standards and negative control. • The tubes must be contiguously placed so that the first empty position is immediately after the final tube in the run.

	Important Each rack must be completely filled before starting to place tubes into the next rack.

Section 13.1.3.5 Pre-run HIDEVO150 Checks and Maintenance

Frequency

These checks and maintenance procedures should be run prior to each sample run.

Pre-run Check/ Maintenance Procedure

Perform the following procedure for pre-run checks and maintenance on the HIDEVO150.

Step	Action
1	Boot up the computer connected to the HIDEVO 150 system.
2	Load the appropriate sample input files for the run onto the computer.
3	As needed, perform the following maintenance tasks: • Empty and replace the DiTi waste bag. • Remove any plasticware (troughs, plates, etc.) that might have been left on the EVO deck. Properly dispose of any waste. • Empty the Liquid Waste container. • Replenish the System Liquid with de-ionized water. – ***NOTE:*** Ideally, this is done on the day preceding the automated run, so that the liquid has had an opportunity to de-gas.
4	Turn the Tecan power on by pressing in the green triangular button to the lower, right side of the deck.
5	Start up the EVOware software on the instrument computer and login to the software.
6	• Select the **Run maintenance** button • According to the table below, run the appropriate script(s) from EVOware:

If before starting the run...	Then run the script...
there are one or more DiTis on the liquid handling arm (LiHa)	*Combo_Drop_DiTis*
it is the first run of the day	*Combo_DailyStartUp*
it is not the first run of the day	*Combo_Flush* • enter **10 mL** in the user interface or • enter **50 mL** if there has been service that required reconnecting the Sytem Liquid tubing to the

	System Liquid carboy (*e.g.,* EVOScrub or filling the carboy)
when you run *Combo_DailyStartUp* or *Combo_Flush*, you see: • air bubbles in the syringe barrels or any supply lines and/or • intermittent flow from a DiTi cone	*Combo_Flush* one or more times until: • there are no visible air bubbles and • flow from the DiTi cones is constant

Notes:
- To exit the maintenance script, click the Cancel button in the software.
- The daily start-up script steps the user through a series of checks and system priming, including checking system liquid and waste, checking syringe and valve fittings, checking DiTi cones, and lastly checking placement of carriers and appropriate racks and labware for the run.
- The flush script simply primes and flushes the liquid system.

Section 13.1.3.6 Setting Up the HIDEVO150 Deck

Deck Setup Perform the following to set up the HIDEVO150 Deck.

Step	Action
1	• Confirm that the deck is set up with the proper carriers and labware for qPCR amplification setup (as shown in Figure 4). • Change out carriers and labware items as needed. ***Note:*** The tube racks (S1-S5) (sample tubes) and 96-well plate containing samples (plate format) do not need to be in place at this time.

2	Place a new 96-well optical reaction plate at position 3, shown in Figure 4. **Important** Ensure that the reaction plate is *centered* in its aluminum plate holder and that the plate is oriented so that well A1 is situated in the far left-hand corner, as you face the deck. **Important** When handling the 96well qPCR plate, as much as possible, do not let the bottom of the plate touch surfaces that might transfer contaminants to the plate. Such contaminants, if subsequently transferred to the AB7500 block, could lead to persistent raised background fluorescence in the qPCR data.
3	Ensure that the DiTi racks at positions 11-14 (see Figure 4) are properly loaded with 4 racks of 50 µL conductive, filtered disposable tips.
4	Use the maintenance script *Combo_Set_50tip_Position* to re-set the 50 µL DiTi position. Alternatively, this may be skipped if there are sufficient tips remaining from the previous run or if empty racks are replaced.

Figure 4

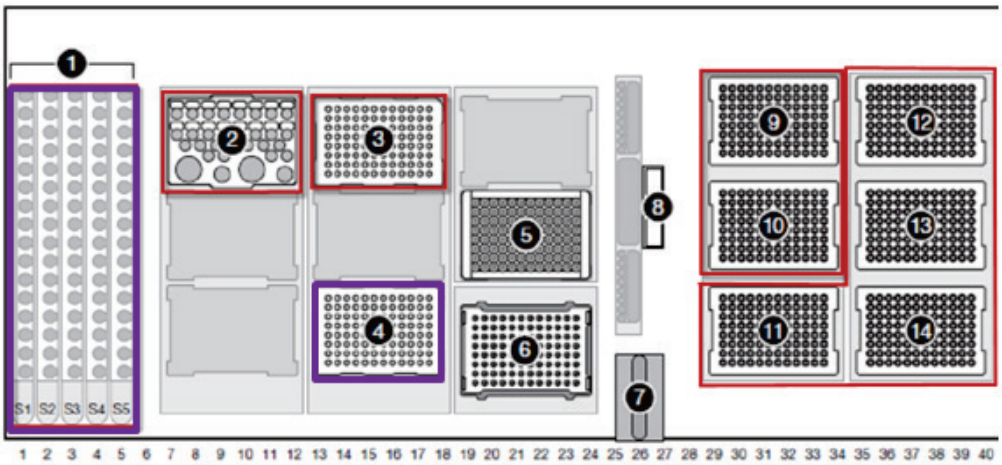


Figure 4. Tecan Deck Configuration for HIDEVO150 qPCR Setup (modified from AB User Bulletin: HID EVOlution™ – Combination System). The numbering key for positions utilized with this script is as follows: **1.** 1.5 mL tube racks for extracted DNA samples in tube format (not all racks required), **2.** qPCR Reagent Carrier, **3.** 96-well optical reaction plate for qPCR processing, **4.** 96-well plate containing samples for plate format, and **11.-14.** 50 µL DiTi Racks.

Note: The remaining numbered positions are not utilized with the Quadruplex qPCR Setup script.

Section 13.1.3.7 Preparation of the CA DOJ Quadruplex qPCR Master Mix

Make the master mix

Perform the following to prepare the Quadruplex qPCR master mix.

Step	Action
1	Obtain the Quadruplex qPCR kit reagents from the refrigerator and freezer. Record the lot number of each reagent to be used.
2	Determine the number of samples to be amplified: # of samples for qPCR amp = **(# of samples in tube racks) + 19** = _____
3	Prepare the qPCR amplification master mix cocktail by combining the following volumes in a 5 mL "VWR" tube and mix thoroughly: # of samples x 11 µL x 1.1 = # of samples x 12.1 µL = _____ µL of TaqMan Master Mix # of samples x 0.55 µL x 1.1 = # of samples x 0.605 µL = _____ µL of AmpliTaq Gold DNA Polymerase # of samples x 5.5 µL x 1.1 = # of samples x 6.05 µL = _____ µL of Quadruplex Primer/Probe Mix # of samples x 2.2 µL x 1.1 = # of samples x 2.42 µL = _____ µL of TE-4 ***Note:*** Each quantity is multiplied by 1.1 to provide excess due to pipetting loss.
4	Close the VWR tube with a screw cap lid and place in the qPCR Reagent Block according to Figure 5.

Section 13.1.3.8 Preparation of the Genomic DNA Standard Dilutions

Make the master mix

Perform the following to prepare the DNA standard dilution series.

Step	Action
1	Obtain the Human Genomic DNA (male) standard from the refrigerator. Record the lot number. Vortex and spin down.
2	Prepare standard DNA dilutions in 1.5 mL microfuge tubes. Standard — Prepare by mixing: 16.0 ng/µL — 12 µL of 32 ng/µL Genomic DNA (male) + 12 µL TE^{-4} 5.33 ng/µL — 8 µL of 16 ng/µL dilution + 16 µL TE^{-4} 1.78 ng/µL — 8 µL of 5.33 ng/µL dilution + 16 µL TE^{-4} 0.59 ng/µL — 8 µL of 1.78 ng/µL dilution + 16 µL TE^{-4} 0.20 ng/µL — 8 µL of 0.59 ng/µL dilution + 16 µL TE^{-4} 0.066 ng/µL — 8 µL of 0.20 ng/µL dilution + 16 µL TE^{-4} 0.022 ng/µL — 8 µL of 0.066 ng/µL dilution + 16 µL TE^{-4}
3	Add 16 µL of TE^{-4} to a new 1.5 mL microfuge tube, which will serve as the negative control for the qPCR reagents.

Section 13.1.3.9 Configure the qPCR Reagent Block

Reagent block

Perform the following to configure the qPCR Reagent Block.

Step	Action
1	Place the DNA Standard dilution tubes and the TE^{-4} negative control tube into the qPCR Reagent Block according to Figure 5.
2	Make sure that the liquid is located at the bottom of each tube.
3	Uncap all tubes in the qPCR Reagent Block.
4	Place the qPCR Reagent Block into Position 2, as shown in Figure 4. **Important** Be sure to orient the front of the Block toward the front of the deck.

Figure 5

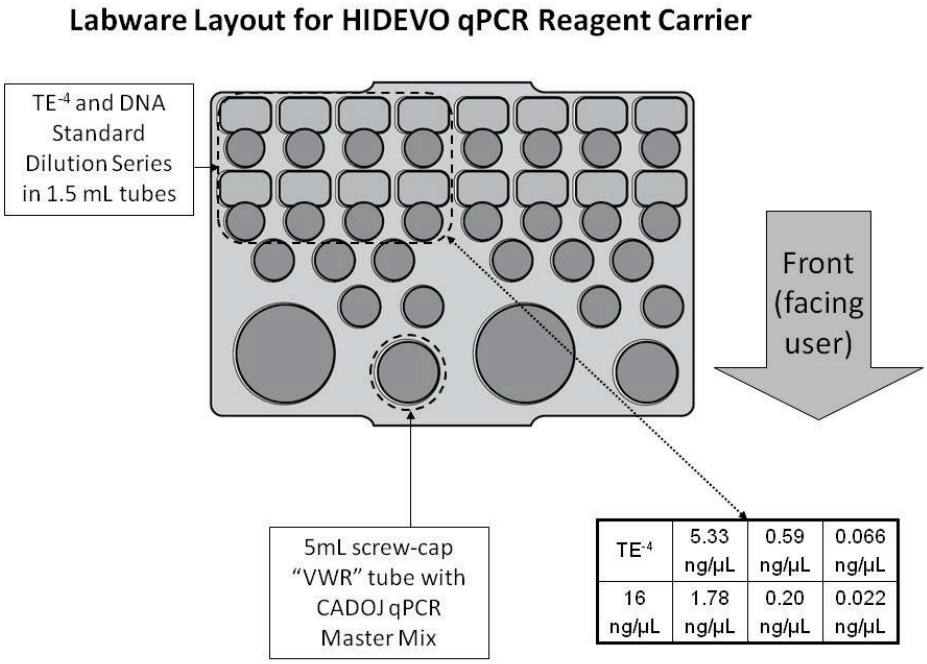


Figure 5. qPCR Reagent Block for the *CA_Quad_tubesCombo_nomix* and *CA_Quad_plateCombo_nomix* scripts.

Section 13.1.3.10 Load the Sample Racks or Sample Plate

Sample Racks For the sample rack format, perform the following to uncap sample tubes and load racks.

Step	Action
1	• Obtain the 16-position sample racks for the run. • Re-confirm that the actual sample positions are in complete correspondence with the data in the sample input file for the run.
2	Taking care not to disturb the original order of the racks or tubes within the racks, open each tube and place it back into its position on the rack.
3	Inspect each tube to ensure that • the sample volume is at the bottom of the tube, • each cap is situated in the rack so that it is not blocking access to the opening of any sample tube, and • each tube is resting (or nearly resting) on the bottom of the rack.

4	Slide each rack into its appropriate position at grids 1-5 on the deck (reference Figure 2).

Note: It is not necessary to put empty racks on the deck.

Plate Format For the sample plate format, perform the following to load the plate.

Step	Action
1	Inspect the sample plate containing extracts to ensure each sample volume is at the bottom of each well and free of potentially interfering bubbles. If needed, quick-spin the plate.
2	Place the plate at position 4 (see Figure 4), making sure to correctly orient well position A1.
3	Remove any plate cover sealing the wells of the plate.

Section 13.1.4 During Run Processing

Section 13.1.4.1 Overview

Introduction This section contains the procedures for performing the HIDEVO150 qPCR Setup run.

Contents This part contains the following topics:

Topic
Section 13.1.4.2 *Opening the script*
Section 13.1.4.3 *Perform the HIDEVO qPCR Setup Run*

Section 13.1.4.2 Opening the script

Before starting If desired, the script may be paused by clicking the button under EVOware, then restarted, at anytime during the run. Keep in mind that when the script is paused, it will stop once it finishes the current operation.

Opening the script Follow the procedure below to open the HIDEVO150 Quadruplex qPCR Setup script (either *CA_Quad_tubesCombo_nomix* or *CA_Quad_plateCombo_nomix*).

Step	Action
1	From the *Runtime Controller* window, select **Edit an existing script.** Do NOT select Run an existing script.
2	**Start your selection** and choose either *CA_Quad_tubesCombo_nomix* or *CA_Quad_plateCombo_nomix.*
3	Click the green arrow to continue.
4	Choose **Run** from the drop-down menu, NOT Run direct.
5	Verify the **Run full script?** box is checked.
6	Click **Run** again.

Section 13.1.4.3 Perform the HIDEVO150 qPCR Setup Run

Performing the run

Follow the procedure below to perform the HIDEVO150 qPCR Setup run.

Step	Action
1	The *HID Evolution EVOware* window should now be open behind the *Runtime Controller*. Move the *Runtime Controller* aside to proceed with the prompts in the *HID Evolution EVOware* window.
2	• At the *Sample Plate Information* window, change the **Number of Samples to Process** in the run from 80 (default) to the number of total samples present in the 16-position tube racks or in the 96-well plate. ***Notes:*** – Do *not* include the 8 tubes on the HIDEVO150 qPCR Reagent Block in the sample count. – For the sample tube format, the starting sample position is typically maintained at rack S1, position #1, although this can be changed. – For the plate format, the starting sample position is well A1 (indicated as "1" by EVOware). • Click the green arrow to continue.
3	• At the *Reagent Information* window, confirm that the **Prepare Standards** button is NOT selected (*i.e.,* confirm that the button is an empty square ☐), then bypass the request for entries of Quantifiler kit lot numbers by clicking the green arrow. • Record the kit and DNA standard lot numbers and all other pertinent information on the HIDEVO150 qPCR Plate Setup checksheet.
4	At the *Load Worktable* window, • click the loaded box for each piece of labware or rack on the deck, or

	• click loaded all. • Click the green arrow to continue.
5	The HIDEVO150 script will now have the POSID (barcode scanner) inventory readable contents of the deck. There will typically be items on the deck that are not barcoded, which will give rise to a POSID error indicated by a red square in the list of loaded labware. • Click the IGNORE button to ignore any such scanning errors, • Next, click the green arrow to continue.
6	At the *Sample Information* window, to enter the sample input file information • click on the box …, • then browse to the appropriate sample input file. – ***Note***: After importing the sample input file, it can be edited before continuing to the next window by clicking the Edit button. • Click the green arrow to continue.
7	A userprompt will appear inquiring if all DiTi racks are loaded. Respond appropriately. The run will now be executed. See **Tecan Processing** below for run information.
8	When the run is complete, a window will appear stating *AB File Generated. 1 file exported: C:\HIDEvolution_qPCRSTRfiles\ ReactionPlate1.txt.* This file is not used for downstream processing and does not need to be saved. Click the green arrow to continue.
9	Next, the *Reporting* window will state *A report has been created and written to file C:\HIDEvolution_qPCRSTRfiles\Quantiler Report_rundate_runtime.pdf.* This file is not used for downstream processing and does not need to be saved. Click the green arrow again to continue. This will cause the *HID Evolution EVOware* window to close.
10	In the *Runtime Controller* window, a message stating "Your script completed with errors!" will be displayed due to one or more sample volumes at < 50 µL. Click **Cancel** to exit the script and to enter the EVOware start-up window.

Tecan Processing

The run will first transfer 18 µL of qPCR Master Mix cocktail into each appropriate well of the 96-well optical plate (in Position 3). The run will proceed by adding 2 µL of sample to each well, beginning with the DNA standard dilution series in the qPCR Reagent Block. The standards are loaded as follows:

Standard Dilution	Amount Loaded	Well Positions
Negative control	0 ng (TE only)	A1,A2
16.0 ng/µL	32.0 ng	B1,B2
5.33 ng/µL:	10.67 ng	C1,C2
1.78 ng/µL:	3.56 ng	D1,D2
0.59 ng/µL:	1.19 ng	E1,E2
0.20 ng/µL:	0.395 ng	F1,F2
0.066 ng/µL:	0.132 ng	G1,G2
0.022 ng/µL:	0.044 ng	H1,H2

After all transfer steps are completed, the script will request an operator response to proceed in generating a report and data files from the run. These are not currently used by CA DOJ.

Section 13.1.5 Post-run Processing

Section 13.1.5.1 Overview

Introduction

The procedures for post-run processing are detailed in this section.

Contents

This part contains the following topics:

Topic
Section 13.1.5.2 *Post-run qPCR Plate Handling and Data Analysis*
Section 13.1.5.3 *Post-run Sample Handling*
Section 13.1.5.4 *Post-run Tecan Clean-up and Maintenance*
Section 13.1.5.5 *Post-run Electronic File Handling*

Section 13.1.5.2 Post-Run qPCR Plate Handling and Data Analysis

Post-run plate handling

Perform the procedures below following the completion of the HIDEVO150 qPCR Plate Setup run.

Step	Action
1	Remove the qPCR plate and inspect to visually confirm the presence of approximately equivalent volumes in the wells. • Some wells may have bubbles; this is normal. • Make appropriate notations of any wells that are obviously problematic (*e.g.,* low volumes). • The plate layout is illustrated below in Figure 6. 1 2 3 4 5 6 7 8 9 10 11 12 A STD 1_1 STD 2_1 S1 S9 S17 S25 S33 S41 S49 S57 S65 S73 B STD 1_2 STD 2_2 S2 S10 S18 S26 S34 S42 S50 S58 S66 S74 C STD 1_3 STD 2_3 S3 S11 S19 S27 S35 S43 S51 S59 S67 S75 D STD 1_4 STD 2_4 S4 S12 S20 S28 S36 S44 S52 S60 S68 S76 E STD 1_5 STD 2_5 S5 S13 S21 S29 S37 S45 S53 S61 S69 S77 F STD 1_6 STD 2_6 S6 S14 S22 S30 S38 S46 S54 S62 S70 S78 G STD 1_7 STD 2_7 S7 S15 S23 S31 S39 S47 S55 S63 S71 S79 H STD 1_8 STD 2_8 S8 S16 S24 S32 S40 S48 S56 S64 S72 S80 *Figure 6.* HIDEVO150 qPCR plate layout. Sample tube positions are indicated (S#) as well as the wells used for the standards (STD). ***Note:*** When handling the qPCR plate, use precautions to keep the bottom of the plate clean so that fluorescent substances are not transferred into the AB 7500 heat block.
2	Place an optical adhesive seal on the qPCR plate.
3	Spin down the plate and visually inspect for possible problems with amplification volumes.
4	• Place the qPCR plate in an AB 7500 and turn on the instrument. • Record the run in the instrument log and the instrument number on the Tecan HIDEVO150 qPCR Plate Setup checksheet.
5	Login to the 7500 control PC, and start the 7500 System SDS Software.

6	• Open the *Quadruplex Assay.sdt* template file. • From the dropdown menu bar, use *File/Import Sample Setup* to open the 7500 platemap file for the run. – This file was previously generated for the HIDEVO150 run by either Excel template: *CADOJ_HIDEVO_TubesInput_v2_0_3.xlt* or *CADOJ_HIDEVO_qPCR_Sample_PlateInput_v1_0_2.xltm* (or higher version) (*i.e. 7500PlateMap_<instrument name>_<MMDDYY>_<operator initials>, SF_7500PlateMap_<instrument name>_<MMDDYY>_<operator initials>*, or NSF_*7500PlateMap_<instrument name>_<MMDDYY>_<operator initials>*). • Confirm that the samples are accurately placed. • Omit unused wells.
7	Select "Instrument" tab to confirm that the Thermal Profile parameters are accurate for the Quadruplex qPCR run: <u>7500 Thermal Profile parameters:</u> Stage 1: 1 Rep (95°C, 10 minutes) Stage 2: 40 Reps of 2-step PCR (95°C, 15 sec; 60°C, 1 min) Sample Volume: 20 µL Run Mode: 9600 Emulation Data Collection: Stage 2, Step 2 (60°C, 1 min)
8	Use *File/Save As* to name and save the .sds run file as Quadruplex Assay *<MMDDYY> <operator initials>-<run 1,2,…>*)..
9	• Close the file and re-open to initialize the qPCR instrument. • Click *Start* to begin the run.
10	After the run has completed, • Transfer the .sds file, • remove the 96-well amplification plate from the instrument block, • turn off the instrument, and • proceed to the data analysis portion of Section 2.1, procedure for nuTH01-nuSRY-nuCSF-IPC Quadruplex qPCR Assay, for the data analysis procedure. – Use the qPCR Quadruplex Analysis checksheet to document the analysis. – ***Notes:*** ▪ When checking ROX signal, check wells B1, E1, and G1 (instead of A3, A6, and A9). ▪ An early cycle baseline artifact ("ECBA") may be periodically observed, typically resulting in a false positive quantitation value in one detector of a negative control. This type of qPCR result can be readily identified by a large quantitation value in one detector and no signal in the other two (for "unknowns"). The resulting inauthentic

	amplification curve can be verified by the shape of the curve and by early baseline elevation in the raw component data.

Section 13.1.5.3 Post-Run Sample Handing

Post-run sample handling

Perform the following after the HIDEVO150 qPCR Plate Setup run is complete.

Step	Action
1	Remove the sample tube racks or plate from the deck and re-cap each sample or re-seal the plate. ***Note:*** If proceeding to a Normalization and STR Setup run, samples may be left on deck.
2	Place the samples in appropriate storage.

Section 13.1.5.4 Post-Run Tecan Clean-Up and Maintenance

Post-run Tecan clean-up/ maintenance

Perform the following after the HIDEVO150 qPCR Plate Setup run is complete.

Step	Action
1	Run the *Combo_Flush.esc* maintenance script: • Choose the script • Click **Run** • Respond with **10** in the user interface to wash with 10 mL
2	If it is the last run of the day: • Exit EVOware • Respond **Yes** to moving all arms to their home positions • Turn off the HIDEVO150 by depressing the **ON** button
3	Discard the qPCR master mix cocktail tube and the DNA standard dilution tubes from the qPCR Reagent Block.
4	If needed: • re-fill the System Liquid carboy with de-ionized water (*e.g.,* Millipore), • empty the Waste Liquid carboy, • empty/replace the DiTi waste bag, and • wipe down the deck.

	Note: When cleaning the HIDEVO150, **<u>do not ever pour or squirt liquids (not even water) on the deck</u>**. Liquids could leak below the deck and short out electronic circuit boards. Instead, wipe the deck with towels dampened with appropriate liquids (*e.g.,* weak detergent, then water, then alcohol, as needed). Tecan carriers and reagent blocks should be treated similarly, in order to avoid exposing them unduly to corrosive agents. **Important** Never clean the instrument while it is powered on.
5	As needed, remove any carriers from the deck and clean with weak detergent, water and/or alcohol. Allow to dry.

Section 13.1.5.5 Post-Run Electronic File Handling

All output files generated by the HIDEVO150 run are archived automatically on the Tecan control PC, in the folders or respective subfolders of either

- ***C:\HIDEvolution_qPCRSTRfiles***\ or
- ***C:\Program Files\Tecan\EVOware\output\SOE***\.

Typically, these files are not utilized or retained.

If proceeding to HIDEVO150 Normalization and STR Setup (procedure Section 13.2), the .sds file from the Quadruplex qPCR run should be retained temporarily for file format conversion. The converted file will be the quantitative input file for this downstream processing on the Tecan. Samples may also be added and deleted at this point.

Section 13.1.6 References

Reference documents

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Fang, R., Liu, J.Y., Kijenski, H.L., Benfield, J., Wong, A., Lagacé, R., Cassel, M.J., Nguyen, V.T., Lauber, W.M., Abeln, D., Treat-Clemons, L., Furtado, M.R., and Shewale, J.G. "The HID EVOlution System for Automation of DNA Quantification and Short Tandem Repeat Analysis," Journal of the Association for Laboratory Automation, Volume 15, Pages 65-73, 2010.

Section 13.1.7 Appendix I - Electronic File Naming Conventions, Storage, and Organization

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Introduction

Electronic file naming and organization should be as described herein to facilitate the tracking and transfer of such files. Files not mentioned within this appendix, do not need to be retained. If proceeding with an HIDEVO150 Normalization and STR Setup run, see also Appendix I of the corresponding procedure (Section 13.2).

Files created by the operator

The sample input and 7500 plate map files created by the operator of the Tecan using Excel macros are named using both common and unique identifiers to facilitate tracking. These files are as follows:

- qPCR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv
- 7500PlateMap_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.txt

Notes:

- Identifiers represented within < > may change from run to run and therefore collectively represent a unique identifier.
- MMDDYY represents the date (month, day, year) of the run.
- Instrument name represents a unique name for the particular Tecan used to perform the run.
- The files may have "SF" or "NSF" prefixes for sperm fraction or non-sperm fraction plates; this is generally paired with the alkaline differential extraction.

File organization

The aforementioned files should ultimately be stored within each Case or Batch Folder in a subfolder named *<instrument name>*_*<MMDDYY>*_*<operator initials>*. Until this subfolder has been copied to the Case or Batch Folder , it may be temporarily stored in another folder named *<instrument name>*_HIDEVO and this second folder may reside on the desktop of the Tecan computer.

<u>For example</u>:
*<instrument name>*_HIDEVO **OR** LLYY#### CF <analyst initials>

- *<instrument name>*_*<MMDDYY>*_*<operator initials>*
  - qPCR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv
  - 7500PlateMap_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.txt

Note: The Case or Batch Folder is created for long-term storage of electronic files associated with a particular case. The naming convention is:

- For Case Folder: LLYY#### CF <analyst initials>, where LLYY#### represents a BFS laboratory case number.
- For Batch Folder: RunFolderUniqueIdentifier BF <analyst initials>

File storage

The electronic files saved to the Case or Batch Folder are copied to one CD for the case file following data analysis. The Case or Batch Folder is additionally stored on a secure network.

Multiple files

In the circumstance where two or more of the same file/folder types are generated on the same day, they will be distinguished by the addition of -2, -3, etc. to the end of the relevant file/folder name(s). For example, qPCR_*<instrument name>*_*<MMDDYY>*_*<operator initials*-2*>*.csv.

Section 13.1.8 Appendix II - Tecan Biweekly Maintenance

Introduction

The EVOScrub procedure is used to clean and condition the system liquid lines and syringes on the Tecan Freedom HIDEVO150 Combo system. The tightness of the DiTi cones and diluter screws is important for the accuracy of liquid transfer.

These procedures should be performed at least every two weeks or at least within two weeks prior to a run. It may be beneficial to run these procedures more frequently with increased usage of the Tecan system. Document the performance of this maintenance in a log.

Contents

This part contains the following topics:

Supplies needed

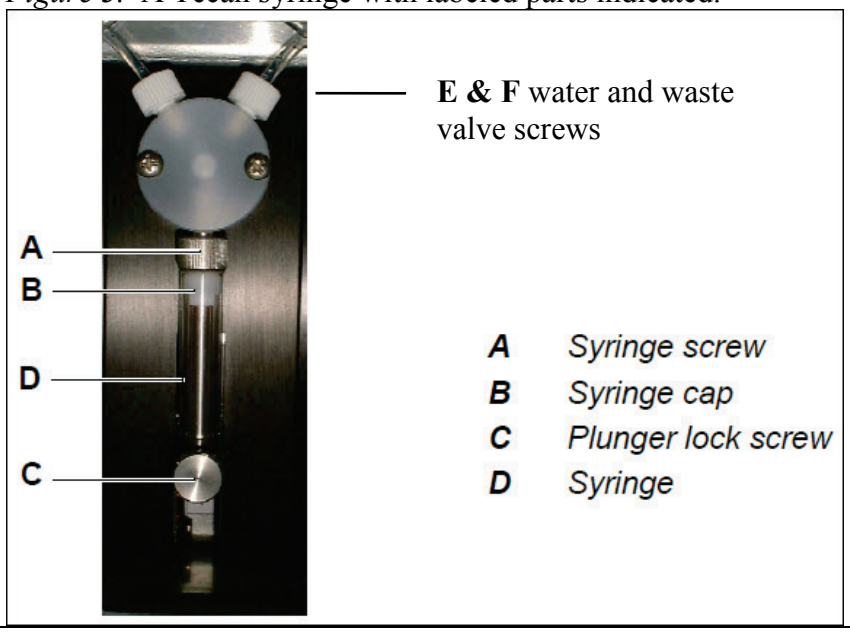
The following supplies are needed to perform Tecan Biweekly Maintenance:

- Tecan RoboScrub or LiquiNox detergent concentrate
- De-ionized water
- A one liter bottle for the detergent dilution
- A one liter bottle for the de-ionized water rinse
- An alcohol squirt bottle (optional)
- A tray (*e.g.,* a kitchen-sink-size plastic container)

General Maintenance

Perform the General Maintenance tasks described below.

Step	Action
1	Ensure the Tecan HIDEVO is turned off. **Important** Never clean the instrument while it is switched on.
2	Check all tubing, tubing connections, syringes, and DiTi cones for leakages. The liquid system is leaking when liquid droplets are hanging on the DiTi cones before the instrument is switched on. If the system is leaking, proceed to **System Leakage** in Section 5.2.7.2 of DNA Technical

	Procedures TP-21 (Volume IV).
3	• Wearing gloves, manually spread the DiTi "fingers" on the LiHa arm. • Check that each gold DiTi cone is finger-tight. If a cone is loose, tighten it to be finger-tight, then further tighten one quarter of a revolution using the DiTi cone wrench. **Important** Over-tightening can cause O-ring compaction and/or breakage inside the DiTi cone, resulting in leakage and/or pipetting inaccuracy.
4	Check the tightness of each syringe and plunger lock screw as well as the water and waste screws. These screws should be *at least* finger-tight. These screws are indicated by A, C, E and F in Figure 1 shown below. *Figure 3.* A Tecan syringe with labeled parts indicated. 
5	• Reboot the Tecan to minimize step loss which is related to the motors and accumulates over time, to as much as at least 1 mm. The motors are reset when turned off. • Reboot the computer to keep it running well and rectify any software driver communication errors.
6	For Tecans running PrepFiler scripts, verify the RoMa is functioning appropriately by running the *Combo_Test_All_RoMa_Vectors_Only* script. This will test both Processing Plate and DiTi box transfers. See the PrepFiler procedure (Section 1.5.5.2 of TP-06) for more information.

EVOScrub Maintenance

Follow the procedure below to perform EVOScrub Maintenance.

Step	Action
1	Dilute ~ 8 mL of the concentrated detergent (Tecan RoboScrub or LiquiNox) in ~ 800 mL de-ionized water.
2	Mix so that the diluted detergent is homogeneous and allow the diluted solution to stand at room temperature for 15 minutes. ***Note:*** It is recommended to use the diluted detergent solution on the same day that it is diluted.
3	Fill another bottle with ~500 mL of de-ionized water.
4	Turn on the HIDEVO150 system.
5	Start up EVOware on the instrument PC and login to the software.
6	Select the **Run maintenance** button and run the *Combo_Flush* (10 mL), or another maintenance script to initialize the Tecan.
7	Place the tray near the system liquid carboy and put the bottles of diluted detergent and de-ionized water in it. This is to prevent spilled liquids from getting on the floor or counter.
8	Transfer the system liquid tubing from the system liquid carboy to the bottle of diluted detergent, making sure that the open end of the tubing is at the bottom of the detergent bottle.
9	Select the **Edit an existing script** radio button in EVOware, choose the *Combo_ RoboScrub* script, and begin the run. ***Notes:*** • This script is very similar to the *Combo_Flush*, except that the flush volume is fixed to be smaller in order to avoid excessive foaming during the EVOScrub procedure. • The RoboScrub and EVOscrub scripts are the same, so either can be used for this purpose.
10	Run the *Combo_RoboScrub* script a total of five times (~100 mL used per run). This is most easily accomplished by using the "NEW" button to re-start the script when it finishes, rather than using the Cancel button to exit then re-start the script. While running the script, • make sure that the end of the tubing remains near the bottom of the EVOScrub bottle so that air is not sucked into the system, and • keep an eye out for excessive foaming in the wash troughs. Excessive foaming can be minimized by squirting 70% ethanol or isopropanol into the troughs between runs, as needed.
11	Remove the tubing from the diluted detergent solution, and place it into

	the bottle of de-ionized water previously prepared in Step 3.
12	Run the *Combo_RoboScrub* script (as in Step 10), repeating the procedure for a total of five runs.
13	Remove the tubing and return it to its original position in the system liquid carboy. Make sure the tubing is at/near the bottom of the carboy.
14	Again, as in Step 10, run the *Combo_RoboScrub* script five times, or longer as needed until no bubbles are seen in the system liquid lines or in the syringes. ***Note:*** Alternatively, the *Combo_Flush* may be run at this point using 50 mL.
15	Exit the script by clicking the Cancel button.
16	Rinse all bottles. Allow to air dry.

Section 13.2 DNA Normalization and STR Amplification Setup Using the Tecan Freedom HIDEVO150 Combo System

Section 13.2.1 Overview

Introduction

This section describes the use of the Tecan Freedom HIDEVO150 Combo System for DNA normalization and STR amplification setup using either the Identifiler_tubesComboManualMasterMix script (for 1.5mL tubes) or the Identifiler_plateComboManualMasterMix script (for 96-well plates) for amplification plate setup with the AmpFℓSTR® Identifiler Plus® PCR Amplification Kit, manufactured by Life Technologies/Applied Biosystems.

Following normalization and amplification, capillary electrophoresis and detection of the STR loci are performed using either a 3130, 3130*xl*, or 3500/3500xL Genetic Analyzer. The data from these instruments is then analyzed through GeneMapper ID /ID-X.

Contents

This procedure contains the following topics:

Hazard

To avoid potential crushing and piercing injuries from moving parts, do NOT reach into the Tecan work space when a script is running. If it is necessary to access the deck or work space during a script, either pause the script (wait for the green indicator light to begin flashing), stop the script (the indicator light turns off), or power down the Tecan.

Hazard

The barcode reader (*i.e.,* PosID) uses laser technology for scanning. Do not stare into the laser beam or its reflection.

Warning

Electromagnetic waves from a cellular phone may affect the function of liquid detection. Faulty detection of the liquid surface may cause the system to produce incorrect results. It is recommended to keep a distance of at least 6.5 feet from the instrument when using a cellular phone.

Section 13.2.2 Materials, Reagents, and Equipment

Materials & reagents

- AmpFℓSTR® Identifiler Plus® PCR Amplification Kit
- TE^{-4}
- Deionized water
- Weak detergent (*e.g.,* Liquinox or RoboScrub)

Equipment

- Tecan Freedom HIDEVO150 Combo System
- 1.5 mL Microfuge tubes – DNA/DNase/RNase free
- Tecan 100 mL reagent trough – DNA/DNase/RNase free
- Mini centrifuge
- Vortex
- Centrifuge with plate rotor or alternative plate spinner
- 96-well reaction plates – DNA/DNase/RNase free, PCR compatible
- 96-well amplification plate covers, or other appropriate cover for 96-well reaction plates – DNase/RNase free, PCR compatible
- VWR 5 mL screw-cap tubes
- Conductive, filtered 50 µL and 200 µL tips – DNA/DNase/RNase free
- Pipettors
- Pipet tips – DNA/DNase/RNase free
- Miscellaneous laboratory supplies

Section 13.2.3 Pre-run Processing

Section 13.2.3.1 Overview

Introduction This section describes the procedures for setting up an HIDEVO150 Normalization and STR Plate Setup run.

Contents This part contains the following topics:

Section 13.2.3.2 About the Input Files

Required files The HIDEVO150 Normalization and STR Amplification Setup procedure requires two input files:

1. a sample input file, which specifies the name and position of each sample tube in the 16-position tube racks or in the 96-well sample plate; and
2. a quantification input file, which specifies the DNA concentration (in ng/uL) for each sample.

Important The HID EVOware software assumes all concentrations are ng/uL.

Figure 1a

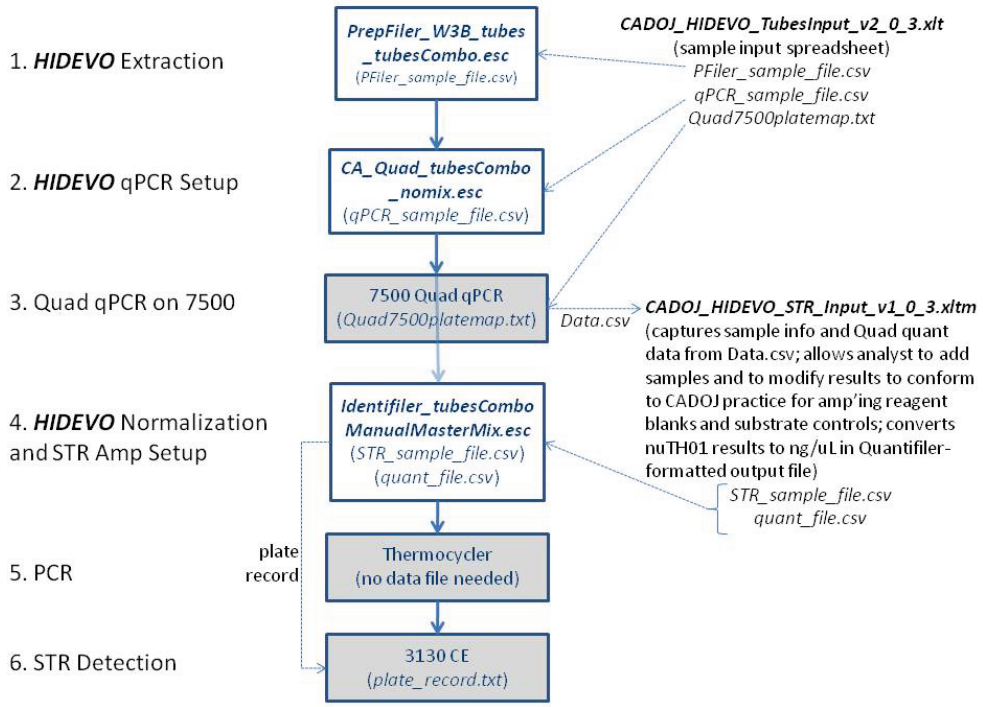


Figure 1a. An overview of HIDEVO150 sample tube processing at the CA DOJ.

- The bold arrows represent the rough flow of the actual samples through the processing.
- The thin, dotted arrows represent the flow of data files generated to connect the processes.
- Steps 1, 2, and 4 represent the three HIDEVO150 automation modules (extraction, quantification setup, normalization/amp setup), while steps 3, 5, and 6 represent operations performed on AB instrument platforms.

Figure 1b

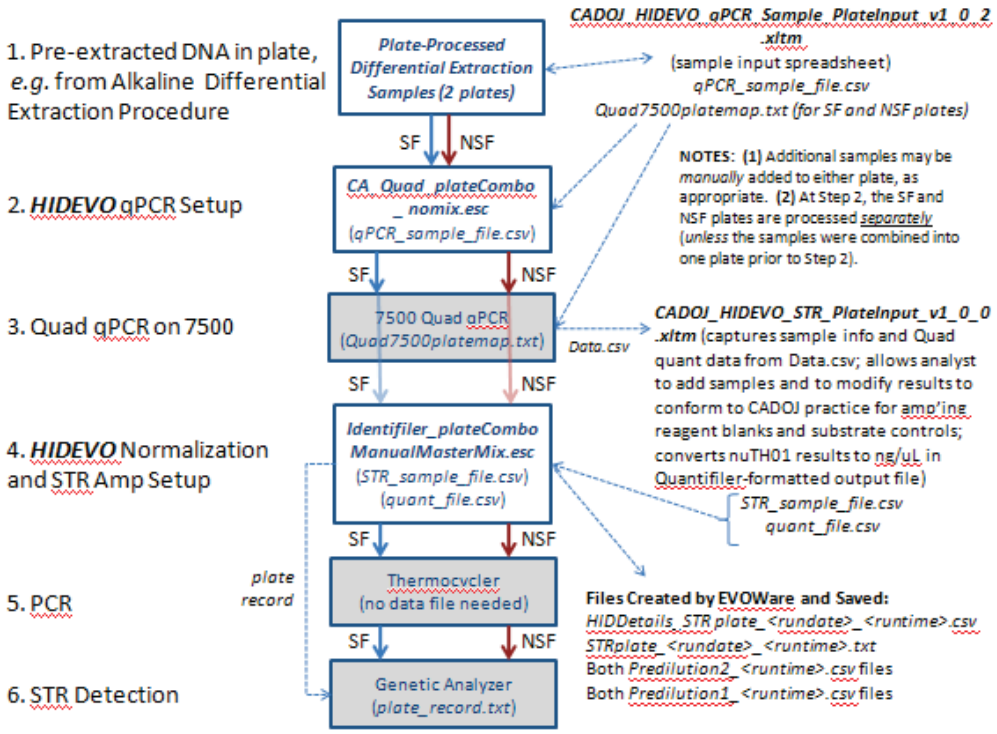


Figure 1b. An overview of HIDEVO150 sample plate processing at the CA DOJ. Differentially-extracted sample plates are illustrated as an example.

- The bold arrows represent the rough flow of the actual samples through the processing.
- The thin, dotted arrows represent the flow of data files generated to connect the processes.
- Steps 1, 2, and 4 represent the three HIDEVO150 automation modules (extraction, quantification setup, normalization/amp setup), while steps 3, 5, and 6 represent operations performed on AB instrument platforms.

Background

Each HIDEVO150 automation module is identified by a specific EVOware script file (.esc), which requires at least one input file (in parentheses in Figure 1) for processing.

Due to the custom nature of our quadruplex qPCR protocol, our data files are not created only by the EVOware software, but are also created or modified by locally-developed VBA-enabled Excel templates (right-hand side of Figures 1a and 1b).

***Sample Tube Format* - Sample and Quantification Input Files**

Both the sample and quantification input files for Step 4 of Figure 1a are generated after step 3 using the *CADOJ_HIDEVO_ STR_Input_v1_0_3.xltm* Excel template, or higher version.

Upon opening, this Excel template should appear as shown in Figure 2a below.

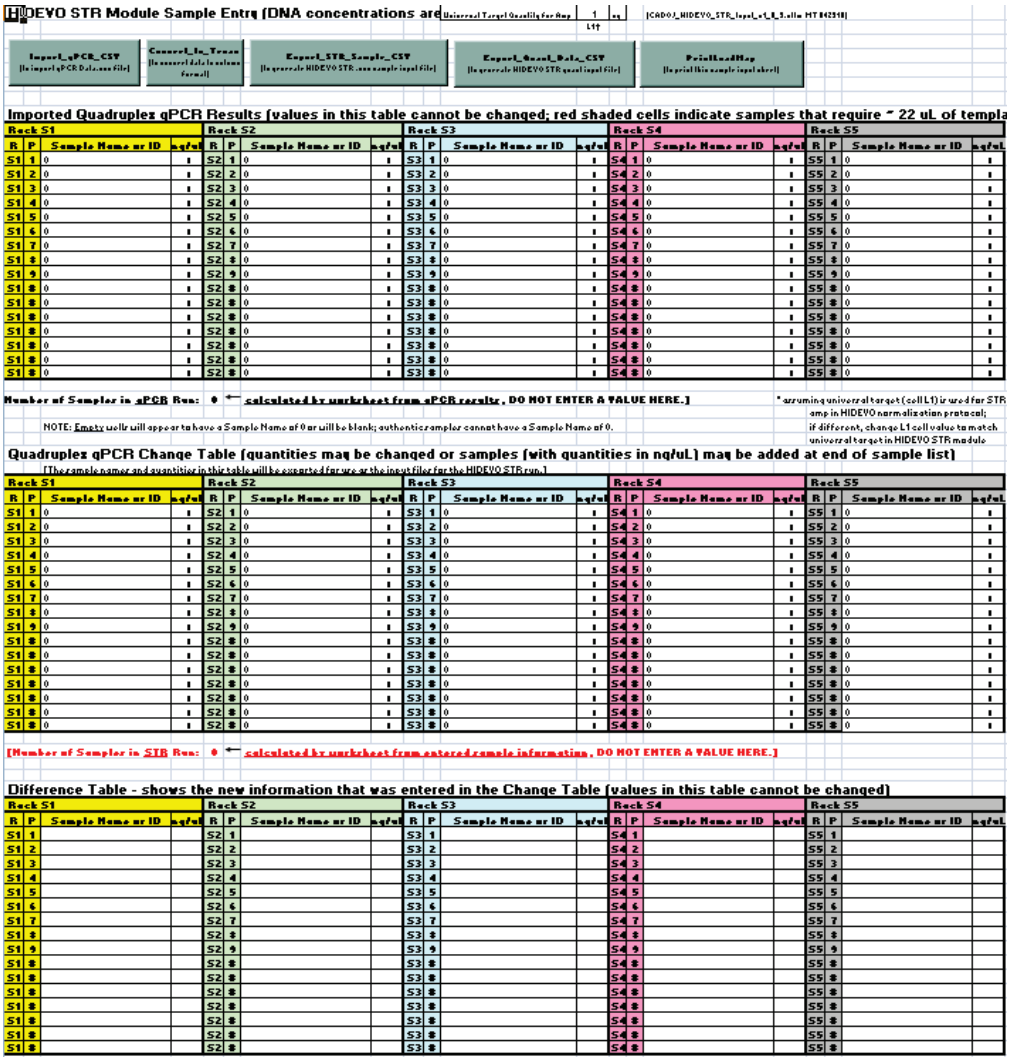


Figure 2a. The user interface of the CADOJ_HIDEVO_STR_Input_ v1_0_3.xltm Excel template for the sample tube format. Upon opening, the five buttons across the top (Import_qPCR_CSV, Convert_to_Tecan, Export_STR_Sample_CSV, Export_Quant_Data_CSV, and PrintLoadMap) and 3 tables (Imported Quadruplex qPCR Results, Quadruplex qPCR Change Table, and Difference Table) should appear. The tables should not initially be populated.

***Plate Format* - Sample and Quantification Input Files**

Both the sample and quantification input files for Step 4 of Figure 1b are generated after step 3 using *CADOJ_HIDEVO_STR_PlateInput_v1_0_0.xltm* Excel template, or higher version.

Upon opening, this Excel template should appear similar to Figure 2b below.

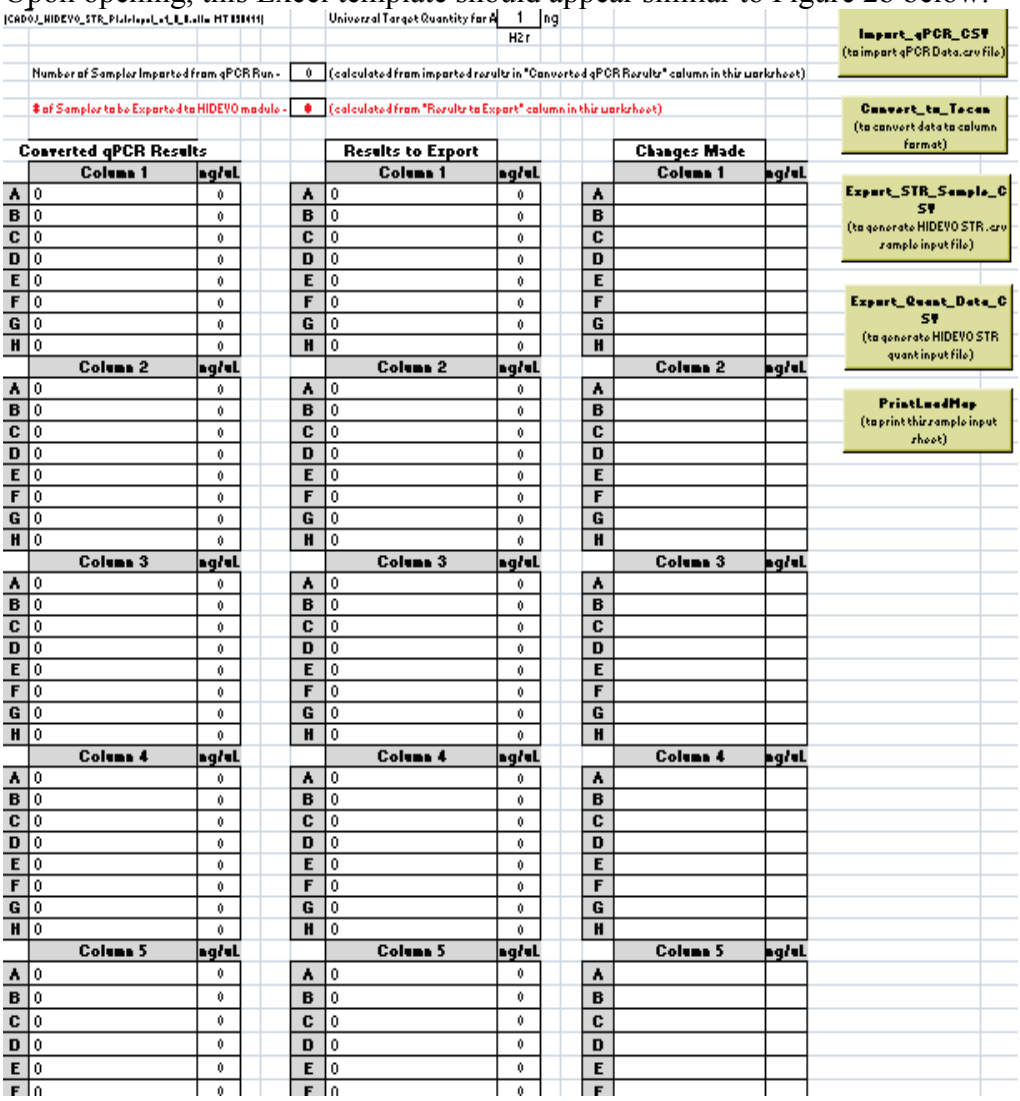


Figure 2b. The user interface of the CADOJ_HIDEVO_STR_PlateInput _v1_0_0.xltm Excel template. Upon opening, the five buttons to the right (Import_qPCR_CSV, Convert_to_Tecan, Export_STR_Sample_CSV, Export_Quant_Data_CSV, and PrintLoadMap) and 3 tables (Converted qPCR Results, Results to Export, and Changes Made) should appear. The tables should not initially be populated.

Notes & Limitations

These Excel templates cannot be used to process qPCR data from runs that were performed following the manual Quadruplex procedure (Section 2.1) as the standards are placed in different wells.

These Excel templates cannot be used to process qPCR data from Duplex runs.

Samples quantified by the Quadruplex but to be omitted from STR amplification should *not* be removed from the Excel spreadsheet *nor* the racks/plate. Instead, simply omit them from processing while in the Sample Normalization Adjustment Table of the script when setting up the run (refer to Steps 8 and 9 of Perform the HIDEVO150 Normalization and STR Amplification Setup run). Note the omission in the bench notes and adjust the sample number accordingly when preparing the Identifiler Plus master mix.

Section 13.2.3.3 About Normalization

Normalization Volumes

The HIDEVO150 normalization script typically uses between 1.5 and 11 µL of sample to dilute the DNA to the desired target concentration for PCR amplification. It will always add a 10 µL volume to the 96-well amplification plate so if the target amount to be amplified requires less than 10 µL of extract, the Tecan will dilute the sample and add 10 µL of the dilution.
For one dilution scheme (1:1.3), 18.55 µL of extract is used to achieve the target DNA concentration for STR amplification. For this particular situation, it is important to ensure there is sufficient volume to make the dilution. Alternatively, the targeted DNA input quantity for PCR can be adjusted to accommodate a lower volume of needed extract.

See Appendix II for more details on dilution volumes using a 1 ng target amount of DNA.

Normalization Dilutions

Using the HIDEVO150 normalization script, a 1:4000 dilution is the maximum dilution factor that will be performed. When needed, the Tecan will perform a one-step dilution up to a maximum of 1:20 on a sample. It will perform a two-step dilution on samples concentrated enough such that a 1:20 is not sufficient (*e.g.,* ≥ 2.297 ng/µL for a 1 ng amplification). See Figure 8 in Appendix II for more details on the dilution schemes.

Table 1 below shows the range of concentrations within which a sample can be processed at a particular target amount. If the DNA concentration of a sample is below the Min concentration, then 10 µL of neat extract will be used for amplification.

Target Amount (ng)	**Min concentration (ng/µL)**	**Max concentration (ng/µL)**
0.1	0.01	40
0.125	0.013	50
0.5	0.05	200
1.0	0.1	400
1.5	0.15	600
2.0	0.2	800
2.5	0.25	1000
5.0	0.5	2000
10.0	1.0	4000

Table 1. The range of extract concentrations for STR amplification at the corresponding target amounts.

For example, a maximum DNA concentration of 400 ng/ µL can be processed for a 1 ng target amount. If the sample exceeds 400 ng/ µL, either a higher target amount can be chosen or the sample can be manually diluted prior to the Tecan Normalization and STR Amplification Setup run. Otherwise, the Tecan will treat the sample as if the exceeding concentration is simply at the maximum of 400 ng/ µL.

See Appendix II for more details on normalization dilutions.

Note: If the sample is manually diluted, the operator must make sure to change the DNA concentration so that it represents that of the diluted extract. This can *only* be done in the Excel template (*CADOJ_HIDEVO_STR_Input_ v1_0_3.xltm* or *CADOJ_HIDEVO_STR_PlateInput_v1_0_0.xltm,* or higher versions).

Section 13.2.3.4 Generate Input Files

Generating input files

Perform the following to generate the sample and quantification input files.

Note: If the qPCR was performed manually, proceed to *Appendix V, Manual Data Entry.*

Step	Action
1	Open the appropriate Excel template and enable macros. The template should appear as shown in either Figure 2a or Figure 2b. **If samples are in** / **Then use** 1.5mL sample tubes: *CADOJ_HIDEVO_STR_v1_0_3.xltm* 96-well PCR plate: *CADOJ_HIDEVO_STR_PlateInput_v1_0_0.xltm* ***Note:*** Higher version Excel template(s) may be used if performanced-checked.
2	Click **Import_qPCR_CSV** to import the Quadruplex qPCR results. This is the Data.csv file that was exported during the qPCR run data analysis. **If samples are in** / **Then** 1.5mL sample tubes: The top table (Imported Quadruplex qPCR Results) will populate after the data is converted in the next step. 96-well PCR plate: The left-hand table (Converted qPCR Results) will populate after the data is converted in the next step.
3	Click **Convert_to_Tecan** to populate the appropriate tables. **If samples are in** / **Then** 1.5mL sample tubes: The top and middle (Quadruplex qPCR Change Table) tables will populate. 96-well PCR plate: The left and middle (Results to Export) tables will populate. ***Note:*** The concentrations are converted from ng/2µL in the Data.csv file to ng/µL in these tables.
4	Verify the samples in the middle table are in the desired order and at the correct concentrations.

5	If desired, use the Universal Target Quantity (in cell L1 or H2) to assist in determining whether sufficient volume is present in each sample tube for dilutions to be prepared by the Tecan robot. This value defaults to 1 ng in each template but may be changed. Samples requiring 18.55 µL for normalization (*i.e.* a 1:1.3 dilution) based on the particular target quantity will be shown in red in the top table. ***Note:*** Target amount changes are *not* transferred to the HIDEVO150 scripts. The Tecan script will still default to 1 ng. It is simply used here to assist in assuring sufficient volumes.

6	Manually enter any additional samples to be run into the middle table and the corresponding concentrations.
	
	Notes on Sample Entry:
	• Include the case number with each sample. The format *LLYY####_sample name* is suggested, where LLYY#### represents a BFS laboratory case number.
	• No samples in the same run can have identical names.
	• A sample name *cannot* include spaces or particular characters, such as colons, commas, asterisks, and slashes.
	  o Permissible characters include -_(){}#.+
	• The sample name *cannot* be a single, numeric 0 (zero).
	• DNA concentrations should be entered as ng/µL
	• Be sure to enter only numeric symbols for DNA concentration values. A zero (0) is *not* the same as the letter O.
	
	Sample tube format:
	• If 2 amplifications of the same sample are desired (*e.g.,* 1 ng and 0.5 ng), a separate 1.5 mL tube must be placed in the rack for each target quantity and the additional tube must be given a different name .
	  o *The Tecan robot will not enter a sample tube more than once.*
	• All samples must be loaded contiguously; sequential tube and rack positions cannot be skipped.
	• Position 1 is Rack S1, P1 in the Excel template; see next section for tube placement in racks.
	
	96-well format:
	• If 2 amplifications of the same sample are desired (*e.g.,* 1 ng and 0.5 ng), extract for the additional amplification must be placed in another well of the plate and the well must be given a different name.
	  o *The Tecan robot will not enter a sample well more than once.*
	• All samples must be loaded contiguously; sequential wells and columns cannot be skipped.

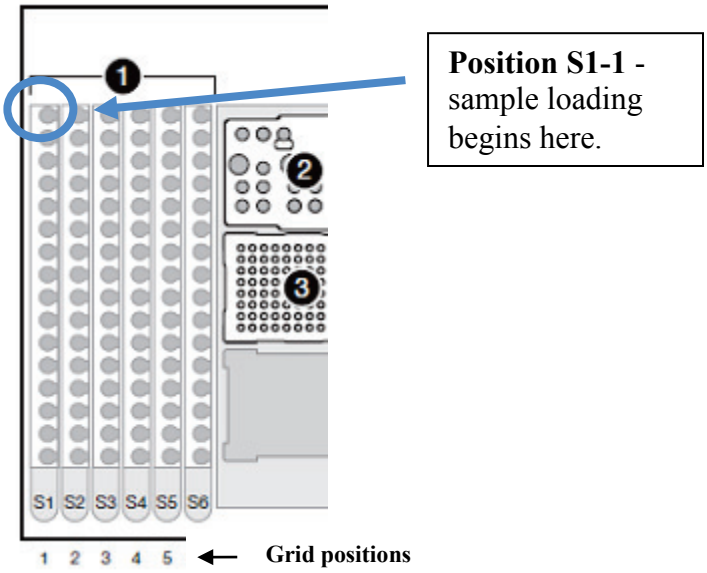
	• Position 1 for plate format is well A1, followed by B1, C1, and so forth.
7	Make any necessary adjustments to reagent blanks and substrate samples. • To match volumes, it is easiest to input matching concentrations to get the Tecan to add the correct dilution to the PCR 96-well plate. These modifications will be reflected in the third table (Difference Table for sample tube format or Changes Made for plate format). • For example, if a reagent blank goes with a sample at 5 ng/µL, change the concentration for the reagent blank to be 5 ng/µL in the second table. It will then be diluted as the sample is and the correct dilution will then be amplified. • Ensure there is sufficient volume present in each adjusted control.
8	Click **Export_STR_Sample_CSV** to generate the sample input file. Name the .csv file as *STR_<instrument name>_ <MMDDYY>_<operator initials>*. ***Note:*** If there is more than one plate to be processed, add identifiers in the file names to differentiate them (*e.g.,* SF_ and NSF_ as prefixes).
9	Click **Export_Quant_Data_CSV** to generate the quantification input file. Name the .csv file as *Quant_<instrument name>_ <MMDDYY>_<operator initials>*. ***Note:*** If there is more than one plate to be processed, add identifiers in the file names to differentiate them (*e.g.,* SF_ and NSF_ as prefixes).
10	Record both file names on the appropriate checksheet and transfer the files to the Tecan computer.
11	Run **PrintLoadMap** to print the sample input sheet for inclusion in the case file.
12	*Optional:* Save and re-name the Excel template as a macro-enabled file.

Section 13.2.3.5 Build Sample Racks or Sample Plate(s) for the Run

Sample Preparation

Perform the following to begin sample setup.

Step	Action
1	Verify that each sample has sufficient volume for the normalization. **For sample tubes:** Generally, a minimum of **15 µL** of sample volume is required for reliable aspiration from the bottom of the 1.5 mL tubes.

	For 96-well format: For 96-well plate format, typically <u>at least</u> **12 µL** is needed to ensure sufficient volume transfer. These volumes may vary, however, based on a laboratory's site validation. **Important** If insufficient volume is present, the Tecan may be unable to detect or transfer *any* sample volume. In other words, it will not necessarily add whatever volume is present. ***Note:*** *Sterile deionized water may be used to reconstitute evaporated samples.*
2	Ensure that the sample volumes are spun down to the bottoms of the 1.5 mL sample tubes or plate wells. **Important** There should be no air bubbles at or near the bottom of any sample.
3	For sample tubes, assemble the *capped* sample tubes for the run into their proper positions in the 16-position tube racks (S1, S2, S3, S4, S5). **Rack Facts** • The tubes must be arranged in the racks sequentially, typically starting at position S1-1. • S1-1 is the furthest back position in the most left-hand rack, as shown in Figure 3.  *Figure 3*. Tecan loading rack positions.

	• A maximum run contains 80 samples (5 filled racks), excluding positive and negative amplification controls. • The tubes must be contiguously placed so that the first empty position is immediately after the final tube in the run. **Important** Each rack must be completely filled before starting to place tubes into the next rack. ***NOTE:*** If the samples are being processed shortly after an HIDEVO150 qPCR setup run, they may already be in their proper positions.

Section 13.2.3.6 Pre-run HIDEVO150 Checks and Maintenance

Frequency

These checks and maintenance procedures should be run prior to each sample run.

Pre-run Check/ Maintenance Procedure

Perform the following procedure for pre-run checks and maintenance on the HIDEVO150.

Step	Action
1	Boot up the computer connected to the HIDEVO150 automated system.
2	Load the appropriate sample and quantification input files for the run onto the computer.
3	As needed, perform the following maintenance tasks: • Empty and replace the DiTi waste bag • Remove any plasticware (troughs, plates, etc.) that might have been left on the EVO deck. Properly dispose of any waste. • Empty the Liquid Waste container. • Replenish the System Liquid with de-ionized water. – ***NOTE:*** Ideally, this is done on the day preceding the automated run, so that the liquid has had an opportunity to de-gas.
4	Turn the Tecan power on by pressing in the green triangular button to the lower, right side of the deck.
5	Start up the EVOware software on the instrument computer and login to the software.
6	• Select the **Run maintenance** button • According to the table below, run the appropriate script(s) from

EVOware:

If before starting the run...	**Then run the script...**
there are one or more DiTis on the liquid handling arm (LiHa)	*Combo_Drop_DiTis*
it is the first run of the day	*Combo_DailyStartUp*
it is not the first run of the day	*Combo_Flush* • enter **10 mL** in the user interface or • enter **50 mL** if there has been service that required reconnecting the Sytem Liquid tubing to the System Liquid carboy (*e.g.,* EVOScrub or filling the carboy)
when you run *Combo_DailyStartUp* or *Combo_Flush*, you see: • air bubbles in the syringe barrels or any supply lines and/or • intermittent flow from a DiTi cone	*Combo_Flush* one or more times until: • there are no visible air bubbles and • flow from the DiTi cones is constant

Notes:

- To exit the maintenance script, click the Cancel button in the software.
- The daily start-up script steps the user through a series of checks and system priming, including checking system liquid and waste, checking syringe and valve fittings, checking DiTi cones, and lastly checking placement of carriers and appropriate racks and labware for the run.
- The flush script simply primes and flushes the liquid system.

Section 13.2.3.7 Setting Up the HIDEVO150 Deck

Deck Setup

Perform the following to set up the HIDEVO150 Deck.

Step	Action
1	• Confirm that the deck is set up with the proper carriers and labware for STR normalization and amplification setup (exactly as shown in Figure 5). • Change out carriers and labware as needed. ***NOTE:*** The tube racks (S1-S5) (sample tubes) and 96-well plate containing samples (plate format) do not need to be in place at this time.
2	Place new 96-well PCR reaction plates at Positions 3, 5, and 6 indicated in Figure 5. **Important** Ensure that each reaction plate is *centered* in its aluminum plate holder and that each plate is oriented so that well A1 is situated in the far left-hand corner, as you face the deck. **Position key:** • Position 6 is for the PCR amplification plate. • Position 5 is for the dilution plate of ≤ 48 samples (*i.e.,* with ≤ 3 filled tube racks) requiring a dilution. • Position 3 is for the second dilution plate of > 48 samples requiring a dilution. In other words, if there are > 48 samples requiring dilution, place 96-well plates in all three positions.
3	Ensure that the DiTi racks at Positions 11-16 (see Figure 5) are properly loaded with conductive, filtered disposable tips (2 racks of 200 µL tips; 4 racks of 50 µL tips).
4	Load a clean 100 mL reagent trough with 15-20 mL of TE^{-4}; place the trough in Position 10. This TE is used for dilutions and should be from the same source as the negative control TE. If there are no dilutions to be made, this trough is not needed.
5	Use the maintenance scripts *Combo_Set__200tip_Position* and *Combo_Set_50tip_Position* to re-set the 200 µL and 50 µL DiTi positions. Alternatively, this may be skipped if there are sufficient tips remaining from the previous run or if empty racks are replaced.

Figure 5

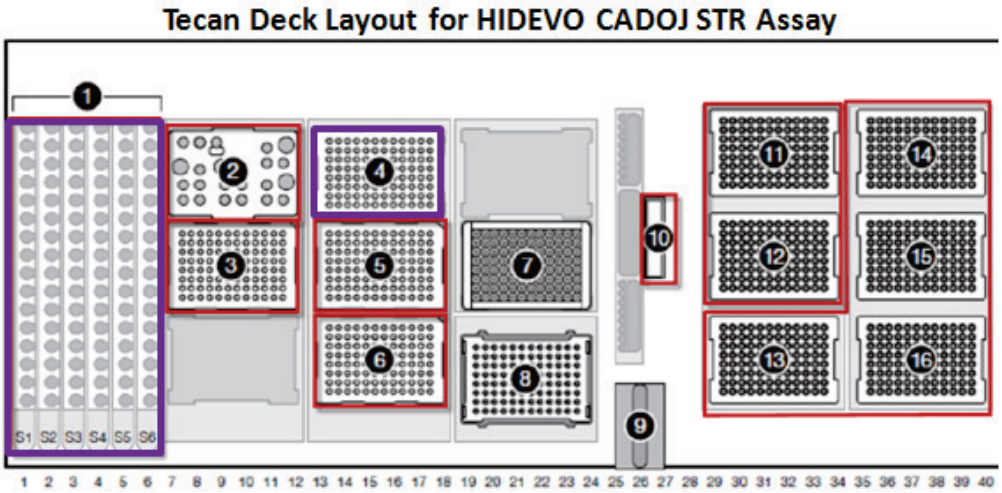


Figure 5. Tecan Deck Configuration for HIDEVO150 DNA Normalization and STR Amp Setup (modified from AB User Bulletin: HID EVOlution™ – Combination System). The numbering key for positions utilized with this script is as follows: **1.** 1.5 mL tube racks for extracted DNA samples in tube format (not all racks required), **2.** STR Reagent Carrier, **3.** Predilution1 plate (not needed if <48 samples), **4.** 96-well plate containing samples for plate format, **5**. Predilution2 plate, **6**. 96-well PCR plate for STR reactions, **10.** 100-mL reagent trough containing ~20 mL TE^{-4}, **11.-12**. 200 µL DiTi Racks, and **13.-16.** 50 µL DiTi Racks.

Note: The remaining numbered positions are not utilized with the Normalization and STR Amplification Setup script.

Section 13.2.3.8 Preparation of the STR Master Mix

Identifiler Plus master mix

Perform the following to prepare Identifiler Plus master mix.

Step	Action
1	Obtain the Identifiler Plus kit reagents from the refrigerator and freezer.
2	Determine the number of samples to be amplified: # of samples for STR amp = # of samples in tube racks (or plate) + 3 = _____
3	Prepare the STR amplification master mix cocktail by combining the following volumes in a 5 mL "VWR" tube and mix thoroughly: # of samples x 11 µL x 1.1 = # of samples x 12.1 µL = _____ µL Identifiler Plus Master Mix # of samples x 5.5 µL x 1.1 = # of samples x 6.05 µL = _____ µL Identifiler Plus Primer Set ***Note:*** Each quantity is multiplied by 1.1 to provide excess due to pipetting loss.
4	Close the VWR tube with a screw cap lid and place in the STR Reagent Block.

Section 13.2.3.9 Configure the STR Reagent Block

Reagent block

Perform the following to configure the STR Reagent Block.

Step	Action
1	Obtain the 9947A tube from the STR reagent kit. Vortex, spin down. ***Note:*** The target range for Control DNA 9947A is 0.5 to 1 ng in a total volume of 10 µL. If amplifying less than 1ng, it should be diluted to the desired quantity within the range and in a total volume of 10 µL. The diluted 9947A should be placed on the Tecan in an equivalent 500 µL skirted tube (Step 3 below).
2	Fill a new 1.5 mL microfuge tube with ~500 µL of TE^{-4}. This is to be used for the negative amplification control.
3	Place the 9947A tube, TE^{-4} tube (negative amplification control), and master mix prepared in the previous section into the appropriate positions of the STR Reagent Block, as shown in Figure 6. Make sure that the liquid in each tube is located at the bottom of each tube. ***Note:*** Positive and negative amplification controls associated with samples being typed shall be amplified concurrently in the same instrument with the samples at all loci and with the same primers as the forensic samples. All samples typed shall also have the corresponding amplification controls typed.
4	Uncap all tubes in the STR Reagent Block.
5	Place the STR Reagent Block into Position 2, as shown in Figure 5. **Important** Be sure to orient the front of the block toward the front of the deck.

Important
The 9947A sample loaded onto the STR Reagent Block *must* be present in the original tube from the kit, or in an equivalent 500 µL skirted tube. It should *not* be in a 1.5 mL tube or 2 mL screw-cap tube.

Figure 6

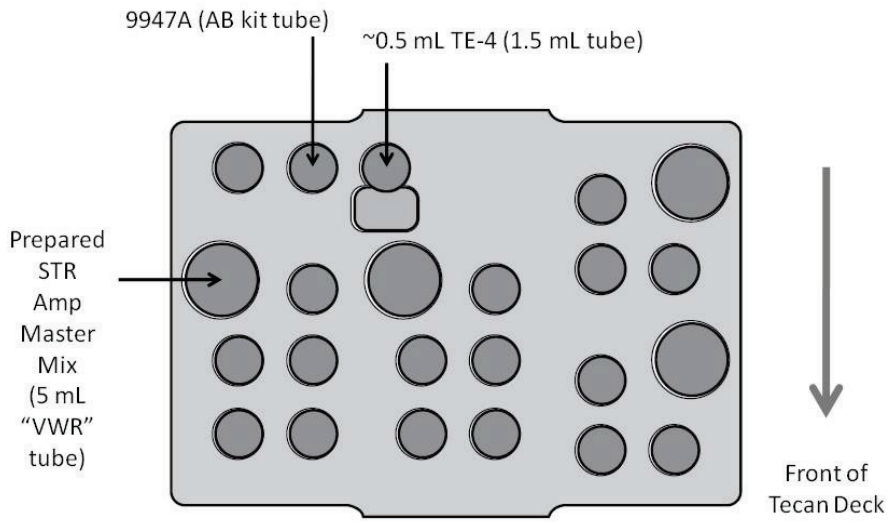


Figure 6. STR Reagent Block for the CA DOJ "*Identifiler_tubesComboManualMasterMix*" and "*Identifiler_plateComboManualMasterMix*" scripts.

Section 13.2.3.10 Load the Sample Racks or Sample Plate

Sample Format For the sample rack format, perform the following to uncap sample tubes and load racks.

Step	Action
1	• Obtain the 16-position sample racks for the run. • Re-confirm that the actual sample positions are in complete correspondence with the data in the sample input file for the run.
2	Taking care not to disturb the original order of the racks or tubes within the racks, open each tube and place it back into its position on the rack.
3	Inspect each tube to ensure that • the sample volume is at the bottom of the tube, • each cap is situated in the rack so that it is not blocking access to the opening of any sample tube, and • each tube is resting (or nearly resting) on the bottom of the rack.

4	Slide each rack into its appropriate position at grids 1-5 on the deck (reference Figures 3 and 5).

***Note**:* It is not necessary to put empty racks on the deck.

Plate Format For the sample plate format, perform the following to load the plate.

Step	Action
1	Inspect the sample plate containing extracts to ensure each sample volume is at the bottom of each well and free of potentially interfering bubbles. If needed, quick-spin the plate.
2	Place the plate at position 4 (see Figure 5), making sure to correctly orient well position A1. **Important** Position 4 for STR Setup is *different* from Position 4 for qPCR Setup!
3	Remove any plate cover sealing the wells of the plate.

Section 13.2.4 During Run Processing

Section 13.2.4.1 Overview

Introduction This section contains the procedures for performing HIDEVO150 Normalization and STR Amplification Setup.

Contents This part contains the following topics:

Topic
Section 13.2.4.2 *Opening the run script*
Section 13.2.4.3 *Performing the HIDEVO150 Normalization and STR Amplification Setup run*

Section 13.2.4.2 Opening the run script

Note: If desired, the script may be paused by clicking the ‖ button under EVOware, then restarted, at anytime during the run. Keep in mind that when the script is paused, it will stop once it finishes the current operation.

Opening the script

Follow the procedure below to open the HIDEVO150 Normalization and STR Amplification Setup script (either "*Identifiler_tubesComboManualMasterMix*" or "*Identifiler_plateComboManualMasterMix"*).

Step	Action
1	From in the *Runtime Controller* window, select **Edit an existing script.** Do NOT select Run an existing script.
2	**Start your selection** and choose either "*Identifiler_tubesComboManualMasterMix*" or "*Identifiler_plateComboManualMasterMix."*
3	Click the green arrow to continue.
4	Choose **Run** from the drop-down menu, NOT Run direct.
5	Verify the **Run full script?** box is checked.
6	Click **Run** again.

Section 13.2.4.3 Perform the HIDEVO150 Normalization and STR Amplification Setup Run

Performing the run

Follow the procedure below to perform HIDEVO150 Normalization and STR Amplification Setup.

Step	Action
1	The *HID Evolution EVOware* window should now be open behind the *Runtime Controller*. Move the *Runtime Controller* aside to proceed with the prompts in the *HID Evolution EVOware* window.
2	• Change the **Number of Samples to Process** in the run from 88 (default) to the number of total samples present in the 16-position tube racks or in the 96-well plate. ***Notes:*** – Do *not* include the 9947A and TE^{-4} on the STR Reagent Block in the sample count.

	– But do include in the count samples that may even be omitted later in the HIDEVO Sample Normalization Adjustment table. – For the sample tube format, the starting sample position is typically maintained at rack S1, position #1, although this can be changed. – For the plate format, the starting sample position is well A1 (indicated as "1" by EVOware). • Click the green arrow to continue.
3	• Bypass the request for entries of the amplification kit lot numbers by clicking the green arrow. • Record the lot numbers and all other pertinent information on the Tecan HIDEVO150 Normalization and STR Amplification Setup checksheet.
4	At the Load Worktable window, • click the loaded box for each piece of labware or rack on the deck, or • click loaded all. • Click the green arrow to continue.
5	The HIDEVO150 script will now have the POSID (barcode scanner) inventory readable contents of the deck. There will typically be items on the deck that are not barcoded, which will give rise to a POSID error indicated by a red square in the list of loaded labware. • Click the IGNORE button to ignore any such scanning errors, • Next, click the green arrow to continue.
6	At the next window, to enter the sample input file information • click on the box …, • then browse to the appropriate sample input file (STR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv) – ***Note***: After importing the sample input file, it can be edited before continuing to the next window by clicking the Edit button. • Click the green arrow to continue.
7	At the next window, to enter the 7500 qPCR quantification "map" for the run, • click Add, • then click the **Quantifiler Human** box, • then click … to browse to the appropriate filename for the run-specific quantification file (Quant_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv). • Click the green arrow to continue.
8	The software will – use data in the quantification file to create a Sample Normalization Adjustment Table for the run,

	– report the number of samples that it has determined to be within the proper range of DNA concentrations for further processing at the target amount, and – prompt the user to inspect the table. • Click the View box to inspect the Sample Normalization Adjustment Table.
9	**Important** Inspect the Sample Normalization Adjustment Table to ensure that: • the desired target amount is set correctly – typically 1 ng/µL, but should be *changed here* for other targeted quantities (*e.g.,* 0.5 ng) – the script will be diluting to within 15% of the set amount • the script will process (dilute or amplify directly) all of the desired samples; click the **Process All** button if you want the script to process even those samples that have been determined to be "out of range" for processing; • there are no surprises, – e.g., samples requiring dilutions that might need higher sample volumes than are available (in particular, samples between 0.113-0.151 ng/µL for a targeted 1 ng amplification; see Appendix II for more information); – where appropriate, the target DNA quantity can be modified (using cells in the "Req. Amt [ng]" column) for individual samples so that lower sample volumes might be used for dilutions. ***Notes:*** • The **Process All** button is used when ***all*** samples are processed in a run. If, however, samples are to be omitted, avoid using the **Process All** button. Instead, include or omit samples for processing by manually selecting each one at a time. • The ratios presented in the dilution column of the Normalization Adjustment Table are rounded by the Tecan script and, therefore, do not always indicate the actual dilution schemes that the Tecan will use for sample processing. The actual dilution schemes used are accurately listed in the printouts from the Excel STR input spreadsheet and from the HIDDetails Excel spreadsheet. • Due to the dilution schemes used by the HIDEVO150 script, the actual target concentrations are *expected* to vary by up to +/- 15% from the ideal target concentration.
10	If there are samples to not process further, omit the samples by clicking off (empty) the "Process" box and note it in the bench notes. – For example, an additional reagent blank to save for future testing of

	the extract.
11	• After reviewing the Sample Normalization Adjustment Table, click OK to continue the run. **Important** Before proceeding, make sure that the Tecan script is accurately reporting the number of samples that you wish to process in the run. If this number is different than you expect, go back to the Normalization Adjustment Table to make changes. • Click the green arrow to continue.
12	A userprompt will appear inquiring if all DiTi racks are loaded. Respond appropriately.
13	A userprompt may appear indicating there is an "SOE Smart Pipetter" error. If so, select **continue** to disregard and continue the run. The run will now be executed. See the run information below, under **Tecan Processing**.
14	Once the run is complete, a message stating *AB file generated, 1 file exported: C:\HIDEvolution_qPCRSTRfiles\AB3130Input\ STRplate_datstamp_runtime.txt* will appear. This is the 3130 plate record for downstream processing. Click the green arrow to continue.
15	Next, a message stating that a pdf report was created will appear. This file is not currently used for downstream processing and does not need to be saved. Click the green arrow again to continue. ***Note:*** This will cause the *HID Evolution EVOware* window to close.
16	In the *Runtime Controller* window, a message stating "Your script completed with errors!" will be displayed due to one or more sample volumes at < 50 µL and because the Tecan could not read a (missing) barcode on the PCR processing plate. Click **Cancel** to exit the script.

Tecan Processing

The run will proceed to dilute the samples into the PreDilution plate(s) to meet the set target DNA concentrations. Unless there are problems with the run, there will be no further user prompts until the end.

After making the appropriate dilutions, the run will proceed by loading 15 µL of prepared master mix cocktail into each appropriate position on the PCR plate. It then adds 10 µL of each template, either from the original tube/plate or from the dilution plate(s).

After this step is completed, the script will request an operator response before it proceeds to generate the report and data files for the run.

Section 13.2.5 Post-run Processing

Section 13.2.5.1 Overview

Introduction The procedures for post-run processing are detailed in this section.

Contents This part contains the following topics:

Section 13.2.5.2 Post-Run PCR Plate Handling

Plate handling Perform the procedures below following the completion of the HIDEVO150 Normalization and STR Amplification Plate Setup run.

Step	Action
1	Remove the PCR plate and visually inspect to confirm the presence of approximately equivalent volumes in the wells (~ 25 µL). • Wells may have bubbles due to mixing; this is normal. • Make appropriate notations of any wells that are obviously problematic (*e.g.,* low volumes). • The specific wells shown below in Figure 7 will be empty because

these particular positions are reserved by the EVOware software for allelic ladder during the Genetic Analyzer run. These wells are H2, H4, H6, H8, H10, and H12. If changes are made to ladder placement, the plate record should be adjusted appropriately prior to the Genetic Analyzer run.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S9	S14	S22	S29	S37	S44	S52	S59	S67	S74	empty
B	S2	S10	S15	S23	S30	S38	S45	S53	S60	S68	S75	empty
C	S3	S11	S16	S24	S31	S39	S46	S54	S61	S69	S76	empty
D	S4	S12	S17	S25	S32	S40	S47	S55	S62	S70	S77	empty
E	S5	S13	S18	S26	S33	S41	S48	S56	S63	S71	S78	empty
F	S6	POS	S19	S27	S34	S42	S49	S57	S64	S72	S79	empty
G	S7	NEG	S20	S28	S35	S43	S50	S58	S65	S73	S80	empty
H	S8	AL	S21	AL	S36	AL	S51	AL	S66	AL	empty	AL

Figure 7. HIDEVO150 STR plate layout. Sample tube positions are indicated (S#) as well as the wells reserved for the positive amplification control (POS), the negative amplification control (NEG), and allelic ladder (AL), the last of which is added during Genetic Analyzer plate setup.

2	Cover the PCR plate with a 96-well amplification mat or other appropriate cover, making sure that the seal is complete for each well.
3	Label the plate with the plate record name. This also serves as the plate unique identifier.
4	Spin down the plate and visually inspect for possible problems with amplification volumes.
5	• Turn on a 9700 thermal cycler (TC). • Confirm the amplification parameters listed below. • Record the appropriate TC information on Tecan HIDEVO150 Normalization and STR Amplification Setup checksheet. Pre-denaturation and enzyme activation: 95°C, 11 minutes Cycle (28 cycles): 94°C, 20 seconds 59°C, 3 minutes Final extension: 60°C, 10 minutes Hold temperature: 4°C
6	Place the plate into the TC 9700 and start the thermal cycler program.

Section 13.2.5.3 Post-Run Sample Handing

Post-run sample handling

Perform the following after the HIDEVO150 Normalization and STR Amplification Plate Setup run is complete.

Step	Action
1	Remove the sample tube racks or plate from the deck and re-cap each sample or re-seal the plate.
2	Store samples appropriately.
3	Remove any Predilution plate(s) from the run.
4	Seal the dilution plate(s) and store appropriately.

Section 13.2.5.4 Post-Run Tecan Clean-Up and Maintenance

Tecan clean-up/ maintenance

Perform the following after the HIDEVO150 Normalization and STR Amplification Plate Setup run is complete.

Step	Action
1	Run the *Combo_Flush.esc* maintenance script: • Choose the script • Click **Run** • Respond with **10** in the user interface to wash with 10 mL
2	If it is the last run of the day: • Exit EVOware • Respond **Yes** to moving all arms to their home positions • Turn off the HIDEVO150 by depressing the **ON** button
3	Remove the TE^{-4} trough from the Waste carrier. Since this trough was not directly exposed to any DNA (all TE transfers from the trough were performed prior to any DNA transfers), it may be re-used following a thorough rinsing with deionized water.
4	If needed: • re-fill the System Liquid carboy with de-ionized water (*e.g.,* Millipore), • empty the Waste Liquid carboy, • empty/replace the DiTi waste bag, and • wipe down the deck.

	Note: When cleaning the HIDEVO150, **<u>do not ever pour or squirt liquids (not even water) on the deck</u>**. Liquids could leak below the deck and short out electronic circuit boards. Instead, wipe the deck with towels dampened with appropriate liquids (*e.g.,* weak detergent, then water, then alcohol, as needed). Tecan carriers and reagent blocks should be treated similarly, in order to avoid exposing them unduly to corrosive agents. **Important** Never clean the instrument while it is powered on.
5	As needed, remove any carriers from the deck and clean with weak detergent, water and/or alcohol. Allow to dry.

Section 13.2.5.5 Post-Run Electronic File Handling

Electronic files

All output files generated by the HIDEVO150 run are archived automatically on the Tecan control PC, in the folders or respective subfolders of either

- ***C:\HIDEvolution_qPCRSTRfiles***\ or
- ***C:\Program Files\Tecan\EVOware\output\SOE***\.

Some of these files are to be saved for downstream processing and archiving with the respective case file electronic data. This is described in detail below and in Appendix I.

Saving files

Perform the following to save the files needed for subsequent processing.

Step	Action
1	Transfer the *HIDDetails_STR plate_<rundate>_<runtime>.csv* file to the Case or Batch Folder . – This file contains the dilution factors, plate positions, and target amplification quantities for each sample in the run. – This file can be found in the ***C:\HIDEvolution_qPCRSTRfiles***\folder.
2	Run the CADOJ_HIDDetailsConvert macro (version 1.0.1 or higher) and print the sheet for inclusion in the case file.

3	Transfer the *STRplate_<rundate>_<runtime>.txt* file to the 3130/3130*xl* Genetic Analyzer. – This file is the plate record for the Genetic Analyzer run. – This file can be found in the ***C:\HIDEvolution_qPCRSTRfiles\AB3130Input***folder.
4	If a dilution plate was created, transfer the *Predilution2_<runtime>.csv* files to the Case or Batch Folder . – There should be two files for each plate of dilutions, both located in the ***C:\Program Files\Tecan\EVOware\output\SOE\CSVHistory\rundate*** folder. ***Note:*** If *no* samples in the run were diluted by the HIDEVO150 procedure, then there will be no *Predilution2_<runtime>.csv* files.
5	If more than 48 samples were diluted for the run, there will also be two *Predilution1_<runtime>.csv* files. Save these files as described in the previous step.

Section 13.2.5.6 Post-PCR Plate Handling

PCR Plate

Perform the following after amplification is complete and the thermal cycler has reached the final hold temperature.

Step	Action
1	Remove the 96-well amplification plate from the instrument block.
2	Either: • proceed to capillary electrophoresis using the appropriate procedure, *or* • place the 96-well plate in storage protected from light with an appropriate seal. – ***Note***: The products can **either** be stored in a refrigerator for short periods of time *or* in a freezer for longer periods.

Section 13.2.6 References

Reference documents

Benfield, J., "Validation of HID EVOlution™ System: Automation and Integration of Quantification and STR Analysis for High Throughput Sample Processing," powerpoint presentation at the 60th Annual Scientific Meeting of AAFS, Washington, DC, 2008.

User Bulletin: HID EVOlution™ – Combination System, July 2009, Applied Biosystems (Part Number 4427707 Rev. A)

HID EVOlution - Combination System Application Manual, 2009-06-02, Tecan (Part number: 30029232.00, ID: 395967, en, Version 1.0)

HID EVOlution™ – qPCR/ STR Setup System Getting Started Guide, July 2009, Applied Biosystems (Part Number 4426903 Rev. A)

Stray, J., Nguyen, V.T., Benfield, J., Fang, R., Brevnov, M., Treat-Clemons, L., Porter, G., Furtado, M.R., and Shewale, J.G., "A dedicated automated system for extraction, quantification and STR amplification of forensic evidence samples," FSI-Genetics Supplement Series, Volume 2, Pages 64-65, 2009 (Progress in Forensic Genetics 13 - Proceedings of the 23rd International ISFG Congress).

Fang, R., Liu, J.Y., Kijenski, H.L., Benfield, J., Wong, A., Lagacé, R., Cassel, M.J., Nguyen, V.T., Lauber, W.M., Abeln, D., Treat-Clemons, L., Furtado, M.R., and Shewale, J.G. "The HID EVOlution System for Automation of DNA Quantification and Short Tandem Repeat Analysis," Journal of the Association for Laboratory Automation, Volume 15, Pages 65-73, 2010.

Section 13.2.7 Appendix I - Electronic File Naming Conventions, Storage, and Organization

Introduction

Electronic file naming and organization should be as described herein to facilitate the tracking and transfer of such files. Files not mentioned within this appendix, do not need to be retained.

Files created by the operator

The sample and quantification input files created by the operator of the Tecan using Excel macros are named using both common and unique identifiers to facilitate tracking. These files are as follows:

- qPCR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv
- 7500PlateMap_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.txt

- STR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv
- Quant_*<instrument name>*_*<MMDDYY>*_*<operator initials>*csv

All four files are created when performing qPCR setup, normalization, and STR amplification setup using the HIDEVO150. The latter two are created when performing only normalization and STR amplification setup using the HIDEVO150 (*i.e.* using a manual qPCR setup).

Notes:

- Identifiers represented within < > may change with each run and collectively represent a unique identifier.
- MMDDYY represents the date (month, day, year) of the run.
- Instrument name represents a unique name for the particular Tecan used to perform the run.
- The files may have "SF" or "NSF" prefixes for sperm fraction or non-sperm fraction plates; this is generally paired with the alkaline differential extraction.

Files created by EVOware

Electronic files created by Tecan EVOware software are automatically named with unique identifiers. The only files of this type that should be saved to the Case or Batch Folder are as follows:

- HIDDetails_STR plate_*<rundate>*_*<runtime>*.csv
- STRplate_*<rundate>*_*<runtime>*.txt (plate record for 3130/3130*xl*)
- Both Predilution2_*<runtime>.csv*
- Both Predilution1_*<runtime>.csv*

Note:

- Runtime and rundate are automatic stamps assigned by EVOware to the HID details and plate record files.

File organization

The aforementioned files should be stored within each Case or Batch Folder in a subfolder named *<instrument name>*_*<MMDDYY>*_*<operator initials>*. Until this folder has been copied to the Case or Batch Folder , it may be temporarily stored in another folder named *<instrument name>*_HIDEVO and this second folder may reside on the desktop of the Tecan computer.

<u>For example</u>:
*<instrument name>*_HIDEVO **OR** LLYY#### CF <analyst initials>

- *<instrument name>*_*<MMDDYY>*_*<operator initials>*
  - qPCR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv
  - 7500PlateMap_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.txt
  - STR_*<instrument name>*_*<MMDDYY>*_*<operator initials>*.csv

- Quant_<*instrument name*>_<*MMDDYY*>_<*operator initials*>csv
- HIDDetails_STR plate_<*rundate*>_<*runtime*>.csv
- Both Predilution2_<*runtime*>*.csv*
- Both Predilution1_<*runtime*>*.csv*

- RF_ STR plate_<*rundate*>_<*runtime*>
  - STRplate_<*rundate*>_<*runtime*>.txt

Notes:

- The Case or Batch Folder is created for long-term storage of electronic files associated with a particular case. The naming convention is:
  - For Case Folder: LLYY#### CF <analyst initials>, where LLYY#### represents a BFS laboratory case number.
  - For Batch Folder: RunFolderUniqueIdentifier BF <analyst initials>
- The 3130/3130*xl* plate record is initially stored with the other electronic files but ultimate long-term storage is generally in the 3130/3130*xl* run folder.

File storage

The electronic files saved to the Case or Batch Folder are copied to one CD for the case file following data analysis. The electronic Case or Batch Folder is additionally stored on a secure network.

Multiple runs

In the circumstance where two or more of the same file/folder types are generated on the same day, they may be distinguished by the addition of -2, -3, etc. to the end of the relevant file/folder name(s). For example, STR_<*instrument name*>_<*MMDDYY*>_<*operator initials*-2>.csv.

Section 13.2.8 Appendix II - HIDEVO150 Dilution Schemes & Processing Highlights

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Introduction

When needed, the HIDEVO150 performs normalization of DNA extracts in preparation for STR amplification plate setup. The HIDEVO150 normalization script will typically use ≤ 10 µL of extract to achieve the target DNA concentration for STR amplification. Because the Tecan will always add a 10 µL volume to the 96-well amplification plate, a dilution will be created for those samples where less than 10 µL is required in order to accommodate the target amount.

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1 ng Target Input

Figure 8 is a representation of the dilution scheme used by Tecan EVOware when the ***target input DNA is 1 ng*** for STR amplification in a 25 µL PCR reaction volume and the DNA is normalized for a volume of 10 µL to be added to the reaction (*i.e.,*

Identifiler Plus).

Figure 8. Dilution scheme for 1 ng DNA targetquantity for PCR input

If your sample		then the dilution factor is*	First Dilution Mixture		Second Dilution		Volume of D1 or D2 added to STR plate (uL)	Min Recommended Extract Volume (uL)			
Min (ng/uL)	Max (ng/uL)		Volume of DNA extract added to D1 (uL)	TE-4 added to D1 (uL)	Volume from D1 added to D2 (uL)	TE-4 added to D2 (uL)			Minimum Target Input DNA (ng)	Maximum Target Input DNA (ng)	Average Target Input DNA (ng)
0.000	0.113	no dilution	10uL sample transferred directly to STR plate				n/a	15	0	1.13	
0.113	0.151	1.3	18.55	6.45	0	0	10	21.55	0.84	1.12	0.98
0.151	0.209	1.8	11.00	9.00	0	0	10	15	0.83	1.15	0.99
0.209	0.279	2.5	8.16	11.84	0	0	10	15	0.85	1.14	1.00
0.279	0.369	3.2	6.16	13.84	0	0	10	15	0.86	1.14	1.00
0.369	0.490	4.3	4.66	15.34	0	0	10	15	0.86	1.14	1.00
0.490	0.649	5.7	3.50	16.50	0	0	10	15	0.86	1.14	1.00
0.649	0.857	7.5	2.66	17.34	0	0	10	15	0.86	1.14	1.00
0.857	1.143	10.0	3	27.00	0	0	10	15	0.86	1.14	1.00
1.143	1.500	13.3	3	37.00	0	0	10	15	0.86	1.13	0.99
1.500	1.846	17.2	2.32	37.68	0	0	10	15	0.87	1.07	0.97
1.846	2.297	20	2	38.00	0	0	10	15	0.92	1.15	1.04
2.297	3.097	27	2	38.00	29.68	10.32	10	15	0.85	1.15	1.00
3.097	4.174	36	2	38.00	22.00	18.00	10	15	0.85	1.15	1.00
4.174	5.581	49	2	38.00	16.32	23.68	10	15	0.85	1.14	1.00
5.581	7.385	65	2	38.00	12.32	27.68	10	15	0.86	1.14	1.00
7.385	9.796	86	2	38.00	9.32	30.68	10	15	0.86	1.14	1.00
9.796	12.970	114	2	38.00	7.00	33.00	10	15	0.86	1.13	1.00
12.970	17.140	150	2	38.00	5.32	34.68	10	15	0.86	1.14	1.00
17.140	22.980	200	2	38.00	4.00	36.00	10	15	0.86	1.15	1.00
22.980	30.860	270	2	88.00	6.66	33.34	10	15	0.85	1.14	1.00
30.860	40.760	360	2	88.00	5.00	35.00	10	15	0.86	1.13	0.99
40.760	54.000	470	2	88.00	3.83	36.17	10	15	0.87	1.15	1.01
54.000	72.000	632	2	88.00	2.85	37.15	10	15	0.86	1.14	1.00
72.000	93.910	837	2	88.00	2.15	37.85	10	15	0.86	1.12	0.99
93.910	120.000	1080	2	88.00	2.50	57.50	10	15	0.87	1.11	0.99
120.000	154.290	1337	2	88.00	2.02	57.98	10	15	0.90	1.15	1.03
154.290	205.710	1800	2	88.00	2.00	78.00	10	15	0.86	1.14	1.00
205.710	266.670	2479	1.50	148.50	2.42	57.58	10	15	0.83	1.08	0.95
266.670	342.860	2985	1.50	148.50	2.01	57.99	10	15	0.89	1.15	1.02
342.860	400.000	4000	1.50	148.50	2.00	78.00	10	15	0.86	1.00	0.93

Note: Dilutions should be changed accordingly with changes to target input quantity.

Order of processing

All samples requiring a one-step dilution are processed first, followed by those requiring a two-step dilution.

Placement of dilutions

For two-step dilutions, the first (D1) and second (D2) dilutions occur in the same predilution plate in adjacent wells. Therefore, there may be samples requiring two-step dilutions in a run but only one dilution plate needed. The second dilution plate is used when dilutions are needed for ≥ 48 samples.

When normalization using two-step dilutions occurs, these dilutions are started in a new column of the predilution plate which can therefore leave some empty wells following the one-step dilutions.

The two predilution plates loaded onto the Tecan work surface provide enough wells for a two-step dilution of the maximum number of samples (80).

Additional information

For additional information on DNA normalization on the HIDEVO150 system, see Appendix D, Dilution Protocols of the HID EVOlution – qPCR/ STR Setup System Getting Started Guide.

Section 13.2.9 Appendix III - 3130/3130*xl*-Related Preference Modifications

Introduction

Two modifications are made to software preferences when processing samples on the Tecan Freedom HIDEVO150 Combo System. One change is made in the EVOware software and the other in the 3130/3130*xl* Data Collection software. Both relate to the downstream process of capillary electrophoresis on the Genetic Analyzers. The modifications are described in detail below.

EVOware Preferences

The default names for both the Results Group and Instrument Procedures are HIDEvolution. The BFS Laboratories typically use Identifiler_Generic and Identifiler5sec. Therefore, the plate record creation preferences can be set to reflect this.

HID EVOWizard

Perform the following to edit the default names for the Results Group and Instrument Procedures from HIDEvolution to Identifiler_Generic and Identifiler5sec, respectively.

Step	Action
1	Log out of the EVOware software.
2	Login to the EVOware software again but as an administrator. Login: **Admin** Password: **admin**
3	Choose **Edit an existing script** and click **Start your selection**.
4	Choose the *Identifiler_tubesComboManualMasterMix* script and/or the *Identifiler_plateComboManualMasterMix* script.
5	Scroll-down through the Script Editor window to line 14.
6	Select this line by clicking once on it.
7	Click on **Edit Parameters** under INFOPAD Process.
8	Advance through the wizard until AB Input Parameters is reached; the plate record creation options are available on this “page.”
9	Delete HIDEvolution from the Results Group text box and enter **Identifiler_Generic**.

10	Delete HIDEvolution from the Instrument Protocol text box and enter **Identifiler5sec**.
11	Continue advancing through the wizard. • The wizard will automatically close when you click through the last page.
12	Log out of the EVOware software.

3130/3130*xl* Data Collection Preferences

In order to have a unique identifier for a particular run appended to sample file names, Plate ID should be used along with the Capillary Number and Well Position. This necessitates modifications to the preferences of the Identifiler_Generic results group in Data Collection. The modified results group may be used for both manual and Tecan-automated amplification setup.

Results Group

Perform the following to edit the sample file naming preferences of the Identifiler_Generic results group in Data Collection.

Step	Action
1	In the tree pane of the Data Collection software, go to **GA Instruments > Results Group**.
2	Click once on **Identifiler_Generic** and then on **Edit…** .
3	Select the **Naming** tab.
4	Under the *Sample File Name Format* section, use the *Format* drop-down menus to choose: • Well Position • Sample Name, • Capillary Number • Plate ID
5	Click **OK** to save the changes to the results group.

Section 13.2.10 Appendix IV - Manual Data Entry

Introduction

If normalization and STR amplification setup is desired for a group of samples in which no csv file exists in the correct format with the sample quantitation values, the analyst may proceed following the steps described in this appendix.

Manual Entry

To manually enter sample and quantitation information, proceed with the steps below.

Step	Action
1	Open the Excel template *CADOJ_HIDEVO_STR_v1_0_3.xltm* or *CADOJ_HIDEVO_STR_PlateInput_v1_0_0.xltm*, or higher version, and enable macros.
2	• Run **Import_qPCR_CSV** to import the file *Data_dot_csv_template_for_manual_STR_setup_v1_0.csv* (or higher version if performance checked), which is an empty Data.csv file. • Run **Convert_to_Tecan** to populate the tables.
3	Manually enter the samples to be run into the middle table and the corresponding concentrations. Remember to match reagent blanks and substrate samples to the appropriate concentration. *See Step 6 of Section 13.2.3.4 Generate Input Files for Notes on Sample Entry.*
4	Click **Export_STR_Sample_CSV** to generate the sample input file. Name the .csv file as *STR_<instrument name>_ <MMDDYY>_<operator initials>*.
5	Click **Export_Quant_Data_CSV** to generate the quantification input file. Name the .csv file as *Quant_<instrument name>_ <MMDDYY>_<operator initials>*.
6	Record both file names on the appropriate checksheet and transfer the files to the Tecan computer.
7	Run **PrintLoadMap** to print the sample input sheet for inclusion in the case file.
8	Proceed to **Section 13.2.3.5, Build Sample Racks or Sample Plate(s) for the Run**, to continue with the run setup.

Manual History

Section & Comment	Manual Issue Date	Change No.	Revision	Author/ Reviewer
Previous manual history entries archived with revision 17 of TP-6. From this date forward, all revision dates listed in the manual history will be the date of issue of the document as opposed to the past practice of documenting the various dates of revision by the author(s).	10/10/2014	N/A - Admin	18	BFS QA Unit
-Section 3.2.1: Storage condition information removed while such information in Volume IV (Section 5.1.2) was updated	10/10/2014	CR-14-019	18	GAS/JW
-Section 1.1 & corresponding worksheets (**DNAWS-68,-71**: Added prompt to record type of microscopy. Also added kit/reagent exp.	10/10/2014	CR-14-051	18	GAS/JW
-Sections 13.1.3.5 & 13.2.3.6, Steps 3-5; Section 1.5.5.2, Steps 3-4: moved EVOScrub and DiTi/screw checks from routine procedures, as evaluated by a deviation. Placed in EVOScrub appendix of 13.1. This way, they are collectively performed/check together & biweekly. Made relevant updates to this Biweekly Maintenance appendix. Added Tecan & hard drive reboot based on Tecan training recommendations. Deleted *Before you begin* blocks. Modified corresponding worksheets: **DNAWS-87, -95, -100, -101, -111**.	10/10/2014	CR-14-097 (RIC-14-012)	18	GAS/JW
-Section 3.9.5, 4.3.5, & 11.3.5: Updated *Printing Specifications* & *Notes about Printing* blocks to remove printing of GS600 electropherograms & tabular data. Updated corresponding worksheets **DNAWS-106, -107, -108**.	10/10/2014	CR-14-098 (RIC-14-013)	18	GAS/JW
-Section 1.1: info-mapped; removed sentence to place sample in tube (excessive detail); added note to record lot #s (consistent w/ 1.4 & 1.5); removed phrase to place bone in microfuge tube (excessive detail/sometimes larger tubes); removed extra statements to modify volumes; removed outdated Microcons; incorporated PrepFiler transitions (diff extr); removed references to Extraction Recovery Worksheet (option to not use); moved NSs into procedure body (routinely used) and modified title of appendix remainder accordingly; added brief spin to Step 7 of 1.1.3.4 and changed phenols from "2-3" to "2" to match simple organic; added blocks on concentrating sample & reconstituting with water (same as other extraction procedures); modified batching block in 1.1.3.2 to apply only to manual extractions -Section 1.1.5, Appendix I: added notes block; made minor adjustments to reagent recipes to more closely match corresponding worksheets; edited ddH2O to dH2O in 1M DTT recipe -Section 1.3: info-mapped; added blocks on concentrating sample & reconstituting with water (same as other extraction procedures); clarified that blood samples from autopsy may be extracted with Maxwell; moved Prepared Lysis Buffer from Appendix I into liquid blood steps; deleted Appendix I remainder	10/10/2014	CR-14-108 (RIC-14-014)	18	GAS/JW

(recipe for 1M DTT b/c in Section 1.1.5) -Outdated worksheets **to be archived**: **DNAWS-6, -23, -24, -25, -34** -Section 1.3.3.1: modified reference samples for Maxwell to clearly include blood samples collected at autopsy -Section 1.4: to reflect regular use of kit now (versus in-house prep), flip-flopped reagent contents with name throughout, updated reagent list, modified *Note* to *Abbreviation key*, & deleted Section 1.4.7 reagent appendix; made corresponding changes to **DNAWS-90** and **DNAQCWS-22,** and changed "batch" to "kit" lot; worksheets **to be archived: DNAQCWS-23,-24, -25,-26,-27,-28** -Section 1.4.3.2: removed outdated Step 1 to thaw DNase buffer; made corresponding change to **DNAWS-90** -Sections 1.5, 13.1 & 13.2: Added Tecan warnings/harards -Section 1.5: Clarified TE versus EB on **DNAWS-110**; clarified NS fraction processing on **DNAWS-109**; created **new worksheet DNAWS-114** for diff's using PrepFiler -Section 2.1.1: removed individual tube statement (out of date) -Section 2.1.2: added "or equivalent" to plasticware list, updated vendor info -Sections 2.1.4.5, 2.1.5.3.2, 2.1.5.3.3, 2.1.5.3.4: modified to include R^2=0.98 as acceptable (i.e. ≥); made corresponding change to **DNAWS-28** -Sections 2.1.5.4: modified wording on RBs w/ >LQ signal to match 1.1 & QAS; added wording on ECBA (from 13.1) -Section 3.2.3.2: removed note before steps because repeated verbatim in Step 4 -Sections 3.5.2.1, 3.9.1, 4.2.4.3, 4.3.1, 11.2.4.3, 11.3.1: Inserted "size standard and" to TR block/paragraph, "The reviewer also checks the electronic or printed *size standard and* allelic ladder data." -Section 3.8.3.7: modified section title ("/Import"), combined Steps 7 & 8 in Import a Plate block, adjusted **DNAWS-105** -Seciton 3.8.3.9: Modified Step 5 from "export" to "txt file" and same change to **DNAWS-105** -Sections 3.9.3, 4.3.3, 11.3.3: corrected Step 3 "file" to "edit" & removed from corresponding worksheets DNAWS-106,-107,-108; modified Step 10 size standard default settings statement to be consistent with data processing -Section 3.9.3: modified wording in Step 10 to reflect practice; added note on project naming to Step 11 -Section 3.9.4: corrected allelic ladder figure # from 3 to 2; added stutter to list of extraneous peaks for editing -Sections 3.9.4, 4.3.4, 11.3.4, Steps 1-2: corrected IDX allele edit wording -Section 3.9.8, Step 5: modified to not select bringing low quality samples to the top due to IDX 1.4 bug -Section 3.9.11 Appendix V: newly added to manage audit records in ID-X -Section 3.10.5: Updated N+4 to tiers and updated offscale wording to include 3500				

-Section 3.10.10, Step 8: added "Plots" for IDX -Section 4.4.4.3.2: added IPA language to stutter/somatic mutation bullet (inadvertently left out of YF TP when added but checksheet updated with others) -Section 7: added dH_2O & ECBA -Section 13.1 & 13.2: updated hazards & warnings based on Tecan training; added note to 13.1 Appendix re: EVOscrub v. RoBoScrub -Fixed headers, typos, & cross-references and made minor grammar edits throughout; added or replaced volume numbers with TP document number throughout -Where relevant throughout, updated Applied Biosystems to Life Technologies/Applied Biosystems, or simply removed Applied Biosystems				
-Sections 1.1.5 Appendix I, 1.4.7 Appendix I, 1.5.7 Appendix I, & 1.5.8 Appendix II: updated to reflect minimum quality requirements, even though components not "critical" -Section: updated to reflect minimum quality requirements -Reagent and Equipment (supply) lists throughout: although supplies are not critical, added minimum quality requirements to relevant plasticware and made minor adjustments to lists for consistency; added manufacturer to critical equipment when unique; note that quality requirements for reagents & services are described in TP Vol.IV -Removed ethanol (& bleach) from procedure reagent lists when listed for the purpose of cleaning to not be confused with "critical" ethanol	10/10/2014	CR-14-091	18	GAS/JW
Section 4.4: -Modified Sections 4.4.5 and 4.4.6 of Y-STR Interpretation and Conclusions to align more closely with ASCLD/LAB-Int. Standard 5.10.3.5 and SWGDAM Y-STR guidelines on haplotype mixture comparisons and statistics -Section 4.4.7 changed "should" to "must" for providing a statistic in the report when there is an inclusion -Section 4.4.8 added 2009 and 2014 SWDGAM Y-STR interpretation guidelines references	1/29/2015	RIC-15-008 (CR-15-027)	19	GAS/JW
-Section 1.2 – Inserted QIAcube procedure, equipment, reference manual, and Appendix; listed new & edited diff worksheets for clarity – these include **DNAWS-68, DNAWS-114, NEW DNAWS- 115, NEW DNAWS- 116**; additionally created microscopy sheet for new modular slide 1.1.3 section – **NEW DNAWS- 117**	9/30/2015	CR-15-118 (RIC-15-018)	20	GAS/JW
-Section 1.1 – Split and modularized into two sections 1.1 and 1.2; edited titles, info-mapping, steps, cross-references as appropriate; added overviews and intros as needed for clarity; created new section Making a Slide -Sections 1.1/1.2 – Made slide creation optional, including for differentials -Section 1.1.1 – Edited trace DNA swabbing to clarify; deleted "Cases may be batched where the type of evidence is similar and the number of total samples is manageable."	9/30/2015	CR-15-119 (RIC-15-020)	20	GAS/JW

-Section 1.1.2 – Modified must to should: At least one QC sample should be run; added statement about when to introduced QC sample during a differential extraction; deleted now irrelevant note on slides -Section 1.1.3 – Edited PBS volume to match worksheets; added note regarding use of MN spin baskets; treat RBs same with extra washes; added detail when performing extra digestion -Section 1.1.4 – Shortened note on avoiding contamination -Section 1.1.5 – PCIA clarification -Section 1.2 – Edited "epithelial" and "E. cell" to "non-sperm" when referring to fraction for document uniformity -Section 1.2.3 – Edited note about slides; Step 7: Added note about using Machery-Nagel spin baskets; Step 13: Added clarification to use same type of clean-up on all samples, & also for Step 7 of Organic processing in Differential Extraction -Section 1.2.4, 1.3.3.3 - Added note regarding ease of use of MN spin baskets -Section 1.3.3.3 – Removed reference to lysis buffer prep appendix deleted in previous version – this info was incorporated into the procedural steps for liquid sample and inadvertently left out of dried sample steps, so corrected here. -Section 1.4.2 – Modified reagent blank requirement from "must…" to "should contain at least two"; added sterile water to abbreviation/kit list **-DNAWS-90** – Step reference typo fixed; removed lot info for water since included with kit **-DNAWS-91** – Changed "QC Batch" to "Alk Diff Kit lot" since all reagents used come from the kit -Section 1.5 – Removed teeth and tissue from list of unvalidated substrates since training samples show these to work fine with PrepFiler; edited E. cell to non-sperm; inserted tube-to-plate option throughout, including editing **DNAWS-111**; edited cell phone warning distance from meters to feet; corrected Tecan isopropanol volume from 345ul to 207ul; updated Excel worksheet version reference; added naming restriction on spaces; added note about importance of emptying DiTi waste; added note to periodically check RoMa; "block" versus "position" clarification; added newer EVOware (v.2.4) script names; made automated/Tecan overview more generic since 4 scripts now; added "of dH2O" to post clean-up for completeness **-DNAWS-109** – Added discard/save substrate -Section 2.1.1: Added two notes regarding potential data loss from runs over the network and mousing -Section 2.1.8: Added troubleshooting appendix -Section 3.7.2.1 – Added option to use 3-allele loci when calculating Mx without an assumed donor for major/minor peaks with exclusive pairing -Sections 3.9.3, 4.3.3, 11.3.3 – Updated batch project wording -Sections 3.9.5, 4.3.5, 11.3.5 – Updated wording about printed ID-X pages containing unique identifier -Section 3.10 – added FBI pop data erratum reference -Section 4.1.3.2 – added to Step 3: "*Note: Less than 0.5 ng*				

positive amplification control may be amplified" -Section 7: Added FRS, LTS abbreviations -Section 13.1.1, 13.2.1 - edited cell phone warning distance from meters to feet -Section 13.1.3.3 – added note to skip this section if proceeding from PrepFiler; removed script references since several now (added table instead) -Section 13.1.8 – Added Step 6 in General Maintenance of Biweekly Maintenance to run RoMa script, moved from Step 12 of Maintenance and Cleaning in Section 5.2.7.2 of TP-21 -Updated **DNAQCWS-10** -**DELETED** *Section 3.5* IDP/GMID v.3.2, *Section 4.2* YF/3130/GMID v.3.2, *Section 11.2* MF/3130/GMID v.3.2 -**Archive DNAWS-5** 3130 (for MF), **DNAWS-10** YF GMID, **DNAWS-37** YF 3130, **DNAWS-85** MF GMID, **DNAWS-98** IDPlus GMID -General grammar and cross-reference corrections throughout; corrected EVOWare to EVOware; added "sterile" where absent in references of dH2O used with evaporated samples				
-NEW Section 2.1.7 Appendix I inserted on using new HID 7500 software for Quadruplex; Added notes within Section 2.1 to reference new appendix (& modified #s of other appendices); modified **DNAWS-28, -29, and -30** for analysis with new software	9/30/2015	CR-15-120 (RIC-15-021)	20	GAS/JW