The purpose of this validation was to develop a more refined approach to applying N+4 stutter thresholds to Identifiler Plus / 3500 Genetic Analyzer data. It has previously been demonstrated in both peer-reviewed literature and in-house BFS studies that N+4 stutter, while lacking reproducibility in detection, does show a clear trend of increasing N+4 percentage with decreasing parent allele peak height. Thus it was decided that the existing global 9.2% filter, which was based on the highest peaks observed in the BFS Identifiler Plus Internal Validation data, could be modified to a tiered approach.

This work included the collection of N+4 Identifiler Plus 3500/3500xl Genetic Analyzer data, the determination of the tiers, and the testing of a modified MixMaster version that incorporated the tiers. It additionally included an assessment of the N+4 filtering capabilities of GeneMapper ID-X 1.4.
N+4 Percentage Tiers for Identifiler Plus 3500/3500xl Genetic Analyzer Data

To collect N+4 data points from Identifiler Plus 3500/3500xl Genetic Analyzer data, five previously generated runs were reanalyzed using a reduced analytical threshold (Blue, Green, Yellow-40rfu, Red-50rfu, Orange-150rfu) to maximize data capture. All other analysis parameters followed the standard technical procedures. The runs were from analyst 3500 training competencies (3), the PrepFiler reproducibility validation (1), and a 3500 software performance check (1); these runs collectively included > 50 single-source sample extractions and amplifications. All potential N+4 stutter peak data was exported from ID-X and compiled in Excel. N+4 percentages were calculated as the N+4 peak height divided by parent allele peak height. Demonstrating the anticipated trend of increasing N+4 percentage with decreasing parent allele peak height, it was then evaluated for tier threshold placement. A total of 479 N+4 data points were included. Following this analysis and discussion with the DNA Technical Advisory Group, it was determined that the thresholds for Identifiler Plus data run on the 3500/3500xl Genetic Analyzer will be as follows:

- 9.2% for parent allele peak heights of ≤ 2499 RFU
- 5% for parent allele peak heights between 2500 – 5200 RFU
- 2% for parent allele peak heights ≥ 5200 RFU

The four data points (indicated in red) deviating from the trend are likely due to a somatic mutation, all derived from the same allele of the same locus from the same person; note that only one exceeds the tiers. Furthermore, no overall locus-specific trends were observed.
MixMaster IDP 3500 (Rel. 1.2) Modified Software Validation

MixMaster IDP 3500 was then modified to Rel. 1.2 which applies the three tiered filters for N+4 stutter peaks, rather than the one overall global filter of 9.2%. Specifically, the MixMaster Rel. 1.2 version accounts for N+4 stutter by subtracting at each cycle a randomly assigned value within:

- 0-9.2% of the parent peak from the N+4 position peak if the parent peak is within the GMID-X peak amplitude/analytical threshold (150 RFU in this case) to 2499 RFU.
- 0-5.0% of the parent peak from the N+4 position peak if the parent peak is within 2500 RFU – 5199 RFU.
- 0-2.0% of the parent peak from the N+4 position peak if the parent peak is at least 5200 RFU.

MixMaster Rel. 1.2 additionally includes a minor designation change of Major/Minor to Donor 1/Donor 2.

Testing of the various N+4 tiers in both N+4 positions as well as N-4/N+4 overlap positions was assessed by evaluating one cycle (2500th) of mock data in two ways. First, mock data was evaluated at two loci (D7S820 and CSF1P0) which use a 10% N-4 stutter filter (for ease of calculations). This test evaluated indirectly whether MixMaster was subtracting N+4 stutter within the appropriate peak height range. In all 19 instances, it appeared to function as expected.

Secondly, MixMaster Rel. 1.2 was evaluated by modifying the default N-4 stutter settings to 0% for the first four loci (D8S1179, D21S11, D7S820, CSF1P0). One-, two-, three-, and four-allele contrived data was entered into these four loci, respectively, to assess N+4 subtraction recorded on the different Excel allele pages. MixMaster was run a total of 6 times, resulting in 16 checks of the ≤ 2499 RFU tier, 10 checks of the 2500-5199 RFU tier, and 10 checks of the ≥ 5200 RFU tier. All data was within the correct N+4 range.

The change from Major/Minor to Donor 1/Donor 2 was assessed for correct designation in the MixMaster report and for functionality of the “Assumed Donor Override.” Donor 1 was correctly swapped for Donor 2 when the override switch was requested. Furthermore, the No Assumed/No Filtered report was correctly filtered and reduced when tested.

Therefore, all results were correct and as expected for this evaluation of MixMaster IDP 3500 (Rel. 1.2).

GMID-X Evaluation

Because GMID-X (version 1.4) has the ability to apply global (and locus-specific) N+4 stutter filters, this functionality was assessed for the purpose of using it at 2% for casework. This is the lowest filter of the three tiers and therefore can be applied globally to all data. The analyst would then apply the 5% and 9.2% filters manually, as relevant. The evaluation revealed that GMID-X applies the N+4 filter correctly. However, it does not apply N-4 and N+4 filters additively for a peak in an N-4/N+4 overlap stutter position; rather, the filters are applied independently. Thus, while the use of a 2% N+4 global filter performed by GMID-X will speed and simplify analysis, the analyst will need to manually calculate the percentages for peaks in question that reside in N-4/N+4 overlap positions.