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The following summary report is a compilation of the conclusion sections in the Identifiler Plus Internal Validation conducted by the Forensic Biology Unit of the Department of Forensic Sciences Forensic Science Laboratory. This validation was conducted and completed during the transition of the Unit from the Metropolitan Police Department to the Department of Forensic Sciences.

Applied Biosystems® AmpF*l*STR® Identifiler® Plus PCR Amplification Kit Validation Using the Applied Biosystems® 3130*xL* Genetic Analyzer

I. Precision Study

The measurement error inherent in any sizing method can be defined by the degree of precision in sizing an allele multiple times. Precision is measured by calculating the standard deviation in the size values obtained for an allele that is run in several plate injections on a capillary instrument (or in several lanes of one gel).

To assess precision of the 3130*xl* instrument and the genotyping software, 16 Identifiler® Plus allelic ladder aliquots were injected six (6) times. Sizing averages, minimum sizes, maximum sizes and standard deviations were calculated for each allele for all loci within a plate injection and across all plate injections. No samples were deleted from the precision study.

All samples were analyzed using GeneMapper® ID Software Version 3.2.1 with the Identifiler® Plus Analysis Method supplied by Applied Biosystems®.

Within injections, the data shows no markers and/or alleles with high standard deviation values (exceeding 0.15 base pairs). Across injections, the data indicates that high standard deviation values were obtained from the following loci: CSF, D2, D18, and FGA. These are the largest loci in the Identifiler® Plus ladder and the high standard deviation values can be attributed to the wide range of base pair sizes at these loci.

This data supports the preference of exporting the data from the instrument as a run folder after each injection. However, it is still acceptable to export the data as multiple injections in one run folder. Placement of controls will need to be considered depending on the method of data export chosen by the analyst. A further evaluation of these two possible methods for data export was conducted in the Accuracy Study of this validation.

II. Sensitivity Study

a. Sensitivity_1 (28 cycles)

For this sensitivity study, serial dilutions of four (4) DNA samples were prepared to assess amplification and instrument performance, determine the optimal quantity of template DNA for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit and to help understand interpretational limitations of the PCR-based genotyping system over a range of input DNA amounts. All samples were amplified for 28 cycles.

Genotyped data from this study was evaluated for genotype concordance, allelic dropout, peak height, heterozygote peak height ratios and potential artifacts to help establish standard operating parameters, optimal DNA input amount(s) and interpretation guidelines.

This sensitivity study was performed prior to the annual planned maintenance of the 3130*xl* A. An optical adjustment was made and a performance check was completed. This check demonstrated an overall increase in the sensitivity of the instrument. An additional sensitivity study (sensitivity_3) was performed to evaluate the Identifiler® Plus kit under the current laboratory conditions.

The recommended target DNA input for amplification using the Identifiler® Plus kit at 28 cycles is approximately 1.0 ng. The 1.0 ng target produced normalized peak height from 650 to 2100 rfu. Offscale data and signs of overamplification are present for targets greater than 1.0 ng.

For the peak height ratio (PHR), sample 1001 produced low peak height ratios at FGA. Without this sample's data, a PHR of 55% is recommended.

The majority of OL and non-matching alleles at the 0.5-1.0 ng target range were eliminated by setting the peak amplitude threshold (PAT) at 70 rfu. The remaining OL and non-matching alleles include stutter (n+4 and n-4), elevated baseline and pull up. At this PAT, allelic dropout began at the 0.125 ng target. The PAT will be further evaluated at the conclusion of each study.

Using the data obtained in this study, an appropriate stochastic threshold for a target input of 1.0 ng is ~200 rfu's. This threshold will also be further evaluated at the conclusion as more data is obtained.

b. Sensitivity_2 (29 cycles)

For this sensitivity study, serial dilutions of four (4) DNA samples were prepared to assess amplification and instrument performance, determine the optimal quantity of template DNA for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit and to help understand interpretational limitations of the PCR-based genotyping system over a range of input DNA amounts. All samples were amplified for 29 cycles.

Genotyped data from this study was evaluated for genotype concordance, allelic dropout, peak height, heterozygote peak height ratios and potential artifacts to help establish standard operating parameters, optimal DNA input amount(s) and interpretation guidelines.

This sensitivity study was performed prior to the annual planned maintenance of the 3130*xl* A. An optical adjustment was made and a performance check was completed. This check demonstrated an overall increase in the sensitivity of the instrument. An additional sensitivity study (sensitivity_4) was performed to evaluate the Identifiler® Plus kit under the current laboratory conditions.

An increase in sensitivity was observed for samples amplified at 29 cycles. The recommended target DNA input for amplification using the Identifiler® Plus kit at 29 cycles is 0.5-1.0 ng. The 1.0 ng target produced normalized peak heights at 1500-4000 rfu. The 0.5 ng target produced normalized peak heights between 600-2000 rfu. Offscale data and signs of overamplification were present for targets above 1.0 ng.

For the peak height ratio (PHR), sample 1005 produced low peak height ratios at FGA. Excluding sample 1005 at FGA, all other samples at 1.0 ng demonstrated PHRs above 60%. Samples at 0.5 ng and lower produced PHRs below 55%. Lower PHR will need to be considered when interpreting low level DNA samples amplified for 29 cycles.

The majority of OL and non-matching alleles at the 0.5-1.0 ng target range were eliminated by setting the peak amplitude threshold (PAT) at 70 rfu. The remaining OL and non-matching alleles include elevated stutter (n+4 and n-4), elevated baseline and pull up. At this PAT, allelic dropout began at 0.125 ng. The PAT will be further evaluated at the conclusion of each study.

Using the data obtained for this study, an appropriate stochastic threshold for a target input of 1.0 ng is 225 rfu. These values will be further evaluated at the conclusion as more data is obtained.

c. Sensitivity_3 (28 cycles)

For this sensitivity study, serial dilutions of two (2) DNA samples were prepared to assess amplification and instrument performance, determine the optimal quantity of template DNA for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit and to help understand interpretational limitations of the PCR-based genotyping system over a range of input DNA amounts. All samples were amplified for 28 cycles.

Genotyped data from this study was evaluated for genotype concordance, allelic dropout, peak height, heterozygote peak height ratios and potential artifacts to help establish standard operating parameters, optimal DNA input amount(s) and interpretation guidelines.

This part of the second set of sensitivity studies was conducted after an optical adjustment was made as a part of the annual planned maintenance of the 3130*xl* A. A subsequent performance check demonstrated an increase in the sensitivity of the instrument. The data obtained from this study was used to determine the performance of the Identifiler® Plus kit under current laboratory conditions. The previous data (Sensitivity_1) was also considered prior to the final conclusions regarding the sensitivity and performance of the Identifiler® Plus kit.

The recommended target DNA input range for amplification using the Identifiler® Plus kit at 28 cycles is 0.5-1.0 ng. The 1.0 ng target produced normalized peak heights of 1000-3000 rfu and peak height ratios of 60% and higher. The 0.5 ng target produced normalized peak heights of 400-1500 rfu and peak height ratios of 60% and higher. Offscale data and signs of overamplification were present for targets of 2.0 ng and higher.

The majority of OL and non-matching alleles were eliminated by setting the peak amplitude threshold at 70 rfu. The remaining OL and non-matching alleles include pull up and one stutter peak (n+4). At this PAT, allelic dropout began at 0.125 ng.

Using the data obtained for this study, an appropriate stochastic threshold for a target input of 0.5-1.0 ng is 130 rfu. These values will be further evaluated at the conclusion as more data is obtained.

The data obtained in this study indicates an increase in sensitivity from the study performed prior to the annual planned maintenance (Sensitivity_1). This is consistent with the increase in

sensitivity documented in the performance check of 3130*xl* A.

d. Sensitivity_4 (29 cycles)

For this sensitivity study, serial dilutions of two (2) DNA samples were prepared to assess amplification and instrument performance, determine the optimal quantity of template DNA for the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit and to help understand interpretational limitations of the PCR-based genotyping system over a range of input DNA amounts. All samples were amplified for 29 cycles.

Genotyped data from this study was evaluated for genotype concordance, allelic dropout, peak height, heterozygote peak height ratios and potential artifacts to help establish standard operating parameters, optimal DNA input amount(s) and interpretation guidelines.

This part of the second set of sensitivity studies was conducted after an optical adjustment was made as a part of the annual planned maintenance of the 3130*xl* A. A subsequent performance check demonstrated an increase in the sensitivity of the instrument. The data obtained from this study was used to determine the performance of the Identifiler® Plus kit under current laboratory conditions. The previous data (Sensitivity_2) was also considered prior to the final conclusions regarding the sensitivity and performance of the Identifiler® Plus kit.

The recommended target DNA input range for amplification using the Identifiler® Plus kit is 0.5 ng. The 0.5 ng target produced normalized peak heights of 600-2500 rfu. At this target, all peak height ratios are above 55% (except one at the D19 locus). Offscale data and signs of overamplification were present for targets of 1.5 ng and higher.

The majority of all non-matching and off ladder alleles were eliminated by setting the peak amplitude threshold (PAT) at 70 rfu. The remaining OL and non-matching alleles at the 0.5-1.0 ng target range include elevated baseline, pull up and stutter (n+4 and n-4). At this PAT, allelic dropout began at 0.0625 ng.

Using the data obtained for this study, an appropriate stochastic threshold for a target input of 0.5 ng at 29 cycles is 300 rfu. These values will be further evaluated at the conclusion as more data is obtained.

e. Sensitivity Overall Conclusion

Using the data from this set of studies, the Identifiler® Plus kit had an optimal input target of 1.0 ng for 28 amplification cycles and 0.5 ng for 29 amplification cycles. At 1.0 ng at 28 cycles, normalized peak heights ranged from 1000-3000 rfu with peak height ratios of 60% or higher. At 0.5 ng at 29 cycles, normalized peak heights ranged from 600-2500 rfu with peak height ratios of 55% or higher.

A peak amplitude threshold of 70 rfu eliminated the majority of non-matching and OL alleles for both the 28 and 29 cycle amplifications. At this PAT, allelic dropout occurred at 0.125 ng for the 28 cycle study and 0.0625 ng for the 29 cycle study. While the majority of alleles are detected at the 0.125 ng and 0.0625 ng targets, peak height ratio and stochastic effects were observed for both the 28 and 29 cycle studies. Further studies need to be conducted on low level inputs (0.03125 and less) to determine (a) if additional interpretation guidelines are necessary for low level samples and/or (b) if quantitation can reliably detect a level at which amplification is not

required.

Using all the data, an appropriate stochastic threshold for samples amplified for 28 cycles is 200 rfu. An appropriate stochastic threshold for samples amplified for 29 cycles is 300 rfu.

Sensitivity_3 and Sensitivity_4 demonstrated an increase in sensitivity from Sensitivity_1 and Sensitivity_2 which is consistent with the increase in sensitivity demonstrated by the performance check of 3130*xl* A after the annual planned maintenance.

The following will be further evaluated in subsequent studies prior to making any final recommendations: optimal target range, peak amplitude threshold, peak height ratio, and stochastic threshold.

III. Accuracy Study

a. Accuracy_1 (1.0 ng, 28 cycles)

For this accuracy study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 1 ng were analyzed and genotyped. All samples were amplified for 28 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

For 1.0 ng at 28 cycles, all alleles within an injection fell inside of the +/- 0.5 bp size difference. However, due to injection migration and temperature fluctuations, several alleles (17) fell outside of the +/- 0.5 bp size range when being sized with allelic ladders from one or more injections away. Based on the data obtained from this study, it is preferred that each injection on the capillary electrophoresis plate contain an allelic ladder. However, this data also suggests that an average of allelic ladders spaced across the entire plate is also acceptable.

b. Accuracy_2 (1.0 ng, 29 cycles)

For this accuracy study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 1 ng were analyzed and genotyped. All samples were amplified for 29 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

For 1.0 ng at 29 cycles, all alleles within an injection and across the plate fell within the +/- 0.5 bp size range when being sized with allelic ladders from up to three injections away. Based on the data obtained from this study, both methods of data export and ladder placement are acceptable.

c. Accuracy_3 (0.5 ng, 28 cycles)

For this accuracy study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 0.5 ng were analyzed and genotyped. All samples were amplified for 28 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

For 0.5 ng at 28 cycles, all alleles within an injection fell inside of the +/- 0.5 np size difference. However, due to injection migration and temperature fluctuations, several alleles (9) fell outside of the +/- 0.5 bp size range when being sized with allelic ladders from one injection away. Based on the data obtained from this study, it is preferred that each injection on the capillary electrophoresis plate contain an allelic ladder. However, this data also suggests that an average of allelic ladders spaced across the entire plate is also acceptable.

d. Accuracy_4 (0.5 ng, 29 cycles)

For this accuracy study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 0.5 ng were analyzed and genotyped. All samples were amplified for 29 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

For 0.5 ng at 29 cycles, all alleles within an injection and across the plate fell within the +/- 0.5 bp size range when being sized with allelic ladders from up to three injections away. Based on the data obtained from this study, both methods of data export and ladder placement are acceptable.

e. Accuracy Study Overall Conclusion

Based on the four plates run for these studies, it is preferred to place a ladder in each injection and export the data as a run folder with each injection. However, it is acceptable to prepare ladders across the plate and export the data as a run folder for all samples in the plate. This method may lead to sample alleles falling outside of the +/- 0.5 bp size range. These samples will need to be re-injected or re-run to determine the final allele assignment.

IV. Reproducibility Study

a. Reproducibility_1 (1.0 ng, 28 cycles)

For this reproducibility study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 1 ng were analyzed and genotyped. All samples were amplified for 28 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

At 1.0 ng of target DNA, discordant alleles can be attributed to artifacts such as pull-up, elevated baseline, -A and n+4 stutter. Samples 1031 and 1032 were not amplified due to a dilution preparation error.

Overall peak heights were higher for this study than the 1.0 ng target of the Sensitivity_3 study. Loci with the highest average peak heights include TH01, D16, D8, and D13. Loci with the lowest average peak heights include Amelogenin, D18 and FGA. Peak height ratios were 59% and higher which was lower than the 65% peak height ratio obtained in the Sensitivity_3 study. The 70 rfu peak amplitude threshold (PAT) suggested by the sensitivity study eliminated 75% of non-matching and off ladder alleles; however, raising the PAT to 80 rfu eliminated 90%.

This data suggests that a lower target input (~0.5 ng) and higher peak amplitude threshold may be necessary for optimal kit performance. These values will be further evaluated in subsequent studies prior to making a final conclusion.

b. Reproducibility_2 (1.0 ng, 29 cycles)

For this reproducibility study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 1 ng were analyzed and genotyped. All samples were amplified for 29 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

At 1.0 ng of target DNA, discordant alleles can be attributed to artifacts such as pull-up, elevated baseline, -A and n+4 stutter. Samples 1049 and 1050 were not amplified due to a dilution preparation error.

Overall peak heights were higher for this study than the 1.0 ng target of the Sensitivity_4 study. Loci with the highest average peak heights include TH01, D3, and D13. Loci with the lowest average peak heights include D18 and FGA. Peak height ratios were 60% and higher which was lower than the 65% peak height ratio obtained in the Sensitivity_4 study. No conclusions can be made regarding an appropriate peak amplitude threshold for this study due to signs of overamplification including excessive pull up, stutter, elevated baseline and background.

This data suggests that a lower target input (0.5 ng or less) may be necessary for optimal kit performance. These values will be further evaluated in subsequent studies prior to making a final conclusion.

c. Reproducibility_3 (0.5 ng, 28 cycles

For this reproducibility study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 0.5 ng were analyzed and genotyped. All samples were amplified for 28 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

Overall peak heights were higher for this study than the 0.5 ng target of the Sensitivity_3 study. Loci with the highest average peak heights include TH01, D8, D3, and D13. Loci with the lowest average peak heights include D18 and FGA. Peak height ratios were 52% and higher which was lower than the 60% peak height ratio obtained in the Sensitivity_3 study. The 70 rfu peak amplitude threshold (PAT) suggested by the sensitivity study eliminated 92% of non-matching and off ladder alleles.

This data suggests that a lower peak height ratio may be necessary for interpretation if the target input is lowered to 0.5 ng. These values will be further evaluated in subsequent studies prior to making a final conclusion.

d. Reproducibility_4 (0.5 ng, 29 cycles)

For this reproducibility study, to demonstrate the ability to correctly genotype samples, 15 different DNA samples amplified three (3) times with a target DNA input of 0.5 ng were analyzed and genotyped. All samples were amplified for 29 cycles.

Allele sizes for each sample and allelic ladder were determined by the use of an internal size standard. Genotype concordance and size difference of each allele within a sample were calculated against the respective allele in each allelic ladder(s). Peak height, heterozygote peak height ratios and artifacts were also examined to establish standard operating procedures and interpretation guidelines.

Overall peak heights were higher for this study than the 0.5 ng target of the Sensitivity_4 study. Loci with the highest average peak heights include TH01 and D8. Loci with the lowest average peak heights include D18 and FGA. Peak height ratios were 47% and higher which is consistent with the 50% peak height ratio obtained in the Sensitivity_4 study. The 70 rfu peak amplitude threshold (PAT) suggested by the sensitivity studies eliminated 71% of non-matching and off ladder alleles; however, raising the PAT to 80 rfu eliminated 83%.

This data suggests that a lower peak height ratio may be necessary for interpretation if the target input is lowered to 0.5 ng. These values will be further evaluated in subsequent studies prior to making a final conclusion.

e. Reproducibility Study Overall Conclusion

Using the data from this set of reproducibility studies, the optimal target for a 28 cycle Identifiler® Plus amplification may need to be lowered to 0.5 ng. The 0.5 ng target suggested by the

sensitivity study is still appropriate for a 29 cycle amplification. At the 1.0 ng target, both the 28 cycle and 29 cycle samples showed signs of overamplification.

Both 28 and 29 cycle samples had peak height ratios of approximately 60% or greater with a 1.0 ng target. Both 28 and 29 cycle samples had peak height ratios of approximately 50% or greater with a 0.5 ng target. The peak height ratios obtained from the 1.0 ng target are consistent with the peak height ratios obtained in the sensitivity study. The peak height ratios obtained from the 0.5 ng target are lower than the peak height ratios obtained in the sensitivity study. This data suggests that a lower peak height ratio may be needed for interpretation of data especially if a 29 cycle amplification is used.

The data from this set of studies also suggests that a peak amplitude threshold of 80 rfu may be necessary for analysis if a 1.0 ng target is amplified; however, 70 rfu may be appropriate to prevent allelic dropout at lower targets. This threshold will be further evaluated once all studies have been completed.

The stochastic threshold was not evaluated in this study because no alleles were missing.

V. Mixture Study

a. Mixture_1 (0.5 ng, 28 cycles)

For this mixture study, six (6) mixed DNA sample sets were prepared in the following proportions so that the total DNA input was 0.5 ng: 1:0, 1:1, 1:3, 1:7, 1:10, 1:15, 1:20, and 0:1. The minor contributor was 0.5, 0.25, 0.13, 0.06, 0.05, 0.03, 0.02, and 0.0 ng, and respectively, the major contributor was 0.0, 0.25, 0.38, 0.44, 0.45, 0.47, 0.48, and 0.5 ng for the mixture ratios listed above. Each mixed DNA sample was amplified in replicates of three (3). All samples were amplified for 28 cycles.

The genotyped data was analyzed for the following: genotype concordance, allelic dropout, heterozygote peak height ratios, mixture ratios and potential artifacts to help determine standard operating procedures, analysis thresholds and develop interpretation guidelines.

For this mixture study, a 0.5 ng target input of DNA was used for amplification. All mixture contributors were reliably detected for mixture ratios of 1:0, 0:1, 1:1 and 1:3. At a ratio of 1:7 and below, alleles from the minor contributors begin to drop out. At a peak amplitude threshold of 70 rfu, dropout is observed at the 1:3 ratio and below. This data is consistent with the levels at which dropout was observed in Sensitivity_3.

The peak amplitude threshold suggested by the sensitivity study (70 rfu), eliminated all but six of the non-matching and off ladder alleles. An 80 rfu peak amplification threshold eliminated all but two non-matching and off ladder alleles; however, more dropout would be observed in the 1:7 ratio.

Single source samples produced peak height ratios of 49% and higher. This was inconsistent with the peak height ratios observed in the sensitivity studies (60% and above), however, consistent with the peak height ratios observed in the accuracy/reproducibility studies (52% and above).

The highest observed peak missing its heterozygous partner was at 214 rfu. Values greater than this were observed, however could not be used as an indicator of allelic dropout due to

stutter from the major contributor's allele. This value is higher than the value observed in the 28 cycle sensitivity studies (130 rfu).

b. Mixture_2 (0.5 ng, 29 cycles)

For this mixture study, six (6) mixed DNA sample sets were prepared in the following proportions so that the total DNA input was 0.5 ng: 1:0, 1:1, 1:3, 1:7, 1:10, 1:15, 1:20, and 0:1. The minor contributor was 0.5, 0.25, 0.13, 0.06, 0.05, 0.03, 0.02, and 0.0 ng, and respectively, the major contributor was 0.0, 0.25, 0.38, 0.44, 0.45, 0.47, 0.48, and 0.5 ng for the mixture ratios listed above. Each mixed DNA sample was amplified in replicates of three (3). All samples were amplified for 29 cycles.

The genotyped data was analyzed for the following: genotype concordance, allelic dropout, heterozygote peak height ratios, mixture ratios and potential artifacts to help determine standard operating procedures, analysis thresholds and develop interpretation guidelines.

For this mixture study, a 0.5 ng target input of DNA was used for amplification. All mixture contributors were reliably detected for mixture ratios of 1:0, 0:1, 1:1 and 1:3 except for one 1:3 ratio sample (1122D_1123D). For this sample, dropout was observed due to an allele from the minor contributor located in stutter position. At 70 rfu peak amplitude threshold, dropout was observed at the 1:7 ratio and below. Overall, in comparison to the Mixture_1 (28 cycles) study, less allelic dropout was observed. This data is consistent with the level of dropout which was observed in Sensitivity_4.

The peak amplitude threshold suggested by the sensitivity study (70 rfu), eliminated the majority of the non-matching and off ladder alleles. An 80 rfu peak amplification threshold would eliminate more non-matching and off ladder alleles; however, more dropout would be observed in the 1:7 ratio.

Single source samples produced peak height ratios of 46% and higher. This was consistent with the peak height ratios observed in the sensitivity study (50% and above) and accuracy/reproducibility studies (47% and above).

The highest observed peak missing its heterozygous partner was at 329 rfu. Values greater than this were observed, however could not be used as an indicator of allelic dropout due to stutter from the major contributor's allele. This value is higher than the value observed in the 29 cycle sensitivity studies (300 rfu).

c. Mixture_3 (1.0 ng, 28 cycles)

For this mixture study, six (6) mixed DNA sample sets were prepared in the following proportions so that the total DNA input was 1.0 ng: 1:0, 1:1, 1:3, 1:7, 1:10, 1:15, 1:20, and 0:1. The minor contributor was 1.0, 0.5, 0.25, 0.13, 0.09, 0.06, 0.05, and 0.0 ng, and respectively, the major contributor was 0.0, 0.5, 0.75, 0.88, 0.91, 0.94, 0.95, and 1.0 ng for the mixture ratios listed above. Each mixed DNA sample was amplified in replicates of three (3). All samples were amplified for 28 cycles.

The genotyped data was analyzed for the following: genotype concordance, allelic dropout, heterozygote peak height ratios, mixture ratios and potential artifacts to help determine standard operating procedures, analysis thresholds and develop interpretation guidelines.

For this mixture study, a 1.0 ng target input of DNA was used for amplification. All mixture contributors were reliably detected for mixture ratios of 1:0, 0:1, 1:1 and 1:3. At a ratio of 1:7 and below, alleles from the minor contributors begin to drop out. At a peak amplitude threshold of 70 rfu, additional dropout was observed at the 1:7 ratio; however, all 1:3 ratio alleles were still detected. The level at which dropout was observed in this mixture study (0.125ng) is consistent with the level which was observed in Sensitivity_3.

The majority of the non-matching and off ladder alleles were eliminated with a 70 rfu peak amplitude threshold. The remaining peaks can be attributed to pull up, background, elevated baseline and stutter (both n-4 and n+4). It is recommended to use caution when evaluating low level peaks in the stutter positions when interpreting mixture samples.

All peak height ratios were consistent with the sensitivity study. Single source samples produced peak height ratios of 62% and higher. 0.5 ng targets (1:1 mixture ratios) produced peak height ratios of 50% and higher.

The highest observed peak missing its heterozygous partner was at 229 rfu. Values greater than this were observed, however could not be used as an indicator of allelic dropout due to stutter from the major contributor. This value is higher than the value observed in the 28 cycle sensitivity studies (130 rfu); however, it is consistent with the 0.5 ng mixture study (214 rfu).

d. Mixture_4 (1.0 ng, 29 cycles)

For this mixture study, six (6) mixed DNA sample sets were prepared in the following proportions so that the total DNA input was 1.0 ng: 1:0, 1:1, 1:3, 1:7, 1:10, 1:15, 1:20, and 0:1. The minor contributor was 1.0, 0.5, 0.25, 0.13, 0.09, 0.06, 0.05, and 0.0 ng, and respectively, the major contributor was 0.0, 0.5, 0.75, 0.88, 0.91, 0.94, 0.95, and 1.0 ng for the mixture ratios listed above. Each mixed DNA sample was amplified in replicates of three (3). All samples were amplified for 29 cycles.

The genotyped data was analyzed for the following: genotype concordance, allelic dropout, heterozygote peak height ratios, mixture ratios and potential artifacts to help determine standard operating procedures, analysis thresholds and develop interpretation guidelines.

For this mixture study, a 1.0 ng target input of DNA was used for amplification. All mixture contributors were reliably detected for mixture ratios of 1:0, 0:1, 1:1 and 1:3. At a ratio of 1:7 and below, alleles from the minor contributors begin to drop out. At a peak amplitude threshold of 70 rfu, no additional dropout is observed at the 1:7 ratio. The level at which dropout was observed in this mixture study (0.125ng) is higher than the level which was observed in Sensitivity_4 (0.0625 ng) due to competitive amplification of the major contributor.

The majority of the off ladder and non-matching alleles were eliminated with a 70 rfu peak amplitude threshold. The remaining peaks can be attributed to pull up, background, elevated baseline and stutter (both n-4 and n+4). It is recommended to use caution when evaluating low level peaks in the stutter positions when interpreting mixture samples.

All peak height ratios were consistent with the sensitivity study. Single source samples produced peak height ratios of 62% and higher. 0.5 ng targets (1:1 mixture ratios) produced peak height ratios of 54% and higher.

The highest observed peak missing its heterozygous partner was at 297 rfu. Values greater than this were observed, however could not be used as an indicator of allelic dropout due to stutter from the major contributor. This value is consistent with the value observed from the 29 cycle sensitivity studies (300 rfu) and the 0.5 ng mixture study (329 rfu).

e. Mixture Study Overall Conclusion

Using the data from this set of studies, both 0.5 ng and 1.0 target input mixtures contained alleles from both contributors at ratios of 1:0, 0:1, 1:1 and 1:3. Dropout was observed at mixtures 1:7 and below. A 70 rfu peak amplitude threshold eliminated most non-matching and off ladder alleles; however, dropout occurred at a ratio of 1:3 in the 28 cycle samples. This data is consistent with the data obtained in the sensitivity and accuracy/reproducibility studies.

In both the 28 and 29 cycle samples for both 0.5 ng and 1.0 ng target input mixtures, peak height ratios below 50% were observed. This data is consistent with the data obtained from the 29 cycle sensitivity study and both 28 and 29 cycle accuracy/reproducibility studies; however, the 28 cycle sensitivity study demonstrated peak height ratios of 60% or higher. A target input of 0.5-1.0 ng may be necessary to obtain higher and more consistent peak heights.

The suggested stochastic threshold from the sensitivity study was 200 rfu for 28 cycles and 300 rfu for 29 cycles. In these studies, alleles missing their heterozygous partners were observed at higher values, an average of 222 rfu (for the 0.5 ng and 1.0, 28 cycle mixture studies) and an average of 313 rfu (for the 0.5 ng and 1.0 ng, 29 cycle mixture studies). Missing heterozygous partner alleles were observed at sufficient peak heights; however, they were located in the stutter position of the major contributor and not called.

For both cycle numbers and both target input amounts, the lower the ratio of Contributor1:Contributor2, the less accurate the calculation of the targeted Contributor1:Contributor2 ratio using peak heights. This indicates that minor contributors may be difficult to determine in mixtures with high level major contributors due to allelic dropout, low peak height ratios, masking by the major contributor and stutter position.

Of note, in both the 28 and 29 cycle 1.0 ng target mixture studies, additional instances of n+4 stutter were observed, especially at D18, in samples with high peak heights and/or offscale data and in locations where the major contributor alleles are separated by eight base pairs. The result may be an elevated stutter peak due to the additive effects of n+4 and n-4 stutter. Mixtures with these characteristics should be interpreted with caution.

VI. Overall Conclusion

All samples were analyzed using GeneMapper® ID Version 3.2.1 with the Identifiler® Plus Analysis Method supplied by Applied Biosystems®.

Based on the studies and tests performed in this internal validation, the following conclusions regarding the Applied Biosystems® AmpFISTR® Identifiler® Plus PCR Amplification Kit using the Applied Biosystems® 3130*xL* Genetic Analyzer can be made:

- The 3130*xl* instrument is precise and within injections no markers and/or alleles with high standard deviation values (exceeding 0.15 base pairs) were observed. However, high standard deviation values were observed across the plate which indicated the need

for multiple ladders. This data was seen in the Precision Study and verified by the Accuracy Study.

- The recommended template DNA range for Identifiler Plus at 28 and 29 cycles is 0.5-1.0 ng. For samples amplified at 28 cycles, peak heights averaged 700 rfu for 0.5 ng and 1700 rfu for 1.0 ng. For samples amplified at 29 cycles, peak heights averaged 1600 rfu for 0.5 ng and 3600 rfu for 1.0 ng. At all targets below 0.5 ng, peak height ratios were low which could lead to difficulty during interpretation. At targets above 1.0 ng, signs of overamplification were observed. This range should be regularly evaluated and is dependent on consistency of sensitivity between lot numbers of both Identifiler® Plus and Quantifiler® Duo kits.

- The recommended peak amplitude threshold for samples amplified with Identifiler® Plus at 28 and 29 cycles is 70 rfu. At this level, the majority of non-matching and off ladder alleles are eliminated. Artifacts observed above 70 rfu threshold include stutter (n-4 and n+4), pull up, and elevated baseline. For both 28 and 29 cycles with a threshold of 70 rfu, dropout was observed consistently at 0.06 ng.

- The recommended peak height ratio for a target range of 0.5-1.0 ng amplified with Identifiler® Plus at 28 cycles is 55%. Samples amplified at 0.5 ng produced some peak height ratios just below this level; however, samples amplified at 1.0 ng produced peak height ratios of 60% and higher.

- The recommended peak height ratio for a target range of 0.5-1.0 ng amplified with Identifiler® Plus at 29 cycles is 50%. Samples amplified at 0.5 ng produced peak height ratios just below this level; however, samples amplified at 1.0 ng produced peak height ratios at 55% and higher.

- The recommended stochastic threshold for samples amplified with Identifiler® Plus at 28 cycles is 200 rfu. The highest peak missing its heterozygous partner in the Sensitivity Study was 130 rfu and was an average of 222 rfu in the Mixture Study. Mixture samples may produce higher peaks missing heterozygous partners due to stutter. This should be considered while interpreting mixture samples.

- The recommended stochastic threshold for samples amplified with Identifiler® Plus at 29 cycles is 300 rfu. The highest peak missing its heterozygous partner in the Sensitivity Study was 309 rfu and an average of 313 rfu in the Mixture Study. Mixture samples may produce higher peaks missing heterozygous partners due to stutter. This should be considered while interpreting mixture samples.

- At mixture ratios of 1:7 or less, alleles from the minor contributor begin to dropout. Therefore, low level mixtures should be interpreted with caution.

In comparison to the amplification kit currently in use at the time of this internal validation study, Identifiler®, the recommended target input for samples amplified using the Identifiler® Plus kit is consistent with the increased sensitivity which occurred after the 3130*xl* planned maintenance. The recommended analytical threshold for use with the Identifiler® Plus kit is consistent with the analytical threshold recommended based on the Identifiler® internal validation study. The peak height ratios observed in the Identifiler® Plus kit internal validation study were consistent with those in the Identifiler® kit study for 28 cycles; however, lower

peak height ratios were observed in samples amplified for 29 cycles with Identifiler® Plus. During the internal validation study the Identifiler® Plus kit produced results at levels of inhibition in which Identifiler® was not able to produce results. More results were obtained from degraded samples amplified with Identifiler® Plus than degraded samples amplified with Identifiler®. Using both the Identifiler® and Identifiler® Plus kits, concordant results were obtained for samples provided by the laboratory (known and non-probative) and NIST. While both kits provide accurate and reproducible results, this internal validation demonstrates that Identifiler® Plus is better able to handle inhibition and degradation. It should be noted that the samples amplified for 29 cycles may produce more results at lower inputs; however, those results, especially mixtures, should be interpreted with caution. Low level samples amplified for 29 cycles were demonstrated in this internal validation study to have poor peak height ratios and/or dropout of heterozygous partner alleles. A review of this internal validation and all associated documentation confirmed that no contamination within the controls used was evident during all of the studies. Additionally, all profiles were found to be in concordance with expected typing results, confirming that no cross-contamination between samples occurred.