Listed below is a summary of stated validation. Additional information can be provided upon request.

STATE OF MINNESOTA DEPARTMENT OF PUBLIC SAFETY
BUREAU OF CRIMINAL APPREHENSION FORENSIC SCIENCE SERVICE
VALIDATION SUMMARY

PROJECT TITLE: Yfiler Plus Validation

The following studies were completed as part of the Yfiler Plus Validation (in alphabetical order):

1. Baseline Noise Protocol

PURPOSE: To assess an alternate protocol that could be used to minimize sporadic elevated baseline noise in Yfiler Plus Kit data.

RESULTS SUMMARY

The positive control DNA was previously amplified with Yfiler Plus targeting a total DNA of 0.60 ng and the average peak heights obtained from three re-preps are consistent with the target amount. The average peak height of the positive control does slightly decline with increased denaturation time; however, the average peak heights are approximately 90% of the positive control with no denaturation. The positive controls show minimal peak height differences and the quality of the profile was not negatively affected.

Positive Sample Name	Protocol	Avg Peak Height
POS121918-VL-RP	No denaturation	5856
POS121918-VL-RP(3min)	3 min denaturation	5425
POS121918-VL-RP(10min)	10 min denaturation	5289

Compared to the no denaturation protocol, the 3 min denaturation step did not minimize elevated baseline noise but produced additional noise and artifacts. The 10 min denaturation step generally reduced elevated baseline noise and artifacts compared to both the 3 min and no denaturation protocols. Based on these observations, the 10 min denaturation protocol is an option to minimize elevated baseline noise in casework samples and negative controls at the scientist's discretion.

2. Contamination

RESULTS SUMMARY: No alleles were obtained from the negative controls or reagent blanks. Based on these results, cross contamination does not appear to occur at levels that would affect interpretation of Yfiler Plus results.

3. Female Mixtures

PURPOSE: To determine what effect increasing amounts of female DNA has on the ability to type the male DNA in a mixed sample

Listed below is a summary of stated validation. Additional information can be provided upon request.

RESULTS SUMMARY:

Table 1. Average Peak Heights

M:F Mixture	M002	M005	Average
1to0	3911	3715	3813
1to1	3412	3616	3514
1to100	3230	3648	3439
1to1000	3029	3666	3347
1to5000	1859	2286	2073
1to10000	1802	1790	1796

Table 2. Percentage of Expected Alleles Detected

M:F Mixture	M002	M005	Average
1to0	100	100	100
1to1	100	100	100
1to100	100	100	100
1to1000	100	100	100
1to5000	100	100	100
1to10000	70	74	72

1:0 to 1:5000 mixture series: Complete male profiles were obtained in all 1:0, 1:1, 1:100, 1:1000 and 1:5000 mixture samples. Peak heights decreased and inhibition increased as the amount of female DNA increased. Average peak heights only decreased slightly until the 1:5000 mixtures, where the average peak height dropped to approximately 54% of the 1:0 mixtures. Inhibition was also observed in the 1:5000 mixtures, with some loci amplifying worse than others and causing noticeable peak imbalance between loci. Beginning in the 1:100 mixtures, a cross-reactive peak occurred at approximately 412 bp in the TAZ (red) dye channel but it fell outside of the read region and did not affect interpretation in any sample. In addition, there were two minor artifact peaks in a 1:1000 mixture that could be attributed to non-specific amplification but due to their low rfu values, they did not impact interpretation.

1:10,000 mixture series: Both samples in this mixture set were inhibited by the high amounts of female DNA. Average peak heights were approximately 47% of the 1:0 mixtures and complete locus drop-out was observed at 5-6 loci with 72% of the expected alleles recovered on average. Loci effected by this level of female DNA that dropped out below MDT were DYS389II (both samples), DYS627 (both samples), DYS518 (one sample), DYS385 (both alleles in both samples), DYS449 (both alleles in both samples), and DYF387S1 (both alleles in both samples). There were also additional loci that were affected by this level of female DNA with a significant decrease in peak heights and poor peak balance throughout both samples. There was not any non-specific amplification observed from the female DNA.

CONCLUSIONS: Complete male DNA profiles were obtained in all mixtures except the 1:10,000 mixture set. Despite inhibition in the 1:10,000 mixture set, a majority of the male profile was still interpretable. Caution should be used when interpreting Yfiler Plus male DNA profiles when the male to female ratio is less than 1:1000 and be aware that non-specific amplification may occur. It should be noted that 500 pg of male DNA was amped for all samples in this study. Reference the Yfiler Plus - Sensitivity in the presence of high concentrations of female DNA study for further information on varying amounts of male DNA in high female DNA.

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4. Low Level DNA Evaluation

PURPOSE: To provide information on how many peaks would be expected if a blank is actually contaminated with an extremely low amount of DNA and also to provide guidance in the evaluation of peaks in reagent blanks or negative controls.

RESULTS SUMMARY:

Table 1. Number of Peaks Detected

	Min # of peaks detected	Max # of peaks detected	Average # of peaks detected
5pg (MDT)	1	8	5
5pg (LOD)	10	17	14
Controls (MDT)	0	1	0
Conrols (LOD)	0	3	0

From the samples with 5pg of DNA, 1 to 8 alleles were detected at MDT with an average number of 5 alleles detected. When analyzed at LOD, 10 to 17 peaks were observed with an average of number of 14 peaks detected.

For the reagent blanks and negative control samples, one peak was detected at MDT in a single negative control but all other samples had no peaks calling at MDT. When analyzed at LOD, 1 peak was observed in three different control samples and 3 peaks were detected in an additional negative control; however, a majority of the samples had no peaks calling at LOD.

ADDITIONAL COMMENTS: This study shows that a minimal amount of DNA (approximately 1.5 times the amount of DNA in a haploid cell) resulted in at least 10 peaks detected, and more typically around 14 peaks, at or above LOD. This result is comparable to Globalfiler, where a similar amount of DNA resulted in a minimum of 7 peaks above LOD, with typically 10 to 20 peaks detected. This study also showed the potential of at least 3 peaks to be detected in a negative control or reagent blank sample at or above LOD. where no DNA should be present in the sample. Unless all types at LOD in the blank are consistent with an associated sample, 6 or fewer peaks at or above LOD (consistent with Globalfiler) will not require re-testing the batch associated with the affected blank.

5. MDT

PURPOSE: To determine the level of background noise detected on the Applied Biosystems® Genetic Analyzer 3500 and establish a minimum detection threshold (at what RFU a peak will be labeled as an allele) for Yfiler Plus data.

RESULTS SUMMARY:

Analysis results were consistent between instruments. Baseline noise, the associated LOD value, and the associated LOQ value were higher for positive controls than for negative controls. Based

on the positive control baseline data obtained from both instruments, a minimum detection threshold was determined using the highest LOQ value in each dye channel. The following thresholds will be used for the analysis of Yfiler Plus amplification data on all 3500 Genetic Analyzers:

Dye	MDT
6-FAM	50
VIC	67
NED	70
TAZ	65
SID	72

6. Mixtures

PURPOSE: To analyze mixture profiles from 2 and 3 male contributors to determine mixture interpretation guidelines for Y-chromosomal DNA profiles and determine at what level minor types can be detected in mixtures.

CONCLUSIONS:
Based on the observations in this study, a minor:major peak height ratio of 50% is appropriate for determining major types in mixtures with a input DNA of 150 pg or more. In low level mixtures where input DNA is below 150 pg, a 30% minor:major peak height ratio should be used. Additional caution should be exercised when calling major types in low level, degraded, or inhibited mixtures and in mixtures that appear to have more than two contributors.

There are circumstances in which overlap of the minor and major contributor can be assumed: if the peak height of the smallest observed minor type is greater than 250 RFU (Yfiler Plus MIT), the total input is at a minimum of 300 pg, and the rfu values of the minor types are significantly greater than the rfu values of the stutter peaks from the major profile. In general, this indicates approximate mixture ratios are less than 1:5. No assumption of allele sharing can be made for input values of 300 pg or less. However, in these instances, the minor contributor can still be ascertained for those loci in which a minor type is present. In mixtures where the rfu values of the observed minor types are approximately the same as the rfu values of the stutter peaks (indicating approximate mixture ratios between 1:5 and 1:10 or greater), overlap of minor and major will not be assumed at loci where only one type is observed regardless of total DNA input or absolute peak heights. The quality of the profile and amount amplified should be taken into account when approximating mixture ratios.

7. Mock Case Samples

PURPOSE: To evaluate profiling results obtained from a variety of samples (skin and fingernail swabs and post-coital swabs) that would typically be encountered in YSTR casework on the Applied Biosystems® Genetic Analyzer 3500 using the Yfiler Plus kit.

Listed below is a summary of stated validation. Additional information can be provided upon request.

CONCLUSIONS:

Y-chromosomal DNA profiles can be obtained from a variety of sample types, and after extended time intervals. The amount of male DNA obtained and the profile quality varies between sample types but is also dependent on post-deposit activities conducted by the female subject. Allelic loss is expected in the presence of high amounts of female DNA (800 to 1000 ng), even when the male DNA input amount is well above what normally should result in a full Y-chromosomal profile in the absence of female DNA.

8. NIST Traceable Samples

RESULTS SUMMARY: Full profiles were obtained from both NIST samples tested. The profiling results were concordant with the previous Yfiler results for the loci that are present in both kits.

9. Precision

RESULTS SUMMARY:
The largest standard deviation by locus observed for the five instruments is outlined in the table below:

Instrument	Highest standard deviation	Locus
3500A	0.3721	DYS576
3500B	0.2440	YGATAH4
3500C	0.2318	DYS570
3500D	0.1288	DYS439
3500E	0.3481	DYS576

ADDITIONAL COMMENTS:
The standard deviation for all loci was below the 0.5bp required for correct allele designation with GMIDX.

10. Related Males

PURPOSE: To assess Y-STR DNA profiles obtained from paternally related males for any mutation events due to the presence of seven rapidly mutating Y-STR loci (DYF387S1a/b, DYS449, DYS518, DYS570, DYS576, and DYS627) in the Yfiler Plus kit.

RESULTS SUMMARY:
All 73 related male samples and the Y-STR DNA profiles generated from them were organized into 24 different family groups (A through X). Comparisons of profiles within each family group were made to observe any apparent mutation events and to determine if family members could be

Listed below is a summary of stated validation. Additional information can be provided upon request.

distinguished from each other. One pair of convicted offender twin samples (Family X) produced profiles that did not match at 12 out of 27 tested locations when 100% concordance is expected for identical twins. It was determined that the two individuals in Family X were not twins, and these samples were not used for related male comparisons. All other convicted offender identical twin sets (Families O through W) showed 100% concordance as expected. Out of the remaining 53 typed male relatives from 14 different families (A through N), 6 apparent mutation events were observed in 5 of those families and they are summarized in the table below.

Locus	DYS576	DYS627	DYS458	DYS449	DYS481	DYF387S1
RM YSTR?	Y	Y	N	Y	N	Y
Observed mutation	18 -> 17	19 -> 20	16 -> 15	30,31 -> 31	25 -> 26	36,37 -> 37
Family	M	B	F	K	M	A

Family A: The first generation male is differentiated from the second and third generation males at the DYF387S1 locus, one of the rapidly mutating loci.

Family B: One brother out of six in the second generation is differentiated from the family profile at the DYS627 locus, one of the rapidly mutating loci.

Family F: The first generation male is differentiated from the second and third generation males at the DYS458 locus, which is not considered a rapidly mutating locus.

Family K: The first generation male is differentiated from the second and third generation males at the DYS449 locus, one of the rapidly mutating loci.

Family M: All three males are differentiated from each other. One second generation male displays a difference from the first generation at the DYS576 locus, one of the rapidly mutating loci. The other second generation male displays a difference from the first generation male at DYS481, which is not considered a rapidly mutating locus. The two second generation males are differentiated from each other at both the DYS576 locus and the DYS481 locus.

ADDITONAL COMMENTS:

DYS481 and DYS458 are the two loci not considered to be rapidly mutating where mutation events occurred. It should be noted that these loci have the two highest mutation rates outside of the rapidly mutating loci.

Assuming that the results from these family groups are typical, it is generally expected for paternally related individuals to have the same Yfiler Plus DNA profile. However, mutations may be observed that allow family members to be differentiated, especially at the rapidly mutating loci.

Listed below is a summary of stated validation. Additional information can be provided upon request.

11. Sensitivity

CONCLUSIONS:
Optimum DNA input is 250 pg to 750pg for a single source male DNA sample and a full profile was obtained for all samples with 60 pg and greater. At 30 pg of DNA a few instances of allele dropout were noted however on average about 90% of the expected alleles were detected. The frequency of allele dropout increases at 15 pg with an average of about 60% of the expected alleles detected; however, at this DNA input stutter peaks and artifacts sometimes amplified at the same rfu or in place of the true allele at this DNA input. Incomplete profiles were obtained from all samples with less than 15 pg of DNA. This indicates that in the absence of other factors affecting profile quality (such as degradation or the presence of female DNA) profiling with Yfiler Plus may be attempted on samples with a male quantitation value of 30 pg or more. A number of instances of pull-up and artifacts were observed at 1500 and 1250pg, and to a lesser degree at 1000pg and 750pg, but not enough to interfere with interpretation of single source samples.

Generally, the peak height ratio for DYS385 and DYF387S1 is expected to be >60% for samples with at least 125pg. Peak height ratios for DNA input amounts of 60 pg or lower are more variable, with minimum peak height ratios of 25 to 36% for DNA input amounts of 15 to 60 pg. Sister allele or complete locus drop-out (below MDT) was observed at DYS385 and DYF387S1 for input levels of 5, 10, and 15 pg. Caution will be used when determining single source or major profiles at 60pg or less due to decreased peak height ratios below 60%, possible drop-out, or the inability to differentiate an artifact from a true peak.

12. Sensitivity- High Female DNA

PURPOSE: To determine the effect of large amounts of female DNA on the quality of DNA profiles obtained from varying concentrations of male DNA on the Applied Biosystems® Genetic Analyzer 3500 using the Yfiler Plus kit.

CONCLUSIONS:
Based on the Sensitivity study that was conducted for samples with only male DNA present, the minimum male DNA input amount for amplification with Yfiler Plus is 30 pg. For samples with significant amounts of female DNA present (1000 ng or more), a minimum input amount of 60 pg of male DNA must be amplified to prevent drop-in or other artifacts from interfering with profile interpretation that may lead to incorrect results. For more on the effect of female DNA see “Yfiler Plus Female Mixtures.”

13. Stochastic Threshold
RESULTS SUMMARY:

Single-source samples:
For single-source samples, the lowest observed PHR was 30%. Based on this PHR and the MDT for the dye channel where this was observed, the minimum for the sister peak where both alleles would still be observed above the MIT would be 241 (rounded to 250) rfu (see table below).

Listed below is a summary of stated validation. Additional information can be provided upon request.

Table 1. MIT summary for DYS385 and DYF387S1 based on sensitivity data

DNA input	Lowest PHR	Color Channel	MDT	Sister Peak Height Expected
1500pg	0.95	Red	65	68.35
1250pg	0.89	Red	65	72.66
1000pg	0.85	Red	65	76.21
750pg	0.71	Purple	72	101.22
500pg	0.74	Purple	72	96.83
250pg	0.63	Purple	72	114.15
125pg	0.58	Red	65	111.24
60pg	0.36	Purple	72	201.14
30pg	0.30	Purple	72	240.85

Mixtures:
The 2-person male mixtures were analyzed for the peak height ratios of minor contributors for DYS365 and DYF387S1 where the minor contributor was heterozygous and no sharing occurred with the major contributor. Peak height ratios and rfu values were evaluated to determine if any drop-out below MDT was observed for allele pairs where the sister allele was detected above MIT. Three instances of drop-out were observed which all occurred in the same degraded mixture series with 600 pg of total DNA input at mixture ratios of 1:5, 1:10, and 1:15 (minor DNA input of 100 pg, 55pg, and 38 pg, respectively). Also, in all three instances the sister allele that dropped out was located in a stutter position to the major contributor, which can cause the minor allele to be masked by the stutter filter especially at high mixture ratios. (see also Stutter Evaluation Summary). No additional drop-out of sister alleles was observed for alleles at or above MIT.

RECOMMENDATIONS: For the 3500 instrument, if two peaks are present at DYS385 or DYF387S1 in a single source sample, the minimum detection threshold (MDT) for the corresponding dye channel is appropriate. A stochastic threshold of 250 RFU is appropriate for a single peak at DYS385 and DYF387S1 in a single source sample.

Listed below is a summary of stated validation. Additional information can be provided upon request.

14. Stutter

RESULTS SUMMARY:

Single-source samples:

A total of 308 samples was included in the evaluation, from 2 different male donors. Samples with DNA input amounts of 5, 10, 15, 30, 60, 125, and 250 pg were each amplified in triplicate. No stutter was observed in the 5 pg and 10 pg samples, likely because the peak heights were generally so low that any stutter would typically fall below the MDT. The following table shows the occurrence of peaks attributable to elevated n-1 repeat, elevated n+1 repeat, elevated n-2 nt, and n-2 repeat in the single-source samples:

DNA (pg)	# samples	# of n-1	average high n-4/ sample	# n+1	average high n+4/ sample	# n-2nt	average high n-2nt/ sample	# n-2	average n-2/ sample
5	30	0	0.000	0	0.000	0	0.000	0	0.000
10	30	0	0.000	0	0.000	0	0.000	0	0.000
15	34	2	0.059	12	0.353	0	0.000	0	0.000
30	36	13	0.361	5	0.139	0	0.000	0	0.000
60	38	24	0.632	11	0.289	0	0.000	0	0.000
125	38	9	0.237	17	0.447	0	0.000	0	0.000
250	38	4	0.105	1	0.026	5	0.132	1	0.026
500	24	1	0.042	1	0.042	0	0.000	1	0.042
750	10	0	0.000	1	0.100	0	0.000	0	0.000
1000	10	0	0.000	2	0.200	0	0.000	6	0.600
1250	10	0	0.000	9	0.900	0	0.000	0	0.000
1500	10	0	0.000	8	0.800	2	0.200	0	0.000

Although ABI did not determine a plus stutter filter for DYF387S1, the occurrence of plus stutter is common at this locus especially in samples with optimal or higher DNA input and averaged at about 1.5% of the allelic peak.

Mixture Samples:

Instances of stutter were observed for 2 and 3-person male mixtures. The occurrence of elevated n-1, elevated n+1, elevated n-2 nt, elevated n+2 nt, and n-2 stutter in the male mixtures are summarized in the following table:

# of contributors	Total DNA (pg)	# samples	# of n-1	average high n-1/ sample	# n+1	average high n+1/ sample	# n-2nt	average high n-2nt/ sample	# n+2nt	average high n+2nt/ sample	# n-2	average n-2/ sample
2	75	30	7	0.233	2	0.067	0	0.000	0	0.000	0	0.000
2	150	40	13	0.325	7	0.175	2	0.050	1	0.025	1	0.025
2	300	60	19	0.317	9	0.150	3	0.050	1	0.017	2	0.033
2	600	60	3	0.050	14	0.233	4	0.067	0	0.000	4	0.067
3	150	36	8	0.222	0	0.000	5	0.139	0	0.000	0	0.000
3	300	36	5	0.139	4	0.111	3	0.083	1	0.028	0	0.000
3	600	18	1	0.056	2	0.111	2	0.111	0	0.000	0	0.000

Listed below is a summary of stated validation. Additional information can be provided upon request.

The effect of stutter on minor type detection:
Minor types have the potential to be masked by stutter from peaks contributed by major contributors or by peaks that are shared by multiple contributors. Multiple instances were observed where a minor allele (above MDT) was potentially masked by the stutter filter (indicated in the allele tables). In the 2-person male mixtures, the number of allelic peaks masked by stutter increased as the mixture ratio decreased, with the 1:20 mixture ratio having the most occurrences of allelic peaks being masked by stutter. Generally, at mixture ratios of 1:5 or 1:3, you can expect to obtain the majority of minor alleles. In the instances where an allele was masked by stutter at these mixture ratios, there was either a degraded contributor or low DNA input.

RECOMMENDATIONS:
High stutter (above ABI stutter filter) is not uncommon, especially for low DNA input levels (60 pg or less), degraded samples, or low male DNA input in the presence of high female DNA. The possible additive effect of stutter and masking of minor alleles by stutter as well as overall profile quality should be considered when determining whether comparisons can be made to minor types, taking into account rfu values of observed minor types relative to rfu values of stutter. See also Yfiler Plus - Male Mixtures.