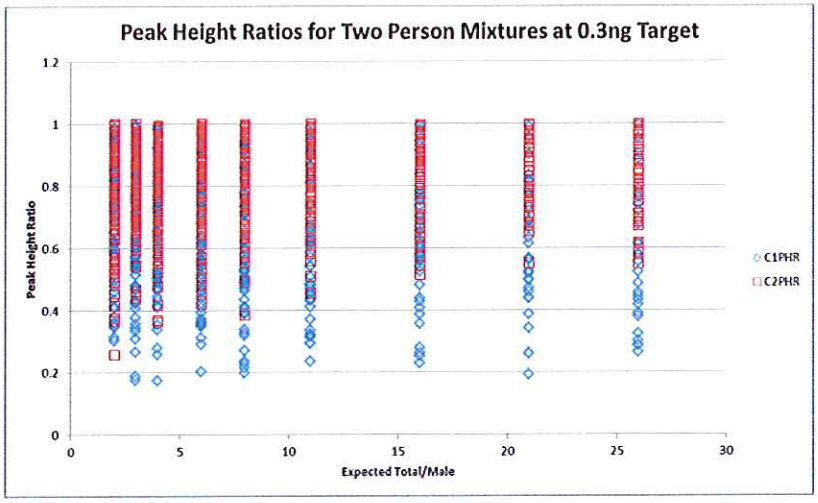
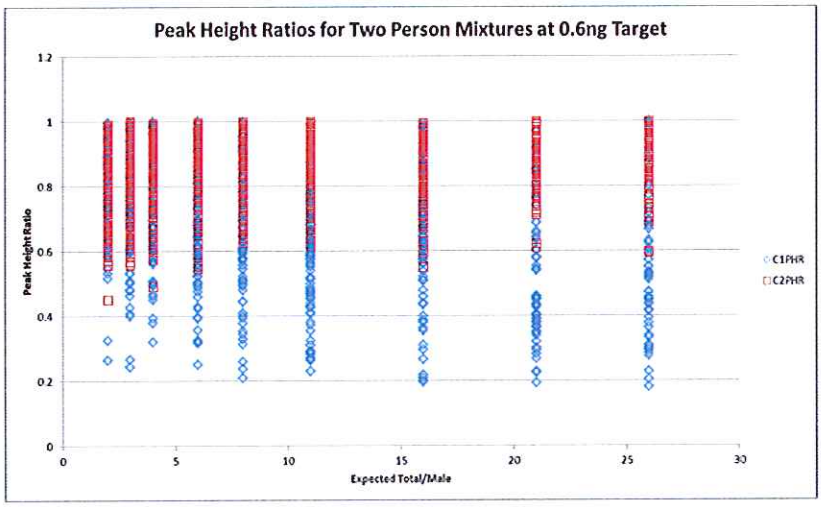
v. Peak Height Ratios





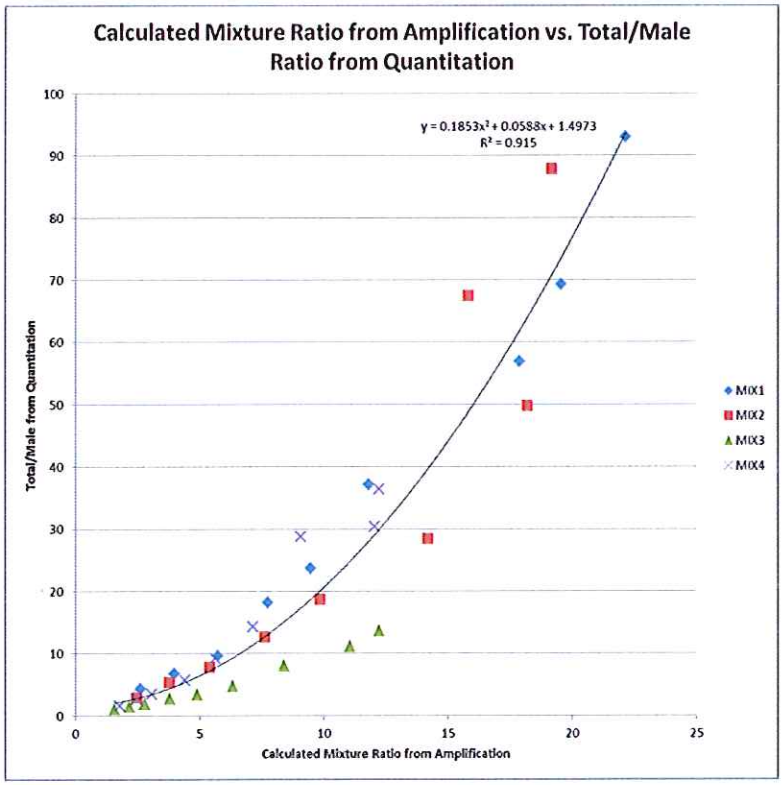
All peak height ratios for the two person mixtures were consistent with the peak height ratios observed for single source samples of similar targets in the sensitivity study. With the exception of two loci where extreme peak imbalance was observed, a minimum peak height ratio of 45% was obtained for the 1:1 0.6ng targets which is consistent with the minimum peak height ratios obtained from the 0.375ng targets. As the targets decreased for C1 in each mixture ratio, so did the peak height ratios. As the targets increased for C2 in each mixture ratio approaching a 0.6ng target, the peak height ratios also increased to approximately 55-60%.

vi. Total/Male DNA Quantity Amplification Cutoff

Total/Male DNA quantity calculations for each 0.6ng two person mixture were performed and compared in the charts below. Each mixture was then plotted and a best fit line generated.

Quantitation - Total/Male				
Expected	MIX1	MIX2	MIX3	MIX4
2	4.26333333	2.956333	1.066867	1.599333
3	6.75366667	5.335667	1.499	2.651667
4	9.64333333	7.834333	1.860333	3.508
6	18.1633333	12.71667	2.715667	5.672
8	23.7066667	18.75	3.406	9.138333
11	37.1933333	28.45	4.770667	14.21667
16	56.91	49.73667	8.033	28.77
21	69.22	67.45667	11.06667	30.32333
26	93.0466667	87.92	13.65	36.45

Amplification - Total/Male				
Expected	MIX1	MIX2	MIX3	MIX4
2	2.60565253	2.46053	1.571635	1.764122
3	3.97110871	3.77707	2.16396	2.449508
4	5.72518921	5.39603	2.76729	3.058326
6	7.73721673	7.607045	3.792529	4.380294
8	9.47655288	9.849787	4.885729	5.626366
11	11.8124849	14.18907	6.318748	7.139815
16	17.8898316	18.19874	8.40793	9.08205
21	19.5419823	15.79668	11.03387	12.04958
26	22.1522496	19.1638	12.22206	12.21319



In order to establish an appropriate cutoff for Total/Male DNA quantity at quantitation, the following conclusions were previously established by this validation and/or the previous Identifiler® Plus Amplification Cutoff Validation.

- While this data was generated from 0.6ng mixtures, some mixtures may be amplified at higher targets (up to 1.0ng) without a significant enough increase in peak heights to affect interpretation (Sensitivity Study above).

- Significant dropout of alleles was observed in the two person mixture study at similar levels to the Sensitivity Study above.

- The previously recommended amplification cutoff for Total DNA is 100pg (Sensitivity Study above).

- The total quantity of input DNA does not affect the way the calculated Total/Male DNA quantity from the amplification compares to the Total/Male DNA quantity from the quantitation (previous Identifiler® Plus Amplification Cutoff Validation). Therefore, the best fit line generated above can be applied to a sample regardless of the Total DNA quantity.

Based on these conclusions, an appropriate Total/Male DNA quantity cutoff was calculated using the following:

Amplification Total/Male DNA quantity = x = 1.0ng/0.1ng = 10.

Using equation from best fit line above, $y = 0.1853x^2 + 0.0588x + 1.4973$, y = 20.62. For simplicity, this value will be rounded to 20.

If the highest target of Total DNA in the ideal range (0.5-1.0ng) can be amplified, then the quantitation result for Total/Male DNA quantity must be at least 20 to obtain an interpretable minor male contributor DNA profile.

To verify that this recommendation was consistent with the data obtained, the average peak heights for the minor male contributor were calculated for the 0.6ng target two person mixtures and listed in the chart below.

0.6ng Average Peak Height of Minor Male Contributor				
Total/Male	MIX1	MIX2	MIX3	MIX4
2	1025.98611	928.9744	4775.178	1251.575
3	1020.40278	613.2821	2739.7	1461.317
4	696.666667	372.5385	2058.144	1074.983
6	410.861111	398.4359	1630.5	540.575
8	454.695707	250.0778	986.1667	547.9685
11	362.078072	232.9759	836.9778	416.2685
16	199.145833	192.6167	663.6	279.2648
21	218.645833	223.8889	393.6404	261.1595
26	159.875	184.9167	508.2897	203.975

While average peak heights below the recommended stochastic threshold (indicated in yellow) were obtained at ratios well below 20, dropout for these samples was not significant until this level. Additionally, the amplification target for these two person mixtures was 0.6ng. A 1.0ng amplification target is expected to produce higher peak heights and less minor contributor dropout.

d. Conclusions

Based on this study, the 90rfu analytical threshold showed detection of a full major and minor contributor for ratios down to 1:5 when the target input is 0.6ng. At 1:7, a small amount of dropout was observed in the minor contributor profile, however significant dropout occurred at 1:20. As expected, the lower target input (0.3ng) produced more dropout at all the ratios which was consistent with the expected target inputs from the sensitivity study. Conversely, a higher target would be expected to produce less dropout, however, care should be taken not to produce oversaturated data.

Discordant alleles were mostly attributed to stutter which will be further assessed in the stutter study covered by the STRmix™ validation. The remaining discordant alleles were easily identified artifacts with the exception of two peaks which will be further evaluated in the contamination study of this validation.

Mixture ratios were able to be accurately calculated for ratios down to 1:7 for the 0.3ng target and 1:15 for the 0.6ng target. At lower ratios, significant dropout of the minor contributor caused more variability in the calculated mixture ratios.

The highest alleles missing their heterozygous partner were higher than what was observed in the sensitivity study, however the majority were consistent with the recommended 500-600rfu. The peak height ratios observed in this study were consistent with the peak height ratios observed in the sensitivity study.

Using the quantitation and amplification results from the two person mixtures, the recommended Total/Male DNA quantity cutoff is 20. At higher ratios, it is not expected

to obtain an interpretable minor male contributor DNA profile regardless of amplification target.

VII. Mock Samples

a. Objective

33 samples from previously analyzed validations, training and proficiency tests were amplified by two separate individuals. Sample results at loci consistent with Identifiler® Plus were then compared to determine concordance and assess parameters recommended by previous studies in this validation.

The following SWGDAM Validation Guideline was addressed in this study:

"4.1 Known and nonprobative evidence samples or mock evidence samples: Methods intended for casework samples should be evaluated and tested using known samples and nonprobative evidence samples or mock case samples. ...Results from these studies should be compared to the previous results of known samples and/or nonprobative evidence or mock case samples to ensure concordance."

b. Materials and Methods

Extracts from previously analyzed Identifiler™ Plus samples from validations, training and proficiency tests were gathered from different analysts within the laboratory. See Appendix for extraction, quantitation, dilution, amplification and 3500 set-up worksheets and reagents used. Some samples were previously extracted and their original Plexor® HY quantitation results used to calculate amplification targets. Other samples were previously extracted and re-quantified using Plexor® HY. Instrument maintenance was documented on worksheets in the post-amplification laboratory. The instrument settings suggested by the Globalfiler™ User Manual were used for all injections.

Two amplifications by separate analysts were performed and run on the 3500xL A. All samples with sufficient quantity were amplified at a 0.75ng target. For samples with previous indications of inhibition or degradation, targets were adjusted based on results from Identifiler® Plus amplifications to attempt to obtain optimal amplification results with Globalfiler™.

All samples were analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 70rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual

for panels, bins, stutter and analysis method. As with sensitivity, 70rfu was used to ensure that the analytical threshold would not need to be lowered for samples at lower target quantities and to provide a good assessment of the artifacts which may be obtained at or around the analytical threshold. Alleles, sizes and peak heights were exported from GMID-X. All calculations for the results section below were then performed using Microsoft® Excel®.

c. Results

For this study, all quantitation, amplification and detection controls produced expected results. All samples which were previously extracted, quantified, amplified and run using Identifiler® Plus produced expected results for their associated reagent blanks during their initial analysis. No re-amplification of these controls was conducted for this study.

The original data for this study included the NIST samples. All calculations below were performed with these samples included, however the specific results of the NIST samples have previously been discussed in the Accuracy Study of this validation.

As with the previous studies, a table of all the detected peaks which were discordant or non-stutter (based on the default parameters suggested by the Globalfiler™ User Manual) was created. Each peak was evaluated and its cause, if determinable, recorded. The total number of artifacts was determined for an analytical threshold of 70rfu, 80rfu, 90rfu and 100rfu.

Total Number of OL/non-matching (≥70rfu)	**678**
Total Number ≥80rfu	504
Total Number ≥90rfu	384
Total Number ≥100rfu	306
Total Number of pull up	312
Total Number of n-4/n+4	14
Total Number of n-4	15
Total Number of n+4	224
Total Number of n-2	2
Total Number of n-8	16
Total Number of n-6	2
Total Number of offscale	4
Total Number of artifact	23
Total Number of background	40
Total Number of minus a	4
Total Number of unknown	22

Most discordant peaks were attributed to stutter or another easy to identify artifact. Because all samples were not re-quantified for this study and/or some samples displayed signs of degradation/inhibition in previous amplifications, higher than expected variability was observed in allele counts and/or peak heights overall and between replicates.

Of the 22 unknown peaks, 12 were attributed to sample M10-00116P-2 which displayed a clear, unexpected minor contributor in both amplifications. These peaks were not considered discordant, however, the sample was eliminated from any additional calculations. Of the remaining 10 peaks, only six were above the recommended 90rfu analytical threshold. One of the six was previously discussed in the Accuracy Study of this validation. The other five will be further evaluated in the Contamination Study of this validation.

The remaining alleles were evaluated by two separate analysts and 29 of 33 samples were determined to be concordant. The four samples with discordant genotypes were further evaluated and determined to be mock sexual assault samples. The additional alleles detected were previously not detected using the Identifiler® Plus amplification and all were consistent with the expected minor contributor. Three samples showed dropout of some concordant alleles which were previously detected using the Identifiler® Plus amplification kit. These samples were known to be degraded/inhibited tooth and tissue samples and no re-amplifications were performed to try and improve the profiles.

Average peak heights were calculated for all samples, but were not used for reproducibility or repeatability calculations. The sample set used for this study was determined to be from such variable sources and time frames that the effects may have produced lower or higher than expected results. Additionally, not all samples were quantified in replicates or re-quantified prior to amplification.

d. Conclusions

Overall, concordant results were obtained for the mock samples tested for this study. Samples which previously demonstrated signs of degradation/inhibition using the Identifiler® Plus amplification kit showed similar signs of degradation/inhibition using the Globalfiler™ amplification kit (overall low peak heights, dropout of alleles at larger loci, etc).

VIII. Contamination/Drop-In

a. Objective

For this study, reagent blanks, positive controls, negative controls and samples were evaluated to determine whether contamination occurred during the Globalfiler™ amplification or 3500xL A detection steps of the procedure. Additionally, unknown peaks documented in the studies above were further evaluated to determine whether they were truly instances of drop-in.

Drop-in will be defined in this study as non-reproducible, unexplained peaks observed within a profile (STRmix™ Validation and Implementation Guide v2.4).

The following SWGDAM Validation Guideline was addressed in this study:

"4.5 Contamination assessment: The laboratory should evaluate, using both controls and known samples, the detection of exogenous DNA (including allele drop-in and heteroplasmy) originating from reagents, consumables, operator and/or laboratory environment."

b. Materials and Methods

For this study, the results for all reagent blanks, positive controls, negative controls and samples run on 3500xL A for the sensivity, accuracy, reproducibility/repeatability, two person mixture and mock casework studies were evaluated. No new samples or controls were amplified or run. Only samples amplified at 2ng or less with unknown peaks greater than the recommended 90rfu analytical threshold will be considered as possible drop-in.

A peak will be evaluated as drop-in using the following requirements:

- It must be demonstrated to be "sporadic" or non-reproducible. Example: If peaks at the same locus in the same base pair range of different heights (above or below analytical threshold) were detected in multiple replicates, the peak will not be considered drop-in. These peaks may be sample specific and not sporadic in nature.
- It may not be associated with a sample with off-scale data or peak heights that were demonstrated in the sensitivity study to cause background or baseline problems (>10,000rfu).
- Peak morphology must be good (tall and sharp).
- Internal Lane Standard (ILS) must display appropriate peak heights with good morphology indicating acceptable injection conditions for that sample.
- The peak must not be labeled as OL (Off-Ladder). The laboratory would re-run or re-amplify the sample to confirm sizing. If the peak did not re-appear, it

would not be included in the interpretation. If the peak did re-appear, it would be confirmed and included in interpretation.

c. Results

i. Controls

No peaks were observed above the 90rfu analytical threshold for all reagent blanks and negative controls. All positive controls produced appropriate profiles with no unidentifiable peaks above the recommended 90rfu analytical threshold. The chart below documents all controls which were extracted, amplified or run on 3500xL A as a part of this validation.

Sample Name	Extraction Batch	Quantitation Batch	Amplification Batch	Detection Batch	Expected Results
060216JS-RB1	060216JS-EXT1	060316JS-QNT1	060616JS-AMP1	060616JS-RUN1	YES
081616YP-RB1	081616YP-EXT1	081716YP-QNT1	090616YP-AMP2	090716YP-RUN1	YES
POS			060616JS-AMP1	060616JS-RUN1	YES
NEG			060616JS-AMP1	060616JS-RUN1	YES
POS			060816JS-AMP1	060916AF-RUN1	YES
NEG			060816JS-AMP1	060916AF-RUN1	YES
POS			060816JS-AMP2	060916AF-RUN2	YES
NEG			060816JS-AMP2	060916AF-RUN2	YES
POS			060816JS-AMP3	060916AF-RUN3	YES
NEG			060816JS-AMP3	060916AF-RUN3	YES
POS			071116JS-AMP2	071316WK-RUN2	YES
NEG			071116JS-AMP2	071316WK-RUN2	YES
POS			071216WK-AMP1	071316WK-RUN3	YES
NEG			071216WK-AMP1	071316WK-RUN3	YES
POS			091616AF-AMP1	091616AF-RUN1	YES
NEG			091616AF-AMP1	091616AF-RUN1	YES
POS			091616AF-AMP2	091616AF-RUN2	YES
NEG			091616AF-AMP2	091616AF-RUN2	YES
POS			091516YP-AMP1	091616YP-RUN1	YES
NEG			091516YP-AMP1	091616YP-RUN1	YES
POS			081916JS-AMP1	082516AF-RUN1	YES
NEG			081916JS-AMP1	082516AF-RUN1	YES
POS			081916JS-AMP2	082516AF-RUN2	YES
NEG			081916JS-AMP2	082516AF-RUN2	YES
POS			082316AF-AMP1	090816YP-RUN1	YES
NEG			082316AF-AMP1	090816YP-RUN1	YES
POS			090616YP-AMP1	090716YP-RUN1	YES
NEG			090616YP-AMP1	090716YP-RUN1	YES
POS			082416YP-AMP2	082916YP-RUN1	YES
NEG			082416YP-AMP2	082916YP-RUN1	YES
POS			082416YP-AMP1	082916AF-RUN1	YES
NEG			082416YP-AMP1	082916AF-RUN1	YES
POS			090616YP-AMP2	090716YP-RUN1	YES
NEG			090616YP-AMP2	090716YP-RUN1	YES
POS			091216YP-AMP1	MIX_4P_1b	YES
NEG			091216YP-AMP1	MIX_4P_1b	YES
POS			091416YP-AMP1	MIX_5P_1	YES
NEG			091416YP-AMP1	MIX_5P_1	YES

ii. Drop-In

Discordant peaks from the previous studies run on 3500xL A were evaluated for possible inclusion as drop-in. Each peak was detailed below along with a conclusion as to its cause.

Sample File	Marker	Peak Height (rfu)	Comments	Drop-In
BKG-2_01_B02_3500 Instrument.hid	D10S1248	111	present in other replicates just under 90rfu analytical threshold; poor peak morphology; peak heights at this locus >10,000rfu	No
CML-1.5_01_D02_3500 Instrument.hid	D2S441	90	present in other replicates and targets just under 90rfu analytical threshold; locus in green dye channel off scale; peak heights at this locus >10,000rfu	No
CML-1.5_01_E02_3500 Instrument.hid	CSF1PO	91	present in other replicates and targets just under 90rfu analytical threshold; peaks heights at this locus >10,000rfu; adjacent pull up peaks similar in peak height	No
CML-2_01_A02_3500 Instrument.hid	D2S441	112	present in other replicates and targets just under 90rfu analytical threshold; locus in green dye channel off scale; peak heights at this locus >10,000rfu	No
CML-2_01_A02_3500 Instrument.hid	D3S1358	154	present in other replicates and targets just under 90rfu analytical threshold; poor peak morphology; locus in green dye channel off scale; peak heights at this locus >10,000rfu	No
CML-2_01_B02_3500 Instrument.hid	D2S441	116	present in other replicates and targets just under 90rfu analytical threshold; locus in green dye channel off scale; peak heights at this locus >10,000rfu	No
CML-2_01_B02_3500 Instrument.hid	CSF1PO	107	present in other replicates and targets just under 90rfu analytical threshold; peaks heights at this locus >10,000rfu; adjacent pull up peaks similar in peak height	No
CML-2_01_B02_3500 Instrument.hid	D2S1338	99	present in replicate A02 at 73rfu; poor peak morphology; peak heights at this locus >10,000rfu	No
CML-2_01_H01_3500 Instrument.hid	D3S1358	97	present in other replicates and targets just under 90rfu analytical threshold; poor peak morphology; peak heights at this locus >10,000rfu	No
GG-1.5_01_C02_3500 Instrument.hid	D8S1179	131	present in other replicates; peak heights at this locus >10,000rfu	No
GG-1.5_01_D02_3500 Instrument.hid	Yindel	93	present in other replicates and targets under 90rfu analytical threshold; peak heights at this locus >10,000rfu	No
GG-1.5_01_E02_3500 Instrument.hid	D8S1179	146	present in other replicates; peak heights at this locus >10,000rfu	No
GG-2_01_A02_3500 Instrument.hid	Yindel	95	present in other replicates and targets under 90rfu analytical threshold; peak heights at this locus >10,000rfu	No
GG-2_01_B02_3500 Instrument.hid	Yindel	91	present in other replicates and targets under 90rfu analytical threshold; peak heights at this locus >10,000rfu	No
KM-1.5_04_E02_3500 Instrument.hid	D2S441	98	present in other replicates and targets under 90rfu analytical threshold; peak heights at this locus >10,000rfu	No
KM-2_04_A02_3500 Instrument.hid	D2S441	179	present in other replicates and targets under 90rfu analytical threshold; peak heights at this locus >10,000rfu	No
KM-2_04_B02_3500 Instrument.hid	D2S441	148	present in other replicates and targets under 90rfu analytical threshold; locus in green dye channel off scale; peak heights at this locus >10,000rfu	No
A0116.2_01_F02_3500A.hid	D12S391	97	good peak morphology; locus peak heights ~2,500rfu; not reproduced in replicate, however, replicate amplified poorly overall (locus peak heights ~500rfu)	Yes
M10-00116P-2_02_H04_3500A.hid	TH01	115	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	TH01	307	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	SE33	142	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D2S441	103	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D3S1358	124	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D16S539	110	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D8S1179	120	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D2S1338	209	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	TH01	135	extract contamination; discordant minor profile present in both amplifications	No
M10-00116P-2_02_H04_3500A.hid	D3S1358	165	extract contamination; discordant minor profile present in both amplifications	No
M11-0020P-2_02_F05_3500A.hid	D19S433	110	present in replicate sample under 70rfu; peak height at this locus >10,000rfu; background and pull up observed throughout sample	No
NISTB_01_C01_3500A.hid	D2S1338	204	poor peak morphology; locus peak height >20,000rfu; present in replicate sample below 50rfu however replicate locus peak height ~10,000rfu	No
TR140008-22_02_H05_3500A.hid	D1S1656	139	good peak morphology however locus peak height >10,000rfu and other loci in sample with offscale data; not present in other replicate however locus peak height <10,000rfu	No
B_01_D01_3500A.hid	D13S317	91	present in other replicates; poor peak morphology; locus peak height >20,000rfu	No
C_01_A02_3500A.hid (actually D - sample naming error on import document)	D5S818	107	present in 2 of 9 replicates; good peak morphology; locus peak height ~6,000rfu; other replicates with higher average peak heights show no peak	Yes
C_02_B02_3500A.hid (actually D - sample naming error on import document)	D5S818	127	present in 2 of 9 replicates; good peak morphology; locus peak height ~6,000rfu; other replicates with higher average peak heights show no peak	Yes
D_02_G01_3500A.hid (actually C - sample naming error on import document)	AMEL	187	Y allele; good peak morphology; locus peak height >10,000rfu however other replicates also >10,000rfu and no indication of Y allele	Yes
E_01_D02_3500A.hid	D2S1338	130	good peak morphology; locus peak heights ~6,500rfu; not produced in other 8 replicates and some replicates have higher peak heights	Yes
H_01_A03_3500A.hid	Yindel	105	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	AMEL	93	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D8S1179	124	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D2S441	95	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D5S818	367	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D13S317	170	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D10S1248	100	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D1S1656	257	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D12S391	92	extract contamination; discordant minor profile present throughout profile	No
H_01_A03_3500A.hid	D2S1338	96	extract contamination; discordant minor profile present throughout profile	No
MIX1_1_02_0_6_02_A05_3500A.hid	D10S1248	102	not present in other replicates or targets; good peak morphology; locus peak heights ~5,000rfu; OL allele so would have been rerun	No
MIX2_1_02_0_3_03_E08_3500A.hid	SE33	121	not present in other replicates or targets; good peak morphology; locus peak heights ~200rfu (minor) and ~1500rfu (major); in stutter position for minor contributor however % too high (45%)	Yes

d. Conclusions

Contamination due to the Globalfiler™ amplification or 3500xL A detection was not observed during this validation. One extract and one amplification showed signs of contamination that were related to user technique and not the Globalfiler™ amplification kit or 3500 detection system.

The six drop-in alleles confirmed by this study will be used to inform the parameters section of the STRmix™ validation. The maximum observed drop-in peak was 187rfu, however, the drop-in cap will be rounded up to 200rfu to account for possible variability in peak height. The total number of concordant alleles observed in this validation is listed in the chart below along with the total number of loci evaluated.

Study	Observed Concordant Alleles
Sensitivity	7378
Accuracy/Mock Casework	3066
Reproducibility/Repeatability	2175
2 Person Mixture	13746
Total	**26365**

Study	Number of samples	Number of loci	Total loci evaluated
Sensitivity	216	24	5184
Accuracy/Mock Casework	72	24	1728
Reproducibility/Repeatability	53	24	1272
2 Person Mixture	209	24	5016
Total			**13200**

IX. 3500 ("B") Performance Check

An additional 3500 Genetic Analyzer will be utilized by the laboratory for the detection of samples amplified using Globalfiler™. This instrument has been named 3500 B. The precision and sensitivity studies below will be individually evaluated and compared to the results obtained from 3500xL A to demonstrate a full performance check of 3500 B.

The following SWGDAM Validation Guideline was addressed in this study:

"6.2 After an internal validation has been performed on a critical instrument, each additional critical instrument of the same make and model shall require a performance check. The performance check should demonstrate that results are reproducible on the new critical instrument and that values from the internal validation can still be obtained. For example, the

performance check of a new critical instrument should demonstrate that the sensitivity level is consistent with the sensitivity level obtained from an internal validation, but need not demonstrate whether or not the new critical instrument is more sensitive. "

a. Precision

i. Objective

To assess the ability of the instrument to reproducibly measure the size of an amplified DNA fragment, 16 Globalfiler™ allelic ladder aliquots were injected three times on 3500 B. Allele sizes were calculated using an internal lane standard (ILS). Precision was expressed as a standard deviation calculated for each allele within each locus.

ii. Materials, Methods and Data Analysis

See Appendix for 3500 set-up worksheets and reagents used. Instrument maintenance was documented on worksheets in the post-amplification laboratory. The instrument settings suggested by the Globalfiler™ User Manual were used for all injections.

All data was analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 50rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual for panels, bins, stutter and analysis method.

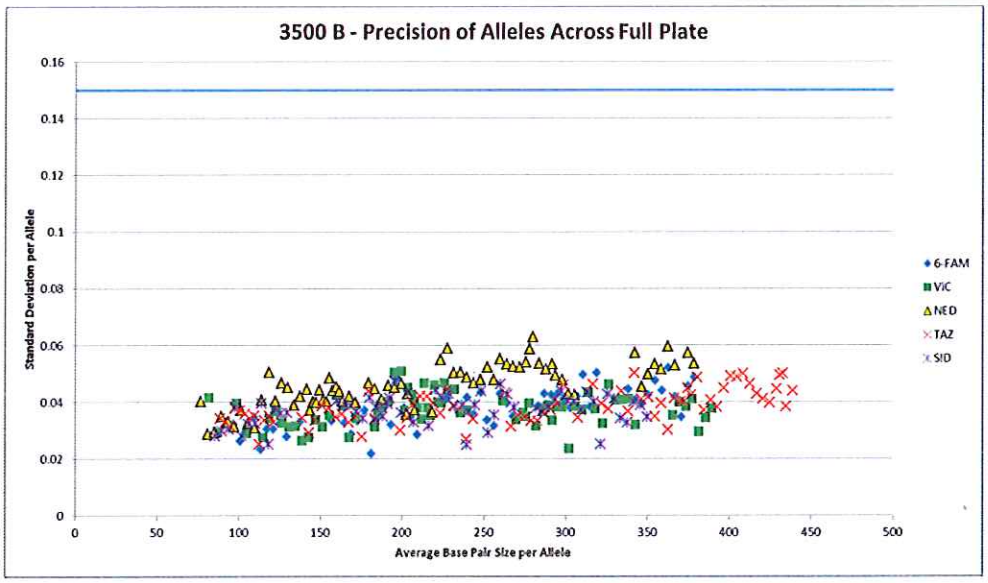
Alleles and sizes were exported from GMID-X. Averages and standard deviations were calculated using Microsoft® Excel® for all data as a plate and per injection.

iii. Results

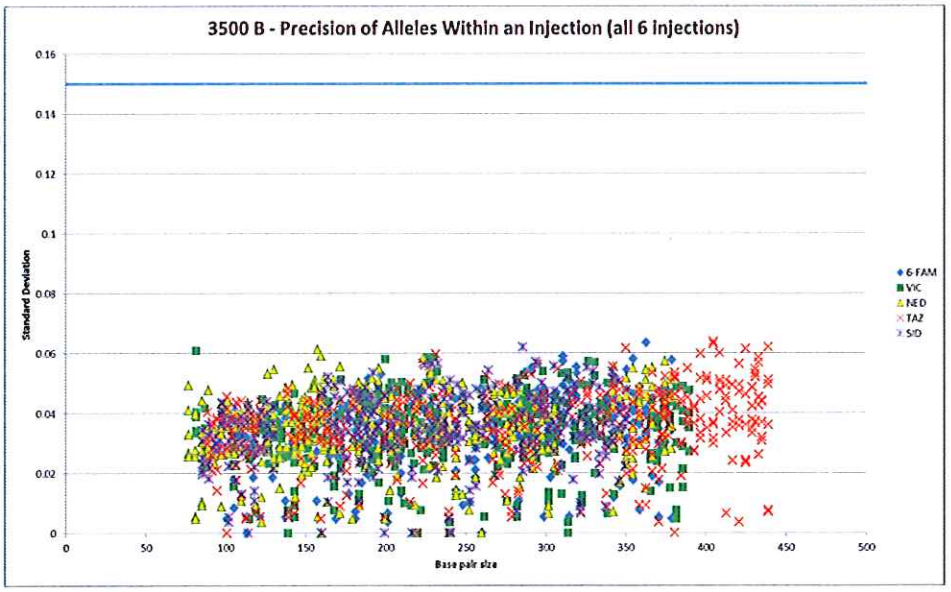
All ladder samples were included in calculations and all alleles were observed. One allelic ladder sample showed lower than expected peak heights for its size standard, however, complete data from all loci was still obtained and able to be used for calculations.

In order to assess the precision of the instrument, the standard deviation of size values was calculated for each allele. Occasionally, a measurement error may occur if a sample allele sizes outside the +/- 0.5 base pair window for a respective allelic ladder. The frequency of such an occurrence is lowest in detection systems having the smallest standard deviations in sizing. The

instance of a sample allele sizing outside of the +/- 0.5 base pair window because of measurement error is relatively rare when the standard deviation in sizing is approximately 0.15 base pairs or less.



All data was analyzed as a plate. The 0.15 base pair standard deviation threshold is shown in blue. All alleles sized well below the 0.15 base pair threshold.



All six injections were analyzed individually. The 0.15 base pair standard deviation threshold is shown in blue. All alleles sized well below the 0.15 base pair threshold.

iv. Conclusions

The data obtained from this study demonstrated that the precision of 3500 B is sufficient for measuring the DNA fragments from sample amplified with the Globalfiler™ kit. All standard deviations were well below the recommended 0.15 base pair threshold indicating that as few as one to two ladders can be used to determine allele calls for an entire plate. This was lower than the one ladder per three injections recommendation made by the manufacturer.

This data is consistent with the data obtained from the 3500xL A previously documented in this validation.

b. Sensitivity/Reproducibility

i. Objective

Amplification plates from the sensitivity study above and the 10x10 study for the Model Maker section of the STRmix™ Validation were run on 3500xL A and 3500 B. Data was analyzed to evaluate reproducibility and verify instrument sensitivity levels are similar. Additionally, an assessment of the analytical threshold for 3500 B was conducted.

ii. Materials and Methods

See Appendix for extraction, quantitation, dilution, amplification and 3500 set-up worksheets and reagents used. Instrument maintenance was documented on worksheets in the post-amplification laboratory. The instrument settings suggested by the Globalfiler™ User Manual were used for all injections. It is important to note that the recommended injection parameters for 3500xL A and 3500 B are different due to the number of capillaries.

Two amplification sets (Sensitivity study and 10x10 study for STRmix™ Validation) were performed and run on both the 3500xL A and 3500 B. For sensitivity plates, each plate for the 3500s was prepared individually. For the 10x10 amplification plates, the same 3500 plate was used on both 3500xL A and 3500 B. For this performance check, only data from 3500 B will be evaluated to

establish the analytical threshold and then compared to the calculations from 3500xL A. Data from both 3500xL A and 3500 B will be compared for reproducibility and sensitivity.

For the analytical threshold calculations, 36 samples amplified at target inputs of 0.5ng to 1.0ng were analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 1rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual for panels, bins, stutter and analysis method. All real alleles, known artifacts, elevated stutter and pull up peaks were eliminated. Alleles, sizes and peak heights were exported from GMID-X. Calculations for maximum, minimum, average and standard deviation were then performed by Microsoft® Excel® for each dye channel.

After a general analytical threshold was determined, data for all samples was analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 70rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual for panels, bins, stutter and analysis method. Alleles, sizes and peak heights were exported from GMID-X. All calculations for the results section below were then performed using Microsoft® Excel®.

iii. Results

For this study, all extraction, quantitation, amplification and detection controls produced expected results.

The following samples were not included in sensitivity or reproducibility calculations due to loss of resolution: CML-0.0234375_03_A07 (3500xL A), CML-0.0234375_08_A07 (3500 B), GG-0.046875_06_B06 (3500 B), and GG-0.375_01_H03 (3500xL A).

1. Analytical Threshold

Multiple definitions and expressions exist for the lower limit of detection for analyzing data. For this laboratory, the term analytical threshold will be defined as the minimum signal at which a peak can reliably be distinguished from noise. At this time, there are two generally accepted ways to calculate analytical threshold. Equation 1 (shown below) is suggested by the Scientific Working Group on DNA Analysis Methods (SWGDAM) in section 1.1. of the Interpretation

Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories .

Equation 1: Analytical Threshold = 2 (Maximum Peak Height - Minimum Peak Height)

Equations 2 and 3 were developed by the International Union of Pure & Applied Chemists (IUPAC). Limit of detection is defined as the smallest measure that can be detected with reasonable certainty. This equation is believed to result in an analytical threshold with 89-99.86% confidence that noise will be below this value.

Equation 2: Limit of Detection = Average Peak Height + (3 × Standard Deviation Peak Height)

Another important calculation is the Limit of Quantitation (LOQ). The LOQ is the estimated limit in which the signal is not only reliably detected but also the peak height is reliably measured.

Equation 3: Limit of Quantitation = Average peak height + (10 × Standard Deviation peak height)

Based on definition, all three equations were used to calculate an analytical threshold for 3500 B and compare it to the previously determined analytical threshold calculated for 3500xL A. While the SWGDAM equation has been used across the forensic community for the analysis of data from capillary electrophoresis instruments, the IUPAC equations are a mathematically supported approach for any type of analytical procedure.

Calculations using negative amplification controls were not calculated for this instrument based on the results obtained in the sensitivity study for 3500xL A. Using the negative control calculations was determined to be impractical for data analysis.

All three equations were applied to the samples with ideal targets and the following results were obtained:

Samples with Ideal Targets 0.5-1.0ng
SWGDAM
Analytical Threshold = 2 x (Maximum Peak Height - Minimum Peak Height)

	Min	Max	2*(max-min)
Blue	1	28	54
Green	1	38	74
Yellow	1	53	104
Red	1	42	82
Purple	1	42	82
		Average	**79.2**

IUPAC
Limit of Detection (LOD) = Average Peak Height + (3 x Standard Deviation)
Limit of Quantitation (LOQ) = Average Peak Height + (10 x Standard Deviation)

	Average	Standard Deviation	LOD	LOQ
Blue	4.947169162	2.615812532	12.79460676	31.10529448
Green	10.37057912	4.60309343	24.17985941	56.40151342
Yellow	4.752180233	3.191163554	14.32567089	36.66381577
Red	7.579620353	3.373994331	17.70160335	41.31956366
Purple	8.053592561	4.363125436	21.14296887	51.68484692

The following results were obtained in the 3500xL A analytical threshold assessment:

Samples with Targets 0.5ng-1.0ng
SWGDAM
Analytical Threshold = 2 x (Maximum Peak Height - Minimum Peak Height)

	Min	Max	2*(max-min)
Blue	1	47	92
Green	1	43	84
Yellow	1	49	96
Red	1	44	86
Purple	1	47	92
		Average	**90**

IUPAC
Limit of Detection (LOD) = Average Peak Height + (3 x Standard Deviation)
Limit of Quantitation (LOQ) = Average Peak Height + (3 x Standard Deviation)

	Average	Standard Deviation	LOD	LOQ
Blue	4.85734	4.99559311	19.84411981	54.81327
Green	7.719072	4.53857132	21.33478577	53.10479
Yellow	4.387467	3.792262397	15.76425434	42.31009
Red	6.115715	3.671940096	17.13153549	42.83512
Purple	7.423103	5.065069444	22.61831097	58.0738

Using the SWGDAM formula, similar values (+/- 10rfu) were calculated for the analytical threshold for all colors except for the blue. The overall average analytical threshold for 3500 B was lower than the analytical threshold calculated for 3500xL A. The same pattern was observed when using the IUPAC formulas. As with the sensitivity study from 3500xL A, the samples from this study will be analyzed at 70rfu and then

further assessed in the conclusions section of this study to determine a final appropriate analytical threshold for 3500 B.

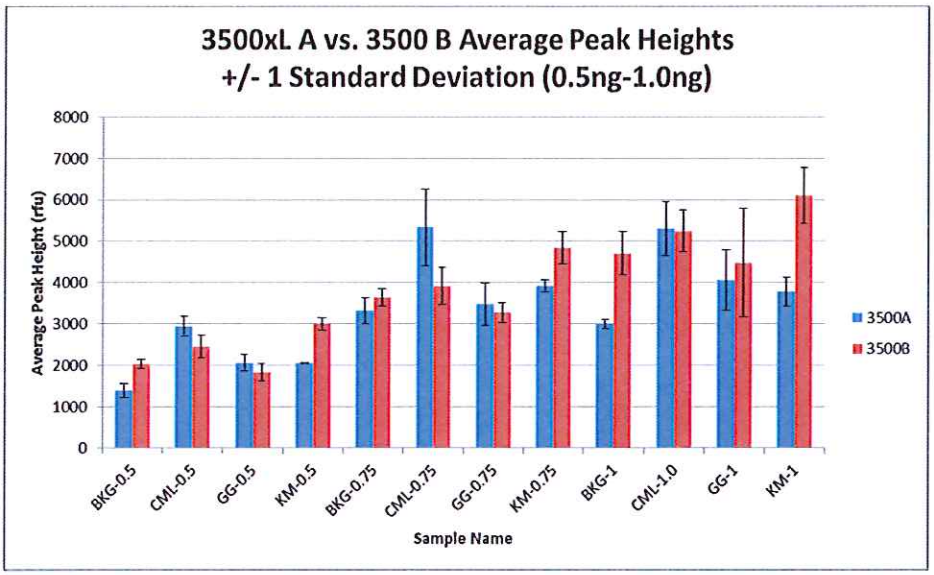
2. Sensitivity

Using a 70rfu analytical threshold for both data from both instruments, the number of alleles detected was similar for both 3500xL A and 3500 B. The chart below shows 100% of expected alleles in green, greater than 50% in yellow and 50% or less in red. All four sample sets produced dropout of the first allele and dropout of approximately half of the expected alleles within one target level. This data demonstrated similar sensitivity for 3500xL A and 3500B.

	3500A			3500B		
Sample Name	Allele Count	Expected Allele Count	% detected	Allele count	Expected Allele Count	% detected
BKG-0.005859375	5	132	3.79%	13	132	9.85%
BKG-0.0078125	9	132	6.82%	4	132	3.03%
BKG-0.01171875	5	132	3.79%	21	132	15.91%
BKG-0.015625	19	132	14.39%	26	132	19.70%
BKG-0.0234375	41	132	31.06%	38	132	28.79%
BKG-0.03125	33	132	25.00%	48	132	36.36%
BKG-0.046875	94	132	71.21%	90	132	68.18%
BKG-0.0625	87	132	65.91%	109	132	82.58%
BKG-0.09375	120	132	90.91%	122	132	92.42%
BKG-0.125	129	132	97.73%	127	132	96.21%
BKG-0.1875	132	132	100.00%	132	132	100.00%
BKG-0.25	132	132	100.00%	132	132	100.00%
BKG-0.375	132	132	100.00%	132	132	100.00%
BKG-0.5	132	132	100.00%	132	132	100.00%
BKG-0.75	132	132	100.00%	132	132	100.00%
BKG-1	132	132	100.00%	132	132	100.00%
BKG-1.5	132	132	100.00%	132	132	100.00%
CML-0.005859375	21	120	17.50%	16	120	13.33%
CML-0.0078125	31	120	25.83%	22	120	18.33%
CML-0.01171875	38	120	31.67%	30	120	25.00%
CML-0.015625	45	120	37.50%	33	120	27.50%
CML-0.0234375	40	80	50.00%	32	80	40.00%
CML-0.03125	80	120	66.67%	82	120	68.33%
CML-0.046875	101	120	84.17%	92	120	76.67%
CML-0.0625	114	120	95.00%	112	120	93.33%
CML-0.09375	120	120	100.00%	119	120	99.17%
CML-0.125	120	120	100.00%	120	120	100.00%
CML-0.1875	120	120	100.00%	120	120	100.00%
CML-0.25	120	120	100.00%	120	120	100.00%
CML-0.375	120	120	100.00%	120	120	100.00%
CML-0.5	120	120	100.00%	120	120	100.00%
CML-0.75	120	120	100.00%	120	120	100.00%
CML-1.0	120	120	100.00%	120	120	100.00%
CML-1.5	120	120	100.00%	120	120	100.00%
GG-0.005859375	11	135	8.15%	12	135	8.89%
GG-0.0078125	11	135	8.15%	10	135	7.41%
GG-0.01171875	5	135	3.70%	6	135	4.44%
GG-0.015625	37	135	27.41%	38	135	28.15%
GG-0.0234375	75	135	55.56%	63	135	46.67%
GG-0.03125	83	135	61.48%	78	135	57.78%
GG-0.046875	122	135	90.37%	76	90	84.44%
GG-0.0625	123	135	91.11%	117	135	86.67%
GG-0.09375	132	135	97.78%	131	135	97.04%
GG-0.125	135	135	100.00%	134	135	99.26%
GG-0.1875	135	135	100.00%	135	135	100.00%
GG-0.25	135	135	100.00%	135	135	100.00%
GG-0.375	90	90	100.00%	135	135	100.00%
GG-0.5	135	135	100.00%	135	135	100.00%
GG-0.75	135	135	100.00%	135	135	100.00%
GG-1	135	135	100.00%	135	135	100.00%
GG-1.5	135	135	100.00%	135	135	100.00%
KM-0.005859375	14	114	12.28%	18	114	15.79%
KM-0.0078125	32	114	28.07%	30	114	26.32%
KM-0.01171875	30	114	26.32%	39	114	34.21%
KM-0.015625	45	114	39.47%	46	114	40.35%
KM-0.0234375	65	114	57.02%	63	114	55.26%
KM-0.03125	71	114	62.28%	79	114	69.30%
KM-0.046875	88	114	77.19%	93	114	81.58%
KM-0.0625	103	114	90.35%	106	114	92.98%
KM-0.09375	114	114	100.00%	114	114	100.00%
KM-0.125	114	114	100.00%	114	114	100.00%
KM-0.1875	114	114	100.00%	114	114	100.00%
KM-0.25	114	114	100.00%	114	114	100.00%
KM-0.375	114	114	100.00%	114	114	100.00%
KM-0.5	114	114	100.00%	114	114	100.00%
KM-0.75	114	114	100.00%	114	114	100.00%
KM-1	114	114	100.00%	114	114	100.00%
KM-1.5	114	114	100.00%	114	114	100.00%

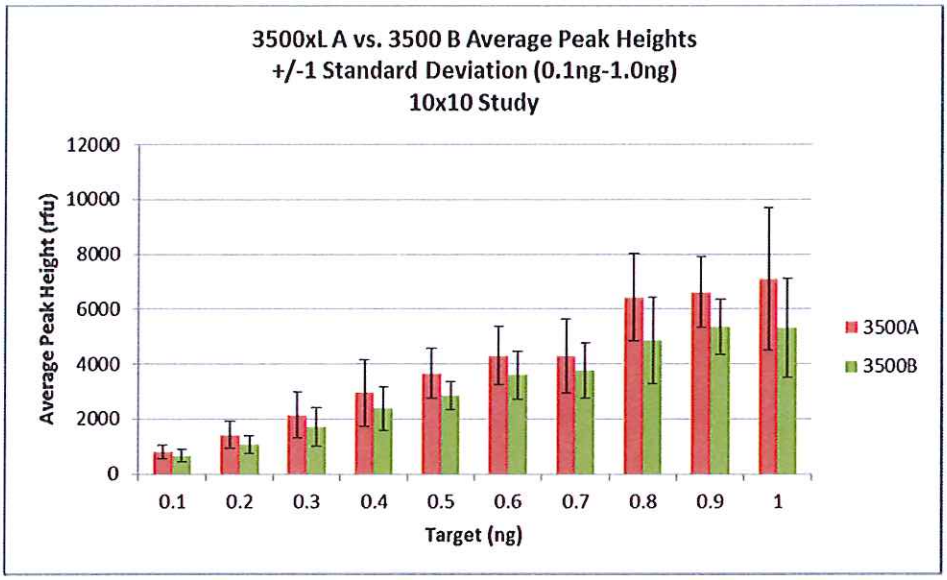
3. Reproducibility

For the first data set, two separate 3500 plates were prepared from each sample sensitivity amplification plate. Average peak heights and standard deviations were calculated from the replicates and plotted in the graph below.



Most samples were within three standard deviations of each other, however some showed significant variability. This may be related to the expected pipetting differences which may occur when plates are separately prepared and run. Additionally, no pattern was observed where one instrument demonstrated a clear higher or lower sensitivity than the other instrument.

To eliminate pipetting differences, an additional set of reproducibility data was produced using the 10x10 plate required for the STRmix validation. One plate was prepared and run on both instruments. Average peak heights and standard deviations were calculated and plotted in the graph below. It is important to note that these samples were not amplified in replicates. Therefore, all ten samples amplified at each target were averaged and used to determine standard deviation.



While 3500xL A showed slightly higher overall average peak heights, all sample targets were within one or two standard deviations.

iv. Conclusions

Based on the studies above, the performance of 3500 B was consistent with the performance of 3500xL A. Similar sensitivities were observed indicating that despite a difference in the analytical threshold calculation for the blue dye channel, a single analytical threshold of 90rfu for both instruments can be recommended. This value will not only provide the reliable detection of true alleles, but also an efficient approach to casework analysis. The precision, sensitivity and reproducibility studies also demonstrate that all other recommended Globalfiler™ settings, parameters, and interpretation guidelines from the 3500xL A portion of this validation are applicable to any data produced by 3500 B.

X. Final Conclusions

Based on this set of studies, the Globalfiler™ PCR Amplification Kit has been verified as precise, sensitive, accurate, reproducible, repeatable and capable of amplifying mixed DNA samples. Additionally, samples amplified with the Globalfiler™ kit may be reliably detected and interpreted from data produced on either 3500xL A or 3500 B. Both instruments will be run using the manufacturer's recommended settings for run modules, run conditions, and injection conditions. For accurate sizing of alleles, as few as one to two ladders may be included on each detection plate for either 3500xL A or 3500 B. Analysis in GeneMapper™ *ID-X* Version 1.5 will be

performed using a 90rfu analytical threshold and the manufacturer's recommendations for all other settings except stutter filters. While this validation was conducted with the default settings for stutter, a set of laboratory specific filters will be further defined in the Stutter Study of the STRmix™ Validation.

The target range for amplifications is 0.5-1.0ng with an ideal target of 0.75ng. At this target, average peak heights are approximately 4,000rfu and peak height ratios are 55% or greater. Depending on sample type, this ideal target may need to be adjusted higher to evaluate low level contributors or raise peak heights of large base pair fragments in degraded samples, however, caution should be taken when peak heights are 10,000rfu or greater. At this level, additional artifacts were observed which may interfere with the resolution of a true contributor.

Dropout was first observed at the 0.1875ng target however significant dropout was not observed until the 0.03125ng target. Using quantitation data, an amplification cutoff of 100pg is recommended to allow the laboratory to only amplify samples where interpretable results may be obtained. This recommendation is based on the level at which the average peak heights of low level DNA samples fall below a recommended stochastic threshold range of 500-600rfu.

A mixture study was prepared and generally evaluated to demonstrate that two person contributors could be resolved up to a 1:15 ratio when amplified at 0.6ng. However, because mixed samples may be amplified at slightly higher targets, 20 was recommended as an amplification cutoff for the Total/Male DNA quantity in sexual assault kit samples with an expected female major contributor and male minor contributor. This value was determined based on the quality of the profile and quantity of alleles which can be expected from the minor male contributor. Three, four and five person mixtures were also amplified and typed in this study, however further evaluation of all mixtures (two, three, four and five person) will be conducted in the STRmix™ Validation.

Typically, specific recommendations for laboratory interpretation guidelines are given based on the data obtained in the amplification kit validation. However, because the laboratory will be utilizing a probabilistic genotyping software (STRmix™) to interpret and apply statistics to results, further detail regarding these recommendations for initial and final profile interpretation will be covered in the STRmix™ validation. The values provided above for Total DNA amplification cutoff, analytical threshold, target input range, and Total/Male DNA quantity cutoff will not be further evaluated and will be included in laboratory protocols. The values recommended in this validation for stochastic threshold and peak height ratio minimums will be considered general guidelines for the following types of decisions:

- Determining whether additional amplifications (replicates or adjusted targets) of a sample may improve the quality of a sample interpretation.

- Making initial gross observations regarding the overall suitability of a profile for interpretation.
- Determining whether a set of matching profiles may be run in STRmix one time to represent the statistic for all obtained profiles.

XI. Appendix

a. Documentation of observed artifacts during analysis including acknowledgement by LifeTechnologies.
b. All set-up worksheets, results, analysis and calculations spreadsheets will be maintained as electronic copies on CD after completion of technical review.
    i. Instrumentation and Maintenance records will remain in the post-amplification laboratory.

XII. References

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