Internal Validation

Applied Biosystems™

Globalfiler™ PCR Amplification Kit

using the Applied Biosystems™

3500/3500xL Genetic Analyzer

This validation has been technically reviewed and approved for use by:

Susan E. Welti — 121916

Susan Welti, FBU Technical Leader | Date

This validation was conducted and written by:

121916

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I. Introduction

The following report describes the internal validation of the Applied Biosystems™ Globalfiler™ PCR Amplification Kit using the Applied Biosystems™ 3500/3500xL Genetic Analyzer at the District of Columbia Department of Forensic Sciences (DC DFS), Forensic Biology Unit. All studies were conducted in accordance with the requirements and guidelines issued by:

- International Organization for Standardization/International Electrotechnical Commission International Standard 17025 General Requirements for the Competence of Calibration and Testing Laboratories (ISO/IEC 17025:2005) and any supplemental requirements for Accreditation of Forensic Testing Laboratories
- Federal Bureau of Investigation Quality Assurance Standards for Forensic DNA Testing Laboratories
- Scientific Working Group on DNA Analysis Methods (SWGDAM) Validation Guidelines for DNA Analysis Methods.
- DC DFS Department Operation Manual – Procedures for Validating Technical Procedures
- DC DFS Forensic Science Laboratory Quality Assurance Manual
- DC DFS Forensic Biology Unit Quality Assurance Manual

II. Precision

a. Objective

To assess the ability of the instrument to reproducibly measure the size of an amplified DNA fragment, 24 Globalfiler™ allelic ladder aliquots were injected four times on the 3500xL Genetic Analyzer ("A"). Allele sizes were calculated using an internal lane standard (ILS). Precision was expressed as a standard deviation calculated for each allele within each locus.

The following SWGDAM Validation Guideline was addressed in this study:

"4.3 Precision and accuracy of the assay should be demonstrated:

Precision characterizes the degree of mutual agreement among a series of measurements, values and/or results. Precision depends only on the distribution of random errors and does not relate to the true value or specified value. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results..."

b. 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.

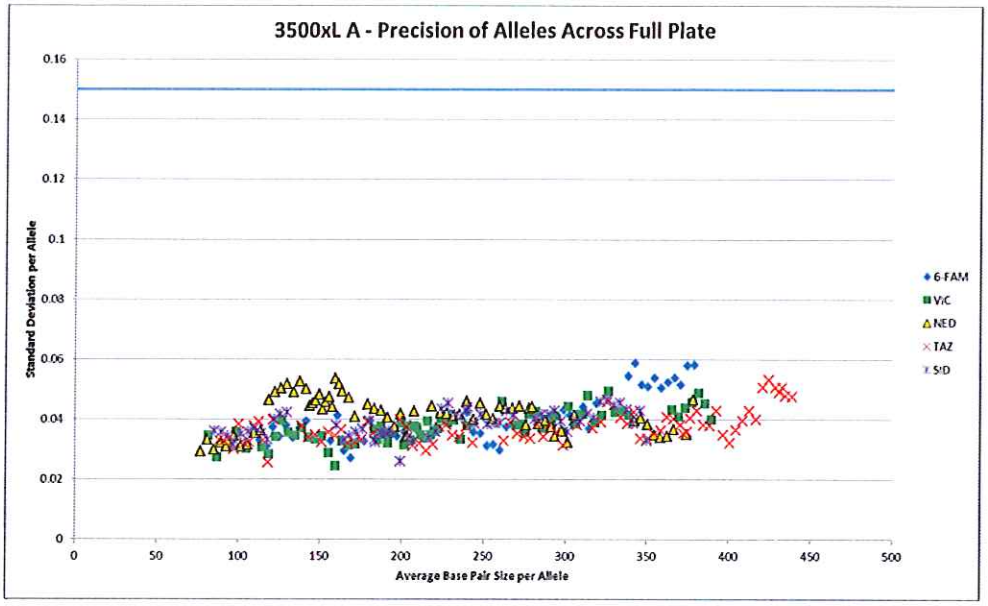
All data was analyzed using Applied Biosystems™ GeneMapper™ *ID-X* (GMID-X) Version 1.5 with a peak amplitude threshold of 50rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual for panels, bins, stutter and analysis method.

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.

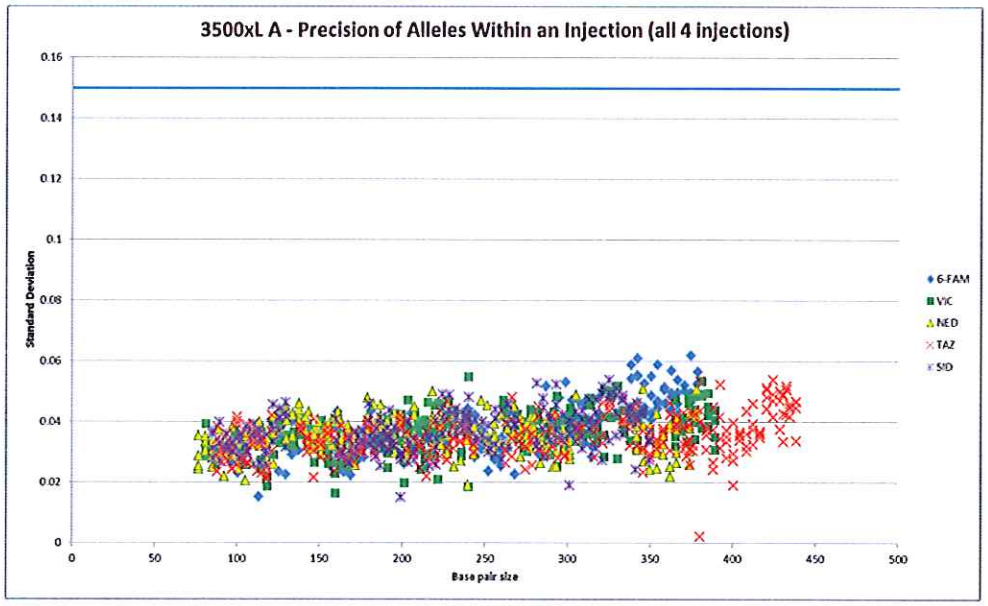
c. Results

Two allelic ladder samples were not included in calculations due to an instrument artifact or loss of resolution. All alleles were observed for all other allelic ladder samples. Additional allelic ladder samples showed lower than expected peak heights for size standards, 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.



In the graph above, 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.



In the graph above, all four 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.

d. Conclusions

The data obtained from this study demonstrated that the precision of 3500xL A is sufficient for measuring the DNA fragments from samples 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 is lower than the one ladder per injection recommendation made by the manufacturer.

Precision data for the additional 3500 Genetic Analyzer ("B") is contained in the performance check section of this validation.

III. Sensitivity

a. Objective

For sensitivity, serial dilutions of four samples were prepared to assess amplification and instrument performance, determine an optimal quantity of template DNA, determine a target at which interpretable results are no longer obtained, and help understand any interpretational limitations over a range of DNA input amounts. While this study will conclude with a set of recommendations for ideal target input, analytical threshold, stochastic threshold and peak height ratio minimums, all settings will be re-evaluated and possibly adjusted in subsequent studies. Additionally, no specific values were recommended for stochastic threshold or peak height ratio minimums due to the intended use of STRMix™ for interpretation.

The following SWGDAM Validation Guideline was addressed in this study:

"4.2 Sensitivity and Stochastic Studies: The laboratory should demonstrate sensitivity levels of the test. Sensitivity studies are used to determine the dynamic range, ideal target range, limit of detection, limit of quantitation, heterozygote balance (e.g., peak height ratio) and the signal to noise ratio associated with the assay. Sensitivity studies can also be used to evaluate excessive random (stochastic) effects generally resulting from low quantity and/or low quality samples."

b. 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.

Amplifications were performed and run on both 3500xL A and 3500 B. Sample dilution series were also quantified in triplicate using Promega Plexor® HY on the 7500. For this sensitivity study, only data from 3500xL A will be evaluated. See the performance check section of this validation for a comparison study and results from 3500 B.

For the analytical threshold calculations, 36 samples amplified at target inputs of 0.5ng to 1.0ng, four negative amplification controls and one reagent blank 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. One set of calculations was obtained for samples containing DNA (36) and one set of calculations was obtained from samples not containing DNA (5).

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. 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. Quantitation results were analyzed and exported from the Plexor® HY software. All calculations for the results section below were then performed using Microsoft® Excel®.

c. Results

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

i. 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 x (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 determine possible analytical thresholds for this laboratory's forensic DNA analysis. 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.

All three equations were applied and the following results were obtained:

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**

Negative Samples
SWGDAM
Analytical Threshold = 2 x (Maximum Peak Height - Minimum Peak Height)

	Min	Max	2*(max-min)
Blue	1	13	24
Green	2	17	30
Yellow	1	8	14
Red	1	17	32
Purple	1	14	26
		Average	**25.2**

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	4.85734	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

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	3.742310889	1.629112325	8.629647865	20.03343414
Green	7.05238829	2.455531668	14.41898329	31.60770497
Yellow	3.243243243	1.174225191	6.765918818	14.98549516
Red	5.630173565	1.939329024	11.44966064	25.02846381
Purple	6.155656109	2.131379416	12.54979436	27.46945027

The SWGDAM and IUPAC calculations above using Negative Samples were significantly lower than the manufacturer's recommendation (50 rfu). It was not expected that these values for analytical threshold would be practical for data analysis. The IUPAC and SWGDAM calculations using samples amplified from 0.5-1.0ng produced analytical thresholds ranging from 40-100rfu. Data will therefore be evaluated in subsequent studies for a variety of analytical thresholds in this range to determine the most effective value for casework analysis.

To evaluate the calculated analytical threshold, all sensitivity samples (including the positive control) amplified at or below 2ng were analyzed at 70rfu and a table of all the detected peaks which were discordant or non-stutter (based on 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)	**513**
Total Number ≥80rfu	376
Total Number ≥90rfu	280
Total Number ≥100rfu	207
Total Number of pull up	207
Total Number of n-4	28
Total Number of n+4	159
Total Number of n-2	5
Total Number of n-8	17
Total Number of n-6	1
Total Number of offscale	15
Total Number of unknown	33
Total Number of background	12
Total Number of artifact	36

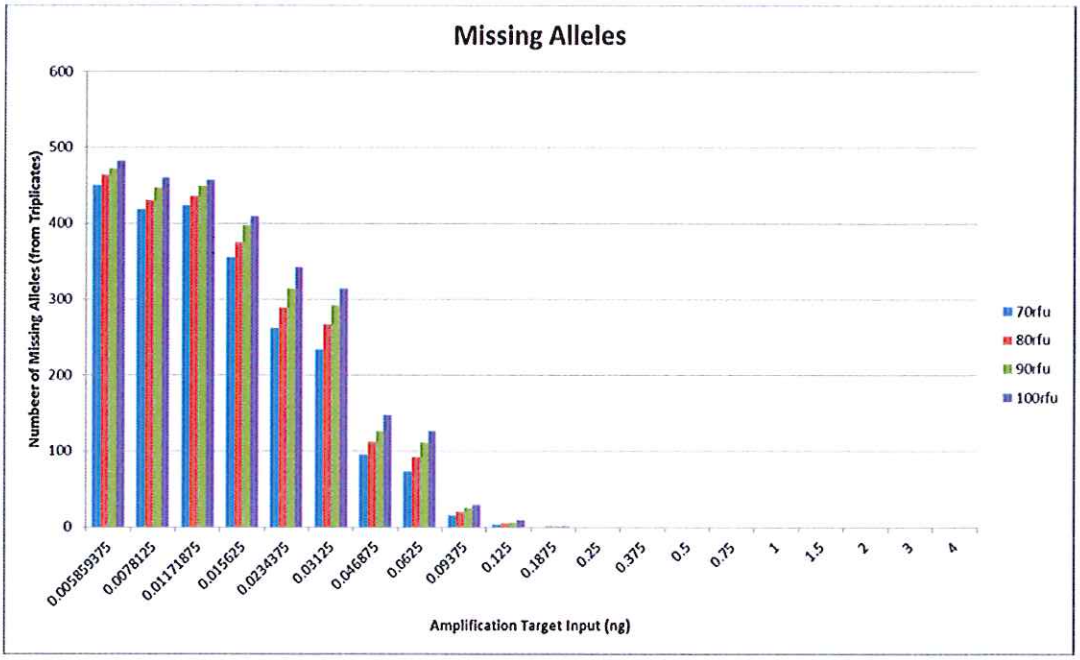
Most of the artifacts observed were easy to identify and eliminate (pull up, background, known artifacts, and stutter) or would be re-amplified at a lower template due to offscale data. The elevated stutter peaks in the n+4 and n-4 positions may be appropriately modeled with the laboratory's interpretation software, STRMix™. The n-2, n-8, and n-6 stutter peaks will be further

evaluated in subsequent studies to determine whether or not a filter in GMID-X is needed.

Of the 33 peaks with an unknown cause, 16 are below the 90rfu analytical threshold calculated using the SWGDAM formula. The remaining alleles range from 90-179rfu. A more detailed analysis of these discordant alleles is discussed in the Concordance section below and the Contamination study of this validation.

The 36 peaks identified as "artifact" are supported by published data in the manufacturer's user manual. The majority of these edits were at the D5S818 and TH01 loci.

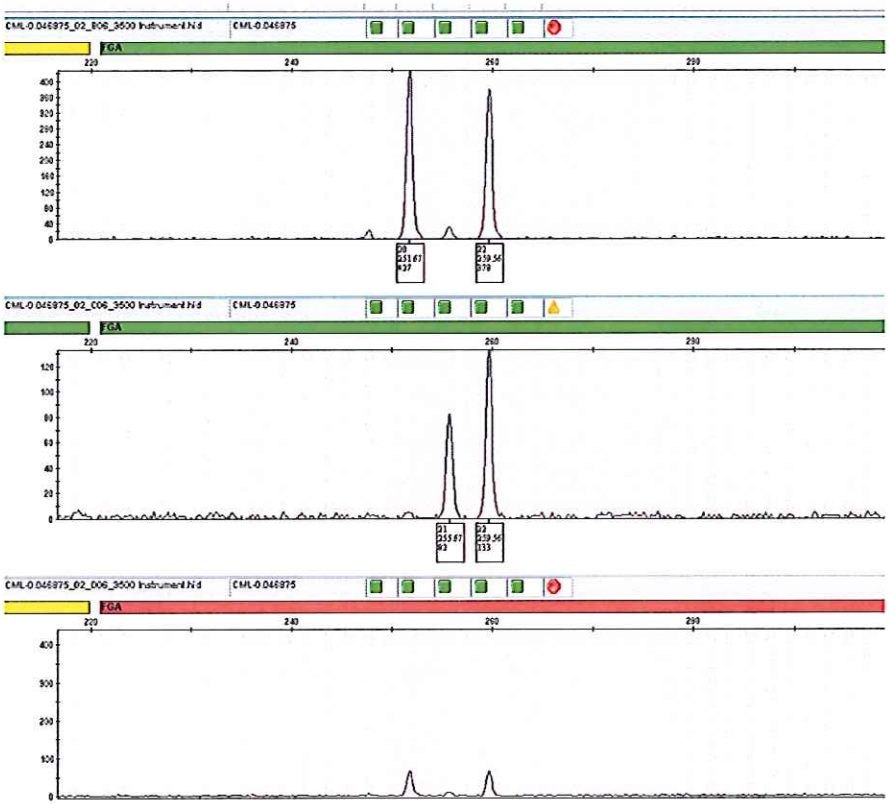
Additionally, the number of observed and expected alleles was calculated for analytical thresholds of 70rfu, 80rfu, 90rfu and 100rfu.



Using a 70rfu analytical threshold, dropout was not observed until the 0.125ng target, however significant edits (513) would be needed during sample analysis. Using an 80rfu or higher analytical threshold, dropout of one allele was observed at the 0.1875ng target and sample analysis became less difficult with a significant decrease in edits at each step (376 at 80rfu, 280 at 90rfu and 207 at 100rfu). Despite the analytical threshold, significant dropout was observed at the 0.03125ng target.

ii. Concordance

18 samples produced discordant peaks which could not be attributed to the corresponding known profile or an identifiable artifact. All were amplified at targets of 1.5ng or higher and may be associated with sample and/or locus peak heights above approximately 10,000rfu with the exception of one sample amplified at 0.046875ng. All three replicates of this sample are shown in the diagram below. The first replicate (B06) produced the concordant genotype of 20,22. The second replicate (C06) produced a 21,22, however, the 21 allele would not have been detected if the analytical threshold was raised to 90rfu. The third replicate (D06) shows activity below threshold consistent with a 20,22.



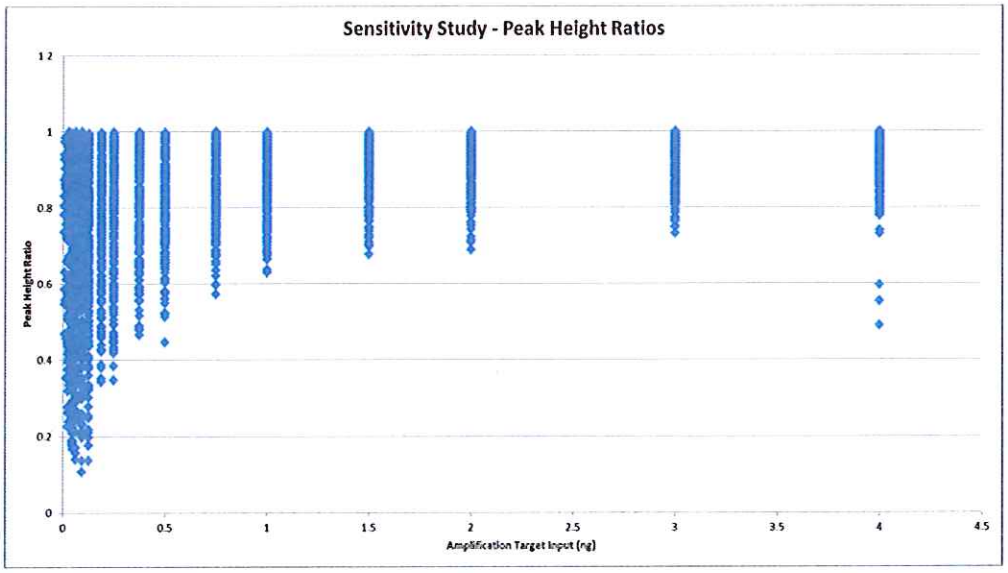
iii. Average peak height

Amp Target (ng)	APH per target (rfu)	Standard Deviation	Observed Alleles	Expected Alleles	Missing Alleles
0.005859375	87.44565217	28.30493248	51	501	450
0.0078125	103.5	36.44420393	83	501	418
0.01171875	101.1111111	36.39902319	78	501	423
0.015625	112.9580153	52.87867238	146	501	355
0.0234375	136.5212766	62.70214006	239	501	262
0.03125	138.9240196	66.47000406	267	501	234
0.046875	206.1634241	96.32995129	405	501	96
0.0625	210.2671756	95.18458168	427	501	74
0.09375	360.7025547	160.5162745	486	501	15
0.125	533.4746377	256.9718967	498	501	3
0.1875	625.9782609	266.1735686	501	501	0
0.25	1192.38587	457.3197594	501	501	0
0.375	1672.538043	793.5724962	501	501	0
0.5	2090.655797	813.786582	501	501	0
0.75	3981.110507	1426.345348	501	501	0
1	4006.369565	1407.390881	501	501	0
1.5	8722.032609	3211.847613	501	501	0
2	8711.474638	3567.522778	501	501	0
3	14026.69384	4743.817505	501	501	0
4	19048.72283	5383.85733	501	501	0

Average peak height (APH) was calculated by averaging the peak height of both alleles at heterozygote loci and halving the peak height at homozygous loci. The Y Indel and DYS391 loci were both included as their single allele peak height. This method of calculating the APH was used throughout this validation. Offscale data was observed in the 1.5ng and higher targets. While no data from the 1ng target samples was indicated by GMID-X as offscale, typical signs of overamplification were observed including pull up, elevated stutter, background, and known artifacts.

With a 70rfu analytical threshold, dropout was observed at the 0.125ng target with an average peak height of 533rfu. With a 90rfu analytical threshold, dropout was observed at the 0.1875ng target with an average peak height of 626rfu. This demonstrates that stochastic effects may be observed when a sample is amplified at these targets or less and peak heights are 500-600rfu or less. A further evaluation of stochastic threshold is discussed below.

iv. Peak Height Ratio



Peak height ratios for all eligible loci were calculated and plotted on the above chart. At the 0.5ng target, one locus had a peak height ratio below 50%. All peak height ratios for the 0.75ng target and greater were above 57%.

Amp Target (ng)	Average PHR by target	minimum PHR	standard deviation
0.005859375	0.806607531	0.737373737	0.051692348
0.0078125	0.725262932	0.46875	0.214181492
0.01171875	0.850111307	0.558139535	0.151527312
0.015625	0.732717211	0.352941176	0.192280733
0.0234375	0.670788986	0.225626741	0.218566785
0.03125	0.657855407	0.239350913	0.2306742
0.046875	0.642951585	0.168831169	0.241096325
0.0625	0.626162257	0.139805825	0.203555984
0.09375	0.666545778	0.108043217	0.21220359
0.125	0.699527046	0.136678201	0.197428
0.1875	0.760917248	0.343629344	0.163285439
0.25	0.768020814	0.347950429	0.158799869
0.375	0.81512728	0.465331279	0.126470252
0.5	0.822296191	0.447121034	0.116636324
0.75	0.860033067	0.571960298	0.098797416
1	0.871044918	0.629925961	0.085188001
1.5	0.895070563	0.677021617	0.072891314
2	0.904082852	0.689756736	0.068051633
3	0.911554687	0.73176047	0.06302022
4	0.914408778	0.489354235	0.071038962

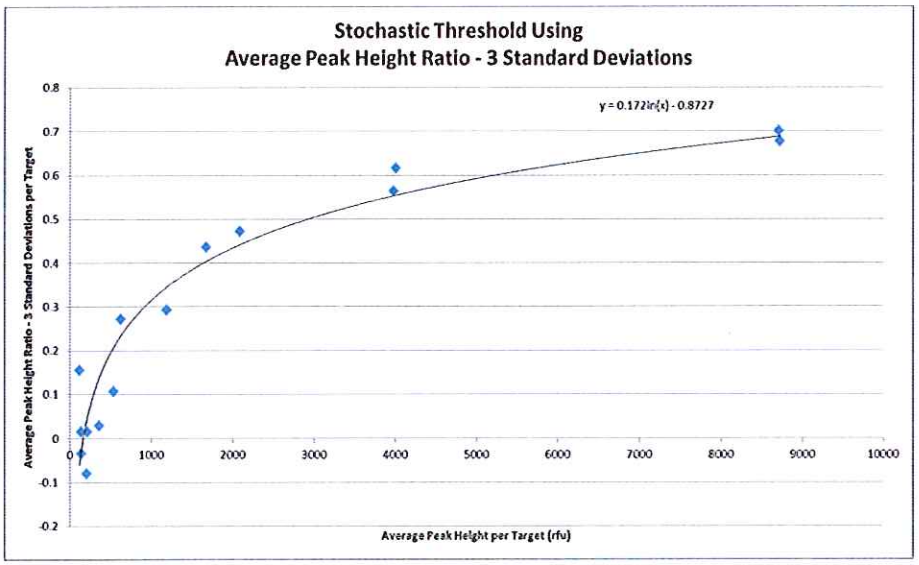
Note: The minimum peak height ratios for 3ng and 4ng samples are not considered accurate due to significant oversaturation.

v. Stochastic Threshold

For each proposed analytical threshold, the highest allele missing its heterozygous partner was determined visually and confirmed by an additional individual. Similar values of 570 and 578rfu were obtained for the 70, 80 and 90rfu analytical thresholds. For the 100rfu analytical threshold, the highest allele missing its heterozygous partner was 833rfu.

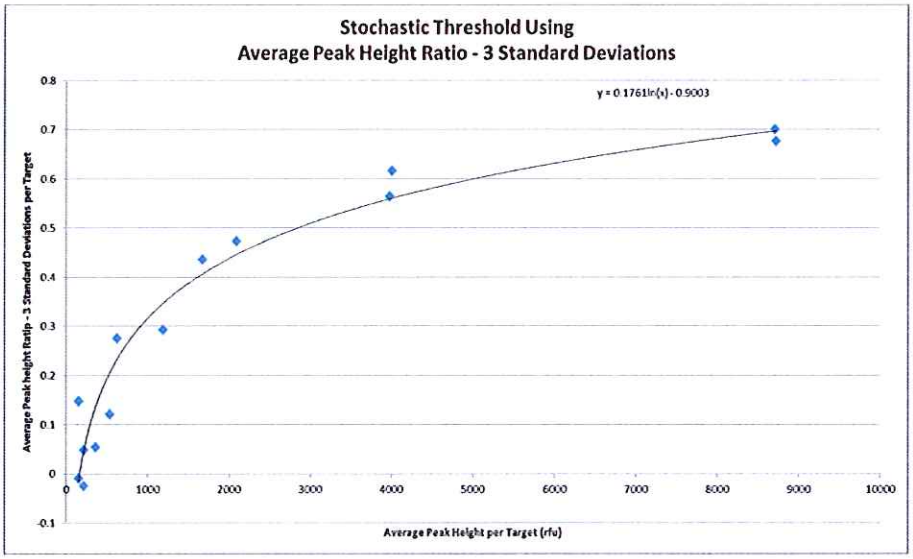
Additionally, a comparison of the above values was done to a stochastic threshold calculated by plotting the average peak heights by the average peak height ratios minus three standard deviations. These values then represent the lowest peaks which would be expected from an average peak height.

With a 70rfu analytical threshold, the following results were obtained:



Target (ng)	Average Peak Height (rfu)	Minimum Expected Peak Height Ratio (Average PHR - 3SD)	Lowest Expected Heterozygous Partner (rfu)
0.015625	112.9580153	-0.059653213	-6.738308547
0.0234375	136.5212766	-0.027065358	-3.694997267
0.03125	138.9240196	-0.024064528	-3.343140975
0.046875	206.1634241	0.043831098	9.036369277
0.0625	210.2671756	0.047221186	9.929065347
0.09375	360.7025547	0.140045231	50.51467273
0.125	533.4746377	0.207358783	110.6206517
0.1875	625.9782609	0.234862291	147.0186883
0.25	1192.38587	0.34569838	412.2058634
0.375	1672.538043	0.403900777	675.5394146
0.5	2090.655797	0.442280089	924.6554315
0.75	3981.110507	0.553062366	2201.802396
1	4006.369565	0.554150212	2220.130542
1.5	8722.032609	0.687960505	6000.413958
2	8711.474638	0.687752174	5991.335619

With a 90rfu analytical threshold, the following results were obtained:



Target (ng)	Average Peak Height (rfu)	Minimum Expected Peak Height Ratio (Average PHR - 3SD)	Lowest Expected Heterozygous Partner (rfu)
0.0234375	153.9713376	-0.013325425	-2.051733577
0.03125	157.4640719	-0.009375352	-1.476281175
0.046875	217.3272358	0.047365283	10.29376593
0.0625	220.729249	0.050100576	11.05866257
0.09375	365.1985294	0.138767682	50.67775324
0.125	536.1909091	0.206398737	110.6691263
0.1875	626.1431159	0.233709856	146.3358173
0.25	1192.38587	0.347141597	413.9267352
0.375	1672.538043	0.406731376	680.2737006
0.5	2090.655797	0.446025544	932.48589
0.75	3981.110507	0.559448562	2227.226548
1	4006.369565	0.560562339	2245.819893
1.5	8722.032609	0.697562296	6084.161093
2	8711.474638	0.697348999	6074.938118

Using this information, dropout of the heterozygous partner allele could be expected at peak heights as high as 500rfu regardless of an analytical threshold at 70 or 90rfu. This is consistent with the observed data where the highest alleles observed missing their heterozygous partner were at 570 and 578rfu.

vi. Amplification Cutoff

The table below shows the amplification targets at which results interpretation may not be possible due to significant allelic dropout or stochastic effects. Samples were color coded red, yellow or green based on the number of alleles obtained from each set of replicates. The previous amplification cutoff was established using allelic dropout as the criteria, however, an additional approach was also considered for this validation using average peak height and stochastic effects. The amplification target at which average peak heights fall below the recommended 500-600rfu stochastic threshold from above were color coded purple. An average of these values was then calculated in the chart below along with a conversion to quantitation values from organic extracts (32µl) or unconcentrated EZ1 extracts (50µl).

Quant Sample Name	Amp Target- before quant	Amp target - after quant	Observed Alleles	Expected Alleles	% alleles obtained	Average peak height
BKG-0.000391	0.00585938	0.0026585	1	132	0.76%	141
BKG-0.000521	0.0078125	0.009424	3	132	2.27%	85.666667
BKG-0.000781	0.01171875	0.004008	3	132	2.27%	76.75
BKG-0.00104	0.015625	0.0128805	12	132	9.09%	125.54167
BKG-0.0015625	0.0234375	0.011304	30	132	22.73%	121.07407
BKG-0.00208	0.03125	0.02555	21	132	15.91%	131.75
BKG-0.003125	0.046875	0.03114	80	132	60.61%	157.94915
BKG-0.00417	0.0625	0.04969	72	132	54.55%	178.36364
BKG-0.00625	0.09375	0.066185	113	132	85.61%	253.88971
BKG-0.00833	0.125	0.077855	127	132	96.21%	379.11972
BKG-0.0125	0.1875	0.12433	131	132	99.24%	465.04861
BKG-0.0167	0.25	0.1824	132	132	100.00%	871.3125
CML-0.000391	0.00585938	0.005544	12	120	10.00%	104.04167
CML-0.000521	0.0078125	0.0086205	18	120	15.00%	118.08824
CML-0.000781	0.01171875	0.008172	25	120	20.83%	126.08696
CML-0.00104	0.015625	0.016146	35	120	29.17%	132.93333
CML-0.0015625	0.0234375	0.028845	44	120	36.67%	162.4875
CML-0.00208	0.03125	0.03658	71	120	59.17%	192.64
CML-0.003125	0.046875	0.04119	98	120	81.67%	253.70492
CML-0.00417	0.0625	0.07964	111	120	92.50%	263.43182
CML-0.00625	0.09375	0.075445	120	120	100.00%	510.94697
CML-0.00833	0.125	0.110515	120	120	100.00%	799.17424
CML-0.0125	0.1875	0.21055	120	120	100.00%	870.02273
CML-0.0167	0.25	0.24085	120	120	100.00%	1683.1591
GG-0.000391	0.00585938	0.00937125	7	135	5.19%	100.42857
GG-0.000521	0.0078125	0.0070275	6	135	4.44%	107.5
GG-0.000781	0.01171875	0.0126115	3	135	2.22%	96.4
GG-0.00104	0.015625	0.0126185	23	135	17.04%	122.925
GG-0.0015625	0.0234375	0.029675	64	135	47.41%	159.90816
GG-0.00208	0.03125	0.0412	58	135	42.96%	138.71739
GG-0.003125	0.046875	0.04926	117	135	86.67%	224.02941
GG-0.00417	0.0625	0.06427	109	135	80.74%	208.45588
GG-0.00625	0.09375	0.11296	131	135	97.04%	363.74306
GG-0.00833	0.125	0.15352	134	135	99.26%	437.34722
GG-0.0125	0.1875	0.2094	135	135	100.00%	585.125
GG-0.0167	0.25	0.23425	135	135	100.00%	1123.8056
GG-0.025	0.375	0.4406	135	135	100.00%	1479.6181
GG-0.0333	0.5	0.4481	135	135	100.00%	2055.5347
GG-0.05	0.75	0.77795	135	135	100.00%	3470.1319
GG-0.0667	1	1.01345	135	135	100.00%	4058.3611
GG-0.1	1.5	1.44995	135	135	100.00%	9415.875
GG-0.133	2	1.913	135	135	100.00%	6753.9861
GG-0.2	3	3.05	135	135	100.00%	12428.243
GG-0.267	4	3.713	135	135	100.00%	17741.042
KM-0.000391	0.00585938	0.007182	9	114	7.89%	101.14286
KM-0.000521	0.0078125	0.0081435	27	114	23.68%	121.5
KM-0.000781	0.01171875	0.00816825	21	114	18.42%	108.35
KM-0.00104	0.015625	0.018405	33	114	28.95%	116.5
KM-0.0015625	0.0234375	0.021063	49	114	42.98%	160.23171
KM-0.00208	0.03125	0.0215755	59	114	51.75%	149.97059
KM-0.003125	0.046875	0.022935	79	114	69.30%	231.9569
KM-0.00417	0.0625	0.06131	97	114	85.09%	226.14063
KM-0.00625	0.09375	0.07095	114	114	100.00%	332.34848
KM-0.00833	0.125	0.08977	114	114	100.00%	550.00758
KM-0.0125	0.1875	0.12608	114	114	100.00%	602.75
KM-0.0167	0.25	0.1927	114	114	100.00%	1126.6894
Average Amplification Cutoff		0.10711 ng				
Quantitation Cutoff for 50µl extract (EZ1)		0.0042844 ng/µl				
Quantitation Cutoff for 32µl extract (organic)		0.00714067 ng/µl				

	67-100% alleles obtained
	34-66% alleles obtained
	0-33% alleles obtained
	amp target at which APH shifts to greater than 500rfu

d. Conclusions

While several methods of establishing an appropriate analytical threshold were evaluated, the SWGDAM suggested equation applied to samples in the ideal target range (0.5-1.0ng) produced the most effective values. Analyzed data using a 90rfu

analytical threshold resulted in sufficient sensitivity to obtain reliable results for low level samples AND did not produce unreliable data that would require significant analyst time and attention for analysis, interpretation and/or re-work. The equation was applied and calculated per dye, however the differences in the values obtained are not significant enough to warrant a per dye set of analytical thresholds. Therefore, for this study, a 90rfu analytical threshold will be recommended for all dyes as an optimum parameter for the analysis of Globalfiler™ data on 3500xL A. A range of analytical thresholds will continue to be evaluated in subsequent studies to verify whether any adjustments are necessary.

While 33 discordant peaks were observed, only 17 of those peaks were over the recommended 90rfu analytical threshold. All 17 peaks were also associated with samples amplified at 1.5ng or higher and contained peak heights at or above 10,000rfu. These samples showed evidence of oversaturation and may be reamplified in normal casework to confirm genotypes. All discordant alleles will be further evaluated in the Contamination study of this validation.

Full profiles and acceptable peak height ratios were obtained for samples amplified above 0.5ng. Offscale data was observed in some 1.5ng samples. Therefore, the ideal target range determined by this study was consistent with the manufacturer's recommended 0.5-1.0ng. Average peak heights are expected to be 2000-4000rfu with peak height ratios greater than 50-60%. With a 90rfu analytical threshold, dropout may occur at amplification targets as high as 0.1875ng and significant dropout is expected at amplification targets of 0.03125ng and lower.

Additionally, a stochastic threshold was assessed but will not be closely defined since the laboratory will be using STRmix™ for interpretation. At a 90rfu analytical threshold, the highest allele observed missing its heterozygous partner was 578rfu. A calculated value of approximately 500rfu was also determined using the average peak heights obtained for each amplification target and the expected peak height ratio minus three standard deviations. Based on both approaches, a stochastic threshold range of 500-600rfu was recommended to guide analysts when interpreting whether a set of single source profiles need to be individually interpreted by STRmix™ or can be interpreted as one profile.

While two approaches were used to calculate a Total DNA amplification cutoff, a value of 107pg value was calculated from the level at which stochastic effects are observed. For ease of use, it is recommended to establish the Total DNA amplification cutoff as a rounded value of 100pg. The approach used in the previous Identifiler® Plus Amplification Cutoff Validation was based on the level at which results shift from high to medium (green to yellow). This approach may not have sufficiently considered that the

samples used for validation are higher quality than typically encountered in casework. By utilizing the approach based on stochastic effects, sample quality would be taken into account and allow analysts to only amplify samples where interpretable profiles can be obtained without needlessly amplifying samples with no chance of providing useful information.

IV. Accuracy

a. Objective

Both profile and sizing accuracy were addressed in this study by amplifying a set of 37 samples which included the NIST SRM 2391c (A-D) and 33 mock casework samples from a variety of sources. For the purposes of this study, these samples were assessed to confirm concordant genotypes were obtained when compared to published documentation and previously obtained results. Additionally, the size difference of each allele when compared to the allelic ladder was calculated to confirm each allele sized within the recommended +/- 0.5 base pair window.

The following SWGDAM Validation Guideline was addressed in this study:

"4.3 Precision and accuracy of the assay should be demonstrated...

...Accuracy is the degree of conformity of a measured quantity to its actual (true) value. Accuracy of a measuring instrument is the ability of a measuring instrument to give responses close to a true value."

b. Materials and Methods

See Appendix for extraction, quantitation, dilution, amplification and 3500 set-up worksheets and reagents used. Some samples were previously extracted and their original 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™.

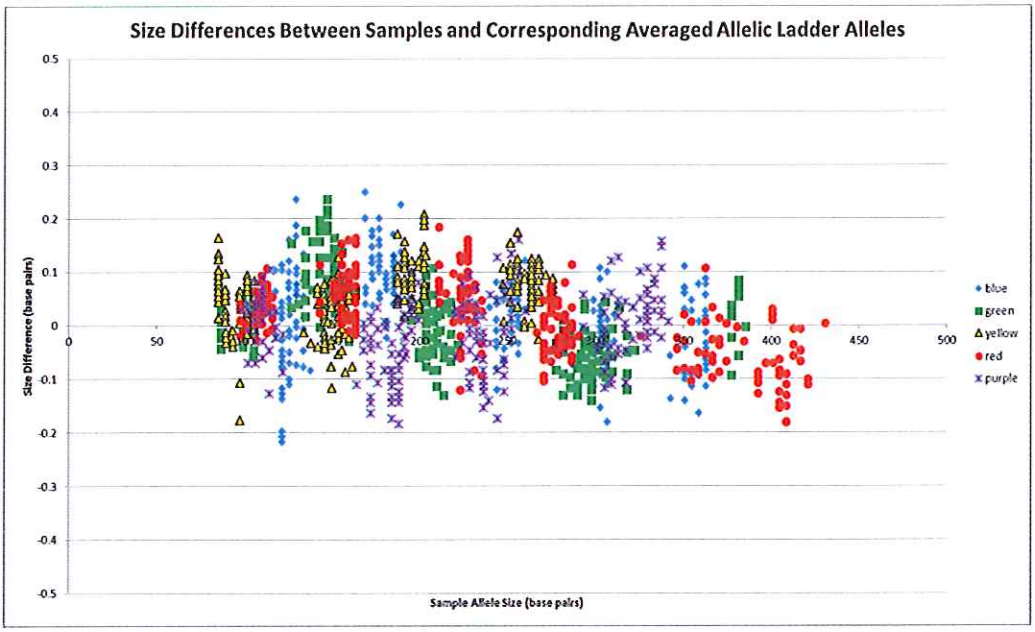
All samples were analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 70rfu for all dye channels and the suggested settings from the Globalfiler™ User Manual for panels, bins, stutter and analysis method. As with sensitivity, 70rfu was used to ensure that the analytical threshold would 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 extraction, quantitation, amplification and detection controls produced expected results.

i. Sizing Accuracy

To determine sizing accuracy, allelic ladder alleles across the plate were averaged and then compared to the sizes obtained from the corresponding concordant alleles in each sample. In the plot below, all alleles sized within the +/-0.5 base pair "window" required for the detection and correct assignment of alleles. If an allele sizes outside of the +/-0.5bp "window" either the allele is a true "off-ladder" and must be re-injected to confirm an appropriate assignment is made or there is a possible measurement error by the instrument and further investigation must be made to determine cause.



ii. Profile Accuracy

Because the mock casework samples from these amplification plates came from a variety of sources and included such issues as inhibition, degradation and limited template, they will not be used in the assessment of profile accuracy or reproducibility/repeatability. Please see the section titled "Mock Casework" for an analysis of the obtained results.

Both replicate amplifications of the NIST SRM 2391c samples (A-D) were evaluated for accuracy using their published results. Concordant genotypes were obtained at all loci with a total of 96 allele edits. While this is a higher than expected number of allele edits, peak heights for these samples were also higher than those obtained from the 0.75ng target samples in the sensitivity study. The sensitivity study 0.75ng target samples produced average peak heights of approximately 4,000rfu, while the range of average peak heights obtained in this study for the single source NIST SRM 2391c samples (A-C) ranged from 4,100-9,400rfu.

Nearly all allele edits were attributed to those expected from higher than optimal peak heights (pull up, background, elevated stutter and minus A) and one off-ladder allele (204rfu) with no known cause was detected at a locus where the concordant allele was greater than 23,000rfu. This peak was not present in the replicate of this sample at or below the analytical threshold, however, the peak height was significant lower at this locus (3,400rfu). With a

90rfu analytical threshold, the total number of artifacts lowered to 49 and no concordant alleles were missing.

Total Number of OL/non-matching (≥70rfu)	96
Total Number ≥80rfu	64
Total Number ≥90rfu	49
Total Number ≥100rfu	35
Total Number of pull up	45
Total Number of n-4/n+4	3
Total Number of n-4	1
Total Number of n+4	30
Total Number of n-2	1
Total Number of n-8	0
Total Number of n-6	0
Total Number of offscale	0
Total Number of artifact	2
Total Number of background	12
Total Number of minus a	1
Total Number of unknown	1

d. Conclusions

Based on the results from this study, accurate results were obtained from the NIST SRM 2391c samples (A-D). Additionally, the sizing accuracy using ladders averaged across the plate is sufficient for reliable allele calls to be made.

V. Reproducibility/Repeatability

a. Objective

To evaluate the reproducibility and repeatability of Globalfiler™, six samples were amplified in triplicate at an ideal target on two separate occasions by the same analyst and a third time by a separate analyst. Results were evaluated and compared to each other as well as the sensitivity study.

The following SWGDAM Validation Guidelines were addressed in this study:

"4.3.1 Repeatability: Precision and accuracy of results (e.g., quantitation and/or qualitative) of the same operator and/or detection instrument should be evaluated.

4.3.2 Reproducibility: Precision and accuracy of results (e.g., quantitative and/or qualitative) among different operators and/or detection instruments should be evaluated."

b. 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.

Six samples were amplified at a 0.75ng target in triplicate for three separate amplifications (two by the same analyst and one by a separate analyst). All three were separately run on the 3500xL A.

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. After artifact removal, concordance was verified by two separate individuals and all calculations for the results section below were then performed using Microsoft® Excel®.

c. Results

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

i. Concordance

All samples produced full profiles with concordant genotypes with the exception of the following:

A single Y allele at 187rfu was detected at Amelogenin in one replicate of sample C on 091616AF-RUN2. Because all other replicates were clear single source samples and the sample itself did not produce any other indications of contamination, the sample was still used for calculations.

One replicate of sample H on 091616YP-RUN1 showed low level contamination and was not included in calculations. All other replicates of this sample produced clear single source profiles.

ii. Analytical Threshold

As with the sensitivity study, all samples were analyzed at 70rfu and a table of all the detected peaks which were discordant or non-stutter (based on 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)	**461**
Total Number ≥80rfu	330
Total Number ≥90rfu	244
Total Number ≥100rfu	181
Total Number of pull up	185
Total Number of n-4	1
Total Number of n+4	137
Total Number of n-2	1
Total Number of n-8	7
Total Number of artifact	17
Total Number of background	53
Total Number of minus a	1
Total Number of spike	29
Total Number of contamination	22
Total Number of unknown	7

Most of the artifacts observed were easy to identify and eliminate (pull up, background, known artifacts, and stutter), however the total number of OL/non-matching alleles at this ideal amplification target was higher than expected from the sensitivity study. An evaluation of the data was conducted if the analytical threshold was raised to 90rfu from the SWGDAM formula in the sensitivity study. The same table as above was generated.

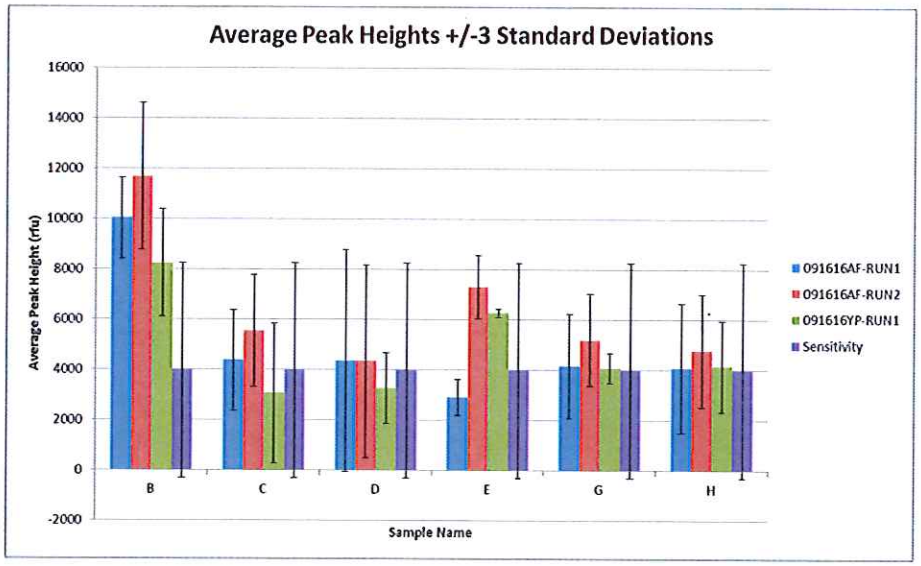
Total Number of OL/non-matching (≥90rfu)	**244**
Total Number ≥100rfu	181
Total Number of pull up	74
Total Number of n-4	1
Total Number of n+4	81
Total Number of n-2	1
Total Number of n-8	2
Total Number of artifact	12
Total Number of background	34
Total Number of spike	24
Total Number of contamination	10
Total Number of unknown	5

The number of pull up artifacts was significantly reduced along with the amount of n+4 stutter. Of the remaining unknown peaks, two were at the same location

for the same sample indicating that the artifact may specific to that sample. All of the unknown peaks will be further evaluated in the Contamination study of this validation.

iii. Average peak height and peak height ratio

Sample Name	Run Name	APH per sample	Standard Deviation
0.75ng	Sensitivity	3981	1426
B	091616AF-RUN1	10039	535
B	091616AF-RUN2	11689	972
B	091616YP-RUN1	8246	714
C	091616AF-RUN1	4372	669
C	091616AF-RUN2	5539	743
C	091616YP-RUN1	3061	930
D	091616AF-RUN1	4364	1469
D	091616AF-RUN2	4341	1279
D	091616YP-RUN1	3269	469
E	091616AF-RUN1	2911	238
E	091616AF-RUN2	7297	421
E	091616YP-RUN1	6263	56
G	091616AF-RUN1	4156	689
G	091616AF-RUN2	5198	611
G	091616YP-RUN1	4081	198
H	091616AF-RUN1	4074	853
H	091616AF-RUN2	4764	745
H	091616YP-RUN1	4150	602



The average peak heights of five of six samples were within three standard deviations when amplified by the same operator on two different occasions or two different operators on two different occasions. Sample E showed lower

values for standard deviations than any other sample demonstrating that the sample itself was very reproducible within its replicates. Sample B showed the most variation from the sensitivity study; however, overall average peak heights and standard deviations were similar to the higher targets with higher standard deviations.

If all samples were used to calculate an average peak height for the reproducibility/repeatability study, average peak heights were within two standard deviations of the overall average peak heights obtained in the sensitivity study.

In addition to sample average peak heights (APH), individual loci were also assessed to demonstrate intralocus balance and peak height ratios (PHR) which can be expected at an ideal target.

Marker	Minimum APH	Maximum APH	Average Peak Height	Minimum PHR	Maximum PHR	Average PHR
D3S1358	2567	15039	6462	0.6973	0.9997	0.8768
vWA	2006	11174	5017	0.7262	0.9969	0.8983
D16S539	1559	9121	3747	0.5881	0.9932	0.8782
CSF1PO	1827	9106	4105	0.7183	0.9961	0.8812
TPOX	1549	8660	3557	0.6748	0.9855	0.8520
Yindel	4359	17664	9480			
AMEL	3065	17959	7311	0.6576	0.9894	0.8328
D8S1179	2876	17086	7108	0.7543	0.9821	0.8874
D21S11	2143	10676	4716	0.6647	0.9958	0.8880
D18S51	1967	9873	4367	0.6474	0.9995	0.8790
DYS391	3052	11880	7111			
D2S441	2971	15804	6968	0.7341	0.9964	0.9036
D19S433	2467	13839	6165	0.6575	0.9973	0.8888
TH01	1944	14533	6092	0.7471	0.9913	0.8967
FGA	2671	14494	6340	0.5958	0.9908	0.8910
D22S1045	2101	11671	4740	0.6470	0.9679	0.8556
D5S818	2520	12081	5431	0.6774	0.9960	0.8716
D13S317	2606	13266	5921	0.6415	0.9877	0.8601
D7S820	2445	12339	5036	0.7076	0.9993	0.8802
SE33	2601	14271	5716	0.6745	0.9913	0.8754
D10S1248	2241	11926	5303	0.6874	0.9853	0.8942
D1S1656	2299	11419	4940	0.6946	0.9988	0.8788
D12S391	1804	9564	4006	0.7307	0.9983	0.8994
D2S1338	2650	14812	6316	0.6212	0.9962	0.8664

d. Conclusions

Five of the six samples amplified for this study showed that the Globalfiler™ kit is reproducible (Samples B, C, D, G, and H) and repeatable (Samples B, C, D, G, and H) at the suggested ideal target of 0.75ng within three standard deviations or less. Five of the

six samples (Samples C, D, E, G, and H) were also reproducible with the data obtained from the same target in the sensitivity study. Using an analytical threshold of 90rfu allowed more efficient analysis of data than an analytical threshold of 70rfu. The observed peak height ratios were consistent with those obtained in the sensitivity study, confirming the recommended 50-60% peak height ratio threshold for samples amplified in the ideal target range of 0.5-1.0ng. Based on this study, if samples are able to be amplified at 0.75ng or higher, a slightly higher peak height ratio (57-60%) can be expected.

VI. Mixtures

a. Objective

For this study, mixtures of two, three, four and five contributors were generated at different ratios and amplification targets. Each two person mixture was closely evaluated to verify concordance and the previously recommended values for analytical threshold, stochastic threshold and peak height ratios. Additionally, the two person mixture samples were evaluated to establish a Total/Male DNA quantity which samples from sexual assault cases may be stopped following the quantitation step since interpretable results of the male contributor are not expected.

Three, four and five person mixtures were only generally evaluated in this study to confirm appropriate results were obtained for controls and no samples would require re-amplification or re-injection. Additional evaluations will be conducted on two, three, four and five person mixtures and discussed in the STRmix™ validation.

The following SWGDAM Validation Guideline was addressed in this study:

"4.4 Mixture studies: Mixed DNA samples that are representative of those typically encountered by the testing laboratory should be evaluated. These studies will assist a casework laboratory to establish guidelines for mixture interpretation, which may include determination of the number of contributors to the mixture, determination of the major and minor contributor profiles, and contributor ratios or proportions."

b. 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.

Four two-person mixture sets were amplified in triplicate at two different targets (0.3ng and 0.6ng) at the following ratios: 1:1, 1:2, 1:3, 1:5, 1:7, 1:10, 1:15, 1:20 and 1:25. 100 three person mixtures, 47 four-person mixtures and 30 five-person mixtures were amplified in duplicate at various targets and ratios. All samples were run on 3500xl A. All two person mixture samples were also quantified in triplicate using Plexor® HY on the 7500.

All two-person mixtures were analyzed using GMID-X Version 1.5 with a peak amplitude threshold of 90rfu 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. After artifact removal, concordance was verified by two separate individuals and all calculations for the results section below were then performed using Microsoft Excel.

For three, four and five-person mixtures, samples were initially analyzed using the settings listed above to verify controls and assess for general amplification and injection quality. Data for all two, three, four and five person mixtures will be re-analyzed and evaluated in the STRmix™ validation.

c. Results

The following samples were not included in the assessments below due to loss of resolution: MIX1_F05, MIX1_B04, MIX2_D10, MIX4_F04, MIX4_G05, MIX4_G02, MIX4_H07. All extraction, quantitation, amplification and detection controls produced expected results with the exception of Ladder_04_E10_3500A from 090716YP-RUN1 which was deleted from the GMID-X project due to indications of a poor injection at D2S441.

i. Analytical Threshold

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

Total Number of OL/non-matching (≥90rfu)	218
Total Number >100rfu	180
Total Number of pull up	11
Total Number of n-4	20
Total Number of n+4	68
Total Number of n-2	4
Total Number of n-4/n+4	44
Total Number of n-2/n+2	3
Total Number of spike	43
Total Number of background	12
Total Number of artifact	11
Total Number of unknown	2

The majority of the discordant peaks observed in these samples can be attributed to stutter. Only two unknown peaks were observed. One unknown peak may be attributed to the unusual number of spikes observed in a few of the samples. The second unknown peak was an off-ladder allele which would be re-injected or reamplified to determine reproducibility. Both of the unknown peaks will be further evaluated in the Contamination study of this validation.

ii. Concordance

Following the removal of the above listed peaks, concordance was verified for the two person mixtures by two separate individuals. The number of missing alleles was then calculated and is represented in the following charts for the 0.3 and 0.6ng targets.

Missing Alleles (0.3ng)						
Ratio	MIX1	MIX2	MIX3	MIX4	Average	Total
1_01	0	1	0	0	0.25	1
1_02	2	2	0	0	1	4
1_03	5	5	0	5	3.75	15
1_05	9	16	1	5	7.75	31
1_07	16	29	0	8	13.25	53
1_10	41	48	1	30	30	120
1_15	56	39	9	45	37.25	149
1_20	70	70	18	67	56.25	225
1_25	43	61	28	66	49.5	198

Missing Alleles (0.6ng)						
Ratio	MIX1	MIX2	MIX3	MIX4	Average	Total
1_01	0	0	0	0	0	0
1_02	0	0	0	0	0	0
1_03	0	0	0	0	0	0
1_05	0	0	0	0	0	0
1_07	2	9	0	1	3	12
1_10	11	15	0	3	7.25	29
1_15	25	38	0	12	18.75	75
1_20	32	61	4	19	29	116
1_25	44	50	4	29	31.75	127

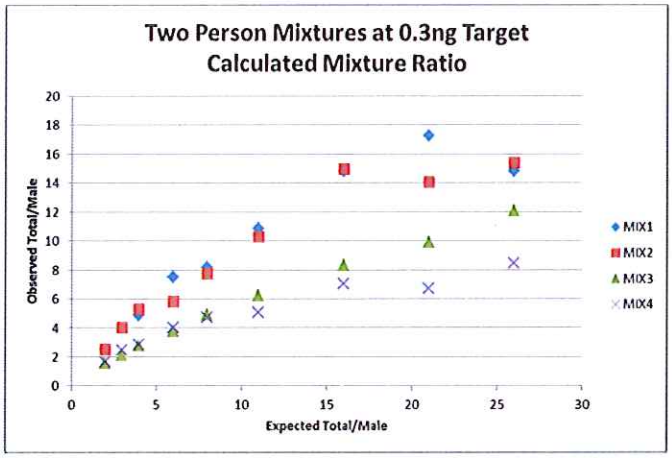
As expected, the 0.6ng target two person mixtures produced less overall dropout than the 0.3ng target two person mixtures. For the 0.6ng, dropout of the minor contributor was not observed until the Total/Male DNA quantity was eight or higher (1_07, minor contributor input of 0.075ng). This was a higher ratio than expected based on the sensitivity study which indicated dropout may be observed as high as 0.1875ng (approximately a 1_03 ratio at 0.6ng). However, the 0.3ng target two person mixtures were consistent with the expected dropout range observed in the sensitivity study. Allele dropout was observed at the 1_01 ratio which is a 0.15ng target for both contributors.

As with the sensitivity study, significant dropout for the 0.3 and 0.6ng target two person mixtures was observed at the 1_10 and 1_20 ratios where the minor contributor target input fell below 0.03125ng.

iii. Calculated Mixture Ratios

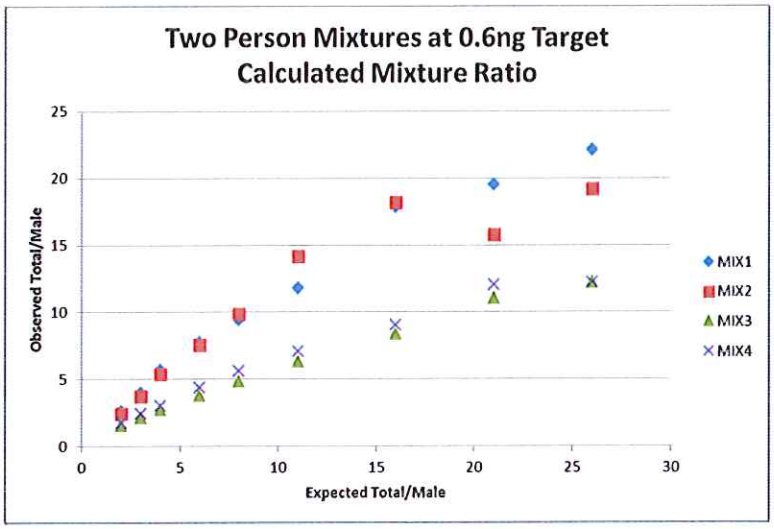
For each mixture, observed percentages of contributor one (C1) were calculated for all loci with discernible genotypes for the two contributors. The per locus percentage was then averaged for each individual sample and ratio. From this averaged percentage, the observed Total/Male DNA quantity was calculated and compared to the expected Total/Male DNA quantity.

Total/Male (0.3ng)		Observed Total/Male				
Ratio	Expected Total/Male	MIX1	MIX2	MIX3	MIX4	Average
1_01	2	2.518612	2.491453	1.58026	1.676236	2.06664
1_02	3	3.975107	4.002596	2.156251	2.466805	3.15019
1_03	4	4.851549	5.300161	2.808035	2.839728	3.949868
1_05	6	7.49506	5.808108	3.808565	4.012275	5.281002
1_07	8	8.179764	7.765758	4.93389	4.724047	6.400864
1_10	11	10.8531	10.27943	6.23434	5.07445	8.11033
1_15	16	14.79046	14.97601	8.313738	7.024831	11.27626
1_20	21	17.28726	14.05587	9.914325	6.729843	11.99683
1_25	26	14.8116	15.35575	12.0638	8.453044	12.67105



For the 0.3ng target two person mixtures the observed Total/Male DNA quantity was similar to the expected Total/Male DNA quantity until eight (7:1 ratio). At this point, significant stochastic effects and/or dropout of the minor contributor caused less data with which to calculate an average for the sample and more variability in the calculated mixture ratio. This data demonstrated the difficulty in determining the actual mixture ratio of a sample as the Total/Male DNA quantity increases.

Total/Male (0.6ng)		Observed Total/Male				
Ratio	Expected Total/Male	MIX1	MIX2	MIX3	MIX4	Average
1_01	2	2.6056525	2.4605302	1.5716346	1.7641219	2.1004848
1_02	3	3.9711087	3.7770698	2.16396	2.4495079	3.0904116
1_03	4	5.7251892	5.39603	2.7672898	3.0583258	4.2367087
1_05	6	7.7372167	7.6070449	3.7925292	4.3802939	5.8792712
1_07	8	9.4765529	9.8497872	4.8857287	5.6263659	7.4596087
1_10	11	11.812485	14.189066	6.3187484	7.1398154	9.8650286
1_15	16	17.889832	18.198744	8.4079302	9.0820504	13.394639
1_20	21	19.541982	15.796678	11.033867	12.049579	14.605527
1_25	26	22.15225	19.163805	12.222056	12.21319	16.437825



For the 0.6ng target two person mixtures the observed Total/Male DNA quantity was similar to the expected Total/Male DNA quantity until 21 (20:1 ratio). At this point, significant stochastic effects and/or dropout of the minor contributor caused less data with which to calculate an average for the sample and more variability in the calculated mixture ratio. In comparison to the 0.3ng target two person mixtures, an accurate calculation of Total/Male DNA quantity may be obtained for mixtures 16 (15:1 ratio) or less when a mixture may be amplified in the ideal target range (0.5-1.0ng).

iv. Stochastic Threshold

Alleles were observed without their heterozygous partner in the two person mixtures as high as 700 and 801rfu, however the majority were under 600rfu. This was consistent with the observed 500-600rfu stochastic threshold recommended by the sensitivity study.