Palm Beach County Sheriff's Office Forensic Biology Unit
Internal Validation with PowerPlex® Fusion 6C, ProFlex™ PCR System, and 3500xL Series Genetic Analyzer
Validation Report (Full Chemistry/Instrument Validation)

Validation Performed by Sorenson Forensics

Introduction

Internal validation of the PowerPlex® Fusion 6C System manufactured by Promega Corporation was performed at the Palm Beach County Sheriff's Office (PBSO) Forensic Biology Unit (FBU). The kit was validated using a ProFlex™ PCR System (Serial number 297807793 (A), a 3500xL Series Genetic Analyzer (Serial number 25363-061 3500xl B), and GeneMapper® *ID-X* (GMID-X) software version 1.5 (all from Applied Biosystems). This combination of polymerase chain reaction (PCR) amplification chemistry and instrument and capillary electrophoresis (CE) instrument was evaluated to determine suitability for use in forensic DNA casework.

The PowerPlex® Fusion 6C System amplifies 27 short tandem repeat (STR) loci on human DNA using a six-dye system. The genetic loci targeted by the kit are: AMEL, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, SE33, D22S1045, DYS391, FGA, DYS576, and DYS570. The internal lane standard used for this validation was WEN Internal Lane Standard 500. The standard was used for sizing of DNA fragments during electrophoresis.

Summary of Validation Studies

The following validation studies/criteria were used to assess the suitability of PowerPlex® Fusion 6C with the ProFlex™ PCR System and the 3500xL Series Genetic Analyzer for use in forensic casework:

(1) Cycle Number
(2) Baseline
(3) Sensitivity
(4) Stochastic Events
(5) Reproducibility/Repeatability
(6) Known Samples
(7) Mock Evidence Samples
(8) NIST SRM Concordance
(9) Precision
(10) Stutter
(11) Mixtures
(12) Contamination Monitoring
(13) Establishing a quantification based cut-off

Results and analyses from the studies indicate that the PowerPlex® Fusion 6C System with the ProFlex™ PCR System and 3500xL Series Genetic Analyzer is suitable for use in forensic casework.

The validation criteria listed above were examined by using quantifications, amplifications, and CE runs of samples to generate sufficient data. This validation was conducted according to the Scientific Working Group for DNA Analysis Methods (SWGDAM) validation guidelines and the FBI Quality Assurance Standards (September 2011 revision). This report includes results, conclusions, and recommendations for each enumerated validation criteria.

Materials and Methods

Samples for the validation were collected by the FBU for analysis during validation. Various substrates including swabs and Whatman® bloodstain cards were used to collect biological material such as blood. Two National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 2391c components (B and C) were used to determine NIST SRM concordance.

Sterile autoclaved water provided by the FBU was used for DNA extract dilutions and amplification reaction setup.

Sample Preparation (Extraction)

DNA samples consisted of extracts from known individuals and extracts from casework-like samples used for the mock evidence sample study. Extracts were generated by the FBU and by Sorenson Forensics at the FBU laboratory using EZ1 Advanced XL Biorobot instruments with EZ1 DNA Investigator Kits (both from QIAGEN).

EZ1 processing was performed in accordance with validated protocol at the FBU. Carrier RNA (cRNA) for extraction was prepared by the FBU with 310 µl TE^{-4} buffer added to individual cRNA tubes for solubilizing cRNA before division into 16-µl aliquots. Master mix consisting of 190 µl Buffer G2, 10 µl of proteinase K, and 1 µl cRNA per sample was prepared with extra volume to account for pipetting discrepancies. Substrates for extraction were put into individual microcentrifuge tubes with NAO spin baskets. Two hundred microliters (200 µl) of master mix were added to each sample. Tubes with baskets, substrates, and master mix were incubated at 56°C in a ThermoMixer for 15 minutes with shaking at 850 rpm.

Following incubation, the tubes were vortexed for 5-10 seconds prior to centrifugation at maximum speed for 2 minutes. Spin baskets with substrates were then discarded. Cap hinges of tubes were severed with sterile scissors to allow for easy removal of caps during EZ1 processing.

Prior to the first EZ1 extraction of the day, a 20-minute UV decontamination was performed on the EZ1 instrument. DNA Investigator Kit cartridges were installed in the instrument cartridge rack following quick mixing of well reagents by inversion and tapping of cartridges to settle reagents in wells. Elution tubes were inserted into position 1 of the EZ1 rack, tip holders with filter tips were inserted into position 2, and sample tubes were inserted into position 4 with the rack inserted into the EZ1 instrument.

The EZ1 instrument was operated using the Trace protocol with TE buffer elution and 40 µl of elution buffer. Following runs, elution tubes with extracts were removed for storage. Cartridges, tip holders with tips, and sample tubes were discarded in biohazard waste containers. Ethanol was used to clean cartridge and tip racks. Ethanol was also used to clean the piercing units.

Each extraction batch was processed with an extraction control negative/reagent blank. A list of all samples used in the validation is included in the companion binder to this report.

Quantification

Select extracts and dilutions for studies were quantified with the PowerQuant® System (Promega). The resulting autosomal and male DNA concentrations were used to characterize extracts used during validation. The validated protocol used by the FBU was used for quantifying extracts. PowerQuant® Male Genomic DNA was used to generate a dilution series for the standard curve. The undiluted standard was used for the 50 ng/µl concentration. Two microliters (2 µl) of the undiluted standard was combined with 48 µl of PowerQuant Dilution Buffer to make the 2 ng/µl concentration (this was in accordance with the protocol's ratio of 4 µl standard with 96 µl of Dilution Buffer).Two micro liters (2 µl) of the 2 ng/µl dilution was combined with 48 µl Dilution Buffer to create a 0.08 ng/µl dilution. Two microliters2 µl of the 0.08 ng/µl dilution was combined with 48 µl Dilution Buffer to create a 0.0032 ng/µl dilution. Standard series were stored at 4°C and used up to five days following generation.

Reagents for PowerQuant reactions were prepared as follows: 2X Master, 20X Primer/Probe/IPC Mix, and Water, Amplification Grade were thawed. Reagents were centrifuged briefly before quick vortex (to reduce settling of reagents); as needed, vortexing before centrifugation was performed. Master mix was prepared for addition to wells of a 96-well plate. Ten micro liters (10 µl) 2X Master Mix, 7 µl Water, Amplification Grade, and 1 µl 20X Primer/Probe/IPC Mix were planned for each well (sample, standard, and no-template control [NTC]) to be used, and extra volumes of reagents were used to account for pipetting variability. Eighteen (18 µl) of master mix were added to each well followed by 2 µl sample, standard, or Dilution Buffer (for NTC). An Optical Adhesive Cover was used to seal each quantitation plate before quick centrifugation and run in a 7500 Real-Time PCR System (Applied Biosystems) in accordance with FBU validated procedures.

Studies

Cycle Number

Three (3) extracts from male donors and the positive control 2800M were examined to determine the optimal cycle number for Fusion 6C amplification. Amplifications were performed on the ProFlex™ PCR System at 28 and 29 cycles. Profile peak heights, peak height ratios, and number of edits made to electropherograms were used to ascertain the optimal cycle number for amplification. Runs were performed on the 3500xL CE instrument designated B (serial number 25363-061).

Baseline

For the 3500xL instrument B, a set of fifteen (15) electropherograms including a range of DNA amounts amplified with Fusion 6C and two (2) negative amplification controls were examined for baseline noise. A list of the samples selected for the baseline analysis can be found in the companion binder under the "Baseline" section. The profiles were analyzed in GMID-X with the analytical thresholds set to one (1) Relative Fluorescent Unit (RFU). Peak heights corresponding to alleles or artifacts in profiles were removed so that remaining data represented baseline noise in electropherograms. For each dye used in allele calling, data from loci associated with that dye were analyzed for average (mean) value, maximum value, and standard deviation. Values of average plus three standard deviations, average plus ten standard deviations (limit of quantitation), maximum plus three standard deviations, and maximum plus ten standard deviations were calculated.

Baseline analysis was also performed using a second method. A set of 29 electropherograms including a range of DNA amounts amplified with Fusion 6C were examined visually in GMID-X. For each individual dye channel (including the orange sizing channel), the highest peak height (in RFUs) not attributable to an allele or artifact was determined. The average (mean), maximum value, and standard deviation were calculated for each dye; this information was then used to calculate average plus three standard deviations, average plus ten standard deviations, and maximum plus three standard deviations.

Similar data was collected from the 3500xL instrument designated A (serial number 24343-190). Baseline data from both instruments were compared to determine a set of analytical thresholds usable for both instruments. Values from the GMID-X analyses at 1 RFU analytical threshold were used for the blue, green, yellow, red, and purple channels (where alleles are detected) while values from the visual observation were used for orange (sizing) channel (average plus ten standard deviations used).

Sensitivity

Three (3) series of DNA from known single-source males were amplified in triplicate and used to analyze sensitivity with Fusion 6C. A range of DNA amounts (4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, and 0.0039 ng) were examined.

Average peak height (APH), standard deviation, percent coefficient of variation (%CV), average peak height ratio (PHR), standard deviation for PHR, %CV for PHR, minimum PHR, and average percentage of alleles detected were calculated. Values were calculated for total input DNA amounts.

Extracts used for the sensitivity study were quantified with PowerQuant® to determine autosomal and male DNA concentrations. The observed values were compared with theoretical values calculated from dilutions to ascertain how well observed and theoretical values matched.

Stochastic Events

The sensitivity sample data were used to evaluate stochastic thresholds for the Fusion 6C loci. Stochastic effects can be observed where a heterozygote locus exhibits only one called allele. These events can be

described as "straddle" (both peaks are present with one called and the other falling below the analytical threshold) and "dropout" (one peak is present and the other cannot be distinguished from the baseline noise). Average peak heights, standard deviations, maximum peak heights, and number of events were determined for loci exhibiting stochastic events. The average peak height plus three times the standard deviation for all stochastic events was calculated to determine a proposed stochastic threshold.

Reproducibility/Repeatability

2800M Control DNA (provided as part of the Fusion 6C amplification kit) was amplified as a positive amplification control for each amplification batch used in the validation. One nanogram was used as a target amount. For two amplification plates, the positive control was amplified in triplicate on both plates. All positive controls were evaluated for concordance with the expected profile, and this was used as one measure of kit reproducibility.

Two (2) of the known samples (designated Rep01 and Rep02) were amplified using Fusion 6C and subjected to CE analysis on the 3500xL Genetic Analyzer. The samples were run in triplicate on two separate plates. All electropherograms for these samples were analyzed as part of reproducibility analysis. Average peak heights, standard deviation, and %CV were calculated for individual loci.

Known Samples

A total of ten (10) male single-source samples were amplified using Fusion 6C. The resulting profiles were compared to those from separate genotyping to determine if alleles were concordant with expected results. In some situations, comparison was not possible because of new loci in the Fusion 6C kit which had not been used for prior typing. Allele peak heights were analyzed for average (mean) values, standard deviation, and %CV. Peak height ratios were also analyzed for average (mean) values, standard deviation, and %CV. Minimum PHR values observed were noted.

Mock Evidence Samples

Ten (10) extracts similar to those anticipated in casework were amplified using Fusion 6C. The profiles obtained were compared to expected profiles to determine if alleles were concordant. Some previously typed profiles did not have information for loci included in the Fusion 6C kit so comparisons were made at loci which had been previously typed. For profiles that appeared single source, average allele peak heights with standard deviations and %CV values and average peak height ratios with standard deviations and %CV values were calculated. Minimum PHR observed values and number of called alleles were also noted.

NIST SRM Concordance

Two NIST SRM 2391c components (B and C) were used for validation. For both components (provided as extracts), amplification was performed using Fusion 6C. Observed alleles were compared with alleles

from published profiles to determine concordance and differences, if any, with published or previously typed data.

Precision

Each CE plate with DNA product amplified by the PowerPlex® Fusion 6C kit contained wells with allelic ladder. A total of 74 ladders from 21 run folders were examined. The ladders were used to determine sizing precision based on all alleles in the allelic ladder. All allelic peaks were evaluated for average base pair (bp) size with standard deviation, maximum size, minimum size, and range between maximum and minimum sizes.

Stutter

A total of fifty (50) samples including those used for sensitivity, reproducibility, knowns, mock evidence, and NIST SRM concordance studies were analyzed for stutter. GMID-X analysis was performed using a panel set with no values included for stutter filters. Stutter below analytical/calling thresholds for each dye were not given allele calls, and these data points, along with stutter that was masked by an allele, were not utilized in the calculations. The average percentage of the stutter allele compared to the true allele was calculated for all observed stutter alleles for each locus. The standard deviation, maximum stutter observed, minimum stutter observed, and number of events were also determined. The average percentage plus three times the standard deviation was calculated to determine a proposed stutter percentage at each locus. If a standard deviation value was not calculated at a locus, the average value was used for the locus. This was compared to the manufacturer's guideline, and the higher percentage was noted.

Mixtures

Two-, three-, and four-person mixtures were generated using DNA extracts for processing with Fusion 6C. Three (3) two-person mixtures with male donors were created with 1:0, 20:1, 10:1, 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5, 1:10, 1:20, and 0:1 ratios. These mixtures were amplified at 0.5 and 1.0 ng targets. Two (2) more two-person mixtures with one (1) male and one (1) female donor were created with 20:1, 10:1, 5:1, 2:1, 1:2, 1:5, 1:10, and 1:20 ratios. These were amplified at 0.1, 0.25, 0.5, and 1.0 ng targets.

Two (2) three-person mixtures (one [1] male and two [2] female donors) were created with 10:5:1, 8:1:1, 3:2:1, and 1:1:1 ratios. The mixtures were amplified at 0.1, 0.25, 0.5, and 1.0 ng targets.

Two (2) four-person mixtures (two [2] male and two [2] female donors) were created with 10:5:2:1, 9:3:3:1, 6:3:1:1, 4:4:1:1, 4:3:2:1, and 1:1:1:1 ratios. The mixtures were amplified at 0.1, 0.25, 0.5, and 1.0 ng targets.

Two (2) two-person mixtures (one [1] male and one [1] female donor) were used to test low male to high female ratios. The ratios examined were 1:5, 1:10, 1:20, 1:50, 1:100, 1:250, 1:500, 1:1000, and 1:5000 (major component: female). These mixtures were amplified at 0.75 ng target.

All mixtures with the exception of the low male:high female mixtures were amplified in duplicate. The low male:high female mixtures were amplified a single time.

The low male:high female mixtures were analyzed for the total number of alleles detected as well as the number of unambiguous minor alleles detected. The percentages of alleles detected for both total and minor contributor were also calculated.

For the remaining mixtures, allele peak heights for loci with alleles unshared among contributors were used to determine relative ratios of peak height contributions. This was done for loci where all contributor alleles were above analytical/calling threshold. The observed ratios were compared with expected ratios based on DNA amounts added to determine similarity of the values. Overall sample number of alleles detected was also ascertained as a measure of ability to detect mixed profiles.

The added two-person mixtures with 20:1, 10:1, 5:1, 2:1, 1:2, 1:5, 1:10, and 1:20 ratios and the low male:high female mixtures were quantified with PowerQuant® for autosomal and male DNA concentrations. The observed autosomal:male DNA ratios were compared with theoretical values.

Contamination Monitoring

Amplification and CE plates were set up to run a negative amplification control on each plate. Water was used as the negative control for all amplification runs. The negative controls were analyzed for signs of possible contamination with checking for the presence of alleles above and below analytical thresholds. Additionally, profiles obtained from samples were compared to expected profiles to determine if any cross-contamination or allelic drop-in occurred.

Quantification cut-off threshold

Each dilution from Sens01, Sens02, and Sens03 were quantified. The allele count for each replicate amplification from both the validation and performance check were calculated and compared against the theoretical and observed allele count, as well as the target amount amplified.

Polymerase Chain Reaction (PCR)

For amplification with PowerPlex® Fusion 6C, a total reaction volume of 25 µl (full-volume) was used per reaction. Within each reaction well, 5 µl 5X Master Mix buffer and 5 µl 5X Primer Pair Mix buffer were used. The remaining 15 µl reaction volume per sample consisted of a combination of DNA extract and water (provided by the FBU). As needed, an overage of approximately 10% volume was employed to account for pipetting variability.

Both positive and negative amplification controls were run with each amplification batch. Control DNA 2800M (provided with the PowerPlex® Fusion 6C kit) was used for positive controls. One nanogram was used as the target DNA amount. The original 2800M stock was diluted based on manufacturer's stated concentration.

Reagents and extracts for samples were pipetted into individual wells of a MicroAmp® Optical 96-well Reaction Plate. Following addition of the reagents, the wells were covered with MicroAmp® Cap Strips, and then the plate was briefly centrifuged prior to loading on the thermal cycler.

For PowerPlex® Fusion 6C amplifications, the ProFlex™ PCR System was run according to manufacturer's recommendations with an initial denaturation step of 96°C for 1 minute. This was followed by cycles (described in the following paragraph) of denaturation at 96°C for 5 seconds and annealing and extension at 60°C for 1 minute. A final extension of 60°C for 10 minutes was performed before a final hold at 4°C. The ProFlex™ PCR System was set to 9700 simulation mode.

During the cycle number study, 28 and 29 cycles of PCR were evaluated. The protocol of 29 cycles was selected for validation.

The validation samples were amplified with the ProFlex™ PCR System instrument designated A (serial number 297807793).

Capillary Electrophoresis

For capillary electrophoresis setups, a master mix of Hi-Di™ Formamide and WEN ILS 500 size standard (Promega) was prepared in a 9.5 µl:0.5 µl ratio for each sample. An overage of approximately 10% was used in preparing the master mix to account for pipetting variability. The amount of master mix prepared was designed to prevent injections of unused wells with a 24-capillary array. Ten (10) µl of master mix was added to each used well of a 96-well plate. One (1) µl of amplified sample or allelic ladder was added to appropriate wells.

Prepared plates were centrifuged and then denatured at 95°C for 3 minutes on a hot plate with a 96-well plate adapter followed by a snap cooling with a 96-well cold block for 3 minutes.

All samples run on the 3500xL Genetic Analyzer were injected at an injection time of 24 seconds. Injections were performed at 1.2 kV.

Data Analysis

GeneMapper® ID-X v1.5

All samples were analyzed with Applied Biosystems' GeneMapper® *ID-X* v1.5 (GMID-X) software. Version 1.2 of the PowerPlex® Fusion 6C panels was used. The manufacturer's default values for stutter filters were used in analysis. During baseline analysis, samples were analyzed to determine

analytical/calling thresholds for each dye. The analytical thresholds were then applied to the projects used in this validation. Analysis parameters were the same for all samples unless otherwise stated.

Microsoft Office Excel 2007

All statistical calculations were performed in Microsoft Office Excel 2007. Tables and figures were also prepared using this software.

Results

(1) Cycle Number:

The number of cycles to be used with PowerPlex® Fusion 6C was tested with 28 and 29 cycles. The average peak heights and peak height ratios were examined from the two cycle numbers. The numbers of edits needed in GMID-X to remove artifacts were also noted. Data from these experiments are included in the binder with a subset of the data included below.

Table 1A shows results with the three male extracts and the 2800M positive amplification control used for testing cycle number.

Table 1A: *Average allele peak heights and peak height ratios for DNA amplified using PowerPlex® Fusion 6C with 28 and 29 cycles. "M12", "M13", and "M21" refer to the three male DNA extracts used for testing along with the positive control ("pos ctrl") 2800M DNA. "28 cyc" and "29 cyc" refer to average allele peak heights at the indicated loci with 28 and 29 cycles respectively. "PHR (28)" and "PHR (29)" refer to average peak height ratios with 28 and 29 cycles respectively. The number of edits used in GMID-X to address artifacts is included at the bottom of the table.*

	M12				M13				M21				pos ctrl (2800M)			
Marker	28 cyc	29 cyc	PHR (28)	PHR (29)	28 cyc	29 cyc	PHR (28)	PHR (29)	28 cyc	29 cyc	PHR (28)	PHR (29)	28 cyc	29 cyc	PHR (28)	PHR (29)
AMEL	1139	4234.5	83%	79%	1798	4148.5	91%	88%	2185	4955	100%	85%	2993	7505.5	98%	91%
D3S1358	828	2771.5	83%	98%	1368	3012			1677	4342			3477	6748.5	91%	79%
D1S1656	584.5	1821	85%	94%	1115.5	2227.5	99%	81%	1213	3082.5	95%	82%	2965	6487.5	94%	82%
D2S441	423	1540			1318	2900	93%	93%	1399.5	2843.5			3237.5	6283.5	91%	95%
D10S1248	500.5	1656.5	84%	80%	1595	3307	97%	91%	1282.5	2877.5			3056.5	6231.5	86%	88%
D13S317	466.5	1345.5	86%	94%	1205	2784.5	86%	94%	1668.5	3293	80%	67%	2931	6034.5	80%	97%
Penta E	450	1143	88%	96%	1419.5	2702			1211	3022	74%	82%	3016.5	6989.5	78%	82%
D16S539	872	3482.5	89%	95%	1624	3420.5	88%	91%	1689.5	4583.5			3222.5	7048	98%	92%
D18S51	693.5	2522	83%	82%	1604	3358.5			1807.5	4202	90%	81%	3032.5	6860	94%	93%
D2S1338	478.5	1409.5	97%	88%	1235.5	2515.5	76%	86%	1173	2567	85%	99%	2745.5	5237.5	99%	90%
CSF1PO	405.5	1220	72%	76%	1347	2890.5	95%	97%	1237	2845.5	73%	76%	3330	6898.5		
Penta D	366.5	1527	91%	70%	1362.5	2955	99%	94%	1505	3028			3586.5	7691.5	89%	93%
TH01	586	2170.5	95%	90%	957	2053			1100.5	2827.5	100%	93%	2141.5	4683	90%	95%
vWA	588.5	2240.5	93%	90%	1291	2334.5			1350.5	2891	97%	97%	2322	4920	93%	92%
D21S11	367.5	1422.5	92%	89%	1031.5	2182.5			914.5	2335	93%	96%	1886	4197	94%	93%
D7S820	282	1280.5	85%	83%	1007.5	1745.5	56%	95%	876	1795.5	92%	70%	2015	4550	94%	91%
D5S818	260	807	98%	88%	971.5	1725.5	93%	90%	756.5	1868.5			2039.5	4444		
TPOX	317.5	782	81%	70%	946	1496.5	87%	94%	861	1652			1856	3976.5		
D8S1179	527.5	1860	78%	72%	1036.5	1751	79%	82%	899.5	2402.5	85%	99%	1750.5	4685	95%	96%
D12S391	452	1540	99%	84%	1057.5	1828.5	74%	86%	1130	2669			1681	4077.5	89%	99%
D19S433	502.5	1317.5			1053.5	1647.5	95%	82%	1133	2074.5	69%	94%	1913	4175	91%	96%
SE33	296.5	1061	89%	99%	776	1430	82%	95%	879.5	1805.5	87%	100%	1921	4167.5	93%	93%
D22S1045	206	620	86%	97%	768.5	1308	99%	93%	667	1344.5	85%	74%	1894.5	4084.5		
DYS391	448	1470			586	1453			742	2133			1435	3002		
FGA	271.5	1103.5			820.5	1518	94%	90%	773.5	2099.5	99%	90%	1465.5	3116.5	96%	95%
DYS576	247	921			704	1532			630	1546			1768	4056		
DYS570	129	500			518	871			386	1085			1635	3239		
Edits	0	0			0	0			0	4			2	8		

The average allele peak heights tended to increase at loci when 29 cycles were used compared with 28 cycles. The average peak height ratios did not show a discernible trend difference between the two different cycle numbers. Examples of these effects are illustrated in Figure 1A for allele peak heights with sample M12 and Figure 1B for peak height ratios with sample M12. Similar figures for the M13, M21, and 2800M samples are included in the binder.

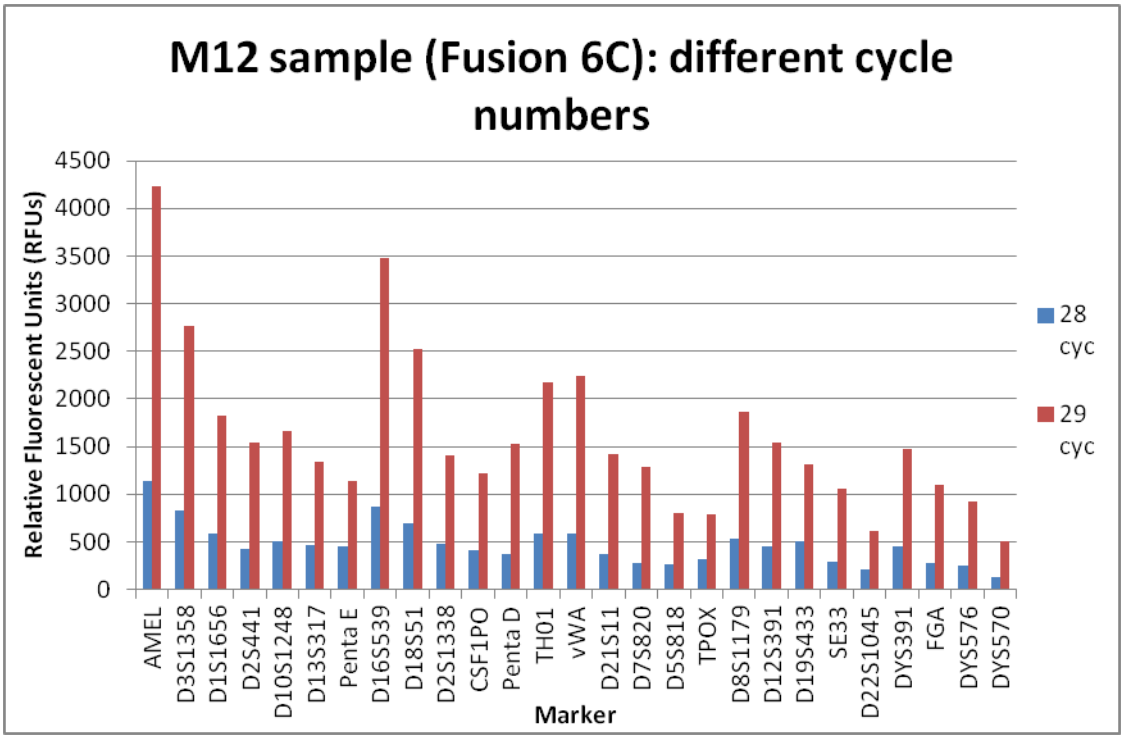

Figure 1A: *Average allele peak heights at PowerPlex® Fusion 6C loci for the M12 sample with different cycle numbers. Blue bars indicate values for 28 cycles while red bars indicate values for 29 cycles.*

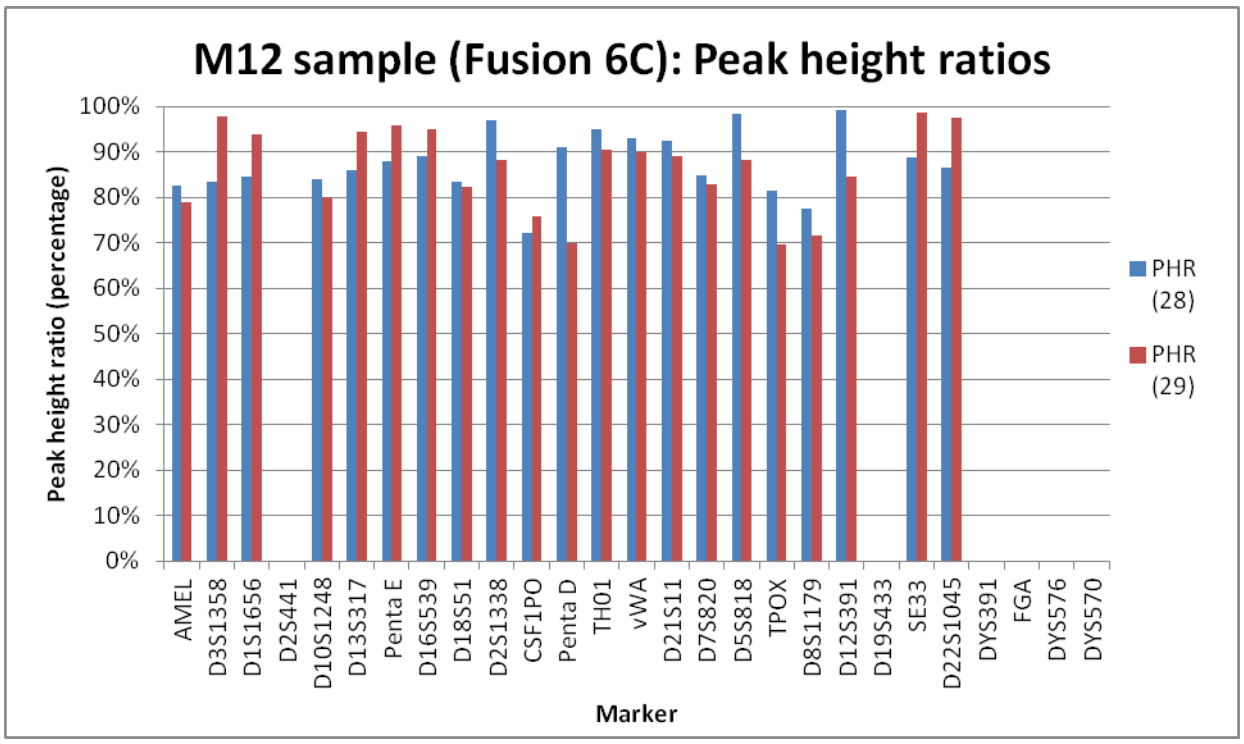

Figure 1B: *Average peak height ratios at PowerPlex Fusion 6C loci for the M12 sample with different cycle numbers. Blue bars indicate values for 28 cycles while red bars indicate values for 29 cycles.*

As shown in Table 1A, the number of edits needed in GMID-X to address artifacts increased for the M21 and 2800M DNA samples when 29 cycles were used for PCR compared with 28 cycles.

Following this testing, the protocol with 29 cycles was selected for further validation. This number of cycles is in line with Promega's observation that 29 cycles worked well with 1.0 ng of template DNA (reference: PowerPlex® Fusion 6C System Technical Manual).

(2) <u>Baseline:</u>

A set of fifteen (15) samples was evaluated for this study. The purpose was to determine the analytical threshold for each of the five dye channels used for allelic analysis at which a true allele can be distinguished from the baseline noise. Additionally, the orange channel was evaluated for determining a threshold for calling sizing peaks.

The results from the analysis are shown in Table 2A. The maximum values plus ten times the standard deviation of the baseline data were used to determine analytical thresholds. Based on the data obtained for 3500xL B, the following analytical thresholds were determined (numbers in RFUs): 65 (blue), 86 (green), 49 (yellow), 59 (red), and 49 (purple). The use of maximum value plus ten times the standard deviation (limit of quantification) should provide a conservative approach for distinguishing true alleles from baseline noise.

Table 2A: *Analysis of the baseline data for allelic channels in PowerPlex® Fusion 6C injections with the 3500xL B instrument. This analysis was performed to determine analytical thresholds. The maximum values plus ten times the standard deviation were selected as the basis for the thresholds. Refer to the corresponding Excel analysis spreadsheet for specific information used in calculations of the baseline.*

Dye	Blue	Green	Yellow	Red	Purple
Average (RFUs):	7	11	5	7	6
Max (RFUs):	34	45	26	34	27
StdDev (RFUs):	3	4	2	3	2
Ave + (3 x StdDev) (RFUs):	16	23	12	15	12
Ave + (10 x StdDev) (RFUs):	37	52	28	33	27
Max + (3 x StdDev) (RFUs):	43	57	33	42	33
Max + (10x StdDev) (RFUs):	65	86	49	59	49
Proposed thresholds (RFUs):	65	86	49	59	49

As GMID-X did not provide peak heights for the orange channel used for sizing, it became necessary to use a different approach for determining the analytical threshold for that channel. A set of 29 electropherograms were examined. Analytical threshold was ascertained based on highest RFU value not attributable to an allele or artifact. Average plus ten times the standard deviation was used to determine a value of 60 RFUs for the orange channel. Tables with the sample data and calculation results for all dye channels including the orange channel are included in the binder.

Similar analyses for baseline were performed with the 3500xL A instrument. A discussion of the proposed thresholds is included in the report for 3500xL A instrument's performance check. Values for the channels from 3500xL A were as follows (numbers in RFUs): 84 (blue), 116 (green), 71 (yellow), 79 (red), 63 (purple), and 40 (orange).

To implement analytical thresholds consistent between both CE instruments, calculations of average values for all dyes were performed. The resulting analytical thresholds for all dye channels were as follows (numbers in RFUs): **75** (blue), **101** (green), **60** (yellow), **69** (red), **56** (purple), and **50** (orange). These values were used for the remainder of the validation.

(3) <u>Sensitivity:</u>

The sensitivity, based on the quality of the profiles obtained at different DNA input levels, of the PowerPlex® Fusion 6C kit with the ProFlex™ PCR System and 3500xL Genetic Analyzer was determined in this study.

Average allele peak heights, peak height ratios, and percentages of alleles detected were used as measures of profile quality. The range of DNA target amounts examined was 4 ng to 0.0039 ng. Data from the three sensitivity sample series used were as shown in Table 3A.

Table 3A: *Sensitivity sample data for various DNA target amounts. Average allele peak heights, peak height ratios, and percentage of alleles detected were as shown. Statistical calculations such as standard deviation ("Std Dev") and percent coefficient of variance ("%CV") were also performed. Minimum peak height ratio ("PHR") percentage values for the different target amounts were as shown.*

	Average Peak Height			Peak Height Ratio				Alleles detected
DNA amount (ng)	APH (RFUs)	Std Dev (RFUs)	%CV	APHR (%)	Std Dev (%)	%CV	Minimum PHR (%)	Percentage
4	8916	3574	40%	92%	5%	6%	77%	100%
2	7900	2591	33%	91%	6%	6%	71%	100%
1	4623	1709	37%	89%	8%	9%	65%	100%
0.5	2445	1085	44%	86%	9%	11%	60%	100%
0.25	1121	486	43%	82%	12%	15%	43%	100%
0.125	593	217	37%	78%	16%	21%	32%	100%
0.0625	224	112	50%	74%	16%	22%	28%	96%
0.03125	124	48	38%	73%	17%	23%	22%	68%
0.0156	104	43	41%	76%	15%	19%	39%	45%
0.0078	91	38	42%	79%	22%	28%	60%	16%
0.0039	79	36	46%	N/A	N/A	N/A	N/A	6%

As expected, average peak height decreased as DNA input amount decreased. This can be seen in Figures 3A and 3B.

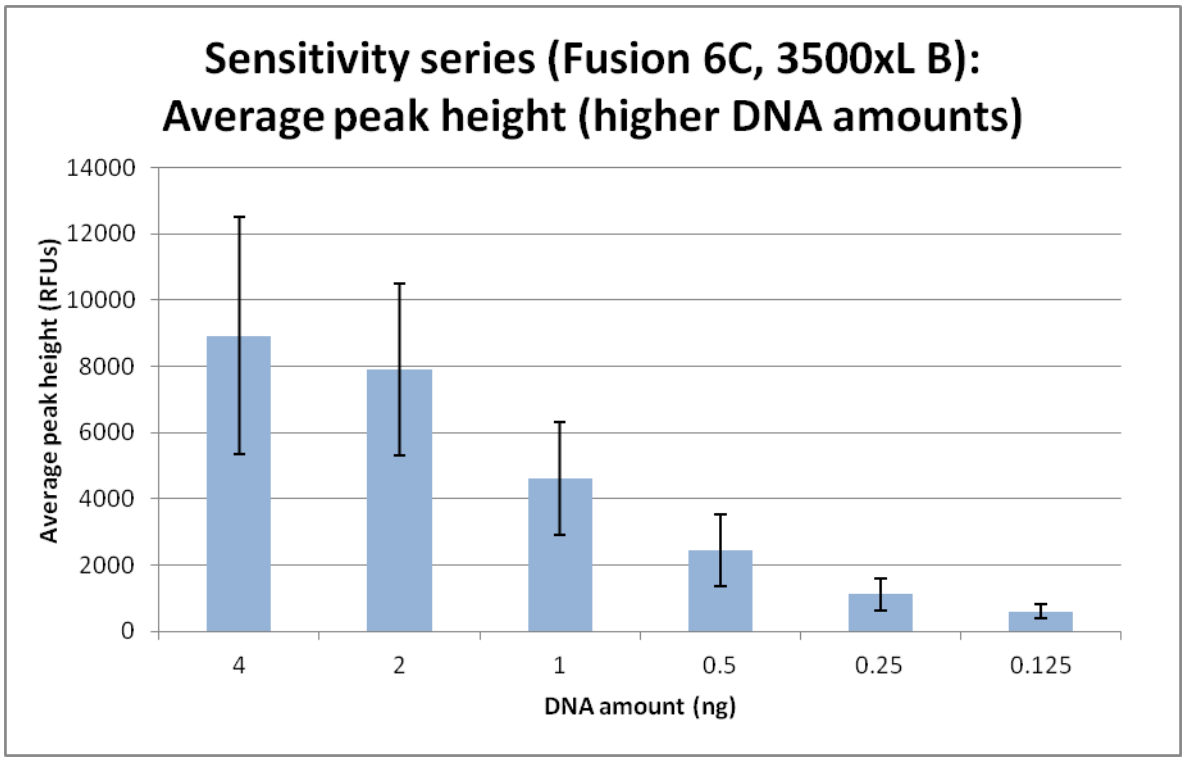

Figure 3A: *Sensitivity by average peak height for higher DNA amounts examined. The average peak height for each dilution was calculated from three sensitivity series amplified in triplicate for nine data points for each input target. Data from the range of DNA amounts of 4 ng to 0.125 ng are shown. Error bars indicate one standard deviation above and below the average.*

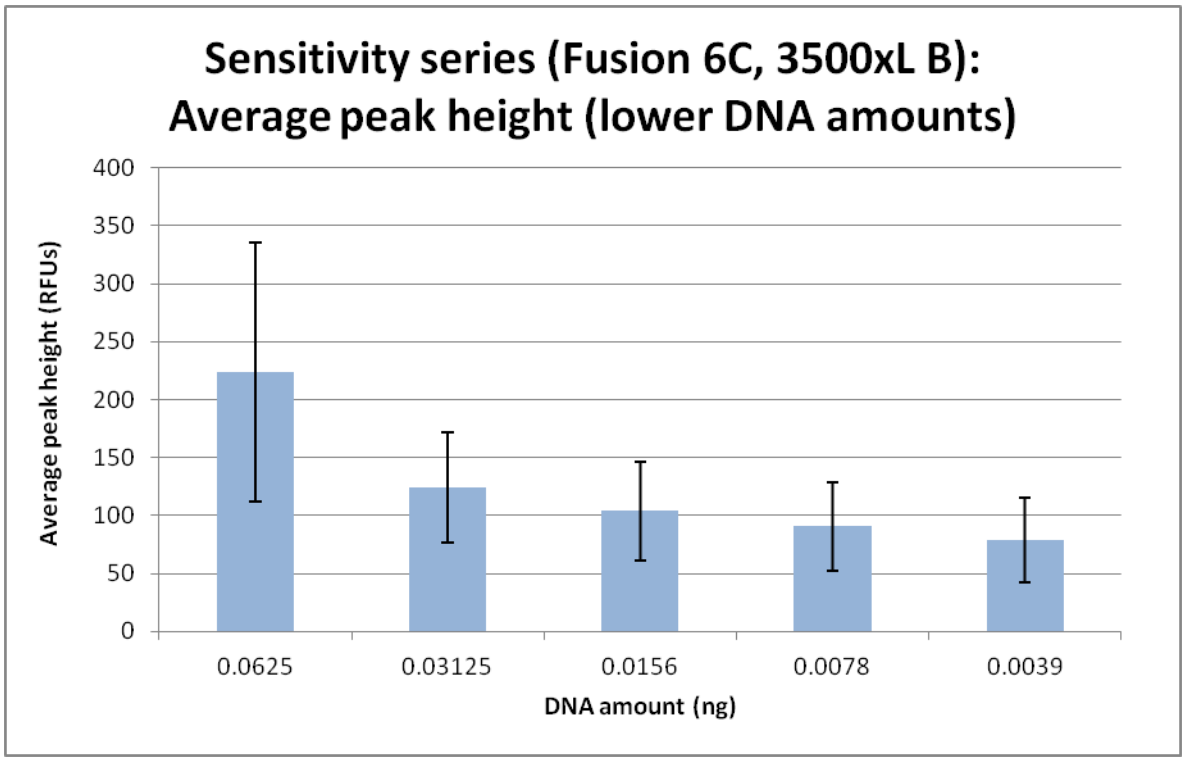

Figure 3B: *Sensitivity by average peak height for lower DNA amounts examined. The average peak height for each dilution was calculated from three sensitivity series amplified in triplicate. Data from the range of DNA amounts of 0.0625 ng to 0.0039 ng are shown. Error bars indicate one standard deviation above and below the average.*

Average peak height ratio (PHR) did not exhibit a discernible trend as target DNA amount changed. Average (mean) values ranged from 73% (at 0.03125 ng) to 92% (at 4 ng). Variability of PHR values as measured by %CV tended to increase as the DNA target amount decreased. Minimum PHR values tended to decrease as DNA amount decreased until 0.0156 ng and 0.0078 ng amounts where the values increased compared with the 0.03125 ng amount. This data can be seen in Figure 3C.

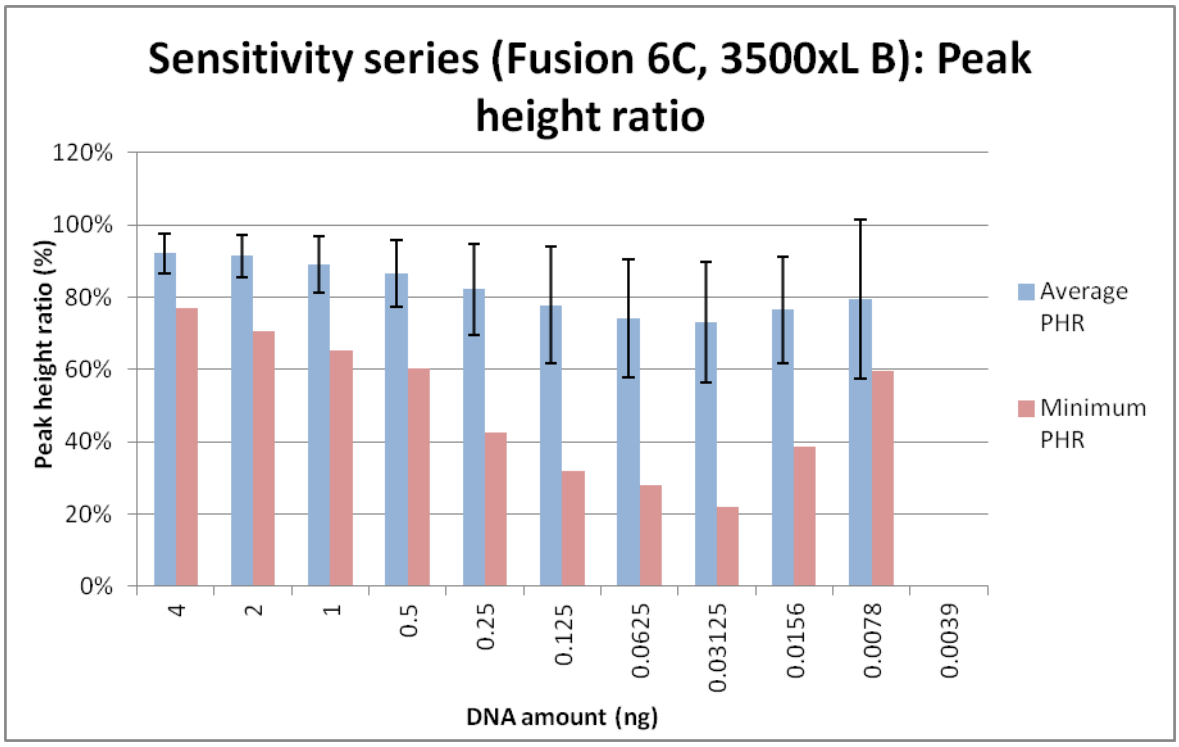

Figure 3C: *Sensitivity by average peak height ratio. The average PHR values (blue bars) for each dilution was calculated from three sensitivity series amplified in triplicate. Error bars indicate one standard deviation above and below the mean. Minimum PHR values (red bars) were also calculated.*

Examining the percentage of alleles detected showed that all expected alleles were detected at 4 ng to 0.125 ng. The percentage of alleles detected started to decrease once the DNA target amount reached 0.0625 ng. Continued decrease of percentage of alleles detected occurred as DNA target amount was lowered to 0.0039 ng. This data is illustrated in Figure 3D.

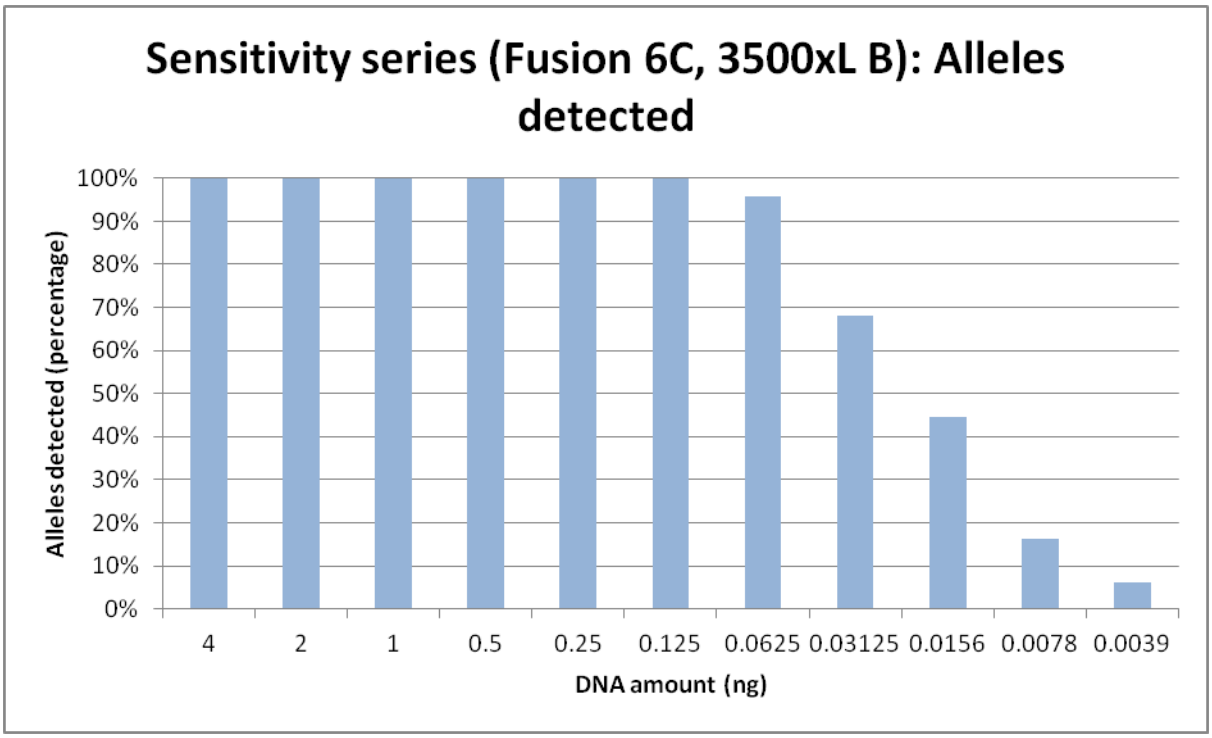

Figure 3D: *Sensitivity by percentage of alleles detected. The percentage of alleles for each dilution was calculated from three sensitivity series amplified in triplicate.*

Consideration of profile quality at the various DNA target amounts led to the selection of an optimal DNA target range for amplification with Fusion 6C. The target range selected was 0.5 ng to 1.0 ng. At this range, complete DNA profiles should be obtained from single-source samples. Average allele peak heights should be from approximately 2400 RFUs to 4600 RFUs and average peak height ratios should be from 86% to 89% (based on the data in Table 3A).

For purposes of validation, 0.75 ng (midway between 0.5 ng and 1.0 ng) was selected as the target amount for amplification with Fusion 6C.

A minimum expected peak height ratio can be calculated based on the data displayed in Table 3A. Using the 0.25 ng target data (to account for possible quantification variance when 0.5 ng is targeted), the average (82%) minus three standard deviations (3*12%) can be determined. That calculation results in a minimum expected peak height ratio of 46%. Using the 0.5 ng target data, the average (86%) minus three standard deviations (3*9%) can be determined. That calculation results in a minimum expected peak height ratio of 59%. It would be expected that sister alleles would have a peak height ratio of approximately 60% or greater when amplified at the target range.

The three sensitivity series samples were quantified using PowerQuant®. The resulting observed autosomal DNA values were compared with theoretical values (based on prior quantification and dilution calculations) to determine how well both sets of values correlated. Results for the Sens01 series are shown in Table 3B. Similar tables for the Sens02 and Sens03 series are included in the binder.

Table 3B: *Observed and theoretical autosomal DNA concentrations for the sensitivity series Sens01. Observed values were from PowerQuant® quantification. Percent differences between the observed and theoretical values were calculated as shown.*

Sens01 series	Auto DNA conc (ng/µl)			
Target DNA amount (ng): theoretical	Theoretical	Observed	DNA amount amplified (ng)	Percent difference (observed vs. theoretical)
4	0.4	0.5599	5.5985	40%
2	0.2	0.2180	2.1804	9%
1	0.1	0.1070	1.0697	7%
0.5	0.05	0.0285	0.2847	-43%
0.25	0.025	0.0118	0.1183	-53%
0.125	0.0125	0.0123	0.1229	-2%
0.0625	0.00625	0.0084	0.0838	34%
0.03125	0.003125	0.0036	0.0356	14%
0.0156	0.00156	0.0008	0.0077	-51%
0.0078	0.00078	0.0004	0.0044	-44%
0.0039	0.00039	0.0008	0.0076	96%

For the Sens01 series, the observed values decreased as the theoretical values decreased with exceptions at 0.0.0625 ng and 0.03125 ng. As expected, the observed DNA concentrations did not exactly match the theoretical concentrations. This could be attributed to variability in real-time PCR signal levels during quantification as well as pipetting variability in generating dilutions and quantification setup.

(4) Stochastic Events:

The sensitivity data was used for determining the stochastic threshold with PowerPlex® Fusion 6C. Table 4A shows the statistical analyses performed on stochastic events.

SWGDAM describes setting this threshold as "the peak height value above which it is reasonable to assume that, at a given locus, allelic dropout from a sister allele has not occurred." Based on this description, a stochastic threshold of 250 RFUs was determined. These values are based upon the average plus three times the standard deviation of the "true dropout" and "straddle" events. It should be noted that no stochastic event was observed at DNA target amounts greater than 0.0625 ng.

Six stochastic events were observed to be greater than the average plus three standard deviations used to calculate the proposed stochastic threshold. Two of the six events were greater than the recommended stochastic threshold of 250 RFUs. Thus, it is possible to note stochastic events with peaks greater than the recommended threshold.

Similar analyses for determining the stochastic threshold were performed with the 3500xL A instrument. A discussion of the proposed stochastic threshold from 3500xL A is included in the validation report.

To implement a stochastic threshold consistent between both CE instruments, the difference in the calculated stochastic thresholds was evaluated. There was a 20 RFU difference between 3500xls. An overall stochastic threshold of 250 RFU was determined for both 3500xlLA and 3500xL B.

DNA extracts (or contributors) amplified within the optimal target range of 0.5 ng to 1.0 ng should not exhibit stochastic events with PowerPlex® Fusion 6C.

Table 4A: *Summary of stochastic events. Straddle represents loci where the sister allele is above background noise but below the analytical threshold. True dropout represents loci where the sister allele is indistinguishable from background noise. Combined stochastic data is the combination of both types of events. Further details including the samples where stochastic effects were observed can be found in the binder.*

Combined stochastic event data

Average (RFUs)	114
Max (RFUs)	288
Std Dev (RFUs)	40
Number of events	201
Average + 3 * Std Dev (RFUs)	233

True dropout event data

Average (RFUs)	114
Max (RFUs)	257
Std Dev (RFUs)	43
Number of events	31
Average + 3 * Std Dev (RFUs)	244

Recommended stochastic threshold: 250 RFUs

(5) <u>Reproducibility/Repeatability:</u>

Two known samples and one positive control were analyzed for reproducibility/repeatability with triplicate amplifications performed on two separate plates. The allele peak heights at all PowerPlex® Fusion 6C loci were analyzed for intraplate and interplate reproducibility.

Results for the interplate average peak heights are shown in Figures 5A through 5C for the positive control 2800M and Rep01 and Rep02 samples respectively. Interplate reproducibility data for the positive control are shown in Table 5A. Other charts and tables including data for intraplate reproducibility are included in the binder.

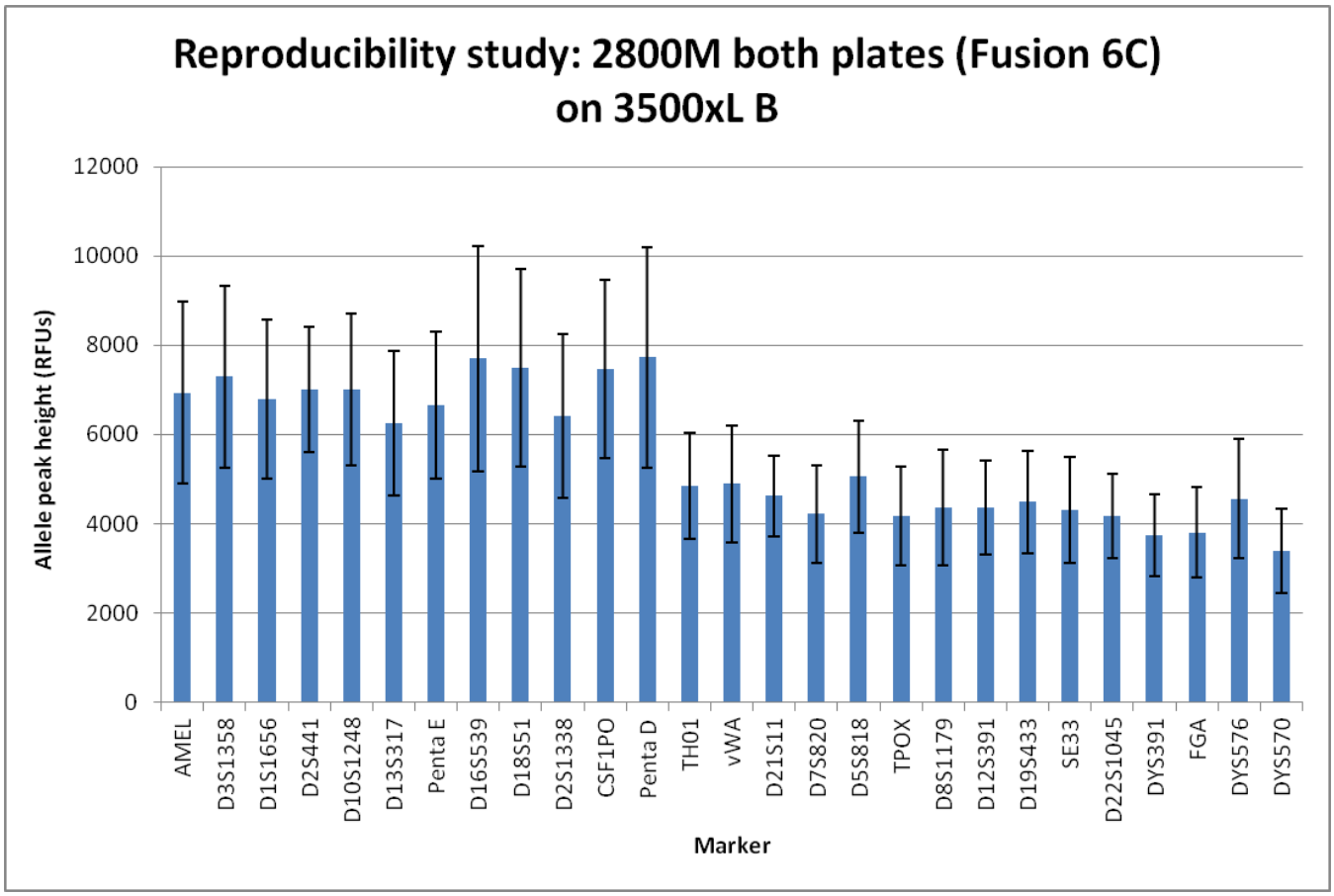

Figure 5A: *Interplate reproducibility for 2800M. Average peak heights for each marker in the PowerPlex® Fusion 6C kit. A total of six amplifications were performed on two separate plates. Error bars indicate one standard deviation above and below the average.*

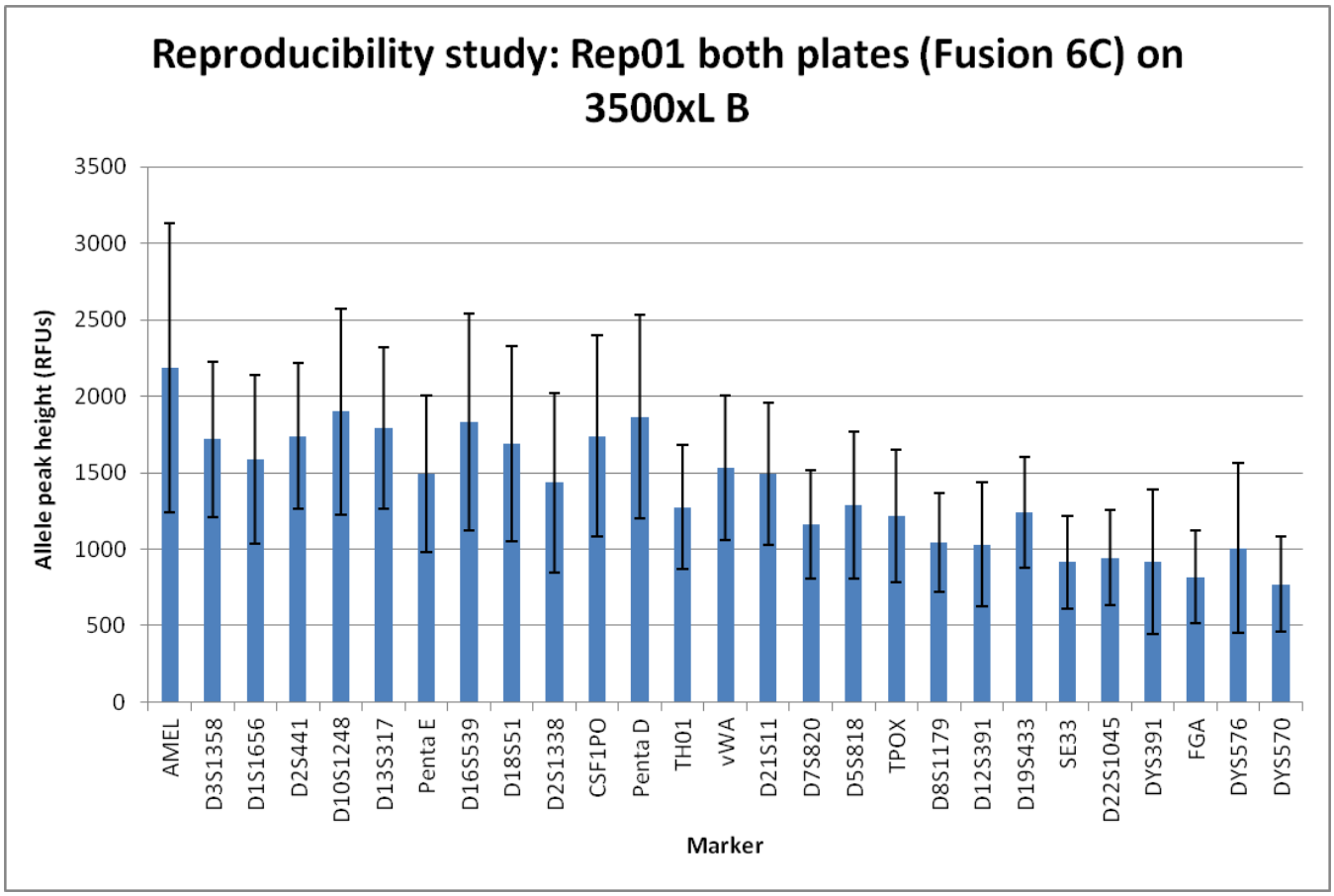

Figure 5B: *Interplate reproducibility for Rep01. Average peak heights for each marker in the PowerPlex® Fusion 6C kit. A total of six amplifications were performed on two separate plates. Error bars indicate one standard deviation above and below the average.*

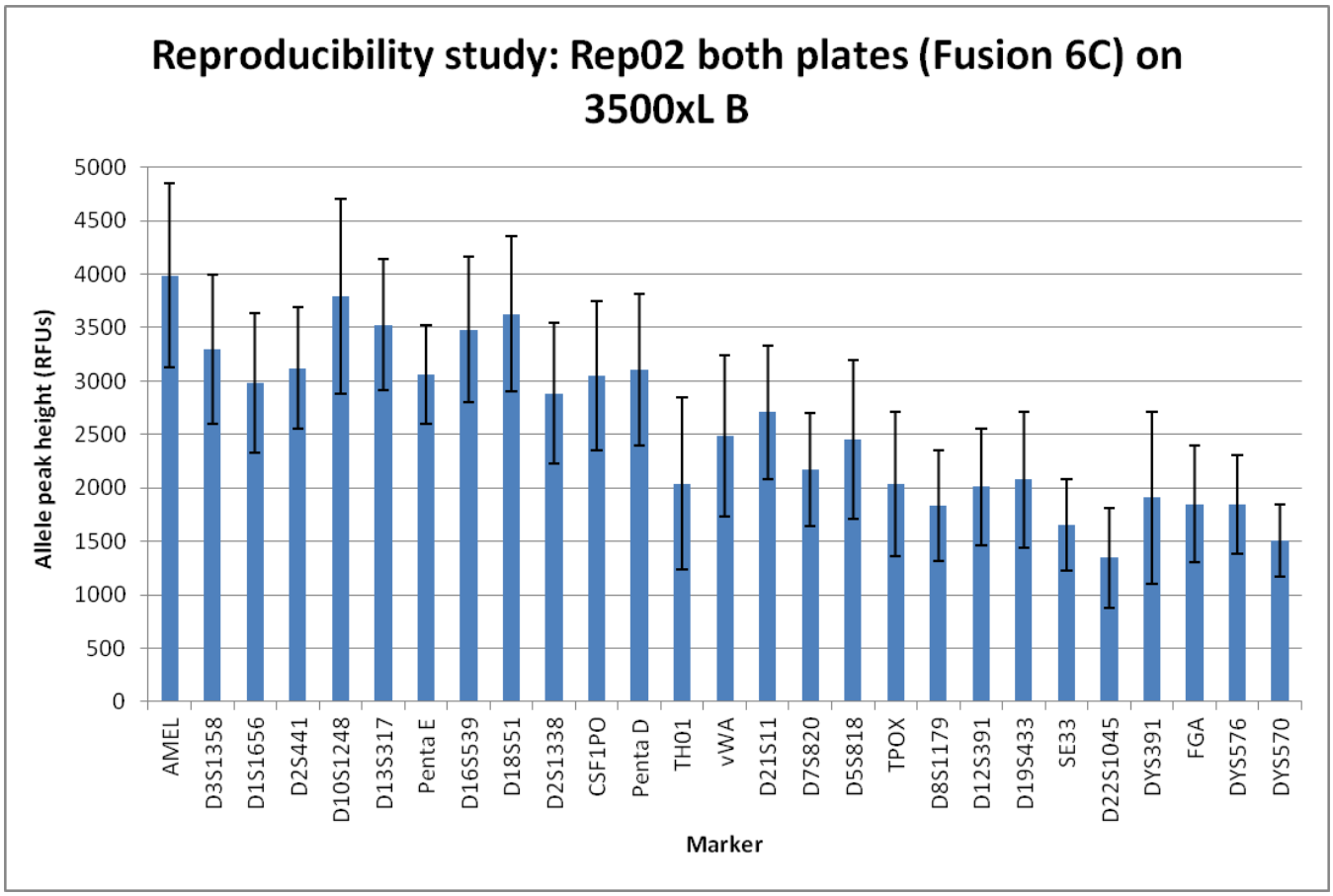

Figure 5C: *Interplate reproducibility for Rep02. Average peak heights for each marker in the PowerPlex® Fusion kit. A total of six amplifications were performed on two separate plates. Error bars indicate one standard deviation above and below the average.*

Table 5A: *Average peak heights with standard deviation and %CV for 2800M amplified for interplate reproducibility analysis. Maximum and minimum %CV values were determined as shown.*

Pos ctrl
both plates

Marker	Average	Std Dev	%CV
AMEL	6929	2040	29%
D3S1358	7299	2039	28%
D1S1656	6792	1771	26%
D2S441	7010	1416	20%
D10S1248	6999	1705	24%
D13S317	6258	1620	26%
Penta E	6665	1643	25%
D16S539	7701	2525	33%
D18S51	7495	2227	30%
D2S1338	6423	1835	29%
CSF1PO	7468	2009	27%
Penta D	7734	2475	32%
TH01	4853	1192	25%
vWA	4899	1308	27%
D21S11	4625	901	19%
D7S820	4222	1093	26%
D5S818	5052	1255	25%
TPOX	4183	1106	26%
D8S1179	4366	1302	30%
D12S391	4367	1054	24%
D19S433	4489	1145	26%
SE33	4297	1189	28%
D22S1045	4172	934	22%
DYS391	3736	911	24%
FGA	3806	1013	27%
DYS576	4562	1340	29%
DYS570	3381	942	28%
		Max %CV	33%
		Min %CV	19%

The profiles obtained from the reproducibility samples were concordant with the expected profiles for all replicates of both the known samples and the positive control.

Variability of average allele peak heights was observed at individual loci examined. This was measured using %CV and ranged from a minimum of 0% (Rep02 second plate at Penta E) to a maximum of 55%

(Rep01 both plates at DYS576). The variation of peak heights was expected, and it should be noted that the peak height variability did not affect allele calling.

The overall results with the reproducibility/repeatability study indicate suitable reproducibility/ repeatability of PowerPlex® Fusion 6C for casework.

(6) Known Samples:

A total of ten (10) single-source known reference samples were examined using PowerPlex® Fusion 6C. All the known samples were from male donors.

The average peak heights and peak height ratios were determined as shown in Table 6A and Figures 6A and 6B. Where possible, alleles were compared with alleles obtained from previous genotyping results.

Three of the samples were not typed separately with the SE33, DYS576, or DYS570 loci in the PowerPlex® Fusion 6C kit. At loci separately typed, detected alleles were concordant with expected profiles.. all ten (10) samples produced full profiles.

Table 6A: *Allele peak heights and peak height ratios for known samples processed with PowerPlex® Fusion 6C. Statistical characteristics such as average, standard deviation ("Std Dev"), percent coefficient of variance (%CV), and minimum were calculated as shown.*

Knowns

	Allele peak height			Peak height ratio			
Sample	Average	Std Dev	%CV	Average	Std Dev	%CV	Minimum
K01	2506	802	32%	86%	11%	13%	57%
K02	2877	826	29%	88%	9%	10%	70%
K03	3572	959	27%	88%	6%	7%	73%
K04	3768	962	26%	88%	7%	8%	69%
K05	4249	1156	27%	89%	7%	8%	72%
K06	2308	742	32%	88%	11%	13%	52%
K07	2946	961	33%	89%	4%	5%	84%
K08	2129	1061	50%	87%	9%	11%	62%
K09	2905	1033	36%	91%	7%	8%	74%
K10	2777	806	29%	91%	7%	7%	77%

The observed peak height ratios from these samples showed that all observed values were greater than the 59% expected minimum peak height ratio (0.5 ng target range) calculated from the sensitivity series samples.

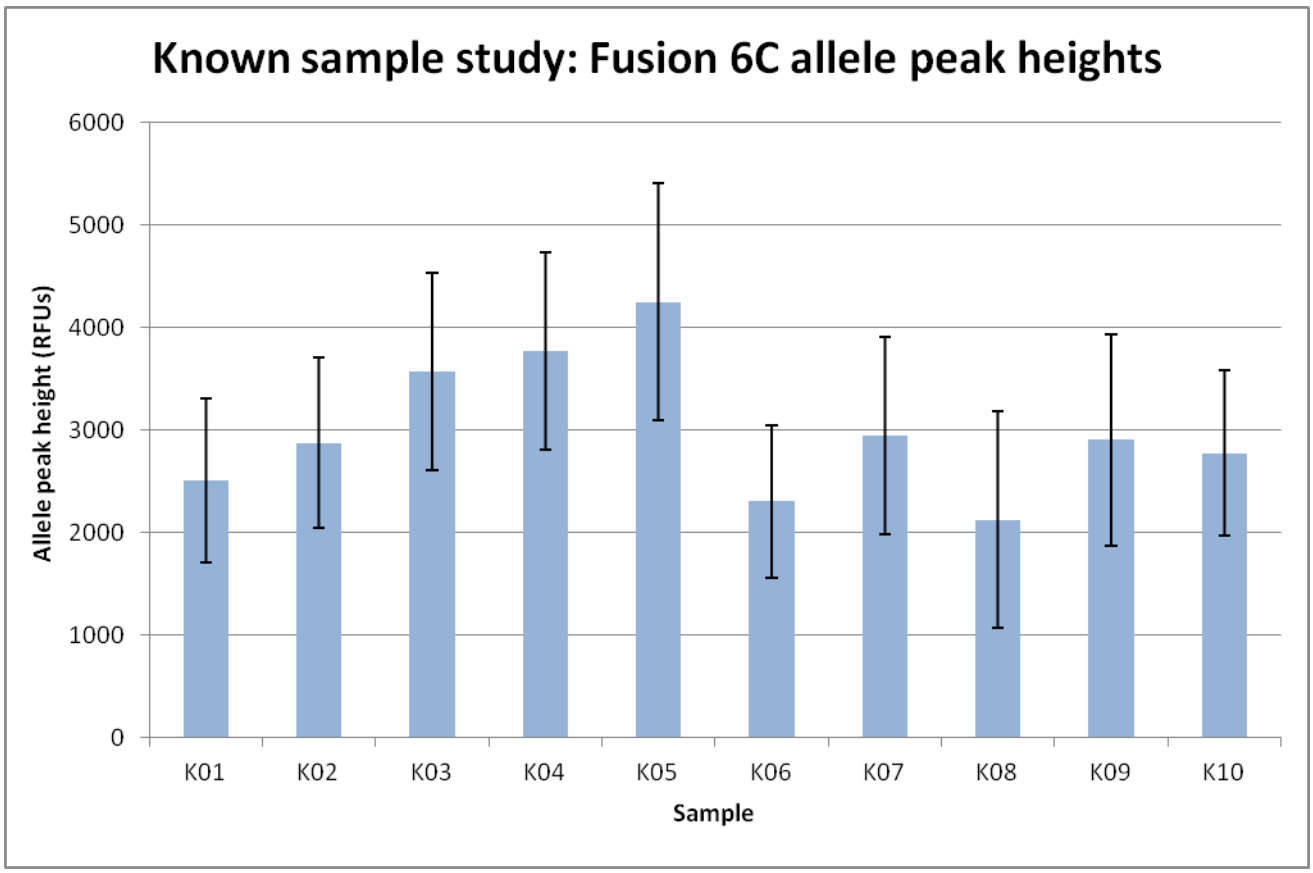

Figure 6A: *Allele peak heights from known samples processed by amplification using PowerPlex® Fusion 6C. Average peak heights were plotted with error bars indicating one standard deviation above and below the mean.*

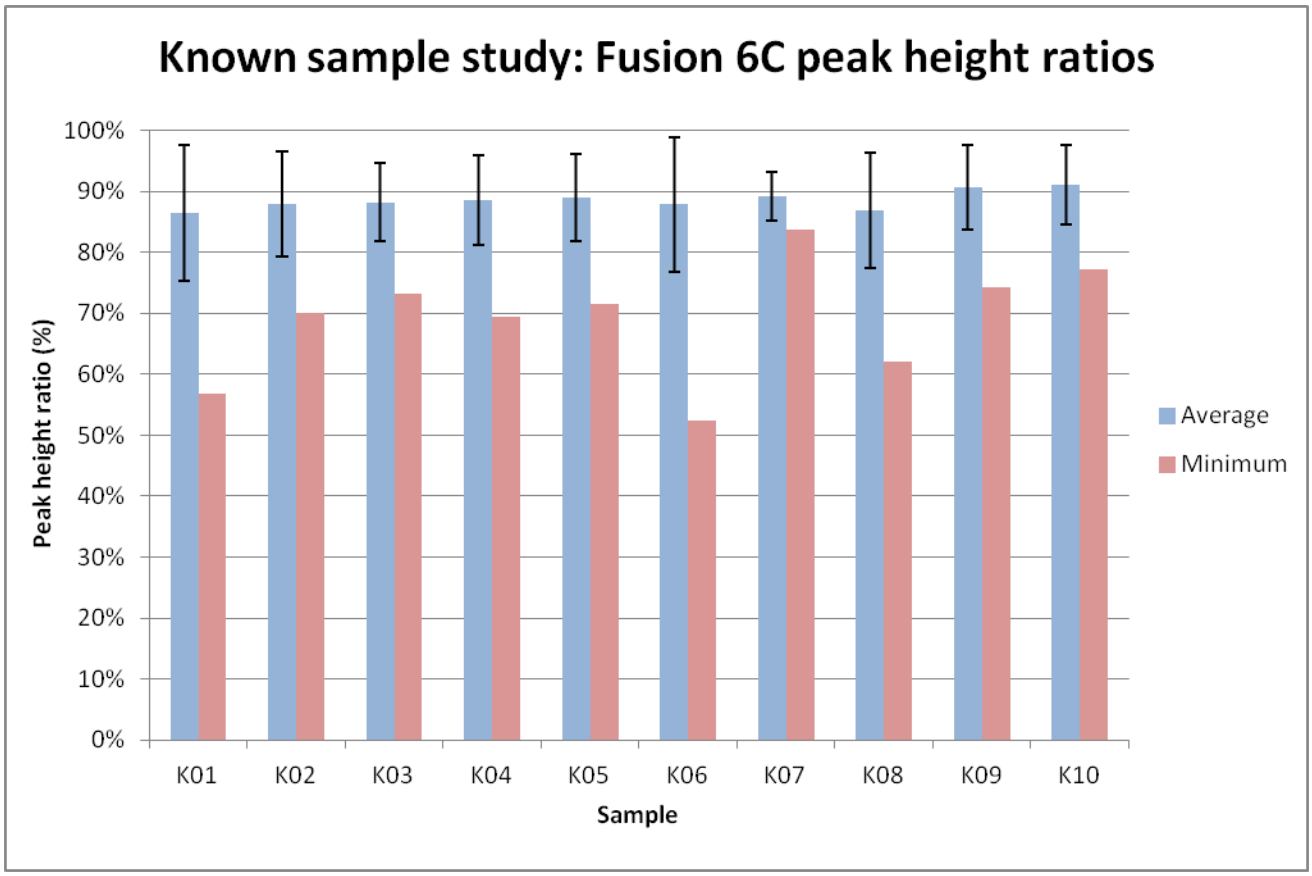

Figure 6B: *Peak height ratios for known samples processed with amplification using PowerPlex® Fusion 6C. Average PHR values were determined as shown with blue bars (error bars indicate one standard deviation above and below the mean). Minimum PHR values observed were as shown in red.*

(7) Mock Evidence Samples:

A total of ten (10) samples were examined. These samples were selected from previously analyzed casework data and prepared samples selected to mimic possible scenarios that could be observed in casework. Of the samples examined, eight (8) were single-source profiles while the remaining two (2) were mixtures.

Where possible, typed alleles were compared with expected alleles (previous typing results). The loci SE33, DYS576, and DYS570 had not been typed previously for these samples so comparisons could not be made at these loci. For eight (8) of the profiles (including both mixed profiles NP05 and NP09), the profiles were concordant with expected alleles at loci previously typed. Sample NP02 appeared single source with Fusion 6C typing but had extra alleles in the expected profile presumably from carry over during differential separation. Sample NP03 typed with Fusion 6C showed an 8 allele in TPOX and a 17 allele in D22S1045 that were below analytical threshold but otherwise had concordant alleles with the expected profile. Sample NP09 showed a 30 peak in D21S11 that could be stutter (the remaining comparable peaks were concordant with the expected profile).

Data for allele peak heights and peak height ratios with the single-source mock evidence sample profiles are shown in Table 7A and Figures 7A and 7B.

Table 7A: *Allele peak height and peak height ratio data for single-source mock evidence sample profiles processed using PowerPlex® Fusion 6C. Statistical characteristics such as average, standard deviation ("Std Dev"), percent coefficient of variation ("%CV"), and minimum were calculated as shown.*

Mock samples

		Allele peak height			Peak height ratio			
Sample	Description	Average	Std Dev	%CV	Average	Std Dev	%CV	Minimum
NP01	16-572_2s_8	2322	876	38%	90%	7%	8%	70%
NP02	Inside of Condom_SP Test tube 10	2381	1119	47%	89%	7%	8%	76%
NP03	Shorts - SP test Tube 2	1313	1135	86%	85%	12%	14%	48%
NP04	16-575 Item 2s_5	1483	444	30%	91%	8%	9%	71%
NP06	17-05702_1s_9	2231	744	33%	85%	10%	12%	63%
NP07	17-05072_4_5	2144	753	35%	88%	10%	12%	61%
NP08	17-5705_item 2s_1	2002	698	35%	85%	10%	12%	60%
NP10	17-5705_item 3sp_2	3393	1113	33%	89%	7%	7%	74%

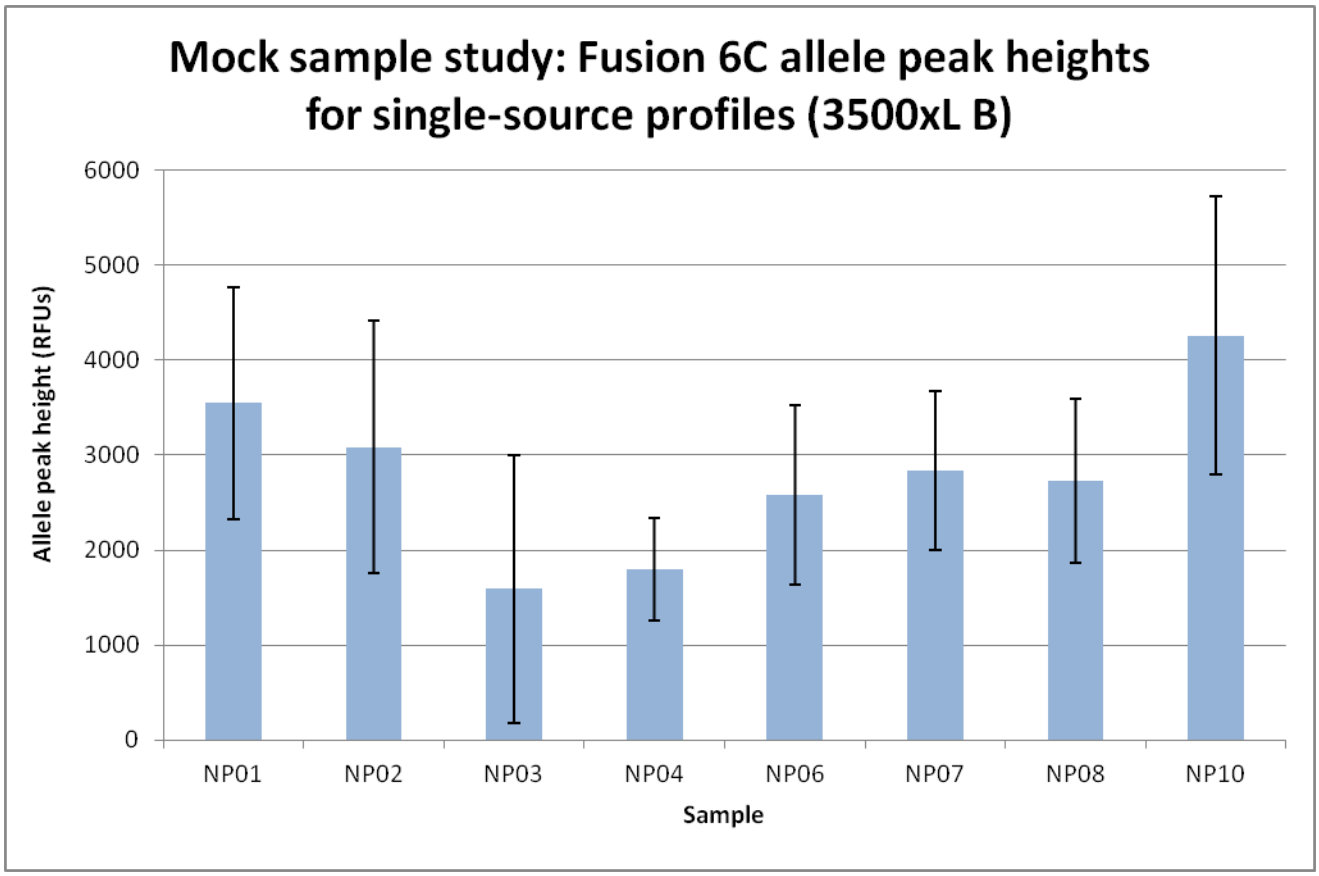

Figure7A: *Allele peak heights for single-source mock evidence samples processed using PowerPlex® Fusion 6C. Average (mean) values were plotted with error bars indicating one standard deviation above and below the mean.*

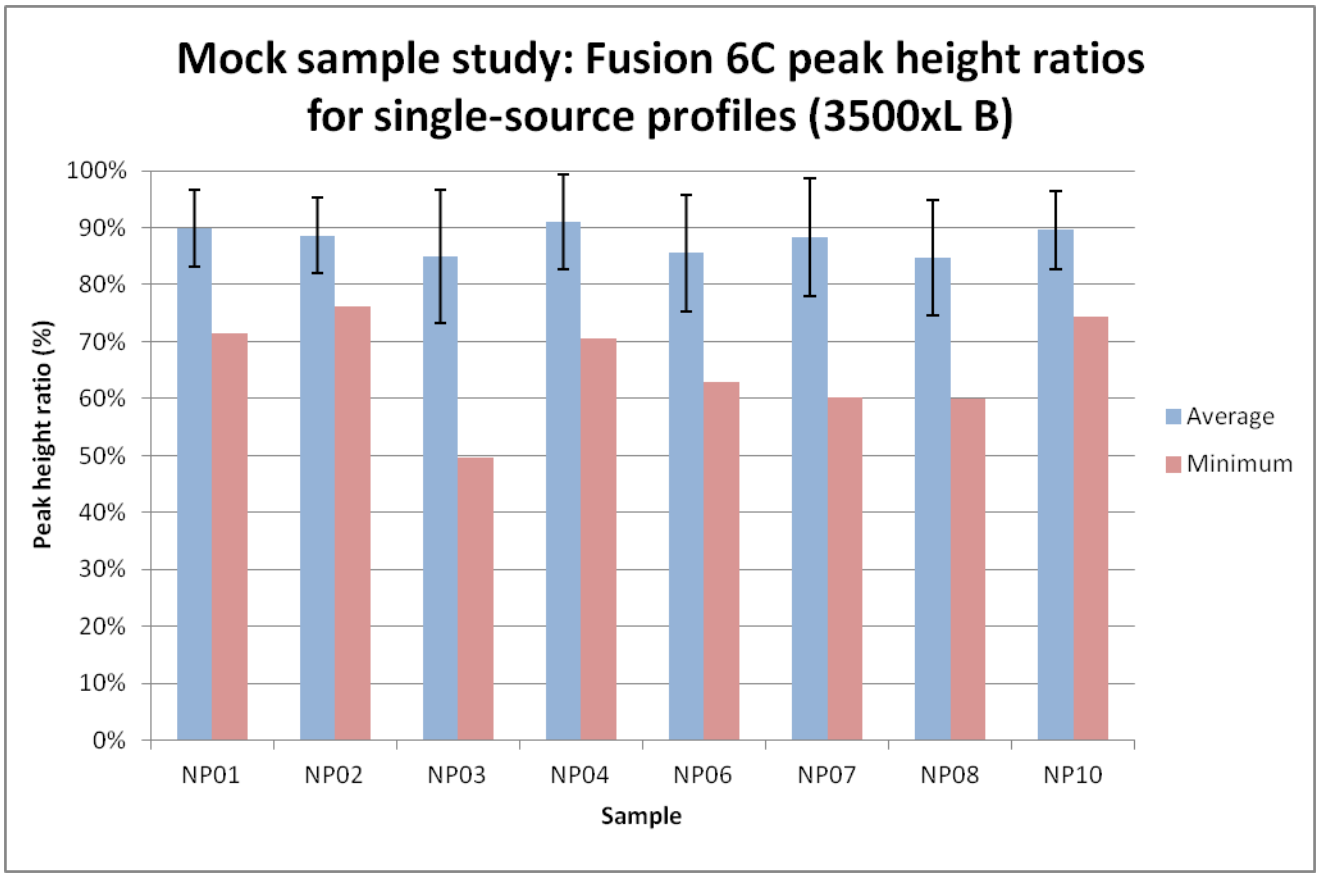

Figure7B: *Peak height ratios for single-source mock evidence samples processed using PowerPlex® Fusion 6C. Average (mean) values were plotted in blue with error bars indicating one standard deviation above and below the mean. Minimum observed values were plotted in red.*

The results for the mock evidence samples indicate casework suitability of PowerPlex® Fusion 6C with the ProFlex™ PCR System and 3500xL Genetic Analyzer if allele peak heights reach analytical thresholds.

(8) <u>NIST SRM Concordance:</u>

Two NIST SRM 2391c components (B and C) were processed with PowerPlex® Fusion 6C. The profiles obtained were compared with published profiles.

Full single-source profiles were obtained for both components. Observed profiles were concordant with expected profiles at all loci of the Fusion 6C kit. Tables 8A and 8B illustrate this data for components B and C respectively.

The results for the NIST SRM 2391c components support the use of PowerPlex® Fusion 6C with the ProFlex™ PCR System and 3500xL Genetic Analyzer in casework.

Table 8A: *Observed and expected profiles for NIST SRM 2391c component B processed with PowerPlex® Fusion 6C. At all loci examined, profiles were concordant.*

NIST01: NIST SRM 2391c component B

Marker	Observed profile	Expected profile
AMEL	X,Y	X,Y
D3S1358	15,19	15,19
D1S1656	11,14	11,14
D2S441	10,14	10,14
D10S1248	13	13
D13S317	9,12	9,12
Penta E	7,15	7,15
D16S539	10,13	10,13
D18S51	13,16	13,16
D2S1338	17	17
CSF1PO	10,11	10,11
Penta D	8,12	8,12
TH01	6,9.3	6,9.3
vWA	17,18	17,18
D21S11	32,32.2	32,32.2
D7S820	10	10
D5S818	12,13	12,13
TPOX	8,11	8,11
D8S1179	10,13	10,13
D12S391	19,24	19,24
D19S433	16,16.2	16,16.2
SE33	17,18	17,18
D22S1045	15,17	15,17
DYS391	10	10
FGA	20,23	20,23
DYS576	17	17
DYS570	18	18

Table 8B: *Observed and expected profiles for NIST SRM 2391c component C processed with PowerPlex® Fusion 6C. At all loci examined, profiles were concordant.*

NIST02: NIST SRM 2391c component C

Marker	Observed profile	Expected profile
AMEL	X,Y	X,Y
D3S1358	16,18	16,18
D1S1656	11,15	11,15
D2S441	10	10
D10S1248	12,16	12,16
D13S317	11	11
Penta E	12,13	12,13
D16S539	10	10
D18S51	16,19	16,19
D2S1338	19	19
CSF1PO	10,12	10,12
Penta D	10,11	10,11
TH01	6,8	6,8
vWA	16,18	16,18
D21S11	29,30	29,30
D7S820	10,12	10,12
D5S818	10,11	10,11
TPOX	11	11
D8S1179	10,17	10,17
D12S391	19,23	19,23
D19S433	13.2,15.2	13.2,15.2
SE33	28.2,31.2	28.2,31.2
D22S1045	16	16
DYS391	11	11
FGA	24,26	24,26
DYS576	16	16
DYS570	20	20

(9) <u>Precision:</u>

Proper allelic designation depends on allelic ladders having sufficient precision such that an allelic peak will be given the same call with every ladder used. Applied Biosystems recommends that an allelic ladder be included on each injection involving a 24-capillary array. During electrophoresis of plates for this validation, at least one allelic ladder was included on each injection set.

Figure 9A gives a graphical view of the standard deviations observed at all loci of the PowerPlex® Fusion 6C kit. All standard deviations for the individual alleles as well as the average standard deviation for all loci were below 0.5 bp. The highest standard deviation was 0.252 (TH01 locus), and the majority of alleles had standard deviation values less than 0.17. As alleles increased in size, the standard deviation tended to decrease. Three times the standard deviation for many alleles would be less than 0.5 bp which would provide sufficient precision for allele calling. For some of the smaller allele sizes included in the ladder, the three times standard deviation value would be greater than 0.5 bp.

The reproducibility, mock evidence, know samples, and the NIST SRM samples all provided the same (correct) allele calls as the previously typed results or published data. Proper allele designation was obtained throughout the course the validation demonstrating that sufficient precision among allelic ladders is present.

A tabular form of the data including maximum size, minimum size, and range of sizes obtained may be found in the companion binder.

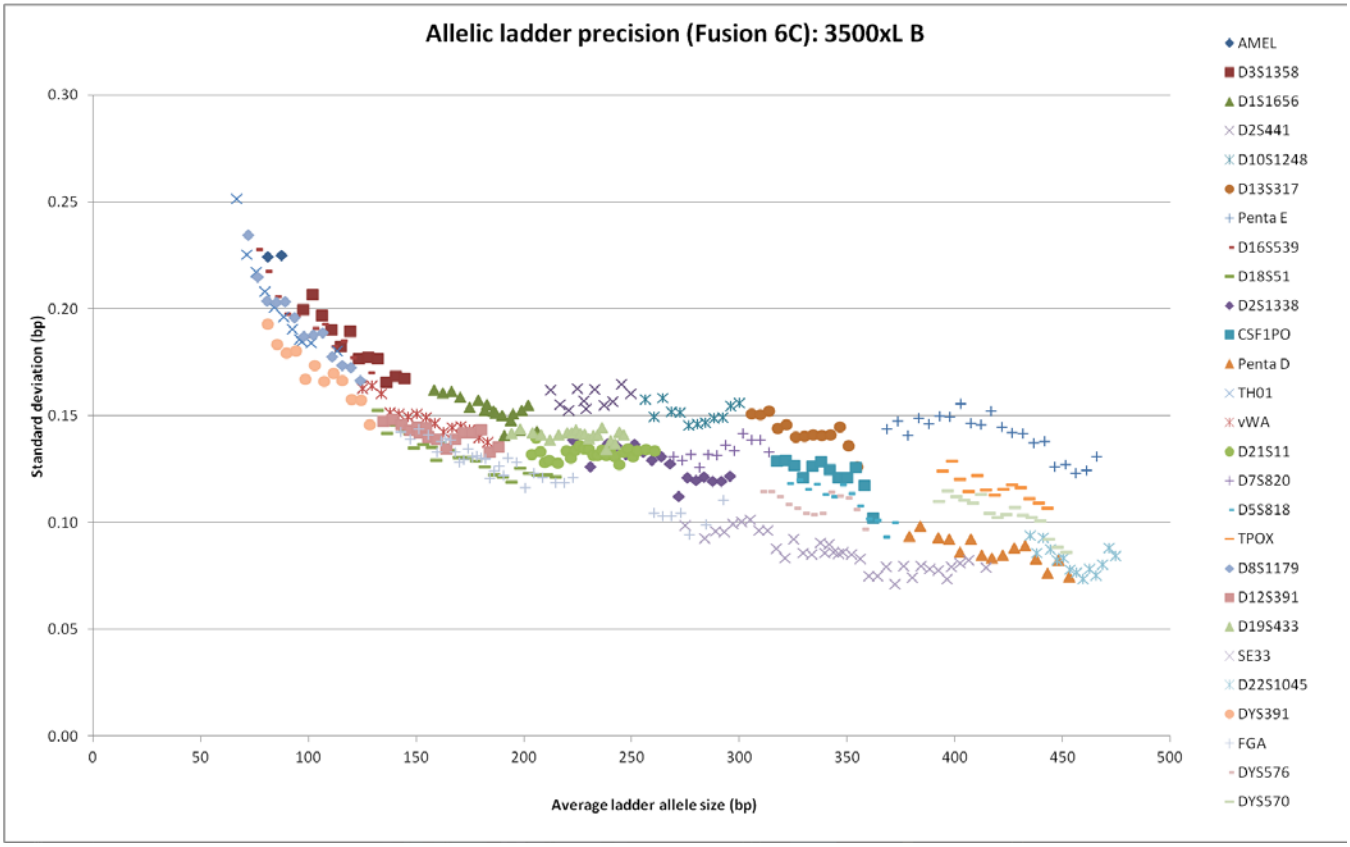

Figure 9A: *Precision of allele sizing as determined from Fusion 6C allelic ladders run during validation. Ladder alleles were analyzed for standard deviation of sizing for alleles at each marker.*

(10) <u>Stutter:</u>

Various samples including those used in the sensitivity, reproducibility, known, mock evidence, and NIST SRM samples (a total of 50 samples) were analyzed for stutter percentages with PowerPlex® Fusion 6C. Alleles from all loci were evaluated.

Table 10A shows the data from reverse stutter of one repeat unit. No stutter was observed at the Amelogenin locus. For the other loci, the average stutter plus three times standard deviation values were calculated. The minimum value observed was 4.2% for Penta D, and the maximum value observed was 19.7% for D22S1045. At some loci, the calculated stutter values exceeded the manufacturer's guideline values. The highest difference in stutter percentage for the loci exceeding manufacturer's guidelines was 3.60%. Figure 10A illustrates the reverse stutter data in graphical form.

Table 10A: *Reverse stutter (one repeat unit) observed during amplification using Fusion 6C. Average plus three times standard deviation column illustrates the observed values from the validation. The manufacturer's guidelines are included for comparison. The highlighted cells indicate the higher value from comparing both values at each locus.*

Stutter (reverse one repeat unit)								
Locus	Average	Standard Deviation	Maximum	Minimum	Number of Events	Avg + 3 * Std Dev	Manufacturer's Guideline	Number of Events > Guideline
AMEL	not applicable							
D3S1358	9.6%	1.8%	14.2%	6.1%	74	15.0%	13.5%	1
D1S1656	9.4%	1.7%	12.9%	5.9%	71	14.5%	14.3%	0
D2S441	5.0%	1.3%	7.4%	2.6%	60	8.9%	9.0%	0
D10S1248	8.7%	1.5%	13.6%	6.4%	61	13.3%	13.0%	1
D13S317	5.8%	1.3%	7.8%	2.5%	54	9.7%	10.3%	0
Penta E	4.3%	1.1%	6.4%	1.5%	48	7.6%	7.2%	0
D16S539	7.2%	1.7%	10.2%	3.7%	60	12.2%	12.0%	0
D18S51	9.4%	2.9%	19.0%	3.6%	74	18.2%	14.6%	1
D2S1338	8.8%	1.9%	13.8%	5.8%	77	14.5%	13.6%	1
CSF1PO	6.3%	1.5%	9.4%	3.6%	53	10.9%	11.1%	0
Penta D	2.2%	0.6%	3.1%	1.4%	11	4.2%	4.5%	0
TH01	2.4%	0.7%	3.8%	1.5%	27	4.6%	4.8%	0
vWA	8.1%	2.4%	12.9%	1.4%	65	15.4%	14.4%	0
D21S11	8.5%	1.2%	11.9%	5.4%	55	12.0%	12.7%	0
D7S820	5.7%	1.9%	11.0%	2.2%	63	11.2%	9.7%	1
D5S818	6.8%	1.3%	10.5%	4.8%	50	10.8%	11.0%	0
TPOX	2.8%	1.0%	4.8%	1.5%	35	5.8%	5.4%	0
D8S1179	7.6%	1.5%	11.7%	3.8%	58	12.1%	11.8%	0
D12S391	10.1%	2.0%	15.1%	5.0%	69	16.2%	17.4%	0
D19S433	8.0%	1.9%	13.5%	4.6%	69	13.6%	12.1%	5
SE33	12.1%	2.3%	18.4%	7.8%	82	19.2%	16.1%	4
D22S1045	9.2%	3.5%	20.5%	3.1%	53	19.7%	16.8%	2
DYS391	7.0%	1.1%	8.8%	3.4%	41	10.4%	9.4%	0
FGA	7.3%	1.9%	11.9%	4.0%	70	12.9%	12.4%	0
DYS576	8.8%	1.5%	12.5%	6.6%	42	13.2%	12.5%	0
DYS570	9.1%	1.6%	12.1%	5.3%	38	14.0%	13.0%	0

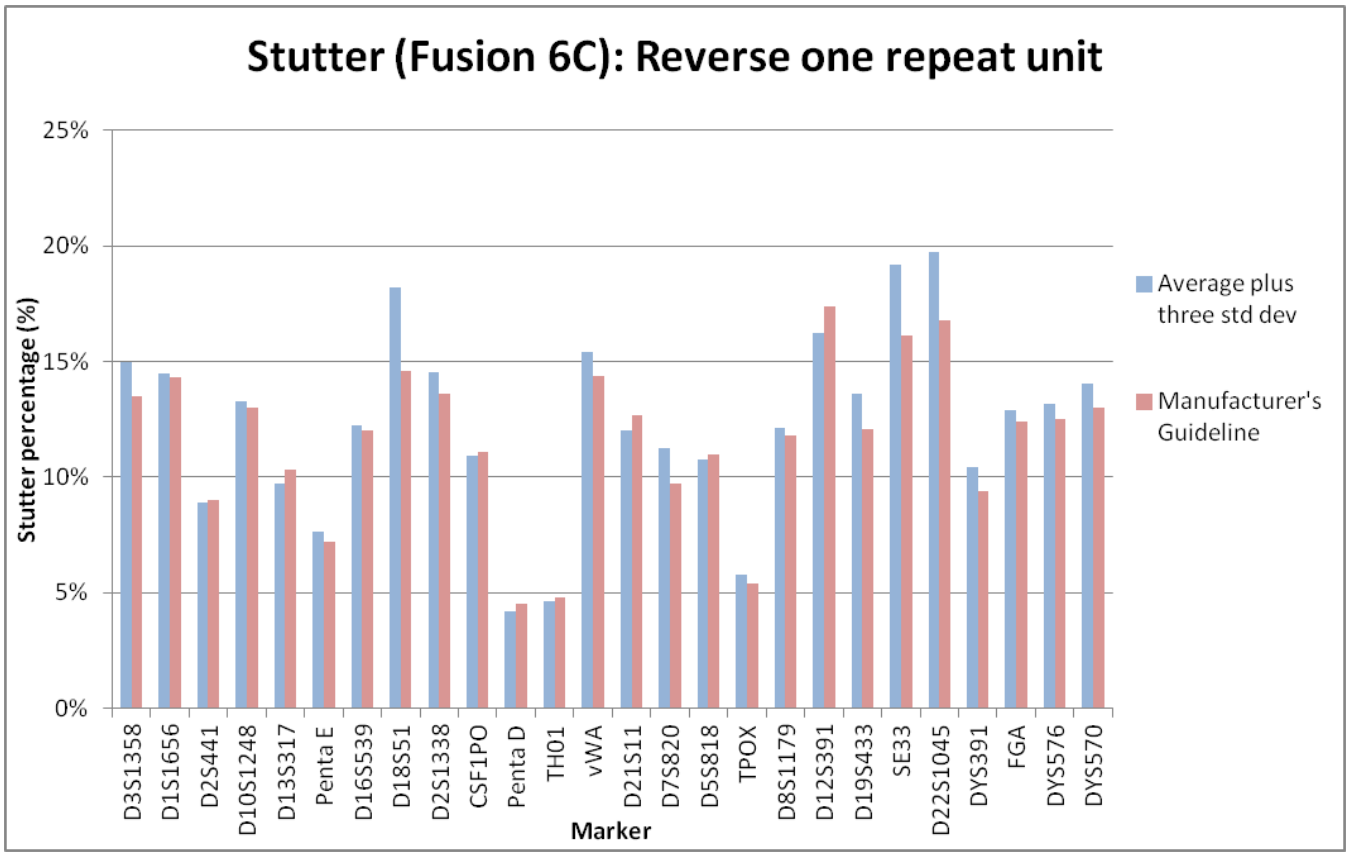

Figure 10A: *Reverse stutter (one repeat unit) observed during amplification using Fusion 6C. Average plus three times standard deviation (blue bars) illustrates the observed values from the validation. The values from the manufacturer's guidelines (red bars) are included for comparison.*

Table 10B illustrates the data from analysis of forward stutter of one repeat unit using amplification with PowerPlex® Fusion 6C. No forward stutter events were observed at some loci with filter values designated by the manufacturer, and at other loci, not enough events were observed to calculate standard deviation values so the average values were used for comparisons. At several loci (D1S1656, D16S539, vWA, D21S11, and D22S1045), the observed forward stutter percentages were greater than the values from the manufacturer's guidelines. With the exception of vWA, the difference between the calculated stutter percentage and the manufacturer's guidelines was less than 1.0% . vWA had a difference of 8% between the calculated stutter value and the manufacture's guidelines. It should be noted that during the internal validation only five events for forward stutter were observed in which one event was greater than the manufacturer's guidelines. Figure 10B illustrates the forward stutter data in graphical form.

Table 10B: *Forward stutter (one repeat unit) observed during amplification using PowerPlex® Fusion 6C. Average plus three times standard deviation column illustrates the observed values from the validation. The values from the manufacturer's guidelines are included for comparison. The highlighted cells indicate the higher value across both columns. Red percentage values indicate the average observed value used for comparison because no standard deviation value was calculated.*

Stutter (forward one repeat unit)								
Locus	Average	Standard Deviation	Maximum	Minimum	Number of Events	Avg + 3 * Std Dev	Manufacturer's Guideline	Number of Events > Guideline
AMEL	not applicable							
D3S1358	1.0%	0.2%	1.2%	0.8%	4	1.6%	1.7%	0
D1S1656	1.5%	0.6%	2.7%	1.1%	6	3.2%	2.3%	1
D2S441	0.8%	N/A	0.8%	0.7%	2	0.8%	1.8%	0
D10S1248	no events observed						1.3%	N/A
D13S317	1.2%	0.3%	1.5%	0.7%	6	2.1%	2.2%	0
Penta E	no events observed						1.9%	N/A
D16S539	1.9%	0.5%	2.5%	1.2%	4	3.5%	3.0%	0
D18S51	1.5%	N/A	1.6%	1.4%	2	1.6%	2.8%	0
D2S1338	no events observed						2.2%	N/A
CSF1PO	1.4%	0.5%	1.9%	1.1%	3	2.8%	3.7%	0
Penta D	no events observed						3.7%	N/A
TH01	no events observed						1.5%	N/A
vWA	3.0%	2.6%	7.6%	1.4%	5	10.7%	2.7%	1
D21S11	1.5%	0.7%	2.3%	0.8%	4	3.7%	2.8%	0
D7S820	0.8%	N/A	0.8%	0.8%	1	0.8%	1.8%	0
D5S818	1.3%	N/A	1.8%	0.8%	2	1.3%	2.3%	0
TPOX	no events observed						1.1%	N/A
D8S1179	1.2%	N/A	1.7%	0.7%	2	1.2%	3.4%	0
D12S391	2.2%	N/A	2.2%	2.2%	1	2.2%	2.7%	0
D19S433	no events observed						2.6%	N/A
SE33	2.5%	N/A	2.5%	2.5%	1	2.5%	3.3%	0
D22S1045	5.6%	1.3%	7.1%	1.9%	37	9.6%	9.0%	0
DYS391	no events observed						2.0%	N/A
FGA	no events observed						2.8%	N/A
DYS576	3.0%	N/A	3.0%	3.0%	1	3.0%	3.4%	0
DYS570	no events observed						2.4%	N/A

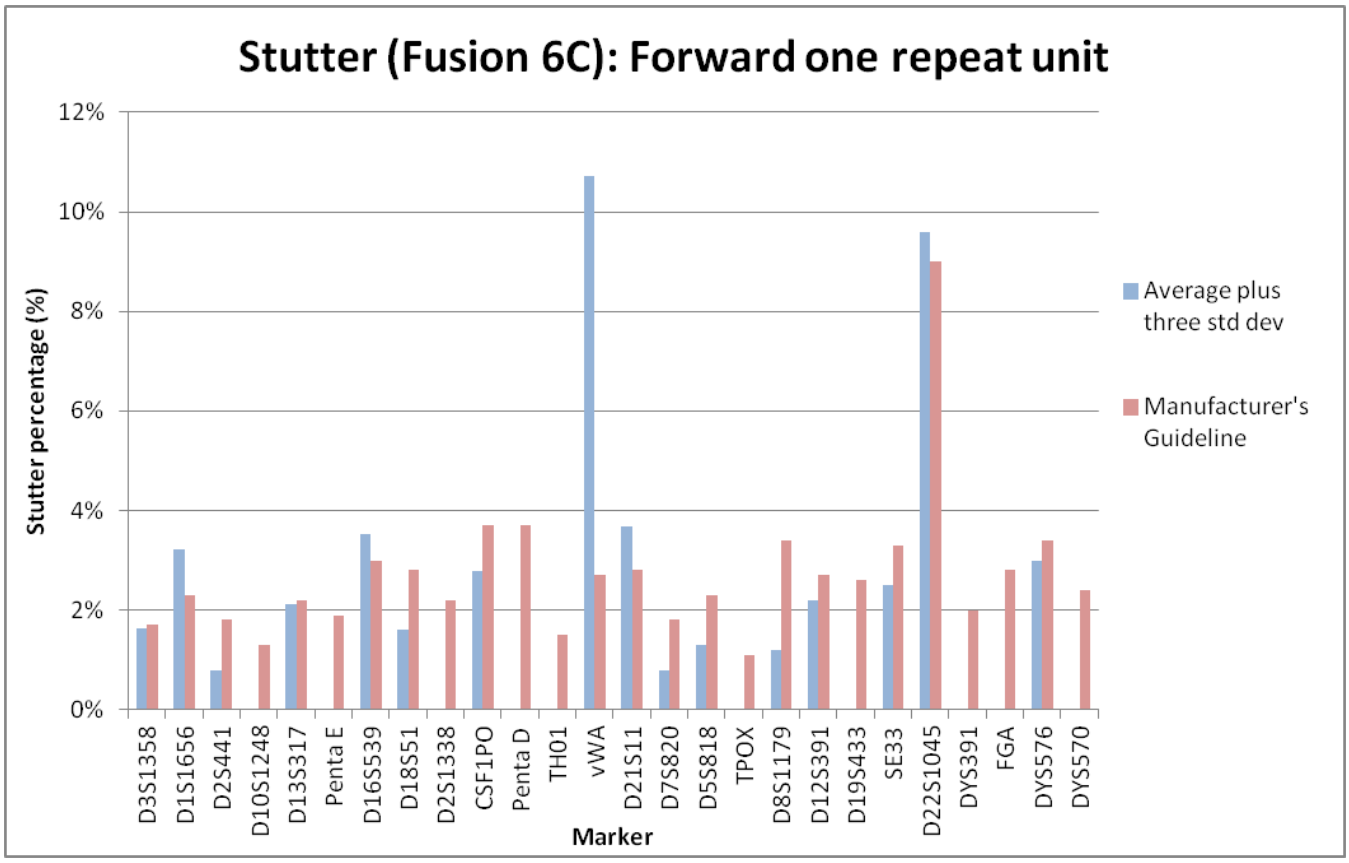

Figure 10B: *Forward stutter (one repeat unit) observed during amplification using Fusion 6C. Average plus three times standard deviation or average values when no standard deviation was calculated (blue bars) illustrates the observed values from the validation. The values from the manufacturer's guidelines (red bars) are included for comparison.*

Besides reverse and forward stutter of one repeat unit, other types of stutter were observed during validation. Reverse half repeat stutter (n-2; half back stutter) has been described by the manufacturer and was observed during validation as shown in Table 10C. There were instances of n-2 stutter at loci (D21S11 and D5S818) not referenced in the manufacturer's guidelines. At SE33 the observed forward stutter percentages were greater than the values from the manufacturer's guidelines. The difference was 0.6% and based on 65 events. Two of these events had calculated stutter percentages that were greater than the manufacture's guidelines.

Table 10C: Reverse *half repeat stutter (n-2) observed during amplification using Fusion 6C. Average plus three times standard deviation column illustrates the observed values from the validation. The manufacturer's guidelines are included for comparison. The highlighted cells indicate the higher value from the comparison. Red percentage values indicate the average observed value used for comparison when no standard deviation value was calculated.*

Stutter (reverse n-2)								
Locus	Average	Standard Deviation	Maximum	Minimum	Number of Events	Avg + 3 * Std Dev	Manufacturer's Guideline	Number of Events > Guideline
D1S1656	2.1%	0.3%	3.0%	1.7%	19	3.1%	3.6%	0
D21S11	6.1%	N/A	7.8%	4.5%	2	6.1%	N/A	N/A
D5S818	1.0%	N/A	1.2%	0.9%	2	1.0%	N/A	N/A
D19S433	no events observed						1.4%	N/A
SE33	5.3%	0.6%	7.7%	4.2%	65	7.2%	6.6%	2
FGA	no events observed						1.2%	N/A

In addition, there was observed reverse stutter of two repeat units (double back stutter) as shown in Table 10D. There are no manufacturer's guideline values for this type of stutter.

Table 10D: *Reverse stutter two repeat units (double back stutter) observed during amplification using Fusion 6C. Average plus three times standard deviation column illustrates the observed values from the validation. Red percentage values indicate the average observed value used for comparison because no standard deviation value was calculated.*

Stutter (reverse two repeat units)						
Locus	Average	Standard Deviation	Maximum	Minimum	Number of Events	Avg + 3 * Std Dev
D3S1358	1.0%	0.4%	1.5%	0.6%	4	2.3%
D1S1656	1.2%	0.1%	1.3%	1.1%	5	1.5%
D10S1248	0.9%	0.2%	1.0%	0.7%	3	1.4%
D16S539	0.8%	N/A	0.8%	0.8%	1	0.8%
D18S51	1.2%	N/A	1.2%	1.2%	1	1.2%
D21S11	1.0%	N/A	1.1%	0.8%	2	1.0%
D8S1179	0.7%	N/A	0.7%	0.7%	1	0.7%
D19S433	1.6%	N/A	1.6%	1.6%	1	1.6%
SE33	1.3%	N/A	1.4%	1.1%	2	1.3%
D22S1045	1.1%	N/A	1.1%	1.1%	1	1.1%
DYS570	1.8%	N/A	1.8%	1.8%	1	1.8%

There were two instances of forward two repeat stutter observed. These were seen in the D21S11 and SE33 loci. As with the reverse two repeat stutter, there are no manufacturer's guideline values for the forward two repeat stutter.

It is recommended that analysts be aware of stutter peaks that can occur other than reverse and forward stutter of one repeat unit and reverse half repeat stutter. No stutter filters for reverse two repeat unit stutter (double back stutter) filter will be added to the GMIDX panels and bins for PowerPlex® Fusion 6C analysis. It should be noted that reverse two repeat stutter (double back stutter) for the loci D3S1358, D1S656, D10S1248, D16S539, D18S51, D21S11, D8S1179, D19S433, SE33, and D22S1045 will be modelled in STRmix v.2.6.

(11) Mixtures:

Two-, three-, and four-person mixtures were evaluated with PowerPlex® Fusion 6C.

Three (3) series of two-person mixtures (designated Mix01, Mix02, and Mix03) were analyzed with 0.5 ng and 1.0 ng target DNA amounts. Various ratios of the contributors were examined. Ratios of peak heights for loci with unshared alleles were used to compare with expected ratios of DNA contribution based on concentration. The average numbers of observed alleles were determined as a measure of detecting minor contributors. Tables 11A and 11B illustrate the results for Mix01 with 0.5 ng and 1.0 ng targets respectively. Similar tables for the Mix02 and Mix03 samples are included in the binder. As the Mix03 series had no loci with unshared alleles (the donors were related), only the average number of alleles detected was analyzed for that set.

Table 11A: *Mixture analysis for Mix01 (two-person mixture) with 0.5 ng DNA target. Allele peak heights for loci with alleles unshared between the contributors were used for calculating observed mixture ratios. The observed ratios were then compared with the expected theoretical ratios. Average numbers of observed alleles were also determined as shown. Total number of alleles in mixture: 79.*

Mix01: Fusion 6C (0.5 ng target)

	Marker												Observed	Expected	Average number
Ratio	D1S1656	D2S441	D13S317	TH01	vWA	D21S11	D7S820	D12S391	D19S433	SE33	FGA	DYS576	average	value	of alleles
1:0	not applicable														46.5
20:1	N/A	N/A	N/A	20.09	10.33	N/A	N/A	N/A	N/A	N/A	N/A	N/A	15.21	20	55
10:1	4.45	5.48	4.14	18.56	5.10	N/A	5.12	4.94	N/A	N/A	N/A	N/A	6.83	10	56
5:1	3.10	1.86	2.78	4.52	3.11	3.37	2.76	2.06	2.81	1.56	3.19	2.78	2.83	5	77.5
3:1	2.90	1.52	1.16	2.81	1.83	3.61	1.55	4.49	2.62	N/A	2.18	1.59	2.39	3	60
2:1	1.48	0.98	0.73	2.14	1.19	0.90	0.87	1.43	1.22	0.75	1.60	1.25	1.21	2	79
1:1	1.25	2.88	2.60	0.80	1.36	1.43	1.96	1.31	1.26	2.53	1.23	2.07	1.72	1	78.5
1:2	2.20	6.89	6.29	1.16	2.16	5.06	3.55	2.14	2.91	4.46	3.02	4.69	3.71	2	75.5
1:3	3.75	7.10	7.72	2.56	3.78	9.06	6.90	4.29	4.23	6.83	7.50	9.89	6.13	3	78.5
1:5	6.95	15.40	8.91	4.11	5.71	7.74	8.12	5.77	6.76	N/A	7.04	N/A	7.65	5	64
1:10	9.81	29.05	13.24	13.43	N/A	N/A	11.55	N/A	9.43	N/A	26.60	12.33	15.68	10	66.5
1:20	29.28	58.47	N/A	N/A	16.79	N/A	N/A	N/A	13.93	N/A	39.33	N/A	31.56	20	58
0:1	not applicable														48

Table 11B: *Mixture analysis for Mix01 (two-person mixture) with 1.0 ng DNA target. Allele peak heights for loci with alleles unshared between the contributors were used for calculating observed mixture ratios. The observed ratios were then compared with the expected theoretical ratios. Average numbers of observed alleles were also determined as shown. Total number of alleles in mixture: 79.*

Mix01: Fusion 6C (1.0 ng target)

	Marker												Observed	Expected	Average number
Ratio	D1S1656	D2S441	D13S317	TH01	vWA	D21S11	D7S820	D12S391	D19S433	SE33	FGA	DYS576	average	value	of alleles
1:0	not applicable														48
20:1	N/A	N/A	N/A	19.79	11.04	N/A	N/A	N/A	N/A	4.70	N/A	5.79	10.33	20	61
10:1	N/A	N/A	N/A	3.40	2.80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3.10	10	41.5
5:1	4.80	1.76	1.72	8.07	3.20	4.60	2.51	5.10	3.17	3.09	4.50	2.98	3.79	5	78
3:1	N/A	0.58	N/A	2.51	0.72	N/A	N/A	1.50	N/A	N/A	0.86	N/A	1.23	3	51
2:1	1.66	1.32	1.02	4.66	1.69	1.53	1.33	2.31	2.28	1.39	1.36	1.07	1.80	2	79
1:1	0.96	2.13	2.84	0.63	1.13	1.06	1.52	0.88	1.02	1.31	1.59	2.98	1.50	1	79
1:2	1.72	3.30	4.16	1.37	1.83	3.35	3.48	1.54	2.39	N/A	2.67	2.65	2.59	2	71.5
1:3	5.65	8.43	8.37	2.55	3.50	6.60	5.87	3.97	4.18	6.44	5.03	6.99	5.63	3	77.5
1:5	6.78	8.53	7.94	2.23	6.36	N/A	N/A	4.11	4.53	4.98	9.04	13.87	6.84	5	74
1:10	12.25	27.31	12.25	8.45	9.89	N/A	12.22	6.82	8.78	N/A	17.38	N/A	12.82	10	69.5
1:20	28.57	33.31	N/A	21.31	N/A	31.32	N/A	N/A	15.75	29.83	43.86	48.58	31.57	20	65
0:1	not applicable														48

Comparing the observed average ratios with the expected values for Mix01 in Tables 11A and 11B demonstrated that the ratios of peak heights can give an approximate ratio of DNA contributions. As the ratios became more skewed, the observed and expected values became less concordant. Mixtures with more even ratios of contributed DNA (closer to 1:1) are more likely to have the complete mixture profiles with all expected alleles called (for example, 79 total alleles in Mix01). As the ratios of DNA contributed became more skewed, fewer alleles were called. Increasing the total amount of DNA amplified from 0.5 ng to 1.0 ng did not necessarily increase the likelihood of observing full mixed profiles. Similar trends were observed for Mix02 and Mix03 series.

Additional two-person mixture series (designated Mix04 and Mix05) were processed with Fusion 6C. Four different DNA target amounts (0.1, 0.25, 0.5, and 1.0 ng) were used. As with the Mix01 and Mix02 series, ratios of peak heights at loci with unshared alleles were used to compare with theoretical ratios, and average numbers of detected alleles were determined. Table 11C shows the results for Mix04. The Mix05 series involved related donors, and so no loci with unshared alleles were present. The analysis for Mix05 focused on the average number of alleles detected. A table with that data is included in the binder.

Table 11C: *Mixture analysis for Mix04 (two-person mixture) with varying DNA target amounts. Allele peak heights for loci with alleles unshared between the contributors were used for calculating observed mixture ratios. The observed ratios were then compared with the expected theoretical ratios. Average numbers of observed alleles were also determined as shown. Total number of alleles in mixture: 75.*

Mix04 (Fusion 6C)

DNA amount (ng)	Ratio	Marker											Observed average	Expected value	Average number of alleles
		D3S1358	D2S441	D13S317	D18S51	Penta D	D21S11	D7S820	TPOX	D12S391	SE33	FGA			
0.1	20:1	12.83	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	12.83	20	47.5
	10:1	6.07	10.77	5.68	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.50	10	53
	5:1	5.86	4.63	3.01	N/A	N/A	2.06	N/A	N/A	N/A	N/A	3.24	3.76	5	56.5
	2:1	1.36	3.88	1.52	1.70	2.18	1.23	2.43	2.61	1.36	N/A	1.98	2.02	2	70.5
	1:2	1.46	2.24	1.06	2.03	0.70	1.25	2.01	1.46	2.04	1.31	1.14	1.52	2	67
	1:5	N/A	N/A	3.30	N/A	N/A	7.84	N/A	5.45	N/A	N/A	N/A	5.53	5	52.5
	1:10	N/A	9.99	N/A	N/A	N/A	N/A	N/A	5.47	N/A	N/A	6.17	7.21	10	50
	1:20	N/A	N/A	N/A	N/A	N/A	11.30	N/A	N/A	N/A	N/A	N/A	11.30	20	46
0.25	20:1	22.71	21.46	26.65	N/A	8.81	N/A	N/A	N/A	N/A	N/A	N/A	19.91	20	53.5
	10:1	11.58	10.25	11.50	N/A	13.56	6.10	N/A	N/A	7.09	2.84	N/A	8.99	10	64.5
	5:1	8.28	7.78	5.31	6.30	5.13	3.92	4.85	6.73	4.00	2.13	4.56	5.36	5	72.5
	2:1	2.00	2.16	3.26	1.94	1.84	1.60	2.10	1.85	1.97	1.49	2.29	2.05	2	75
	1:2	1.54	1.40	3.41	2.32	1.37	2.17	1.56	1.88	1.98	1.28	1.66	1.87	2	74.5
	1:5	3.32	4.20	4.58	N/A	4.92	4.74	N/A	4.01	5.93	1.68	4.77	4.24	5	66.5
	1:10	3.69	5.38	N/A	N/A	N/A	6.67	N/A	10.71	6.21	N/A	8.02	6.78	10	63
	1:20	N/A	12.91	N/A	N/A	N/A	16.68	N/A	12.39	N/A	N/A	13.82	13.95	20	53
0.5	20:1	15.92	26.49	25.44	13.69	25.51	6.38	N/A	N/A	N/A	N/A	N/A	18.90	20	60.5
	10:1	6.81	11.84	7.37	12.39	8.55	4.32	6.36	8.75	9.21	1.89	4.34	7.44	10	72
	5:1	3.75	5.22	5.20	4.98	3.43	3.44	5.63	7.58	5.42	1.81	3.29	4.52	5	75
	2:1	2.19	2.11	3.16	1.58	2.49	1.55	2.23	1.83	1.98	1.29	1.83	2.02	2	75
	1:2	1.93	1.73	1.95	1.74	1.68	2.05	1.84	2.38	2.13	1.17	2.12	1.88	2	75
	1:5	3.75	4.61	4.37	5.43	4.31	5.92	3.82	5.24	4.42	2.05	6.38	4.57	5	74
	1:10	4.88	10.82	6.80	9.20	6.91	8.84	6.86	11.10	5.05	3.04	16.70	8.20	10	71.5
	1:20	N/A	25.19	13.79	N/A	N/A	24.08	9.13	11.68	N/A	N/A	30.17	19.01	20	59
1.0	20:1	14.60	36.05	20.04	15.15	13.11	N/A	11.14	N/A	13.43	N/A	N/A	17.65	20	64
	10:1	9.58	10.78	10.28	12.49	9.10	4.18	9.89	10.70	8.16	2.14	4.61	8.35	10	74
	5:1	4.29	5.99	5.62	5.76	6.54	3.49	4.42	5.31	4.19	1.79	3.03	4.58	5	75
	2:1	2.04	1.94	2.75	2.66	2.08	1.80	2.47	2.47	2.01	1.48	1.93	2.15	2	75
	1:2	1.64	1.90	1.61	2.02	1.83	2.23	1.70	1.52	2.14	1.22	2.35	1.83	2	75
	1:5	2.98	4.63	4.21	4.50	4.14	4.23	4.25	3.73	3.92	2.64	4.63	3.99	5	75
	1:10	N/A	9.75	14.24	9.82	6.66	8.90	6.04	8.98	7.53	3.32	12.08	8.73	10	72
	1:20	N/A	21.77	N/A	18.74	N/A	12.98	12.86	17.20	N/A	4.43	21.94	15.70	20	64.5

As with the Mix01 series, observed average ratios can provide an approximate ratio of DNA contributions as the values were relatively similar to expected values. Mixtures with ratios having more even DNA contributions (closer to 1:1) tended to have more called alleles than mixtures with more skewed DNA contributions. The samples with 0.1 ng DNA amplified exhibited no ratios where the complete mixture profile was detected on average. As the DNA amount increased, more samples showed full mixture profiles though the change from 0.5 ng to 1.0 ng was not as substantial (similar result as with the Mix01 series).

Two (2) sets of three-person mixtures (designated Mix06 and Mix07) were amplified with Fusion 6C. Four different DNA target amounts (0.1, 0.25, 0.5, and 1.0 ng) were used. At loci with unshared alleles, the relative peak heights were used to calculate the relative contributions among the three donors. The observed ratios were compared with the theoretical ratios (based on DNA amounts added). The average numbers of alleles observed were also noted. Table 11D illustrates the results for the Mix06 series.

Table 11D: *Mixture analysis for Mix06 (three-person mixture) with varying DNA target amounts. Allele peak heights for the locus (SE33) with alleles unshared between the contributors were used for calculating observed mixture ratios. The observed ratios were then compared with the expected theoretical ratios. Average numbers of observed alleles were also determined as shown. Total number of alleles in mixture: 93.*

Mix06 (Fusion 6C): relative ratios

SE33 locus		Relative contributor ratio			Observed	Average number
DNA amt (ng)	Ratio name	1	2	3	ratio	of alleles
0.1	10:5:1	N/A	N/A	N/A	N/A	74.5
	8:1:1	N/A	N/A	N/A	N/A	70
	3:2:1	N/A	N/A	N/A	N/A	81
	1:1:1	N/A	N/A	N/A	N/A	87.5
0.25	10:5:1	4.1	1.9	1.0	4.1:1.9:1.0	85.5
	8:1:1	N/A	N/A	N/A	N/A	83.5
	3:2:1	2.1	1.2	1.0	2.1:1.2:1.0	92
	1:1:1	1.0	1.6	1.4	1.0:1.6:1.4	93
0.5	10:5:1	7.2	3.7	1.0	7.2:3.7:1.0	91.5
	8:1:1	9.5	1.4	1.0	9.5:1.4:1.0	92.5
	3:2:1	2.6	1.7	1.0	2.6:1.7:1.0	93
	1:1:1	1.0	1.2	1.3	1.0:1.2:1.3	93
1.0	10:5:1	6.8	3.5	1.0	6.8:3.5:1.0	93
	8:1:1	7.6	1.2	1.0	7.6:1.2:1.0	93
	3:2:1	2.9	1.8	1.0	2.9:1.8:1.0	93
	1:1:1	1.0	1.4	1.6	1.0:1.4:1.6	93

As shown in Table 11D, observed ratios of the three contributors can give an approximate ratio of DNA contributors as values were relatively similar to expected ratios. Mixtures with more even ratios of contributors (e.g. 1:1:1) tended to show more observed alleles than mixtures with more skewed ratios (e.g. 10:5:1). Additionally, as the DNA target amount increased from 0.1 ng to 1.0 ng, the average number of observed alleles increased with the maximum number of 93 alleles observed with all ratios at 1.0 ng. The observed ratios of the mixtures showed some variation from the theoretical ratios, and this can be attributed to variability in quantification of DNA concentration ,variability of pipetting in generating the mixtures, as well a variability in the amplification process.

An addition three person mixture set containing related indivuals Mix07 was analyzed in a similar manner to that used for Mix06 was performed. A table with data is included in the binder.

Two (2) four-person mixtures (designated Mix08 and Mix09) were analyzed with Fusion 6C. Four different DNA target amount (0.1, 0.25, 0.5, and 1.0 ng) were amplified. Neither series of mixtures had any loci with unshared alleles so analysis focused on the average number of alleles observed. The results for the Mix08 mixture are shown in Table 11E. A similar table for Mix09 is included in the binder.

Table 11E: *Mixture analysis for Mix08 (four-person mixture) with varying DNA target amounts. The average numbers of observed alleles were determined as shown. Total number of alleles in mixture: 109.*

Mix08: Fusion 6C

DNA amount (ng)	Ratio	Average number of alleles
0.1	10:5:2:1	75.5
	9:3:3:1	80
	6:3:1:1	80
	4:4:1:1	80.5
	4:3:2:1	84
	1:1:1:1	89
0.25	10:5:2:1	99
	9:3:3:1	97.5
	6:3:1:1	96
	4:4:1:1	100
	4:3:2:1	104
	1:1:1:1	109
0.5	10:5:2:1	102
	9:3:3:1	103.5
	6:3:1:1	107.5
	4:4:1:1	108
	4:3:2:1	109
	1:1:1:1	109
1.0	10:5:2:1	107.5
	9:3:3:1	107.5
	6:3:1:1	108
	4:4:1:1	108
	4:3:2:1	108
	1:1:1:1	109

In results similar to that for Mix06, mixtures with more even ratios of contributors (e.g. 1:1:1:1) tended to show more alleles than mixtures with more skewed ratios (e.g. 10:5:2:1). As the DNA target amount increased from 0.1 ng to 1.0 ng, more complete mixture profiles were observed.

Two (2) series of mixtures with low male to high female ratios (designated MixMF1 and MixMF2) were examined using Fusion 6C. Ratios from 1:5 to 1:5000 (female major) were analyzed to determine the ratio at which the male component would most likely drop out. Table 11F shows results with MixMF1 for the total alleles detected and the unambiguous male minor alleles detected at the various ratios. A similar table for MixMF2 is included in the binder.

Table 11F: *Mixture analysis for MixMF1 (low male to high female mixture) with varying ratios (female major in all samples of the series). The total number of mixture alleles and the number of unambiguous male minor alleles were determined as shown.*

MixMF1 series

	Total alleles		Minor alleles	
Ratio	Number	Percentage	Number	Percentage
1:5	72	100%	30	100%
1:10	65	90%	23	77%
1:20	64	89%	22	73%
1:50	52	72%	10	33%
1:100	45	63%	3	10%
1:250	42	58%	0	0%
1:500	42	58%	0	0%
1:1000	42	58%	0	0%
1:5000	42	58%	0	0%

The results indicate that full unambiguous male minor male alleles were detected at the 1:5 ratio. As the proportion of male contributor decreased, the percentage of minor male alleles decreased starting with the 1:10 ratio. The majority of minor male alleles can still be detected at the 1:20 ratio. At the 1:50 ratio, the minor male profile starts dropping substantively, and by the 1:250 ratio, no minor male alleles can be distinguished. Similar results can be seen with the MixMF2 data in the table included in the binder. The results suggest that in a mixture with a lower ratio of male to female DNA than 1:20, an alternative approach to analyzing the mixture (such as typing Y-STRs) should be considered.

The data from the low male to high female mixtures were also used to determine when a sample would benefit for proceeding directly to Y-STR analysis. The mixture profiles were evaluated for the presence of unambiguous male alleles. The Auto:Y ratios were tracked and the average, standard deviation, and average plus 3X the standard deviation were calculated to determine a cut-off. At an Auto:Y ratio of greater than or equal to 100 the number of unambiguous male alleles detected ranged between 1 and 3 (data can be found in companion binder). Samples with an Auto:Y ratio of greater than or equal to 100 would benefit from Y-STR analysis.

Four mixture series were quantified with PowerQuant®. The autosomal and male concentrations were used to calculate the autosomal:male DNA ratio, and the observed ratios were compared with theoretical ratios (based on DNA concentrations at the time of mixture generation) to determine similarity of values. Table 11G and Figure 11A show the results for the two-person Mix04 and Mix05 mixtures.

Table 11G: *Quantification analysis for Mix04 and Mix05 (male to female mixtures) with varying ratios. Autosomal (Auto), degradation (Deg), and male (Y) targets were quantified with PowerQuant®. Autosomal:degradation (Auto:D) and autosomal:male (Auto:Y) ratios were calculated. Observed Auto:Y ratios were compared with expected/theoretical values.*

Sample	Ratio (M:F)	Auto	Deg (D)	Y	Auto:D	Auto:Y (observed)	Auto:Y (expected)
Mix04	20:1	0.1017	0.0575	0.0892	1.7683	1.1401	1.0500
	10:1	0.0813	0.0537	0.0745	1.5128	1.0905	1.1000
	5:1	0.0744	0.0446	0.0694	1.6689	1.0721	1.2000
	2:1	0.0667	0.0314	0.0419	2.1260	1.5916	1.5000
	1:2	0.0923	0.0388	0.0260	2.3825	3.5540	3.0000
	1:5	0.0897	0.0367	0.0102	2.4403	8.7703	6.0000
	1:10	0.1003	0.0436	0.0098	2.3008	10.2512	11.0000
	1:20	0.0845	0.0351	0.0037	2.4098	22.5448	21.0000
Mix05	20:1	0.0904	0.0697	0.0740	1.2968	1.2228	1.0500
	10:1	0.1217	0.0857	0.0876	1.4194	1.3886	1.1000
	5:1	0.1137	0.0691	0.0738	1.6444	1.5400	1.2000
	2:1	0.0636	0.0385	0.0394	1.6493	1.6122	1.5000
	1:2	0.0501	0.0287	0.0147	1.7455	3.4148	3.0000
	1:5	0.0705	0.0429	0.0106	1.6413	6.6541	6.0000
	1:10	0.0743	0.0447	0.0077	1.6629	9.6999	11.0000
	1:20	0.0653	0.0364	0.0016	1.7969	40.3897	21.0000

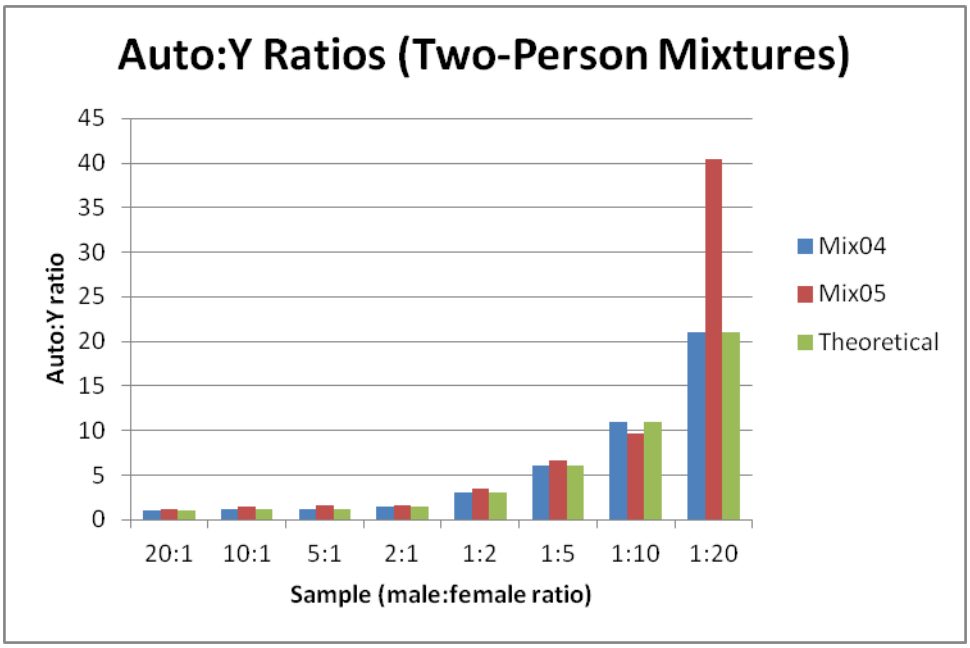

Figure 11A: *Quantification analysis for Mix04 and Mix05 (male to female mixtures) with varying ratios. Autosomal:male (Auto:Y) ratios were calculated and plotted for comparison with expected/theoretical values.*

The Mix04 autosomal:male ratios tended to be very similar to the expected ratios as shown in Table 11G and Figure 11A while the ratios were more different between Mix05 and the theoretical set. The differences could be attributed to variability in quantitation of the initial extracts used to prepare the dilutions.

The low male to high female series (MixMF1 and MixMF2) were also quantified with PowerQuant®. The autosomal:male ratios were determined as shown in Table 11H and Figure 11B.

Table 11H: *Quantification analysis for MixMF1 and MixMF2 (low male to high female mixtures) with varying ratios. Autosomal (Auto), degradation (Deg), and male (Y) targets were quantified with PowerQuant®. Autosomal:degradation (Auto:D) and autosomal:male (Auto:Y) ratios were calculated. Observed Auto:Y ratios were compared with expected/theoretical values.*

Sample	Ratio (M:F)	Auto	Deg (D)	Y	Auto:D	Auto:Y (observed)	Auto:Y (expected)
MixMF 1 F	1:5	0.0985	0.0477	0.0181	2.0660	5.4534	6.0000
	1:10	0.0828	0.0397	0.0073	2.0850	11.3814	11.0000
	1:20	0.0837	0.0432	0.0057	1.9375	14.5716	21.0000
	1:50	0.0830	0.0413	0.0032	2.0100	25.7318	51.0000
	1:100	0.0764	0.0428	0.0008	1.7824	97.5268	101.0000
	1:250	0.0754	0.0447	0.0009	1.6884	79.5262	251.0000
	1:500	0.0815	0.0412	0.0006	1.9772	139.6250	501.0000
	1:1000	0.0811	0.0398		2.0359	N/A	1001.0000
	1:5000	0.0518	0.0278		1.8625	N/A	5001.0000
MixMF 2 F	1:5	0.0927	0.0564	0.0126	1.6446	7.3703	6.0000
	1:10	0.0795	0.0542	0.0071	1.4659	11.2161	11.0000
	1:20	0.0778	0.0548	0.0030	1.4210	26.2445	21.0000
	1:50	0.0856	0.0530	0.0016	1.6171	54.4651	51.0000
	1:100	0.0982	0.0587	0.0011	1.6728	90.4289	101.0000
	1:250	0.0795	0.0467		1.7030	N/A	251.0000
	1:500	0.0966	0.0556		1.7361	N/A	501.0000
	1:1000	0.0783	0.0502		1.5591	N/A	1001.0000
	1:5000	0.0802	0.0517		1.5516	N/A	5001.0000

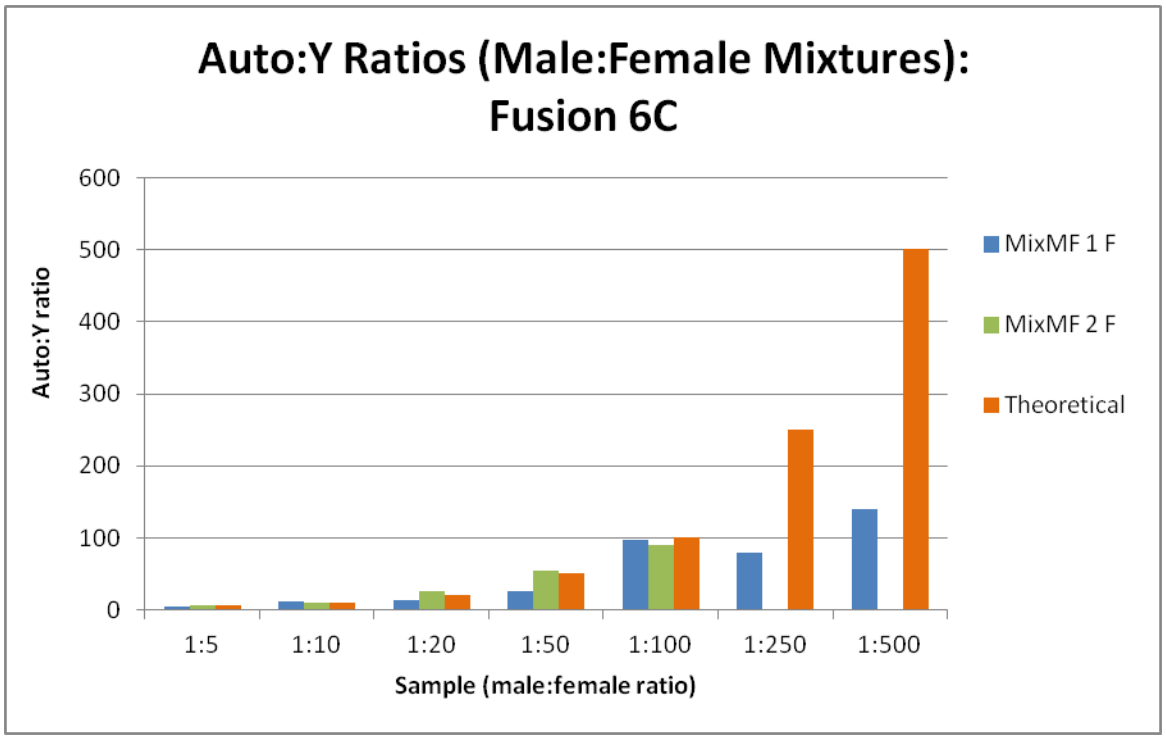

Figure 11B: *Quantification analysis for MixMF1 and MixMF2 (low male to high female mixtures) with varying ratios. Autosomal:male (Auto:Y) ratios were calculated and plotted for comparison with expected/theoretical values.*

As the ratio of male to female DNA increased in the MixMF1 and MixMF2 mixtures, the amount of male DNA present was reduced to undetectable levels at 1:1000 for the MixMF1 series and 1:250 for the MixMF2 series. The observed Auto:Y ratios were relatively similar to the theoretical ratios for mixtures with more equal contributions (e.g. 1:5) with differences becoming more pronounced as the ratios became more skewed (e.g. 1:500).

The overall results with mixtures support the use of PowerPlex® Fusion 6C with the ProFlex™ PCR System and 3500xL Genetic Analyzer for analyzing mixed DNA samples in casework.

(12) <u>Contamination Monitoring:</u>

Each PCR amplification plate contained a negative amplification control treated in the same manner as other samples included on the plate. A total of 19 negative amplification controls were examined for the presence of any alleles. No alleles were detected supporting the idea that amplification with Fusion 6C can be performed without contamination provided sufficient safeguards are taken.

No drop-in alleles were observed with samples during analysis.

This data indicate that amplification with the PowerPlex® Fusion 6C kit, ProFlex™ PCR System, and 3500xL Genetic Analyzer will be suitable for use in casework.

(13) Establishing a Quantification Based Cut-off

The allele count for each sensitivity replicate was compared to the quantification value from the stock dilution. Not enough quantification data was generated during the course of the validation to assign a quantification based cut-off for stopping the DNA process. Allele drop out was first observed for the sensitivity data at 0.01 ng/µl (0.125 ng target). The laboratory will monitor the DNA profiles obtained from quantification values above zero and at or below 0.01 ng/µl from casework samples to determine a quantification based cut-off. Based on the Forensic Biology Unit's internal validation of the PowerQuant system, the sensitivity study demonstrated that extremely low amounts of DNA can be reliably detected using the PowerQuant system. It is reasonable that when no autosomal quantification value is obtained from a sample, no interpretable STR profile will be obtained. As a result the DNA process may be stopped when a quantification value of zero (undetermined) is obtained.

Conclusions/Recommendations:

The results of this validation indicate that the PowerPlex® Fusion 6C kit with the ProFlex™ PCR System and 3500xL Genetic Analyzer is suitable for use on forensic casework. The results and conclusions from the different studies of the validation are summarized below:

1. **Cycle Number –** Analysis was performed with samples amplified at 28 and 29 cycles with PowerPlex® Fusion 6C. The protocol with 29 cycles was selected for validation based on the peak heights and overall quality of profiles observed and will be implemented for casework

2. **Baseline –** It is recommended that different analytical thresholds be set for the various dyes used in analyzing PowerPlex® Fusion 6C data. The following values were used for analytical thresholds: 75 (blue), 101 (green), 60 (yellow), 69 (red), 56 (purple), and 50 (orange).

3. **Sensitivity –** Decreasing the amount of DNA amplified with PowerPlex® Fusion 6C reduced average allele peak heights and the percentage of alleles detected in profiles. Full profiles were detected as low as 0.125 ng for all replicates examined. Average peak height ratios remained relatively constant throughout the range of DNA amounts amplified though variability tended to increase with lower DNA amounts. The minimum peak height ratio observed decreased from 4 ng target to 0.03125 ng before increasing at 0.0156 ng and 0.0078 ng amounts. The optimal range for DNA amplification with Fusion 6C was determined to be 0.5 ng to 1.0 ng. The amount of DNA selected for further validation was 0.75 ng. Quantification of sensitivity series extracts showed general corresponding decrease in observed DNA concentrations as the theoretical DNA concentrations decreased with some differences detected (as expected) between observed and theoretical values. A minimum expected peak height ratio was calculated from the 0.25 ng and 0.5 ng target. That calculation results in a minimum expected peak height ratio of 46% and 59% respectively. It would be expected that sister alleles would have a peak height ratio of approximately 60% or greater

when amplified in the target range. Based on the validation results it is recommended that a target of 1 ng be utilized for the 280M amplification control positive (ACP).

4. **Stochastic Events –** Stochastic effects (with allelic dropout) were detected in DNA sensitivity samples with DNA targets of 0.0625 ng or less. At the recommended range of DNA target amounts from 0.5 ng to 1.0 ng, no stochastic events were observed. A stochastic threshold of 250 RFUs was determined.

5. **Reproducibility/Repeatability –** Analysis of a positive control and two (2) known samples demonstrated that the PowerPlex® Fusion 6C, ProFlex™ PCR System, and 3500xL Genetic Analyzer system produce reproducible allele calling. Concordant alleles were detected with intraplate and interplate replicate samples.

6. **Known Samples –** Amplification of extracts obtained from ten (10) known samples resulted in expected profiles for those samples at loci previously typed. Full single-source profiles were obtained for all known samples examined.

7. **Mock Evidence Samples –** Amplification with ten (10) mock evidence extracts demonstrated that single-source and mixed profiles can be successfully processed with PowerPlex® Fusion 6C. Obtained profiles were concordant with expected profiles with a note that NP02 had extra alleles in the expected profile that could be from incomplete differential separation and NP03 had two subthreshold alleles.

8. **NIST SRM Concordance –** Concordance was obtained with the two NIST SRM 2391c components B and C using PowerPlex® Fusion 6C in terms of observed profiles compared to expected profiles.

9. **Precision** – The majority of alleles at loci showed sufficiently low variability (as measured by three times standard deviation of less than 0.5 bp) to demonstrate precision of allele calling. For some shorter length alleles, the three times standard deviation value exceeded 0.5 bp. All standard deviations for the individual alleles as well as the average standard deviation for all loci were below 0.5 bp. The reproducibility, mock evidence, know samples, and the NIST SRM samples all provided the same (correct) allele calls as the previously typed results or published data. Proper allele designation was obtained throughout the course the validation demonstrating that sufficient precision among allelic ladders is present.

10. **Stutter –** Reverse and forward stutter of one repeat unit were observed with PowerPlex® Fusion 6C. In some instances, the observed stutter values (calculated as average plus three standard deviations or average where no standard deviation was calculated) were greater than

the manufacturer's guideline values. During developmental validation[1] 652 samples were evaluated to determine the manufacturer's stutter recommendation. The number of reverse stutters events range between 450 and 800 and 75 and 750 events for forward stutter. The observed stutter values obtained from the internal validation provided similar results with the highest difference between observed reverses stutter percentage and manufacture's guidelines was 3% and 8% between the observed forward stutter percentage and the manufacturer's guidelines. The manufacturer's recommended stutter values will be utilized. Reverse half repeat stutter (n-2) was observed during validation for D1S1656 and SE33. No observed instances of reverse half repeat stutter (n-2) were observed during validation for D19S433 and FGA. Stutter filter percentages for reverse half repeat stutter (n-2) were calculated for D1S1656, and SE33. Manufacturer's recommendations will be used for assigning reverse half repeat stutter (n-2) percentages for D1S1656, SE33, D19S433, and FGA. It should be noted that reverse half repeat stutter (n-2) will not be modelled in STRmix for D19S433 and FGA. No stutter filters for reverse two repeat unit stutter (double back stutter) will be added to the GMIDX panels and bins for PowerPlex® Fusion 6C analysis. It should be noted that reverse two repeat stutter (double back stutter) for the loci D3S1358, D1S656, D10S1248, D16S539, D18S51, D21S11, D3S1358, D1S656, D10S1248, D16S539, D18S51, D21S11, D8S1179, D19S433,SE33, and D22S1045 will be modelled in STRmix v.2.6.

11. **Mixtures** – Two-, three-, and four-person mixtures were successfully analyzed with PowerPlex® Fusion 6C. Ratios of peak heights at loci with unshared alleles can give an approximate ratio of DNA contributions. The number of detected alleles in mixtures increased as target DNA amount increased to 1.0 ng and as the ratio of contributors became more equal in the mixtures. Quantification of mixture series extracts demonstrated some concordance between observed and expected autosomal:male DNA concentration ratios with less concordance at more skewed mixture ratios. It is recommended that a target amount of 1ng of DNA be amplified for mixed samples.

    At an Auto:Y ratio of greater than or equal to 100 the number of unambiguous male alleles detected ranged between 1 and 3 (data can be found in companion binder). Samples with an Auto:Y ratio of greater than or equal to 100 would benefit from proceeding directly to Y-STR analysis.

12. **Contamination Monitoring** – Contamination monitoring using negative amplification controls demonstrated that the PowerPlex® Fusion 6C kit reagents were appropriate for use with the amplification of casework samples. No signs of contamination from the negative controls or drop-in alleles were detected during validation. QA/QC techniques typical for forensic DNA laboratories are sufficient to minimize contamination from sample setup.

[1] Developmental validation of the PowerPlex® Fusion 6C System. Ensenberger, M. G., et al. Forensic Science International: Genetics 21 (2016) 134-144.

13. **Quantification Based cut-off-** The DNA process may be stopped for samples with a quantification value of zero (undetermined).