The New York City Office of the Chief Medical Examiner

PowerPlex® Fusion System Amplification Kit on the Applied Biosystems® 3500xL Genetic Analyzer with GeneMarker® HID 2.9.5

Validation Report

July 2019

Validation Work completed by: Promega GI Validation Services and The New York City Office of the Chief Medical Examiner

Table of Contents

1. Introduction

The PowerPlex® Fusion system is a 24-locus system that includes all CODIS and ESS loci. The loci included are: Amelogenin, D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, D22S1045, DYS391, and FGA.

Before an established method or procedure may be employed in a forensic laboratory, an internal validation must be completed to show that the method performs as expected. This validation summary outlines a set of experiments that shows conformance with the current versions of the FBI Quality Assurance Standards for Forensic DNA Testing Laboratories and the validation guidelines outlined by the Scientific Working Group on DNA Analysis Methods (SWGDAM). These experiments were designed to demonstrate the sensitivity and reliability of the PowerPlex® Fusion system on the Applied Biosystems® 3500xL Genetic Analyzer. The studies completed were sensitivity and stochastic studies, precision, reproducibility/repeatability, non-probative evidence samples, mixture samples, direct amplification of known samples, and contamination assessment.

2. FBI Quality Assurance Standard

Standard 8.3.1: Internal validation studies conducted after the date of this revision shall include as applicable: known and non-probative evidence samples or mock evidence samples, reproducibility and precision, sensitivity and stochastic studies, mixture studies, and contamination assessment. Internal validation studies shall be documented and summarized. The technical leader shall approve the internal validation studies.

3. Materials and Methods

3.A Processing of Swabs with SwabSolution™

Processing of buccal swabs was performed following the instructions described in the SwabSolution™ Kit technical manual (TMD037). Five hundred microliters of SwabSolution™ Reagent was added to each ½ swab cutting in a 1.5ml tube. Each tube was then incubated on a thermomixer at 70°C for 30 minutes. Except where noted, a 0.1 dilution of the lysate in water was performed prior to amplification.

3.B Processing of Bloodstain cards with PunchSolution™

Processing of bloodstain cards was performed following the instructions described in the PunchSolution™ Kit technical manual (TMD038). Ten microliters of PunchSolution™ Reagent was added to each 1.2mm punch in a 0.2ml tube. Each open tube was then incubated on a thermal cycler at 70°C for 30 minutes or until PunchSolution™ Reagent was completely evaporated. Following evaporation, each sample was re-suspended by adding 8µl of water to each tube.

3.C DNA Quantification

All extracted DNA samples were quantified by the laboratory with Quantifiler Trio Quantification kit on the 7500 Real-Time PCR Instrument using standard OCME protocols.

3.D DNA Amplification

DNA amplification was performed following standard OCME protocol for extracted DNA. Direct amplification samples were process using the OCME protocol to determine if the same dilution could be utilized for PunchSoution™ and SwabSolution™. Please refer to Appendix A for the lot numbers of the reagents and equipment numbers that were used during the validation. Appendix B contains the plate layouts for the amplification plates run during the validation.

3.E Data Analysis

Spectral resolution was established by the laboratory using the PowerPlex® 5C matrix standard (TMD049) prior to the start of the validation. One microliter of amplified DNA or PowerPlex® Fusion allelic ladder was combined with 9.5µl of Hi-Di™ formamide and 0.5µl of the WEN ILS 500. The samples were heat denatured at 95°C for 3 minutes and chilled for 3 minutes at 4°C. All samples were run on the 3500xL Genetic Analyzer instrument referred to as Carmody. Sensitivity, contamination, precision, and reproducibility samples were run on the 3500xL Genetic Analyzer instrument referred to as Pavlov. All samples were injected at 1.2kV for 24 seconds using a 13kV run voltage and 1500 seconds run time as per manufacturers recommendation. The data was analyzed using GeneMarker® HID v 2.9.5 software using the analysis methods listed in Appendix C. Upon completion of the sensitivity study, the analytical thresholds (AT) were calculated. These thresholds (Section 4.A), along with a 10% global filter were used for analysis of all direct amplification samples.

Average peak height was calculated by adding the sum of all detected peak heights divided by the total number of peaks expected. Allele drop-out was treated as a 0 RFU peak in the sum which resulted in average peak height below the analytical threshold for low template samples.

Peak height ratio (PHR) was calculated at heterozygote loci by dividing the peak height of the allele with the lower RFU value by the peak height of the sister allele with the higher RFU value, then multiplying this value by 100 to express the PHR as a percentage.

3.F Sensitivity Study

A sensitivity study was performed using six extracted DNA samples (buccal swabs): 14F, 34F, 35F, 12M, 21M, and 25M. The DNA template amounts tested were 750pg, 525pg, 250pg, 125pg, 100pg, 75pg, 50pg, 37.5pg, 25pg, 15pg, 7.5pg, and 3.25pg. Each of these dilutions was amplified in duplicate. A subset of these samples were used to establish the analytical thresholds as described in section 3.G.

3.G Analytical Threshold

The analytical threshold (AT) is the point above which a peak can reliably be distinguished from baseline noise. The peak is either allelic or attributable to some known artifact such as pull-up or stutter. If a peak is not above the analytical threshold, it cannot be reliably considered to be a part of any developed DNA profile. If an allelic peak is below the analytical threshold, that peak is considered to have "dropped out" of the profile.

To establish analytical thresholds the following samples from the amplification of the sensitivity study were used: 750pg, 525pg, and 250pg except those 750pg samples that showed saturation. The determination of the analytical threshold was conducted by setting the reading threshold to 1 RFU in the GeneMarker® HID software. All peaks and artifacts associated with the profile or known artifacts in the PowerPlex® Fusion amplification kit were removed. The average height of the remaining noise peaks and standard deviation was determined for each dye channel. After combining noise peak height data from both instruments, analytical thresholds were calculated for each dye by determining an RFU threshold that was greater than 99.9% of observed noise peaks in that dye.

3.H Stochastic Threshold

A stochastic threshold is the RFU level above which a single peak representing an allele at a locus can be determined to be a homozygote. If a single peak is observed below the stochastic threshold, then it can be assumed that a paired peak has potentially "dropped out" of the DNA profile. The stochastic threshold was calculated using the sensitivity series. The average peak height of the remaining alleles were determined along with the standard deviation. Three times the standard deviation was added to the average. The analytical threshold for each dye channel was then divided by the minimum observed

peak height ratio for each target amount at 37.5pg, 50pg, and 75pg. The maximum value found per dye channel was then rounded up to the nearest 100 to obtain the stochastic threshold.

3.I Non-Probative Casework Samples

A total of 28 non-probative casework samples were amplified in duplicate targeting 525pg (when possible), otherwise maximum volume allowed was utilized (Table 1). All non-probative samples were analyzed with saturation artifacts excluded from genotype evaluation. Additional known artifacts (elevated stutter, etc.) were only excluded from genotype consideration from apparent single source samples along with epithelial and sperm fractions (EC or SF).

Table 1: Sample key of non-probative casework samples.

Sample Name	Sample Description	Sampling Type	Extraction Method
Mock_1	Blood (cut)	PT blood evidence sample - IQAS # 17330	EZ1
Mock_2	Blood (cut)	PT blood evidence sample - IQAS # 16287	EZ1
Mock_3	can	cotton swab	EZ1
Mock_4	straw	cotton swab	EZ1
Mock_5	can	cotton swab	EZ1
Mock_6	straw	cotton swab	EZ1
Mock_7	cup	cotton swab	EZ1
Mock_8	mouse	cotton swab	EZ1
Mock_9	glasses	SDS	EZ1
Mock_10	hair clip	SDS	EZ1
Mock_11	mug handle	SDS	EZ1
Mock_12	stress ball	SDS	EZ1
Mock_13	spoon handle	SDS	EZ1
Mock_14	bottle and cap	cotton swab	DNAIQ
Mock_15	bottle and cap	cotton swab	DNAIQ
Mock_16	bottle and cap	cotton swab	DNAIQ
Mock_17	drawer handle	cotton swab	DNAIQ
Mock_18	ear pods	SDS	DNAIQ
Mock_19	lipstick tube	SDS	DNAIQ
Mock_20	phone case	SDS	DNAIQ
Mock_21	arm rest	SDS	DNAIQ
Mock_22	Semen Only (SF Fraction)	denim	AutoDiff
	Semen Only (EC Fraction)	demin	AutoDiff
Mock_23	Semen and Saliva (SF Fraction)	cotton swab	AutoDiff
	Semen and Saliva (EC Fraction)	cotton swab	AutoDiff
Mock_24	Semen and Blood (SF Fraction)	fabric 92% Polyester, 8% Spandex	AutoDiff
	Semen and Blood (EC Fraction)	fabric 92% Polyester, 8% Spandex	AutoDiff
Mock_25	Semen Only	denim	Zygem
Mock_26	Semen and Saliva	cotton swab	Zygem
Mock_27	Semen and Blood	fabric 92% Polyester, 8% Spandex	Zygem
Mock_32	Hole punch	cotton swab	DNAIQ

3.J Precision

Sizing precision is critical for accurate genotyping. The migration of each allele in the PowerPlex® Fusion allelic ladder was evaluated on one CE plate . The average base pair size, standard deviation, minimum size, maximum size and range of base pair size was calculated for each allele present in the allelic ladder.

3.K Repeatability and Reproducibility

The 2800M DNA sample supplied in the PowerPlex® Fusion amplification kit was used for the repeatability study. 2800M profiles were evaluated across all amplification plates set up by different analysts.

Components A-D of NIST SRM 2391c were used for the reproducibility study. The NIST samples were amplified in duplicate. A concordance table was generated for all 2800M and NIST samples amplified during the validation study.

3.L Mixture Study

Mixture samples are commonly encountered in forensic casework samples. This study demonstrates the ability to detect major and minor profiles in samples, as well as the ability of the amplification kit to show mixtures at different ratios of contributors. Each mixture was tested at 5 template amounts: 750pg, 525pg, 150pg, 75pg, 37.5pg, and 15pg.

3.L.1 Two-Person Mixtures

For this study, two series of 2-person mixtures were generated. Four different individuals were utilized (two per series). 14F and 12M were combined for mixture 1 series. 22F and 1M were combined for mixture 2 series. The mixture ratios tested were as follows: 1:1, 1:2, 1:4, 1:10, 1:15, 1:20, 1:50, 1:75, 1:100, 100:1, 75:1, 50:1, 20:1, 15:1, 10:1, 4:1, and 2:1. Mixtures were amplified in duplicate and were evaluated for % profile obtained from the unique minor contributor using only unshared minor alleles. In addition, genotype tables were generated.

3.L.2 Three-Person Mixtures

For this study, two series of 3-person mixtures were generated. Six different individuals were utilized (three per series). 9F, 21F, and 22M were combined for mixture 1 series. 4M, 8F, and 34F were combined for mixture 2 series. The mixture ratios tested were as follows: 20:5:1, 10:5:1, 10:2:1, 5:5:1, 5:2:1, 5:1:1, 3:2:1, 1:2:1, and 1:1:1. Mixtures were amplified in duplicate. The percent profile was calculated for each of the mixture samples. In addition, genotype tables were generated.

3.L.3 Four-Person Mixtures

For this study, two series of 4-person mixtures were generated. Eight different individuals were utilized (four per series). 3F, 5F, 7M, and 16F were combined for mixture 1 series. 12F, 17F, 23F, and 25M were combined for mixture 2 series. The mixture ratios tested were as follows: 20:10:5:1, 20:5:2:1, 10:5:3:1, 10:5:1:1, 10:1:1:1, 1:3:5:1, 1:3:3:1 and 1:1:1:1. Mixtures were amplified in duplicate. The percent profile was calculated for each of the mixture samples. In addition, genotype tables were generated.

3.L.4 Five-Person Mixtures

For this study, two series of 5-person mixtures were generated. Ten different individuals were utilized (five per series). 26M, 27M, 36F, 37F, and 38F were combined for mixture 1 series. 21M, 28M, 35F, 39F and 40F were combined for mixture 2 series. The mixture ratios tested were as follows: 10:5:1:1:1, 5:5:1:1:1, 5:1:1:1:1 and 1:1:1:1:1. Mixtures were amplified in duplicate. The percent profile was calculated for each of the mixture samples. In addition, genotype tables were generated.

3.M Contamination Assessment

Negative controls were run to show the quality of each set-up. Negative controls were evaluated across all of the amplification plates.

3.N Direct Amplification of Known Samples

Twenty blood and twenty buccal samples were direct amplified in duplicate. The samples were evaluated for peak heights, peak height ratios, first pass success rate and concordance between profiles.

4. Results

4.A Analytical Threshold

The samples that were used in the calculation were the 750pg, 525pg, and 250pg samples. These samples were chosen because they did not exhibit any drop out. Note that any 750pg samples with saturation were excluded from analysis. The average noise peak height plus 10 X SD ranged from 73RFU-235RFU between the dye channels (Table 2). The maximum noise peak was 254RFU. In addition, calculations based on coverage were provided for each dye channel (Table 3). Based on the results the laboratory chose an analytical threshold for each dye channel (Table 4). An AT of 85RFU for FL, 120RFU for JOE, 130RFU for TMR and 160RFU for CXR were used for the remainder of the validation. Appendix C contains the analysis methods used throughout the validation. Analytical threshold data tables are provided in Appendix D. Noise distribution by dye can be found in Appendix E.

Table 2: Average baseline noise by dye channel for samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL using a 1.2kV, 24 second injection on both Carmody and Pavlov.

Dye	Instrument	Average Noise Peak Height	Standard Deviation	Average + 10 X SD	Maximum Noise Peak Height
Fluorescein	**Carmody**	**7**	**7**	**73**	**94**
	Pavlov	**7**	**7**	**79**	**121**
JOE	**Carmody**	**11**	**10**	**112**	**254**
	Pavlov	**12**	**12**	**134**	**222**
TMR	**Carmody**	**16**	**14**	**156**	**184**
	Pavlov	**16**	**14**	**159**	**185**
CXR	**Carmody**	**21**	**21**	**235**	**205**
	Pavlov	**18**	**15**	**170**	**208**

Table 3: Average baseline noise by dye channel for samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL using a 1.2kV, 24 second injection on both Carmody and Pavlov.

Dye	# of Noise Peak	Mean	Std	Min	Max	AT	Carmody < AT	Pavlov < AT	Total < AT	Coverage	SDs
FL	7956	7.0	6.9	1	121	**85**	3667	4281	7948	**99.9%**	11.3
Joe	9451	11.7	11.3	1	254	**120**	4265	5177	9442	**99.9%**	9.60
TMR	9524	15.8	14.2	1	185	**130**	4279	5236	9515	**99.9%**	8.05
CXR	11196	19.7	18.3	1	208	**160**	5072	6112	11184	**99.9%**	7.68

Table 4: Laboratory analytical thresholds by dye channel for PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500 using a 1.2kV, 24 second injection.

Dye	AT
Fluorescein	85
JOE	120
TMR	130
CXR	160

4.B Stochastic Threshold

The sensitivity series was examined for dropout of one of two heterozygous peaks. The average peak height of the surviving sister allele was calculated on both 3500xL instruments. The average peak height of the surviving sister allele by dye channel ranged from 208RFU to 356RFU (Table 5). The maximum peak height of the surviving sister allele by dye channel ranged from 587RFU to 1218RFU (Table 5). By taking the maximum value observed for each dye channel using the AT divided by the minimum peak height ratio and rounding to nearest 100, the following stochastic thresholds were chosen: 900RFU for FL, 1000RFU for JOE, 900RFU for TMR, and 900RFU for CXR (Table 6). Appendix F has the stochastic threshold tables.

Table 5: Average peak height of surviving sister allele by dye channel for samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL using a 1.2kV, 24 second injection on both Carmody and Pavlov.

	Overall By Dye Channel	Surviving Sister Allele Count	Surviving Sister Allele Average Peak Height	Surviving Sister Allele Standard Deviation	Surviving Sister Allele Max Height	Surviving Sister Allele Average Peak Height +3SD
Carmody	Fluorescein	71	220	127	668	601
	JOE	79	296	179	1052	832
	TMR	68	286	165	915	782
	CXR	64	356	215	1218	1000
Pavlov	Fluorescein	81	208	110	587	538
	JOE	86	293	168	899	798
	TMR	70	269	134	745	670
	CXR	72	350	196	1154	937

Table 6: The analytical threshold divided by the minimum observed peak height ratio for low level samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL using a 1.2kV, 24 second injection on both Carmody and Pavlov.

37.5pg	AT/Min(PHR)	50pg	AT/Min(PHR)	75pg	AT/Min(PHR)
Fluorescein	509	Fluorescein	825	Fluorescein	429
JOE	958	JOE	577	JOE	391
TMR	534	TMR	511	TMR	827
CXR	699	CXR	596	CXR	854

AT	AT	Population	Results	Avg	Max	Max (Round Up 100)
Fluorescein	85	267	Fluorescein	588	825	900
JOE	120	255	JOE	642	958	1000
TMR	130	276	TMR	624	827	900
CXR	160	246	CXR	716	854	900

4.C Sensitivity

Full profiles were observed in all replicates on both CE instruments when template amounts were between 75pg and 750pg (Table 7, 8). Dropout was first observed in both instruments at 50pg of DNA template. Three template amounts of 34F (15pg, 25pg, and 37.5pg) resulted in peak heights higher than expected and were therefore not included in calculations (Figure 2). One possible reason for these unexpected results is due to a pipetting error. One 35F replicate of 525pg had loss of resolution in two injections and therefore was not included in calculations (data not shown). The peak heights from all other samples showed a linear correlation between template amount and average peak height with an R-squared value of 0.93 and above (Appendix G). The average peak heights were similar between Carmody and Pavlov across the various template amounts (Figure 1, 2, 3, 4, 5, and 6). The average peak heights for 525pg ranged from 5883RFU to 10146RFU (Table 9). The average peak heights by locus can be found in Appendix H, I. The average overall peak height ratio for 525pg was 80% or greater for all samples on both instruments (Appendix J, K). In general, the peak height ratios decreased with decreasing template amount (Figures 7,8).

Table 7: Percent profile obtained for samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. Samples were analyzed with a dye-specific analytical threshold. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed. Gray cells represent samples not included in analysis.

	Carmody % Profile of Sensitivity Series					
	14F	**34F**	**35F**	**12M**	**21M**	**25M**
3.25pg	57%	8%	51%	24%	9%	23%
	14%	24%	56%	29%	18%	31%
7.5pg	59%	54%	33%	50%	36%	54%
	39%	49%	38%	50%	36%	56%
15pg	77%		82%	66%	69%	74%
	52%		89%	63%	67%	69%
25pg	95%		89%	92%	89%	97%
	91%		96%	87%	91%	85%
37.5pg	98%		100%	100%	96%	97%
	98%		100%	100%	96%	100%
50pg	98%	100%	100%	100%	100%	100%
	98%	97%	100%	100%	98%	100%
75pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
100pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
125pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
250pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
525pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
750pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%

Table 8: Percent profile obtained for samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Pavlov) using a 1.2kV, 24 second injection. Samples were analyzed with a dye-specific analytical threshold. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed. Gray cells represent samples not included in analysis.

	Pavlov % Profile of Sensitivity Series					
	14F	**34F**	**35F**	**12M**	**21M**	**25M**
3.25pg	52%	30%	47%	29%	18%	36%
	14%	27%	44%	34%	11%	28%
7.5pg	55%	49%	33%	45%	44%	51%
	45%	49%	38%	26%	33%	51%
15pg	70%		82%	63%	64%	77%
	52%		84%	74%	64%	67%
25pg	84%		89%	87%	91%	97%
	91%		96%	82%	89%	79%
37.5pg	95%		100%	100%	93%	97%
	98%		100%	100%	93%	97%
50pg	93%	100%	100%	100%	100%	100%
	91%	97%	98%	100%	96%	100%
75pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
100pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
125pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
250pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%
525pg	100%	100%	100%	100%	100%	100%
	100%	100%		100%	100%	100%
750pg	100%	100%	100%	100%	100%	100%
	100%	100%	100%	100%	100%	100%

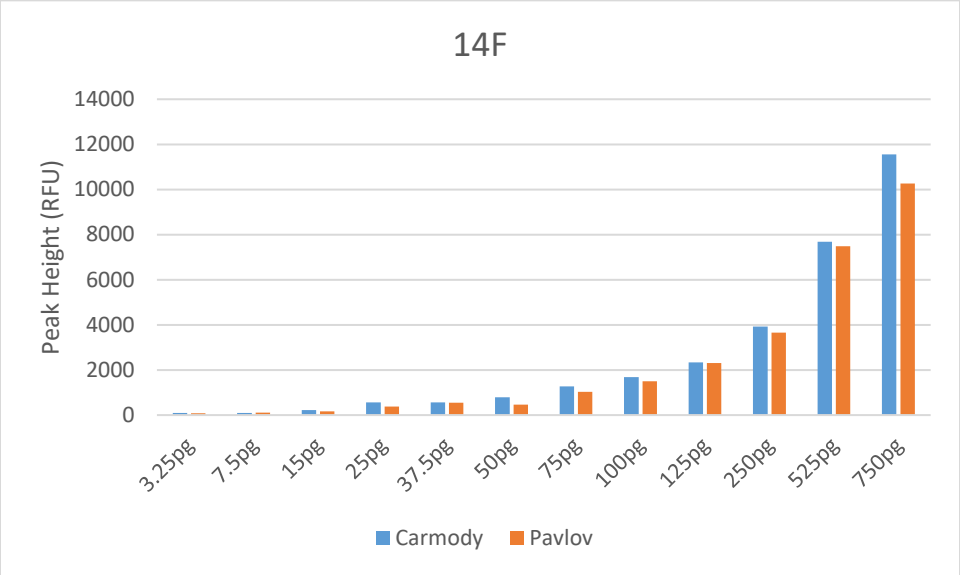

Figure 1: Average peak height for sensitivity sample 14F at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

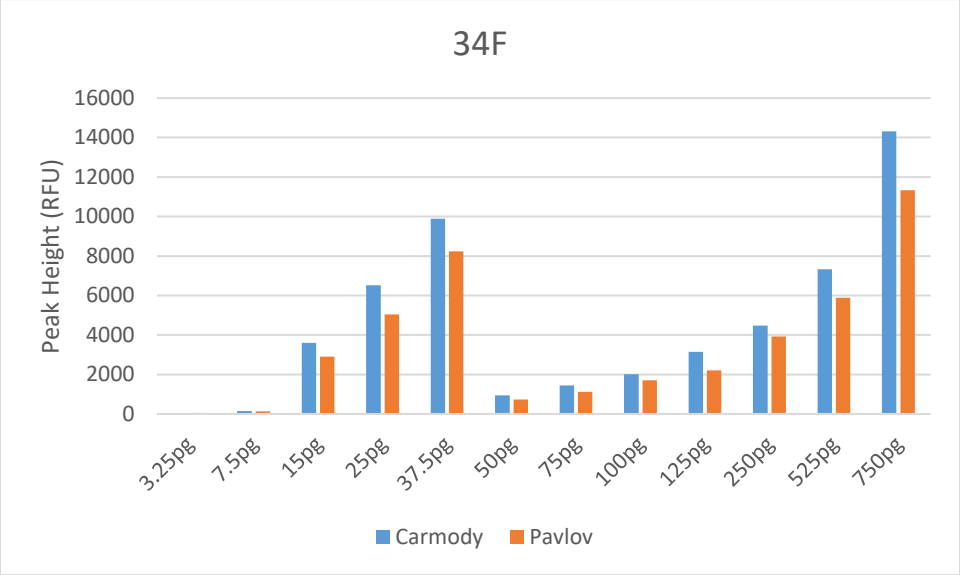

Figure 2: Average peak height for sensitivity sample 34F at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

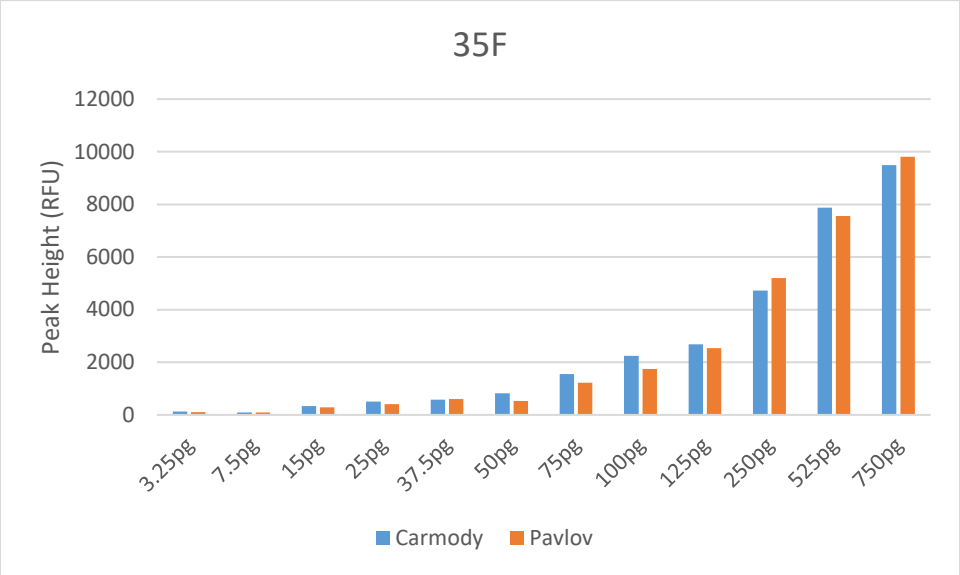

Figure 3: Average peak height for sensitivity sample 35F at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

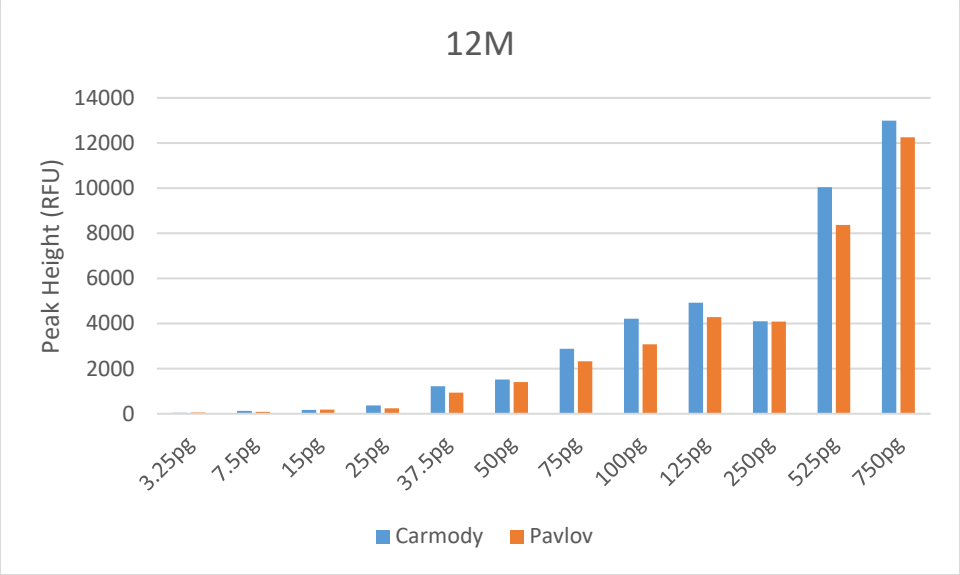

Figure 4: Average peak height for sensitivity sample 12M at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

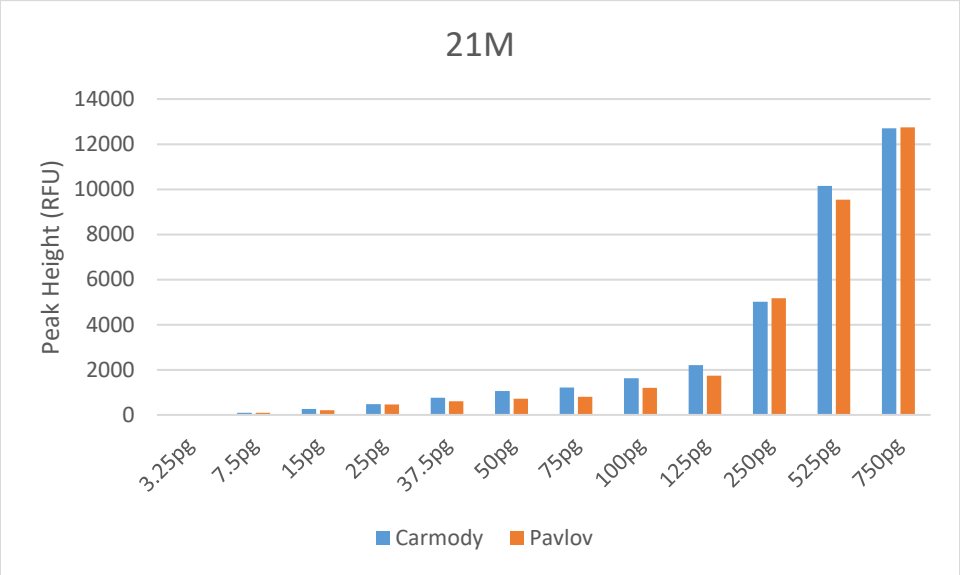

Figure 5: Average peak height for sensitivity sample 21M at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

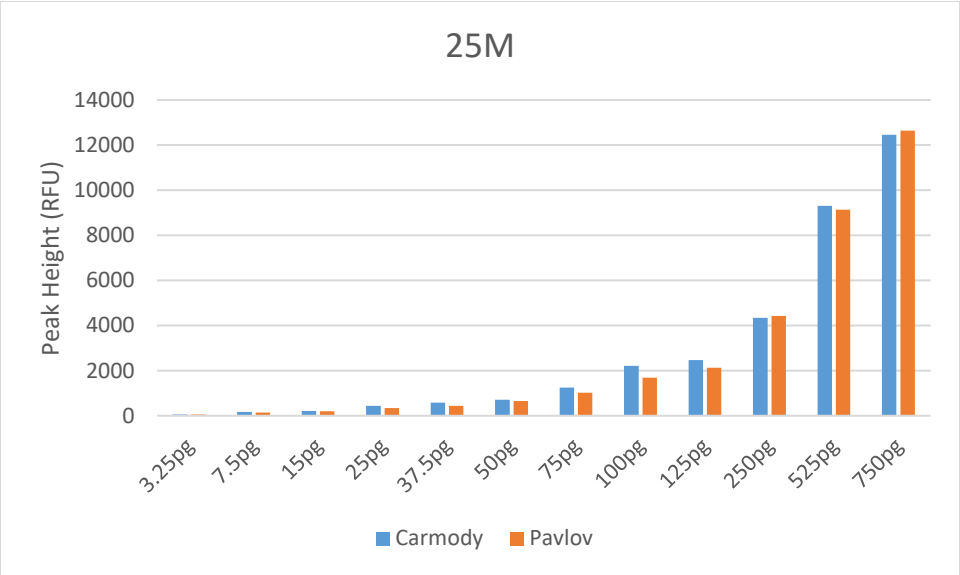

Figure 6: Average peak height for sensitivity sample 25M at various template amounts when run on both Carmody (blue) and Pavlov (orange). Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

Table 9: Average peak heights for six genomic DNA samples amplified using various template amounts. Samples were amplified using PowerPlex® Fusion for 29 cycles on the Applied Biosystems® 9700. Samples were run on the Applied Biosystems®3500xL using a 1.2kV, 24 second injection.

		3.25pg	7.5pg	15pg	25pg	37.5pg	50pg	75pg	100pg	125pg	250pg	525pg	750pg
14F	**Carmody**	95	99	222	558	568	795	1279	1681	2333	3918	7680	11560
	Pavlov	76	106	165	384	545	463	1031	1505	2310	3656	7479	10270
34F	**Carmody**	25	139	3593	6515	9889	944	1440	2018	3143	4480	7331	14315
	Pavlov	55	124	2906	5047	8234	732	1127	1700	2210	3919	5883	11330
35F	**Carmody**	127	95	330	506	583	816	1547	2245	2679	4721	7869	9493
	Pavlov	99	91	283	406	606	536	1226	1744	2538	5198	7563	9805
12M	**Carmody**	48	130	177	374	1223	1521	2881	4207	4927	4104	10049	12987
	Pavlov	57	82	178	243	941	1402	2320	3079	4284	4083	8362	12257
21M	**Carmody**	21	90	258	471	755	1055	1209	1625	2207	5022	10146	12702
	Pavlov	23	88	213	468	602	722	803	1202	1735	5164	9543	12739
25M	**Carmody**	53	167	209	435	586	702	1255	2215	2463	4338	9307	12447
	Pavlov	58	147	204	341	436	650	1024	1687	2124	4420	9138	12635

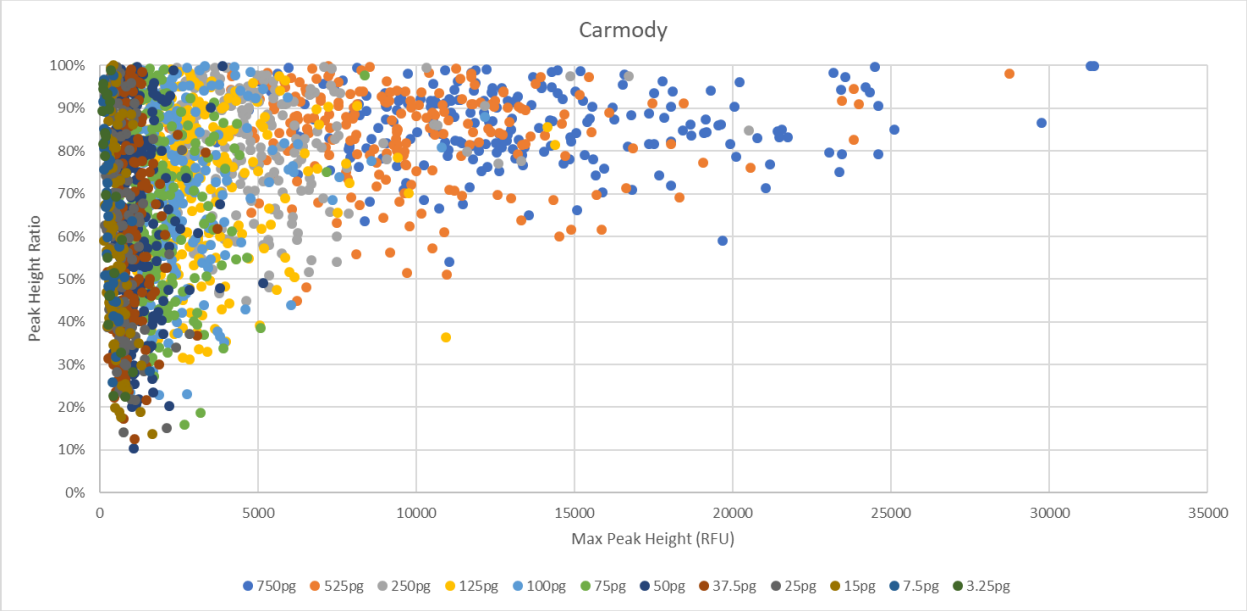

Figure 7: Peak height ratios vs. max peak heights at heterozygous loci for samples amplified with PowerPlex® Fusion with varied template amounts. Samples were amplified on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection.

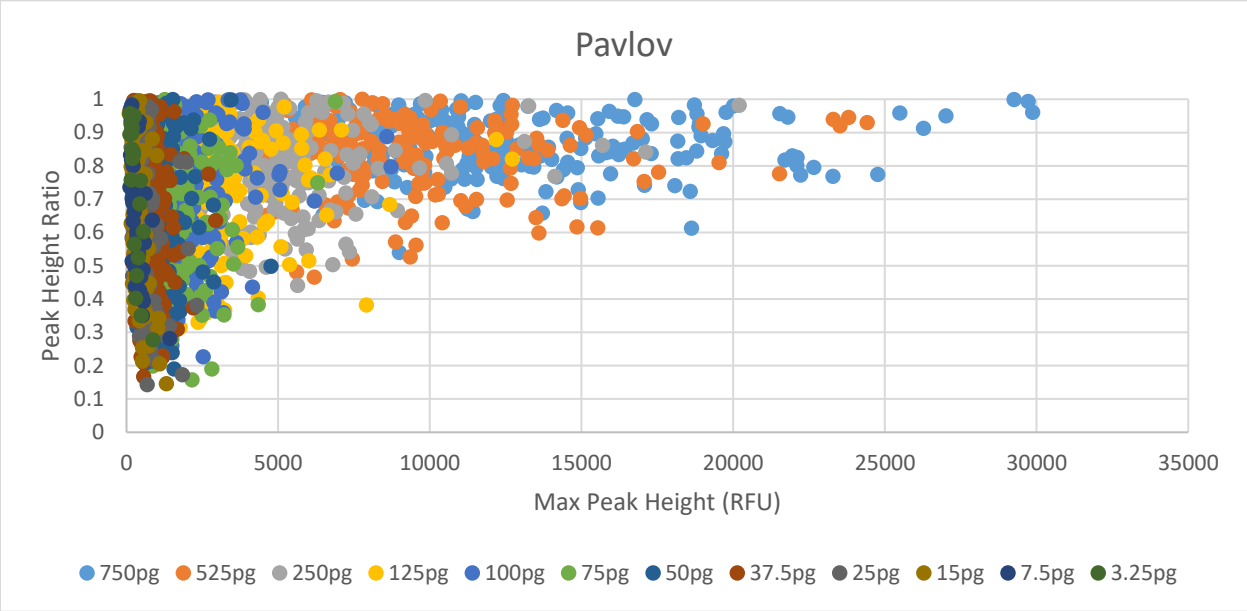

Figure 8: Peak height ratios vs. max peak heights at heterozygous loci for samples amplified with PowerPlex® Fusion with varied template amounts. Samples were amplified on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Pavlov) using a 1.2kV, 24 second injection.

4.D Non-Probative Casework Samples

Twenty-eight non-probative casework samples that incorporated a variety of sample types and substrates were amplified. All of the samples produced STR profiles that were then compared to profiles for the expected contributors (Table 10, Appendix L). All of the samples produced profiles consistent with the expected contributor.

Table 10: Summary of STR profile and concordance results for 28 non-probative casework samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection.

Sample	Sample Description	STR Profile Notes	Concordance Notes
Mock_1	Blood	Single source female.	Concordant.
Mock_1_rep	Blood	Single source female.	Concordant.
Mock_2	Blood	Single source male.	Concordant.
Mock_2_rep	Blood	Single source male.	Concordant.
Mock_3	Can	Single source male.	Concordant.
Mock_3_rep	Can	Single source male.	Concordant.
Mock_4	Straw	Single source female.	Concordant.
Mock_4_rep	Straw	Single source female.	Concordant.
Mock_5	Can	Single source female.	Concordant.
Mock_5_rep	Can	Single source female.	Concordant.
Mock_6	Straw	Single source female.	Concordant.
Mock_6_rep	Straw	Single source female.	Concordant.
Mock_7	Cup	Single source female.	Concordant.
Mock_7_rep	Cup	Single source female.	Concordant.

Mock_8	Mouse	Mixture. Major male contributor.	Major matches provided contributor.
Mock_8_rep	Mouse	Mixture. Major male contributor.	Major matches provided contributor.
Mock_9	Glasses	Mixture. Major male contributor.	Major matches provided contributor.
Mock_9_rep	Glasses	Mixture. Major male contributor.	Major matches provided contributor.
Mock_10	Hair Clip	Mixture. Major female contributor.	Major matches provided contributor.
Mock_10_rep	Hair Clip	Mixture. Major female contributor.	Major matches provided contributor.
Mock_12	Stress Ball	Mixture. Major female contributor.	Major matches provided contributor.
Mock_12_rep	Stress Ball	Mixture. Major female contributor.	Major matches provided contributor.
Mock_13	Spoon Handle	Single source male.	Concordant.
Mock_13_rep	Spoon Handle	Single source male.	Concordant.
Mock_16	Bottle and Cap	Single source male.	Concordant.
Mock_16_rep	Bottle and Cap	Single source male.	Concordant.
Mock_17	Drawer Handle	Mixture. Major female contributor. Dropout present.	Provided contributor present in Alleles >AT.
Mock_17_rep	Drawer Handle	Mixture. Major female contributor. Dropout present.	Provided contributor present in Alleles >AT.
Mock_18	Ear Pods	Single source male. Dropout present.	Alleles > AT are Concordant.
Mock_18_rep	Ear Pods	Single source male. Dropout present.	Alleles > AT are Concordant.
Mock_19	Lipstick Tube	Mixture. Major female contributor.	Major matches provided contributor.
Mock_19_rep	Lipstick Tube	Mixture. Major female contributor.	Major matches provided contributor.
Mock_20	Phone Case	Mixture. Major female contributor.	Major matches provided contributor.
Mock_20_rep	Phone Case	Mixture. Major female contributor.	Major matches provided contributor.
Mock_21	Armrest	Mixture. Major male contributor.	Major matches provided contributor.
Mock_21_rep	Armrest	Mixture. Major male contributor.	Major matches provided contributor.
Mock_22_EC	Semen (Diff)	Single source male.	Concordant.
Mock_22_EC_rep	Semen (Diff)	Single source male.	Concordant.
Mock_22_SF	Semen (Diff)	Single source male.	Concordant.
Mock_22_SF_rep	Semen (Diff)	Single source male.	Concordant.

Mock_23_EC	Saliva (Diff)	Mixture. Major male contributor.	Major matches expected contributor. 2nd contributor consistent with SF.
Mock_23_EC_rep	Saliva (Diff)	Mixture. Major male contributor.	Major matches expected contributor. 2nd contributor consistent with SF.
Mock_23_SF	Semen (Diff)	Single source male.	Concordant.
Mock_23_SF_rep	Semen (Diff)	Single source male.	Concordant.
Mock_24_EC	Blood (Diff)	Mixture.	Provided contributor present. 2nd contributor consistent with SF.
Mock_24_EC_rep	Blood (Diff)	Mixture.	Provided contributor present. 2nd contributor consistent with SF.
Mock_24_SF	Semen (Diff)	Single source male.	Concordant.
Mock_24_SF_rep	Semen (Diff)	Single source male.	Concordant.
Mock_25	Zygem	Single source male.	Concordant.
Mock_25_rep	Semen (Diff)	Single source male.	Concordant.
Mock_26	Semen and Saliva	Mixture. Major male contributor. Dropout of 2nd contributor.	Provided contributors present in Alleles >AT.
Mock_26_rep	Semen and Saliva	Mixture. Major male contributor. Dropout of 2nd contributor.	Provided contributors present in Alleles >AT.
Mock_27	Semen and Blood	Mixture.	Concordant.
Mock_27_rep	Semen and Blood	Mixture.	Concordant.
Mock_32	Hole Punch	Mixture. Major female contributor.	Major matches provided contributor. Minor contributor present.
Mock_32_rep	Hole Punch	Mixture. Major female contributor.	Major matches provided contributor. Minor contributor present.

4.E Precision

The allelic ladder precision calculations had values of <0.16 standard deviation and <0.5bp range when 9 ladders were injected across one plate on both Carmody and Pavlov (Figure 9, 10). The maximum standard deviation observed was 0.07 with a maximum size range of 0.2bp (Appendix M,N).

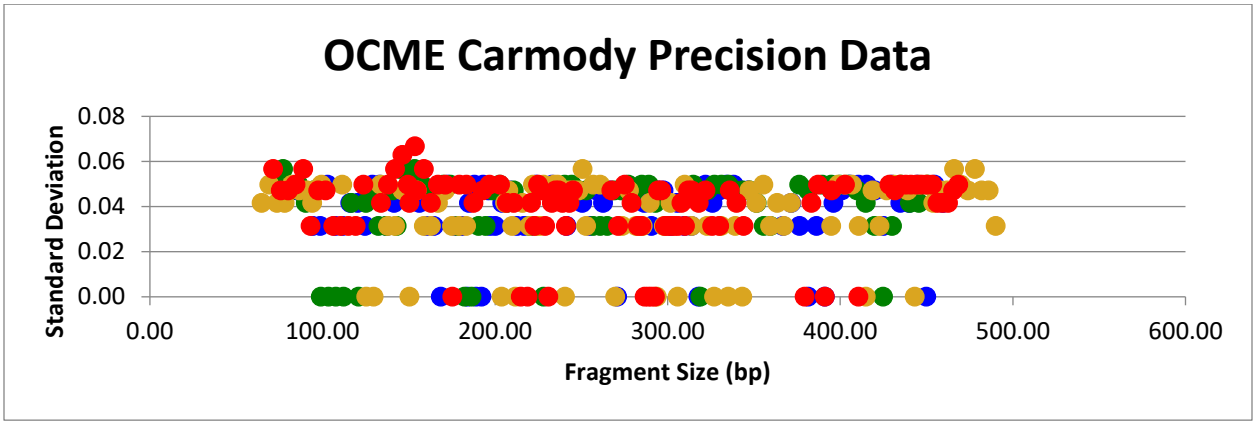

Figure 9: Precision of the PowerPlex® Fusion system for 9 ladders on the Applied Biosystems® 3500xL (Carmody) using a 1.2kv, 24 second injection. For each allele, the average fragment size (in bases) was plotted against the standard deviation of the mean size observed.

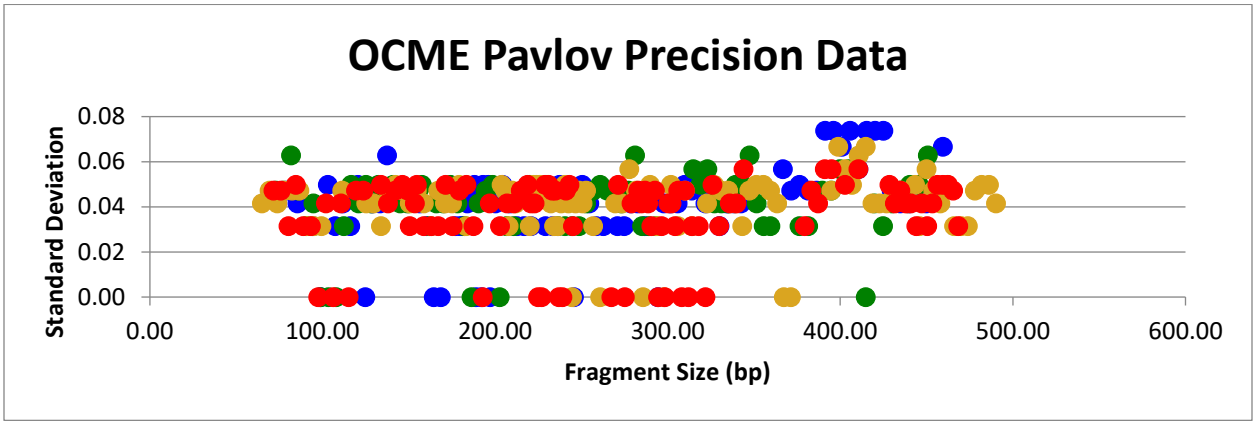

Figure 10: Precision of the PowerPlex® Fusion system for 9 ladders on the Applied Biosystems® 3500xL (Pavlov) using a 1.2kv, 24 second injection. For each allele, the average fragment size (in bases) was plotted against the standard deviation of the mean size observed.

4.F Repeatability and Reproducibility

Positive controls were amplified on each amplification plate and run on both Carmody and Pavlov. Twenty-four samples were run on Carmody and ten samples were run on Pavlov. All positive control genotype results were in concordance with the expected 2800M profile provided in section 9.A of the PowerPlex® Fusion technical manual (TMD039). The genotype results for the 2800M samples are found in Table 11, 12, and 13. All NIST SRM 2391c samples ran on Carmody and Pavlov produced profiles concordant with the expected profiles provided in the NIST certificate of analysis (Table 14 and 15).

Table 11: Summary of 2800M genotype and concordance results for positive control samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection.

		Direct Amplification Plates			Casework	Sensitivity Amplification Plates						
	2800M Amp_pos	Amp Pos_03182019.134050	Amp Pos_03182019.134050	Amp Pos_03202019.141325	Amp_Pos_05172019.124544	Amp Pos_02062019.143004	Amp Pos_01302019.092233	Amp Pos_02042019.115931	Amp Pos_02042019.120104	Amp Pos_01302019.092440	Amp Pos_02042019.135347	Amp Pos_02042019.141008
AMEL	X Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y
D3S1358	17 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18
D1S1656	12 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
D2S441	10 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14
D10S1248	13 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15
D13S317	9 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11
Penta E	7 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14
D16S539	9 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13
D18S51	16 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18
D2S1338	22 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25
CSF1PO	12	12	12	12	12	12	12	12	12	12	12	12
Penta D	12 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
TH01	6 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3
vWA	16 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19
D21S11	29 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2
D7S820	8 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11
D5S818	12	12	12	12	12	12	12	12	12	12	12	12
TPOX	11	11	11	11	11	11	11	11	11	11	11	11
DYS391	10	10	10	10	10	10	10	10	10	10	10	10
D8S1179	14 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15
D12S391	18 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23
D19S433	13 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14
FGA	20 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23
D22S1045	16	16	16	16	16	16	16	16	16	16	16	16

Table 12: Summary of 2800M genotype and concordance results for positive control samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection.

	Mixture Amplification Plates												
	Amp_Pos	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Amp_Pos**	**Positive Control**
AMEL	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y
D3S1358	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18
D1S1656	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
D2S441	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14
D10S1248	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15
D13S317	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11
Penta E	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14
D16S539	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13
D18S51	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18
D2S1338	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25
CSF1PO	12	12	12	12	12	12	12	12	12	12	12	12	12
Penta D	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
TH01	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3
vWA	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19
D21S11	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2
D7S820	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11
D5S818	12	12	12	12	12	12	12	12	12	12	12	12	12
TPOX	11	11	11	11	11	11	11	11	11	11	11	11	11
DYS391	10	10	10	10	10	10	10	10	10	10	10	10	10
D8S1179	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15
D12S391	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23
D19S433	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14
FGA	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23
D22S1045	16	16	16	16	16	16	16	16	16	16	16	16	16

Table 13: Summary of 2800M genotype and concordance results for positive control samples amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Pavlov) using a 1.2kV, 24 second injection.

		Direct Amplification Plates			Sensitivity Amplification Plates						
	2800M Amp_pos	Amp Pos_032020 19.141325	Amp Pos_0318201 9.134050	Amp Pos_03182019.134 050	Amp Pos_02062019. 143004	Amp Pos_01302019.0 92233	Amp Pos_02042019 .115931	Amp Pos_020420 19.120104	Amp Pos_01302019.0 92440	Amp Pos_02042019.1 35347	Amp Pos_02042019.141 008
AMEL	X Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y
D3S1358	17 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18	17, 18
D1S1656	12 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
D2S441	10 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14	10, 14
D10S1248	13 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15	13, 15
D13S317	9 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11	9, 11
Penta E	7 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14	7, 14
D16S539	9 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13	9, 13
D18S51	16 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18	16, 18
D2S1338	22 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25	22, 25
CSF1PO	12	12	12	12	12	12	12	12	12	12	12
Penta D	12 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13	12, 13
TH01	6 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 9.3
vWA	16 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19	16, 19
D21S11	29 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2	29, 31.2
D7S820	8 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11	8, 11
D5S818	12	12	12	12	12	12	12	12	12	12	12
TPOX	11	11	11	11	11	11	11	11	11	11	11
DYS391	10	10	10	10	10	10	10	10	10	10	10
D8S1179	14 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15	14, 15
D12S391	18 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23	18, 23
D19S433	13 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14	13, 14
FGA	20 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23	20, 23
D22S1045	16	16	16	16	16	16	16	16	16	16	16

Table 14: Summary of genotype and concordance results for NIST SRM 2391c components amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection.

	NIST_A			NIST_B			NIST_C			NIST_D		
	Reference	Determined	Determined	Reference	Determined	Determined	Reference	Determined	Determined	Reference	Determined	Determined
AMEL	X	X	X	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y
D3S1358	15, 16	15, 16	15, 16	15, 19	15, 19	15, 19	16, 18	16, 18	16, 18	15, 16, 18	15, 16, 18	15, 16, 18
D1S1656	17.3	17.3	17.3	11, 14	11, 14	11, 14	11, 15	11, 15	11, 15	11, 15, 17.3	11, 15, 17.3	11, 15, 17.3
D2S441	10	10	10	10, 14	10, 14	10, 14	10	10	10	10	10	10
D10S1248	15, 16	15, 16	15, 16	13	13	13	12, 16	12, 16	12, 16	12, 15, 16	12, 15, 16	12, 15, 16
D13S317	8	8	8	9, 12	9, 12	9, 12	11	11	11	8, 11	8, 11	8, 11
Penta E	5, 10	5, 10	5, 10	7, 15	7, 15	7, 15	12, 13	12, 13	12, 13	5, 10, 12, 13	5, 10, 12, 13	5, 10, 12, 13
D16S539	10, 11	10, 11	10, 11	10, 13	10, 13	10, 13	10	10	10	10, 11	10, 11	10, 11
D18S51	12, 15	12, 15	12, 15	13, 16	13, 16	13, 16	16, 19	16, 19	16, 19	12, 15, 16, 19	12, 15, 16, 19	12, 15, 16, 19
D2S1338	18, 23	18, 23	18, 23	17	17	17	19	19	19	18, 19, 23	18, 19, 23	18, 19, 23
CSF1PO	10	10	10	10, 11	10, 11	10, 11	10, 12	10, 12	10, 12	10, 12	10, 12	10, 12
Penta D	9, 13	9, 13	9, 13	8, 12	8, 12	8, 12	10, 11	10, 11	10, 11	9, 10, 11, 13	9, 10, 11, 13	9, 10, 11, 13
TH01	8, 9.3	8, 9.3	8, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 8	6, 8	6, 8	6, 8, 9.3	6, 8, 9.3	6, 8, 9.3
vWA	18, 19	18, 19	18, 19	17, 18	17, 18	17, 18	16, 18	16, 18	16, 18	16, 18, 19	16, 18, 19	16, 18, 19
D21S11	28, 32.2	28, 32.2	28, 32.2	32, 32.2	32, 32.2	32, 32.2	29, 30	29, 30	29, 30	28, 29, 30, 32.2	28, 29, 30, 32.2	28, 29, 30, 32.2
D7S820	11	11	11	10	10	10	10, 12	10, 12	10, 12	10, 11, 12	10, 11, 12	10, 11, 12
D5S818	11, 12	11, 12	11, 12	12, 13	12, 13	12, 13	10, 11	10, 11	10, 11	10, 11, 12	10, 11, 12	10, 11, 12
TPOX	8	8	8	8, 11	8, 11	8, 11	11	11	11	8, 11	8, 11	8, 11
DYS391	-	-	-	10	10	10	11	11	11	11	11	11
D8S1179	13, 14	13, 14	13, 14	10, 13	10, 13	10, 13	10, 17	10, 17	10, 17	10, 13, 14, 17	10, 13, 14, 17	10, 13, 14, 17
D12S391	18.3, 22	18.3, 22	18.3, 22	19, 24	19, 24	19, 24	19, 23	19, 23	19, 23	18.3, 19, 22, 23	18.3, 19, 22, 23	18.3, 19, 22, 23
D19S433	13, 14	13, 14	13, 14	16, 16.2	16, 16.2	16, 16.2	13.2, 15.2	13.2, 15.2	13.2, 15.2	13, 13.2, 14, 15.2	13, 13.2, 14, 15.2	13, 13.2, 14, 15.2
FGA	21, 23	21, 23	21, 23	20, 23	20, 23	20, 23	24, 26	24, 26	24, 26	21, 23, 24, 26	21, 23, 24, 26	21, 23, 24, 26
D22S1045	15	15	15	15, 17	15, 17	15, 17	16	16	16	15, 16	15, 16	15, 16

Table 15: Summary of genotype and concordance results for NIST SRM 2391c components amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Pavlov) using a 1.2kV, 24 second injection.

	NIST_A			NIST_B			NIST_C			NIST_D		
	Reference	Determined	Determined	Reference	Determined	Determined	Reference	Determined	Determined	Reference	Determined	Determined
AMEL	X	X	X	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y	X, Y
D3S1358	15, 16	15, 16	15, 16	15, 19	15, 19	15, 19	16, 18	16, 18	16, 18	15, 16, 18	15, 16, 18	15, 16, 18
D1S1656	17.3	17.3	17.3	11, 14	11, 14	11, 14	11, 15	11, 15	11, 15	11, 15, 17.3	11, 15, 17.3	11, 15, 17.3
D2S441	10	10	10	10, 14	10, 14	10, 14	10	10	10	10	10	10
D10S1248	15, 16	15, 16	15, 16	13	13	13	12, 16	12, 16	12, 16	12, 15, 16	12, 15, 16	12, 15, 16
D13S317	8	8	8	9, 12	9, 12	9, 12	11	11	11	8, 11	8, 11	8, 11
Penta E	5, 10	5, 10	5, 10	7, 15	7, 15	7, 15	12, 13	12, 13	12, 13	5, 10, 12, 13	5, 10, 12, 13	5, 10, 12, 13
D16S539	10, 11	10, 11	10, 11	10, 13	10, 13	10, 13	10	10	10	10, 11	10, 11	10, 11
D18S51	12, 15	12, 15	12, 15	13, 16	13, 16	13, 16	16, 19	16, 19	16, 19	12, 15, 16, 19	12, 15, 16, 19	12, 15, 16, 19
D2S1338	18, 23	18, 23	18, 23	17	17	17	19	19	19	18, 19, 23	18, 19, 23	18, 19, 23
CSF1PO	10	10	10	10, 11	10, 11	10, 11	10, 12	10, 12	10, 12	10, 12	10, 12	10, 12
Penta D	9, 13	9, 13	9, 13	8, 12	8, 12	8, 12	10, 11	10, 11	10, 11	9, 10, 11, 13	9, 10, 11, 13	9, 10, 11, 13
TH01	8, 9.3	8, 9.3	8, 9.3	6, 9.3	6, 9.3	6, 9.3	6, 8	6, 8	6, 8	6, 8, 9.3	6, 8, 9.3	6, 8, 9.3
vWA	18, 19	18, 19	18, 19	17, 18	17, 18	17, 18	16, 18	16, 18	16, 18	16, 18, 19	16, 18, 19	16, 18, 19
D21S11	28, 32.2	28, 32.2	28, 32.2	32, 32.2	32, 32.2	32, 32.2	29, 30	29, 30	29, 30	28, 29, 30, 32.2	28, 29, 30, 32.2	28, 29, 30, 32.2
D7S820	11	11	11	10	10	10	10, 12	10, 12	10, 12	10, 11, 12	10, 11, 12	10, 11, 12
D5S818	11, 12	11, 12	11, 12	12, 13	12, 13	12, 13	10, 11	10, 11	10, 11	10, 11, 12	10, 11, 12	10, 11, 12
TPOX	8	8	8	8, 11	8, 11	8, 11	11	11	11	8, 11	8, 11	8, 11
DYS391	-	-	-	10	10	10	11	11	11	11	11	11
D8S1179	13, 14	13, 14	13, 14	10, 13	10, 13	10, 13	10, 17	10, 17	10, 17	10, 13, 14, 17	10, 13, 14, 17	10, 13, 14, 17
D12S391	18.3, 22	18.3, 22	18.3, 22	19, 24	19, 24	19, 24	19, 23	19, 23	19, 23	18.3, 19, 22, 23	18.3, 19, 22, 23	18.3, 19, 22, 23
D19S433	13, 14	13, 14	13, 14	16, 16.2	16, 16.2	16, 16.2	13.2, 15.2	13.2, 15.2	13.2, 15.2	13, 13.2, 14, 15.2	13, 13.2, 14, 15.2	13, 13.2, 14, 15.2
FGA	21, 23	21, 23	21, 23	20, 23	20, 23	20, 23	24, 26	24, 26	24, 26	21, 23, 24, 26	21, 23, 24, 26	21, 23, 24, 26
D22S1045	15	15	15	15, 17	15, 17	15, 17	16	16	16	15, 16	15, 16	15, 16

4.G Mixture Study

4.G.1 Two-Person Mixtures

For each mixture set, the two profiles were compared, and a unique minor profile was generated containing only unshared alleles in the minor contributor. The mixtures were then compared to this unique minor profile to determine what percentage of the unique minor profile was detected in each mixture ratio. The 1:1 mixtures had the unique minor profile calculated using both the male and female as the minor. Both contributors had full profiles in 1:1 mixtures and 1:2 mixtures when 750pg, 525pg, and 150pg were amplified (Table 16A,B, Appendix O). In general, as the ratios increased and templates decreased, less minor profile was detected.

Table 16A: Percent Profile for the unique minor male profile for two-person mixture sets amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed.

	Percent Unshared Minor Alleles-Male											
	15pg		37.5pg		75pg		150pg		525pg		750pg	
	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2
1:1	42%	10%	88%	17%	100%	90%	100%	100%	100%	100%	100%	100%
1:1	42%	28%	75%	14%	96%	93%	100%	100%	100%	100%	100%	100%
1:2	25%	28%	63%	76%	88%	90%	100%	100%	100%	100%	100%	100%
1:2	46%	21%	63%	69%	92%	90%	100%	100%	100%	100%	100%	100%
1:4	25%	24%	54%	55%	79%	62%	88%	97%	100%	100%	100%	100%
1:4	33%	24%	63%	59%	63%	76%	100%	97%	100%	100%	100%	100%
1:10	13%	10%	17%	17%	29%	34%	79%	66%	88%	83%	92%	90%
1:10	8%	17%	4%	14%	46%	45%	67%	62%	92%	97%	92%	83%
1:15	0%	0%	29%	10%	46%	41%	50%	59%	75%	79%	79%	79%
1:15	13%	0%	21%	17%	29%	41%	42%	45%	67%	86%	79%	79%
1:20	8%	10%	4%	21%	29%	28%	38%	41%	79%	79%	75%	83%
1:20	21%	59%	4%	10%	21%	17%	46%	62%	63%	69%	67%	55%
1:50	0%	0%	8%	10%	21%	10%	21%	28%	46%	52%	42%	72%
1:50	4%	0%	0%	3%	4%	14%	21%	21%	33%	45%	38%	62%
1:75	0%	0%	0%	0%	4%	7%	8%	7%	21%	41%	38%	34%
1:75	8%	0%	4%	0%	4%	10%	13%	14%	29%	48%	21%	48%
1:100	4%	0%	0%	0%	4%	7%	17%	0%	29%	24%	25%	24%
1:100	4%	0%	0%	0%	0%	0%	17%	7%	17%	21%	29%	31%

Table 16B: Percent Profile for the unique minor female profile for two-person mixture sets amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed.

	Percent Unshared Minor Alleles-Female											
	15pg		37.5pg		75pg		150pg		525pg		750pg	
	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2
1:1	50%	35%	90%	23%	100%	96%	100%	100%	100%	100%	100%	100%
1:1	47%	38%	97%	35%	100%	92%	100%	100%	100%	100%	100%	100%
2:1	37%	42%	80%	62%	93%	96%	100%	100%	100%	100%	100%	100%
2:1	50%	42%	73%	81%	93%	96%	100%	100%	100%	100%	100%	100%
4:1	37%	19%	63%	38%	90%	73%	100%	100%	100%	100%	100%	100%
4:1	30%	0%	57%	50%	73%	58%	100%	85%	100%	100%	100%	100%
10:1	7%	4%	33%	15%	40%	38%	57%	85%	97%	96%	97%	92%
10:1	3%	15%	13%	35%	3%	62%	53%	62%	93%	96%	93%	96%
15:1	17%	0%	30%	15%	40%	31%	53%	69%	93%	81%	90%	88%
15:1	0%	8%	13%	8%	33%	27%	57%	46%	87%	88%	93%	88%
20:1	0%	8%	10%	4%	37%	12%	50%	62%	87%	92%	90%	88%
20:1	3%	0%	0%	23%	30%	15%	60%	38%	97%	88%	90%	81%
50:1	3%	0%	13%	8%	17%	19%	33%	12%	63%	46%	73%	58%
50:1	0%	0%	20%	0%	20%	12%	37%	15%	63%	42%	77%	73%
75:1	7%	0%	7%	4%	3%	12%	20%	23%	43%	31%	70%	62%
75:1	3%	4%	10%	4%	23%	12%	17%	12%	40%	42%	80%	50%
100:1	7%	0%	3%	0%	10%	4%	0%	19%	50%	38%	63%	50%
100:1	3%	0%	10%	4%	7%	4%	23%	15%	50%	35%	57%	19%

4.G.2 Three-Person Mixtures

Three-person mixtures were evaluated for percent profile. One hundred percent of the alleles were detected in at least one replicate for both mixture series in the 750pg and 525pg samples with ratios 3:2:1, 1:2:1, and 1:1:1. 100% of alleles were detected in at least one replicate for mixture series 2 in the 750pg and 525pg samples with ratios 10:5:1, 10:2:1, 5:5:1, 5:2:1, and 5:1:1. (Table 17 and Appendix P). The 1:1:1 ratio amplified with 150pg also yielded full profiles for mixture series 2. As the template amount decreased, there was also a decrease in the percent profile.

Table 17: Percent profile obtained for the three-person mixture sets amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed.

	Percent Profile Obtained Three-Person Mixtures											
	15pg		**37.5pg**		**75pg**		**150pg**		**525pg**		**750pg**	
	Mix 1	**Mix 2**	**Mix 1**	**Mix 2**	**Mix 1**	**Mix 2**	**Mix 1**	**Mix 2**	**Mix 1**	**Mix 2**	**Mix 1**	**Mix 2**
20:5:1	18%	37%	55%	51%	56%	76%	78%	89%	88%	97%	90%	96%
20:5:1	23%	19%	47%	69%	65%	77%	84%	91%	87%	96%	89%	96%
10:5:1	20%	25%	67%	64%	73%	81%	82%	93%	93%	99%	97%	100%
10:5:1	16%	32%	48%	71%	64%	81%	88%	87%	96%	100%	98%	99%
10:2:1	24%	39%	37%	29%	67%	75%	81%	97%	95%	100%	96%	99%
10:2:1	44%	39%	40%	19%	69%	75%	80%	92%	95%	97%	95%	100%
5:5:1	27%	49%	67%	73%	71%	75%	93%	95%	98%	100%	99%	100%
5:5:1	44%	49%	64%	75%	73%	73%	95%	93%	98%	100%	98%	100%
5:2:1	30%	31%	71%	79%	86%	95%	96%	93%	97%	100%	97%	100%
5:2:1	23%	49%	68%	87%	88%	95%	90%	99%	99%	100%	99%	100%
5:1:1	12%	13%	42%	69%	75%	85%	88%	89%	99%	100%	99%	100%
5:1:1	16%	15%	47%	60%	86%	93%	84%	91%	99%	100%	100%	100%
3:2:1	58%	48%	70%	80%	82%	69%	99%	97%	98%	91%	100%	100%
3:2:1	35%	56%	75%	81%	86%	95%	98%	96%	100%	100%	100%	100%
1:2:1	52%	52%	71%	73%	84%	85%	99%	99%	100%	100%	100%	100%
1:2:1	40%	45%	79%	77%	92%	91%	97%	97%	100%	100%	100%	100%
1:1:1	42%	35%	69%	64%	97%	95%	99%	100%	100%	100%	100%	100%
1:1:1	38%	51%	70%	68%	92%	95%	98%	100%	100%	100%	100%	100%

4.G.3 Four-Person Mixtures

Four-person mixtures were evaluated for percent profile. One hundred percent of the alleles were detected in at least one replicate for both mixture series in the 750pg samples with ratios 1:3:5:1, 1:3:3:1, and 1:1:1:1. In addition, 100% alleles were detected in at least one replicate for mixture series 2 in the 750pg samples with ratio 10:5:3:1. (Table 18 and Appendix Q). All alleles were detected in 525pg samples in at least one replicate for both mixtures with ratios of 1:3:3:1, and 1:1:1:1. Mixture series 1 has one replicate where all alleles were detected at the 1:3:5:1 ratio. One replicate of mixture series 1 at 150pg had all alleles detected for the 1:1:1:1 ratio. As the template amount decreased, there was also a decrease in the percent profile.

Table 18: Percent profile obtained for the four-person mixture sets amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed.

	Percent Profile Obtained Four-Person Mixtures											
	15pg		37.5pg		75pg		150pg		525pg		750pg	
	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2
20:10:5:1	31%	35%	72%	65%	70%	77%	86%	89%	92%	97%	97%	99%
20:10:5:1	39%	40%	69%	60%	78%	77%	83%	85%	94%	96%	96%	97%
20:5:2:1	24%	6%	54%	61%	75%	68%	87%	67%	94%	94%	97%	98%
20:5:2:1	35%	7%	57%	55%	78%	70%	85%	85%	95%	95%	95%	96%
10:5:3:1	38%	30%	62%	60%	86%	76%	89%	92%	98%	99%	99%	100%
10:5:3:1	35%	32%	66%	61%	90%	76%	87%	91%	98%	99%	98%	98%
10:5:1:1	27%	28%	54%	58%	61%	78%	88%	88%	96%	95%	97%	98%
10:5:1:1	27%	31%	46%	45%	61%	73%	91%	86%	95%	97%	98%	98%
10:1:1:1	20%	19%	42%	47%	74%	58%	80%	81%	96%	94%	98%	98%
10:1:1:1	28%	23%	38%	55%	79%	68%	69%	84%	96%	94%	99%	97%
1:3:5:1	31%	30%	60%	59%	82%	78%	60%	95%	98%	99%	100%	100%
1:3:5:1	29%	21%	71%	66%	76%	80%	94%	86%	100%	99%	100%	99%
1:3:3:1	42%	28%	52%	56%	88%	84%	94%	91%	100%	100%	99%	100%
1:3:3:1	41%	41%	68%	58%	83%	83%	95%	96%	100%	94%	100%	100%
1:1:1:1	46%	33%	72%	66%	91%	91%	100%	98%	100%	100%	100%	100%
1:1:1:1	39%	42%	62%	65%	81%	79%	98%	99%	100%	100%	100%	100%

4.G.4 Five-Person Mixtures

Five person mixtures were evaluated for percent profile. One hundred percent of the alleles were detected in at least one replicate for both mixture series in the 750pg and 525pg samples with the 1:1:1:1:1 ratio. 98% of alleles or greater were detected in at least one replicate for both mixture series in the 750pg and 525pg samples with the remaining ratios. (Table 19 and Appendix R). One replicate of 1:1:1:1:1 had a full profile when amplified with 150pg of DNA in mixture series 1. As the template amount decreased there was also a decrease in the percent profile.

Table 19: Percent profile obtained for the five-person mixture sets amplified with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on the Applied Biosystems® 3500xL (Carmody) using a 1.2kV, 24 second injection. The three-color heat map was used to represent the lowest percent profile (red), the average percent profile (yellow) and the highest percent profile (dark green). The heat map allows for a quick visualization of the range of the percent profile observed.

	Percent Profile Obtained Five-Person Mixtures											
	15pg		37.5pg		75pg		150pg		525pg		750pg	
	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2
10:5:1:1:1	34%	30%	54%	46%	67%	74%	86%	84%	95%	98%	97%	98%
10:5:1:1:1	34%	34%	57%	61%	63%	61%	89%	86%	98%	98%	99%	98%
5:5:1:1:1	27%	25%	64%	58%	72%	78%	84%	89%	98%	98%	100%	100%
5:5:1:1:1	13%	40%	56%	61%	80%	80%	93%	94%	100%	99%	99%	98%
5:1:1:1:1	39%	31%	52%	51%	81%	72%	92%	93%	100%	100%	99%	100%
5:1:1:1:1	28%	34%	60%	62%	83%	75%	88%	92%	99%	100%	99%	100%
1:1:1:1:1	28%	19%	57%	58%	83%	92%	97%	96%	100%	100%	100%	100%
1:1:1:1:1	43%	47%	63%	2%*	80%	85%	100%	98%	100%	100%	100%	100%

*Sample is outlier, possible misload.

4.H Contamination Assessment

A total of 41 negative controls were run on Carmody and 15 on Pavlov. Five samples had peaks called above the analytical threshold (Table 20, Appendix S). Multiple samples on the Carmody casework plate have a 30 allele present at D21S11. No samples on the sensitivity amplification plate have a 4 allele at TH01. Negative controls on the direct amplification plate could not directly attributed to a sample on the amplification plate because all samples have an X allele present and none have a known 17.3 allele at D3S1358.

Table 20: Peaks called above AT in negative controls

Instrument	Sample	Dye (Allele Call)	Size	Height
Carmody Casework Re-Run	Amp Neg 05172019.124544	TMR (30 at D21S11)	228.1bp	196 RFU
Pavlov Sensitivity Plate	Amp_Neg 01302019.902440	TMR (4 at TH01)	68.7bp	144 RFU
Pavlov Direct Amplification Plate	E Neg1_03182019.110046	FL (X at AMEL)	79bp	91 RFU
Pavlov Direct Amplification Plate	Amp_Neg 03182019.134050	FL (X at AMEL)	78.3bp	113 RFU
Pavlov Direct Amplification Plate	E Neg 1 03182019.110046	FL (OL, OB (17.3) in Amel and D3S1358)	77.9bp, 131.8bp	122 RFU, 91 RFU

4.I Direct Amplification of Known Samples

The average peak heights for the direct amplification of buccal swabs ranged from 453 RFU to 16283 RFU on Carmody (Appendix T). The same samples ranged from 378 RFU to 13723 RFU on Pavlov (Appendix T). The peak height ratios were generally above 60% although some samples did have loci that dropped below (Appendix U). The first pass success rate for buccal samples was 85% on Carmody and 90% on Pavlov (Table 21). The genotypes and notes for the buccal samples can be found in Appendix V.

A profile is considered passing if all peaks in the ILS are correctly labeled and interpretable allele calls are assigned to the profile. A single peak at any locus is required to meet the stochastic threshold for the sample to pass.

Table 21: Success rate of 20 buccal samples direct amplified in duplicate with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on two Applied Biosystems® 3500xL instruments (Carmody and Pavlov) using a 1.2kV, 24 second injection.

	Buccal Samples	
Buccal Samples	**Carmody**	**Pavlov**
Total Sample Injections	40	40
Failed Samples	6	4
Pass Rate Total	**85%**	**90%**

The average peak heights for the direct amplification of blood samples ranged from 6073 RFU to 21587 RFU on Carmody (Appendix W). The same samples ranged from 4514 RFU to 17580 RFU on Pavlov (Appendix W). The peak height ratios were generally above 60% although some samples did have loci that dropped below (Appendix X). The first pass success rate for blood samples was 65% on Carmody and 82% on Pavlov (Table 22). The lower success rate for blood samples on Carmody can be attributed to saturation causing the size standard to fail. The genotypes for blood samples can be found in Appendix Y. We are no longer processing blood samples through direct amplification because less than 50% of the samples that we ran gave complete profiles.

Table 22: Success rate of 20 blood samples direct amplified in duplicate with PowerPlex® Fusion on an Applied Biosystems® 9700 for 29 cycles. Samples were run on two Applied Biosystems® 3500xL instruments (Carmody and Pavlov) using a 1.2kV, 24 second injection.

	Blood Samples	
Blood Samples	**Carmody**	**Pavlov**
Total Sample Injections	40	40
Failed Injections	0	1
New Total Samples	40	39
Failed Samples	14	7
Pass Rate Total	**65%**	**82%**

5. Conclusion

The PowerPlex® Fusion system was evaluated using a half volume reaction and 29 cycles on the Applied Biosystems® GeneAmp® PCR 9700 thermal cycler. The amplified product was run using a 1.2kv, 24 second injection on two of the Applied Biosystems® 3500xL genetic analyzers. An analytical threshold for each dye channel was chosen for the validation with 29 cycles. A stochastic threshold for each dye channel was determined for 29 cycles. The results showed that the system was capable of producing reliable and reproducible results. The precision of the system is within the recommended range. The studies performed in this validation meet the criteria for an internal validation and have shown that the PowerPlex® Fusion system is suitable for use in a forensic casework laboratory on the Applied Biosystems® 3500xL genetic analyzer.

6. References

Quality Assurance Standards for Forensic DNA Testing Laboratories (effective September 1, 2011)

Scientific Working Group on DNA Analysis Methods Validation Guidelines for DNA Analysis Methods – Approved December 2016 (SWGDAM.org)

PowerPlex® Fusion System Technical Manual TMD039, Revision Date 4/17 from Promega Corporation

SwabSolution™ Kit Technical Manual TMD037, Revision Date 9/16 from Promega Corporation

PunchSolution™ Kit Technical Manual TMD038, Revision Date 9/16 from Promega Corporation

PowerPlex® 5C Matrix Standard Technical Manual TMD049, Revision Date 10/15 from Promega Corporation

approved 2/17/2020