SDPD Forensic Science Section – Forensic Biology Unit **December 17, 2010**

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Validation Summary

Introduction

The AmpFℓSTR® Identifiler® Plus (ID Plus) PCR Amplification Kit from Applied Biosystems is a multiplex DNA typing kit that amplifies the 13 core CODIS STR loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D16S539, vWA, TPOX, D18S51, D5S818, and FGA), two additional STR loci (D2S1338 and D19S433), and a sex determining locus (Amelogenin). These are the same loci that the AmpFℓSTR® Identifiler® kit amplifies and the primer sequences are identical. The ID Plus kit has been optimized in an effort to overcome inhibition, increase sensitivity, increase efficiency, and reduce artifacts. This is accomplished via optimization of the master mix formulation, PCR cycling conditions, reduction in amplification time, cleaner baselines, and improved balance within a locus and between loci.

The following validation experiments were designed to assess the performance of ID Plus: Baseline, Sensitivity, Stutter, Concordance, Peak Height, Stochastic, Mixture, Challenged Samples, Increased Injection Time, Contamination Assessment, Reproducibility, and Precision.

Baseline Study

The baseline study established the detection threshold. Blanks and samples containing DNA were amplified for both 28 and 29 cycles and capillary electrophoresis was performed on all 3130 instruments. The baseline noise was evaluated for each dye channel and for the entire profile. For all sample types, amplification cycles, 3130 instruments, and dye channels the baseline mean peak height + 10 standard deviations was less than 30 RFU. Therefore, 30 RFU was determined to be an appropriate value to use as the detection threshold.

Spectral pull-up was observed when using a 30 RFU detection threshold. If spectral inadequacies prove continually problematic it may be necessary to slightly increase the detection threshold.

Sensitivity Study

The sensitivity study determined the limit of detection for complete profiles and optimal DNA input range. Full DNA profiles were obtained down to ~300 pg. Partial DNA profiles were observed down to the minimal input tested (~50 pg). The optimal DNA input range is between 1.5 ng and 2.0 ng as profiles in this range demonstrated the most desirable peak heights and peak height ratios.

Stutter Study

The levels of n-4 stutter were evaluated for known samples amplified within the optimal DNA input range. The mean stutter + 3 standard deviations was compared to the GMID-X marker specific stutter filters. These values were comparable demonstrating that the GMID-X filters are sufficient for filtering out the vast majority of stutter without being too high.

Stutter peaks may occasionally exceed the GMID-X filters. If this occurs and there is sufficient indication that the peak truly is stutter the allele call may be manually edited.

Concordance Study

Known samples previously typed with the Identifiler kit were re-analyzed with ID Plus. Twenty known profiles were 100% concordant between the kits for a total of 584 concordant alleles.

Since the Identifiler and ID Plus kits utilize the same STR loci with the same primer sequences it is expected that results obtained with both kits are fully concordant.

Peak Height Study

Samples amplified within the optimal DNA input range with ID Plus demonstrated good inter and intra-locus balance. Robust samples can be expected to routinely generate peak height ratios of greater than 80% with imbalance occasionally reaching as low as 60%. As expected, samples amplified with DNA input amounts below this range will begin to show imbalance. As input amounts approach the stochastic range peak height ratios of less than 30% may be observed.

Peak height balance between loci has been greatly improved with ID Plus compared to Identifiler.

Stochastic Study

Known samples were amplified with ID Plus using a low level of input DNA in an effort to generate DNA profiles that straddle the 30 RFU detection threshold. Peak heights as tall as 177 RFU were observed at known heterozygous loci where one peak was detected and the sister allele dropped below the 30 RFU detection threshold. When a single peak reaches above 200 RFU it can reliably be assumed that this represents a true homozygote. Below 200 RFU drop-out is a possibility. It is also possible that peaks in this range will have a sister allele that cannot even be distinguished from the baseline noise, therefore, so-called "blips" are not always expected to be visually detectable. Extreme peak height imbalance (observed as low as 21.3%) is also possible for samples in this range.

Mixture Study

Robust and low level two, three, and four person mixtures were prepared in various ratios. The goal was to re-create the mixture study previously performed with the Identifiler kit (see

"Summary of the SDPD Mixture Study") with the addition of low level mixtures. The 200 RFU stochastic threshold was verified to be appropriate for use with mixtures. The current guidelines for Identifiler with regards to determining whether or not a mixture is balanced and for designating major/minor contributor alleles were shown to be appropriate for robust mixture data obtained with ID Plus. When the tallest peak at a locus is less than 350 RFU these guidelines no longer apply as extreme imbalance (less than 33%) can be observed at these levels.

The data also shows that an individual's percent contribution to a mixture can fluctuate to a small degree across loci but in general remains fairly consistent throughout the entire profile. Factors such as stutter, stacking, and peak imbalance can complicate the determination of percent contribution to a mixture.

Challenged Samples Study

Challenging adjudicated casework and mock casework samples previously analyzed with the Identifiler and Minifiler kits were re-analyzed with ID Plus. The ID Plus results were typically equal to or superior than the Identifiler kit with regards to quantity of genetic information obtained and quality of the DNA profile. At least a partial profile was obtained from all samples analyzed with ID Plus.

Increased Injection Time Study

Samples in the stochastic range were re-injected with 7 and 10 second injection times in order to increase the amount of signal detected. The average increases in peak heights were ~1.35 and 1.83 times compared with that of the 5 second injection, respectively. These increases did not appear to be locus dependent.

Increasing injection time showed that it is necessary to increase the stochastic threshold proportionally. Stochastic thresholds of 280 and 400 RFU are recommend for 7 and 10 second injection times respectively. The data from this study shows that the detection threshold of 30 RFU does not need to be adjusted for increased injection times. In all instances peak heights increased and the number of alleles detected either increased or remained the same (but never decreased) when injection time was increased.

Contamination Assessment Study

No extraneous DNA was detected in any of the amplification blanks over the course of this validation. One sample from the Stochastic Study had a single extra DNA peak detected. No other known samples had extraneous DNA detected. The occurrence of this single peak is sample specific and not systemic.

Contamination is not an inherent problem associated with the ID Plus kit. QC of new kits and amplification controls will serve as ongoing verification that contamination is not an issue with ID Plus.

Reproducibility Study

The 9947A positive control and many known samples were amplified multiple times over the course of this validation. The results for these samples were always consistent between amplifications. Therefore, DNA typing results obtained using ID Plus have proven to be reproducible.

Precision Study

Precision was assessed by examining the variability of the 250 base pair peak of the GSS500 internal size standard. The standard deviation of this peak for 62 samples was 0.0427 base pairs. Although precision is more closely associated with the capillary electrophoresis instrument than the DNA typing kit this data confirms that ID Plus demonstrates acceptable levels of precision.

Conclusions

The AmpFℓSTR® Identifiler® Plus (ID Plus) PCR Amplification Kit from Applied Biosystems has undergone extensive internal validation studies in accordance with the SWGDAM guidelines and DAB requirements. The kit has been validated for 28 amplification cycles using manual set up as well as automated set up on the Qiagen Universal. The kit is therefore approved for use in DNA casework in the SDPD Forensic Biology Unit.

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SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Baseline Study

Purpose

The goal of this study is to empirically determine the peak amplitude threshold that allows for reliable interpretation of AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) data.

Materials and Methods

Sensitivity Study Samples

Two employee reference samples and the 9947A positive control were amplified at variable input volumes along with corresponding blanks for both 28 and 29 cycles using ID Plus. Capillary electrophoresis was performed on "3130B" and the resultant data was analyzed in GMID-X with a detection threshold of 1 RFU. Alleles corresponding to the genotypes of known contributors and the associated stutter peaks were ignored and the baseline was evaluated.

Instrument Comparison

Three TE amplification blanks were amplified for both 28 and 29 cycles using ID Plus. Capillary electrophoresis was performed on "3130A", "3130B", and "3130C" and the resultant data was analyzed in GMID-X with a detection threshold of 1 RFU. The baseline was evaluated.

Results and Discussion

Sensitivity Study Samples

Summary statistics were generated for the baseline of only the blanks as well as all samples at both 28 and 29 cycles (Table 1). The data indicates that blanks have similar baseline levels as samples containing DNA.

Table 1: Mean peak heights (RFU) and standard deviation separated by dye channel (red, green, yellow, red) and combined (white).

Description	28 CYCLES				29 CYCLES			
	Mean (M)	Std. Dev. (SD)	M+3SD	M+10SD	Mean (M)	Std. Dev. (SD)	M+3SD	M+10SD
Sensitivity Study Blanks (3130B)	3.87	1.97	9.78	**23.56**	4.21	2.15	10.66	**25.71**
	2.43	1.19	6.01	**14.36**	2.98	2.17	9.48	**24.65**
	2.53	1.02	5.59	**12.75**	3.15	1.34	7.17	**16.52**
	5.01	1.51	9.53	**20.07**	5.15	1.88	10.79	**23.96**
	5.35	1.85	10.89	**23.83**	5.42	1.81	10.84	**23.49**
Sensitivity Study All Samples (3130B)	4.10	2.01	10.13	**24.19**	4.43	2.17	10.93	**26.09**
	2.85	1.65	7.80	**19.36**	3.29	2.08	9.53	**24.10**
	3.11	1.46	7.49	**17.71**	3.60	1.63	8.50	**19.93**
	5.07	1.76	10.36	**22.70**	5.34	2.13	11.72	**26.61**
	5.20	1.86	10.78	**23.78**	5.37	1.87	11.00	**24.12**

Instrument Comparison

Summary statistics were generated for the baseline at both 28 and 29 cycles for each instrument (Table 2). There is variability in the baseline between capillary electrophoresis instruments. The baseline for "3130A" is lowest, followed by "3130B", and "3130C" has the highest baseline. This trend corresponds to the sensitivity of the instruments as demonstrated in previous studies.

Table 2: Mean peak heights (RFU) and standard deviation separated by dye channel (red, green, yellow, red) and combined (white).

Description	28 CYCLES				29 CYCLES			
	Mean (M)	Std. Dev. (SD)	M+3SD	M+10SD	Mean (M)	Std. Dev. (SD)	M+3SD	M+10SD
Blanks (3130A)	3.15	1.58	7.88	**18.91**	3.32	1.70	8.42	**20.33**
	1.93	0.80	4.32	**9.92**	2.04	1.19	5.60	**13.92**
	2.26	1.11	5.60	**13.40**	2.38	1.15	5.83	**13.88**
	3.69	1.21	7.31	**15.76**	4.02	1.33	8.01	**17.31**
	4.47	1.47	8.89	**19.20**	4.59	1.55	9.25	**20.13**
Blanks (3130B)	3.89	1.93	9.68	**23.17**	3.69	1.94	9.52	**23.12**
	2.60	1.66	7.59	**19.22**	2.59	2.10	8.88	**23.54**
	2.74	1.10	6.05	**13.78**	2.69	1.10	5.99	**13.68**
	4.99	1.57	9.72	**20.74**	4.57	1.63	9.45	**20.84**
	5.09	1.67	10.10	**21.77**	4.80	1.65	9.76	**21.31**
Blanks (3130C)	4.78	2.16	11.25	**26.35**	4.59	2.07	10.79	**25.25**
	3.01	1.23	6.69	**15.27**	3.26	1.80	8.66	**21.24**
	3.58	1.36	7.66	**17.16**	3.64	1.33	7.63	**16.94**
	6.03	1.76	11.32	**23.67**	5.55	1.76	10.84	**23.18**
	6.33	1.89	12.00	**25.25**	5.85	1.94	11.68	**25.30**

The mean baseline does demonstrate differences between dye channels (Figure 1). In all instances the mean baseline increased down the electropherogram (6-FAM < VIC < NED < PET).

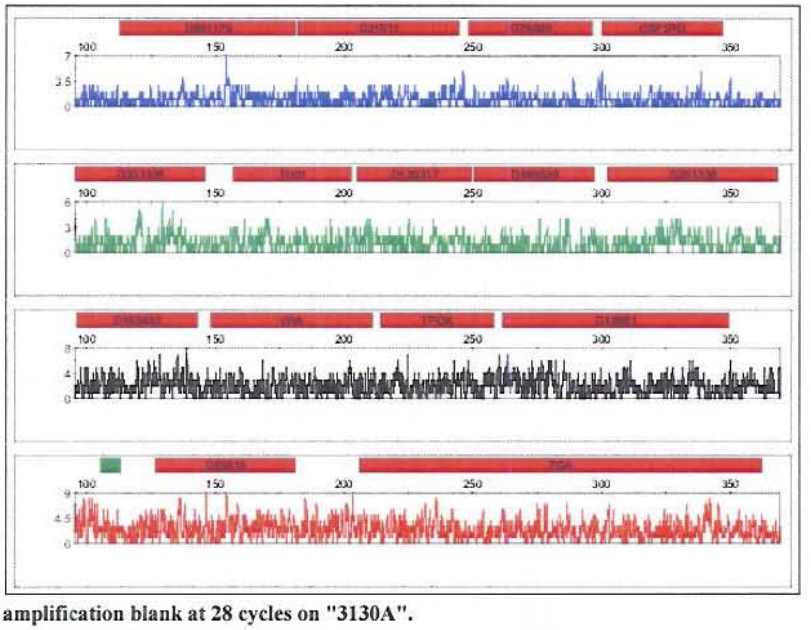


Figure 1: Baseline of TE amplification blank at 28 cycles on "3130A".

There does not appear to be a clear trend in baseline differences for samples amplified at 28 cycles versus 29 cycles.

A comparison of the baseline noise from amplification blanks with Identifiler and ID Plus clearly demonstrates that the ID Plus baseline is cleaner (Figures 2 and 3).

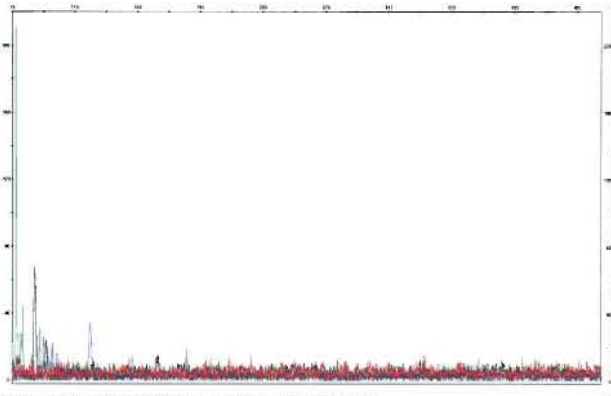


Figure 2: Identifiler amplification blank.

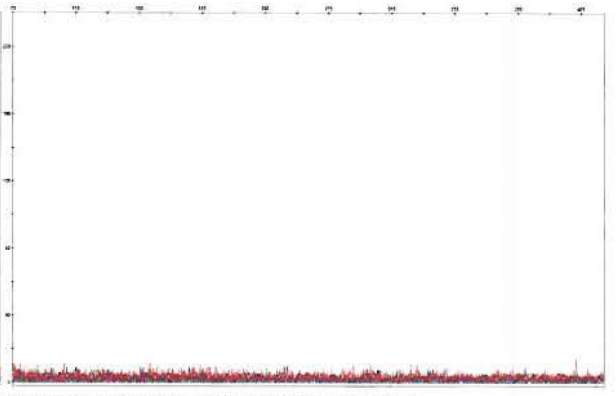


Figure 3: Identifiler Plus amplification blank.

Conclusions

The highest baseline mean peak height + 10 standard deviations observed was 26.61 for the NED dye channel using all of the "Sensitivity Study" samples amplified for 29 cycles. Based on the observed means + 10 standard deviations a peak amplitude threshold of 30 RFU is appropriate for both 28 and 29 cycles and will be utilized in the ID Plus validation experiments.

An issue with this detection threshold was elucidated during analysis of the "Sensitivity Study" samples at 30 RFU. On occasion the spectral calibration is insufficient to resolve the dyes at this level resulting in "spectral pull-up" detected above 30 RFU (Figure 4). The occurrence of pull-up and the level at which it occurs was further examined (Figure 5 and Table 3). Green pulling up into yellow was the main type of pull-up observed. Pull-up in the other dye channels was much less common and occurred mainly when samples were overamplified. It is possible that the pull-up is a result of an inadequate spectral calibration used during validation and may be reduced with an improved spectral calibration. However, if an improved spectral calibration does not resolve the issue it may be necessary to raise the detection threshold slightly in order to compensate for spectral inadequacies.

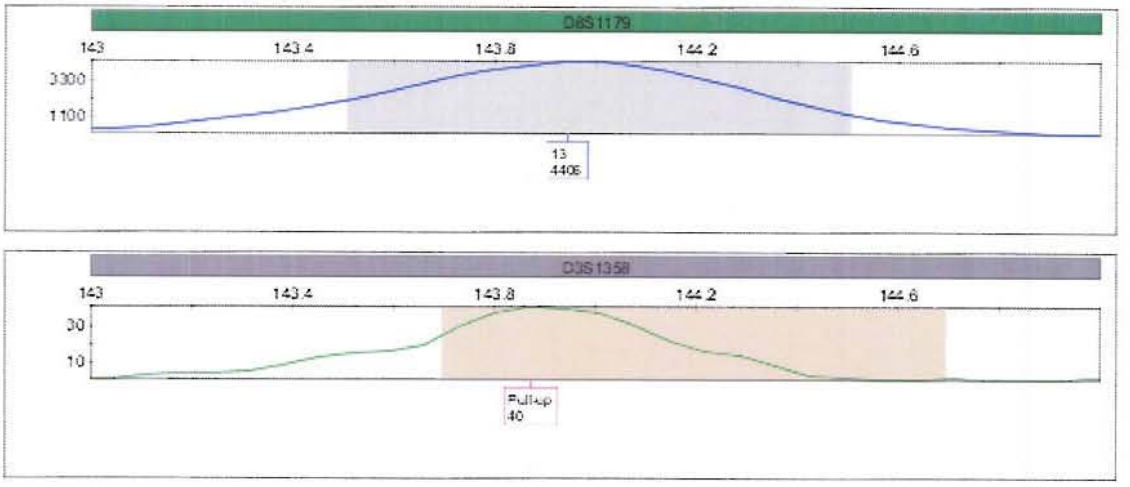


Figure 4: Pull-up peak observed in green at 40 RFU from 13 allele at D8.

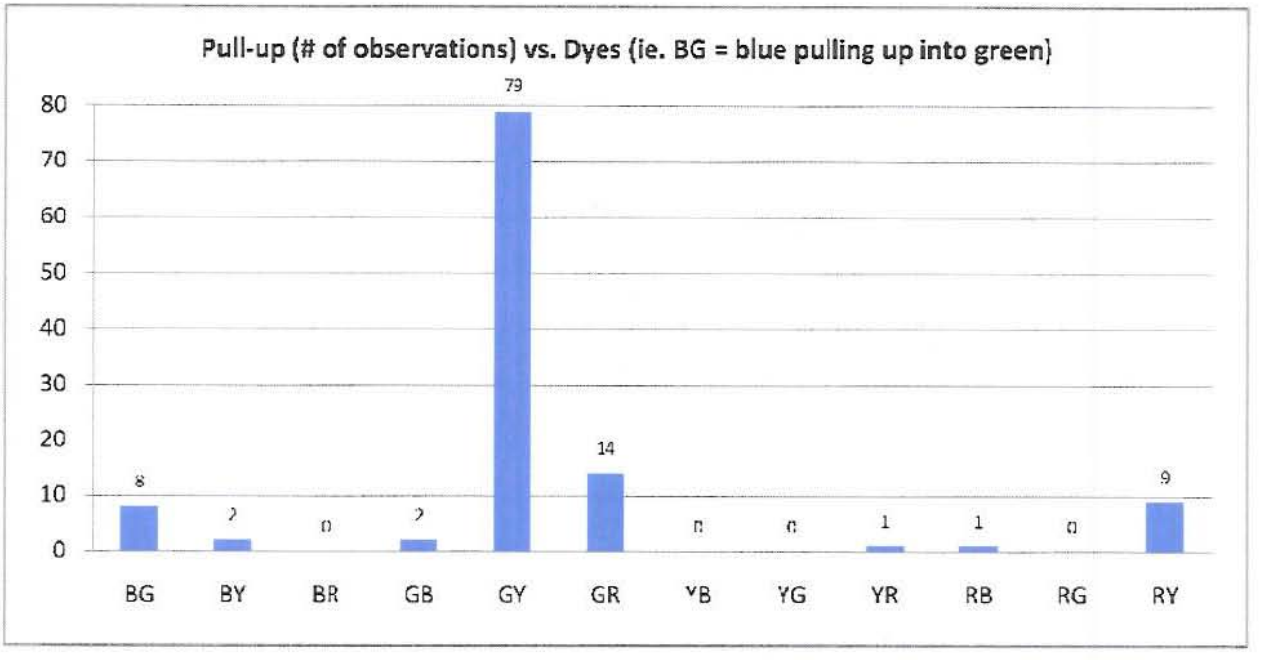


Figure 5: The number of times each type of pull-up was observed during validation of ID Plus.

Table 3: The level at which each type of pull-up occurred during validation of ID Plus

Type	Count	Min	Max	Avg	SD
BG	8	3050	8503	5658	2237
BY	2	7923	8503	8213	410
BR	0	n/a	n/a	n/a	n/a
GB	2	1902	2464	2183	397
GY	79	1112	3949	1956	634
GR	14	2004	7879	3745	1758
YB	0	n/a	n/a	n/a	n/a
YG	0	n/a	n/a	n/a	n/a
YR	1	1112	1112	1112	n/a
RB	1	2572	2572	2572	n/a
RG	0	n/a	n/a	n/a	n/a
RY	9	2435	6078	3853	1622
Total	116	1112	8503	2685	1701

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Sensitivity Study

Purpose

The goal of this study is to determine the detection limit and optimal DNA input range of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

Two employee reference samples and the 9947A positive control were amplified at variable input volumes for both 28 and 29 cycles using ID Plus. Capillary electrophoresis was performed on "3130B" and the resultant data was analyzed in GMID-X with a detection threshold of 30 RFU.

Results and Discussion

At 28 and 29 cycles full DNA profiles were obtained down to ~330 pg for 48-RL and ~340 pg for 61-KM based on the Quantifiler Human results (Table 1). At 28 cycles a full DNA profile was obtained down to ~130 pg for 9947A and down to ~60 pg at 29 cycles based on the Quantifiler Human results.

Table 1: Identifiler Plus sensitivity study data summary. The inputs outlined in red represent the optimal range.

Sample Name	Target Input (ng)	Actual Input (ng) - Quantifiler Human	Actual Input (ng) - Quantifiler Duo	Alleles Expected	28 CYCLES		29 CYCLES	
					Alleles Detected	Total RFU	Alleles Detected	Total RFU
48-RL	0.05	0.06	0.05	29	17	779	21	1700
	0.10	0.11	0.10		22	1669	28	2438
	0.30	0.33	0.29		29	4996	29	12427
	0.80	0.88	0.78		29	17664	29	31254
	1.00	1.10	0.97		29	19291	29	40897
	1.50	1.54	1.36		29	20815	29	58719
	2.00	2.20	1.94		29	44355	29	78782
	3.00	3.30	2.91		29	61720	n/a	n/a
	5.00	5.50	4.85		29	115257	n/a	n/a
61-KM	0.05	0.06	0.04	29	18	895	18	656
	0.10	0.11	0.09		25	2239	24	2766
	0.30	0.34	0.27		29	5211	29	8340
	0.80	0.91	0.71		29	12784	29	25785
	1.00	1.14	0.89		29	19028	29	33503
	1.50	1.60	1.24		29	24414	29	65099
	2.00	2.28	1.78		29	39530	29	69559
	3.00	3.42	2.66		29	58607	n/a	n/a
	5.00	5.70	4.44		29	91588	n/a	n/a
9947A	0.05	0.06	0.12	26	25	2330	26	5834
	0.10	0.13	0.25		26	4730	26	10204
	0.30	0.38	0.74		26	12901	26	24064
	0.80	1.02	1.97		26	40234	26	75274
	1.00	1.28	2.46		26	37114	26	89563
	1.50	1.92	3.69		26	51186	26	88199
	2.00	2.56	4.92		26	47767	26	124665
	3.00	3.84	7.38		26	85845	n/a	n/a
	5.00	6.40	12.30		26	106104	n/a	n/a

Conclusions

The detection limit for full DNA profiles is ~300 pg. Partial DNA profiles were observed down to the minimal input tested (~50 pg). The optimal input range is between 1.5 ng and 2.0 ng of total DNA. Profiles in this range demonstrated the most desirable peak heights and peak height ratios.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Stutter Study

Purpose

The goal of this study is to evaluate the levels of n-4 stutter that are observed with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

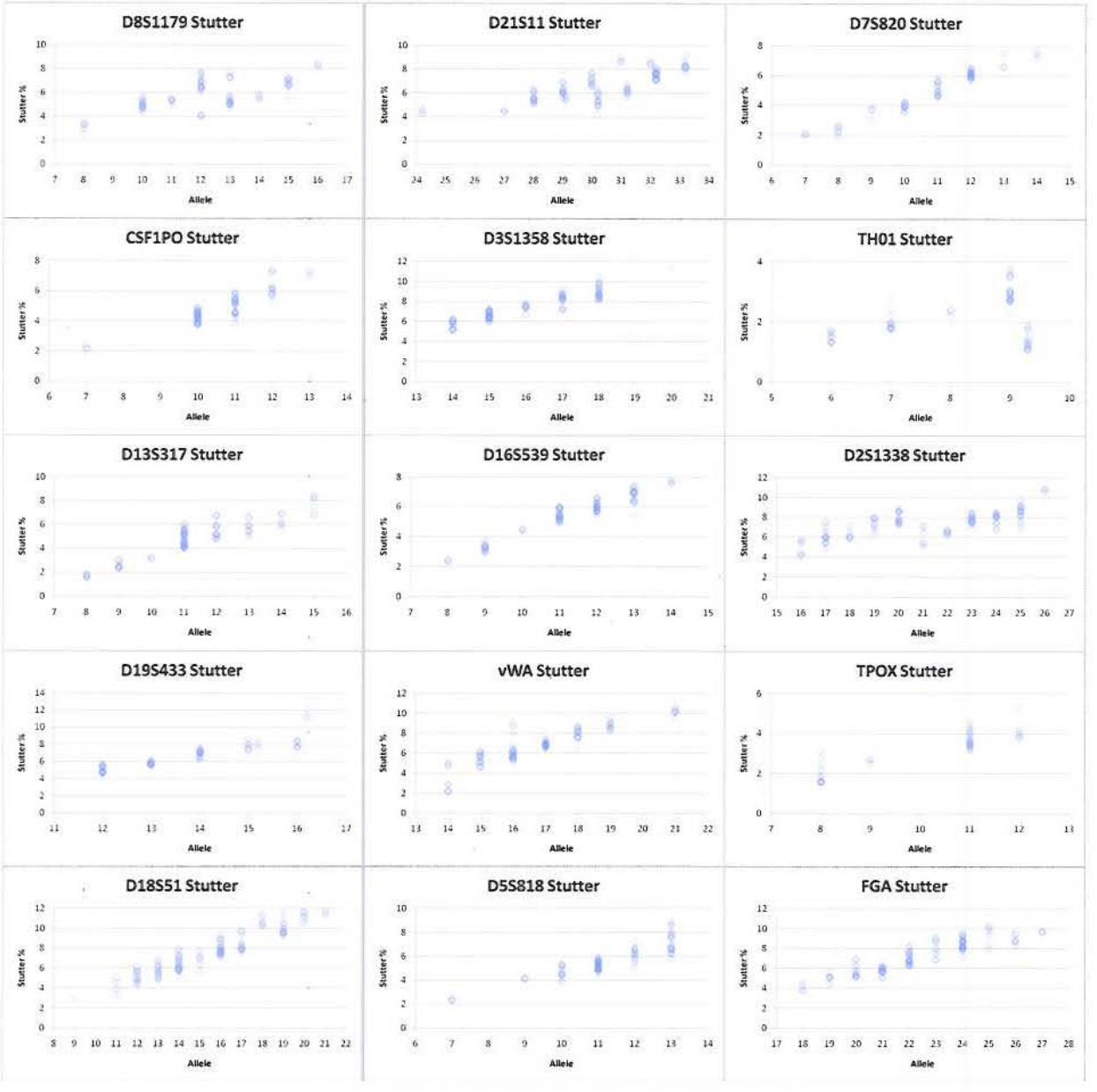
Twenty employee reference samples were amplified at quantities within the optimal input range for 28 cycles using ID Plus. Capillary electrophoresis was performed and the resultant data was analyzed in GMID-X with a detection threshold of 1 RFU and the marker specific stutter ratio filters turned off. The employee reference samples were chosen in effort to maximize allele variation.

Results and Discussion

Summary statistics were generated for the experimentally derived stutter percentages (Table 1). The amount of stutter is marker dependent as well as allele dependent (Figures 1 to 15). Larger alleles typically exhibit the highest stutter peaks. The only stutter observed above the GMID-X marker specific stutter ratio filter was for a 16.2 allele at D19S433 (Figure 16).

Table 1: Summary statistics for the Identifiler Plus stutter study.

LOCUS	STUTTER PERCENTAGE					
	Minimum	Maximum	Average	Std. Dev.	Avg.+3S.D.	GMID-X
D8	2.48	8.44	5.81	1.28	9.66	10.32
D21	4.20	9.11	6.58	1.24	10.29	10.67
D7	1.95	7.63	4.94	1.50	9.46	9.69
CSF	2.18	7.43	4.93	1.09	8.19	9.20
D3	5.14	11.34	7.39	1.35	11.43	12.27
TH01	1.07	3.77	2.14	0.76	4.44	4.08
D13	1.52	8.46	4.83	1.59	9.59	9.93
D16	2.31	7.78	5.51	1.37	9.64	10.39
D2	4.09	10.87	7.37	1.30	11.28	12.44
D19	4.58	12.14	6.64	1.62	11.51	11.21
vWA	2.19	10.56	6.70	1.88	12.33	12.45
TPOX	1.56	5.24	3.15	1.02	6.21	6.38
D18	2.96	11.76	7.66	2.17	14.18	13.68
D5	2.32	8.88	5.61	1.30	9.51	10.06
FGA	3.65	10.26	7.21	1.59	11.97	13.03



Figures 1 to 15: Observed allele specific stutter percentages.

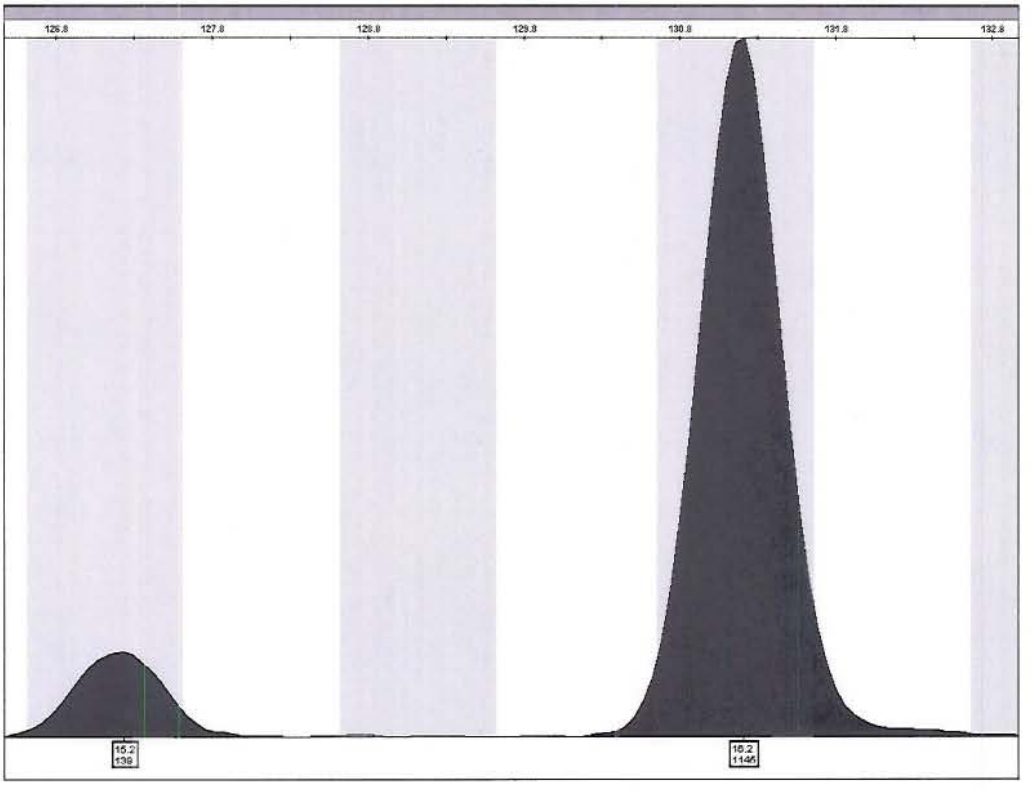


Figure 16: The 15.2 allele is elevated stutter from the 16.2 allele at D19. The stutter is 12.14% and the GMID-X filter is 11.21%.

Conclusions

The GeneMapper ID-X pre-defined marker specific stutter ratios are capable of filtering out the vast majority of observed n-4 stutter peaks without being excessively high. As expected a stutter peak may occasionally exceed the filter by a small percentage, however, this occurrence is rare and these peaks can be manually edited if there is sufficient evidence suggesting that the peak is truly stutter.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Peak Height Study

Purpose

The goal of this study is to evaluate the inter and intra-locus balance by examining peak heights and peak height ratios observed with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

Optimal Range

Twenty employee reference samples were amplified at quantities within the optimal DNA input range (~1.5 to 2.0 ng) for 28 cycles using ID Plus. Capillary electrophoresis was performed with triplicate injections and the resultant data was analyzed in GMID-X.

Sub-Optimal Range

Three employee reference samples were amplified at multiple quantities below the optimal DNA input range (~200, 400, 600, and 800 pg) for 28 cycles using ID Plus. Capillary electrophoresis was performed and the resultant data was analyzed in GMID-X.

Results and Discussion

Optimal Range

The total average peak height ratio for samples in the optimal DNA input range was 88.0% (Figure 1 and Table 1). The average peak height ratio did not vary substantially between loci or dye channels. The minimum peak height ratio observed was 61.3%, however, more than 84% of all peak height ratios observed were greater than 80%. Additionally, the number of repeat units between sister alleles at heterozygous loci did not appear to have much of an impact on the peak height ratio between those alleles.

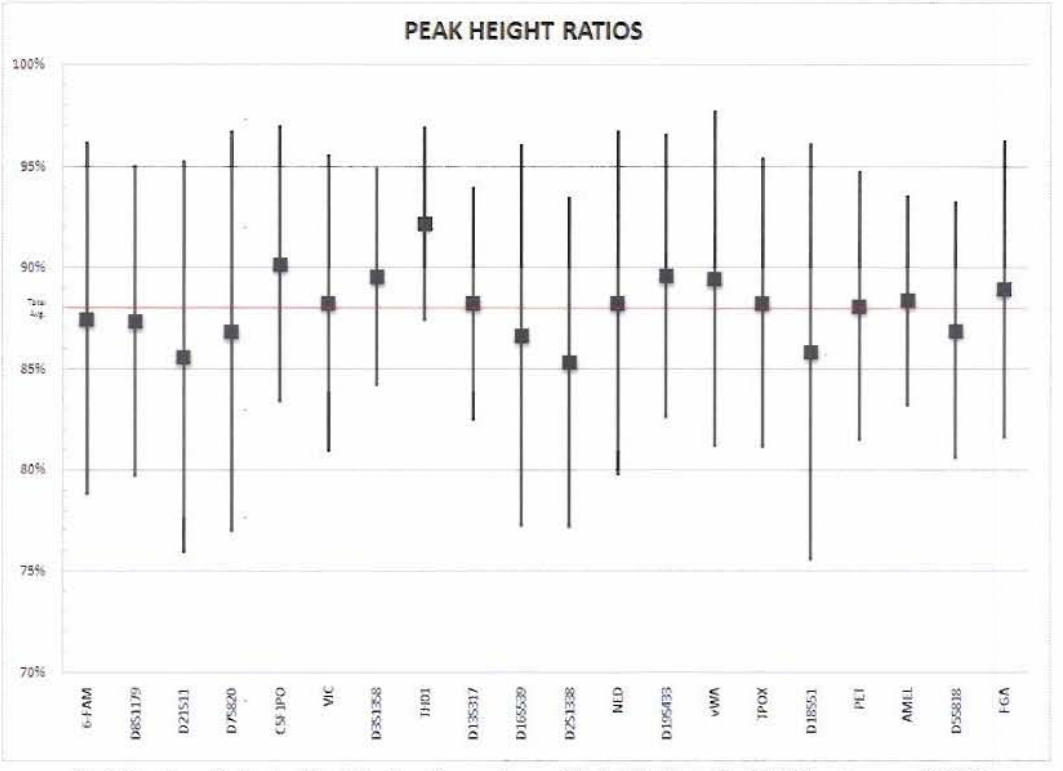


Figure 1: Average peak height ratios with standard deviation bars for samples amplified within the optimal DNA input range with ID Plus separated by locus and dye channel.

Table 1: Summary statistics table for peak height ratio/peak height study of samples amplified within the optimal DNA input range with ID Plus.

Marker/Dye	6-FAM	D851179	D21S11	D7S820	CSF1PO	VIC	D3S1358	TH01	D13S317	D16S539	D2S1338	NED	D19S433	vWA	TPOX	D18S51	PET	AMEL	D5S818	FGA	TOTAL
Avg PH Ratio	87.5%	87.3%	85.6%	86.8%	90.2%	88.3%	89.5%	92.2%	88.2%	86.6%	85.3%	88.3%	89.6%	89.5%	88.3%	85.8%	86.1%	88.4%	86.9%	88.9%	88.0%
Std Dev	8.7%	7.7%	9.7%	9.9%	6.8%	7.3%	5.4%	4.8%	5.7%	9.5%	8.2%	8.5%	7.0%	8.3%	7.2%	10.3%	6.7%	5.2%	6.4%	7.4%	8.0%
Max PH Ratio	100.0%	98.7%	100.0%	99.9%	98.6%	100.0%	97.1%	99.9%	99.0%	100.0%	99.3%	99.8%	99.8%	98.7%	99.8%	99.6%	95.9%	98.3%	96.9%	99.9%	100.0%
Min PH Ratio	64.7%	64.7%	65.9%	67.6%	75.3%	66.7%	77.1%	82.6%	78.0%	66.7%	68.5%	61.3%	74.1%	63.3%	76.1%	61.3%	72.4%	80.8%	73.5%	72.4%	61.3%
# Hetero Loci	216	54	57	51	54	249	48	48	48	48	57	207	51	57	42	57	120	24	42	54	792
60-70%	16	3	6	7	0	9	0	0	0	6	3	9	0	3	0	6	0	0	0	0	34
	7.4%	5.6%	10.5%	13.7%	0.0%	3.6%	0.0%	0.0%	0.0%	12.5%	5.3%	4.3%	0.0%	5.3%	0.0%	10.5%	0.0%	0.0%	0.0%	0.0%	4.3%
70-80%	26	3	12	5	6	23	5	0	3	3	12	28	5	4	9	10	16	0	5	10	92
	12.0%	5.6%	21.1%	9.8%	11.1%	9.2%	10.4%	0.0%	6.3%	6.3%	21.1%	13.5%	9.8%	7.0%	21.4%	17.5%	13.3%	0.0%	11.9%	18.5%	11.6%
80-90%	67	24	15	16	12	98	16	12	27	19	24	67	23	14	16	14	51	16	21	14	283
	31.0%	44.4%	26.3%	31.4%	22.2%	39.4%	33.3%	25.0%	56.3%	39.6%	42.1%	32.4%	45.1%	24.6%	38.1%	24.6%	42.5%	66.7%	50.0%	25.9%	35.7%
90-100%	107	24	24	23	36	119	27	36	18	20	18	103	23	36	17	27	54	8	16	30	383
	49.5%	44.4%	42.1%	45.1%	66.7%	47.8%	56.3%	75.0%	37.5%	41.7%	31.6%	49.8%	45.1%	63.2%	40.5%	47.4%	45.0%	33.3%	38.1%	55.6%	48.4%
Avg PH	1058	1449	1072	797	916	1189	1407	1343	1263	1083	850	879	1037	1043	778	656	844	996	874	724	1014
Std Dev PH	384	366	264	244	295	380	305	358	337	302	285	281	264	255	197	191	226	219	212	193	362
Max PH	2642	2642	1730	1511	1627	2300	2300	2226	2058	1687	1583	1692	1692	1509	1234	1114	1392	1379	1392	1218	2642
Min PH	345	742	494	348	345	364	617	631	568	491	354	295	573	435	348	295	342	474	446	342	295

Diff (BP)	2	3	4	5	6	8	10	11	12	14	15	16	18	20	24	26	28	32	40
Diff (Repeat)	0.50	0.67	1.00	1.33	1.50	2.00	2.50	2.67	3.00	3.50	3.67	4.00	4.50	5.00	6.00	6.50	7.00	8.00	10.00
# Occurences	5	3	78	1	5	45	2	5	51	2	4	29	2	10	7	1	4	1	1
N	15	9	234	3	15	135	6	15	153	6	12	87	6	30	21	3	12	3	3
Avg PH Ratio	90.0%	92.7%	87.7%	83.8%	81.9%	90.5%	88.5%	93.5%	87.6%	93.2%	89.9%	88.4%	90.3%	81.6%	79.5%	91.8%	92.6%	63.5%	97.1%
Std Dev	4.3%	6.6%	8.1%	0.3%	10.2%	6.2%	11.9%	4.0%	8.1%	3.9%	4.4%	7.7%	1.7%	8.5%	6.2%	1.4%	6.5%	1.8%	0.5%
Max PH Ratio	97.8%	99.9%	100.0%	84.0%	96.6%	99.8%	100.0%	99.8%	99.9%	97.5%	94.9%	98.7%	92.0%	98.5%	87.1%	93.4%	97.7%	64.7%	97.8%
Min PH Ratio	84.6%	84.1%	64.7%	83.4%	65.9%	73.5%	76.6%	87.5%	63.3%	89.4%	82.6%	65.8%	88.0%	69.2%	68.5%	90.8%	80.8%	61.3%	96.8%

Peak height balance between loci has been greatly improved with the Identifiler Plus kit when compared to Identifiler (Figures 2 and 3). The blue (6-FAM) and green (VIC) dye channels are

still somewhat more robust than the yellow (NED) and red (PET) dye channels. Peak heights at the smaller loci are also somewhat more robust than those at larger loci with the ID Plus kit. Although the former trend was believed to also be true for the Identifiler kit, Figure 2 suggests that this is not the case by demonstrating that peak heights were less closely linked to locus size. The overall average peak height per allele with ID Plus was 1,014 RFU for samples amplified within the optimal DNA input range.

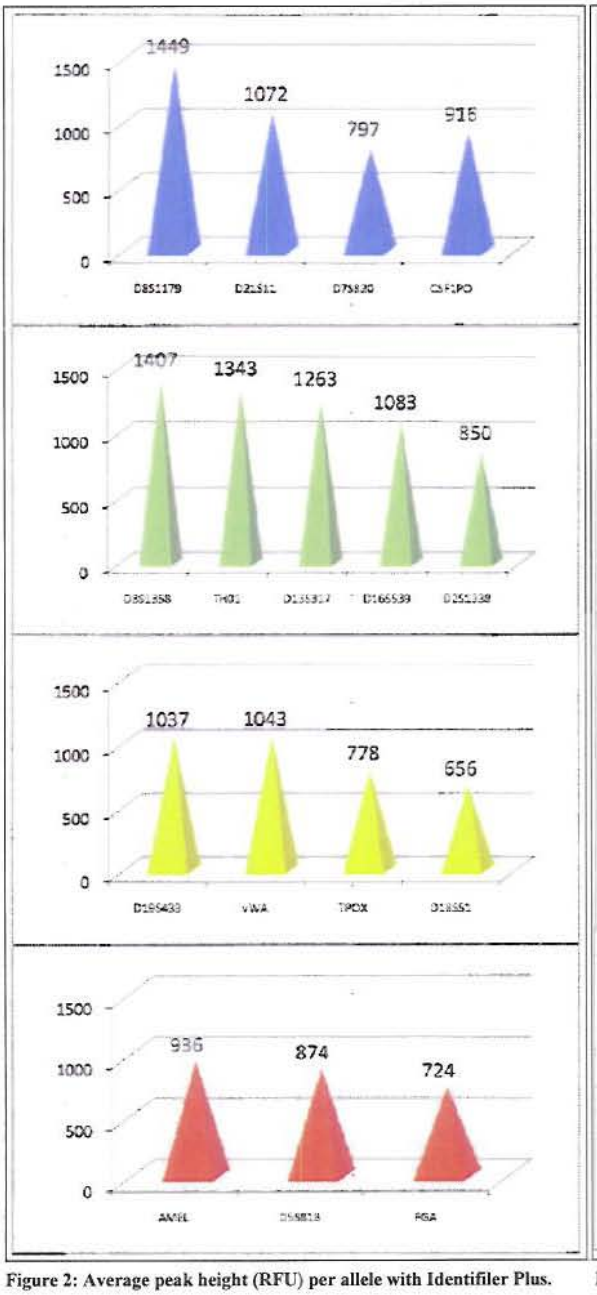


Figure 2: Average peak height (RFU) per allele with Identifiler Plus.

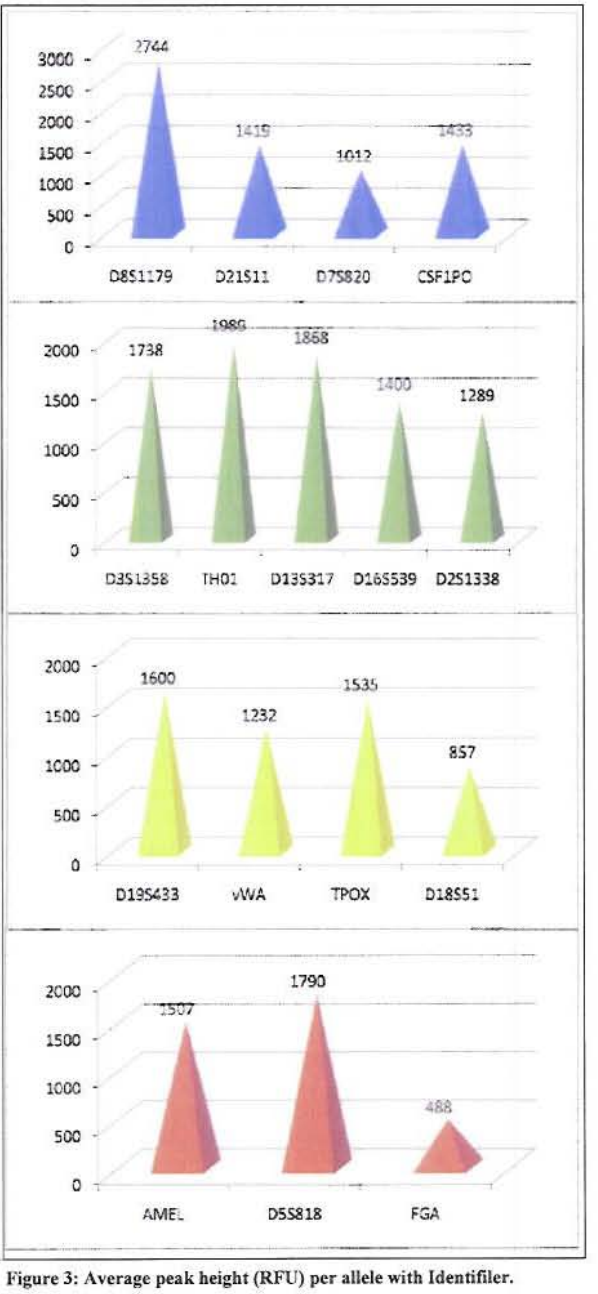


Figure 3: Average peak height (RFU) per allele with Identifiler.

Sub-Optimal Range

Samples amplified with quantities below the optimal DNA input range demonstrate more peak height imbalance than those within the optimal range (Table 2). While the majority of peak height ratios observed were still greater than 60%, there were a number of ratios observed below this. The lower the input DNA amount the greater the imbalance became. One heterozygous locus demonstrated a peak height ratio of as low as 28.8% when only 203 pg of input DNA was amplified (Figure 4).

Table 2: Summary statistics table for peak height ratios of samples amplified below the optimal DNA input range with ID Plus.

PH Ratio (%)	200 pg		400 pg		600 pg		800 pg	
	Count	Percent	Count	Percent	Count	Percent	Count	Percent
0-10	0	0.00	0	0.00	0	0.00	0	0.00
10-20	0	0.00	0	0.00	0	0.00	0	0.00
20-30	1	2.78	0	0.00	0	0.00	0	0.00
30-40	2	5.56	0	0.00	2	5.00	0	0.00
40-50	3	8.33	2	5.00	1	2.50	2	5.00
50-60	3	8.33	6	15.00	1	2.50	2	5.00
60-70	3	8.33	3	7.50	7	17.50	5	12.50
70-80	10	27.78	11	27.50	12	30.00	5	12.50
80-90	6	16.67	11	27.50	10	25.00	15	37.50
90-100	8	22.22	7	17.50	7	17.50	11	27.50
Total	36	100.00	40	100.00	40	100.00	40	100.00
Min	28.76		41.32		32.92		47.37	
Max	98.84		98.51		100.00		99.80	
Avg	72.50		76.49		76.25		80.64	
Std Dev	19.39		14.67		15.59		13.89	

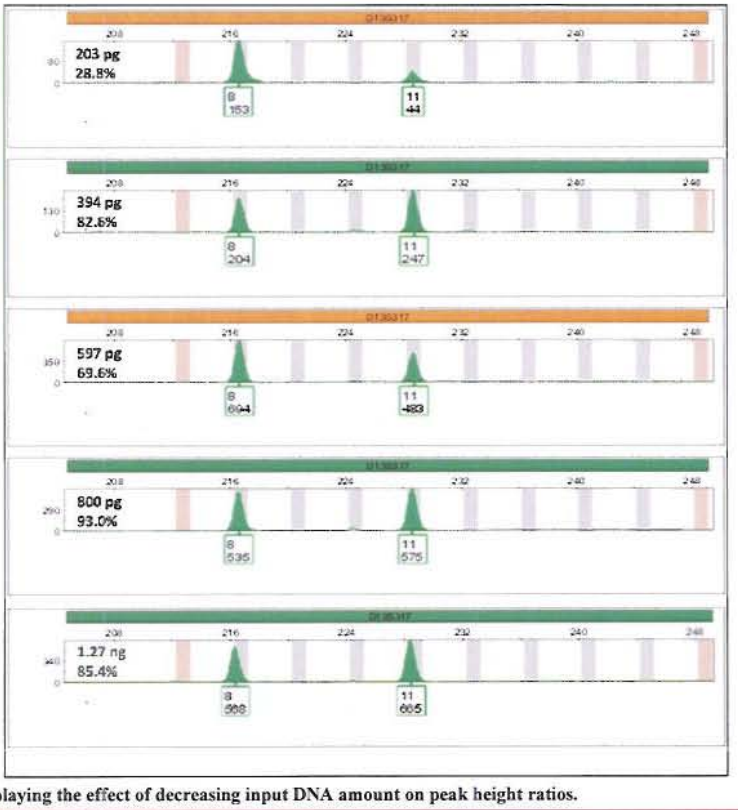


Figure 4: Electropherogram displaying the effect of decreasing input DNA amount on peak height ratios.

Conclusions

Samples amplified within the optimal DNA input range with ID Plus demonstrated good inter and intra-locus balance. Robust samples can be expected to routinely generate peak height ratios of greater than 80% with imbalance occasionally reaching as low as 60%. The previously determined optimal DNA input range of ~1.5 to 2.0 ng has been verified to be appropriate since samples within this range produced peak heights of ~1,000 RFU per allele on average. As expected, samples amplified with DNA input amounts below this range will begin to show more imbalance. As input amounts approach the stochastic range peak height ratios of less than 30% may be observed, therefore, low level samples should be interpreted with caution. Peak height ratios in these low level samples were further explored by correlating the peak height ratio with the height of the tallest peak for all single source samples from the ID Plus validation (Table 3 and Figure 5).

Table 3: Peak height ratios separated by the height of the tallest peak for all single source heterozygotes from the ID Plus validation.

Max. PH	Min. Ratio	Max. Ratio	Avg. Ratio	Std. Dev.
0-100	33.33	100.00	71.94	16.81
100-200	22.16	100.00	66.97	18.92
200-300	21.29	99.52	68.89	20.64
300-400	21.50	96.53	73.93	20.11
400-500	49.21	99.56	83.61	12.41
500-600	52.88	100.00	87.15	10.83
600-700	57.72	98.23	88.25	8.25
700-800	62.91	100.00	89.35	8.52
800-900	48.30	99.77	86.52	9.12
900-1000	66.73	99.59	86.91	8.04
1000+	63.32	99.93	88.10	7.40

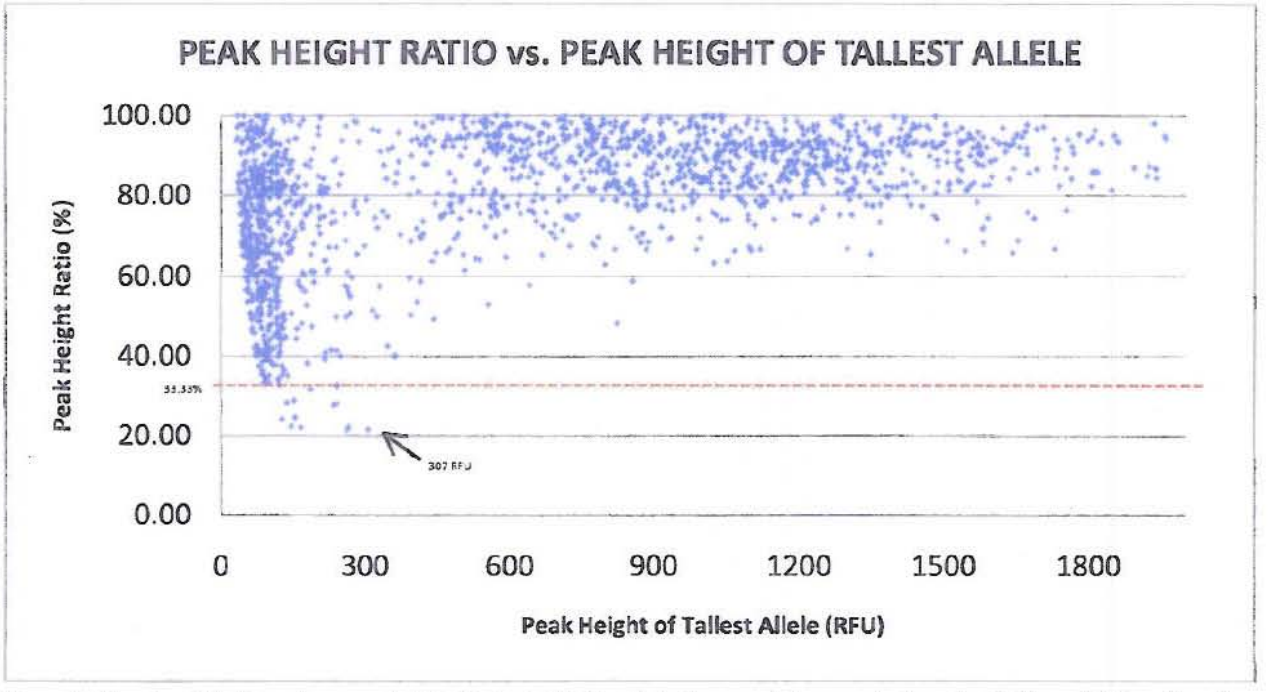


Figure 5: Plot of peak height ratio versus height of tallest allele for all single source heterozygotes from the ID Plus validation. Samples with peak heights greater than 2000 RFU are not shown.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Concordance Study

Purpose

The goal of this study is to verify that DNA typing results obtained with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) are concordant with those obtained with the Identifiler kit.

Materials and Methods

Twenty employee reference samples were amplified at quantities within the optimal input range for 28 cycles using ID Plus. Capillary electrophoresis was performed and the resultant data was analyzed in GMID-X. The employee reference samples were previously amplified and typed using Identifiler. The results of the two kits were compared.

Results and Discussion

The genotypes obtained following ID Plus amplification of twenty employee reference samples were 100% concordant with the genotypes previously obtained using Identifiler. A total of 584 concordant alleles were observed.

Conclusions

DNA typing results obtained using ID Plus are fully concordant with those obtained using Identifiler. This confirms the expected results due to the fact that ID Plus uses the same primer sequences as the earlier generation Identifiler kit.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Stochastic Study

Purpose

The goal of this study is to determine the appropriate stochastic threshold for the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) and evaluate profiles in the stochastic range.

Materials and Methods

Six employee reference samples were amplified for 28 cycles using ID Plus at multiple low level DNA inputs (~50, 90, and 140 pg) in an effort to generate profiles that straddle the 30 RFU detection threshold. Capillary electrophoresis was performed with triplicate injections and the resultant data was analyzed in GMID-X using both a 30 RFU and 10 RFU detection threshold.

Results and Discussion

When allelic dropout was observed at known heterozygous loci with a 30 RFU detection threshold the average height of the single detected peak was ~60 RFU (Table 1). Approximately 5% of the time the detected peak reached 100 RFU or more. The tallest peak detected was 177 RFU when there was known to be allelic dropout of the sister peak (Figure 1).

Table 1: Summary of allelic dropout data utilizing both a 30 RFU and 10 RFU detection threshold.

Known heterozygous loci where a single allele peak was detected above the analysis threshold with allelic dropout of the sister peak.				
RFU	30 RFU Threshold		10 RFU Threshold	
	#	%	#	%
10-20	n/a		29	38.16
20-30			14	18.42
30-40	56	30.11	8	10.53
40-50	35	18.82	6	7.89
50-60	15	8.06	6	7.89
60-70	22	11.83	1	1.32
70-80	24	12.90	7	9.21
80-90	16	8.60	4	5.26
90-100	8	4.30	0	0.00
100-110	3	1.61	1	1.32
110-120	1	0.54	n/a	
120-130	0	0.00		
130-140	0	0.00		
140-150	1	0.54		
150-160	2	1.08		
160-170	2	1.08		
170-180	1	0.54		
Total	186	100.00	76	100.00
SUMMARY				
Min	30		10	
Max	177		100	
Avg	59.34		35.38	
SD	27.81		23.78	
Avg +3SD	142.78		106.73	

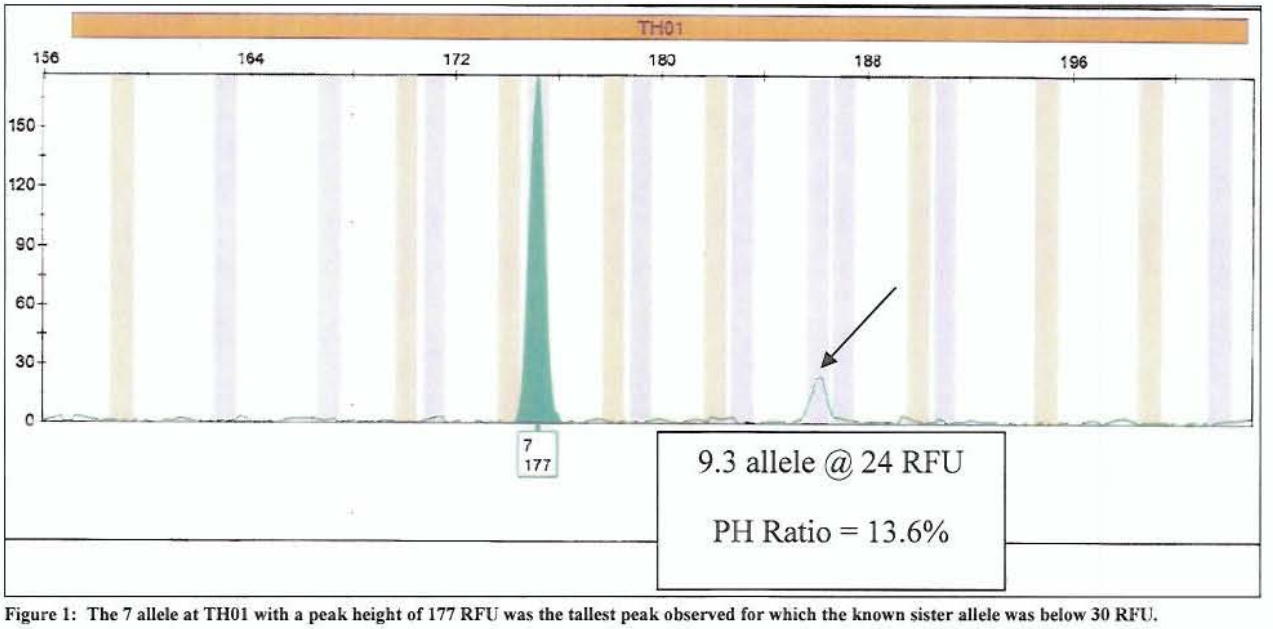


Figure 1: The 7 allele at TH01 with a peak height of 177 RFU was the tallest peak observed for which the known sister allele was below 30 RFU.

The data was also analyzed with a 10 RFU detection threshold because data less than 10 RFU is not expected to be visually discernible from baseline noise. When this was done there were multiple instances where a single allele peak would be detected with a peak height greater than 30 RFU and the known sister peak was less than 10 RFU and therefore visually indistinguishable from the baseline. The tallest peak observed with the sister allele below 10 RFU was 100 RFU (Figure 2).

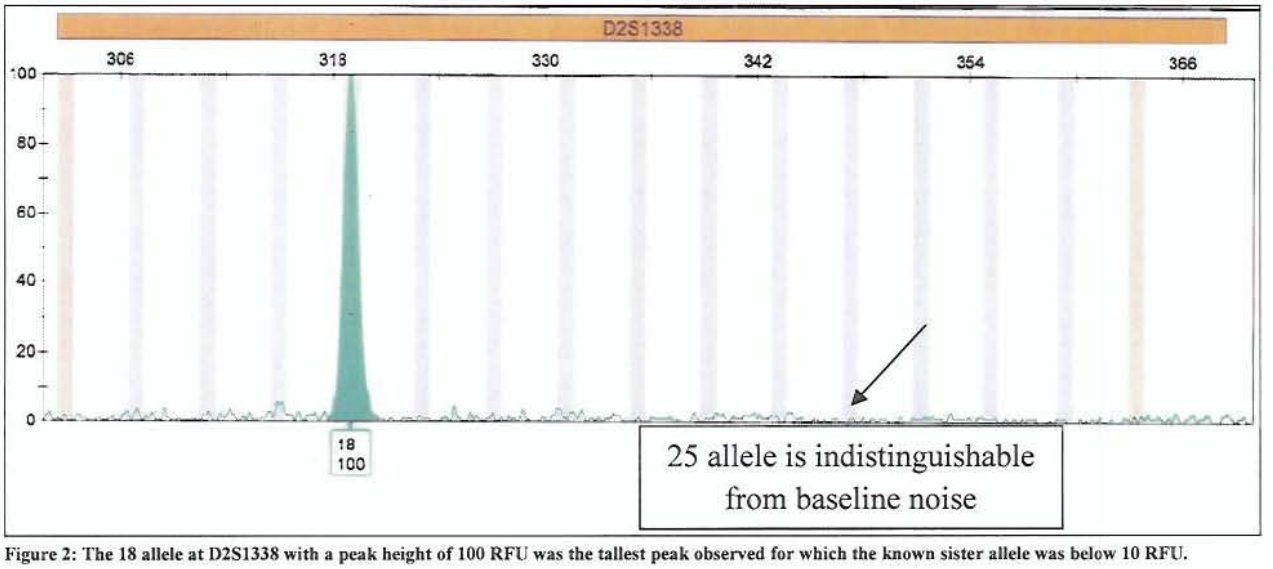


Figure 2: The 18 allele at D2S1338 with a peak height of 100 RFU was the tallest peak observed for which the known sister allele was below 10 RFU.

Peak height ratios were also calculated for heterozygous loci where both alleles were above the 30 RFU detection threshold in order to further investigate the behavior of samples within the

stochastic range (Table 2). Samples at this low level demonstrated peak height ratios of greater than 60% nearly 70% of the time. A peak height ratio of as low as 21.3% was observed (Figure 3).

Table 2: Peak height ratio data from the ID Plus stochastic study.

Summary of peak height ratio results from the Identifiler Plus stochastic study at 30 RFU.	
Ratio	**%**
20-30	1.86
30-40	4.87
40-50	11.14
50-60	13.46
60-70	17.40
70-80	17.87
80-90	22.04
90-100	11.37
Total	100
MIN	21.29
MAX	100.00
AVG	68.90
STD DEV	18.07

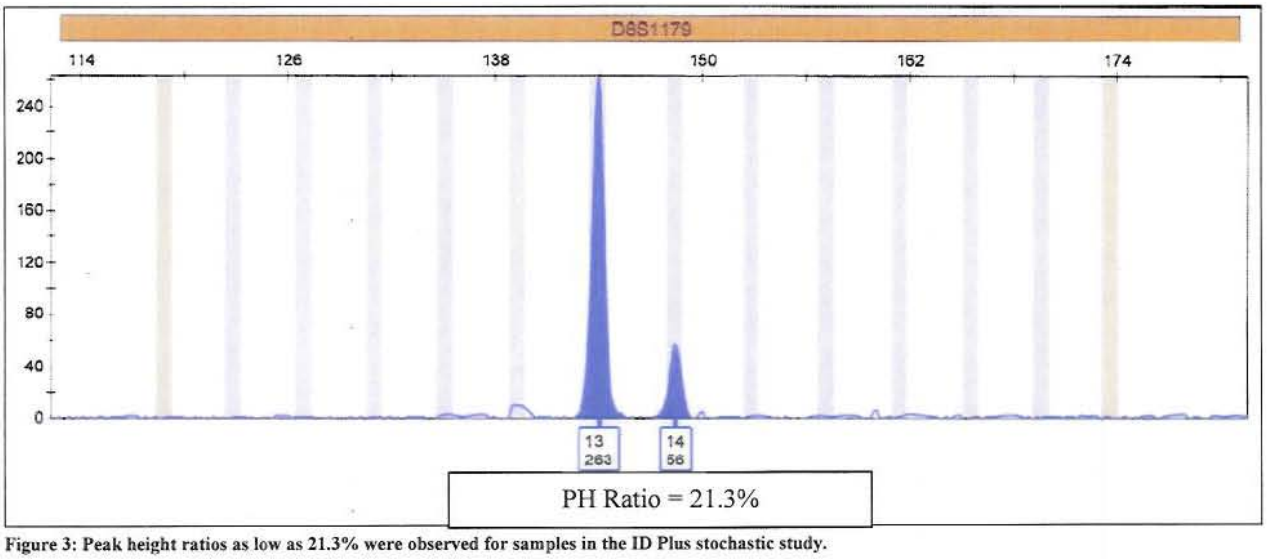


Figure 3: Peak height ratios as low as 21.3% were observed for samples in the ID Plus stochastic study.

The DNA profiles generated with the 30 RFU detection threshold were examined for completeness in order to supplement the previously completed Sensitivity Study (Table 3). Full

profiles were obtained at ~140 pg in half of the samples. Partial profiles were obtained at ~90 pg and ~50 pg.

Table 3: Alleles detected are presented for comparison to the expected number of alleles obtained during the ID Plus stochastic study.

Summary of sensitivity results from the Identifiler Plus stochastic study at 30 RFU.								
Sample	**Target (pg)**	**QH (ng)**	**QD (ng)**	**Expected**	**Inj. #1**	**Inj. #2**	**Inj. #3**	**Inj. #4**
14	50	0.051	0.043	29	9	4	8	n/a
	90	0.090	0.076		20	21	20	n/a
	140	0.139	0.117		26	21	26	26
41	50	0.048	0.039	27	19	19	17	n/a
	90	0.090	0.074		23	23	23	n/a
	140	0.138	0.113		27	27	27	n/a
48	50	0.050	0.044	28	17	14	13	n/a
	90	0.090	0.079		27	27	25	n/a
	140	0.143	0.126		24	25	23	n/a
52	50	0.050	0.055	29	18	16	18	n/a
	90	0.087	0.097		19	19	20	n/a
	140	0.141	0.156		29	29	29	n/a
67	50	0.051	0.051	27	8	8	8	n/a
	90	0.089	0.090		17	16	14	n/a
	140	0.140	0.141		25	24	24	n/a
80	50	0.049	0.078	26	19	19	19	n/a
	90	0.090	0.144		24	24	24	n/a
	140	0.139	0.222		26	26	26	n/a

Conclusions

A stochastic threshold of 200 RFU is suitable for use with ID Plus. A single allele detected above 200 RFU in an apparent single source profile can reliably be considered a homozygote. If a single allele is detected between 30 and 200 RFU then it is possible the sister allele of a heterozygote has dropped below the detection threshold.

The absence of visual evidence of an allele peak below the 30 RFU threshold (so-called "blips") is not sufficient to assume that no data is missing. Analysts should not attempt to look below the detection threshold due to the unreliability of this low level data.

Conclusions from the previously completed Peak Height and Sensitivity Studies are upheld by the data generated in this Stochastic Study.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Mixture Study

Purpose

The goal of this study is to evaluate simple and complex DNA mixtures in order to determine the appropriate parameters for interpreting mixtures resulting from amplification with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

Two, three, and four person DNA mixtures were created by combining employee reference samples in various ratios (Table 1). Robust mixtures as well as low level mixtures were created. The mixtures were amplified for 28 cycles using ID Plus followed by capillary electrophoresis and the resultant data was analyzed in GMID-X.

Table 1: Description of samples used in the ID Plus mixture study.

Two Person Mixtures		
Ratio	Target	Low-Level
8:1	1.6ng:200pg	400pg:50pg
5:1	1.5ng:300pg	300pg:60pg
2:1	1.2ng:600pg	200pg:100pg
1:1	900pg:900pg	100pg:100pg
1:2	600pg:1.2ng	100pg:200pg
1:5	300pg:1.5ng	60pg:300pg
1:8	200pg:1.6ng	50pg:400pg
Three Person Mixtures		
Ratio (%)	Target	Low-Level
33.3:33.3:33.3	600pg-600pg-600pg	100pg-100pg-100pg
70:20:10	1.4ng-400pg-200pg	350pg-100pg-50pg
60:30:10	1.2ng-600pg-200pg	300pg-150pg-50pg
50:40:10	1.0ng-800pg-200pg	250pg-200pg-50pg
50:30:20	1.0ng-600pg-400pg	250pg-150pg-100pg
45:45:10	900pg-900pg-200pg	225pg-225pg-50pg
40:40:20	800pg-800pg-400pg	100pg-100pg-50pg
35:35:30	700pg-700pg-600pg	70pg-70pg-60pg
60:20:20	1.2ng-400pg-400pg	300pg-100pg-100pg
50:25:25	1.0ng-500pg-500pg	100pg-50pg-50pg
40:30:30	800pg-600pg-600pg	200pg-150pg-150pg
Four Person Mixtures		
Ratio (%)	Target	Low-Level
25:25:25:25	500pg-500pg-500pg-500pg	100pg-100pg-100pg-100pg
60:20:10:10	1.2ng-400pg-200pg-200pg	300pg-100pg-50pg-50pg
50:20:20:10	1.0ng-400pg-400pg-200pg	250pg-100pg-100pg-50pg
70:10:10:10	1.4ng-200pg-200pg-200pg	350pg-50pg-50pg-50pg
40:20:20:20	800pg-400pg-400pg-400pg	200pg-100pg-100pg-100pg
40:40:15:5	800pg-800pg-300pg-100pg	400pg-400pg-150pg-50pg
35:35:20:10	700pg-700pg-400pg-200pg	350pg-350pg-200pg-100pg
40:40:10:10	800pg-800pg-200pg-200pg	200pg-200pg-50pg-50pg
35:35:25:5	700pg-700pg-500pg-100pg	350pg-350pg-250pg-50pg
30:30:20:20	600pg-600pg-400pg-400pg	150pg-150pg-100pg-100pg
30:30:30:10	600pg-600pg-600pg-200pg	150pg-150pg-150pg-50pg

Results and Discussion

The previously determined stochastic threshold of 200 RFU (see "Stochastic Study") was evaluated to determine if any unique (without stacking) detected alleles at known heterozygous loci in mixtures ever exceeded this threshold. The tallest such peak was determined to be 192 RFU (Figure 1). The mixture data was also evaluated to determine the height of the tallest unique peak for which the sister allele is indistinguishable from the baseline (Figure 2).

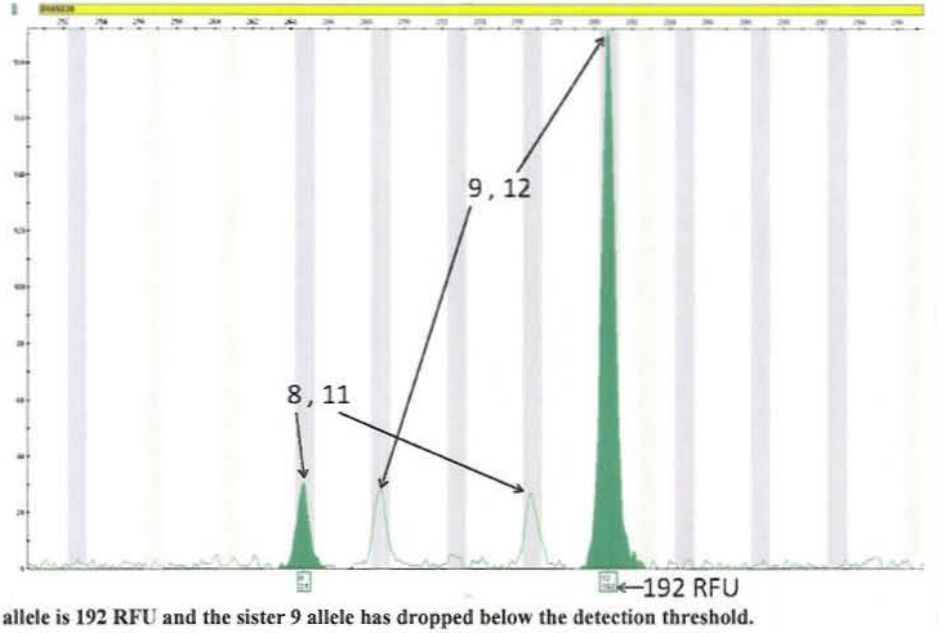


Figure 1: The 12 allele is 192 RFU and the sister 9 allele has dropped below the detection threshold.

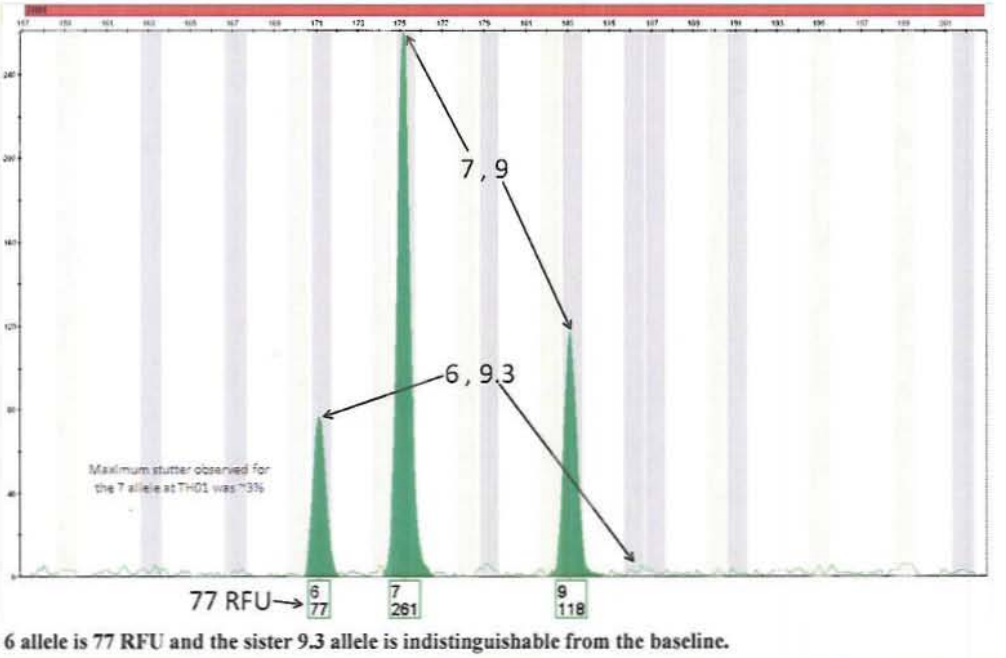


Figure 2: The 6 allele is 77 RFU and the sister 9.3 allele is indistinguishable from the baseline.

The mixture guidelines currently in place with respect to designating minor alleles were assessed for applicability to the ID Plus mixture data. The rules for determining balance (one locus with 5 alleles above 33.3% of the tallest peak and/or 3 loci with 4 alleles within 50% of the tallest peak denote balance in the mixture between at least two contributors) and for designating minor alleles (less than 1/3 the height of the tallest peak at a locus for unbalanced mixtures and less than 10% of the total RFU at a locus for balanced mixtures) proved to be applicable to mixture data from ID Plus. However, these guidelines breakdown for low level samples. This is demonstrated by the fact that for low level single source samples peak height ratios can be less than 33% (Figure 3); therefore, the 1/3 method loses interpretational significance for these samples. A peak height ratio of less than 33% was observed when peak heights reached as high as 307 RFU.

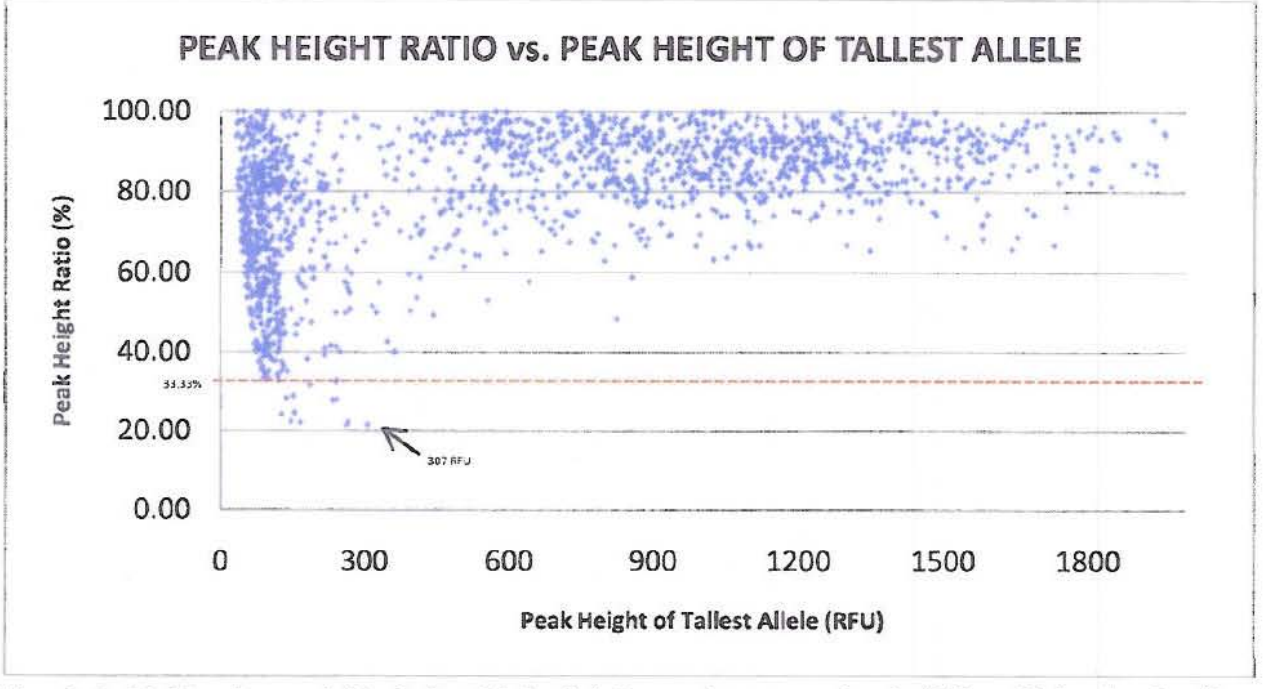


Figure 3: Peak height ratio versus height of tallest allele for all single source heterozygotes from the ID Plus validation. Samples with peak heights greater than 2000 RFU are not shown.

The mixtures were evaluated to determine if percent contribution remains constant across a profile for individual contributors. In general it was shown that percent contribution does remain fairly constant across a profile (see figures labeled 2-1 thru 3-11 at the end of this document). There is some deviation as expected, however, it is never so extreme as to have an individual appear to be a minor contributor at some loci and switch to a major contributor at others. Because of the complicating factors inherent in DNA mixtures (stutter, stacking, peak height imbalance, etc.) it is difficult to assess percent contribution at all markers. Percent contribution

can only be assessed where unique alleles are present and sometimes there are complications even here. This type of analysis requires assumptions with respect to peak height ratios which can be impossible to achieve (Figure 4).

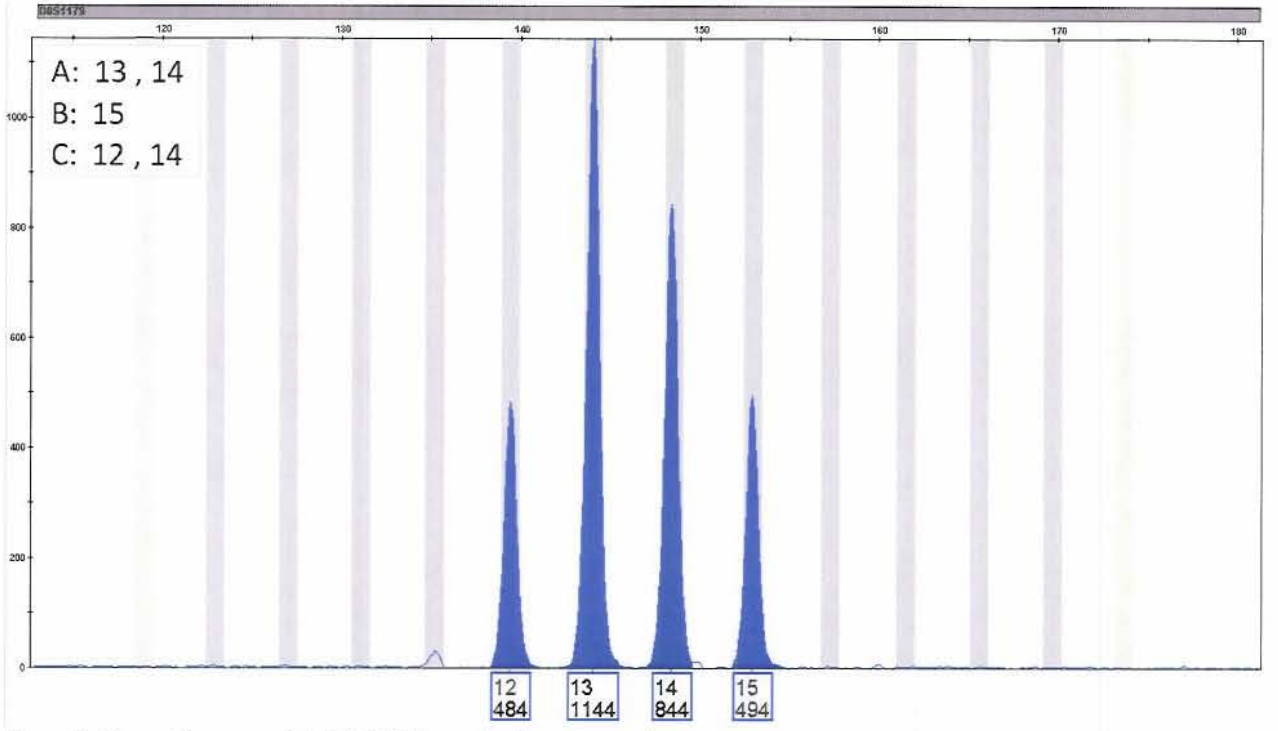


Figure 4: Electropherogram of the D8S1179 locus of a three person mixture where the known contributor genotypes are displayed in the upper left corner. The 14 allele is not tall enough to satisfy the necessary assumptions about peak height balance.

Conclusions

Following the release of the SWGDAM guidelines in 2009 a mixture study was performed at the SDPD using the Identifiler kit (see "Summary of the SDPD Mixture Study"). The ID Plus mixture study sought to recreate this work with the addition of low level mixtures. For robust mixtures the findings and conclusions of the Identifiler mixture study have proven to also apply to mixture data generated using ID Plus. As the Identifiler mixture guidelines largely rely on peak height ratios within a locus it is not surprising that these guidelines also hold for ID Plus as both kits have demonstrated similar levels of balance within a locus.

The addition of low level mixtures elucidated the fact that minor alleles cannot be reliably predicted for such samples. Single source samples had peak height ratios of less than 33% when the tallest peak was as high as 307 RFU. Therefore, a threshold of 350 RFU will be set below which minor alleles will not be designated.

The mixture study also shows that an individual's percent contribution to a mixture will tend to remain fairly consistent throughout the entire profile. However, there will be some minor

variation even for robust and pristine mixtures. This variation may be effected by the nature of evidentiary profiles which often display degradation that may have a greater impact on some contributors than others. Confounding factors such as stutter, stacking, and assumptions about peak height ratios can make it difficult to assess an individual's percent contribution at certain markers. However, in general one can expect percent contribution to remain fairly constant thereby supporting the validity of the theory behind the "Total RFU" model for evaluating the possibility of allelic drop out for a given contributor (i.e. the loci where the total RFU is higher than the total RFU of the strongest locus where the individual drops out can be relied upon for statistical purposes).

Additionally, this study confirms that the previously determined 200 RFU stochastic threshold is also applicable to DNA mixtures.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Increased Injection Time Study

Purpose

The goal of this study is to evaluate the use of extended injection times during capillary electrophoresis of samples amplified with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) and to determine the appropriate analysis parameters to use for these samples.

Materials and Methods

One amplification blank containing only TE buffer was amplified for 28 cycles using ID Plus. Capillary electrophoresis was performed using 5, 7, and 10 second injection times. The resultant data was analyzed in GMID-X with a 1 RFU detection threshold.

Three employee reference samples were amplified for 28 cycles using ID Plus at two low level DNA inputs (~50 and 90 pg) in an effort to generate profiles that straddle the 30 RFU detection threshold. Capillary electrophoresis was performed using 5, 7, and 10 second injection times.

Eight samples previously amplified for 28 cycles using ID Plus as part of the Stochastic Study were re-prepared for capillary electrophoresis. Capillary electrophoresis was performed with duplicate injections using 5, 7, and 10 second injection times.

Two samples from the Challenged Samples Study (84b and (3-1)) were amplified for 28 cycles using ID Plus. Capillary electrophoresis was performed using 5 and 10 second injection times.

Results and Discussion

The baseline noise for the amplification blank was evaluated for the 5, 7, and 10 second injection (Figure 1). In all instances the average plus 10 standard deviations was less than 30 RFU (Table 1).

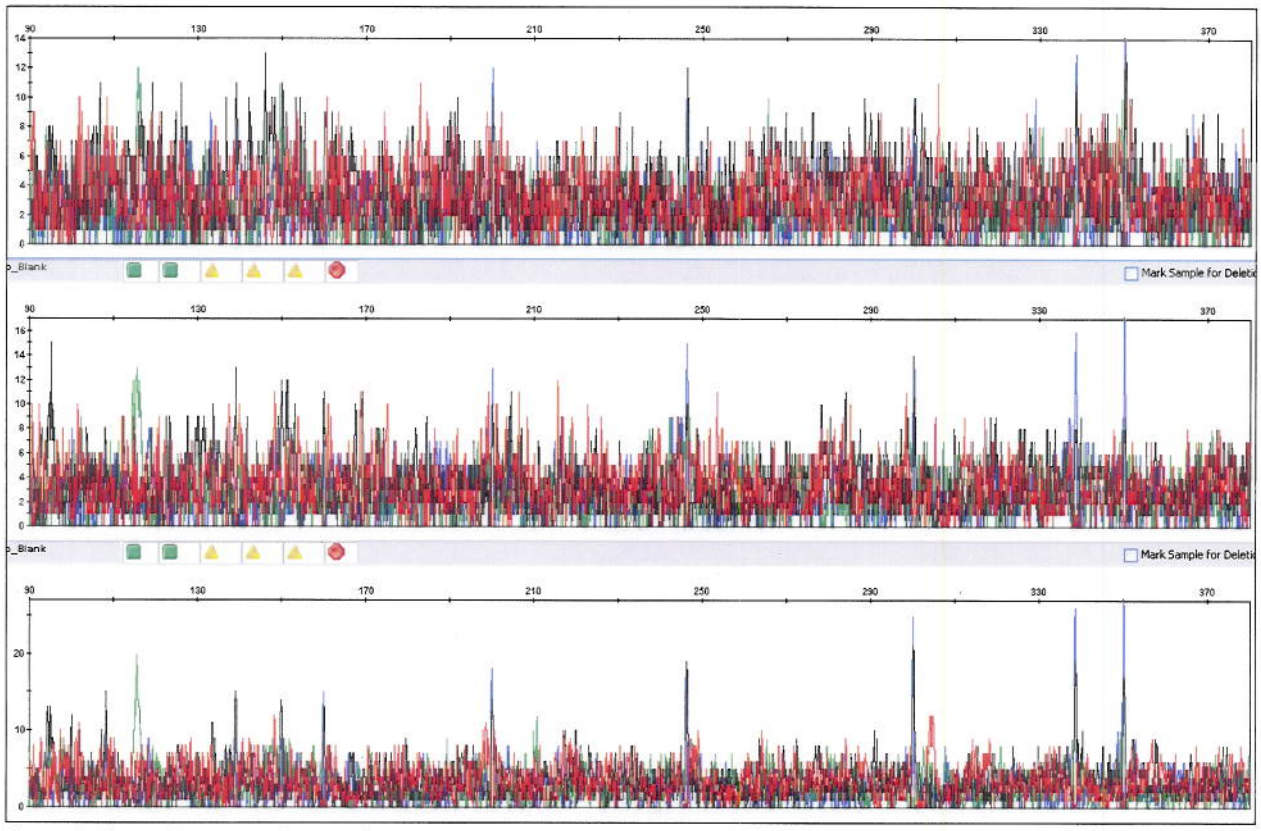


Figure 1: Electropherograms for amplification blank injected for 5, 7, and 10 seconds respectively.

Table 1: Baseline levels for amplification blank at three different injection times.

Injection Time (s)	Average (RFU)	Standard Deviation	Avg + 3SD	Avg + 10SD
5	5.30	1.94	11.12	24.72
7	5.42	1.99	11.41	25.37
10	5.52	2.24	12.25	27.94

The increase in signal intensity for all samples containing DNA was examined by comparing the peak heights of alleles which were detected at all three injection times (Table 2 and Figure 2). This data was further evaluated to determine whether or not the increase in peak heights is marker specific (Figures 3 and 4).

Table 2: Fold increase in peak height for samples injected for 7 and 10 seconds versus the standard 5 second injection time. In a perfect system the fold increase for the 7 second injection versus the 5 second injection would be 1.4 and for the 10 second injection would be 2.0.

Injection Time (s)	Peak Height Increase			
	Average	Standard Deviation	Minimum	Maximum
7	1.349	0.111	1.086	1.604
10	1.833	0.164	1.129	2.206

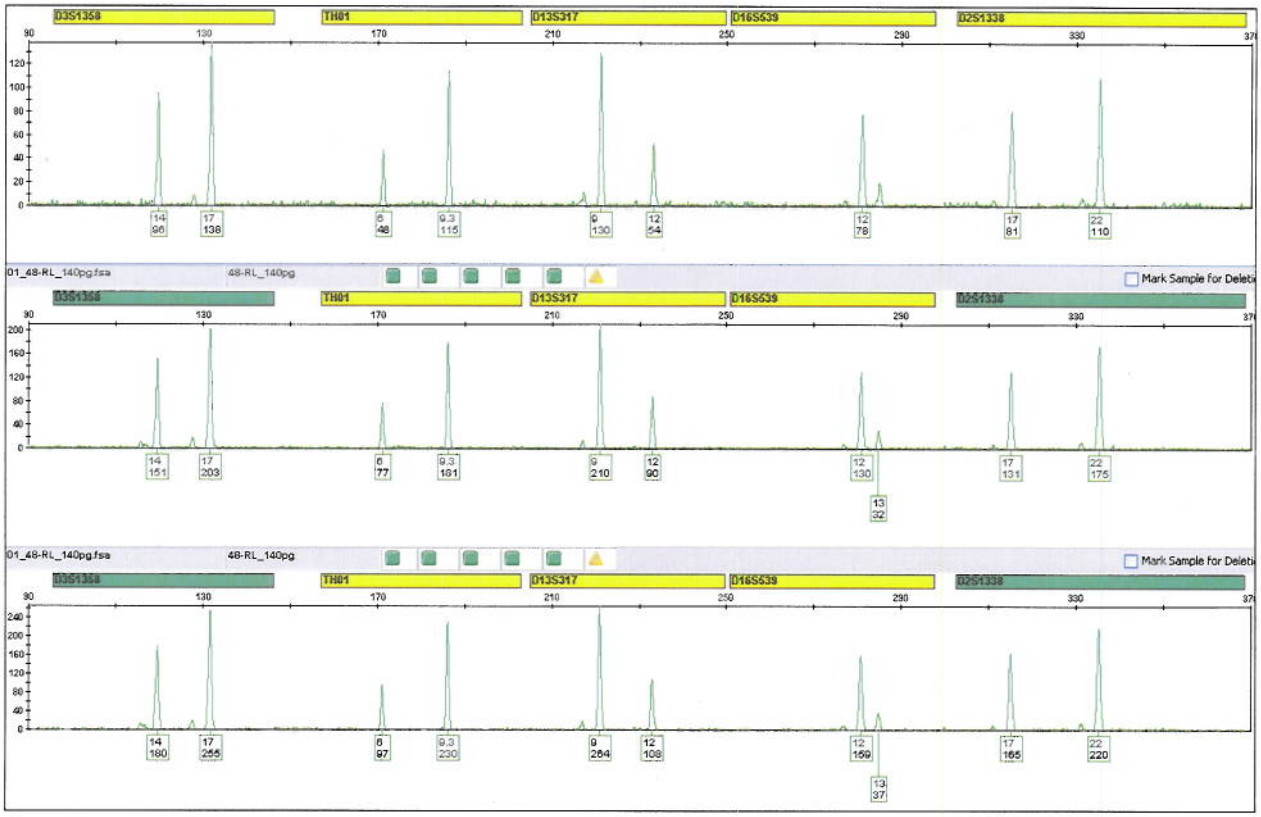


Figure 2: Electropherograms showing the green (VIC) dye channel of the same sample injected for 5, 7, and 10 seconds respectively.

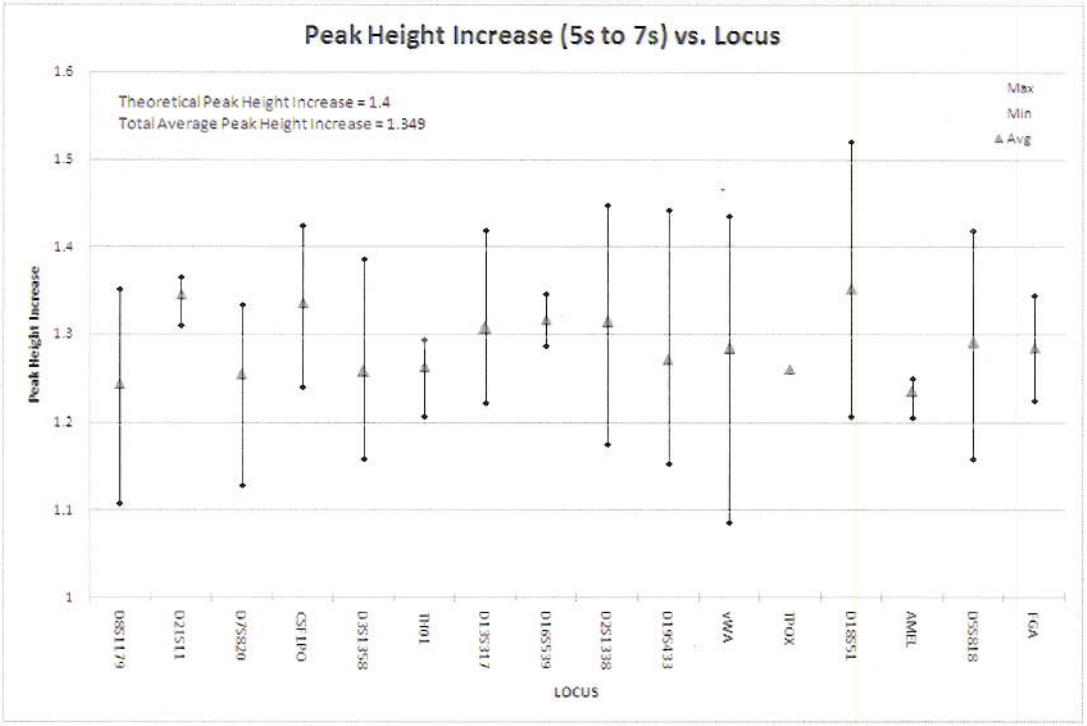


Figure 3: Peak height increase at each locus when going from a 5 to 7 second injection.

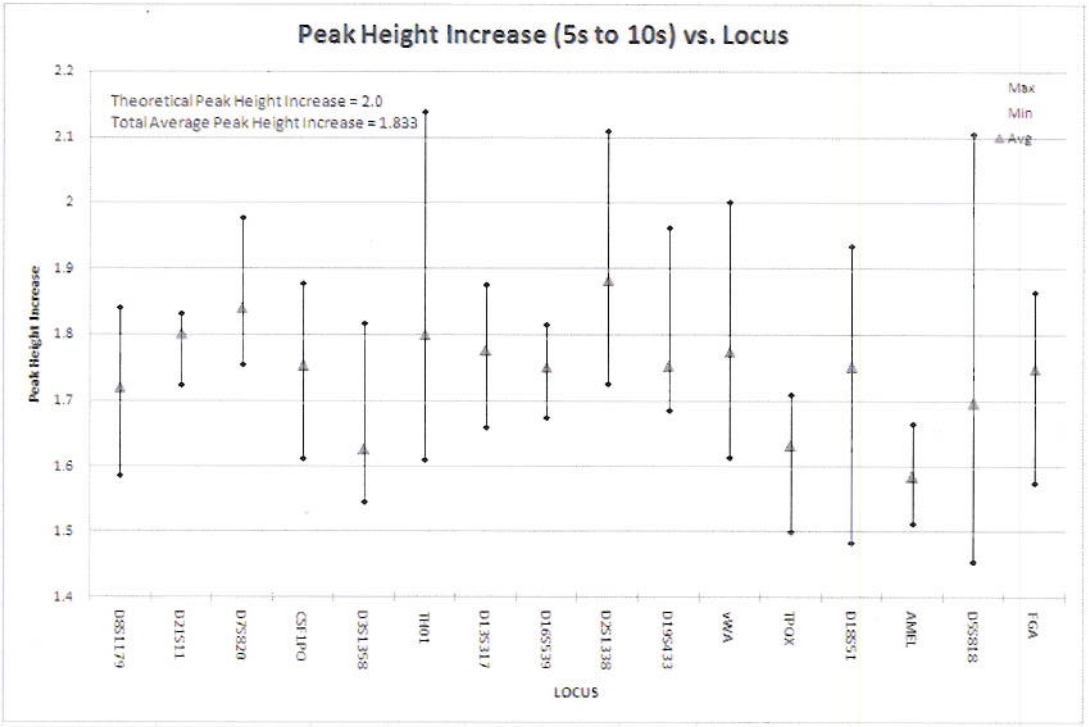


Figure 4: Peak height increase at each locus when going from a 5 to 10 second injection.

In all instances the number of alleles detected either increased or remained the same as injection time increased (Table 3).

Table 3: Comparison of alleles detected at three different injection times.

ID	Input DNA (~pg)	Number of Alleles							
		Expected	Injection Time (s)						
			5		7		10		
14-JS	50	29	2		3		8		
14-JS	90	29	3		-		8		
48-RL	140	28	25	25	27	26	28	28	
52-KK	50	29	19	17	19	19	21	21	
67-FH	50	27	9	8	14	12	19	18	
67-FH	50	27	2		5		10		
67-FH	90	27	18	15	20	21	23	23	
67-FH	90	27	10		17		20		
67-FH	140	27	25	25	26	26	26	26	
80-DT	50	26	18	17	20	19	22	24	
80-DT	50	26	21		22		23		
80-DT	90	26	25	24	25	25	25	26	
80-DT	90	26	25		26		26		
80-DT	140	26	26	26	26	26	26	26	
(3-1)	3000	n/a	15		-		18		
84b	1400	n/a	18		-		20		

The tallest single peak detected when there was known to be allelic dropout of the sister peak was 172, 227, and 315 RFU using 5, 7, and 10 second injection times respectively (Table 4 and Figure 5).

Table 4: Peak heights of the single detected allele of a known heterozygote when the sister allele has dropped out.

Injection Time (s)	Peak Height (rfu)				
	Minimum	Maximum	Average	Standard Deviation	Avg. + 3 S.D.
5	30	172	59.95	28.47	145.37
7	30	227	66.22	40.83	188.72
10	30	315	80.06	60.56	261.74

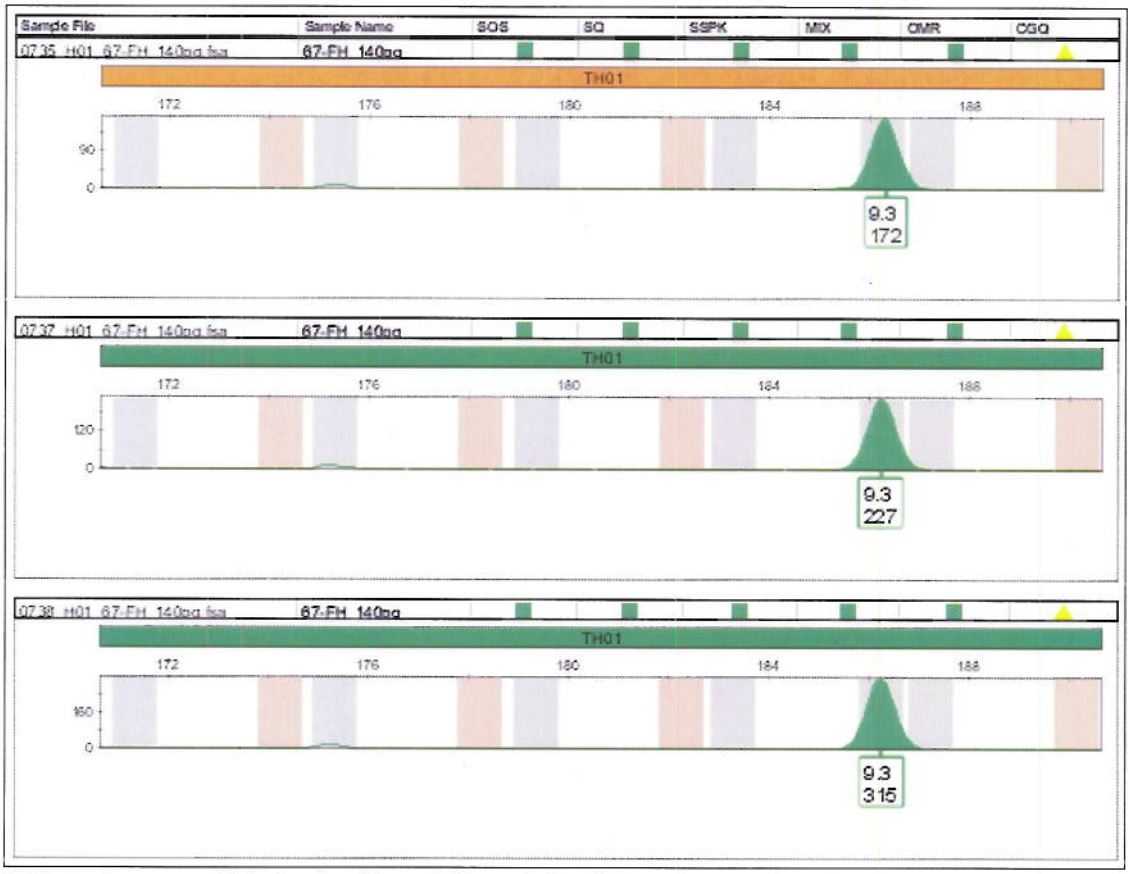


Figure 5: Electropherograms displaying the tallest peak detected when there was known to be allelic dropout of the sister peak (7 allele) at 5, 7, and 10 second injection times respectively.

One sample displayed a single extra peak that was not detected when injected for only 5 seconds during the Stochastic Study (Figure 6). This extra peak was called in one of the 7 second injections and both of the 10 second injections. The peak was visible but not called in the 5 second injections. The peak does not appear to be an artifact. There are no other extra peaks called in the sample. The extra peak corresponds to a known allele for all of the other samples in this run; therefore, sample to sample contamination is a possibility.

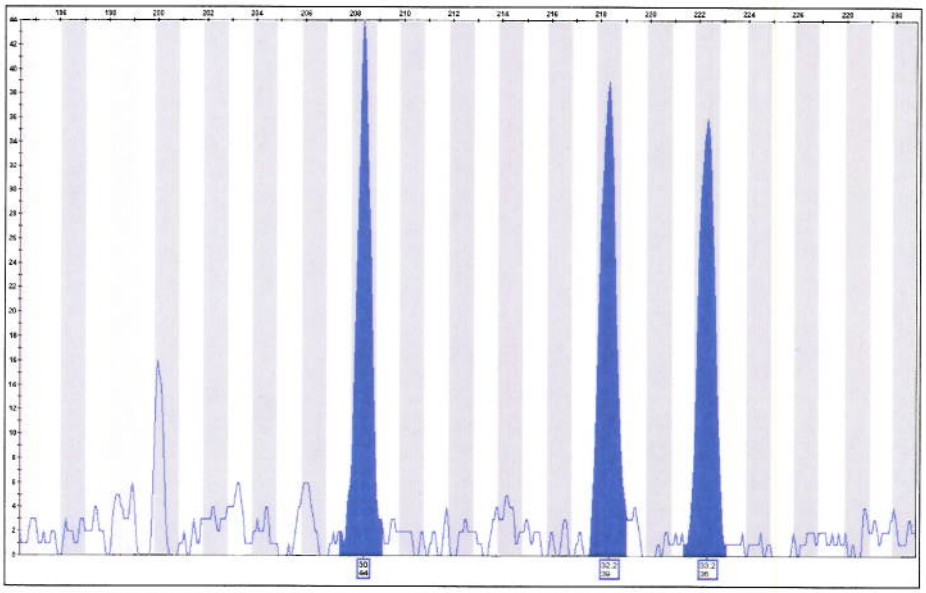


Figure 6: An extra peak (33.2 allele at D21S11) was detected in one of the samples (67-FH_90pg).

Conclusions

Evaluation of the baseline noise levels from the amplification blank verifies that the 30 RFU detection threshold is suitable for use with 5, 7, and 10 second injection times.

In theory, peak heights should double when the injection time is increased from 5 seconds to 10 seconds and should increase 1.4 times for samples injected for 7 seconds. In this study the actual average increases were ~1.83 and 1.35 times respectively. These increases did not appear to be locus dependent. The tallest peaks detected when there was known to be allelic dropout of the sister peak were close to the average increases with peaks of 172, 227, and 315 RFU for the 5, 7, and 10 second injection times respectively. For increased injection times it is necessary to elevate the stochastic threshold in order to account for this increase in peak heights. The conservative approach is to apply the theoretical signal increases. Therefore, stochastic thresholds of 280 and 400 RFU are recommended for 7 and 10 second injection times respectively.

Increased injection times are expected to result in an increase in the number of alleles detected for a sample and the data from this study supports this.

A single extraneous peak was observed with increased injection time of one sample in this study. Upon further inspection of the 5 second injections this peak was visible but not detected. This contamination event appears to be sample specific and not a systematic problem. This observation re-iterates the idea that the data from the 5 second injection should be closely evaluated for apparent single source samples prior to injecting for a longer duration.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Challenged Samples Study

Purpose

The goal of this study is to demonstrate the utility of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) on challenging adjudicated casework and mock casework samples.

Materials and Methods

Seven challenging adjudicated casework and mock casework samples were previously amplified and analyzed using the Identifiler and Minifiler amplification kits (Table 1). The DNA extracts were amplified for 28 cycles using ID Plus. Capillary electrophoresis was performed and the resultant data was analyzed in GMID-X.

Table 1: Description of samples used for the Challenged Samples Study.

Sample	Description
AC	Known sample spiked with cola (potential PCR inhibitor).
P_1(50)	Known sample spiked with Pepsi (PCR inhibitor).
84b	Bloodstain from a t-shirt in a 1977 homicide (inhibited and degraded).
27	Reference hair sample from a decomposed homicide victim.
(2-1)	Bone sample (degraded).
(2-2)	Bone sample (degraded).
(3-1)	Bone sample from a 1992 helicopter crash (degraded).

Results and Discussion

Table 2 presents the DNA typing results obtained using Minifiler, Identifiler, and ID Plus for comparison. For five of the seven samples (samples P_1(50), 84b, 27, (2-1), and (2-2)) more genetic information was obtained with ID Plus when compared to the Identifiler results. In one of the remaining two (sample (3-1)), ID Plus resulted in three fewer alleles (16) detected than Identifiler (19). Only five alleles were obtained for sample AC with ID Plus, even though a full profile was obtained with Identifiler (there was only a small amount of DNA extract remaining and it was dark brown in color, so it is possible that these results reflect the quality of the remaining extract as much or more so than the capabilities of ID Plus).

Table 2: Minifiler (MF), Identifiler (ID), and Identifiler Plus (ID +) DNA typing results for Challenged Samples Study. ND = No DNA types obtained.

Kit	Sample	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	Amel	D5S818	FGA
MF	AC	ND	28	8	11,12	ND	ND	8,12	9,11	17,19	ND	ND	ND	9,19	X,Y	ND	20,23
ID	AC	12,15	28,29.1	8	11,12	15	6,8	8,12	9,11	17,19	14	15,16	8,11	9,19	X,Y	13,14	20,23
ID+	AC	12,15	ND	ND	ND	ND	ND	ND	ND	ND	14	ND	ND	ND	XY	ND	ND
MF	P_1(50)	ND	28,30,32.2	8,10,12	12	ND	ND	8,11	9,10	17,19	ND	ND	ND	14,20	X,Y	ND	21,22
ID	P_1(50)	11,12,15	30,32.2	10,12	12	15	6,9	11	9,10	17,19	12,13	15,18	8,11	14,20	X	11,12	21,22
ID+	P_1(50)	11,12,15,14	28,30,32.2	8,10,12	12,13	15	6,8,9	8,11	9,10,11	17,19	12,13	15,16,18	8,11	14,20	X	11,12,14	21,22
MF	84b	ND	28,30	11,12	10,12	ND	ND	11,12	12	17,26	ND	ND	ND	12,17	X	ND	23,24
ID	84b	12,15	ND	ND	ND	15,19	ND	ND	ND	ND	12,14	16,19	ND	ND	X	11,13	23
ID+	84b	12,15	28,30	ND	ND	15,19	8,9	ND	12	ND	12,14	16,19	8	ND	X	11,13	23,24
MF	27	ND	28,30	8,10	10,12	ND	ND	9,12	11,14	16,24	ND	ND	ND	12,17	X,Y	ND	21,23
ID	27	13,15	ND	ND	ND	15,17	8,9	9,12	ND	ND	14,15.2	19	8	ND	X,Y	9,11	ND
ID+	27	13,15	28,30	8,10	10,12	15,17	8,9	9,12	11,14	16,24	14,15.2	16,19	8	12,17	XY	9,11	21,23
MF	(2-1)	ND	29,30,31	11,12	11,12	ND	ND	9,11,12,13	11,12,13,14	17,20,24	ND	ND	ND	13,15	X,Y	ND	19,23,25
ID	(2-1)	13	ND	ND	ND	15,18	6	ND	ND	ND	12,13,14	14	ND	ND	X,Y	9	ND
ID+	(2-1)	13,14,15	29,30,31	11	11	15,16,18	6,9.3	9,11,13	11,12	ND	12,13,14	14,16,17,18	8	15	XY	9,10,12,13	19,23,25
MF	(2-2)	ND	29,30,31,32.2	11,12	11,12	ND	ND	9,11,12,13	11,12,13,14	17,20,24	ND	ND	ND	13,15	X,Y	ND	19,23,25
ID	(2-2)	13	ND	ND	ND	15,16	6	ND	ND	ND	12,13,14	18	ND	ND	X,Y	9,13	ND
ID+	(2-2)	13,14,15	29,30,31,32.2	11	11	15,16,18	6,9.3	9,11,12,13	10,11,12,13,14	17,24	10,11.2,12,12.2, 13,14,14.2	14,15,16,17,18	8,11	13,15	XY	9,10,12,13	19,23,24,25
MF	(3-1)	ND	31.2,35.2	9,10	10	ND	ND	8,12	12,13	21,23	ND	ND	ND	14,17	X,Y	ND	19,24
ID	(3-1)	13,15	31.2	ND	ND	15,18	6,8	8	12	ND	11,13	18	8	ND	X,Y	11,12	19,24
ID+	(3-1)	13,15	ND	ND	ND	15,18	6	8	12	ND	13	18	8	ND	XY	11,12	19,24

A comparison of the electropherograms for sample 27 generated from the Identifiler, Minifiler, and ID Plus analyses demonstrates the superior quality of the ID Plus profile (Figures 1, 2, and 3).

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Contamination Assessment Study

Purpose

The goal of this study is to verify that contamination is not a concern for samples amplified with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

Over the course of the validation a total of 191 samples have been amplified for either 28 or 29 cycles using ID Plus. Fourteen of these samples were amplification blanks containing only TE buffer. The remaining 177 samples were either from known sources (9947A positive control and employee reference samples) or previously analyzed with the Identifiler kit (adjudicated casework samples).

Results and Discussion

No DNA types were detected in any of the 14 TE amplification blanks.

One sample had a single extra peak detected during the Increased Injection Time Study. The peak was not detected when the sample was originally injected for only 5 seconds during the Stochastic Study. However, the extra peak was called in one of the 7 second injections and both of the 10 second injections (Figure 1). Upon further inspection of the 5 second injections the peak was visible but not called. The peak does not appear to be an artifact. There are no other extra peaks called in the sample. The extra peak corresponds to a known allele for all of the other samples in this run; therefore, sample to sample contamination is a possibility.

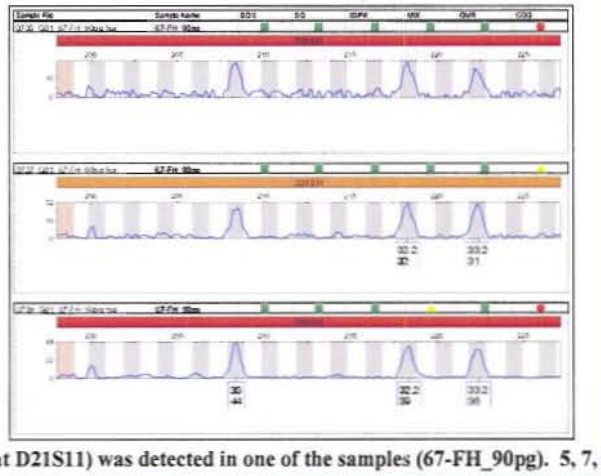


Figure 1: An extra peak (33.2 allele at D21S11) was detected in one of the samples (67-FH_90pg). 5, 7, and 10 second injections shown.

No extraneous DNA was detected in any of the remaining 176 samples containing amplified DNA.

Conclusions

A single extraneous peak was observed with increased injection time of one sample in this validation. Upon further inspection of the 5 second injections this peak was visible but not detected. This contamination event appears to be sample specific and not a systematic problem.

Contamination is not an inherent problem associated with the ID Plus kit. Each new lot # of ID Plus kits received into the laboratory will have a quality control ("QC") run performed prior to use in casework. This QC and the TE amplification blank included in each amplification will serve as ongoing verification that the components of the ID Plus kits do not exhibit inherent contamination.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Reproducibility Study

Purpose

The goal of this study is to verify that the results obtained from samples amplified with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus) are reproducible.

Materials and Methods

Over the course of the validation a total of 177 samples containing DNA have been amplified for either 28 or 29 cycles using ID Plus. Included in each amplification was at least one 9947A positive control. The same 20 employee reference samples were used throughout the validation, therefore, many of these samples were amplified multiple times.

Results and Discussion

The results from the 9947A positive control were consistent between each amplification with the ID Plus kit and were consistent with the known profile published by the manufacturer. Replicates of the same employee reference sample produced concordant results between multiple ID Plus amplifications and with previously obtained Identifiler results.

Conclusions

Results obtained with ID Plus are reproducible.

SDPD Forensic Science Section – Forensic Biology Unit

Validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit from Applied Biosystems

Precision Study

Purpose

The goal of this study is to evaluate the sizing precision of data generated from samples amplified with the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit (ID Plus).

Materials and Methods

The 250 base pair (bp) peak of the GS500 internal size standard shows variability between samples. To evaluate the extent of this variability the 250 bp peaks from 62 samples and controls from the Mixture Study were compared.

Results and Discussion

An overlay view of the 250 bp peaks for the 62 samples visually illustrates the small amount of variability between samples (Figure 1). The very small standard deviation of 0.0427 further demonstrates the precision of the results (Table 1).

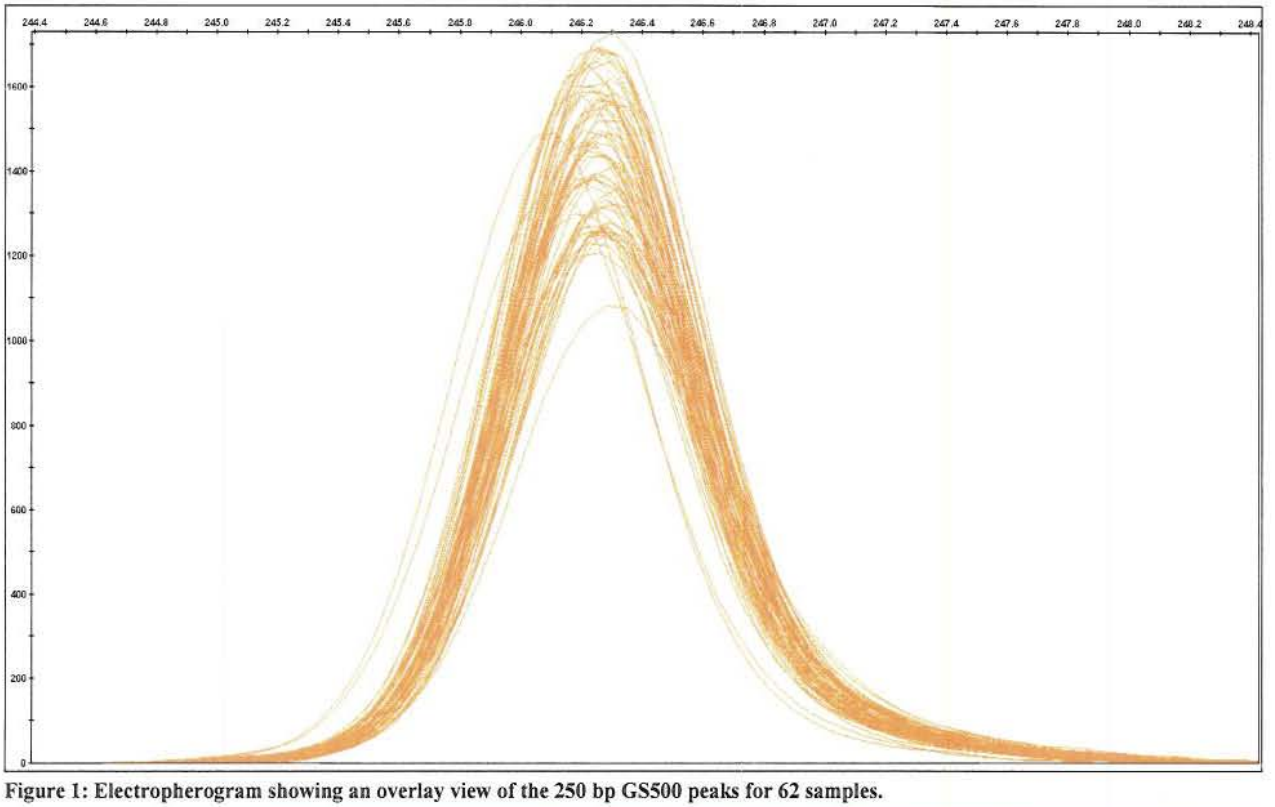


Figure 1: Electropherogram showing an overlay view of the 250 bp GS500 peaks for 62 samples.

Table 1: Minimum, maximum, average, and standard deviation size of the 250 bp GS500 peaks for 62 samples.

Description	Size (bp)
Minimum	246.11
Maximum	246.32
Average	246.25
Standard Deviation	0.0427

Conclusions

Precision is more closely associated with the capillary electrophoresis instrument than the DNA typing kit. With this in mind, the results obtained following ID Plus amplification demonstrated acceptable levels of precision.