Internal validation and implementation of the PowerPlex® Fusion amplification system for forensic DNA casework

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Introduction

The impending expansion of the CODIS core loci in the U.S. and the European Standard Set in Europe has created the need for new STR amplification kits. In response, Promega Corporation has developed the PowerPlex® Fusion kit. PowerPlex® Fusion is a 24 loci multiplex reaction that includes the existing 13 CODIS core loci, all of the new required loci, two of the three recommended loci, plus Penta D and Penta E [1-3]. PowerPlex® Fusion is a five dye chemistry which is compatible with existing validated equipment and software (i.e. AB® 3100/3130xl genetic analyzers and GeneMapper® v3.2/IDX). This is a critical benefit as it will greatly simplify the validation, implementation, and training process.

Our laboratory recognizes the impending need for an expanded STR amplification kit to satisfy upcoming NDIS requirements. In addition to complying with new standards in the field, we also realize the practical benefits of an improved and expanded multiplex reaction and believe implementation of the PowerPlex® Fusion system will offer superior quality STR typing results. An internal validation was conducted to assess the capabilities of the PowerPlex® Fusion amplification kit for use in forensic DNA casework. The following topics were addressed: concordance, reproducibility and precision, sensitivity, mixtures, species specificity, artifacts, stutter percentages, contamination, analytical and stochastic thresholds, and statistics. A variety of sample types were tested with a focus on challenged samples (i.e. inhibited and/or degraded, low level, and aged samples).

Materials and Methods

DNA extractions were conducted using organic or Chelex extraction protocols. Quantitation was performed using the Applied Biosystems Quantifiler® kit on the AB 7000 Sequence Detection System. Identifiler® Plus, Minifiler®, and PowerPlex® Fusion amplification were performed on the GeneAmp® 9700. Amplified samples were run on the AB 3130xl and analyzed with GeneMapper® v3.2 software. All previously validated methods were performed according to protocol in the TDS Procedures Manual [4]. An analytical threshold of 50 RFU was used. PowerPlex® Fusion amplification and capillary electrophoresis were conducted based on the recommendations in the Promega PowerPlex® Fusion manual as detailed below in figures 1 and 2 [3].

Component	Volume Per Sample
PowerPlex® Fusion 5x Master Mix	5 uL
PowerPlex® Fusion 5x Primer Pair Mix	5 uL
Template DNA (0.25 – 0.50 ng)	up to 15 uL
Amplification Grade Water	to a final volume of 25 uL

Figure 1: Amplification reaction setup for a 25uL PowerPlex® Fusion amp.

For samples, template DNA was added (up to 15 uL) to reach a 0.25 - 0.5 ng target concentration. 2800M positive control DNA (10 ng/uL) was diluted 1:100 (.10 ng/uL) and 5 uL was added with 10 uL of water for an amplification positive control of 0.5ng concentration. An amplification blank (negative control) was run with 15 uL of the provided amplification grade water.

Initial Incubation Step	Denature/Anneal/Extend			Final Extension	Final Hold
HOLD	30 CYCLES			HOLD	HOLD
96° C 1 min	94° C 10 sec	59° C 1 min	72° C 30 sec	60° C 10 min	4° C ∞

Figure 2: Thermal cycling protocol for PowerPlex Fusion amp. As specified in the manual, the protocol was run with Max Ramp speed selected.

Capillary electrophoresis fragment analysis was carried out in a similar manner to previous amplification systems. The PowerPlex® Fusion manual outlines the necessary steps to set up the reaction as well as prepare the GeneMapper® software for analysis [3]. The reaction consisted of a master mix of CC5 internal lane standard (1 uL per sample) and deionized formamide (10 uL per sample) with 1 uL of amplified DNA or allelic ladder being added [3]. Prior to running and analyzing PowerPlex® Fusion samples, the provided panels/bins, size standard, analysis method, and stutter files must be imported according to the manuals instructions. In addition, there must be an active PowerPlex® Fusion spectral calibration file selected prior to running the plate. No changes to the reagents required for fragment analysis (i.e. capillary array, POP4, buffer, etc) were needed and the run parameters (i.e. injection time, voltage, etc) also remain the same.

Results and Discussion

Concordance

Six previously typed standards (using Identifiler® Plus) and one NIST standard (NIST 2391c_A) were processed with PowerPlex® Fusion and a comparison of all allele calls were made to determine if there were any discrepancies. All seven of the known standard profiles produced concordant results with no different allele calls observed.

Precision and Reproducibility

Precision (within the same sample run) of the PowerPlex® Fusion results was assessed by amplifying identical sample extracts at the same time in triplicate (Sens4 and Sens5) and running the amplified products on the same 3130xl run. Reproducibility (between different sample runs) was assessed by amplifying identical sample extracts on separate days and running the amplified products on separate 3130xl runs. The data was compared to determine if the results for the identical samples were comparable within the same sample run (precision) and between separate runs (reproducibility). The data charts below (figure 3) illustrate the base pair sizing variance and standard deviation as well as the number of alleles called and total RFU heights for the different amps/runs.

AMP 1:

	Base Pair Sizing - Average Sizing Variance
Sens4 (x3)	0.0009
Sens5 (x3)	0.0015

	Base Pair Sizing - Average Sizing StDev
Sens4 (x3)	0.0247
Sens5 (x3)	0.0339

AMP2:

	Base Pair Sizing - Average Sizing Variance
Sens4 (x3)	0.0007
Sens5 (x3)	0.0016

	Base Pair Sizing - Average Sizing StDev
Sens4 (x3)	0.0237
Sens5 (x3)	0.0355

# of Alleles Called	Sum of RFUs

	# of Alleles Called	Sum of RFUs
Sens4	38	21015
Sens4	38	20243
Sens4	38	16045
Sens5	30	6439
Sens5	30	5777
Sens5	32	7798
Sens4	38	14459
Sens4	38	14442
Sens4	38	12731
Sens5	23	2748
Sens5	26	2993
Sens5	30	4517

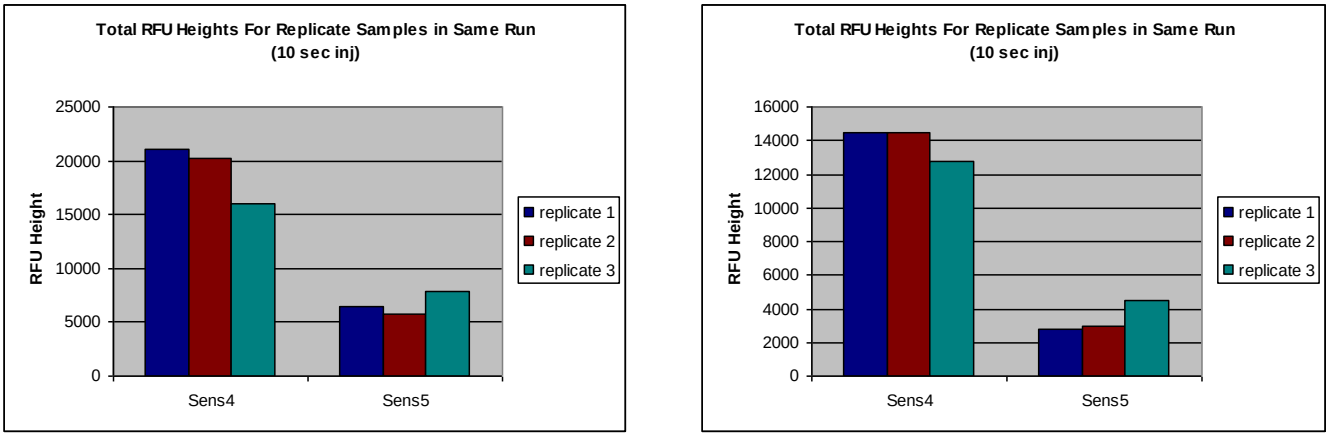

Figure 3: Precision and reproducibility data charts for replicate PowerPlex® runs.

Base pair sizing within the same amplification run as well as between the two separate amplifications was nearly equivalent. The number of alleles called and total RFU heights did have some minor variation but nothing unexpected. These differences could be attributed to pipetting error in amplification or 3130xl set up.

Sensitivity

Sensitivity of the PowerPlex® Fusion kit was assessed by amplifying a series of diluted 9947A (quantified at 0.366 ng/ul) positive control samples as shown in the chart below (figure 4). The maximum allowed 15uL of each sensitivity sample was amplified and all samples were run at 10 second injections.

	9947A dilution	estimated Qt (ng/uL)
Sens1	1:2	0.183
Sens2	1:4	0.0915
Sens3	1:8	0.0458
Sens4	1:16	0.0229
Sens5	1:32	0.0114
Sens6	1:64	0.0057
Sens7	1:128	0.0029
Sens8	1:256	0.0014
Sens9	1:512	0.0007
Sens10	1:1024	0.00035
Sens11	1:2048	0.000175

Figure 4: 9947A dilution series sensitivity samples.

The total number of alleles called and the percent of alleles called (of the expected full positive control profile) were determined. Eight of the lower level sensitivity samples (Sens4 – Sens11) were also processed with Minifiler® for comparison. See figures 5-7 below.

			PowerPlex® Fusion		Minifiler®	
	Qt ng/uL	**ng**	**# of Alleles Called (out of 38)**	**% called**	**# of Alleles Called (out of 15)**	**% called**
Sens1	0.183	2.745	38	100.00	NA	NA
Sens2	0.0915	1.3725	38	100.00	NA	NA
Sens3	0.0458	0.687	38	100.00	NA	NA
Sens4	0.0229	0.3435	37	97.37	13	86.67
Sens5	0.0114	0.171	34	89.47	6	40.00
Sens6	0.0057	0.0855	26	68.42	9	60.00
Sens7	0.0029	0.0435	15	39.47	1	6.67
Sens8	0.0014	0.021	3	7.89	2	13.33
Sens9	0.0007	0.0105	2	5.26	1	6.67
Sens10	0.00035	0.0053	1	2.63	1	6.67
Sens11	0.000175	0.0026	0	0.00	1	6.67

Figure 5: 9947A dilution series sensitivity samples – number and percentage of alleles called for PowerPlex® Fusion and Minifiler® amplifications.

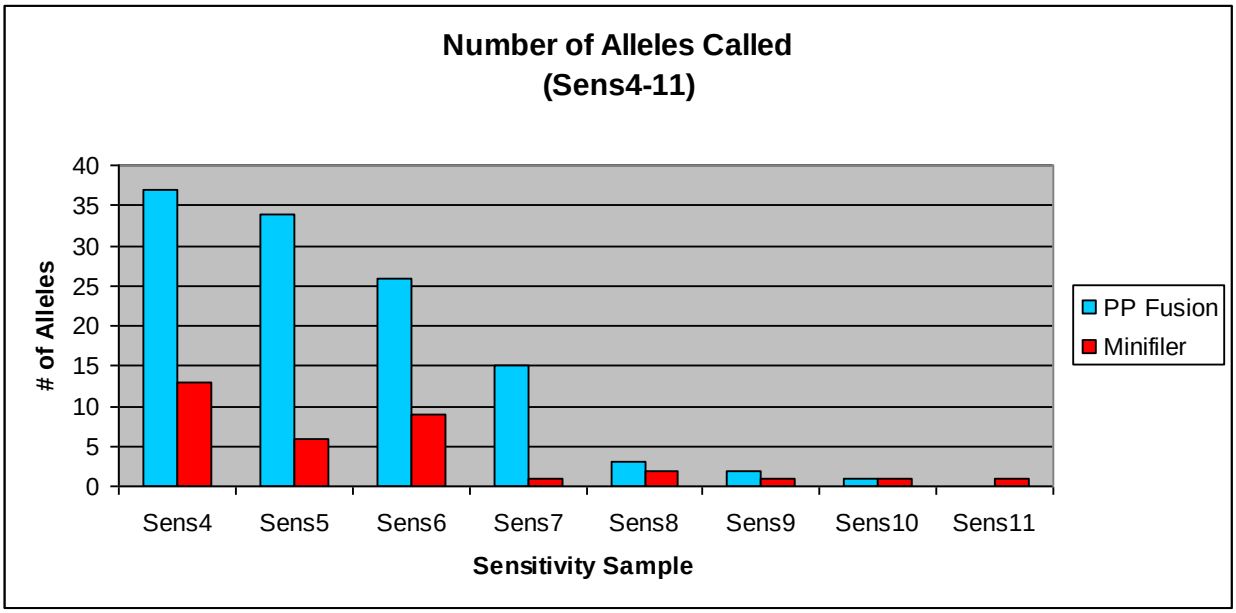

Figure 6: 9947A dilution series sensitivity samples 4-11 – number of alleles called for PowerPlex® Fusion and Minifiler® amplifications.

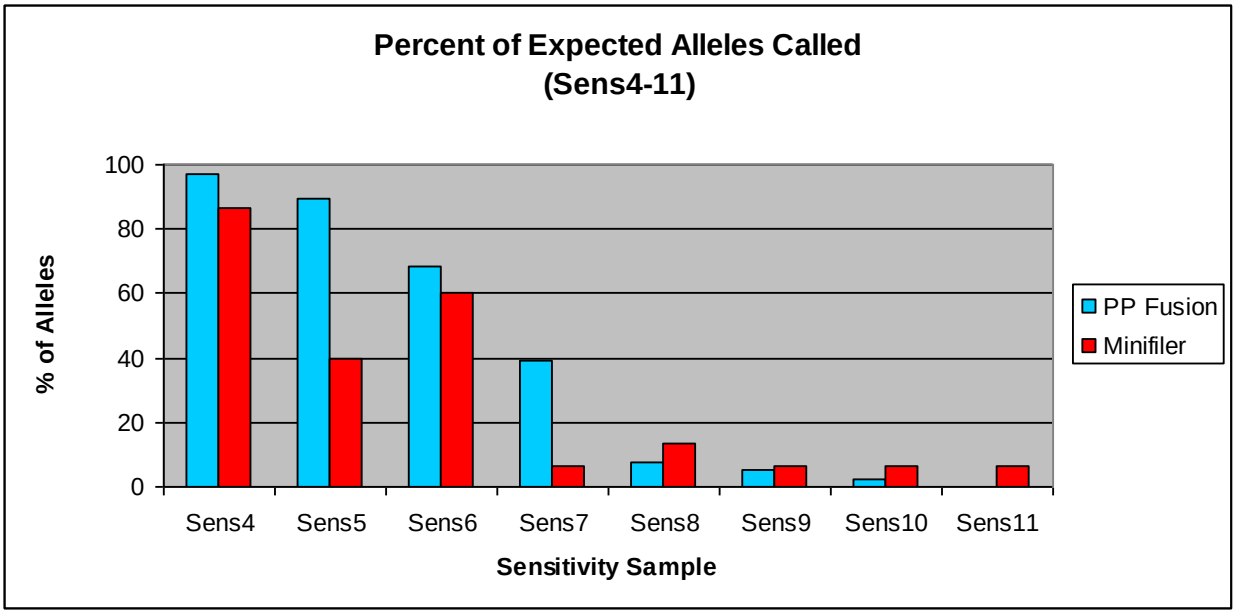

Figure 7: 9947A dilution series sensitivity samples 4-11 – percentage of alleles called for PowerPlex® Fusion and Minifiler® amplifications.

In addition to the above mentioned sensitivity samples, another 1:64 dilution of a different 9947A positive control lot was also processed (Sens7). This sample is from the previous Identifiler® Plus validation and is an approximately two year old frozen extract. The estimated concentration of sample Sens7 is 0.00156 ng/ul based on the manufacturers values. A quant of the sample came up undetected (0 ng/ul). Figures 8 -10 illustrate the total number of alleles called and the percent of alleles called (of the expected full positive control profile) for all four amplification types.

		Sens7 (0.00156 est ng/uL, 0 quanted ng/uL)
PowerPlex® Fusion	# Called	33
	% Called (of 38)	86.84
Minfiler®	# Called	13
	% Called (of 15)	86.67
ID® Plus (28 cycle)	# Called	1
	% Called (of 26)	3.85
ID® Plus (29 cycle)	# Called	10
	% Called (of 26)	38.46

Figure 8: Additional 9947A dilution sensitivity sample (Sens7) – number and percentage of alleles called for PowerPlex® Fusion, Minifiler®, and Identifiler® Plus (28 and 29 cycle) amplifications.

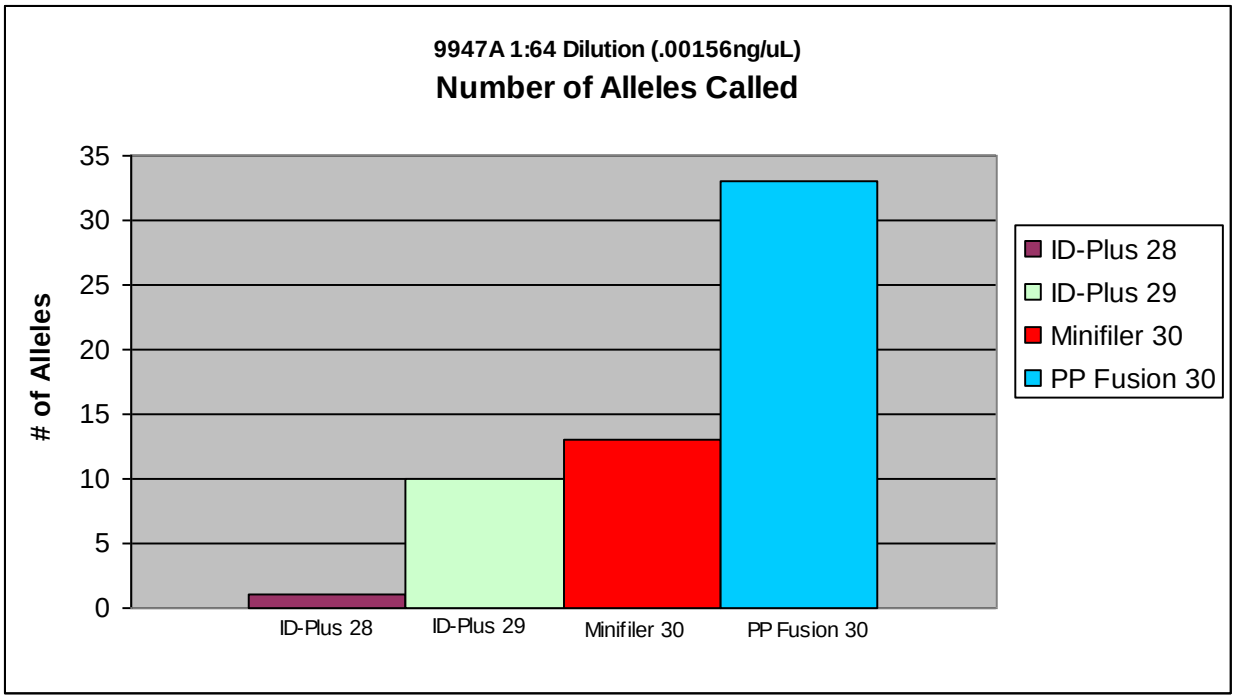

Figure 9: Additional 9947A dilution sensitivity sample (Sens7) – number of alleles called for PowerPlex® Fusion, Minifiler®, and Identifiler® Plus (28 and 29 cycle) amplifications.

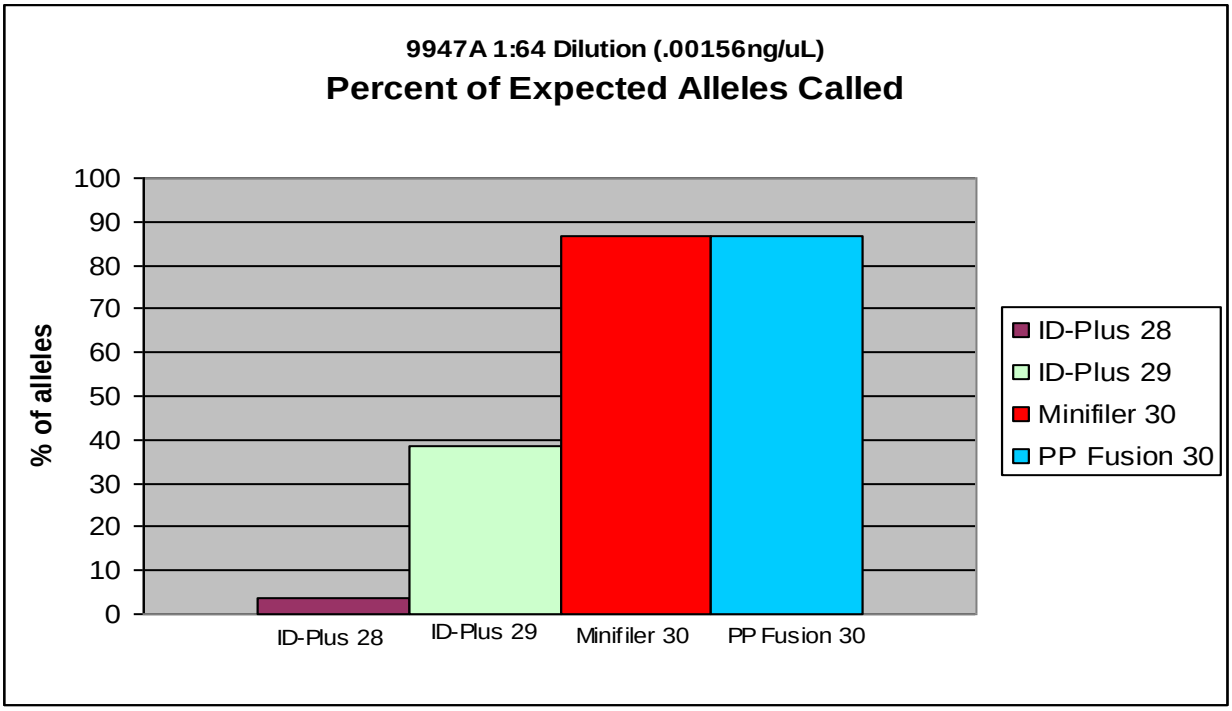

Figure 10: Additional 9947A dilution sensitivity sample (Sens7) – percentage of alleles called for PowerPlex® Fusion, Minifiler®, and Identifiler® Plus (28 and 29 cycle) amplifications.

It is difficult to put a precise number on the sensitivity limits of any amplification kit as the quality of the DNA sample can greatly affect the profile obtained. However, based on the above sensitivity data as well as the mock and

non-probative evidence sample data, it is highly likely that full profiles will be obtained at a target amp concentration of 0.5ng, assuming the DNA sample is of reasonably good quality (i.e. not severely degraded or inhibited). It is expected that target amp concentrations lower than this, down to approximately 0.2ng, also have the capability to obtain full or nearly full profiles. In fact, if a sample is suspected to be of sufficient quantity/quality (i.e. standards or samples where inhibition and degradation are not expected) it may be preferred to lower the target concentration to approximately 0.25ng, as 0.5ng may result in too high RFU values.

One of the advantages of the increased number of loci with this kit is that a partial profile (i.e. 17 loci) can actually be of higher discrimination potential than a full profile (i.e. 15 loci) from previous kits. Therefore, even significantly lower quant values can generate sufficient allele data of statistical significance. Specific quant values aside, it is apparent that PowerPlex® Fusion is significantly more sensitive than current Identifiler Plus® amplification protocols. This can be attributed to the increased PCR cycles (30) and the ability to add 15ul of template DNA vs 10ul for Identifiler® Plus reactions. In addition, while the kit components are proprietary, it has been designed to increase sensitivity and deal with inhibited or otherwise challenged samples. PowerPlex® Fusion's sensitivity is more in line with that of the Minifiler® kit. This is not as surprising as it may seem when you consider the similarities of the thermal cycling protocols and the fact that a number of the PowerPlex® Fusion loci do fall in the "mini" base pair sizing range (i.e. below 250 base pairs).

PowerPlex® Fusion Thermal Cycling Protocol [3]:

Initial Incubation Step	Denature/Anneal/Extend			Final Extension	Final Hold
HOLD	30 CYCLES			HOLD	HOLD
96° C 1 min	94° C 10 sec	59° C 1 min	72° C 30 sec	60° C 10 min	4° C ∞

Minifiler® Thermal Cycling Protocol [5]:

Initial Incubation Step	Denature/Anneal/Extend			Final Extension	Final Hold
HOLD	30 CYCLES			HOLD	HOLD
95° C 11 min	94° C 20 sec	59° C 2 min	72° C 1 min	60° C 45 min	4° C ∞

Figure 11: Comparison of PowerPlex® Fusion and Minifiler® thermal cycling protocols.

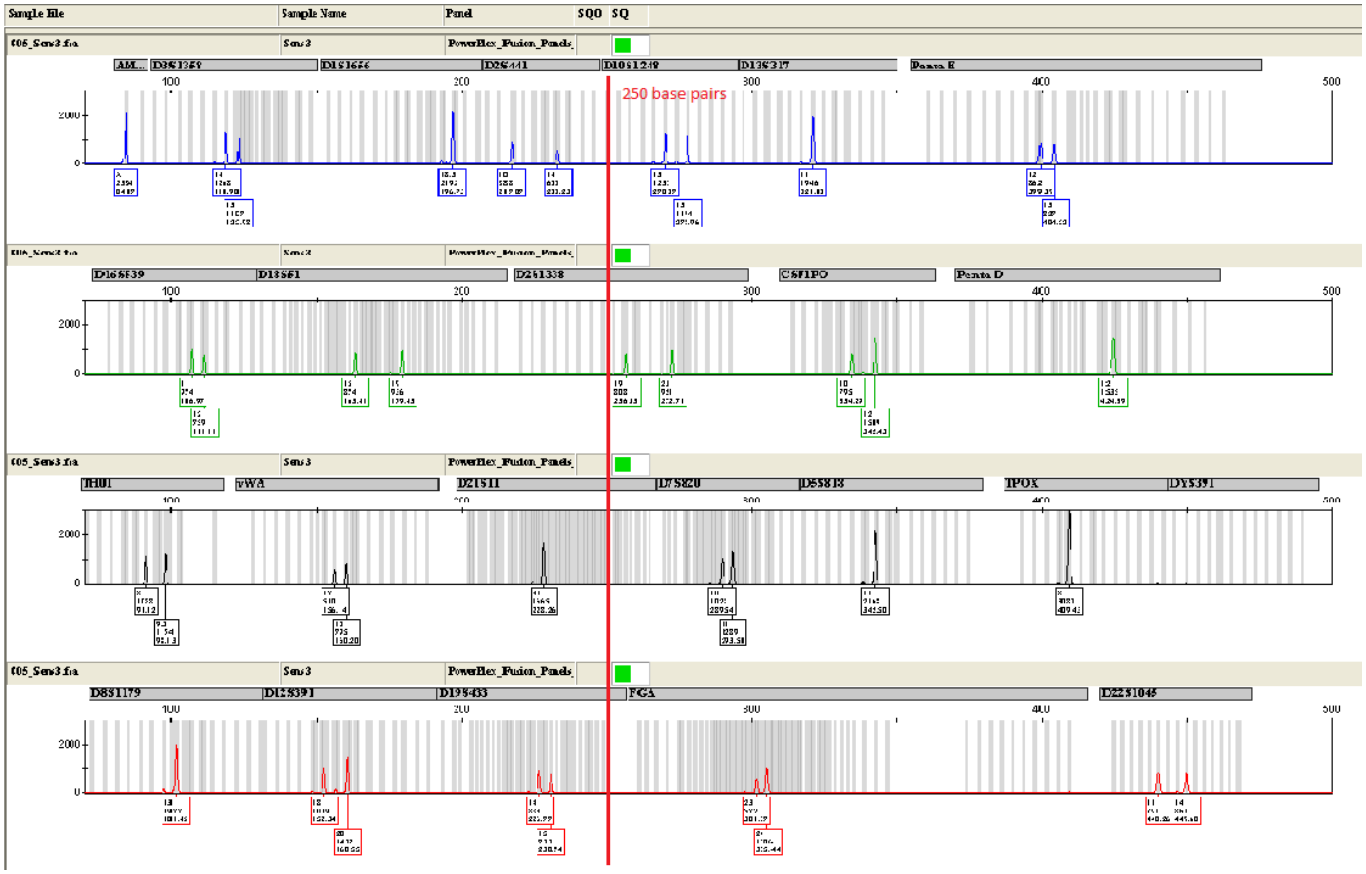

Figure 12: The red line indicates the 250 base pair sizing mark. A good portion of the PowerPlex® Fusion sizing range falls below this 250 base pair mark.

The increased sensitivity of the PowerPlex® Fusion kit is definitely an advantage when dealing with low level or challenged samples (inhibited and/or degraded) as is often the case in DNA casework. However, this increased sensitivity does not come without consequence. The ability to detect DNA down to such low levels means that an increase in stochastic amplification issues can be expected and are often times extreme. This will be covered subsequently in the stochastic threshold section. In addition to stochastic issues, analysts can expect the possibility of more complicated profiles (i.e. mixtures) as adventitious or extraneous profiles are picked up on more profoundly.

Mock and Non-Probative Evidence

Approximately 30 mock or non-probative evidence samples were processed with PowerPlex® Fusion. Majority of these samples were also processed or had previously been processed with Identifiler Plus® (or in some cases Identifiler®) so the results could be compared. The samples were specifically chosen to test the kit's capabilities with low level and/or challenged samples. Most all of the samples were one or more of the following types that we typically encounter with casework evidence: trace/low level, inhibited, degraded, aged, previously processed, sexual assault, firearm, drugs, or unusual evidence.

The PowerPlex® Fusion system performed impressively for a variety of sample types and challenged samples. Concordant profiles from the expected donors were obtained for all samples. In some instances, additional profiles were observed from explainable extrinsic sources. For example, one wearer profile sample from denim blue jeans resulted in a mixture of the known wearer and the wearer's husband's profile as well. The sensitivity and robustness of the system was quite apparent and it generated significantly more data than previous kits.

Mixtures

For the mixture assessment, mixture ratios of 1:1, 1:2, 1:5, 1:10, and 1:20 were created using a combination of known DNA standards. Full mixture profiles were obtained for all 1:1 and 1:2 mixture ratio samples. The other three mixture ratios (1:5, 1:10, and 1:20) showed the minor donor beginning to drop out as expected. By amplifying additional DNA ("blowing out the major"), nearly full minor profiles were able to be obtained. However, the major profiles did exhibit some off scale alleles and this method would not have passed standards for casework. The sensitivity of the PowerPlex® Fusion kit appears to allow for adequate detection of minor donor DNA even in the presence of much higher quantities of major donor DNA. The analyst should be careful when "blowing out" the major donor profile to bring up a minor profile as this may complicate analysis as artifacts become more severe. An attempt should be made to keep RFU values to a level where the minor profile can still be reliably deciphered.

Species Specificity

Promega's developmental validation study did reveal some cross-reactivity with certain species, namely Chimpanzee, Macaque, Gorilla, Chicken, Pig, and Mouse [6]. The following two figures show data from the Promega developmental validation presentation on their species specificity study [6].

Species	Qty	Cross-Reactivity
Chimpanzee (male)	0.5ng	Y
Macaque (male)	0.5ng	Y
Gorilla	0.5ng	Y
Chicken	10ng	Y
Pig	10ng	Y
Mouse	10ng	Y
Cow	10ng	N
Cat	10ng	N
Dog	10ng	N
Rabbit	10ng	N
Deer	10ng	N
Horse	10ng	N

Species	Qty	Cross-Reactivity
Escherichia coli	10ng	N
Enterococcus faecalis	10ng	N
Lactobacillus acidophilis	10ng	N
Streptococcus mutans	10ng	N
Staphylococcus epidermidis	10ng	N
Micrococcus luteus	10ng	N
Fusobacterium nucleatum	10ng	N
Streptococcus salivarius	10ng	N
Streptococcus mitis	10ng	N
Acinetobacter lwoffi	10ng	N
Pseudomonas aeruginosa	10ng	N
Candida albicans	10ng	N
Saccharomyces cervisae	10ng	N

Figure 13: Promega's developmental validation data charts on species specificity [6].

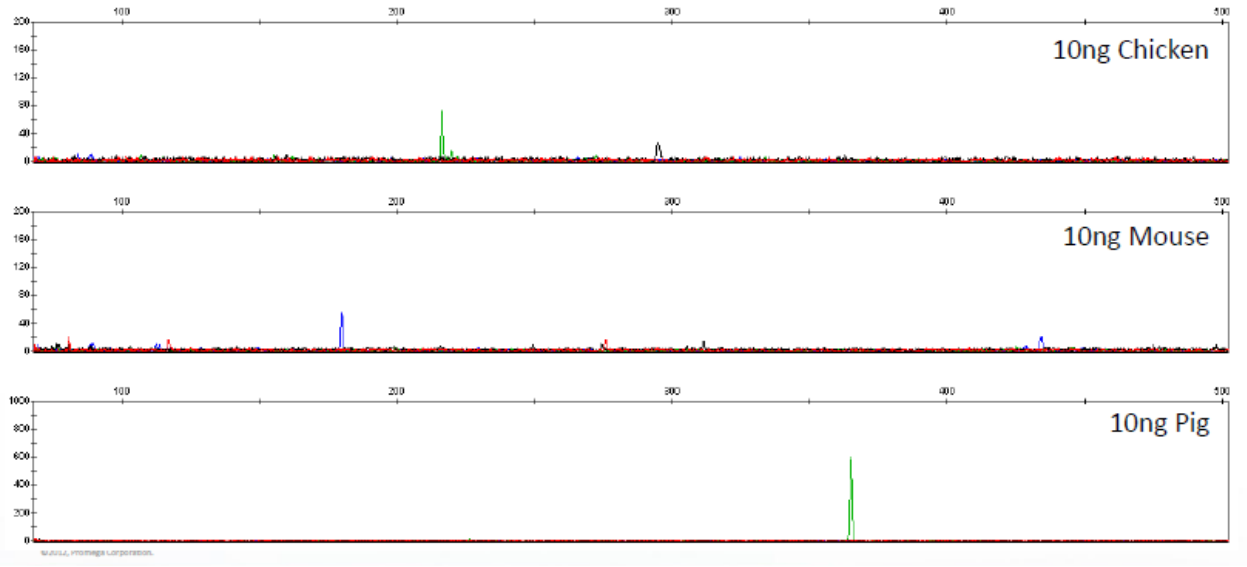

Figure 14: Promega's developmental validation data on chicken, mouse, and pig cross reactivity [6].

Primate cross-reactivity is expected with human STR typing kits due to the similarities in genetic markers. The observed chicken, mouse, and pig cross-reactivity resulted in stray, off-ladder (OL) peaks and not full profile information (as seen above).

Our laboratory's internal species study tested cat and dog blood as these are some of the more commonly encountered animal samples. Two separate dried cat blood samples (cat and cat2) and two separate dried dog blood samples (dog and dog2) were amplified with PowerPlex® Fusion. The first cat sample resulted in three OL peaks at D1S1656, D2S1338, and vWA (figure 15). All of the peaks were reproducible on re-injection. The second cat sample was negative. Promega provided their two cat data samples, neither of which had called peaks [6]. However, on close inspection of the electropherograms, the three OL peaks observed in our internal first cat sample did appear to be present at low levels in the Promega cat data at the same general base pair position. It is possible that these three OL peaks could be due to the presence of cat DNA, and therefore should be anticipated if a sample is thought to contain cat DNA.

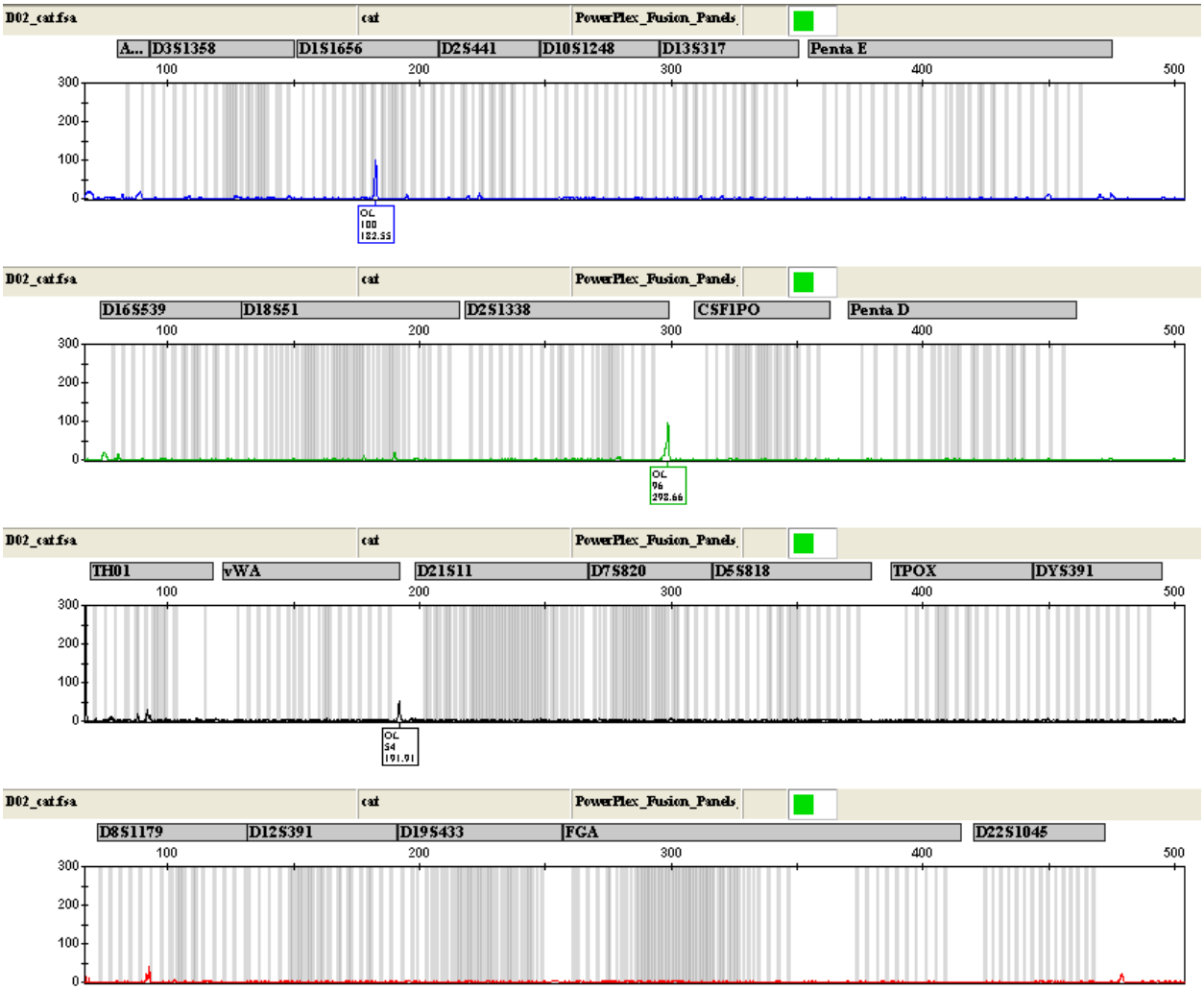

Figure 15: Three OL peaks observed in internal cat blood sample.

The first dog sample resulted in a called 29.1 peak at D21S11 that was reproducible on re-injection. The peak was not observed in the second dog sample or either of the two dog samples provided by Promega from their validation [6]. While all of the above mentioned potential cross-reactivity will be documented and the analysts made aware of, none of the observed cross-reactivity is severe enough to have a significant impact on the analysis of human profiles.

Artifacts

Artifacts, both biological and technological, are a well known part of PCR based STR amplification systems. It is the analyst's responsibility to understand the types of artifacts to expect and interpret them accordingly when analyzing DNA profiles. Artifacts specific to the PowerPlex® Fusion system will be covered hereafter while stutter will be discussed in a separate section.

The PowerPlex® Fusion manual details a number of various artifacts that may be encountered when employing this system [3]. The chart shown below (figure 16) summarizes the artifacts described in the manual as well as any additional artifacts observed during the internal validation study. An image of a PowerPlex® Fusion electropherogram with the artifact positions noted is also shown (figure 17) to help analysts visualize and better recognize where these artifacts are appearing. Print outs of both the artifact chart and electropherogram will be provided to all analysts/technical reviewers to assist with data interpretation. If at any time an artifact is thought to

obscure or compromise the integrity of an allele call the analyst should consult with the technical leader to determine if that allele data should be deemed inconclusive.

It should be noted that most all of these artifacts become more pronounced at higher RFU levels. When RFU values are kept down to reasonable levels (i.e. below 1000 or 2000 RFU) majority of these artifacts will remain below the calling threshold and have less of an affect on analysis. It is therefore exceedingly important to obtain an accurate quantification value and achieve the proper target concentration for amplification. This will help minimize the presence of both biological and technological artifacts and ensure a "cleaner" and easier to interpret profile.

Artifact	Channel or Locus	Comments
n-2, n+2	D1S1656 D13S317 D18S51 D21S11 D7S820 D5S818 D12S391 D19S433	
n-1 (-A)	Amelogenin D2S441	Due to decreased 60° C final extension hold during PCR complete +A addition may not take place
n-3	D12S391	Not a trinucleotide
Allele 5 (84 bp)	D16S539	Occasionally observed around 83 bp as well
71-73 bp 75-77 bp	TH01	
214 bp	D18S51	Falls right before D2S441 locus
247 bp	D2S1338	
64-65 bp 69-71 bp 70-74 bp 88-90 bp	Fluorescein (Blue)	
74-76 bp	JOE (Green)	
66-68 bp	TMR-ET (Yellow/Black)	
58-65 bp	CXR-ET (Red)	
177-183 bp Allele 24.3 (180 bp)	D12S391	Not documented in the current version manual; contacted Promega and they did observe this artifact in higher RFU level samples and may update manual

Figure 16: Artifact summary chart.

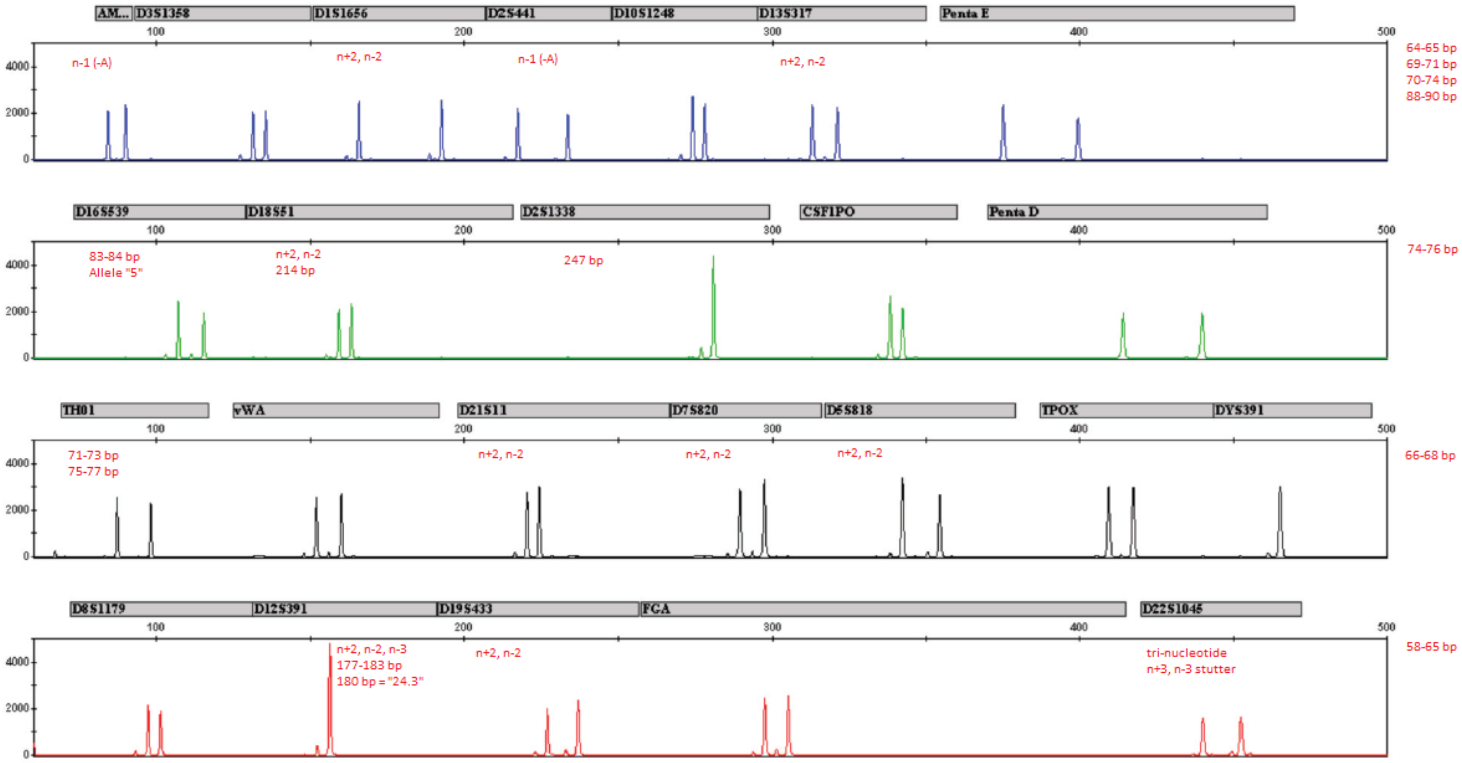

Figure 17: Artifact position electropherogram.

Stutter

Promega provides a default marker specific stutter percentage filter for import into the GeneMapper® analysis software [3]. Figure 18 shows the stutter data from Promega that is used in the provided filter [3]. GeneMapper® v3.2 only allows for n-4 stutter values so the plus stutter and n-2 stutter on the chart would not be imported.

Minus Stutter	Count	Average	Std Dev	Mean +3 Std Dev
AMEL	--	--	--	--
D3S1358	122	0.081	0.013	0.119
D1S1656	151	0.085	0.019	0.142
D2S441	110	0.048	0.015	0.092
D10S1248	123	0.079	0.015	0.124
D13S317	106	0.054	0.015	0.098
Penta E	47	0.039	0.012	0.076
D16S539	132	0.059	0.014	0.102
D18S51	164	0.082	0.021	0.146
D2S1338	177	0.083	0.019	0.139
CSF1PO	116	0.058	0.012	0.095
Penta D	11	0.029	0.013	0.068
TH01	80	0.023	0.008	0.046
vWA	108	0.062	0.017	0.112
D21S11	148	0.079	0.012	0.116
D7S820	118	0.052	0.019	0.110
D5S818	111	0.052	0.014	0.095
TPOX	82	0.027	0.009	0.055
DYS391	101	0.062	0.008	0.087
D8S1179	143	0.069	0.013	0.109
D12S391	157	0.087	0.024	0.158
D19S433	127	0.064	0.015	0.110
FGA	143	0.068	0.017	0.121
D22S1045	123	0.081	0.028	0.164
Plus Stutter	**Count**	**Average**	**Std Dev**	**Mean +3 Std Dev**
D22S1045	103	0.049	0.013	0.086
n-2				
D1S1656	56	0.025	0.004	0.036

Figure 18: Promega provided stutter filter percentages.

All of the validation data was analyzed using these provided values. In addition, stutter values were calculated internally and assessed (no offscale data was used as this could falsely skew stutter percentages). The provided stutter filter values proved to be effective for filtering n-4 stutter for all loci. For all of the validation data, there were only three samples where n-4 stutter was not filtered by the software (each at just one allele). These isolated incidents do no warrant increasing the overall n-4 stutter percentage values. There were also a few isolated cases of n+4 stutter observed at various loci, as well as two instances of possible n-8 and n+8 stutter. Since these occurred infrequently and sporadically it is not possible to reliably calculate a stutter filter percentage for these positions. The analysts should just be aware that stutter is a possibility in the n+4 position as well as the rare chance of n+/- 8 stutter.

Locus D1S1656 will be discussed separately as this locus commonly has n-2 data peaks, as noted above in the Promega data chart. All of the observed n-2 calls were recorded and the average and standard deviation was calculated. The mean plus 3 standard deviations was determined from these values. The values TDS calculated for n-2 at locus D1S1656 are nearly identical to the data obtained by Promega (0.037 vs 0.036 respectively). A rounded value of 0.04 was decided on for data analysis. If using GeneMapper® v3.2, this position cannot be imported into

the software so analysts will have to manually assess stutter for this location. In addition to n-2 stutter, the D1S1656 locus did seem to have a higher occurrence of n+4 stutter. Promega was contacted to see if they had any data for this stutter position. They did not provide any n+4 stutter filter values because they did not have enough data points to accurately calculate a percentage. TDS observed seven instances of n+4 stutter at D1S1656; generally these values were between 1-3%. While this is not enough data to calculate a dedicated filter percentage at this time, the 1-3% observed values may be used as a guideline during data interpretation. As with the n-2 data, this data cannot be entered into GeneMapper v3.2 so the analyst would manually determine stutter peaks at the n+4 position.

As a reminder, if a minor donor is expected during mixture interpretation, the analyst should assume that stutter peaks could be possible minor alleles if they are in the proper RFU height range. It may even be necessary to remove the stutter filter and re-analyze the data. This will become even more important when using PowerPlex® Fusion as there is an increase in possible stutter positions to anticipate.

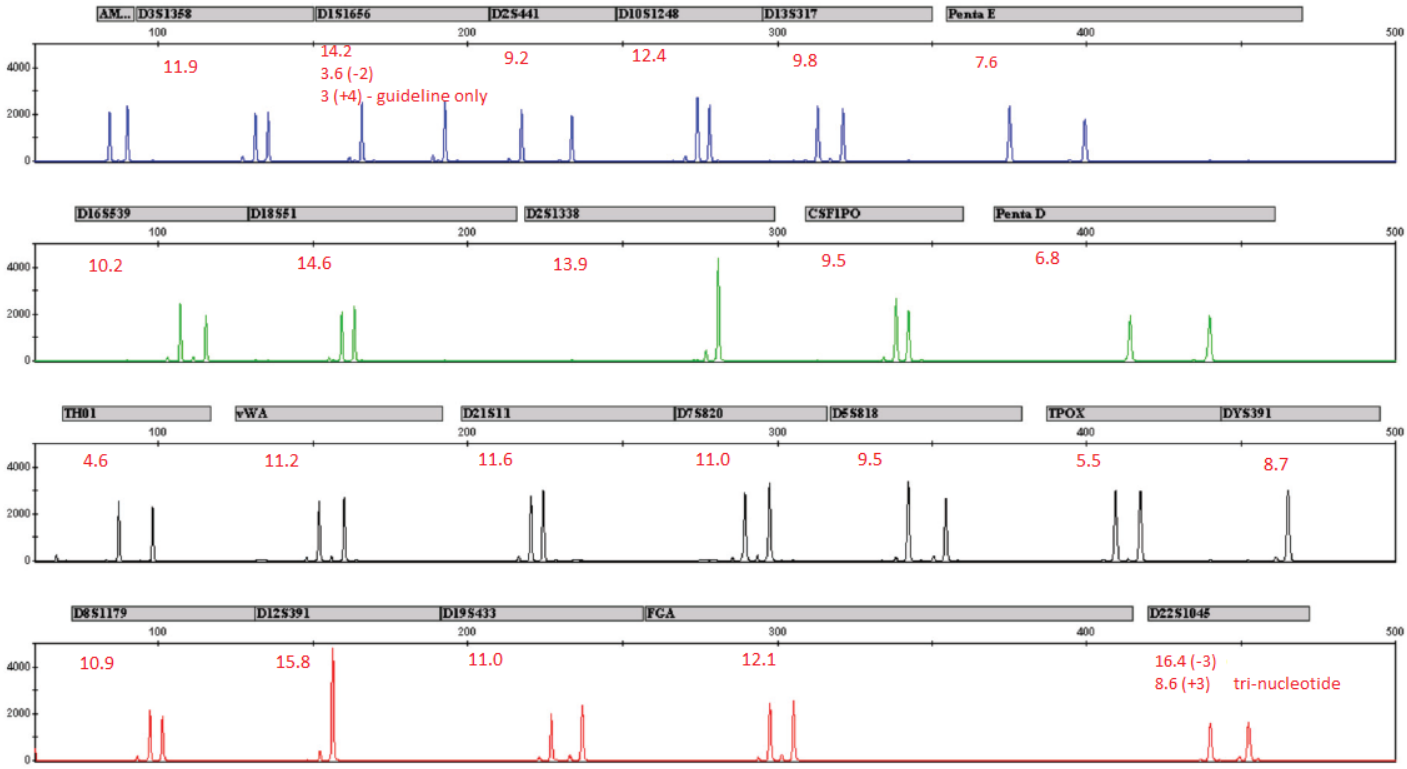

Figure 19: Stutter percentages electropherogram; all values are for n-4 stutter unless otherwise noted.

Analytical Threshold

Trinity DNA Solutions has a previously validated analytical threshold (aka minimum peak height threshold) of 50 RFU. Validation of the PowerPlex® Fusion kit requires an assessment of the kits baseline noise to determine if the current 50 RFU value is suitable. Figures 20 and 21 illustrate the PowerPlex® Fusion baseline for each dye channel for both an amplification blank and positive control sample, respectively. Note that the peak detection threshold was set to 1 RFU in GeneMapper® in order to determine the baseline noise height.

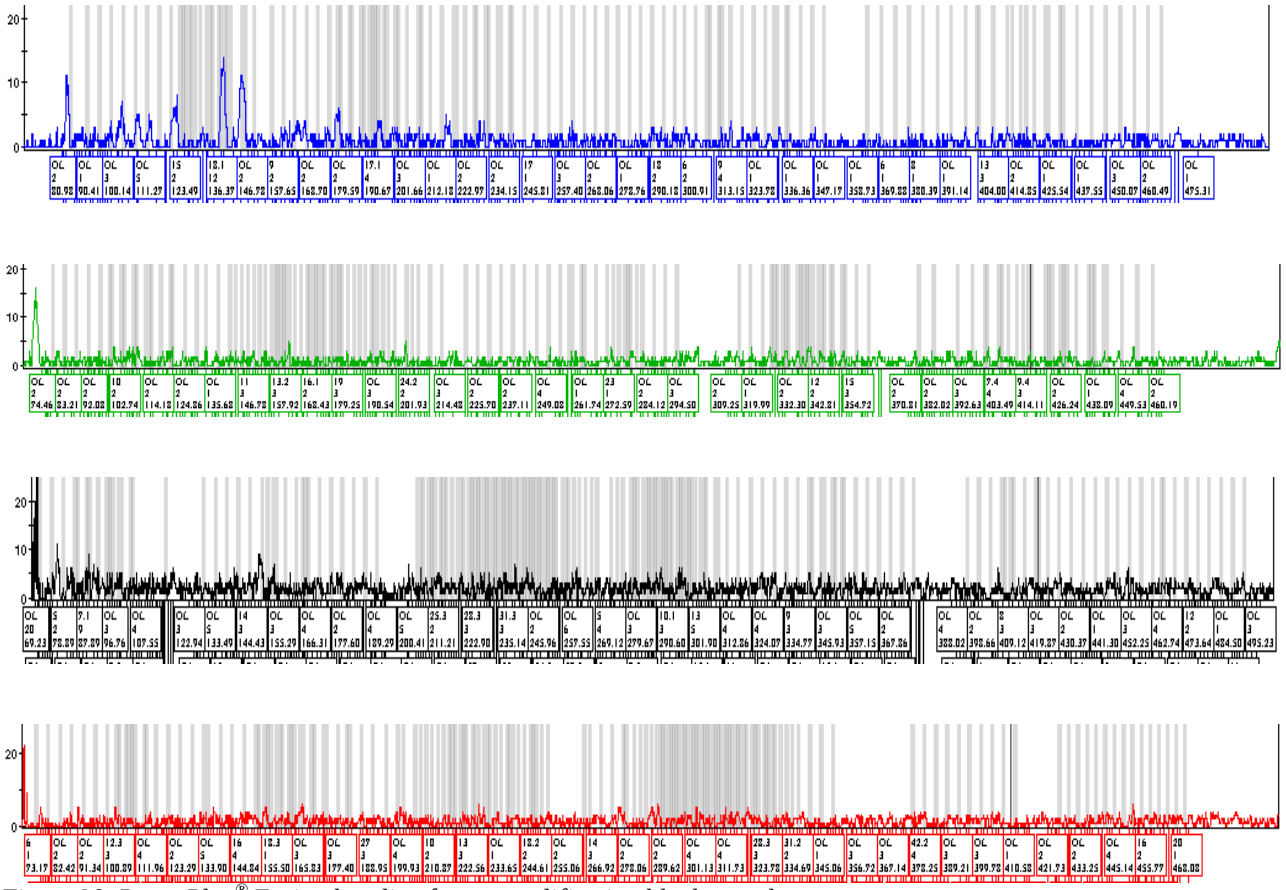

Figure 20: PowerPlex® Fusion baseline for an amplification blank sample.

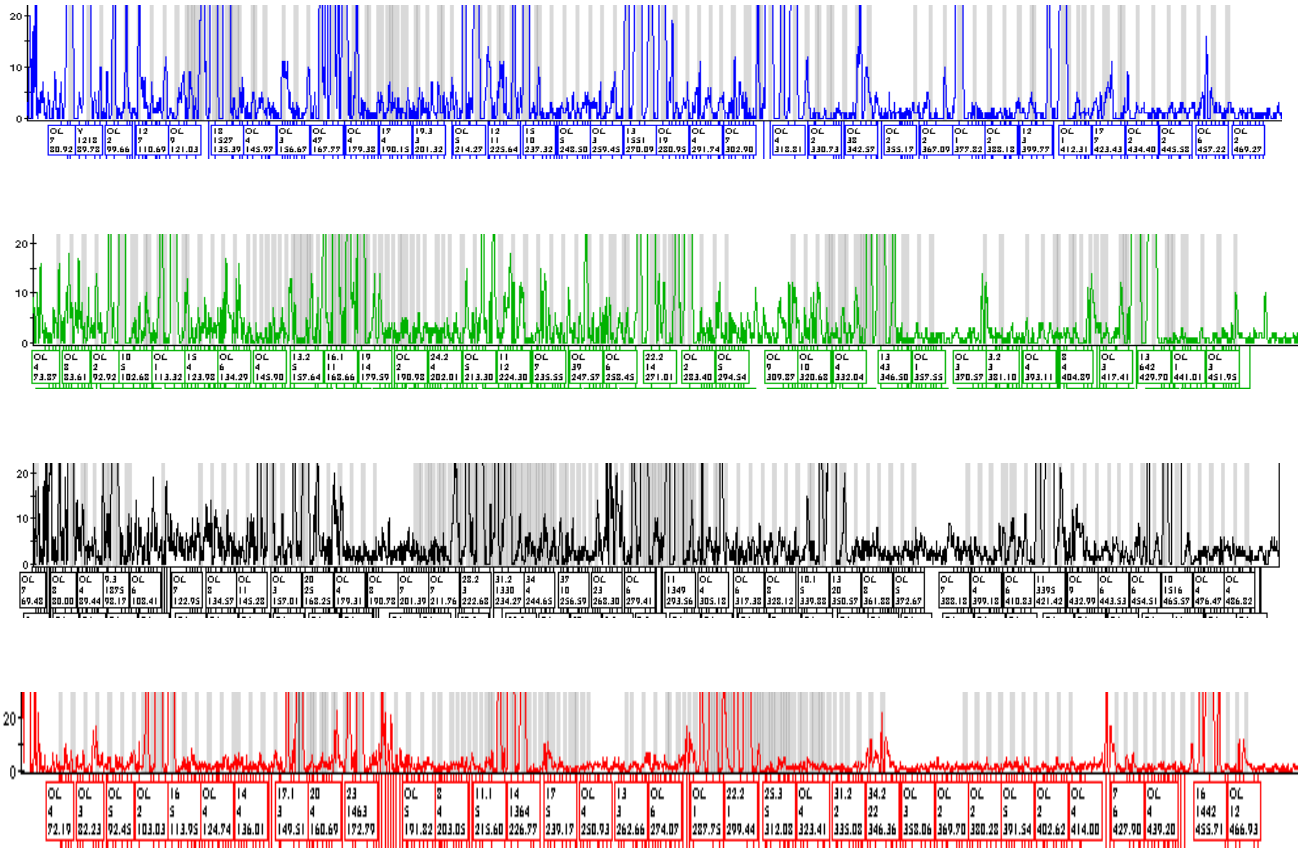

Figure 21: PowerPlex® Fusion baseline for an amplification positive control sample.

Visual observation indicated very low levels of baseline noise. For the blank samples, baseline noise averages appear to be consistently below 5 RFU for all dye channels. For non-blank samples the baseline noise does increase, as expected, due to increased RFU activity and artifacts associated with peaks. Keeping RFU levels down to a reasonable level (i.e. not overloading the sample) will help to keep the baseline low/clean and simplify analysis. Based on the observed data, nothing indicates that the analytical threshold needs adjusting from the previously determined 50 RFU value.

Stochastic Threshold

Due to the increased sensitivity of the PowerPlex® Fusion kit, lower levels of DNA can be successfully amplified. It is known that PCR amplification, particularly with lower quantities of DNA, can have stochastic effects. When analyzing data within this stochastic range, extreme heterozygote peak height imbalance can be expected and allelic dropout can occur (one allele of the heterozygote pair fails to amplify to a detectable level). A stochastic threshold is necessary to assist the analyst with data interpretation for these types of samples. The 2010 SWGDAM guidelines define the stochastic threshold as "the value above which it is reasonable to assume that allelic dropout has not occurred within a single-source sample."[7] In other words, if an allele peak exceeds the stochastic threshold RFU value, then the analyst can be confident that all the allele data is present and no alleles have dropped out. Conversely, if an allele peak falls below the stochastic threshold then the analyst must assume that allele data is potentially dropping out and interpret the profile accordingly. According to the SWGDAM 2010 guidelines, "The laboratory establishes a stochastic threshold based on empirical data derived within the laboratory and specific to the quantitation and amplification systems (e.g., kits) and the detection instrumentation used. It is noted that a stochastic threshold may be established by assessing peak height ratios across multiple loci in dilution series of DNA amplified in replicate."[7]

Replicate sensitivity samples of 9947A positive control dilutions that were know to fall in the stochastic range (i.e. drop out beginning to be observed) were assessed to determine the stochastic threshold. Namely, samples in the approximate 200-1000 RFU range were focused on as this is where drop out for somewhat higher RFU levels was being seen.

# Of Alleles Detected (38 Total) - PPF 3130xl (Run 1)			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens4 1:16 **(0.344ng)**	38	37	36
Sens4 1:16 **(0.344ng)**	38	38	37
Sens4 1:16 **(0.344ng)**	38	38	38
Sens5 1:32 **(0.171ng)**	30	24	20
Sens5 1:32 **(0.171ng)**	30	23	15
Sens5 1:32 **(0.171ng)**	32	30	23

Maximum Observed Peak Height (RFU) for Remaining Heterozygote Allele			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens4 1:16 **(0.344ng)**		196	175
Sens4 1:16 **(0.344ng)**			125
Sens4 1:16 **(0.344ng)**			
Sens5 1:32 **(0.171ng)**	384	193	136
Sens5 1:32 **(0.171ng)**	329	164	106
Sens5 1:32 **(0.171ng)**	308	141	188

# Of Alleles Detected (38Total) - PPF 3130xl (Run 2)			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens4 1:16 **(0.344ng)**	38	35	33
Sens4 1:16 **(0.344ng)**	38	36	33
Sens4 1:16 **(0.344ng)**	37	36	25
Sens5 1:32 **(0.171ng)**	23	14	5
Sens5 1:32 **(0.171ng)**	26	12	0
Sens5 1:32 **(0.171ng)**	30	22	11

Maximum Observed Peak Height (RFU) for Remaining Heterozygote Allele			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens4 1:16 **(0.344ng)**	*	217	144
Sens4 1:16 **(0.344ng)**		179	205
Sens4 1:16 **(0.344ng)**	227	191	100
Sens5 1:32 **(0.171ng)**	181	129	64
Sens5 1:32 **(0.171ng)**	180	83	NA
Sens5 1:32 **(0.171ng)**	178	96	73

* 71 with 409

# Of Alleles Detected (38Total) - PPF 3130xl			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens7 1:64 **(0.0234ng)**	33	15	14
Sens4 1:16 **(0.344ng)**	37	NA	NA
Sens5 1:32 **(0.171ng)**	34	NA	NA
Sens6 1:64 **(0.0855ng)**	26	NA	NA

Maximum Observed Peak Height (RFU) for Remaining Heterozygote Allele			
9947A dilutions	1uL DNA		
	10 sec	5 sec	3 sec
Sens7 1:64 **(0.0234ng)**	281	98	78
Sens4 1:16 **(0.344ng)**	90	NA	NA
Sens5 1:32 **(0.171ng)**	234	NA	NA
Sens6 1:64 **(0.0855ng)**	114	NA	NA

Figure 22: Stochastic threshold charts document the maximum remaining heterozygote peaks where the sister allele had dropped out.

The highest heterozygote peak with drop out of its sister allele was observed at 384 RFU for a 10 second injection (shown below). The missing allele was not visible at all, even below threshold.

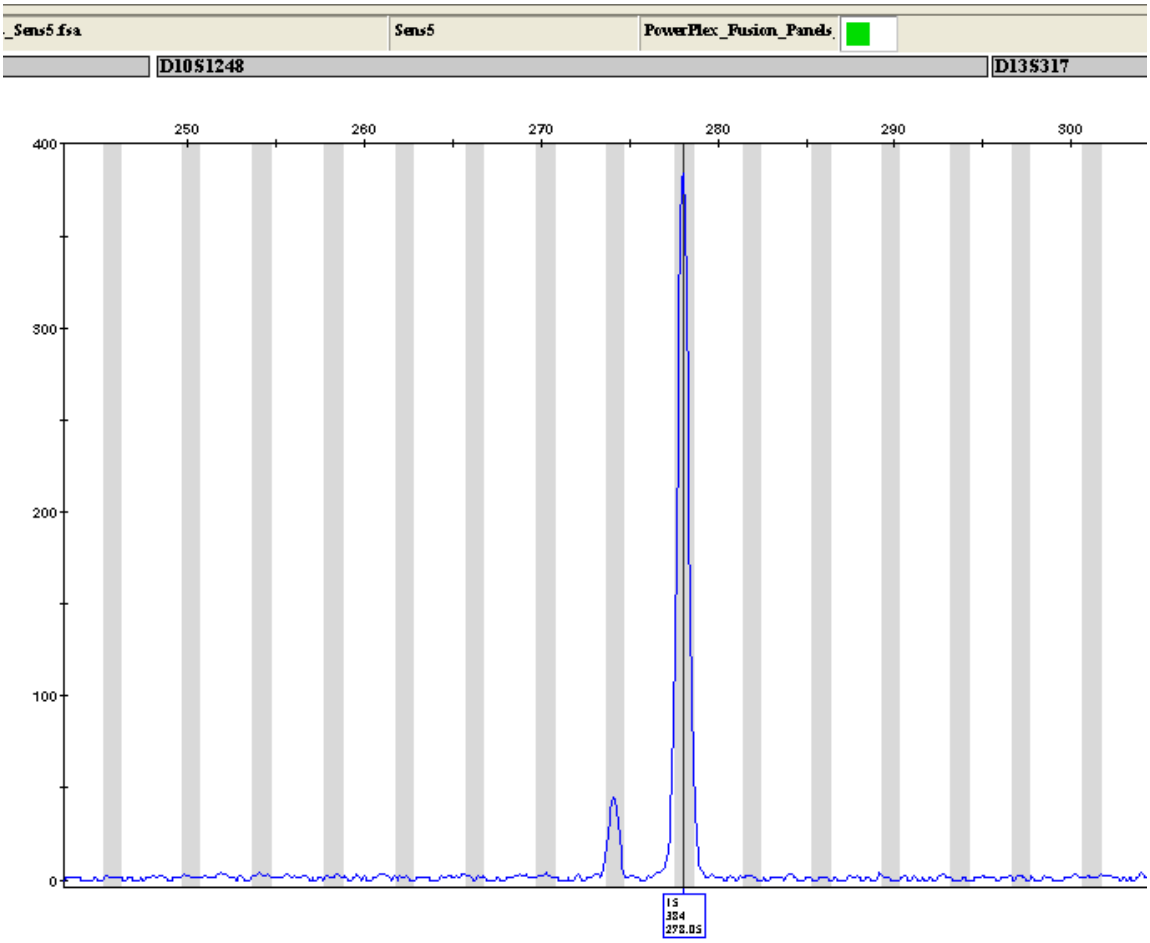

Figure 23: Maximum observed true heterozygote peak with drop out of sister allele; 15 allele = 384 RFU, 13 allele dropped out (no sign of it below threshold).

In addition to determining the highest observed false homozygote peak, two other methods for determining the stochastic threshold were also used: 1.The analytical threshold (50 RFU) was divided by the minimum observed peak height ratio (14.5%) and 2. False homozygote peak heights were averaged plus 3 standard deviations for relevant input amounts. The chart below (figure 24) illustrates the three different stochastic thresholds for each of the above mentioned methods. The maximum observed false homozygote method yielded the highest value so this value was used.

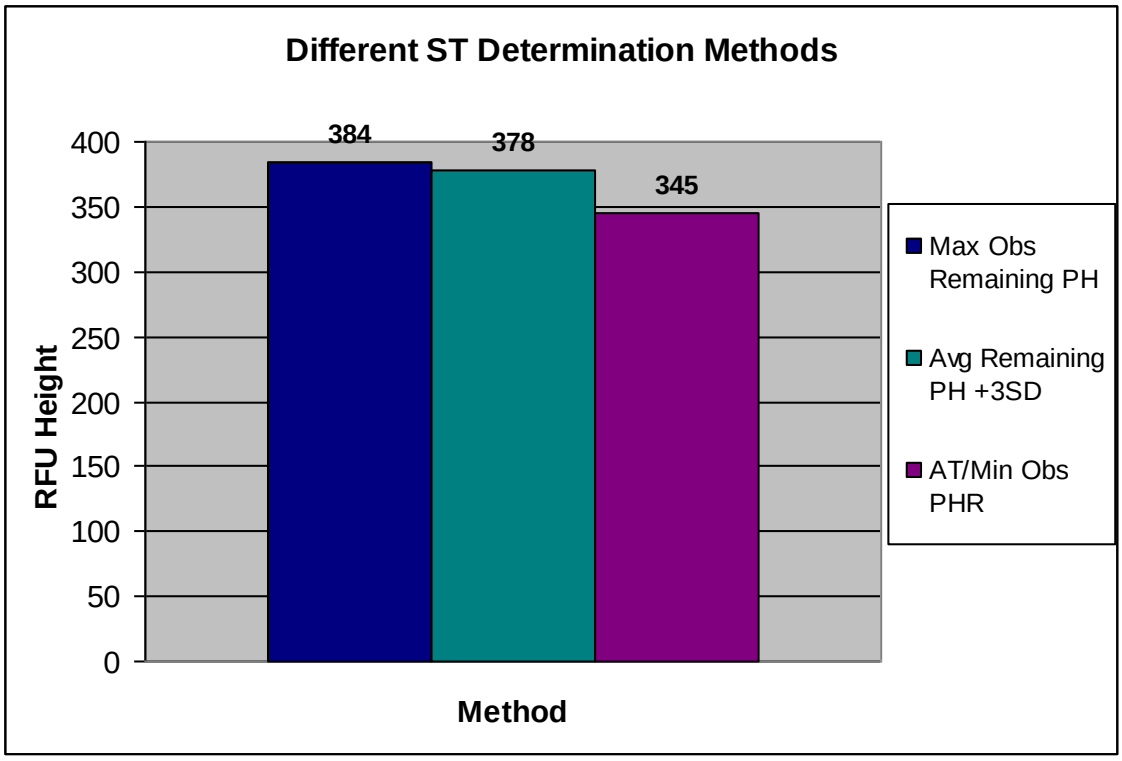

Figure 24: Comparison of different methods for determining the stochastic threshold.

The stochastic threshold values for PowerPlex® Fusion amplification (30 cycle) on the 3100 and 3130xl are as follows:

10 second injection = 400 RFU
5 second injection = 200 RFU
3 second injection = 120 RFU

In an effort to be more conservative, the highest observed value of 384 RFU was rounded up to 400 RFU for a 10 second injection. The 5 second and 3 second injections were then calculated (400/2 and 400/3.33 respectively). These values are higher than the stochastic threshold used for Identifiler® Plus amplification. This is to be expected due to the fact that PowerPlex® Fusion is more sensitive and able to generate data from lower levels of input DNA. Previous internal validation data has shown little difference between data run on the 3100 and 3130xl so separate values are not necessary.

Contamination Assessment

Reagent blanks (aka extraction blanks) and amplification blanks (amplification grade water provided with kit) were run to detect any possible contamination. No instances of contamination were observed within any of the blanks or known samples throughout the validation.

It should be noted that while no true contamination was observed, there were some samples that exhibited additional data from explainable sources. For example, the wearer profile for a pair of jeans gave the wearer's profile and some of her husband's alleles as well. Due to the sensitivity of PowerPlex® Fusion, extraneous profiles may become more common as low level innocent sources are picked up on.

Due to increased sensitivity, clean technique is exceedingly important to help avoid contamination. The current method of sterilizing certain non-disposable utensils between evidence cuttings (i.e. scissors) was evaluated and is still satisfactory. No profile carryover was detected on blank cuttings using sterilized utensils.

PowerPlex® Fusion Statistics

Frequency data for PowerPlex® Fusion loci is available and can be obtained from the NIST population study documented in the following paper: C.R. Hill, et al., U.S. population data for 29 autosomal STR loci, Forensic Sci. Int. Genet. (2013). [8] For casework statistics, TDS has created in house excel worksheets to conduct statistical calculations for PowerPlex® Fusion data. Worksheets using the NIST frequency data can conduct RMP, mRMP, and CPI calculations for the following races: US Caucasian, African American, and Hispanic.

Due to the increased number of loci in the PowerPlex® Fusion kit, it is expected that full profile (or even near full profile) matches will yield some astounding match statistics (i.e. 10^{-33} and beyond) [3]. Below is an example of a full 22 loci single source (excludes Amelogenin and DYS391) random match probability statistic:

RANDOM MATCH PROBABILITY					
African American	1 in	18,730,924,130,759,200,000,000,000,000,000,000	or	1 in	18 decillion
Caucasian	1 in	106,951,148,979,356,000,000,000,000,000,000	or	1 in	106 nonillion
Hispanic	1 in	47,540,893,158,019,000,000,000,000,000,000	or	1 in	47 nonillion

Figure 25: Full female profile single source RMP statistic.

Conclusion

Based on the validation results obtained, the Promega PowerPlex® Fusion amplification kit has been found to perform satisfactorily. The PowerPlex® Fusion kit demonstrates accuracy, precision, reproducibility, and out performs the current Identifiler® Plus kit in sensitivity. It is expected to benefit the laboratory in obtaining better profile results for low level, degraded, inhibited, and/or otherwise problematic samples.

References

[1] D.R. Hares, Expanding the CODIS core loci in the United States, Letter to the Editor Forensic Sci. Int. Genet., 6 (2012) e52-e54.

[2] D.R. Hares, Addendum to expanding the CODIS core loci in the United States, Letter to the Editor Forensic Sci. Int. Genet., 6 (2012) e135.

[3] PowerPlex® Fusion System Technical Manual. Promega Corporation, Revison 10/12, www.promega.com.

[4] Trinity DNA Solutions Forensic Biology Procedures Manual v15.

[5] AmpFlSTR Minifiler PCR Amplification Kit User Guide, Applied Biosystems, Rev. B, 03/2007.

[6] PowerPlex® Fusion: An Expanded STR Multiplex for New Global Standards. Kathryn Oostdik, Promega Corporation, December 2012.

[7] 2010 SWGDAM interpretation guidelines. http://www.fbi.gov/about-us/lab/biometric-analysis/codis/swgdam-interpretation-guidelines

[8] C.R. Hill, et al., U.S. population data for 29 autosomal STR loci, Forensic Sci. Int. Genet. (2013), http://dx.doi.org/10.1016/j.fsigen.2012.12.004