Internal Validation of PowerPlex® 16 HS with the Applied Biosystems® 3500*xL* Genetic Analyzer

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Abstract

An internal validation is performed in order to demonstrate reliability and reproducibility of new instruments and chemistries within a specific laboratory. These studies show that results are accurate and consistent with previous methods. The validation study performed at the Philadelphia Police Department Forensic Science Bureau (PPD) demonstrated the reliability of the PowerPlex® 16 HS amplification chemistry and the use of the Applied Biosystems® 3500*xL* to provide accurate and reliable data. The chemistry and instrument are both novel technologies and platforms to the PPD DNA laboratory, thus requiring an internal validation to be performed prior to use with casework samples.

A variety of studies were performed in this validation in order to demonstrate the reliability of the use of PowerPlex® 16 HS with the Applied Biosystems® 3500*xL* Genetic Analyzer. These studies consisted of injection voltage and time, DNA target, analytical threshold, stochastic threshold, precision, sensitivity, concordance, reproducibility, stutter, mixture, heterozygote peak heights, and non-probative casework samples. All studies were analyzed using Applied Biosystems® GeneMapper *ID-X* v1.2. Each of these studies demonstrated the proper settings to optimize the use of the amplification chemistry on the genetic analyzer. Overall, this validation demonstrated that consistent and reliable results could be obtained through the use of PowerPlex® 16 HS with the AppliedBiosystems® 3500*xL* Genetic Analyzer.

Introduction

An important aspect of forensic science is the need to validate new chemistries and instrumentation for use with STR analysis. An internal validation must be performed on these new methods and technologies prior to implementation in laboratory standard operating

procedures. This validation must demonstrate the ability of the procedure to obtain reliable results, the ideal conditions to obtain these results, and the limitations of this new procedure (4). An internal validation was requested for the Philadelphia Police Forensic Science Bureau through the National Institute of Justice's Technical Assistance Program on the use of PowerPlex® 16 HS amplification chemistry (Promega Corporation, Madison, Wisconsin) with the Applied Biosystems® (Foster City, California) 3500*xL* Genetic Analyzer. The Applied Biosystems® 3500 Genetic Analyzer is a relatively new platform to the field of forensics and offers many advantages to the previously used Applied Biosystems® 3130*xL* such as an improved mechanical pump, new laser technology, more consistent temperature control, prepackaged consumables, and reduced power requirements (5). Data files are also saved in .HID format and analyzed with GeneMapper® *ID-X* (Applied Biosystems). In addition to these advancements, the Applied Biosystems® 3500*xL* offers increased throughput with the ability to perform an injection of 24 samples. The PowerPlex® 16 HS amplification chemistry, released in 2009, offers an advantage over the previously used PowerPlex® 16 kit; the addition of hot-start *Taq* directly to the mastermix. This eliminated the need to purchase the Taq reagent separately (7). PowerPlex® 16 HS also offers the ability to perform direct amplification procedures on known samples, as well as an increased ability to perform in the presence of inhibitors.

Due to the difference in manufacturer between the amplification chemistry and instrument, adjustments are required prior to performing analysis, including matrix calibrations and the creation of the appropriate size standards. The appropriate dye set was confirmed through a spectral calibration with the Promega 4 dye set, and the 60- 600 base pair size standard set was entered into the instrument protocol (6). These procedures are necessary to perform prior to performing the necessary validation studies.

This internal validation incorporates a variety of studies to demonstrate the reliability and reproducibility of this instrument with the PowerPlex® 16 HS chemistry. These studies include the determination of an appropriate analytical threshold through SWGDAM approved methods, a stochastic threshold, an injection time and voltage which provided optimal peak heights and peak height ratios, a sensitivity range to provide the optimal input amount of target DNA, heterozygosity ratios, and stutter percentages for comparison to manufacturer recommended values (7). Studies will be run simultaneously on both the 3130*xL* and 3500*xL* instruments for the duration of the validation study. The results from the 3500*xL* will be compared with data

collected from the 3130*xL* to demonstrate concordance. Results collected for various other studies will also be compared between the two platforms including stutter ratios and mixture interpretations. Samples also will be analyzed to demonstrate reproducibility across multiple amplifications and runs and compared with the expected results from previously analyzed non-probative casework samples. These non-probative casework samples encompass various extraction methods and substrates typically encountered in the Philadelphia Police Forensic Science Bureau. The comparison of these results is meant to establish instrument and chemistry concordance.

Materials and Methods

The studies performed during the validation of the Applied Biosystems® 3500*xL* Genetic Analyzer using PowerPlex 16® HS used extracts previously obtained from reference samples as well as previously analyzed casework samples. These sample extracts were stored at 4°C and were considered non-probative. Prior to use, all extracts were quantitated by a PPD analyst to determine the proper concentration necessary for amplification procedures. All data was analyzed using GeneMapper® *ID-X* version 1.2. The profiles generated were compared to profiles previously obtained by analysts using PowerPlex® 16 on the Applied Biosystems® 3130*xL*.

Analytical Threshold, Injection Time/Voltage, Target, Stochastic Threshold Study

An initial analytical threshold, injection time and voltage, and DNA target study were performed in order to determine the ideal DNA target load to inject at a specific injection voltage and time to produce reliable results which limit artifacts and stochastic events. For this study a dilution series was created from a previously analyzed non-probative reference sample, ranging between 10ng and 0.0156ng. This dilution series was run in triplicate with each injection containing the dilution series, three 2800M positive controls, three allelic ladders, and a row of amplification negative controls. Each sample was setup in triplicate and injected under the following conditions: injection of 24 seconds and 1.2 kV (instrument default setting), 12 seconds and 1.2 kV, 10 seconds and 3 kV, and 5 seconds and 3 kV. The range of injection voltages and injection times were used to determine the ideal injection parameters for the 3500*xL* and the ideal DNA target load to inject at the decided voltage and time.

An analytical threshold is necessary to determine at what level a true peak can be differentiated from background noise. The analytical threshold was calculated by analyzing a run of 24 amplification negative controls (3 rows). Each row was generated from each of the three amplification negative controls included in the amplification of the target load and injection time study. Prior to analysis, an analytical threshold of 1 RFU was selected for all dyes to allow for the calling of "background noise" above this threshold (8). Called peaks were then exported to an Excel spreadsheet. Peaks below a base pair size of 90 were removed to prevent interference from the primer peak (out of marker peaks) as well as all peaks plus or minus two base pairs from the size standard peaks. These peaks were removed to prevent the interference of "pullup" or raised baseline within the background noise levels from these size standard peaks. A variety of methods for calculating the analytical threshold were utilized.

A stochastic threshold level is calculated to determine above what RFU level a homozygote peak can be identified without the consideration of dropout of a heterozygote sister allele. The stochastic threshold was calculated from the target study dilution series data at 24 sec / 1.2 kV (the selected injection voltage and time). This calculation consisted of finding the average peak height ratio for all dye channels and each individual channel and performing the calculation previously used by Sorenson Forensics for the validation of PowerPlex® 16 for the PPD.

Precision Study

A precision study was performed to demonstrate the reliability and degree of precision of the allele size calling. This study was performed on three consecutive days with a new plate prepared each day containing three rows of allelic ladders (24 samples). The plate prepared on day one (June 12, 2012) was ran on day 2 (June 13, 2012) and day 3 (June 14, 2012) to ensure precise size calling when a plate is stored for multiple days after a capillary electrophoresis run. This practice was consistent with PPD standard operating procedures in which a plate can be stored and re-ran within a three day time period. A newly prepared plate was run on June 12 and June 13 as well, also containing three rows of allelic ladder. Prior to exporting the size calling data, one sample was set to allelic ladder and the remaining samples were designated as samples.

Sensitivity Study

A sensitivity study was performed to determine the ideal target DNA load within the recommended range determined by the Target study. Thus, a target dilution series between 0.125 ng and 1.5 ng was created to accurately account for the multitude of amplification targets within the 0.5 to 1.0 ng range. The sensitivity study consisted of analyzing a dilution series between 0.30ng/µl and 0.025ng/µl. These dilutions were created to target 0.125ng, 0.25ng, 0.5ng, 0.75ng, 1.0ng, 1.25ng, and 1.5ng prior to amplification. The same sample utilized for the target, injection time, analytical threshold, and stochastic threshold study was also used for this study. This dilution series was created to more accurately select an optimal concentration range for amplification. Dilutions were quantitated individually with Promega's Plexor® HY System prior to amplification to ensure that dilutions were consistent with the expected concentration values. The dilution samples were run in triplicate creating two injections consisting of 21 samples (seven samples in the dilution series loaded in triplicate), two amplification positive controls (2800M), two amplification negative controls, and three allelic ladders. This data was analyzed, input into an Excel table, and appropriate target recommendations were made based on these results.

Peak Height and Heterozygosity Study

According to PPD standard operating procedures the peak height ratio between two peaks at a locus must be greater than 50% to call those peaks heterozygote sister alleles. For the heterozygosity/peak height ratio study the average, standard deviation, maximum, minimum, and range of the peak height ratios for the sensitivity study were reanalyzed to further confirm the selection of the 1.0 - 0.5 ng range. Average peak heights were expected to be well above the 50% cutoff value for heterozygote alleles within a single source profile.

Concordance, Reproducibility, and NIST Study

A reproducibility study is performed to evaluate a technique performed repeatedly to assess the reproducibility of the method while a concordance study provides an evaluation of a technique performed by a different analyst or piece of equipment to demonstrate the reproducibility of results. Seventeen previously analyzed reference samples were diluted and reamplified with PowerPlex® 16 HS. The results of these samples previously ran with

PowerPlex® 16 on the 3130*xL* were compared to the PowerPlex® 16 HS results for the concordance study. The amplified products were also run on both the 3130*xL* and the 3500*xL*. Data was exported to an Excel spread sheet where allele calls were compared for consistency. Results were compared for concordance, comparing allele calls between instruments.

The reproducibility study consisted of reamplifying the samples from the concordance study (three amplifications) and running these samples in separate runs on the 3500*xL*. Dilutions were prepared from each extract and a DNA target of 0.5ng was loaded for each of the three amplification procedures. The runs were performed on June 28, June 29, and July 3, 2012. Allele calls and peak heights between runs were compared for reproducibility and calculations were compared to identify precision.

The concordance and reproducibility study also contained three genomic NIST samples. The profiles generated with these samples were compared to the expected profiles provided by the NIST literature.

Contamination Study

A contamination study was prepared along with the non-probative casework sample study. The purpose of the study was to determine if contamination occurred due to crossover from the capillaries during sequential injections, as well as contamination during the preparation of a plate for capillary electrophoresis. This study was prepared in concordance with the Promega recommended validation guidelines (2). The plate setup was a checkerboard pattern across a 96 well plate with the samples used for the non-probative casework study along with run negative controls. The plate was set up in this configuration to allow for a capillary which previously injected a sample to perform a sequential injection in a run negative control. Only Negative controls were analyzed using GeneMapper® *ID-X* and the presence of possible contamination was evaluated.

Stutter Study

A stutter study was performed to indicate the appropriate stutter ratios to recommend for future analysis using PowerPlex® 16 HS on the Applied Biosystems® 3500*xL*. This study consisted of 53 single source reference profiles, each diluted to a target of 0.5ng. Samples were analyzed using GeneMapper® *ID-X* with all artifacts removed except for stutter peaks. Positive

and negative stutter peaks were labeled as stutter as well as an indication of the allele from which they originated. Data was exported to an Excel workbook and separated according to marker. The peak height ratio, peak height ratio standard deviation, maximum peak height ratio, and minimum peak height ratio were then calculated within each marker. The recommended stutter ratio for each specific marker was then calculated by adding the average peak height ratio to three times the standard deviation. These recommended stutter ratios were then entered into an Excel table and compared to Promega recommended stutter ratios. Due to the fact that positive stutter ratios from Promega were not obtained, the values calculated for in the study were input directly into GeneMapper® *ID-X* for future analysis.

Mixture Study

A mixture study was performed in concordance with the Promega recommended validation guidelines (2). The mixture study consisted of two male reference samples added in different ratios. The selected ratios were 1:0, 4:1, 9:1, 19:1,1:1,1:4,1:9,1:19, and 0:1. These ratios were utilized to determine the lowest ratio at which a minor contributor could be differentiated from a full major profile. Mixture samples were amplified with the appropriate target determined in previous studies (0.5ng) and ran on the 3500*xL*. Samples were analyzed using GeneMapper® *ID-X* and major and minor alleles were designated when possible. These allele designations consisted of the use of a 60% peak height ratio cutoff to determine the presence of "light" alleles. This cutoff value is incorporated in the standard operating procedures of the Philadelphia Police Forensic Science Bureau DNA Laboratory in the analysis of possible mixture samples.

Non- Probative Casework Samples Study

Non-probative questioned samples which were part of the same cases as the previously used reference samples were used to determine concordance with previously analyzed data. This study was meant to simulate casework procedures with selected settings and recommendations created during the previous validation studies. A quantitation with Plexor® HY was performed on the samples prior to amplification. The samples were then run in a checkerboard pattern with run negative controls to serve as the contamination study. All samples were analyzed in GeneMapper® *ID-X* and allele calls were made, designating both major and "light" alleles. This

data was compared to the results submitted for previous analysis of the samples using PowerPlex® 16 on the 3130*xL*.

Results

Analytical Threshold, Injection Time/Voltage, Target, Stochastic Threshold Study

Initial data analysis determined that the run time of 1210 seconds (default on the 3500*xL*) was not sufficient due to the 600 base pair size standard peak consistently being cutoff. The absence of this peak did not allow for accurate size calling and allele designation. The run was repeated with a run time of 1800 seconds in the instrument protocol. Data was analyzed and it was determined that the increased run time allowed for the 600 base pair peak to be detected, thus allowing for all peaks to be detected within the size standard and allow for accurate size calling and allele designation.

The dilution series samples for the target study and injection time and voltage study were analyzed with a 20% filter and a 150 RFU analytical threshold. The results of the injections created using the 24 sec/1.2 kV, 12 sec/1.2 kV, 5 sec/3 kV, and 10 sec/3 kV parameters were analyzed, all stutter peaks were removed, and peak heights and allele calls were exported to an Excel table. The average peak height, peak height standard deviation, average peak height ratio, and average peak height ratio standard deviation were calculated (**Figure 1**). Dropout was seen to occur in the 0.125ng dilution. All peak height ratios were found to be within the appropriate range for a single source profile (greater than 50%). An ideal standard deviation for peak height ratios was calculated for the 1.0ng, 0.5ng, and 0.25 ng targets. The minimum peak height ratio standard deviation occurred during the 24 sec/1.2 kV injection. The peak heights and peak height ratios within the 1.0 ng, 0.5 ng, and 0.25 ng targets were analyzed due to laboratory expectations based on the previous use of PowerPlex® 16. Based on the results of this study, a target between 1.0 ng and 0.25 ng was further analyzed in the sensitivity study to determine an ideal target for amplification. Based on the average peak height ratio standard deviations, an injection voltage of 24 seconds and 1.2 kV was selected for the remaining validation studies.

The analytical threshold study determined the average baseline noise when an amplification negative was run on the 3500*xL*. The method which provided the analytical threshold relied upon for the remainder of the validation studies consisted of the following equation:

$$AT = 2(Maximum\ peak\ height - Minimum\ peak\ height)$$

The analytical threshold was calculated for each dye channel and the optimal analytical threshold was selected based on these calculations (**Figure 2**). The maximum calculated analytical threshold was found in the blue dye channel (104 RFU). Based on this value, an analytical threshold value of 125 RFU was set for remaining studies. This conservative value prevents background noise interference when determining true peaks.

The determination of a stochastic threshold occurred through the use of the target study peak height data as well as the determined analytical threshold. A method previously utilized by the PPD Forensic DNA Laboratory calculated the stochastic threshold using the following equation:

The stochastic threshold was individually calculated for each dye channel (**Figure 3**). It was determined that a single stochastic threshold should be used across all dye channels. The highest calculated stochastic threshold value was found in the green dye channel (352.17 RFU). Based on this value, a stochastic threshold of 360 RFU was set for remaining validation studies across all dye channels.

Precision Study

The determination of precise allele size calling was confirmed in the precision study. A 20% global cut-off was used for this study. Prior to the samples being exported, a microvariant off ladder was observed in FGA in the samples and was called a 30.2. This artifact was 426.65 base pairs in size and a height of 1205 RFU. This microvariant was not observed in the allelic ladder and was removed prior to being exported. A spike/ pull up was seen in one of the Run1C samples from capillary 11. The 23 in FGA of the sample containing the spike was engulfed and thus could not be manually entered. The size of this allele was not included in calculations. All allele calls were checked for accuracy and the average size, standard deviation of sizes, maximum size, minimum size, and range of sizes was calculated for each allele within each marker across all three of the injections performed. However the precision calculations were only considered for samples falling into the selected 24 second and 1.2 kV injection category. Through the use of this combined data, the average standard deviation of sizes was found to be

0.0539 base pairs with a standard deviation range of 0.03-0.11 base pairs. Based on these calculations, it can be confirmed that the standard deviation was within the acceptable 0.15 base pair window. The average of the range of base pair sizes for each allele was also calculated at 0.24 base pairs. This average was within the acceptable 0.5 base pair window. However, a range of 0.55 base pairs was found at 46.2 in FGA, the only value greater than the acceptable 0.50 base pair window.

Sensitivity Study

A sensitivity study was performed to determine the ideal amplification target to use with the PowerPlex® 16 HS amplification chemistry and the 3500*xL*. The resulting peak heights from a run of each of the amplification targets on the 3500*xL* were analyzed and exported to an Excel table. From these peak heights the minimum, maximum, average peak height ratio, peak height ratio standard deviation, average peak height, and peak height standard deviation were calculated (**Figure 4**). Homozygote peak heights were divided by two, with this value being used for average peak heights but not included within the average peak height ratio calculation. Based on the results of this study the 0.5 to 1.0ng target range was once again found to be ideal due to moderate peak heights, an acceptable average peak height ratio and peak height ratio standard deviation. The samples with amplification targets of 0.25 and 0.125 were found to contain peak height ratios below 50%. Peak height ratios must be above 50% in single source profiles to be designated as a major allele according to PPD standard operating procedures. It was determined that a DNA target of 0.5 ng would be utilized for remaining studies.

Peak Heights and Heterozygosity Study

The sensitivity study samples were further analyzed to indicate an appropriate range where consistent peak height ratios were obtained above 50%. A peak height ratio above 50% is relied upon by the PPD to determine a heterozygote locus within a single source sample. The compiled peak height ratio average, standard deviation, maximum, minimum, and range for each DNA concentration sample within the chosen sensitivity range can be seen in **Figure 5**. Peak height ratios below 50% began in the 0.25 ng target samples. All loci within the sensitivity range of 0.5 – 1 ng of target DNA had peak height ratios above 80%, well above the required 50% peak height ratio for determination of a major contributor in a single source profile.

Concordance, Reproducibility, NIST Study

The concordance and reproducibility studies were performed to demonstrate both the reproducibility of results from multiple amplifications and runs on separate days as well as the consistency of the data collected between different instruments and amplification chemistries. The concordance study consisted of the comparison of 17 reference samples; these samples were amplified and ran on both the 3500*xL* as well as the 3130*xL*. Allele calls were seen to be consistent for both instruments. The allele calls generated with the 3500*xL* were also compared to previously obtained data using the seventeen samples amplified with PowerPlex® 16 and ran on the 3130*xL*. Allele calls proved to be consistent between both runs and across the two amplification chemistries ran on different platforms.

The reproducibility study consisted of the comparison of allele calls, peak heights for each called allele, average peak heights across all runs, minimum peaks heights, and maximum peak heights between multiple amplifications and runs on three separate days. The data was exported to Excel and a table was created for each sample (**Figure 6**). All allele calls were seen to be consistent between the multiple amplifications and runs performed in this study. NIST samples were also amplified and run in the reproducibility and concordance study. NIST profiles were found to be consistent with expected allele calls as indicated in NIST reference material.

Contamination Study

Run negative controls which were run in a checkerboard pattern with the non-probative caseworks study samples were analyzed for contamination. These samples were analyzed for the presence of peaks or other factors within baseline noise levels that would indicate the presence of contamination from either the capillary or the run set-up procedure. The negative controls analyzed did not display contamination and no peaks were indicated.

Stutter Study

The average, standard deviation, minimum, and maximum peak height ratios were calculated for the stutter values obtained within each marker of the 53 analyzed reference samples. These values were then utilized to calculate the stutter value for the specific marker through the use of the following equation:

$$Stutter = 3\ x\ Stutter\ Ratio\ Std.Dev. + Average\ Stutter\ Ratio$$

Calculations were performed for both plus 4 stutter and minus 4 stutter. The calculated stutter values were then entered into a table to assist in the comparison of these values to the provided Promega stutter values (**Figure 7** and **Figure 8**). The obtained minus 4 values were found to be consistent with the Promega values calculated in the PowerPlex® 16 HS validation. The values obtained by the PPD followed the same general trend as the Promega minus 4 values (**Figure 9**). Due to the small degree of variability between the PPD values and Promega values, it was determined that the Promega minus 4 stutter values would be used for future analysis using PowerPlex® 16 HS on the 3500*xL*. Plus 4 stutter values would be used for future analysis due to the fact that plus 4 stutter values are not available for reference.

Mixture Study

A mixture study was conducted through the analysis of two contributor samples in various concentrations as well as a three contributor mixture and a five contributor mixture. Results were analyzed and major and minor components were indicated for each mixture sample **(Figure 10** and **Figure 11)**. Full major component profiles were able to be pulled out in both the 19:1 and 1:19 mixture samples with very few light alleles detected. The majority of the alleles from the major contributor were also detected in the 9:1 and 1:9 ratio samples with only two markers (Penta E and Penta D) not containing a full major profile. An increasing frequency of light alleles were also detected in the 9:1 and 1:9 ratio samples with all markers containing a light allele except five loci (Penta E, CSF1PO, D8S1179, D21S11, and FGA). The 4:1 and 1:4 samples consisted of both the majority of alleles from the major contributor with only seven markers not containing a full major profile. Also, a majority of the light alleles from the minor component were also called except for two markers which did not contain light alleles. The 1:1 sample contained alleles from both contributors and a major contributor could not be determined for any markers other than TH01 and D13S317. A 4:1 two contributor mixture is the apparent optimal mixture to observe both contributors, above this ratio the major contributor is more apparent and less of the minor contributor or light alleles are detected.

The three person mixture contained a major allele in TH01, D21S11, D18S51, D13S317, and D7S820 (**Figure 11**). Major alleles were detected in each locus of the 5 person contributor except the Penta_D and vWA markers. No full major contributor profile could be detected at

either the three or five contributor mixture samples. This result was expected for mixtures of this type due to the fact that samples were added in a 1:1 ratio.

Non-Probative Casework Samples Study

The results of the non-Probative study were analyzed, removing all raised stutter and artifacts. Analysis settings and stutter ratios determined in previous studies were relied upon for analysis of results (125 RFU analytical threshold, 360 RFU stochastic threshold, and 0.5 ng amplification target). Alleles were assigned as major or minor based on the apparent presence or absence of a mixture. The standard 60% peak height ratio was utilized to assign major alleles in apparent mixtures while a 50% peak height ratio was utilized to determine heterozygote sister alleles in an apparent single source profile. Allele calls were exported to a table to assist in comparison **(Figure 12)**. Allele calls were compared between samples which were run in duplicate. The obtained profiles were found to be consistent with previously obtained results. These results were previously obtained through the use of PowerPlex® 16 and the 3130*xL*.

Discussion

The PowerPlex® 16 HS amplification chemistry was found to produce reliable and reproducible results with the use of the Applied Biosystems® 3500*xL* Genetic Analyzer. The reliability and reproducibility of the incorporation of this platform and chemistry are based on the settings and recommendations set forth by the validation studies. The internal validation study determined that an ideal injection load of 1.0 ng, 0.5 ng, and 0.25 ng corresponds with an injection voltage and time of 1.2 kV and 24 seconds. This amplification load was further investigated in the sensitivity study which indicated the ideal target range to be between 0.5 ng and 0.10 ng. These amplification targets were found to produce peak height ratios above the required 50% peak height ratio cut-off for a single source profile. An analytical threshold of 125 RFU and a stochastic threshold of 360 RFU were also determined using the selected injection voltage and time. These settings were used for the remaining validation studies and incorporated in the analysis methods for use with GeneMapper® *ID-X* v1.2.

The performed validation studies also demonstrated consistent and reliable size calling of alleles within each marker, the absence of contamination from both the run set-up and capillary carry over, and concordance between samples previously analyzed using PowerPlex® 16 on the

3130*xL* as well as the same PowerPlex16® 16 HS run setup run on the 3130*xL*. Reproducibility was also demonstrated between three separate days with separate amplifications and runs being performed on each of these days. Stutter ratios were calculated and compared to the values recommended by Promega. It was determined that the Promega recommended stutter values would be used for the remaining studies. Also, a mixture study determined that a two contributor mixture sample in a 1:19 ratio contained a major contributor which could be completely identified. Samples with a concentration of 1:9 and 1:4 contained identifiable major and minor contributors. No full major profile could be determined in the three and five contributor mixture samples.

Finally, a non-probative study was performed at the conclusion of the validation which incorporated the settings and recommendations from all previous validation studies. The samples used in this study were previously analyzed and represented a wide array of sample types encountered by the PPD. The results of this study were found to be consistent with the results previously obtained.

Conclusion

Overall, the instrument provided accurate profiles with few artifacts across a wide range of input target DNA amounts. The results of the performed validation studies demonstrated the robustness and reliability of the kit and instrument. Based on the findings of these studies, specific settings were recommended to be incorporated into the standard operating procedure of the Philadelphia Police Forensic Science Bureau DNA Laboratory. These settings included a set analytical threshold across all dye channels, a stochastic threshold value to assist in the determination of true homozygote peaks, an optimal target DNA range, laboratory specific stutter ratios, and mixture interpretation guidelines. Future studies may be necessary to further confirm stutter ratios. Through the demonstration of these settings and recommendations it was determined that the PowerPlex® 16 HS amplification chemistry and the use of the Applied Biosystems® 3500*xL* Genetic Analyzer could be used to produce accurate and reliable genotypes. The use of this amplification chemistry and instrument is recommended for use with future casework samples to increase both sensitivity and throughput.

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Figures

1 ng/µL	sec / kV	Avg. Ht.	Pk. Ht. Std. Dev.	Avg. PHR	PHR Std. Dev.
	24 sec / 1.2 kV	4144.936	1239.278	0.792	0.078
	12 sec / 1.2 kV	1946.500	584.562	0.766	0.147
	10 sec / 3 kV	4134.064	1218.391	0.792	0.217
	5 sec / 3 kV	2083.256	615.286	0.797	0.078
0.5 ng/µL	**sec / kV**	**Avg. Ht.**	**Pk. Ht. Std. Dev.**	**Avg. PHR**	**PHR Std. Dev.**
	24 sec / 1.2 kV	1688.808	510.221	0.753	0.088
	12 sec / 1.2 kV	792.654	248.531	0.754	0.090
	10 sec / 3 kV	1598.590	525.729	0.700	0.212
	5 sec / 3 kV	834.295	257.651	0.754	0.089
0.25 ng/µL	**sec / kV**	**Avg. Ht.**	**Pk. Ht. Std. Dev.**	**Avg. PHR**	**PHR Std. Dev.**
	24 sec / 1.2 kV	920.974	342.673	0.748	0.156
	12 sec / 1.2 kV	435.038	176.043	0.749	0.156
	10 sec / 3 kV	961.449	369.443	0.695	0.244
	5 sec / 3 kV	460.423	198.198	0.730	0.192

Figure 1: Average peak heights, peak height standard deviations, average peak height ratios, and peak height ratio standard deviations for the DNA target and injection time/voltage study. Targets were selected for analysis based on laboratory expectations. Heterozygote peak heights were not utilized for calculations. Based on values, an injection time/voltage of 24 sec/1.2 kV was selected for future studies. DNA target was further analyzed in the sensitivity study.

DYE	AVERAGE	STDEV	MIN	MAX	Lowest Trough	AT
BLUE	5.07	3.65	1	53	1	104
GREEN	5.58	2.13	1	43	1	84
YELLOW	4.40	1.76	1	39	1	76

Figure 2: Calculated analytical threshold for each dye channel. The average "noise" peak height, standard deviation of these heights, and min and maximum peak heights also used for calculations.

	Stochastic Threshold
All Dyes	323.82
Blue	277.70
Green	352.17
Yellow	349.72

Figure 3: Calculated stochastic threshold for combined dyes and each dye. This calculation utilized the peak height ratios and the peak height ratio standard deviations from the target study, as well as the analytical threshold value (125 RFU).

PowerPlex 16 HS	1.5			1.25			1			0.75			0.5			0.25			0.125		
	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio	Peak 1	Peak 2	Ratio
D3S1358	9109	11652	0.7818	13150	15313	0.85875	9604	9363	0.97491	7075	7300	0.969178	2747	4176	0.65781	1815	2490	0.7289	414	580	0.71379
	8570	10830	0.7913	9922	11585	0.85645	10765	10480	0.97353	7151	7331	0.975447	2767	4244	0.65198	1785	2477	0.7206	389	532	0.7312
	7698	9796	0.7858	12362	14559	0.8491	10285	10002	0.97248	6143	6460	0.950929	2793	4228	0.6606	1871	2621	0.7138	383	527	0.72676
TH01	16528	15096	0.9134	19385	20031	0.96775	16013	11234	0.70155	10157	11273	0.901002	6934	7222	0.96012	3552	3677	0.966	1094	1013	0.92596
	15843	14395	0.9086	14667	15164	0.96723	17951	12772	0.71149	10120	11251	0.899476	6957	7370	0.94396	3559	3744	0.9506	1052	933	0.88688
	14372	12979	0.9031	18699	19289	0.96941	17376	12213	0.70287	8971	10018	0.895488	7033	7399	0.95053	3728	3856	0.9668	1036	919	0.88707
D21S11	10848	10584	0.9757	10889	10803	0.9921	9140	6841	0.74847	6198	6495	0.954273	3644	2904	0.79693	1842	1980	0.9303	214.48	228.69	0.93786
	10193	10203	0.999	8162	8069	0.98861	10359	7845	0.75731	6181	6506	0.950046	3699	3008	0.81319	1849	1977	0.9353	214.52	228.69	0.93804
	9327	9090	0.9746	10573	10425	0.986	9842	7352	0.747	5407	5734	0.942972	3717	3015	0.81114	1924	2072	0.9286	214.53	228.7	0.93804
D18S51	14762	14355	0.9724	24318	18706	0.76922	12201	11645	0.95443	6358	10240	0.620898	4349	4052	0.93171	3022	1686	0.5579	1083	980	0.90489
	14201	13807	0.9723	17683	13567	0.76723	13749	13498	0.98174	6403	10286	0.622497	4493	4136	0.92054	3083	1709	0.5543	997	909	0.91174
	12915	12543	0.9712	23169	17690	0.76352	12975	12573	0.96902	5620	9018	0.623198	4562	4236	0.92854	3219	1779	0.5527	1005	904	0.8995
Penta_E	12898	10446	0.8099	15129	11876	0.78498	12545	8414	0.67071	9506	5058	0.532085	4580	5078	0.90193	2535	3231	0.7846	1581	377	0.23846
	12478	10159	0.8142	10868	8462	0.77862	14232	9467	0.66519	9624	5116	0.531588	4719	5201	0.90733	2611	3288	0.7941	1456	366	0.25137
	11139	9103	0.8172	14491	11304	0.78007	13316	8832	0.66326	8452	4404	0.52106	4837	5347	0.90462	2740	3454	0.7933	1443	361	0.25017
D5S818	21491	10745.5		28378	14189		18972	9486		14148	7074		7846	3923		3393	1696.5		1749	874.5	
	20635	10317.5		21694	10847		21009	10504.5		14182	7091		7892	3946		3390	1695		1646	823	
	18030	9015		27030	13515		20167	10083.5		12261	6130.5		7764	3882		3540	1770		1650	825	
D13S317	19778	9889		21163	10581.5		14407	7203.5		10822	5411		7825	3912.5		3759	1879.5		1600	800	
	19315	9657.5		16055	8027.5		16526	8263		10687	5343.5		7963	3981.5		3857	1928.5		1470	735	
	16633	8316.5		19922	9961		15676	7838		9318	4659		7935	3967.5		3976	1988		1494	747	
D7S820	10007	10251	0.9762	12914	15137	0.85314	6782	7333	0.92486	6261	6520	0.960276	2437	3166	0.76974	1527	1766	0.8647	883	1092	0.80861
	9564	9977	0.9586	9717	11376	0.85417	7735	8275	0.93474	6161	6415	0.960405	2478	3204	0.77341	1597	1808	0.8833	810	1030	0.78641
	8426	8645	0.9747	12284	14474	0.84869	7275	7850	0.92675	5415	5621	0.963352	2492	3262	0.76395	1674	1845	0.9073	832	1028	0.80934
D16S539	11136	10216	0.9174	13838	12898	0.93207	10172	9057	0.89039	7149	6069	0.84893	4894	5006	0.97763	1999	1800	0.9005	697	806	0.86476
	10946	9962	0.9101	10218	9524	0.93208	11637	10378	0.89181	7248	6095	0.840922	4976	5124	0.97112	2082	1846	0.8866	627	744	0.84274
	9607	8712	0.9068	13226	12288	0.92908	10819	9677	0.89444	6240	5283	0.846635	4975	5167	0.96284	2171	1946	0.8964	639	754	0.84748
CSF1PO	8674	10231	0.8478	13233	11419	0.86292	8086	7805	0.96525	7016	6341	0.903791	4302	2566	0.59647	2611	1107	0.424	810	664	0.81975
	8523	9988	0.8533	9659	8264	0.85558	9312	8905	0.95629	7074	6389	0.903167	4411	2633	0.59692	2670	1154	0.4322	739	611	0.82679
	7535	8862	0.8503	12606	10935	0.86744	8654	8240	0.95216	6086	5554	0.912586	4483	2653	0.59179	2796	1194	0.427	755	642	0.85033
Penta_D	14020	10879	0.776	16420	17330	0.94749	12216	11940	0.97741	8403	6921	0.823634	4297	4730	0.90846	2241	2047	0.9134	807	1519	0.53127
	13818	10676	0.7726	11903	12577	0.94641	13831	13578	0.98171	8492	6915	0.814296	4376	4835	0.90507	2293	2107	0.9189	736	1399	0.52609
	12165	9432	0.7753	15731	16631	0.94588	12839	12449	0.96962	7298	5917	0.81077	4477	4922	0.90959	2387	2221	0.9305	741	1418	0.52257
AMEL	12123	12673	0.9566	10944	15231	0.71853	10468	8761	0.83693	6593	8459	0.779407	3624	5311	0.68236	2534	2359	0.9309	252	768	0.32813
	11424	11753	0.972	10340	14546	0.71085	11773	9893	0.84031	6270	8101	0.773979	3697	5326	0.69414	2591	2429	0.9375	279	797	0.35006
	8615	8965	0.961	10548	14683	0.71838	12284	10366	0.84386	6299	8111	0.7766	3610	5335	0.67666	2621	2425	0.9252	259	763	0.33945
vWA	16483	14324	0.869	15201	12309	0.80975	9782	8266	0.84502	8541	7561	0.885259	5061	3914	0.77336	1314	2059	0.6382	1002	508	0.50699
	15545	13438	0.8645	14558	11770	0.80849	11022	9455	0.85783	8183	7216	0.881828	5034	3862	0.76718	1334	2129	0.6266	1052	532	0.5057
	11586	10046	0.8671	14754	11972	0.81144	11692	9923	0.8487	8189	7177	0.87642	5072	3900	0.76893	1379	2132	0.6468	988	512	0.51822
D8S1179	31639	15819.5		32214	16107		21133	10566.5		17107	8553.5		9315	4657.5		3088	1544		2655	1327.5	
	31421	15710.5		32022	16011		24701	12350.5		16399	8199.5		9519	4759.5		3249	1624.5		2807	1403.5	
	24042	12021		32102	16051		25558	12779		16607	8303.5		9499	4749.5		3261	1630.5		2625	1312.5	
TPOX	9836	14117	0.6967	13851	13275	0.95841	11114	8959	0.8061	7485	7038	0.940281	5981	4384	0.73299	2163	3174	0.6815	1412	1862	0.75832
	9547	13616	0.7012	13174	12489	0.948	12533	10222	0.81561	7011	6716	0.957923	5941	4356	0.73321	2288	3353	0.6824	1494	1938	0.7709
	7232	10311	0.7014	13658	12980	0.95036	13064	10509	0.80442	7213	6829	0.946763	5972	4428	0.74146	2281	3323	0.6864	1374	1811	0.7587
FGA	11588	9835	0.8487	12188	10666	0.87512	7414	5406	0.72916	5338	4715	0.88329	2554	3168	0.80619	1458	1540	0.9468	500	958	0.52192
	11204	9513	0.8491	11314	9790	0.8653	8444	6106	0.72312	5003	4504	0.90026	2542	3182	0.79887	1562	1641	0.9519	531	997	0.5326
	8359	7235	0.8655	11846	10436	0.88097	8698	6311	0.72557	5146	4571	0.888263	2601	3234	0.80427	1558	1617	0.9635	489	926	0.52808
Minimum	7232	7235	0.697	8162	8027.5	0.711	6782	5406	0.663	5003	4404	0.5211	2437	2566	0.592	1314	1107	0.424	214.5	228.69	0.2385
Maximum	16528	15820	0.999	24318	20031	0.992	17951	13578	0.982	10157	11273	0.9754	7033	7399	0.978	3728	3856	0.967	1581	1938	0.938
Average Peak HT Ratio			0.87			0.87			0.85			0.85			0.81			0.79			0.68
Peak Height Ratio StDev			0.09			0.08			0.11			0.13			0.12			0.17			0.22
Average Peak Height			11138			13250			10336			7008.89			4312.4			2219			842.51
Peak Height StDev			2330.7			3192.2			2422.1			1565.2			1186.3			673.2			400.65

Figure 4: Peak heights for each of the three samples analyzed for each dilution target within the sensitivity study. Homozygote peak heights were only divided by two for use in the average peak height calculations. Peak height ratios below 50% highlighted. Calculations made for combined peak heights within each amplification target.

	1ng	0.75ng	0.50ng	0.25ng	0.125ng
Average PHR	.85	.85	.81	.79	.68
St Dev	.11	.13	.12	.17	.22
Max	.98	.98	.98	.97	.94
Min	.66	.52	.59	.42	.24
Max/Min	.32	.45	.38	.54	.70

Figure 5: Average peak height ratio, standard deviation of peak height ratios, minimum peak height ratio, maximum peak height ratio, and range of peak height ratios for the sensitivity study samples. These ratios were analyzed to determine if the selected sensitivity range provided peak height ratios above 50%.

33182				Target: 0.5 ng Amp Date: 6/27/12 Run Date: 6/28/12		Target: 0.5 ng Amp Date: 6/28/12 Run Date: 6/29/12		Target: 0.5 ng Amp Date: 7/2/12 Run Date: 7/3/12			
		ALLELE 1	ALLELE 2	HEIGHT 1	HEIGHT 2	HEIGHT 1	HEIGHT 2	HEIGHT 1	HEIGHT 2	AVERAGE 1	AVERAGE 2
	D3S1358	16	18	6796	6232	7089	7240	6909	5157	6931	6210
	TH01	6	9.3	12547	10389	14116	13000	9536	9519	12066	10969
	D21S11	30	32.2	5027	4315	5466	3842	4081	4324	4858	4160
	D18S51	12		15400		13112		12203		13572	
	Penta E	13	16	5655	3991	6316	4645	4883	5346	5618	4661
	D5S818	12		9802		11384		11345		10844	
	D13S317	11	12	4667	3870	3657	4483	4279	3423	4201	3925
	D7S820	10	11	6152	5309	4961	3875	5506	4607	5540	4597
	D16S539	11		12070		12966		10562		11866	
	CSF1PO	11	12	5213	4595	6133	5675	5048	4172	5465	4814
	Penta D	11		14712		14946		12428		14029	
	AMELO	X	Y	6204	6574	5010	7183	5142	7165	5452	6974
	vWA	17	19	7061	5213	6107	5905	4515	5222	5894	5447
	D8S1179	13	15	6512	8501	6415	6428	4870	6905	5932	7278
	TPOX	8	11	7818	6327	6708	7465	7072	6830	7199	6874
	FGA	20	22	4155	4599	5456	3850	3509	3867	4373	4105
				3870	15400	3657	14946	3423	12428	3423	15400
				MIN	MAX	MIN	MAX	MIN	MAX	MIN	MAX

Figure 6: Example of data collected for the reporoducibility study consisting of allele calls, peak heights, average peak heights, and total minimum/maximum peak heights for each allele within multipe amplifications and runs on three separate days.

Locus	Average	Std. Dev.	Min	Max	Stutter Ratio	Promega Stutter Ratio
D3S1358	0.10	0.02	0.02	0.16	16%	13%
TH01	0.03	0.01	0.00	0.05	6%	6%
D21S11	0.09	0.02	0.03	0.17	16%	22%
D18S51	0.09	0.03	0.04	0.18	17%	13%
Penta E	0.04	0.02	0.01	0.11	9%	13%
D5S818	0.07	0.02	0.03	0.12	13%	11%
D13S317	0.07	0.03	0.02	0.15	15%	12%
D7S820	0.06	0.02	0.02	0.11	11%	10%
D16S539	0.08	0.02	0.03	0.14	15%	13%
CSF1PO	0.06	0.02	0.02	0.09	11%	10%
Penta D	0.02	0.02	0.01	0.10	7%	6%
vWA	0.09	0.03	0.01	0.16	16%	14%
D8S1179	0.07	0.02	0.03	0.12	12%	11%
TPOX	0.03	0.01	0.01	0.10	8%	6%
FGA	0.09	0.02	0.05	0.17	16%	14%

Figure 7: Calculated stutter values and Promega recommended stutter values for minus four stutter in PowerPlex® 16 HS. Stutter values were calculated using the standard deviation of the peak height ratios between the stutter peak and the parent peak from which the stutter originated within a marker, as well as the average peak height ratio of the stutter peak and parent peak.

Locus	Average	Std. Dev.	Min	Max	Stutter Ratio
D3S1358	0.02	0.00	0.01	0.03	3%
TH01	0.00	0.00	0.00	0.01	1%
D21S11	0.02	0.01	0.01	0.07	5%
D18S51	0.01	0.01	0.01	0.06	5%
Penta E	0.00	0.00	0.00	0.00	0%
D5S818	0.02	0.01	0.01	0.06	4%
D13S317	0.03	0.02	0.01	0.12	8%
D7S820	0.02	0.01	0.01	0.04	3%
D16S539	0.02	0.01	0.01	0.04	4%
CSF1PO	0.02	0.01	0.01	0.05	6%
Penta D	0.02	0.02	0.01	0.05	8%
vWA	0.01	0.02	0.00	0.11	6%
D8S1179	0.01	0.00	0.01	0.02	2%
TPOX	0.01	0.00	0.01	0.02	2%
FGA	0.03	0.02	0.01	0.09	9%

Figure 8: Calculated stutter values and Promega recommended stutter values for plusfour stutter in PowerPlex® 16 HS. Stutter values were calculated using the standard deviation of the peak height ratios between the stutter peak and the parent peak from which the stutter originated within a marker, as well as the average peak height ratio of the stutter peak and parent peak.

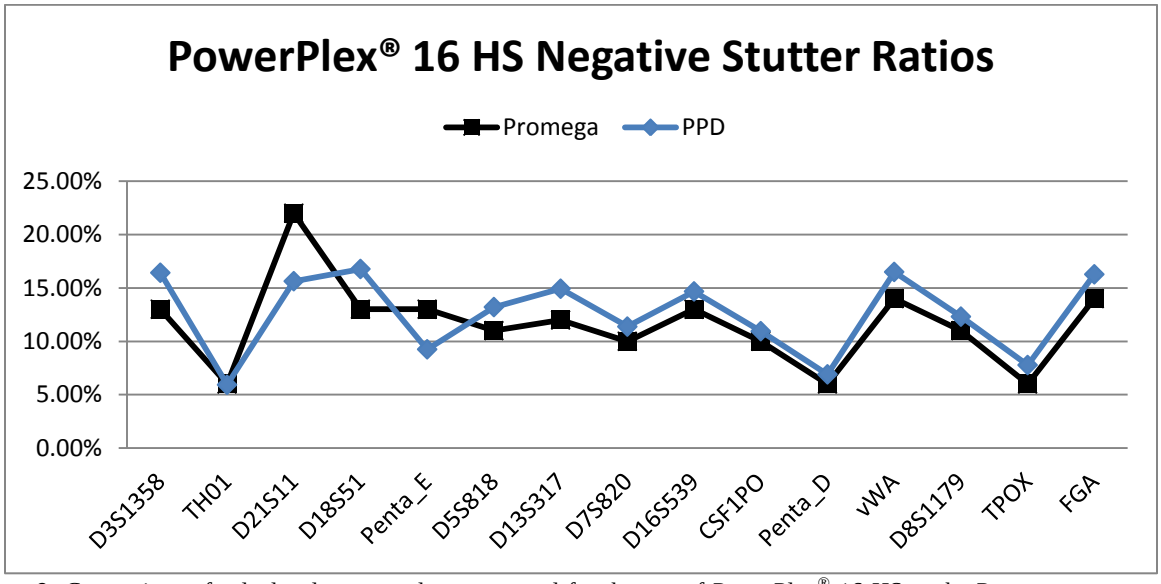

Figure 9: Comparison of calculated stutter values generated for the use of PowerPlex® 16 HS to the Promega recommended stutter values. The use of Promega stutter values for future analysis was selected due to the consistency of generated values to recommended values. Stutter values will be further analyzed with additional samples in a future validation study.

35394 : 35386 Ratio	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	AMEL	vWA	D8S1179	TPOX	FGA
1 to 0	16,17	9,9.3	29,30	10.2,20	5,14	13,13	11,12	11,13	11,13	12,12	13,13	X,Y	15,16	15,16	8,11	23,29
19 to 1	(14),(15), 16,17	(7),9,9.3	29,30	(10.2),20	5,14	13,13	11,12	(10),11,13	11,13	12,12	(11),13	XY	15,16	15,16	8,11,(12)	23,29
MAJOR COMP	16,17	9,9.3	29,30	20	5,14	13,13	11,12	11,13	11,13	12,12	13,13	XY	15,16	15,16	8,11	23,29
9 to 1	(14),(15), 16,17	(7),9,9.3	(28),29,30	10.2,(17),20	5,14	(9),(12),13	11,12	(10),11, (12),13	(9),11,13	12,12	(11),13	(X)*,Y	15,16,(20)	15,16	8,11,(12)	(22),23, (25),29
MAJOR COMP	16,17	9,9.3	29,30	10.2,20	5,14	13,13	11,12	11,13	11,13	12,12	13,13	XY	15,16	15,16	8,11	23,29
4 to 1	(14),(15), 16,17	(7),(9),9.3	29,30	10.2,(17),20	5,(9),14	(9),(12),13	11,12	(8),(10),11, 13	(9),11,13	12,12	13,(14)	XY	15,16,(20)	(14),15,16	8,11,(12)	(22),23, (25),29
MAJOR COMP	16,17	9.3	29,30	10.2,20	5,14	13,13	11,12	11,13	11,13	12,12	13,13	XY	15,16	15,16	8,11	23,29
1 to 1	14,15,16,17	(7),(9)*,9.3	28,29,30	10.2,17,(20)	(5),9,(11),14	(9),12,13	11,12,	8,10,11,13	(9),11,13	12,(13)	11,13,14	XY	(15),16,(20)	(14),15,(16)	8,11,(12)	22,23,25, (29)
MAJOR COMP	-	9.3,9.3	-	-	-	-	11,12	-	-	-	-	XY	-	-	-	-
1 to 4	14,15,(16), (17)	7,(9),9.3	28,30	(10.2),17, (20)	(5),9,11, (14)	9,12,13	11,12	8,10,(11), (13)	(9),11,(13)	12,13	(11),13,14	XY	(15),16,(20)	(14)*,15, (16)	8,(11),(12)	22,(23),25, (29)
MAJOR COMP	14,15	7,9.3	28,30	17,17	9,11	-	11,12	8,10	9,11	12,13	-	XY	16	-	8	22,25
1 to 9	14,15,(16), (17)	(7),(9),9.3	28,30	(10.2),17	9,(11),(14)	9,12,(13)	11,12	(8)*,10,(11)	9,11,(13)	12,13	11,(13),(14)	XY	(15),16,20	14,15	8,(11),12	22,25
MAJOR COMP	14,15	7,9.3	28,30	17,17	9	9,12	11,12	8,10	9,11	12,13	11	XY	16,20	14,15	8,12	22,25
1 to 19	14,15,(16)	7,9.3	28,30	(10.2),17	9,11,(14)	9,12,(13)	11,12	8,10,(11)	9,(11)*	12,(13)*	11,14	XY	(15),16,(20)	14,15	8,(11),(12)	22,25
MAJOR COMP	14,15	7,9.3	28,30	17,17	9,11	9,12	11,12	8,10	9,11	12,13	11,14	XY	16	14,15	8,12	22,25
0 to 1	14, 15	7, 9.3	28, 30	17, 17	9, 11	9, 12	11, 12	8, 10	9, 11	12, 13	11, 14	X, Y	16, 20	14, 15	8, 12	22, 25

Figure 10: Allele calls were generated for two contributor mixture samples. Apparent major and minor contributors were designated within each sample. A 4:1 two contributor mixture is the apparent optimal mixture to observe both contributors, above this ratio the major contributor is more apparent and less of the minor contributor or light alleles are detected. (*signifies a peak height ratio between 55-59%)

Contributors	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	AMEL	vWA	D8S1179	TPOX	FGA
3	14,15,16	7,(8),(9),(9.3)	28,(29),30,(31.2)	(16),17,(18)*,(21),(23)	7,(8),(9),11,17	8,(9),12,(13)	8,11,(12),(13)	(8),10,(11),(12),(13)	8,9,11,12,13	7,10,12,(13)	8,11,13,(14)	XY	16,(17),18,19,20	12,14,15,(16)	6,(7),8,(11),12	(21),22,23,(25)*,30
MAJOR COMP	-	7	28,30	17	-	-	8,11	10	-	-	-	XY	-	-	-	-
5	(14),(15),16,(17),18	(6),(7),8,(9),9.3	(28),29,(30),31.2	(10.2),12,16,(17),(18),(20),(21)	(5),(7),(9),(14),16,(17)	(8),(9),(11),(12),13	(8),(11),12	(8),(10),11,12,(13)	(8),(9),(11),12,(13)	(7),10,11,(12),(13)	9,(11),12,13,(14)	XY	15,16,17,(18),(19),(20)	(12),(14),15,(16)	(6),8,(11)*,(12)	(22),(23),25,(26)*,29,(30)
MAJOR COMP	16,18	8,9.3	29,31.2	12,16	16	13	12	11,12	12	10,11	-	XY	-	15	8	25,29

Figure 11: Allele calls were designated for three and five contributor mixture samples. The three person mixture contained a major allele in TH01, D21S11, D18S51, D13S317, and D7S820 (**Table 10**). Major alleles were detected in each locus of the 5 person contributor except the Penta D and vWA markers. No full major contributor profile could be detected at either the three or five contributor mixture samples. (*signifies a peak height ratio between 55-59%)

Sample	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	AMEL	vWA	D8S1179	TPOX	FGA
33153	15,18	6,8	28,31	14,14	5,7	11,13	11,11	10,11	10,11	10,10	9,13	XY	16,19	13,14	8,8	21,21
33153	15,18	6,8	28,31	14,14	5,7	11,13	11,11	10,11	10,11	10,10	9,13	XY	16,19	13,14	8,8	21,21
33153-.01	15,(18)	6,(8)	28,(31)	14,14	NR	11,13	11	10,11	(10),11	10	9,(13)	XY	(16),19	14	8,8	21,21
33153-.01	15,(18)	6,(8)	28,(31)	14,14	NR	11,13	11,11	10,11	(10),11	10	9,(13)	XY	(16),19	14	8,8	21,21
33153-.02	15,18	6,(8)	28,31	14,14	5,7	11,13	11,11	10,11	10,11	10,10	9,13	(X),Y	16,19	13,14	8,8	21,21
33153-.02	15,18	6,(8)	28,31	14,14	5,7	11,13	11,11	10,(11)	10,11	10,10	9,13	XY	16,19	13,14	8,8	21,21
33158	15,16	6,9.3	29,30	17,18	12,18	11,12	8,10	12,12	9,11	11,12	9,10	XX	15,18	13,13	8,11	21,23
33158	15,16	6,9.3	29,30	17,18	12,18	11,12	8,10	12,12	9,11	11,12	9,10	XX	15,18	13,13	8,11	21,23
33534	(16),17	6,7	28,30,(32.2)	(14),(15),(16),18,(19),(20),21	(8),11,13	(11),12,13	(9),11,12	10,(12)	11,12	(7),8,(10), 12	(2.2),12	XY	(15),(16),18,19	13,14	8,9	(22),(23),24,25
33534	(16),17	6,7	28,30,(32.2)	(16),18,(19),21	(8),11,13	(11),12,13	11,12	10,(11),(12)	11,12	(7),8,(10), 12	(2.2),12	XY	(15),(16),18,19	13,14,(19)	8,9, (12),(13)	(17.2),(22),(23),24,25
33535	16,17	6,7	28,32.2	14,16	10,11	11,11	9,13	11,12	8,11	12,13	10,12	XX	15,15	10,14	8,10	22,24
33535	16,17	6,7	28,32.2	14,16	10,11	11,11	9,13	11,12	8,11	12,13	10,12	XX	15,15	10,14	8,10	22,24
33536	16,17	6,7	28,(29),(30),(32.2)	15,18,19,(20),21	8,11,12,13	11,12,13	(11),12	10,(11),(12)	9,10,11,12	7,8,10,12, (13)	2.2,11,12	XY	(15),(16),18,(19)	(10),(13), 14	8,(9),(10)	23,24,(25)
33536	16,17	6,7	28,(29),(30),(32.2)	15,18,19,(20),21	8,11,12,13	11,12,13	(9),(11),12	10,(11),(12)	9,10,(11), (12)	7,8,10,12, (13)	2.2,(10),11, 12	XY	(15),(16),18,(19)	(10),(13), 14	8,(9),(10)	23,24,(25)
33537	16,17	6,7	28,32.2	14,16	10,11	11,11	9,13	11,12	8,11	12,13	10,12	XX	15,15	10,14	8,10	22,24
33537	16,17	6,7	28,32.2	14,16	10,11	11,11	9,13	11,12	8,11	12,13	10,12	XX	15,15	10,14	8,10	22,24
33879	15,16	(5),7,9	28,33.2	14,16	7,11	12,13	10,12	10,11	12,12	8,11	10,11	XY	17,17	13,15	8,9,(13)	(16),18,(24),(25),26
33879	15,16	7,9	28,33.2	14,16	7,11	12,13	10,12	(7),10,11, (12)	12,12	8,11	10,11	XY	17,17	13,15	8,9,(13)	18,(20.2),(24),(25),26
34318	15,18	6,7	30,31.2	16,18	7,(17)	11,13	8,13	10,11	9,10	8,10	5,12	XY	16,19	(9),13,14, (16)	12,12	(20),22
34318	15,18	6,7	30,31.2	16,18	7,(17),(18)	11,13	8,13	(9),10,11	9,10	8,10	5,12	XY	16,(18),19	13,14,(16)	(10),(11),12	22,22
34347	14,14	7,7	27,32.2	12,14	11	11,11	11,12	10,14	11,12	NR	10	XY	17,(19)	11,13	8,9	23
34347	14,14	7,7	27,32.2	12,14	11	11,11	11,12	10,14	11,12	11	10	XY	17,19	11,13	8,9	23
34571	15,16	7,7	30,30	12,18	11,(17)	12,12	8,9	9,10	9,(12),(13)	9,11	10,14	XX	14,18	11,13	8,8	23,25
34571	15,16	(5),7	30,30	12,18	11,(17)	12,12	8,9	9,10	9,(12),(13)	9,11	10,14	XX	14,18	11,13	8,8	23,25
34572	15,16	7,7	30,30	12,18	11,17	12,12	8,9	9,10	9,13	9,11	10,14	XX	14,18	11,(13)	8,8	23,25
34572	15,16	7,7	30,30	12,18	11,17	12,12	8,9	9,10	9,13	9,11	10,14	XX	14,18	11,(13)	8,8	23,25
34573	15,16	7,(9)	30,30	12,18	11,17	12,12	8,9	9,10	9,13	9,11	10,14	XX	14,18	11,13	8,8	23,25
34573	15,16	7,7	30,30	12,18	11,17	12,12	8,9	9,10	9,13	9,11	10,14	XX	14,18	11,13	8,8	23,25
34574	14,15,(16), (17)	6,8,(9.3)	28,(30),(32.2),35	15,19	(8),12,15	11,11	11,12	8,10	10,11,(12)	10,11,(12)	2.2,(12),14	XY	(16),17,19	14,15	8,9,(11)	22,24
34574	14,15,(16), (17)	6,8,(9.3)	28,(30),(32.2),35	15,19	(8),12,15	11,11	11,12	8,10	10,11,(12)	10,11,(12)	2.2,(12),14	XY	(16),17,19	14,15	8,9,(11)	22,24
35851	14,17	6,8	28,29	16,18	9,10	11,13	12,12	10,12	9,10	12,13	8,11	XY	15,18	13,13	11,11	21,22
35851	14,17	6,8	28,29	16,18	9,10	11,13	12,12	10,12	9,10	12,13	8,11	XY	15,18	13,13	11,11	21,22
35852	14,17	6,8	28,29	16,18	9,10	11,13	12,12	(7),10,12	9,10	12,13	8,11	XY	15,18	(12),13	11,11	21,22
35852	14,17	6,8	28,29	16,18	9,10	11,13	12,12	10,12	(5),9,10	12,13	8,11	XY	15,18	13,13	11,11	21,22
36244	14,(15),16, (18)	(6),(8),9,(9.3)	(28),30,32.2	16,(17),20	11,(15),19	10,11,(12)	8,12,(13), (14)	8,10,(12)	11,13	(10),12	12,13	XY	16,17,(19)	12,13	8,10	20,21,(23)
36244	14,(15),16, (18)	(6),(8),9,(9.3)	(28),30,32.2	16,(17),20	11,(15),19	10,11,(12)	8,12,(13), (14)	8,10	11,13	12,12	12,13	XY	16,17,(19)	12,13	8,10	20,(21),(23)

Figure 12: Allele calls were made based on the analysis of the non-probative casework samples. These samples consisted of a variety of substrates an extraction methods typically encountered at the PPD. Major and minor contributors were designated and results were compared to data previously generated from the used sample extracts.