Internal Validation of the Promega PowerPlex® Fusion System using the Applied Biosystems® 3130*xl* Genetic Analyzer

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Abstract

Prior to placing a new method into service in a crime laboratory setting, accredited laboratories must perform internal validations according to standard 8 of the FBI Quality Assurance Standards to verify that developmentally validated methods work reliably and robustly. An internal validation was performed at the Marshall University Forensic Science Center DNA Laboratory on the PowerPlex® Fusion System amplification kit in conjunction with the Applied Biosystems® 3130*xl* Genetic Analyzer.

Promega recently released a new amplification kit under the name of PowerPlex® Fusion System. This kit is a 24-locus multiplex used for human identification applications. Autosomal STR loci include the 13 core CODIS (U.S.A.) loci as well as the 12 European Standard Set (ESS) core loci in addition to D2S1338, D19S433, Penta D and Penta E. The kit also includes the sex-determining Amelogenin locus and the Y-STR DYS391 to identify null Y alleles from Amelogenin and help with mixture interpretation. The kit utilizes a 5-dye chemistry and is able to function with most Genetic Analyzers including the ABI PRISM® 310 and 3100 as well as the Applied Biosystems® 3130, 3130*xl*, 3500 and 3500*xl*.

Validation studies such as amplification cycle number, injection time and voltage, target DNA load, threshold studies (analytical and stochastic) , peak height ratio for heterozygote alleles, sensitivity, precision, reproducibility, concordance, mixture studies, and non-probative

casework sample studies were performed to ensure the reliable functionality of the kit chemistry.

The results of this validation showed that the PowerPlex® Fusion System produces accurate and reproducible STR profiles. Furthermore, data regarding stutter formation, heterozygote balance and mixture patterns have been evaluated to develop Forensic Interpretation Guidelines as part of the incorporation of the kit into the Marshall University Forensic Science Center's Standard Operating Procedure Manual. Future studies will include a non-human DNA study, and enhancement to the mixture study including mixtures of relatives.

Introduction

Due to the extreme level of scrutiny that forensic science undergoes due to the National Academy of Science report released in 2009, and due to recommendations from FBI Quality Assurance Standards in particular Standard 8, there exists a need to validate every reagent, chemistry and instrument used in the process of evidence handling and analysis. Of these recommendations is the need to internally validate amplification kits to prove their efficiency and accuracy of function intended for. The Internal Validation performed at a laboratory is the foundation of the confidence when employing procedures, moreover an analyst has to have confidence in the reagent in use.

MUFSC is constantly seeking to increase its options and resources for DNA testing. Next Generation kits are hitting the market specifically the new PowerPlex® Fusion System. Promega Corporation (Madison, Wisconsin) recently released a new multiplex aimed at forensic DNA analysis under the name of PowerPlex® Fusion System that is able to amplify and detect 24

different loci including the core 13 Combined DNA Index System-CODIS loci (CSF1PO, FGA, TH01, TPOX, vWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11) and the 12 European Standard Set loci (TH01, vWA, GA, D21S11, D3S1358, D8S1179, D18S51, D10S1248, D22S1045, D2S441, D1S1656, and D12S391), supplemental loci (Penta E, Penta D, D2S1338, D19S433) and sex-determining Amelogenin locus and the Y-STR DYS391 to identify null Y alleles from Amelogenin and help with mixture interpretation. The kit was developed to produce a future bridge between both the european and american databases.

Several studies were done as a part of the internal validation that was done for the PowerPlex® Fusion System chemistry. Initially starting with cycle number and injection time study and then moving to target DNA load. After establishing the initial parameters for DNA typing, studies such as analytical threshold study, and stochastic threshold study as well as peak height ratio study were done. Following that a stutter study was done to identify the frequency and percentage of stutter- a common amplification artifact resulting from DNA to primer slippage resulting in a peak four base pairs either before or after the parent peak known as +4 or -4 stutter. Concordance, precision and reproducibility studies were done to ensure that peaks amplified by the Fusion chemistry are true peaks and that the kit does not falsely call alleles. A mixture study was done to test the limits at which mixtures of different profiles at different ratios could be identified and distinguished from each other. Finally a number of non-probative mock casework samples were run as well as inhibited samples to test out the parameters previously set by the studies and to ensure that the PowerPlex® Fusion System kit is ready for true case-work.

Materials and Methods

The validation of the PowerPlex Fusion ® System amplification kit was performed using a Applied Biosystems® 3130*xl* Genetic Analyzer. Samples used in the validation where extracted using the Qiagen EZ1 DNA Investigator Kit® and the Qiagen EZ1® Advanced XL. After extraction, samples where quantitated using the Qiagen Investigator Quantiplex HYres® quantification kit. Data was analyzed using GeneMapper® ID v3.2.1. The samples used were TF NIST traceable FTA card punches and 35 convicted offenders samples, previously tested, determined to have alleles in several loci at 3 or more STR repeats of difference to aid in stutter studies to be able to distinguish a true peak from a stutter artifact peak.

Cycle Number, DNA Target, Reaction Volume, Injection Time, Sensitivity Study

An initial study to determine the optimal cycle number for amplification as well as the optimal DNA load and injection time was performed. Promega® recommends a 30 amplification cycle with a 0.5 ng DNA load and injected at 5 seconds. The extracted TF punches were quantitated using the Qiagen Investigator Quantiplex HYres® kit. Then the punches were combined together to give a higher volume to help in concentrating the extract to the needed concentration. A Serial dilution of the combined extraction product was created ranging from 10 ng to 0.0156 ng according to the table below.

TF Extracts Dilution Series
10 ng
5 ng
2.5 ng
1 ng
0.5 ng
0.25 ng
0.125 ng
0.0625 ng
0.0313 ng
0.0156 ng

The serial dilution was then re-quantitated to make sure the concentrations were accurate. The serial dilution was then amplified in triplicates at 29 cycles, 30 cycles and 31 amplification cycles. The amplification products were then run on the ABI 3130*xl* genetic analyzer at four different injection times of 3 seconds, 5 sec, 10 sec, and 15 sec all at 3kV of injection voltage with a total run time of 1500 seconds. The results of the four capillary electrophoresis runs were used to identify the optimal amplification and electrophoresis conditions. Results were analyzed using GeneMapper® ID v3.2.1 at a 20 relative fluorescence units (*rfu*) analytical threshold. Since a single source of DNA was used and the aim of the study was to identify true peaks and study their heights a 20% filter was used to remove artifacts 20% lower than the largest peak to aid in the analysis process. The results were then exported from GeneMapper® ID into and excel worksheet, where they were separated according to injection time, cycle number, dye color, and DNA target volume.

Concordance Study

35 samples previously amplified using Promega® PowerPlex® 16 were extracted and quantitated and then diluted down to 0.125 ng./ml. These samples where then amplified at 0.125 ng. target, 0.5 ng. target and 1.875 ng. amplification target at 30 cycles and then where ran at 3kV with a 5 second injection time and a total run time of 1700 seconds. The results where then

compared to the profiles previously obtained from PowerPlex®16 - eliminating the new loci present in the PowerPlex ® Fusion kit to match the 16 loci present in the PowerPlex® 16 kit- to test for the concordance and allele calling consistency of the amplification kit.

Peak Height Ratio Study

For the peak height ratio study results from the 5 second injection time of the TF punches as well as the results from the 35 samples ran were used to calculate the average Peak Height Ratio and the range of Ratios for different amplification loads. Ideally a 70% or greater peak height ratio is acceptable according to SWGDAM standards and according to standard protocol at the Marshall University Forensic Science Center.

Analytical Threshold

Method 1: The analytical threshold was then calculated using two different methods. According to the IUPAC (International Union for Pure and applied Chemistry) that utilizes the following formula:

$$AT = Y_{bI} + ks_{bI}$$

AT= Analytical Threshold

Y_{bI}= Average blank RFU signal

k= 3

S_{bI}= Standard Deviation of the blank signal

Method 2: Section 1.1 of the SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories provided an example of a means to calculate the analytical threshold. It states, "…an analytical threshold may be based on two times the intensity difference between the highest peak and lowest trough within the instrumental noise data." The following formula was used to calculate the analytical threshold:

$$AT = 2(Y_{max} - Y_{min})$$

AT= Analytical Threshold

Y_{max}= Highest peak within instrumental noise

Y_{min}= signal of the lowest trough

Furthermore the Limit of Detection- minimum peak height detected by the chemistry- and Limitof Quantification- minimum peak height that the chemistry can quantify- were also calculated using the following formulas:

LOD= Average noise signal + 3 * Standard Deviation

LOQ= Average noise signal + 10 * Standard Deviation

Reagent Blanks from the extraction step of the 35 convicted offenders samples were used to calculate the analytical threshold. The 3 reagent blanks were set in 5 replicates in 16 columns with 1 run negative and injected 5 times. The results were analyzed using GeneMapper® with an analytical threshold of 1 rfu. The results were then exported to excel, where peaks two base pairs

before and after the Internal Lane Standard (ILS) peaks were deleted to remove any peak that would result from pull-up from the ILS. The range for each dye was then set, where the calculation range started at the beginning of the first loci and ended at the end of the last loci, meaning that peaks outside the calling range of the dye channel were not considered when calculating the analytical threshold.

Stochastic Threshold Study

The stochastic threshold is the limit at which a homozygote peak can be called without the consideration of drop-out occurring. The stochastic threshold was calculated using the following formula:

ST= [1/ (Average PHR- 3x STD)] x AT

where the Peak Height Ratios for every dye channel were calculated from the TF punches as well as the standard deviation for each and the analytical thresholds used from the previous results to give a stochastic threshold unique to every de channel.

Precision Study

For the precision study 16 ladders were injected five times using the ABI® 3130*xl* genetic analyzer. The results of the injections were analyzed using GeneMapper® ID v3.2.1 and the result table exported into an excel workbook. Peak size was analyzed for each allele to ensure that the sizing was within 3 standard deviations, where ideally three times the standard deviation should be less than 0.5bp.

Stutter Study

35 convicted offenders samples imported into CODIS were extracted from FTA cards and swabs using the Qiagen® Investigator EZ1 kit and then quantitated using the Qiagen® HYres quantification kit. Then the samples were then amplified at three different amplification targets of 0.125 ng., 0.5 ng., and at 1.875 ng. And then the amplification tray was run on the ABI® 3130*xl* genetic analyzer. Results were then analyzed using GeneMapper® *ID* v3.2.1, table settings were then altered to call for 50 alleles at and analytical threshold of 20 rfu. with no 20% filter. The result table was then exported into a macro-excel spreadsheet downloaded from the "strbase.com" website. Stutter peaks for compared to the initial peak called and scatter plots of 1. Stutter Peak Height versus Allele Peak Height, 2. Stutter/Allele Peak Height Ratio % versus Allele Peak Height and 3. Percent Stutter versus Allele call were created for 21 of the 24 loci of the PowerPlex® Fusion where no results where identifiable for both Penta E and D loci as well as for the Amelogenin Sex determining loci. To calculate the stutter of each loci the following formula was used:

*Stutter = Average Stutter Ratio + 3 * Stutter Ratio Standard Deviation*

Contamination Study

A checkerboard plate was set-up and run on the ABI 3130*xl* Genetic Analyzer to see if any contamination results from cross-contamination between wells. The set-up was of altering Run Negative and Ladders to be able to detect any peak that would result from cross-contamination. Furthermore all negative samples ran throughout the validation were analyzed for possible contamination that would be a result of either the injection step or the amplification kit itself- contamination that is not a result of analyst error, processing, or extraction.

Mixture Study

In order to test the capability of the amplification chemistry to identify profiles in mixtures at different ratios three mixtures at different ratios were amplified. From the 35 convicted offenders samples previously used four profiles were chosen that had the lowest number of overlapping alleles throughout the 24 loci. Out of those profiles only one was female, so that profile was chosen for the Male:Female mixture. Another male profile was chosen were only four alleles overlapped, which would maximize the ability to calculate the contributor ratio at every loci. Using this male profile another male profile was matched to it with seven alleles overlapping to produce the Male: Male mixtures. For the Male: Male: Male mixtures a third male profile was chosen to match with the first male at eight alleles and the second male at 12 alleles. To produce the mixtures the pre-extracted samples quantitated and then diluted down to 0.1 ng/µl. To produce the mixtures the samples were mixed at the same ratio needed to produces 19:1, 9:1, 4:1, 1:1, 1:4, 1:9, 1:19 ratios for both the male: Female mixtures and the Male: Male mixtures, as for the three male mixtures they were mixed at a 1:1:1 ratio. The mixtures were then amplified at a 1ng. amplification target load at 30 cycles. They were then run on the ABI® 3130*xl* Genetic Analyzer at five second injections and a 1700 second run time. The results were then analyzed using the GeneMapper® ID v3.2.1 at an analytical threshold of 20 rfu. The results were then exported to excel to calculate the ratio for each mixture at each loci and for each dye channel.

Inhibition Study

For the Inhibition study 3 amplification inhibitors were used EDTA (Ethylenediaminetetraacetic acid), Humic acid and Denim Blue Dye. EDTA was serially diluted

into 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM from a stock solution of 0.5 M EDTA at pH 8.0. For the Humic Acid five concentrations were produced using Sigma-Aldrich® stock Humic Acid powder of 100 ng/µl., 150 ng/µl., 200 ng/µl., 250 ng/µl., 300 ng/µl. These concentrations for EDTA and Humic Acid were chosen after reviewing Promega's developmental validation of the Fusion® kit where the lowest concentrations chosen gave a full profile and the lowest concentration gave a partial profile yet the concentrations in between were not tested, therefore these concentrations were narrowed down to test the range at a higher level. As for the Denim Blue Dye the ratios that were used were 1:5, 1:10, 1:50, 1:100, 1:500 of dye:MBG water ratio. A TF punch was extracted and quantitated to produce the DNA needed to amplify. The TF was diluted to 0.1 ng/µl. To produce the amplification plate the TF DNA target was 0.5ng and the needed MBG water that helps get the amplification volume to 25µl. was then replaced with the different inhibitors, hence adding 10µl. of the inhibitors to the 25 µl. total amplification volume. The amplification plate was then amplified at 30 cycles and then run twice on the ABI® 3130*xl.* Genetic Analyzer at five seconds injections time and 1700 second total run time. The results were then analyzed using the GeneMapper® ID v3.2.1 at 20 rfu and then exported to excel.

Mock Casework Samples Study

For the mock casework study 25 samples were chosen to test. Five cigarette butts, five differential samples, four swabs of a mobile phone and the Otter box® protective case(from the speaker, ear piece, Rubber case, screen- five touch evidence swabs)two door handles, face of the microwave in break room, two keyboards- two gum samples, two buccal swabs, and two hair samples. The hair samples were previously extracted and quantitated for a separate validation for hair extraction on the Qiagen® Investigator EZ1 kit , but the 23 other samples were extracted using the Qiagen® Investigator EZ1 kit and quantitated using the Qiagen® Investigator

Quantiplex HYres quantification kit. Those samples were then amplified on the 9700 thermal cycler using the Powerplex® Fusion kit at 30 cycles. After amplification the samples were then run on the ABI® 3130*xl* Genetic Analyzer at 5 second injection and 1700 second total injection time. The results were then analyzed on GeneMapper® *ID* v3.2.1.

Note*: All samples were analyzed with a 20% filter since the aim of the study was to calculate the results based on peak heights rather than allele calls, therefore the filter helped in the analysis process by eliminating artifacts. For the mixture study, the stutter study, and the mock casework samples no filter was used to be able to attain all possible allele calls.*

Results

Troubleshooting for Analytical Threshold

Three different artifacts were noticed in the reagent blanks for the extraction of the convicted offenders' samples that were at TH01(Yellow Channel), D18 (Green channel) and D2(Green Channel). An artifact table was made to pin point the artifact in the samples themselves (table), that showed non-concordance between the presence of the artifact in the reagent blank and presence of the artifact in the samples, where for example samples that were extracted with Reagent Blank 4 that was completely free of any artifact had the highest amount of artifacts present. Moreover the artifact that was found in Reagent Blank 3 was not found in the samples extracted with it. To see if the artifacts were reproducible the three Reagent Blanks (RB3, RB4,RB5) were amplified again and ran using a different PowerPlex® Fusion kit with a different lot number to ensure that the artifacts were not related to the amplification or run process nor related to the chemistry kit as well. The results for the rerun showed clean reagent

blanks for RB3 and RB5 and only 1 artifact was present in RB4 at a different loci this time D16 (Green Channel), but this artifact showed morphology indicating it to be an amplification artifact product rather than contamination which was the case with D18 and D2 artifact previously detected. Based on the troubleshooting it was decided that the peaks in the reagent blanks used to produced the analytical threshold would be cut out of the calculation since they showed to be contamination artifact that was non-reproducible and not related to the true baseline. The TH01 artifact was cut out as well since it was 1-2 basepairs smaller than the first allele call in the yellow dye channel. The artifact that was present in the rerun of the reagent blanks found at D16 was not removed due to its morphology, so it was suspected to be an amplification artifact and thus should be considered when calculating the analytical threshold.

Cycle Number, DNA Target, Reaction Volume, Injection Time Study

A plate set-up of the serial dilutions of the TF punches was initially run at 3 seconds injection time and then the plate was re-heated and snap-cooled and run at 5 seconds and then 10 seconds and 15 seconds. Several samples had failed Internal Lane Standards so the plates were re-run at 3, 5 and 10 seconds again. The failed samples at 15 seconds were due to a high amount of pull up resulting into a failed ILS for the 31 cycle 2.5 ng. DNA target so re-running the plate would have not resulted in a difference. After re-running the plates the results were compiled and analyzed on GeneMapper® ID v3.2.1 at an analytical threshold of 20 rfu with a 20% filter to decrease the amount of peak to click –off since the samples were of single source and of known profile.

The lowest standard deviation for peak height ratio was at 10 second injection time where the results of the 5 second injection time were less than 2 % difference. Yet with the 10 second

injection time extreme allele drop out occurred with one sister peak being at 130 + rfu with the other peak being completely undetectable, whereas in the 5 second injection time results this anomaly was less observable. A re-run of the same amplification plate was set-up again to see whether this occurrence was reproducible, and in fact the allelic drop-out still occurred at 10 seconds of injection. Furthermore results from the 10 second injection time showed extreme peak imbalance in several loci excessive of 50% which was not observed in the results of the 5 second injection time even though the set-up was the same and coming from the same amplification plate.

Drop-out still occurred in the results until 0.125ng, with peaks at 0.25ng. being below the calculated stochastic threshold, hence giving rise to the chance of allelic drop-out.

Therefore the optimal injection time was chosen to be 5 seconds at 3kVolts and a DNA amplification load range of 0.5ng to 1.0ng. amplified at 30 cycles.

Concordance Study

The concordance study was used to check for allele calling consistency between different amplification chemistries used on separate instruments and ran on separate days. 35 samples previously tested and uploaded to CODIS were used for the concordance study. The samples were initially run on a different ABI 3130*xl* genetic analyzer at the laboratory using the PowerPlex® 16 amplification kit. The common loci between PowerPlex® Fusion and PowerPlex® 16 were compared. Calling between both chemistries was consistent with similar profiles given from both sets of results. Of all the 35 samples only 1 sample at 0.125ng target exhibited allelic drop-out at CSF1PO, Penta D, and TPOX with the 10 allele dropping out at CSF, the 11 allele dropping out at Penta D, and no alleles being called at TPOX.

Peak Height Ratio Study

Results from the 35 CODIS samples used to calculate the peak height ratio at 0.125 ng. amplification DNA target showed a wide range of ratios from as low as 0.21 up to a ratio of 1. Whereas for the amplification target of 0.5 ng the minimum ratio was at 0.27 and the maximum at 1, and for the target of 1.875 ng the minimum was at 0.32 and the maximum at 0.99, however only 3 loci were below 0.50 for 1.875 ng.

Analytical Threshold

Using the Method 1 for calculating the analytical threshold set by the IUPAC the following thresholds were calculated for each dye channel (table 1).Whereas using the Method 2 set by SWGDAM Guidelines Section 1.1 the analytical threshold calculated for each dye channel was as follows (table 2).

The limit of detection LOD and Limit of quantification LOQ that were also calculated from this study were as follows (table 3)

Stochastic Threshold Study

The stochastic threshold of each dye channel was calculated using the formula mentioned in the materials and methods section using both analytical threshold results (M1-method 1, and M2-method 2) which resulted in 2 different stochastic thresholds for each dye. The following table shows the value for both thresholds calculated. (table 4)

Precision Study

For the precision study both ladders and TF samples were used to calculate the average size as well as the 3x standard deviation. Results for precision showed high precision for the PowerPlex® Fusion kit with the lowest 3x std being 0.069 that was the size for allele 12 at loci D16S539 for the 31 cycle amplification samples injected at 5 seconds. Whereas the highest 3x std was 0.284 at allele 15 for the loci D12S391 amplified at 31 cycles and injected at 5 second injection. With those results the PowerPlex® Fusion is highly precise with the remaining 3x std ranging between 0.08bp to 0.15bp i.e. being below the recommended 0.5 bp. size difference.

Stutter Study

For the Stutter Study the peak height ratio, average peak height ratio, the standard deviation of the peak height ratios, minimums and maximum ratios were calculated for each locus.

The following table shows the stutter percentages calculated using the afro mentioned formula in the methods and materials section and the stutter percentage chosen by comparing the stutter calculated with the maximum observed stutter. (table 5)

Contamination Study

All the run negatives in the checker board run of ladders and negatives ran initially at 3, 5, 10, 15 second injections did not show any reproducible contamination that would indicate any cross contamination from the ladders present in wells close to the run negatives or from any other sample present in the 96-well tray. Furthermore no contamination that results from the

amplification kit itself was detected in any negative sample whether amplification negatives or run negatives amplified and ran throughout the validation process.

Mixtures Study

The mixture study was conducted by mixing two contributors male:female and male:male together at varying ratio and a mixture of three males. The results of the two profile mixtures resulted in complete detection of the major contributor as well as the minor contributor up until 9:1 ratios were only 100 pg. of the minor contributor was amplified. Drop out was seen at 19:1 mixture ratios with D2S441 being dropped out in the first 19:1 male:male mixture and D10S1248, D13S317, D19S433, and Penta E being dropped out in the 1:19 male:male mixture. In the female:male mixture D12S391 was dropped out in the 1:9 mixture, Penta D was dropped out of the 19:1 mixture, and D2S1338 was dropped from the 1;19 mixture. For the mixture study ratios for the mixtures were calculated for each sample at every dye channel and averaged for each sample. The mixture ratios that resulted in the mixtures averaged to be close to the intended ratio in the 1:1, 1:4, 1:9 and vice versa, whereas in the 1:19 mixtures ratios calculated were offset mainly due to overlapping alleles that had a larger effect on the average ratio for each dye and for the whole profile as well.

Note: Loci that had overlapping alleles were kept for the calculation and the ratio was calculated on the assumption of a peak height ratio of 1 for the major contributor.

Inhibition Study

For the inhibited samples three inhibitors were used at varying concentration from Humic Acid, EDTA, and Denim Blue Dye. Samples inhibited by Humic acid did not produce any results with all five concentrations. With Denim Blue Dye the 1:10 ratio of Dye to MBG water did not

produce any results. At 1:20 ratio drop out of alleles was present with loci larger than about 250 bp in size showing a ski slope effect with the three samples that were injected twice. Ratios 1:50, 1:100, 1:500 full profiles were achieved with increasing total peak heights from 20,564 to 26,498 to 27,572 rfu respectively. With EDTA full profiles were achieved for all the five samples that were injected twice. Total peak heights for the profiles inhibited by EDTA did not show any difference with the total peak heights ranging from 26819 rfu at 0.7mM to 25,134 rfu at 0.8mM which does not show any sign of inhibition. A note about EDTA inhibited samples is that minus A artifacts where especially noted with the loci below 250bp in the red channel. Several artifacts in the baseline were generated from the inhibiting chemicals used, that produced an elevated baseline, or peaks that would resemble a dye blob or other artifacts were noted in the inhibited samples.

Mock Casework Study

Mock Casework samples were analyzed without using the 20% filter that was used throughout the study to be able to detect all possible peaks and the analytical threshold was set at 20 rfu. Various DNA targets were present ranging from about 15 pg. from the microwave swab upto 1 ng. from buccal swabs and other differential samples that were diluted. Complete profiles were generated from all the samples with the exception of one cigarette butt(Penta E and D dropped out), and the two door swabs and microwave swab the three later samples resulted in a mixture of profiles of at least four individuals so it was not possible to evaluate whether the

profiles were without drop out. Results were noted as full profile keeping in mind that the analysis was done at 20 rfu and not the calculated analytical threshold.

Discussion

The PowerPlex® Fusion amplification chemistry produced reliable and reproducible results with the use of the Applied Biosystems® 3130*xl* Genetic Analyzer. The validation showed that the ideal DNA injection target load is a range from 0.5 ng. to 1.0 ng. where these targets would produce full profiles with minimal artifacts and peak height ratios of 50% or better. The optimal injection time using the ABI® 3130*xl* genetic analyzer is 5 seconds and the total injection time of 1700 seconds with a 3kVolt injection voltage. The analytical threshold was set at 70 rfu according to the second method and the resulting stochastic thresholds varied from dye to another due to the large variation between the values.

The performed validation studies performed showed that size calling of alleles within each marker is reliable, absence of contamination from the run set-up, and concordance between the results previously obtained from Promega® PowerPlex 16 amplification kit and the PowerPlex® Fusion. Stutter Ratio was calculated using convicted offenders sample to provide a variety of allele calls. Mixture studies that were performed also showed that minor and major contributor are detected even up to 19:1 mixture ratio, even full profiles were detected with the three male mixtures.

Finally, a non-probative study was conducted to test out the capacity of the amplification chemistry. The results of this study were found to be consistent with the results previously

obtained either through the Marshall University Forensic Science Center or through the validation study itself.

Conclusion

In conclusion, the amplification chemistry of PowerPlex® Fusion provided accurate profiles and a wide range of input target DNA with low amount of artifacts. The results of the performed validation studies demonstrated the robustness and reliability of the kit. Based on the findings of these studies, specific settings were recommended to be incorporated into the standard operating procedures of the Marshall University Forensic Science Center. These settings included an analytical threshold across the spectrum and four separate stochastic thresholds used in the determination of true heterozygote peaks. Furthermore an optimal DNA target load was determined, a laboratory specific stutter percentage table per loci, and a specific mixture interpretation guideline produced. More samples need to be tested to be able to confirm the stutter study since it was based on only 35 samples amplified three times. Future studies will be conducted on this amplification chemistry to test the human specific amplification relative to human normal flora of the mouth and the genital and anal region, as well as a further detailed guideline for mixture interpretation. The use of the Promega® PowerPlex Fusion amplification kit is recommended for the use in future casework samples based on the validation studies and recommendation developed.

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Appendix

Table 1: Analytical Threshold- Method 1

Dye	Average Height	Standard Deviation Height	Minimum Height	Maximum Height	Analytical Threshold
Blue	4.63	1.56	1	18	9.31
Green	6.03	1.87	1	20	11.63
Yellow	8.37	2.46	2	27	15.75
Red	6.34	1.86	2	35	11.93

Table 2: Analytical Threshold- Method 2

Dye	Average Height	Standard Deviation Height	Minimum Height	Maximum Height	Analytical Threshold
Blue	4.63	1.56	1	18	34
Green	6.03	1.87	1	20	38
Yellow	8.37	2.46	2	27	52
Red	6.34	1.86	2	35	68

Table 3: LOD and LOQ

Dye	Average Height	Standard Deviation Height	Minimum Height	Maximum Height	LOD	LOQ
Blue	4.63	1.56	1	18	9.31	20.21
Green	6.03	1.87	1	20	11.63	24.72
Yellow	8.37	2.46	2	27	15.75	32.96
Red	6.34	1.86	2	35	11.93	24.96

Table 4: Stochastic Threshold using both analytical thresholds

Dye	AVG PHR	STD PHR	AT-M1	AT-M2	ST-M1	ST-M2
Blue	0.8191	0.0883	9.31	34	16.8044	61.3696
Green	0.8023	0.0666	11.63	38	19.3093	63.0915
Yellow	0.7874	0.1107	15.75	52	34.603	114.245
Red	0.7611	0.1234	11.93	68	30.517	173.944

Table 5: Stutter percentages

Locus	Min	Max	Avg PHR	STD PHR	Stutter	Promega®
D10S1248	5.556	17.582	8.930	2.620	17%	12.4%
D12S391	4.178	20.619	10.120	3.455	20%	15.8%
D13S317	1.573	28.125	6.431	4.045	28%	9.8%
D16S539	3.287	14.925	5.986	1.950	15%	10.2%
D18S51	3.752	22.472	9.281	3.408	22%	14.5%
D19S433	3.241	13.359	7.479	2.171	14%	11.6%
D1S1656	4.247	19.802	9.093	2.813	20%	16.4%
D21S11	5.395	22.115	9.191	2.533	22%	13.9%
D22S1045	2.581	17.857	10.922	2.992	20%	9.2%
D2S1338	5.040	14.043	8.781	2.061	15%	11.9%
D2S441	2.164	10.313	5.514	1.661	10%	9.2%
D3S1358	5.726	13.043	8.681	2.007	15%	11.9%
D5S818	2.257	21.359	7.176	2.971	21%	9.5%
D7S820	2.379	24.528	6.600	3.578	24%	11%
D8S1179	3.414	13.873	7.656	2.020	14%	10.9%
DYS391	5.157	15.302	8.069	2.056	15%	8.7%
FGA	3.994	17.021	8.017	2.473	17%	12.1%
TH01	1.266	7.014	2.851	1.519	7%	4.6%
CSF1PO	2.558	11.607	7.018	1.747	12%	9.5%
TPOX	1.808	7.962	3.570	1.353	7%	5.5%
vWA	4.895	28.986	9.306	4.040	29%	11.2%