Validation of PowerPlex® Y23 PCR Amplification System for Forensic Casework

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ABSTRACT

Forensic DNA laboratories rely on Y-chromosome specific short tandem repeat (Y-STR) analysis for a variety of cases where the identification of male-specific DNA is essential. The majority of these are sexual assault cases where admixed samples contain low concentrations of foreign male DNA masked by extremely high levels of female DNA. Y-STR analysis allows for the targeted amplification of male DNA, producing only a genetic profile for the male donor(s) in a sample. Y-STR analysis has also proven advantageous in cases where mixed gender DNA cannot be segregated by differential extraction. Y-STR profiles resulting in exclusions are perhaps more helpful to an investigation, as a match can only indicate that the individual and their paternal relatives could have contributed the biological stain. Increasing the discrimination power of the Y-STR amplification system used for analysis increases the exclusionary power and increases the accuracy of profile matches. The newly released PowerPlex® Y23 System (Promega Corporation, Madison, WI) targets 23 loci making it the most discriminating Y-STR kit on the market to date. This study focuses on the validation of the PowerPlex® Y23 System for use in the Colorado Springs Police Department Metro Crime Laboratory (Colorado Springs, CO) following the SWGDAM and FBI validation guidelines for forensic laboratories. Results demonstrated the ability of PowerPlex® Y23 to perform as intended by Promega for use on casework samples. Further studies that focus on the performance of PowerPlex® Y23 with

degraded sample studies, inhibited sample studies, and environmentally stressed sample studies would demonstrate the full capabilities of the amplification system.

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1. INTRODUCTION

Y-chromosome specific short tandem repeat (Y-STR) analysis has been recognized as a valuable tool in forensic DNA casework. Given that the Y-chromosome is exclusive to the male genome, targeting of loci on the Y-chromosome alone can be valuable in cases where the identification of male-specific DNA is essential, such as with sexual assaults (1). Foreign DNA from a male in admixed samples is often masked by extremely high levels of female DNA. During traditional autosomal STR (short tandem repeat) analysis of these admixtures, competition for the primers during PCR amplification can lead to preferential amplification of the major female component. This can result in either a female only profile where amplification and/or detection of the male component has failed (2) or a DNA profile with dominant female peaks and minor male peaks that can be difficult to distinguish from stutter peaks of the major contributor (3). Use of Y-STR analysis, on the other hand, allows for targeted amplification of male DNA with no amplification of female DNA, even when present at high levels (4, 5). Therefore, use of Y-STR analysis on female dominant admixtures as described above would result in a male only profile. Y-STR analysis has also proven advantageous in sexual assault cases involving multiple male assailants where autosomal STR analysis is ambiguous regarding the number of contributors to the mixture

(6, 7). The hemizygous inheritance of Y-STRs results in the presence of only one allele at each locus (excluding DYS385), which greatly simplifies the determination of the number of male contributors in multiple donor samples. Furthermore, Y-STR analysis is valuable in cases where mixed gender DNA cannot be segregated by differential extraction, such as evidence from azoospermic or vasectomized males and blood-blood or blood-saliva mixtures (1, 8).

However, Y-STR testing is not without its limitations. Unlike autosomal chromosomes, the majority of the Y-chromosome's genome remains unchanged during meiosis because it lacks a homologous pair with which it can shuffle its genes. Therefore, a male will exhibit the same Y-STR profile as all of his paternal relatives, barring any mutational events. Any Y-STR testing that results in the failure to exclude a suspect as the donor of a forensic stain must be associated with a statistic that conveys the rarity of that forensic profile within the population(1). For Y-STRs, a random match statistic is calculated using the counting method because Y-chromosome markers are inherited concomitantly, instead of through independent assortment. The counting method takes into account the number of times the profile occurs within a given database in relation to the total number of profiles contained within that database. This method is ideal for forensic application because it provides a very conservative estimate of randomness, especially when combined with an upper confidence limit (9, 10). Increasing the strength of the random match statistic is essential and can be achieved directly, by increasing the number of samples within the reference database, or indirectly, by increasing the discriminating power of Y-STR testing.

In July of 2012, Promega Corporation released their 23-locus Y-STR multiplex assay, PowerPlex® Y23, to the forensic market. The PowerPlex® Y23 System targets the 11 core loci established by the Scientific Working Group on DNA Analysis Methods (SWGDAM), which include *DYS19, DYS385 a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392* and *DYS393*(11). Twelve additional loci are also targeted and include *DYS437, DYS448, DYS456, DYS458, DYS635 (Y GATA C4), Y GATA H4*, *DYS576*, *DYS481*, *DYS533*, *DYS549*, *DYS570*, and *DYS643*, six of which are unique to PowerPlex® Y23 (underlined) (12). This drastic increase in testable loci has significantly increased the discriminating power of Y-STR testing. In a study conducted by Issam Mansour, PhD., numerous 'non-related' individuals with common ancestors were resolved by their PowerPlex® Y23 profile, when previous testing with AmpFlSTR® Yfiler™ resulted in indistinguishable profiles. The resolving power of PowerPlex® Y23 was also shown to be greater among database samples than among localized communities (13). Therefore, incorporation of the PowerPlex® Y23 System into forensic Y-STR testing will likely improve the laboratory's ability to exclude multiple suspects as the donor of a forensic stain, and thus increase the strength of profile matches.

The purpose of this study was to validate the PowerPlex® Y23 System for use in the Colorado Springs Police Department Metro Crime Laboratory (Colorado Springs, CO). Internal validations are crucial to demonstrate the ability of a chemistry to perform as intended by the manufacturer. Strict guidelines for internal validation, set forth by the Scientific Working Group on DNA Analysis Methods and the Quality Assurance Standards, were followed (14, 15).

2. MATERIALS AND METHODS

2.1 Quantification and PCR Amplification

Standard samples from the National Institute of Standards and Technology (NIST) and Promega Corporation, which did not require extraction, as well as non-probative buccal and blood samples previously extracted using Chelex®100 Resin (Bio-Rad Laboratories) were used for validation. All samples were quantified with Quantifiler® Duo prior to amplification. A total reaction volume of 25µL, containing 10.5µL Quantifiler® Duo Primer Mix, 12.5µL Quantifiler® Duo PCR Reaction Mix, and 2µL sample was used. PCR amplification cycling conditions consisted of an initial soak at 50°C for 2 minutes, enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The following parameters were used to determine a passing standard curve: R2 value of 0.99 or greater and a slope range of -3.0 to -3.6. All standard curves required at least one data point for each of the 8 standard concentrations (except for Standard 8, which could be completely removed from the curve). The male component concentration result was used to calculate the amount of sample to be amplified.

All amplification protocols outlined in the PowerPlex Y23 user manual were followed unless stated otherwise. PCR amplification was performed in individual 0.2mL thin-walled tubes on the GeneAmp® PCR System 9700 (Applied Biosystems-Life Technologies) at a total reaction volume of 25µL containing 5.0µL PowerPlex® Y23 5x Master Mix, 2.5µL PowerPlex® Y23 10x Primer Pair Mix, 7.5µL Amplification Grade Water, and 10µL normalized template DNA.

Thermal cycling conditions consisted of enzyme activation at 96°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 61°C for 1 minute, and extension at 72°C for 30 seconds. A final extension was carried out at 60°C for 20 minute with a final hold at 4°C.

2.2 Sample Electrophoresis and Data Analysis

Amplified product was loaded in 1.0uL volumes onto 96-well plates containing 1.0µL CC5 ILS 500 Y23

10.0µL Hi-Di™ Formamide. The plates were denatured at 95°C for 3 minutes and then snap cooled at 4°C for 3 minutes. Amplification products were separated and detected on the Applied Biosystems 3130 Genetic Analyzer in accordance with run parameters outlined in the PowerPlex® Y23 user's manual with one exception. Due to run-to-run migrational variation, the total run time was increased from the manufacturer's recommendation of 1,500 seconds to 2,100 seconds in order to allow for detection of all size standard peaks.

Data were analyzed using GeneMapper® ID v 3.2.1 following the manufacturer's recommended analysis settings outlined in the user manual. All allelic ladders, positive amplification controls, and samples were reviewed for appropriate allele calls at each locus. Female negative controls were reviewed to ensure that amplification of non-male DNA had not occurred, while negative amplification controls and formamide/size standard blanks were reviewed for contamination.

2.3 Precision Study

Precise sizing of Y-chromosome short tandem repeat (Y-STR) fragments generated during amplification is essential to the production of an accurate and reliable haplotype. With an increased number of loci represented within each dye channel of the PowerPlex® Y23 amplification system, the overall size range of Y-STR fragments was largely expanded (86bp to 380bp[1]) in relation to the previous PowerPlex® Y kit. Therefore, PowerPlex® Y23 allelic ladder, which encompasses the kit's entire sizing range, was used to determine precision of the most common alleles found within the population at each locus based on manufacturer studies.

Seven PowerPlex® Y23 allelic ladder samples were injected in triplicate on 3130 Genetic Analyzers in sequence. During validation, migrational variation was observed in first injection data due to preemptive recording of scan points prior to laser activation and attainment of the oven temperature set point. To compensate, the oven was pre-heated prior to each run.

For analysis purposes, one allelic ladder sample was used to bin all other ladder samples producing a total of twenty replicate samples. Average Standard Deviation and Maximum Standard Deviation for each locus as well as Standard Deviation for each allele were calculated.

[1] Fragment size range values based on the minimum and maximum allelic ladder fragment size listed in the PowerPlex® Y23 manual. Calculated sizes of allelic ladder components may vary from these listed values due to acceptable differences in migration during a run.

2.4 Sensitivity/Injection Time/Amplicon Load Study

To determine the DNA template concentration range for which a full genetic profile can be produced, as well as the optimal target DNA concentration range for a standard run protocol of 1uL amplicon load with a 5 second injection time, two male NIST Standard Reference Material 2391c DNA samples (Genomic B and Genomic C) and one male Promega Sensitivity Sample (Sample A) were amplified at concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 ng.

An amplicon load of 1, 2 and 3µL was also investigated in conjunction with 2, 5, and 10 second injection times to determine if alternative protocols could be used to increase the probability of producing a full profile with minimal stochastic artifacts for a wide range of DNA concentrations.

The average peak height value for each sample was calculated using the peak heights of all alleles called within the profile, assigning a zero value for instances of allelic dropout. Maximum and minimum peak heights for each sample were also noted.

Finally, Female control 9947A was run at a concentration of 100ng to confirm amplification would not occur, even in the presence of extremely high concentrations of female DNA.

2.5 Mixture Study

One male/female mixture sample (Promega Mixture Sample B) and one male/male mixture sample (Promega Mixture Sample C) were amplified at ratios of 0:1, 1:0, 1:1, 1:9, 1:19, 2:1, 5:1, 9:1, and 19:1, targeting a male DNA concentration of 0.5ng/µL. Male/male mixture ratios of 1:2, 2:1, 1:5 and 5:1 were also amplified. Male/male ratios of 1:1, 1:2, and 2:1 were amplified in triplicate to determine if the relative peak height ratio between the allele of Male #1 and the allele of Male #4 at each locus varied between amplifications. All mixture samples were run on the 3130 Genetic Analyzer at the standard 5 second injection time as well as an increased injection time of 10 seconds.

Samples were analyzed assuming no prior knowledge of the donor(s) haplotype or number of contributors. The number of persons contributing to the profile was determined by assessing the number of peaks appearing at each locus that could not be identified as an artifact. For samples containing two contributors (all male/male samples), the peak-height ratio of all peaks appearing in stutter position was calculated and compared to the manufacturer's stutter specific ratios. If the peak-height ratio was greater than the stutter specific ratio at that locus, the peak was considered a true peak and remained part of the final profile. If the peak-height ratio was less than the stutter specific ratio, then the peak was marked as stutter and removed from the profile. If a peak could not be distinguished as either a true peak or an artifact, it was not removed from the profile. Profiles obtained from the male/female and male/male mixture rations of 0:1 and 1:0 were used to confirm correct allele calling post-analysis.

2.6 Stutter Study

Twenty-one previously amplified, non-probative samples and eleven previously amplified, standard samples (Promega Sensitivity Sample A and B, Promega Reproducibility Sample A and B, and 2800M) run at standard injections parameters of 1µL amplicon load and a 5 second injection time were evaluated for stutter. Stutter filters were turned off for analysis and the peak threshold value for each dye (Including ILS500 Y23) was set at 25 RFUs. All stutter artifacts were identified and stutter ratio values were calculated for each stutter position at each locus by dividing the peak height of the stutter peak by the peak height of the associated true peak. Stutter ratio values for each stutter position at each locus were then averaged among all samples. The overall stutter ratio value for each locus was calculated by adding the highest average stutter ratio value at that locus to three times the associated standard deviation.

2.7 Analytical Threshold

To determine the RFU level at which a true peak can be detected and distinguished from baseline noise with 99% confidence, previously injected sensitivity data for NIST Standard Reference Material 2391c DNA Samples, Genomic B and Genomic C, as well as Promega Sensitivity Sample A were analyzed at a peak threshold value of 1 RFU. Sizing tables for all samples were exported into an Excel spread sheet, and the data was used to calculated analytical threshold using five different methods (16).

Method 1, Method 2, and Method 3 Data Manipulation and Calculations:

True peaks, stutter peaks, and all other artifact peaks were removed from the exported data. Average peak height, standard deviation, maximum peak height, and minimum peak height were calculated for the combined data as well as for each dye channel. This data was then used in the following analytical threshold formulas:

Method 1: Intentional Union of Pure and Applied Chemists (IUPAC) (1976) - Negatives

$$AT_{M1} = \bar{Y}_{bl} + ks_{bl}$$

AT_{M1} is the analytical signal calculated using Method 1

$\bar{Y}_{bl}$ is the average blank RFU signal

s_{bl} is the standard deviation of the blank signal

k relates to percent confidence. A value of k=3 has been argued to result in 89% confidence (if the noise is not normally distributed) and at most a 99.86% confidence (if the noise is normally distributed) that noise will occur only below this value.

Method 2: IUPAC (1995) - Negatives

$$AT_{M2} = \bar{Y}_{bl} + t_{1-\alpha,v} \frac{S_{bl}}{\sqrt{n}}$$

AT_{M2} is the analytical signal calculated using Method 2

$\overline{Y}_{bl}$ is the average blank RFU signal

$t_{1-\alpha,v}$ is from the student t-table (ex. 30 measurements and 99% confidence, $t_{1-\alpha,v} = 2.46$)

$\frac{S_{bl}}{\sqrt{n}}$ is the estimated standard deviation of the net signal when x=0 (i.e. blanks)

Method 3: Example in SWGDAM Guidelines - Negatives

$$AT_{M3} = 2(Y_{max} - Y_{min})$$

AT_{M3} is the analytical signal calculated using Method 3

Y_{max} is the highest peak within instrumental noise data

Y_{min} is the signal of the lowest trough

Method 4 and 5 Data Manipulation and Calculations:

All peaks, except true peaks, were deleted from the sizing table data. Data were then sorted by input DNA concentration and the average peak height and standard deviation were calculated for each concentration well as for each separate dye channel within each concentration. Analytical threshold calculation methods 4 and 5 rely on the relationship between RFU and input DNA. This relationship is approximately linear from an input DNA concentration of 0.0625ng to 1.0ng. Therefore, only data falling within this concentration range was used. In order to calculated analytical threshold, the slope, Y-intercept, and standard error for the linear regression line of DNA input verses RFU is needed. These

values were obtained by entering the average peak heights and standard deviation values (in total and per dye channel) into the regression template available at www.bumc.bu.edu (17). The regression model values were then used in the following analytical threshold formulas:

Method 4: Miller - DNA Dilution Series

$$AT_{M4} = b + 3S_y$$

AT_{M4} is the analytical signal calculated using Method 4

b is the y-intercept

S_y is the standard error of regression

Method 5: IUPAC CEAC (1997) - DNA Dilution Series

$$AT_{M5} = b + t_{n-1,\alpha}S_y$$

AT_{M5} is the analytical signal calculated using Method 5

b is the y-intercept

S_y is the standard error of regression

$t_{n-1,\alpha}$ is from student t-table ((ex. 5 dilutions and 99% confidence, $t_{n-1,\alpha} = 3.75$)

2.8 Reproducibility Study

To determine the ability of the PowerPlex® Y23 System to produce identical Y-STR profiles for multiple amplifications of the same sample, three non-probative samples (Bobby, Fred, and Pratt) and one standard sample (Male Standard 2800M) were amplified five times. Allele calls were compared across duplicate amplification products to assess concordance.

2.9 Concordance Study

As a means of determining whether the PowerPlex® Y23 System produces genetic profiles that are true in nature, eleven previously extracted non-probative samples and two standard samples (Promega Sensitivity Samples A and B) were amplified using the PowerPlex® Y23 System and the AmpFlSTR® Yfiler™ Amplification Kit at a target DNA concentration of 0.5ng following amplification and capillary electrophoresis procedures outline in their respective user manuals. Allele calls generated at overlapping loci were compared for concordance across kits.

2.10 Contamination Study

Lack of detectable cross-contamination between wells was assessed using negative samples from all runs performed on the 3130 Genetic Analyzers. If contamination was present, the possible origin of the contaminating profile was evaluate by determining whether the contaminating profile was associated with, or independent from, all other samples run on the same 96-well plate.

3.1 RESULTS

3.1 Precision Study

The 3130 Genetic Analyzers correctly binned all alleles within the PowerPlex® Y23 ladder. The average standard deviation at each locus was below 0.15, with a range of 0.017 to 0.097. In addition, all alleles within each locus exhibited a standard deviation value less than 0.15, indicating compliance with the 95% confidence interval (Figure1).

3.2 Sensitivity/Injection Time/Amplicon Load Study

The minimum amount of DNA input required to produce a full profile was investigated using three male DNA samples amplified at a concentration range of 0.0312 to 2.0ng. Allelic dropout was observed in all samples when DNA input concentrations were 0.0625 and 0.0312ng. The loci at which allelic drop out occurred varied overall between samples, but was most commonly observed at DYS437, DYS389I, DYS389II, and DYS456.

Increasing the injection time from 5 to 10 seconds increased the number of allele calls in all samples producing partial profiles; however, it did not result in generation of a full profile. Increasing amplicon load had little to no effect on the number of allele calls made. Decreasing the injection time to 2 seconds decrease peak height and reduced the number of artifacts observed in electropherograms exhibiting off-scale data.

Full profiles were generated when DNA input concentrations were 0.125ng or greater (Table 1). Optimal results (complete profiles, good peak morphology, and minimal baseline noise) were obtained when a concentration range of 0.5-1.0ng DNA input was targeted.

Amplification was not observed with female Standard Sample 9947A.

3.3 Mixture Study

3.3.1 Male/Female Mixture

Full profiles containing only alleles from the male donor were obtained at all ratios. No amplification of female DNA was observed. Genetic profiles generated from a 10 second injection time were identical to those produced from the standard 5 second injection time, with no indication female peaks present.

3.3.2 Male/Male Mixture

Full genetic profiles for both male donors (Male #1 and Male #4) were produced at male/male ratios of 1:1, 1:2, 1:5, 2:1, and 5:1. Profiles generated from 1:1 ratio samples exhibited inconsistent inter-locus peak height balance between donor alleles and fairly consistent patterns of uneven inter-color peak height balance. At loci where alleles were shared between Male #1 and Male #4, peak height was not consistently two times the height of non-shared alleles.

For male/male mixtures exhibiting a 1:2 or 2:1 ratio, inconsistent inter-locus peak height ratios between donor alleles were observed when compared across triplicate amplification products. Hence major and minor donor profiles could not be discerned with a reasonable level of certainty. However, major and minor donor profiles could be determined for ratio samples 1:5 and 5:1, with peak height ratio values of less than 60% between minor and major alleles at all loci.

For male/male mixture ratios 1:9, 1-19, 9:1, and 19-1, individual major and minor donor profiles were discernible. However, many minor donor alleles could not be distinguished from stutter peaks of the major donor. This, in addition to allelic drop out resulted in partial profiles for the minor donor. On average, 21.74% of minor donor alleles were lost for 1:9/9:1 ratio samples, while an average of 50% of minor donor alleles were lost for 1:19/19:1 ratio samples.

Increasing injection time from 5 seconds to 10 seconds did not increase the number of minor donor alleles identified at male/male mixture ratio of 1:9, 9:1, 1:19, or 19:1.

3.4 Stutter Study

All 23 loci were evaluated for stutter using data from twenty-one non-probative samples and eleven standard samples. The majority of loci exhibited overall percent stutter values comparable to those reported by Promega, except for DYS389II, DYS549, DYS390, DYS385, DYS456, and GATTA H4 which exhibited notably higher stutter ratio values (Table 2).

Incidence of stutter generally increased with increasing repeat length. DYS481 (Trinucleotide) exhibited the greatest incidence of stutter, while DYS438, DYS643 (Pentanucleotides) and DYS448 (Hexanucleotide) exhibited exceptionally low incidence of stutter.

The most commonly observed stutter position occurred at one repeat unit below the true allele peak, and was observed at all loci. Stutter product was also observed at two repeat units below and one repeat unit above the true allele peak with varying frequency at select loci. Additional stutter was detected for locus DYS19 at n-2 and n+2 positions. All patterns of stutter are consistent with those reported by Promega. Although stutter was not detected at n-9 to n-15 for locus DYS448, it should be noted that stutter has been reported to occur at these locations. A full stutter position summary can be found in Table 3.

3.5 Analytical Threshold

For the purpose of calculating analytical threshold using Methods 1 through 3, the average peak height, standard deviation, maximum peak height, and minimum peak height for the combined data as well as each dye channel were generated and are displayed in Table 4.

Data needed for the calculation of Methods 4 and 5, including average peak height, standard deviation, and regression data (y-intercept and standard error) for each sensitivity

sample concentration and each dye channel within each concentration are displayed in Table 5.

Methods 1 and 2 produced the lowest overall analytical threshold value of 14.00 and 12.74 RFU respectively. Methods 4 and 5 on the other hand produced much higher overall analytical threshold values of 197.58 and 206.87 respectively. The calculated overall analytical threshold value of 68.00 for Method 3 most closely matches the 50 RFU peak height threshold value recommended by Promega for use with the PowerPlex Y23 kit (Table 6).

3.6 Reproducibility Study

Three non-probative and one standard sample were amplified in quintuplicate to determine if results obtained using the PowerPlex® Y23 system are reproducible. For all replicate amplifications, identical allele calls were produced at each locus for which an allele call was made.

3.7 Concordance Study

Eleven non-probative and two standard samples amplified with PowerPlex® Y23 System and AmpFlSTR® Yfiler™ Amplification Kit were used to determine concordance. Results obtained using PowerPlex® Y23 System were 100% concordant with Yfiler™ amplification data at all shared loci for all samples.

3.8 Contamination Study

A single contamination event was observed during the validation of PowerPlex® Y23, and was associated with a Negative Amplification Control (NAC). The contaminating profile matched that of the standard sample loaded onto the same plate. Subsequent set up and injection of NAC produced clean baseline with no contaminating peaks.

4. DISCUSSION

4.1 Precision Study

Given the number of alleles assessed and the low average standard deviation values observed for all alleles, the 3130 Genetic Analyzer has been found to produce precise migration with PowerPlex® Y23 PCR amplification system.

4.2 Sensitivity/Injection Time/Amplicon Load Study

PowerPlex® Y23 PCR Amplification System proved effective when targeting the manufacturer's recommended DNA input concentration of 0.5ng. However, results indicate that a target range of 0.5-1.0ng DNA input is acceptable for use with the 3130 Genetic Analyzer. Although adhering to these DNA input ranges is recommended for optimal results, full DNA profiles can be obtained from as little as 0.125ng template DNA. In addition, partial profiles can still be generated at concentrations as low a 0.0312ng. Therefore, use of the PowerPlex® Y23 System should not be restricted to only those samples containing male DNA concentrations within the target range.

In relation to increasing amplicon load, no significant difference in peak height or number of allele calls was seen. Therefore, the standard 1µL amplicon load is recommended for all runs using the 3130 Genetic Analyzers. However, manipulating injection time proved to be a beneficial in multiple cases. A 10 second injection time is recommended for DNA input concentrations below 0.125ng as a means of increasing the number of allele calls, although it is not guaranteed to result in generation of a full profile. In addition, decreasing the injection time from 5 to 2 seconds is recommended for blown-out samples where a reduction in peak height will result in fewer artifacts caused by off-scale data.

4.3 Mixture Study

4.3.1 Male/Female Mixture

Although PowerPlex® Y23 has shown no signs of female DNA amplification, additional studies containing higher concentrations of female DNA in comparison to male DNA need to be performed in order to determine whether it is possible for substantially high levels of female DNA to inhibit male DNA amplification. However, thus far, Y-STR analysis has demonstrated to be ideal for casework samples where targeted amplification of male DNA in admixed samples is desired.

4.3.2 Male/Male Mixture

PowerPlex® Y23 is capable of resolving male/male mixture samples into discernible major and minor components at ratios of 1:5, 1:9, 1:19, 5:1, 9:1, and 19:1, with minor allele drop out occurring at ratios less than and equal to 1:9/9:1. However, as the male/male ratio nears equalization, care must be taken to guard against false identification of a major and minor donor illusioned by inconsistent inter-loci peak height ratios between donor alleles.

4.4 Stutter Study

Stutter ratio values presented here are specific to the Colorado Springs Police Department Metro Crime Laboratory's instrumentation. Therefore, all following recommendations apply only to the CSPD laboratory.

For loci exhibiting a calculated stutter ratio value less than the manufacturer's recommended value (DYS576, DYS389I, DYS391, DYS481, DYS533, DYS438, DUS437, DYS570, DYS635, DYS392, DYS458, DYS393), it is suggested that the manufacturer's stutter ratio value be used for analysis. However, loci exhibiting a calculated stutter ratio value greater than the manufacturer's recommended value should be analyzed using the stutter ration value calculated from this study.

4.5 Analytical Threshold

Based on the analytical threshold study, the Colorado Springs Police Department Metro Crime Laboratory will set the analytical threshold at 70 RFU for all dye channels. This 70 RFU

threshold was determined using Method 3, which produced an analytical threshold value closest to recommended peak threshold value recommended by Promega for analysis of PowerPlex® Y23 data.

4.6 Reproducibility Study

The PowerPlex® Y23 System is capable of generating identical Y-STR profiles for multiple amplifications of the same sample, and therefore demonstrates reproducibility.

4.7 Concordance Study

Data generated using the PowerPlex® Y23 System is 100% concordant with data generated using Yfiler™ Amplification Kit for all shared loci. Therefore, it can be concluded that PowerPlex® Y23 System produces genetic profiles that are true in nature.

4.8 Contamination Study

The single contamination event observed during validation was determined to be due to pipetting error during run set-up. Therefore, the PowerPlex® Y23 System has appropriately demonstrated the lack of detectable cross-contamination between wells.

Internal validation of PowerPlex Y23 has verified the amplification system's ability to perform as intended by Promega Corporation. Although all validation studies required by the Quality Assurance Standards were completed, further studies aimed at demonstrating PowerPlex® Y23's ability to perform optimally with a wide variety of sample types as well as determining

PowerPlex® Y23's ability to perform with challenged samples should be performed. Challenged samples could include degraded sample studies, inhibited sample studies, stability studies, or even environmentally stressed sample studies.

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7. TABLES AND FIGURES

Figure 1. Precision of migration of alleles in the PowerPlex® Y23 allelic ladder on a 3130 Genetic Analyzer

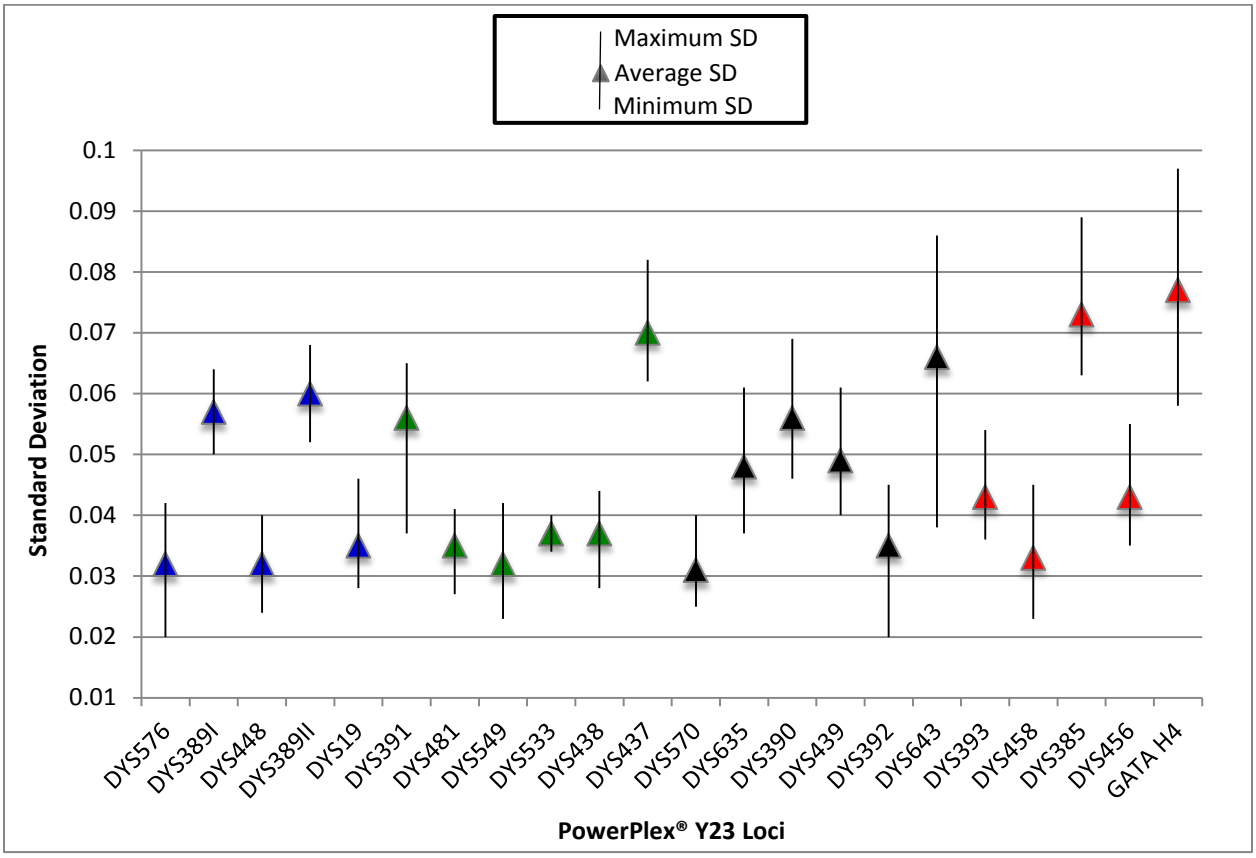

Table 1. Summary of PowerPlex® Y23 Sensitivity Results by input DNA concentration for the recommended run parameters of 1µL amplicon load at a 5 second injection time.

	3130 Genetic Analyzer #1			3130 Genetic Analyzer #2		
	Peak Height			Peak Height		
	Avg	Min	Max	Avg	Min	Max
0.0312ng						
Sample A	143	0	349	89	0	326
Sample B	171	0	467	167	0	369
Sample C	161	0	431	109	0	330
0.0625ng						
Sample A	215	0	359	137	0	413
Sample B	360	0	992	210	0	464
Sample C	339	112	727	199	50	412
0.125ng						
Sample A	448	204	993	294	123	442
Sample B	504	109	886	468	184	1004
Sample C	580	249	1160	464	246	778
0.25ng						
Sample A	926	506	1746	808	457	1291
Sample B	1405	600	2234	1009	602	1645
Sample C	1387	700	1930	1017	657	1934
0.5ng						
Sample A	1875	1471	2940	1435	908	2002
Sample B	2383	1481	3966	2167	1389	3917
Sample C	2731	1852	4356	1872	1057	3049
1.0ng						
Sample A	3496	2242	4929	3063	1322	5246
Sample B	4944	2517	7444	3666	1750	6071
Sample C	4864	2928	7670	3231	1725	5287
2.0ng						
Sample A	N/A	N/A	N/A	5033	2188	8223
Sample B	N/A	N/A	N/A	6553	3035	8753
Sample C	N/A	N/A	N/A	6717	3522	8499

Table 2. Calculated stutter values for all loci.

Locus	Average Stutter Ratio	Standard Deviation	Overall Avg. (Stutter Ratio + 3 SD)	Promega's Recommended Stutter Ratio
DYS576	0.106	0.013	0.144	0.147
DYS389I	0.055	0.008	0.080	0.081
DYS448	0.016	0.010	0.046	0.040
DYS389II	0.114	0.022	0.179	0.152
DYS19	0.068	0.014	0.111	0.107
DYS391	0.074	0.009	0.102	0.124
DYS481	0.200	0.027	0.280	0.298
DYS549	0.078	0.022	0.143	0.114

Locus	Average Stutter Ratio	Standard Deviation	Overall Avg. (Stutter Ratio + 3 SD)	Promega's Recommended Stutter Ratio
DYS533	0.066	0.011	0.099	0.107
DYS438	0.033	0.006	0.049	0.050
DUS437	0.049	0.008	0.074	0.084
DYS570	0.105	0.014	0.147	0.159
DYS635	0.060	0.011	0.093	0.115
DYS390	0.088	0.020	0.148	0.134
DYS439	0.070	0.013	0.108	0.107
DYS392	0.107	0.015	0.152	0.171
DYS643	0.021	0.006	0.040	0.039
DYS393	0.097	0.009	0.123	0.151
DYS458	0.095	0.014	0.136	0.147
DYS385	0.094	0.022	0.161	0.149
DYS456	0.123	0.031	0.216	0.151
GATTA H4	0.070	0.024	0.142	0.111

Table 3. Stutter positions observed at each locus.

Locus	Most Common[2] Stutter Positions	Less Common[3] Stutter Positions
DYS576	n-4	n-8, n+4
DYS389I	n-4	None
DYS448	n-6	None
DYS389II	n-4	n-8, n+4
DYS19	n-2, n-4	n+2, n+4
DYS391	n-4	n-8, n+4
DYS481	n-6, n-3, n+3	None
DYS549	n-4	n+4
DYS533	n-4	n+4
DYS438	n-5	n+5
DUS437	n-4	None
DYS570	n-4	n-8, n+4
DYS635	n-4	None
DYS390	n-4	n+4
DYS439	n-4	n+4
DYS392	n-3, n+3	None
DYS643	n-5	n+5
DYS393	n-4, n+4	n-8
DYS458	n-4	n+4
DYS385	n-4	n+4
DYS456	n-4	n+4
GATTA H4	n-4	None

[2] Most Common – for loci exhibiting more than one stutter position, the most commonly observed stutter positions are those detected in greater than 60% of samples (Exception: DYS438 where n-5 stutter was observed 41% of the time and n+5 was observed 3% of the time, and DYS 643 where n-5 stutter was observed 50% of the time and n+5 was observed 6% of the time)

[3] Less Common - for loci exhibiting more than one stutter position, the least commonly observed stutter positions are those detected in less than 40% of samples. A value on 'None' indicates that no addition stutter positions were observed at that locus.

Table 4. Tabulated average, standard deviation, maximum peak height, and minimum peak height for sensitivity samples used in the negative analytical threshold calculations.

	RFU			
	Average	STDEV	Max	Min
Combined (All 4 channels)	5.54	2.82	35	1
Blue	4.36	2.34	34	1
Green	4.83	2.28	22	1
Yellow	6.44	2.73	35	1
Red	6.51	3.16	29	1

Table 5. Average peak height, standard deviation, y-intercept, and slope for each DNA concentration and each dye channel. These values were used in calculating analytical threshold.

	DNA Input (ng)	Average Peak Height	Standard Deviation	Y-Intercept	Standard Error
All Dyes	0.0625	309.74	171.49	2.82	64.92
	0.125	510.93	216.56		
	0.25	1239.68	440.84		
	0.5	2329.39	659.28		
	1.0	4434.39	1363.49		
Blue	0.0625	274.27	138.22	-7.60	53.86
	0.125	518.40	262.92		
	0.25	1223.73	519.06		
	0.5	2359.47	889.84		
	1.0	4109.00	1766.79		
Green	0.0625	274.06	155.24	-23.38	147.14
	0.125	387.39	157.17		
	0.25	1280.17	490.13		
	0.5	2321.56	668.90		
	1.0	3702.83	864.49		
Yellow	0.0625	400.33	235.80	15.84	76.60
	0.125	651.56	206.46		
	0.25	1371.11	418.01		
	0.5	2560.50	603.04		
	1.0	5634.83	1101.25		
Red	0.0625	282.88	95.58	9.94	32.42
	0.125	487.61	160.03		
	0.25	1081.06	305.98		
	0.5	2081.06	390.77		
	1.0	4236.67	823.62		

Table 6. Analytical threshold values (in RFU) for combined data as well as for each dye channel calculated by Methods 1-5.

	Method 1	Method 2	Method 3	Method 4	Method 5
All Dyes	14.00	12.74	68.00	197.58	206.87
Blue	11.39	10.34	66.00	153.98	161.69
Green	11.68	10.65	42.00	418.05	439.09
Yellow	14.62	13.40	68.00	245.63	256.59
Red	15.99	14.57	56.00	107.21	111.85