Validation of Promega's PowerPlex® Y-23 Amplification Kit for West Virginia State Police

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Abstract

This validation study will allow the West Virginia State Police to replace the current Y-STR kit (PowerPlex® Y) in the case workflow with Promega's PowerPlex® Y23. By performing this internal validation, PowerPlex Y23 can be implemented more quickly into casework than if an analysis would have to divide time between the validation studies and casework. PowerPlex® has an additional 11 loci than PowerPlex® Y , and these loci will contribute to more informative profiles. More information will be gained due to the increase in loci number making the profile more discriminatory (Thompson et al. 2013). A more discriminatory profile would help with analysis, and improve the certainty of if the profile belonged to the person in question. While YSTR will not be as discriminatory as autosomal STRs, it will still increase the ability to more definitively discern between males from different paternal lineages. The West Virginia State Police hope to contribute to the new YSTR database, which will cater to profiles with more loci than the

current YSTR database. The West Virginia State Police Forensic Lab Biochemistry Section was one of the initial alpha testers for Promega's PowerPlex® Y23 kit.

The purpose of this study was to complete the internal validation of the PowerPlex® Y23 amplification kit. An internal validation was required by the FBI quality assurance board to ensure results would be reproducible, accurate, and robust before the amplification kit could be used on casework samples to corroborate that the product worked as reported (SWDAM, 2012). The other aspect of the validation was to determine the best parameters to be utilized for equipment specific to that laboratory. To accomplish these tasks a number of studies were performed including: a sensitivity study, thermal cycler cycle number protocol study, concordance study, reproducibility and precision study, a mixture study, and known and nonprobative sample study. A study was also performed to determine if distilled water could replace using amplification grade water in creating sample dilutions during the amplification step.

At the conclusion of the validation, PowerPlex® Y23 was found to perform optimally when amplified at 30 cycles using a 9700 GeneAmp PCR System thermal cycler with a target DNA concentration of 0.5ng to 1.0ng. The optimal injection times on the 3130XL genetic analyzer were determined to be5 and 15 seconds injection at 3kV. A variation in the migration of DYS437 was observed throughout the validation study, which contributed to a higher but still acceptable standard deviation within the precision study. Since DYS437 has a higher molecular weight it takes longer to migrate through the capillary, which can contribute to varying migration time. The water study showed that using commercially purchased

distilled water was an acceptable alternative to using the amplification grade water included in the amplification kit for dilutions since comparable data was obtained from the two groups of profiles.

In conclusion, PowerPlex® Y-23 performed as expected and will be implemented into the case workflow of the West Virginia State Police Forensic Laboratory Biochemistry Department. Future studies will include: determining in house stutter percentages, amplifying degraded samples, and a more extensive study on DNA extracted from different substrates.

Introduction:

Several unique characteristics of Y-STR testing make it a valuable asset in DNA casework. Similar to the testing performed on autosomal chromosomes, Y-chromosome testing uses short tandem repeat units (STRs) within the Y-chromosomal DNA to produce a profile. The difference between autosomal (STR) testing and Y-chromosome short tandem repeat (Y-STR) testing is that the Y-chromosome is inherited as a single copy and is only inherited paternally through the male line (Butler, 2005); therefore the profile produced is a haplotype profile instead of a diploid profile. This means that the profile produced will only contain one allele call per locus with the exception of DYS385, which is a polymorphic locus. These unique characteristics of Y-STR testing make it a valuable asset in DNA casework.

Y-STR testing is particularly useful in respect to sexual assault cases where a female victim is the major contributor and a male suspect is the minor contributor in the collected samples. Due to the female portion of the mixture being present in a

much higher concentration, it has the potential to out-compete the lower male DNA concentration for primers during the polymerase chain reaction (PCR) of autosomal testing. The results of this preferential amplification of the female component may include obtaining a single source female profile in which the male component is either below the analytical threshold or not present at all. The other alternative is a DNA profile where the female is the major component and the male is the minor component resulting in the male profile either only being partially observed or falling below the stochastic threshold. A solution to this type of challenging sample would be to perform Y-STR testing, which only amplifies DNA found on the Y-chromosome (Decker et al. 2008), thus yielding a single source male profile even with a high concentration of female DNA present. During the developmental validation of Promega's PowerPlex® Y23 amplification kit, a sample comprised of 400ng female DNA to 0.5ng male DNA yielded a full single source male profile (Thompson et al. 2013).

Y-STR testing is also beneficial in cases where there are multiple male assailants. Y-STR testing on such a sample would produce a profile that could be used more easily to determine the number of male assailants present in the sample compared to autosomal STR testing. Since Y-STR testing only produces one allele call per loci (with the possible exception of DSY385), it would also be possible to separate two male profiles if the profile of a known contributor was subtracted from the mixture. Known contributors are profiles generated from samples collected directly from a person. These can be profiles obtained from suspects, victim, consensual partners, ect. A major drawback with Y-STR testing is there is very little

chance for the Y-chromosome to undergo recombination during meiosis since the Y-chromosome only exists as a single copy within the genome. The impact of this is that the Y-profile generated would be the same for every male paternally related with exception of cases of mutation (Butler, 2005).

PowerPlex® Y23 is the Promega Corporation's newest Y-STR amplification kit. This kit improves on the discrimination power of Y-STR profiles produced by adding eleven more loci to their previous PowerPlex® Y amplification kit. PowerPlex® Y23 includes 11 markers recommended by Scientific Working Group of DNA Analysis Method (SWGDAM) (DYS19, DYS385a/b, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS438, DYS439) and then 12 additional loci (DYS437, DYS448, DYS456, DYS481, DYS533, DYS549, DYS570, DYS576, DYS635, DYS643, and Y-GATA-H4). Along with the 11 SWGDAM recommended loci, DYS437 is the other shared locus between the PowerPlex® Y and PowerPlex® Y23 kits. PowerPlex ®Y23 shares an additional four loci with the Applied Biosystems AmpFISTR Yfiler® amplification kit (DYS448, DYS456, DYS635, and Y-GATA-H4) (Life Technologies, 2012). This leaves six loci currently unique to the PowerPlex® Y-23 kit (DYS481, DYS533, DYS549, DYS570, DYS576 and DYS643). The additional loci allow for the greatest discrimination between male profiles of all the current Y-STR amplification kits (Thompson et al. 2013).

This study was performed to validate Promega Corporation's PowerPlex® Y23 amplification kit for the West Virginia State Police Forensic Laboratory's Biochemistry Department. The internal validation process was crucial to show that the chemistry of the amplification kit performs as the manufacturer proposed. The

validation also determined how the kit performed with the instruments currently used by the Biochemistry Department. The validation guidelines of which studies (sensitivity, precision, mixture, reproducibility, and contamination studies) to be conducted was outlined by SWGDAM (Scientific Working Group on DNA Analysis Methods, 2012). In addition to those studies, a comparison between making sample dilutions with commercial distilled water instead of amplification grade water included in the kit was performed to determine if there would be any differences between the results produced at the request of the laboratory.

Materials and Methods:

The testing process involved in this validation included extraction, quantification, amplification, data collection, and data analysis. The validation studies included were sensitivity study, thermal cycler protocol study, water study, reproducibility study, precision study, concordance study, mixture study, and a known and non-probative study. Sample types used for this validation consisted of buccal swabs from 16 men and 10 blood cards for the non-probative samples.

Extraction

The 16 known samples were extracted in four batches, and each batch contained a known reagent control (KRC), which acts as the negative control and was carried throughout all stages of the workflow. After the workstation and EZ1 sample tubes were UV'd for 15minutes, a master mix of 20% Sarkosyl Digestion Buffer, DTT, and proteinase K was created to ensure that each sample receives

200µl of solution. The master mix used was created as specified by the operating procedures of the Biochemistry Department.

Master Mix recipe: (180µl Sarkosyl)(Per samples)
(10µl DTT)(Per samples)
(10µl Proteinase K)(Per samples)

Once the digest buffer was added to each sample tube, half of each swab was added to it's respective tube. The samples were then placed on a heat block set at 56°C for at least an hour after which they were vortexed and then briefly spun down on a microcentrifuge.

The substrate (half of the swab) was then discarded before placing the extract on the QIAGEN's Biorobot EZ1 instrument. To accomplish the purification of the extract, the trace protocol was used with an elution in 50µl of TE.

The same protocol was used when the 10 blood card samples were extracted. The samples were extracted in two groups of five with the addition of a KRC for each extraction. The sample size was a square cutting of approximately 0.5cm x 0.5cm.

Quantification

Quantifiler® Duo DNA Quantification Kit produced by Applied Biosystems was used to determine the concentration of DNA in the sample extracts. A serial dilution of the stock DNA standard was previously prepared following the protocol provided in the West Virginia State Police Biochemistry Procedure Manual. A master mix was then created per the manufacturer's recommendations to accommodate for the eight standards in duplicate, samples, and KRCs.

Master Mix recipe:(12.5µl Quantifiler® Duo PCR Reaction mix) (per samples)

(10.5µl Quantifiler® Duo Primers)(per samples)

23µl of the master mix was dispensed into each well of the 96-wellplate. Then 2µl of the samples were added with the standards being placed in the first two columns. The plate was then covered with a clear optical adhesive and then securely sealed around each well to prevent evaporation. After the plate was briefly centrifuged to remove bubbles, it was placed on Applied Biosystems' 7500 Real-Time PCR System to be analyzed.

Microcon Concentration

Microcons were used to concentrate the amount of DNA in a sample by lowering the liquid volume of the extract. The Microcon tube containing the DNA extract to be concentrated was then placed in a centrifuge for 6 minutes at 2,000RPMs. The amount of liquid that had passed through the microcon was evaluated using pipets, and if necessary the sample went back on the centrifuge for another 6 minutes. When the appropriate amount of liquid had passed through the microcon filter, it was flipped upside down into a clean tube and placed on the centrifuge for 3 minutes at 3,000RPMs in order to collect the concentrated DNA sample.

Amplification

To amplify the samples, the PowerPlex® Y23 kit created by Promega Corporation was used. The appropriate dilutions of the extracts were created after interpreting the quantification data and then amplifed. A master mix was created per the manufacturer's recommendations to include the number of samples,

controls (KRC, amplification positive, and amplification negative) and two extra samples to allow for pipetting error.

Master Mix recipe: (5.0µl PowerPlex Y23 5x Master Mix) (per samples)
(2.5µl PowerPlex Y23 10x Primer Pair Mix) (per samples)

The master mix was vortexed but not centrifuged to prevent the primers from settling to the bottom of the tube due to high molecular weights of primers. Then 7.5µl of the master mix was pipetted to each thin walled sample tube. The appropriate amount of DNA and water was added to the sample tubes to bring the total volume up to 25µl. For example if targeting 0.5ng of DNA and you have a DNA dilution of 0.5ng/µl, then 1µl of the DNA and 16.5µl of water would be added to the master mix.

For the amplification positive, 7.5µl of the master mix, 12.5µl of water, and 5µl of 2800M diluted to a concentration of 0.1ng/µl were added to it's respective tubes. The amplification negative was comprised of 7.5µl of master mix and 17.5µl of water.

Each sample was vortexed to properly mix and homogenize the master mix, sample and water together. The samples were then placed on a GeneAmp® PCR System 9700 (Applied Biosystems thermal cycler) and ran with the protocol outlined in the PowerPlex® Y23 users manual.

Capillary Electrophoresis

A master mix of the internal lane standard (CC5_ILS 500 Y23) and Hi-Di™ formamide was created per the manufacturer's recommendations.

Master mix: (10.0µl Hi-Di™ formamide) (per samples)
(1.0µl CC5 ILS 500Y23) (per samples)

The master mix was then vortexed, and 11µl of the master mix was pipetted into each well. 1µl of samples, controls, and the ladder were pipetted into respective wells. The plate was covered with septa and centrifuged briefly to remove all bubbles from the wells. To allow for denaturation of the amplified DNA, the plate was then placed on a thermal cycler set at 95°C for five minutes, and then immediately placed on a snap cool plate for three minutes.

After the snap cool step, the plate is placed between the holders and loaded onto the 3130xL Genetic Analyzer (Applied Biosystems). Two injections were done for each sample according to the lab's protocol. The first injection was 5 seconds and the second was 15 seconds both at 3kV.

Data Analysis

Data was uploaded to Genemapper® ID software v3.2 (Applied Biosystems) to be analyzed, and then tables including sample name, height, size, loci, and allele calls were exported into Excel for organization and calculations. An analytical threshold of 50RFU as decided by the laboratory was used for all YSTR testing.

Validation Studies

The studies that were used to validate Promega's PowerPlex® Y23 amplification kit used the techniques as described. Each study was performed to test a different aspect of the kit's validity. All samples used in the validation study were extracted on the EZ1 Biorobot, quantified using Applied Biosystems's Quantifiler® Duo, amplified using Promega's PowerPlex® Y23, ran on the 3130xL genetic analyzer (Applied Biosystems), and then analyzed using GeneMapper® ID v3.2.

Thermal Cycler Cycle Number

Serial dilutions were created to achieve the desired concentrations using three samples BC, FI, and control 2800M. Table 1 details the serial dilution of each sample. The water used to make these dilutions was the amplification grade water provided with the amplification kit.

Sample	Initial Concentration (ng)	DNA added (µl)	Water added (µl)	Final Concentration (ng)
BC	4.28	5	21.4	1
BC	4.28	5	417.4	0.0625
BC	4.28	5	838.5	0.0313
FI	4.66	5	23.3	1
FI	4.66	5	452.8	0.0625
FI	4.66	5	838.45	0.0313
2800M	10	3	27	1
2800M	10	2	315	0.0625
2800M	10	2	634	0.0313

Table 1. Initial concentration determined from the quantitation step and the amount of water needed to dilute to the desired concentration.

After the amplification master mix was dispensed into each thin walled tube, the mixture of DNA and water was added to the respective tubes as shown in Table 2.

Serial Dilution (ng)	Dilution Concentration used (ng)	DNA added (µl)	Water added (µl)
5.0	1	5	12.5
2.5	1	2.5	15
1.0	1	1	16.5
0.5	0.0625	8	9.5
0.25	0.0625	4	13.5
0.125	0.0625	2	15.5
0.0625	0.0625	1	16.5
0.0313	0.0313	1	16.5

Table 2: The combination DNA and water used to obtain the 17.5µl sample volume, and from which dilution the DNA was taken from. This held true for all three samples (2800M, BC, and FI).

The samples were then placed on the thermal cycler for 30 cycles. This serial dilution amplification was reproduced two more times. The second set of serial dilution samples was placed on the thermal cycler for 29 cycles and the final set was placed on the thermal cycler for 31 cycles.

Water

The dilutions from Table 1 were recreated the same way as previously described with the exception that distilled water was used instead of the amplification grade water provided with the kit. Those dilutions were then used to create the serial dilutions listed in Table 2. The samples were placed on the thermal cycler for 30 cycles, which was determined in the thermal cycler cycle number study to be the default cycle number for all subsequent studies. The samples were then ran in duplicate on the genetic analyzer to ensure that no unusual or increase in artifact type and numbers were observed.

Sensitivity

Data obtained from the water study and the 30-cycle thermal cycler study was used to perform the calculations of the sensitivity study. Sensitivity was determined by comparing the peak heights found within each dye set and then for all the dye sets. Each dye is analyzed separately to begin with because each channel calls RFUs independently of another dye channel, but to obtain a good overall picture those individual averages are used to create a final average for the entire profile.

Reproducibility and Precision

Seri, which is a NIST traceable positive control created by Serological Research Institute, comes in a stock concentration of 0.1ng/µl. This sample was amplified five times. Each sample tube received 5µl of the stock solution in order to reach a target of 0.5ng of template DNA.

The 96-well plate was set up so the five samples passed through each of the 16 capillaries once. Figure 1 shows the plate set up used for the 16 capillary 3130xL Genetic Analyzer.

Seri1	Seri2	Seri3	Seri4	Seri5	Seri1	Seri2	Seri3	Seri4	Seri5	POS	Blank
Seri2	Seri3	Seri4	Seri5	Seri1	Seri2	Seri3	Seri4	Seri5	Seri1	NEG	Blank
Seri3	Seri4	Seri5	Seri2	Seri1	Seri3	Seri4	Seri5	Seri2	Seri1	Ladder	Blank
Seri4	Seri5	Seri3	Seri1	Seri2	Seri4	Seri5	Seri3	Seri1	Seri2	Ladder	Blank
Seri5	Seri4	Seri1	Seri2	Seri3	Seri5	Seri4	Seri1	Seri2	Seri3	Blank	Blank
Ladder	Seri1	Seri2	Seri3	Seri4	Seri5	Seri1	Seri2	Seri3	Seri4	Seri5	Blank
Seri1	Seri2	Seri3	Seri4	Seri5	Seri1	Seri2	Seri3	Seri4	Seri5	Blank	Blank
Seri2	Seri3	Seri4	Seri5	Seri5	Seri2	Seri3	Seri4	Seri5	Seri5	Blank	Blank

Figure 1. The 3130xL genetic analyzer plate set up for 16 capillary injections. Each of the 5 Seri samples will pass through each capillary once.

Known and Non-probative

Buccal swabs collected from 16 male employees were procured and used as known samples. For the non-probative samples, 10 blood cards from prior proficiency tests were used. The profiles produced with the PowerPlex® Y23 kit were then compared to the profiles produced previously with the PowerPlex® Y amplification kit. This was used to show concordance between the two kits and how the PowerPlex® Y23 kit reacts with mock evidence and reference samples.

Mixture

The mixture study was broken down into three different studies. Samples used for each of the mixtures were chosen based on which profiles had the greatest number of different alleles at the 23 loci. The first mixture study was a series of different concentrations between two male profiles. Mixtures were created by first diluting the extracts to 0.1ng/µl. The dilutions were combined in the ratios described in Table 3. Then 6µl of the mixtures were added to the amplification tube.

Mixture Ratio	M1 (µl)	M2 (µl)
19:1	19	1
9:1	18	2
3:1	15	5
2:1	13.2	6.6
1.5:1	12	8
1:1	10	10
1:1.5	8	12
1:2	6.6	13.2
1:3	5	15
1:9	2	18
1:19	1	19

Table 3. The combination of 0.1ng/µl of M1 and M2 used to create the ratios for the male to male mixture study. Then 6µl of this mixture was added to the amplification tubes.

The second mixture study involved mixing together three male profiles. The two males from the previous mixture study were used in addition to a third male. The selection of the third male was based upon his profile having different allele calls from the previous two male profiles at as many loci that was possible. Again 6µl were then added to the amplification tubes. Table 4 shows how each mixture was created.

Mixture Ratio	M1 (µl)	M2 (µl)	M3 (µl)
1:1:1	3.4	3.4	3.4
1:1:2	2.5	2.5	5.0
1:2:1	2.5	5.0	2.5
2:1:1	5.0	2.5	2.5

Table 4. The combination of .1ng/µl of M1, M2, M3 used to create the ratios for the male:male:male mixture study. Then 6µl of this mixture was added to the amplification tubes.

The final mixture study involved a male to female mixture. Four buccal swabs were collected from a female subject. Three samplings were extracted along with three KRCs. The three extracts were then combined into one tube to give a total of 150µl for the sample. The same process was completed for the KRCs. The samples were then quantified, and the concentration for the female sample was 22.070ng/µl. Afterwards, 100µl of the stock female extract was concentrated down to a volume of 30µl using a microcon, which has an approximate concentration of 110.35ng/µl. Then as with the previous two mixture studies, the mixtures were prepared (Table 5) and the full amount of the mixture was added to the amplification tube. Then enough water was added to each amplification tube containing the sample and 7.5µl of master mix to create a total amplification volume of 25µl.

Ratio (M:F)	M (µl)	F (µl)
1:1	1	1 diluted stock
1:10	1	1 diluted stock
1:100	1	4.5stock
1:500	1	4.5 concentrated
1:1000	1	9 concentrated
.5:300	8	3 concentrated
.25:300	4	3 concentrated
.125:300	2	3 concentrated
0.06:300	1	3 concentrated
0.03:300	1	3 concentrated

Table 5. Shows the volume of male and female sample added together to create the ratios.

Contamination

The Negative controls and KRCs ran with the other studies were analyzed for this study. These controls were examined to ensure that there was no called peak above the analytical threshold of 50RFUs which indicated a clean amplification and run.

Results:

Thermal Cycler

The following figures show the percentage of the profile obtained from the thermal cycler study, which compared a serial dilution of three samples amplified at 29, 30, and 31 cycles. With the five second injections (Figure 2 through Figure 4), 31 cycles consistently produced a more complete profile especially for the lower concentrations of DNA, but it also had an increased amount of pull up and stutter in the 5ng concentration. 30 cycles, which was the manufacturer's recommended number of cycles, produced full profiles from the manufacturer's recommended 0.5ng amplification target. 29 cycles had similar results to that of 30 cycles, however the peak heights were overall lower.

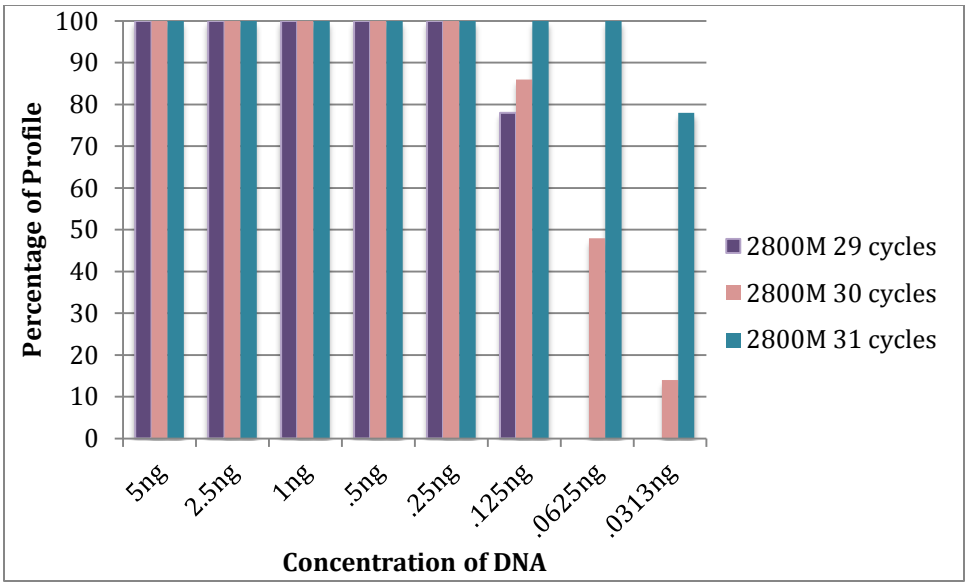

Figure 2. Percentage of the profile obtained for the serial dilution of 2800M ran at 29, 30, and 31 cycles. The injection time was 5 seconds at 3kV on the 3130xL genetic analyzer.

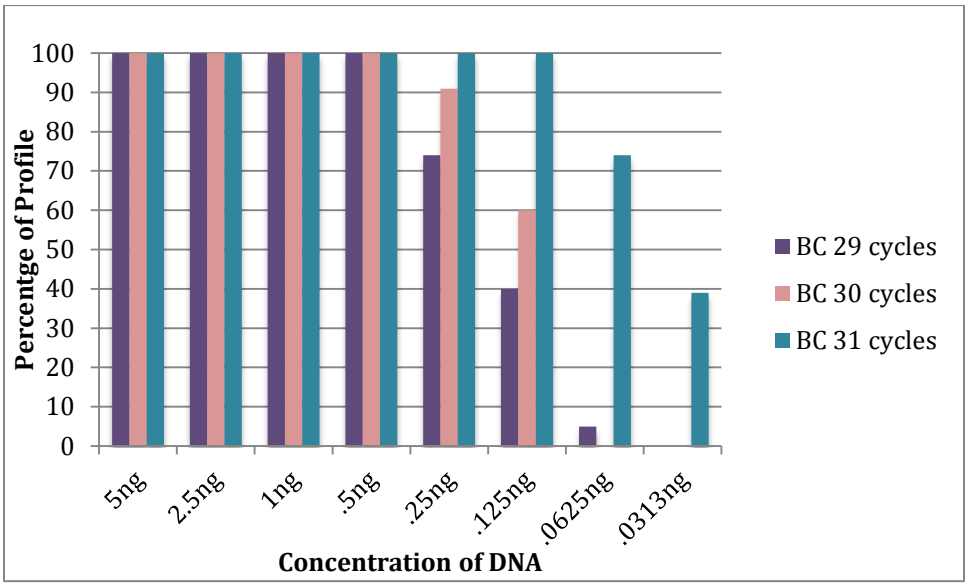

Figure 3. Percentage of the profile obtained for the serial dilution of sample BC ran at 29, 30, and 31 cycles. The injection time was 5 seconds at 3kV on the 3130xL genetic analyzer.

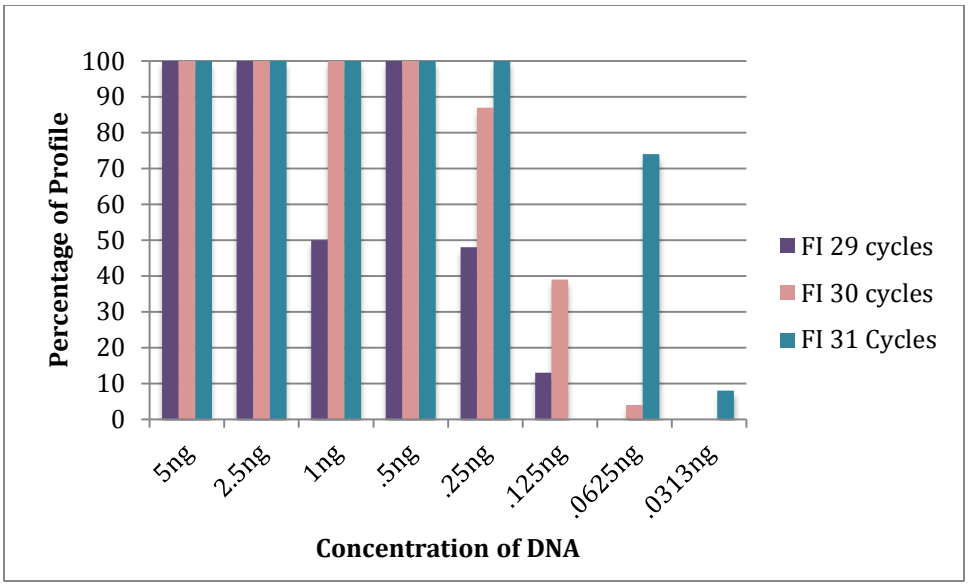

Figure 4. Percentage of the profile obtained for the serial dilution of sample FI ran at 29, 30, and 31 cycles. The injection time was 5 seconds at 3kV on the 3130xL genetic analyzer.

The amplified samples were also injected at 15 seconds. By lengthening the injection time, the peak heights were increased, which allows for a better partial profile than the 5 second injection for lower concentrations. Figure 5 through Figure 7 shows the percentage of the profile obtained at the 15 second injection. The 5ng amplification target almost all failed due to the internal size standard failing, which was due to the high amount of fluorescence from the DNA masking the fluorescence of the ILS making it hard for the camera to detect. Also, there was a substantial increase of pull up and stutter from the amplification concentration of 1ng and up, which would make it unsuitable for DNA reporting.

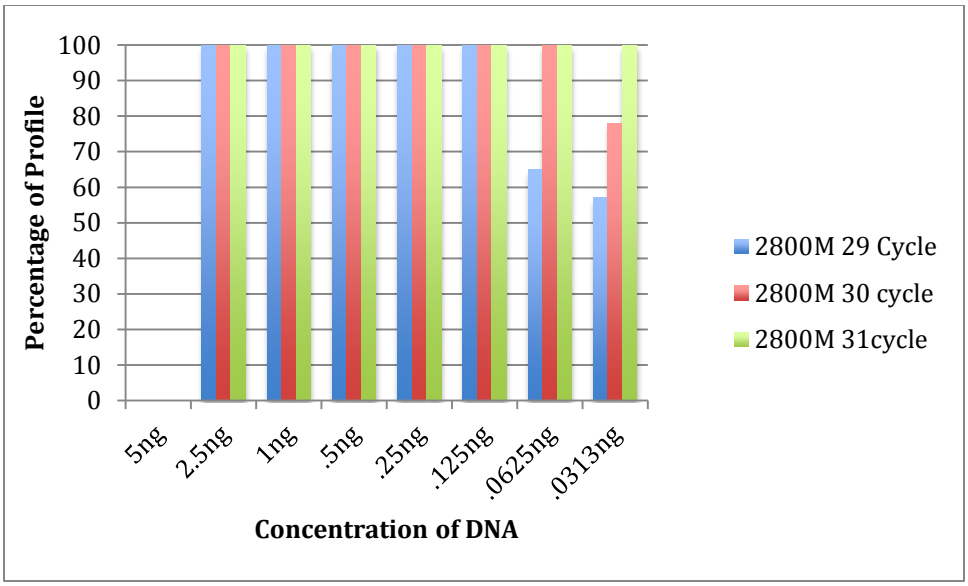

Figure 5. Percentage of the profile obtained for the serial dilution of 2800M ran at 29, 30, and 31 cycles. The injection time was 15 seconds at 3kv on the 3130xL genetic analyzer.

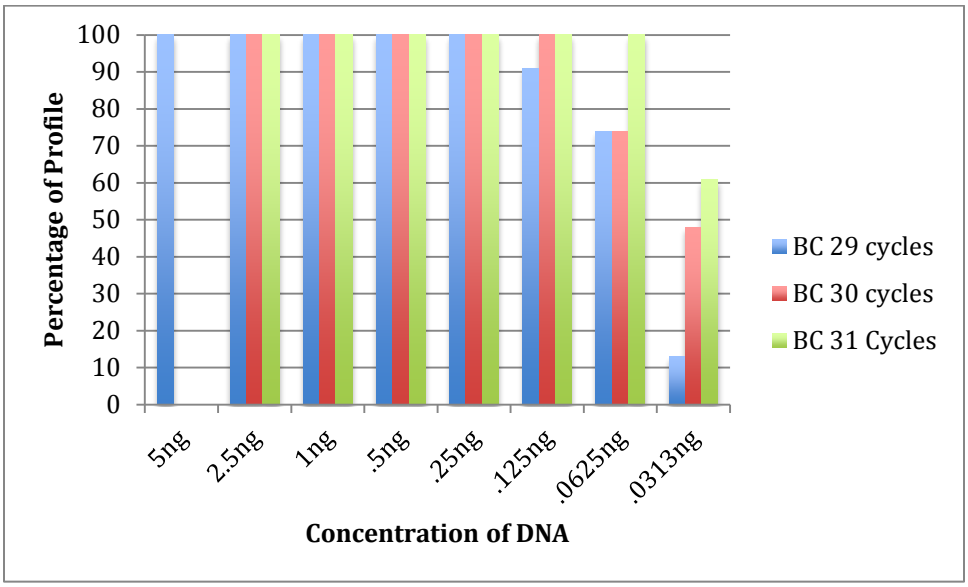

Figure 6. Percentage of the profile obtained for the serial dilution of sample BC ran at 29, 30, and 31 cycles. The injection time was 15 seconds at 3kv on the 3130xL genetic analyzer.

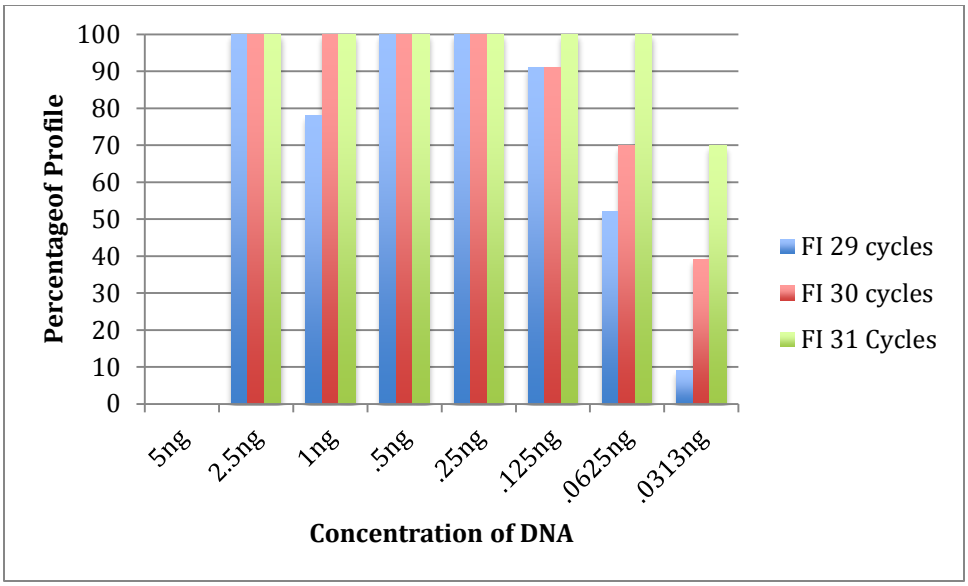

Figure 7. Percentage of the profile obtained for the serial dilution of sample FI ran at 29, 30, and 31 cycles. The injection time was 15 seconds at 3kv on the 3130xL genetic analyzer.

Water

The water study samples showed no significant differences between the serial dilutions created with the amplification grade water and the serial dilutions made with the distilled water. There was no increase in artifacts or pull up observed between the two runs. Data from this and the thermal cycler study were combined to create the three serial dilutions for each sample used in the sensitivity study.

Sensitivity

The sensitivity study was completed to determine the optimal concentration of sample to amplify. Each serial dilution for the three samples was amplified and analyzed on the 3130xL Genetic Analyzer. Peak heights were measured in relative fluorescence units (RFUs), which semi-quantitates the amount of DNA present at each locus. The results in Table 6 shows the average total peak heights for all dye channels combined at each concentration for a sample. 2.5ng and 5ng concentration

of DNA for each sample was also accompanied by an increase of pull up making it more difficult to analyze.

Concentration (ng)	28000M (RFUs)	BC (RFUs)	FI (RFUs)
5	5597.87	3874	4455.45
2.5	4470.9	2419.18	2417.78
1	3386.13	1004.25	986.26
0.5	1008.71	489.53	410.18
0.25	431.43	241.89	212.19
0.125	212.09	157.98	153.89
0.0625	146.76	112.47	142.25
0.0313	128.23	117.5	194.75

Table 6. The average peak heights of all the dye channels measured in RFUs. The samples were amplified at 30 cycles and placed on the genetic analyzer for 5 second injection at 3 kV.

Reproducibility

The Seri control was amplified 5 five times over the course of the validation to complete this study. Table 7 demonstrates that the same profile was obtained over the five different amplifications that took place.

	Seri1	Seri2	Seri3	Seri4	Seri5
DYS576	18	18	18	18	18
DYS389I	13	13	13	13	13
DYS448	19	19	19	19	19
DYS389II	29	29	29	29	29
DYS19	14	14	14	14	14
DYS391	10	10	10	10	10
DYS481	22	22	22	22	22
DYS549	13	13	13	13	13
DYS533	13	13	13	13	13
DYS438	12	12	12	12	12
DYS437	15	15	15	15	15
DYS570	17	17	17	17	17
DYS635	24	24	24	24	24
DYS390	23	23	23	23	23
DYS439	11	11	11	11	11
DYS392	13	13	13	13	13
DYS643	10	10	10	10	10
DYS393	13	13	13	13	13

DYS458	18	18	18	18	18
DYS385	11, 15	11, 15	11, 15	11, 15	11, 15
DYS456	15	15	15	15	15
YGATAH4	12	12	12	12	12

Table 7. The profiles obtained for the sample Seri for the five different amplifications.

Precision

In the precision study, a standard deviation of less than 0.5 at each loci along with a consistent grouping of the five runs at each loci were indicative of good precision. Consistent migration of the DYS437 was observed, which for the Seri control is a fragment size of approximately 366bp. For each Seri sample, the peak was called correctly in each analysis. Figure 8 shows the standard deviation of fragment size for the 5 second injection, and Figure 9 shows the standard deviation for the 15 second injection for each locus.

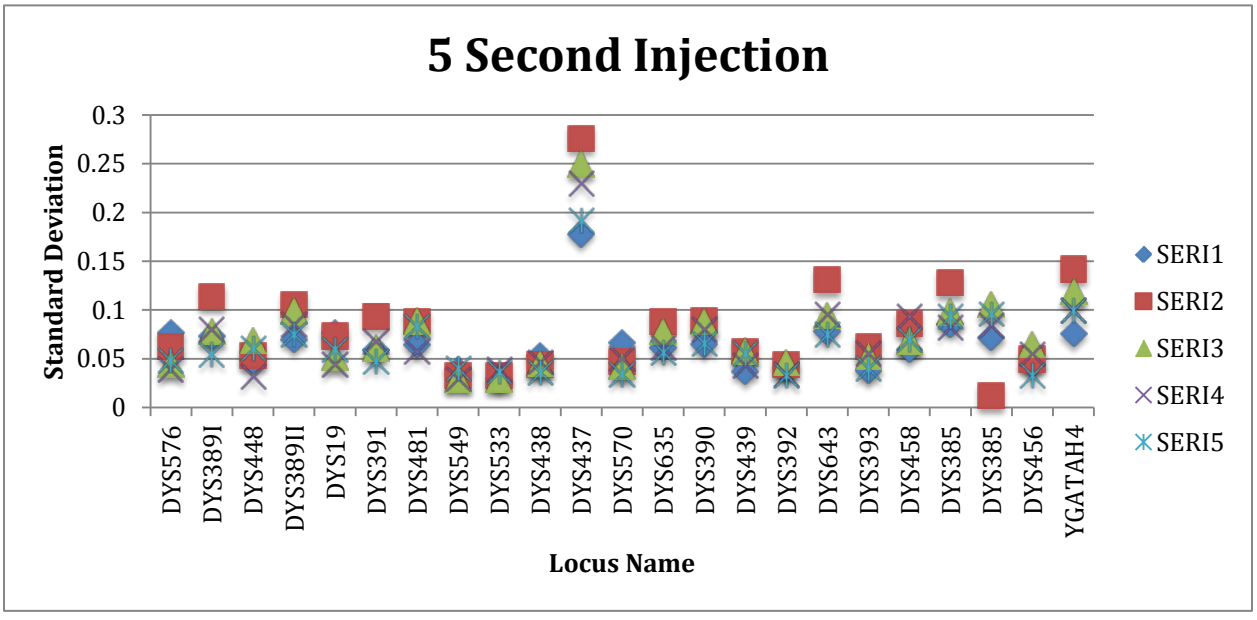

Figure 8. Standard deviation of fragment size of the five Seri samples injected with each of the capillaries at a 5 second injection time. Consistently there is poor migration of the locus DYS437.

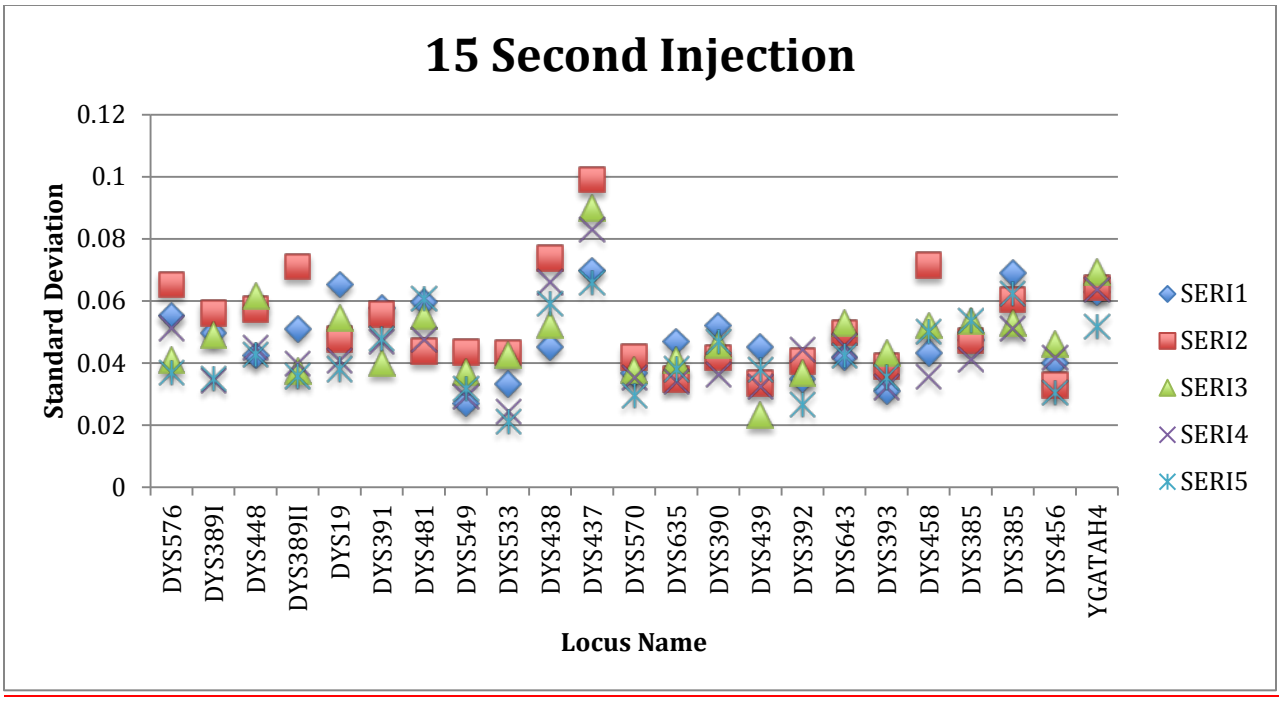

Figure 9. Standard deviation of fragment size of the five Seri samples injected with each of the capillaries at a 15 second injection time. Consistently there is poor migration of the locus DYS437.

Mixture

The mixture study was broken into three studies. The first was a two male mixture at the ratios of 19:1, 9:1, 3:1, 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:3, 1:9, and 1:19. The percentage of the profiles obtained for each ratio was shown in the following two figures (Figures 10 and 11). The two males used for this study have 8 loci in common leaving the remaining15 loci to potentially show multiple peaks indicative of a potential mixture.

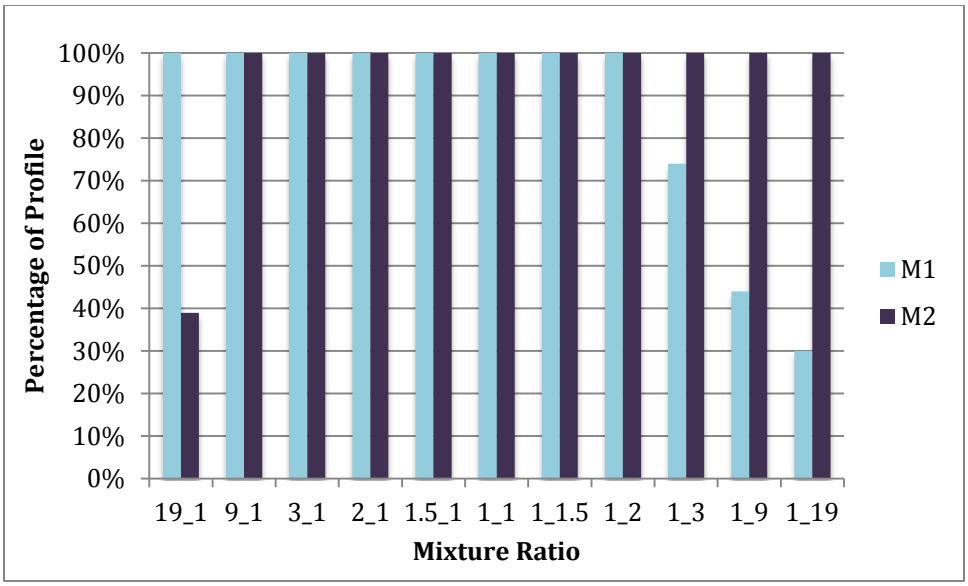

Figure 10. Percentage of each male profile observed at each mixture ratio injected for 5 seconds at 3kV

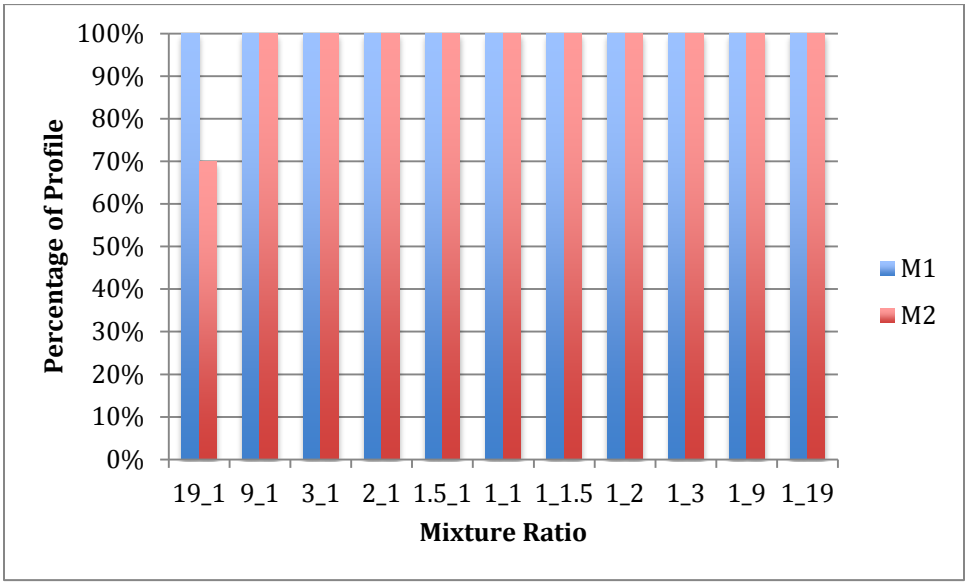

Figure 11. Percentage of the each male profile observed at each mixture ratio injected for 15 seconds at 3kV.

The second part of the mixture study was creating a mixture of 3 males at ratios of 1:1:1, 1:1:2, 1:2:1, and 2:1:1. The results shown in Figure 12 and Figure 13 depict the percentage of the profile for each male. At 6 loci all three males had a different allele which allowed for determination of the number of males involved in the mixture. Of those six loci, five of them were new to the Powerplex® Y23

amplification kit, so with PowerPlex Y the only locus, which would have indicated a three person mixture would have been DYS392. When comparing the third man to the other two male's profiles it was observed that Male 1 and Male 3 possessed different alleles at 14 loci while Male 2 and Male 3 possessed different alleles at 12 loci.

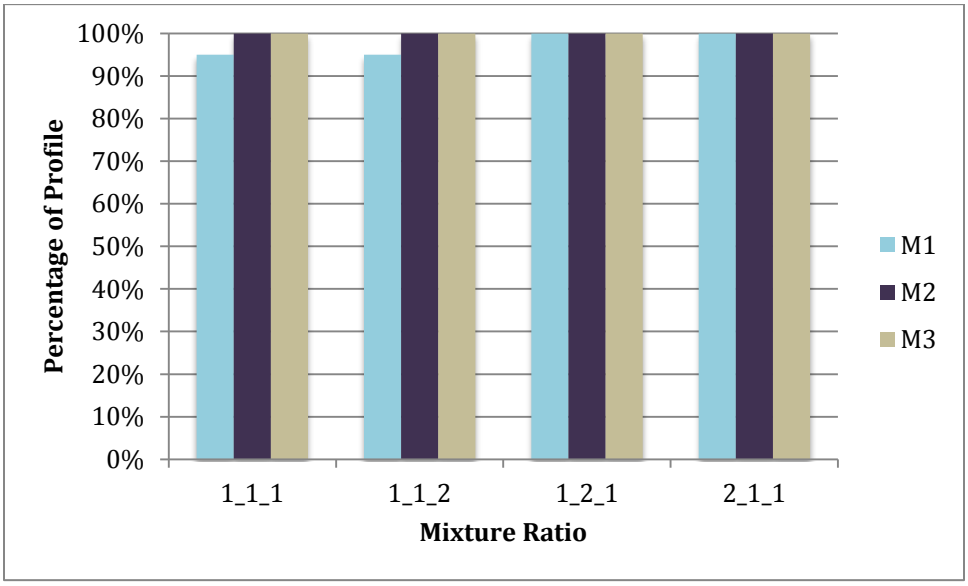

Figure 12. Percentage of each male profile observed at each man at each mixture ratio injected for 5 seconds at 3kV.

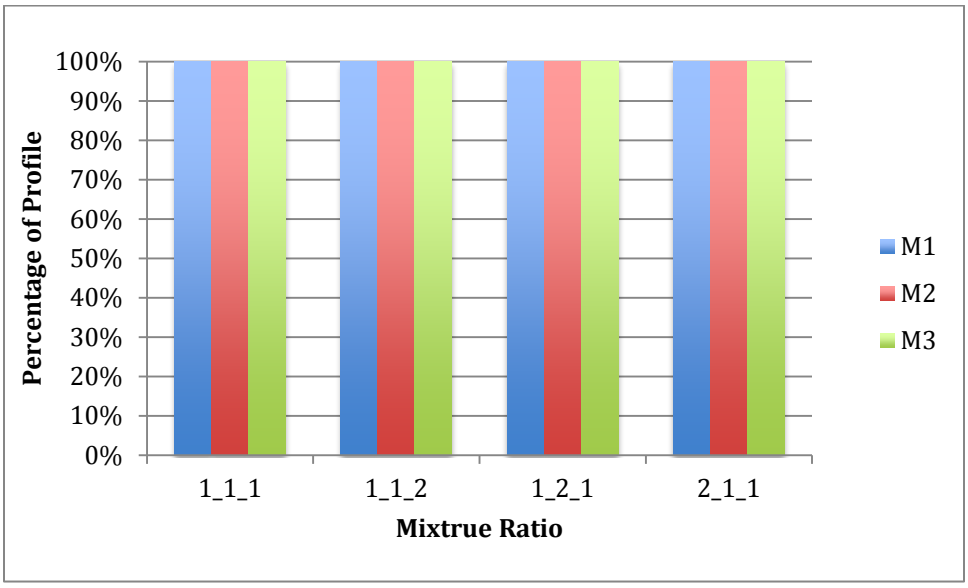

Figure 13. Percentage of each male profile observed at each at each mixture ratio injected for 15 seconds at 3kV.

The final part of the mixture study was a male and female mixture. This was to ensure that the female portion was not amplified, and to see if there was any inhibition with a large concentration of female DNA to that of male DNA. Figure 14 depicts that there was no interference from the female DNA portion. Even though a full profile was not obtained for the .06ng and the .03ng concentration samples, the results were similar to those seen when only male DNA of that concentration was amplified.

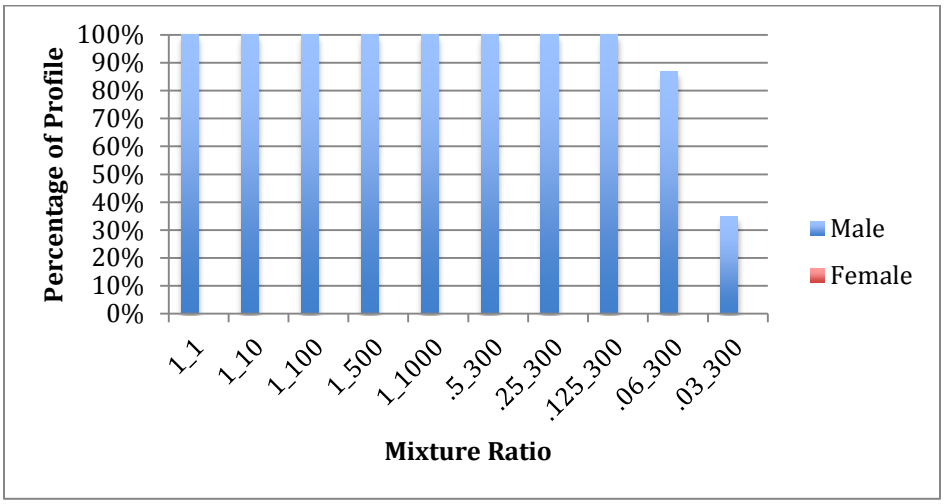

Figure 14. Percentage of the profile that was seen of male DNA at each mixture ratio injected for 5 seconds at 3kV.

Known and Non-Probative and Concordance

The profiles generated with PowerPlex® Y23 for both the 16 known buccal swabs and the 10 non-probative blood card samples matched the previous profiles generated with PowerPlex® Y. PowerPlex® Y23 also produced full profiles for both the buccal and blood cards, which showed that the kit was capable of amplifying profiles from different DNA extracted from different substrates. The two kits are

concordant with each other. Table 8 shows the loci that are shared by both kits and what dye channel those loci are found in for easy reference.

Locus	Promega's PowerPlex® Y dye channel	Promega's PowerPlex® Y-23 dye channel
DYS391	Blue	Blue
DYS389I	Blue	Blue
DYS439	Blue	Yellow
DYS389II	Blue	Blue
DYS438	Green	Green
DYS437	Green	Green
DYS19	Green	Blue
DYS392	Green	Yellow
DYS393	Yellow	Red
DYS390	Yellow	Yellow
DYS385	Yellow	Red

Table 8. The 12 loci that are constant between the Promega's PowerPlex® Y and Promega's PowerPlex® Y-23 along with the dye channel the loci is found in for each kit.

Contamination

There was no contamination or called peaks above the analytical threshold of 50rfu in the 10 negative controls and 7 KRCs. This study showed that there was no contamination found in the techniques used to set up the amplifications or within the kit itself.

Discussion and Conclusion:

Thermal Cycler

The number of cycles suggested by the manufacturer's manual was 30 cycles for an amplification concentration of 0.5ng of DNA. One cycle above and below were tested against the 30 cycles using a serial dilution for three separate samples. The samples were injected for 5 seconds and 15 seconds, which had many ILS failures for the 5ng in the 15 second injection. The ILS failure had to do with the amount of

DNA fluorescence that was interfering with the ILS fluorescence. There was also an obvious difference in the percentage of the profile obtained between the 2800M and that of actual extracted samples, which were represented by sample BC and sample FI.

Overall, 29 cycles and 30 cycles were very similar in the percentage of the profiles that were obtained with the major difference being in the peak heights. 29 cycles had lower peak height values, which could lead to dropout thus creating partial profiles. 31 cycles showed the highest amount of loci called especially in the smaller concentration range, but in the concentrations above 1ng there was substantial pull up and stutter, which would lead to the data being difficult or impossible to analyze. The optimal result for the amplification process was the 30 cycles with the target concentration of DNA between 0.5ng to 1.0ng as shown with the sensitivity study outlined below.

Sensitivity

The sensitivity study was used to determine the optimal concentration range that provided full profiles most consistently. Only the 5 second injection time was analyzed since that is the injection time used for reporting if profiles are complete, show good balance, and no further information is gained from the 15 second injection. This made it important to set the optimal concentration range in order to meet those criteria in the 5 second injection time. The optimal range for creating a full profile would be from 0.5ng to 1.0ng of template DNA.

When analyzing the peak heights for this study it was apparent that the positive control consistently had larger peak heights compared to the two extracted

samples. However, all three samples demonstrated the decline in RFU values as concentration of the sample decreased. The lab uses an analytical threshold of 50 RFUs for YSTR testing. It was determined that amplifying 0.5ng to 1.0ng was acceptable giving approximately 500 RFU and 1000 RFU values respectively. RFU values within that range ensured good profiles with clear defined peaks, and with limited amount of pull up or stutter present in the profile.

Water

This study was performed to demonstrate that the purchased distilled water could be used instead of the amplification grade water that comes with the kit itself to create dilutions and not produce unknown artifacts in the DNA profile. At the conclusion of this study, it was determined that using distilled water to create dilutions of samples had no discernible differences. Both serial dilutions had similar peak heights, pull up, and stutter. Since the distilled performed the same as the amplification grade water, distilled water could be used to create the dilution saving the amplification grade water to set up the amp itself. This would enable the laboratory to not have to purchase amplification grade water with the intent to make dilutions.

Reproducibility

The purpose of the reproducibility study was to show that the amplification kit could produce the same results for a sample amplified at different time periods. Seri which is a positive control purchased by the lab was amplified five times, and

when analyzed the profile was the same for each amplification. This proved PowerPlex® Y-23 does produce reproducible results.

Precision

The precision study was performed to ensure that the migration of the alleles were within an acceptable range (less than a 0.5 standard deviation). Since alleles within a locus are spaced by only one repeat unit (3 or 4 base pairs) it was imperative that migration of fragments be precise for the correct call to be made for an allele. The precision study measured the standard deviation of the fragment size, which is measured in base pairs. The standard deviation was clustered around 0.07 at most loci. The loci are still tightly grouped, which showed that each allele fragment for every loci migrated with approximately the same amount of precision, with one exception at locus DYS437. The standard deviation at DYS437 was closer to 0.2. Since DYS437 is a larger locus it is typical to see migration issues due to the higher molecular weight taking more time to move through the capillary. When the 5 second injection was compared to the 15 second injection, a much tighter grouping was seen within the 15 second injections for all loci.

Mixture

The mixture study was a compilation of three tests each involving different mixture profiles and ratios that included: male:male mixture to ensure that two profiles could be obtained, a male:male:male mixture to test the capabilities of the amplification kit to distinguish three male donors, and a male:female mixture to ensure that female DNA did not affect the male profile generated. In the male:male

mixture test, full profiles were seen for both men in good quality from the 3:1 (M1:M2) ratio to the 1:2 (M1:M2) ratio. While a full profile was observed for the 9:1 ratio, some of the peaks for Male2 were just above the analytical threshold for the five second injections. In the 1:3 and 1:9 mixture studies, the peaks that were not called for Male1 were potentially present, but fell within stutter position of Male 2's alleles and could have been filtered out.

The second part of the mixture study was male:male:male at ratios of 1:1:1, 1:1:2, 1:2:1, 2:1:1. The results demonstrated that there were three males in the mixture although drop out was observed at one locus (DYS643) for Male1 in the 1:1:1 and the 1:1:2 mixture ratios. For this locus Male2 and Male3 had the same allele call so the drop out was not at an allele that would have been used in determining that it was a three person mixture. Alleles that would be used to determine that this was a three person mixture were those loci where all three men had a different allele call.

The final mixture study was a male:female mixture. The results supported that this kit only amplifies the male DNA profile in the presence of large quantities of female DNA. Even in the largest ratio 1:1000 (Male:Female) a full male profile was obtained with average peak heights of 1107RFU. Part of the mixture study included creating a dilution series with the male concentration between 0.5ng to 0.03ng. Each male concentration was then mixed with 300ng of female DNA. Again, only male DNA was observed and, the results correlate to that of the sensitivity study for decreasing concentrations. In regards to male:female mixtures, the female portion of

the sample had no discernible impact on the male profile obtained neither inhibiting the amplification nor adding to the amplified product.

Known and Non-Probative

The purpose of this study was to demonstrate that the kit was capable of producing clean and complete profiles. Known profiles were used to ensure that the correct profile was being created. It also showed that the kit was suitable to amplify extract from different sample types. For both the known buccal swabs and non-probative blood card samples, all samples produced full and correct profiles with PowerPlex® Y-23. This showed that the amplification kit was capable of successfully amplifying DNA extracted from different sample types.

Concordance

The concordance study showed that the results produced by the PowerPlex® Y-23 kit matched those produced from the PowerPlex® Y kit at the 12 shared loci. Data from the Known and non-probative study was used for this study, and it was shown that the two kits were concordant creating the same profile for those 12 loci.

Contamination

The contamination study was completed to exhibit that the kit's reagents, techniques and instruments used did not add any unaccountable peaks or profiles into the samples. The negative controls used throughout the various studies showed no contamination or peaks above the analytical threshold. This indicates that the kit and techniques used were clean and free of potential contamination.

Conclusion

With the conclusion of the internal validation, it was found that the PowerPlex® Y23 amplification kit delivered results consistent with the manufacturer's validations for all studies performed at the West Virginia State Police Biochemistry Department using the EZ1 Biorobot, 7500 Real-Time PCR system, Applied Biosystems'® 9700 Thermal cyclers, 3130xL genetic analyzer, and Genemapper® ID v3.2. The kit performed optimally when 0.5ng to 1.0 ng of male DNA was targeted for amplification, and ran for 30 cycles on the thermal cycler. In data analysis, it was not uncommon to see +3 stutter at the two tri-nucleotide loci (DYS481 and DYS392). This was especially prevalent in DYS392 located in the yellow channel. Other common artifacts seen were the documented n-9 to n-15 peak at DYS481 and n-2 or n+2 at DYS19 (Promega Corporation, 2012). The one added artifact was an n-6 peak also seen in DYS481, which occurs because DYS481 is a hexameric loci. A hexameric loci is a loci that has the four traditional repeat unit, but each unit is separated by an additional two basepairs (Roewer,2012). Following this validation, studies that could be performed in the future to further test the capabilities of this kit include: testing different sample substrate types, degraded samples, inhibited samples, and testing if the kit reacts to non-human DNA especially bacteria typical to the human body

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