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Internal validations are done in forensic laboratories to ensure the methods and procedures are working as expected. For this internship practicum, I conducted an internal validation of Promega's PowerPlex® Fusion 6C System, as well as, a Quarter Plate Method for the Hamilton MICROLAB® STARlet Robotic System. This validation project was done for the Kansas Bureau of Investigation's DNA Databank Unit. This validation consisted of testing a PBS wash versus no wash, a sensitivity study, an injection time study, an analytical threshold study, a stochastic threshold study, a precision study a reproducibility study, and a contamination study. The results of these validation studies demonstrated that both the novel Quarter Plate Method that was created and Promega's PowerPlex® Fusion 6C System can be successfully used by the DNA Databank Unit for sample processing.

VALIDATION OF THE POWERPLEX® FUSION 6C SYSTEM AND ALTERNATE ROBOTIC METHODS ON THE HAMILTON MICROLAB® STAR

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VALIDATION OF THE POWERPLEX® FUSION 6C SYSTEM AND ALTERNATE ROBOTIC METHODS ON THE HAMILTON MICROLAB® STAR

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

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Thank you.

Briana Smith

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CHAPTER I
INTRODUCTION

The Kansas Bureau of Investigation Forensic Science Center has a DNA Databank Section that is responsible for collecting, analyzing, and maintaining offender DNA samples for the state of Kansas. This section is also responsible for maintaining the Combined DNA Index System (CODIS) so that forensic DNA profiles may be compared to offender profiles. The DNA Databank Unit was created to process samples taken from convicted offenders and arrestees in accordance with K.S.A. 21-2511 [1]. This law requires the collection of fingerprints and a biological sample from all individuals arrested or convicted of all felonies and several misdemeanors, including criminal restraint and cruelty to animals. This law also established the Kansas Bureau of Investigation as the central repository for samples taken from convicted offenders and arrestees in accordance with the law [2]. Currently buccal samples are collected; however, in the past blood samples were collected.

The Scientific Working Group on DNA Analysis Methods (SWGDAM) is a group of forensic scientists from various laboratories who provide expertise in the area of forensic DNA analysis. These scientists may be from academia or local, state, federal, or international forensic DNA laboratories. These scientists provide their expertise in different aspects of forensic DNA analysis, and are responsible for setting forth guidelines regarding forensic biology training, methods, protocols, and research. They are also responsible for revising the Quality Assurance Standards for DNA Databasing Laboratories and Quality Assurance Standards for Forensic DNA Testing Laboratories as necessary; as well as, conducting research to develop and validate forensic biology methods [3]. This validation study was conducted according to the Quality

Assurance Standards for DNA Databasing Laboratories and SWGDAM Validation Guidelines for DNA Analysis Methods.

Validation studies are performed in forensic laboratories to ensure procedures are efficient and reliable. In order to introduce a new procedure or instrument, or modify an existing procedure or instrument, two types of validation studies need to be performed: a developmental validation and an internal validation. Developmental validations are conducted before internal validations and may be done by a different laboratory than the one performing the internal validation. Developmental validations require a peer-reviewed publication of the underlying scientific principle(s) of the method or technology. For this type of validation, test data is compiled and the conditions and limitations of the new DNA methodology for use on different types of samples are determined. Developmental validations include characterization of genetic markers, species specificity, sensitivity studies, stability studies, precision and accuracy studies, case-type samples, population studies, mixture studies, Polymerase Chain Reaction (PCR)-based studies, and Next Generation Sequencing (NGS)-specific studies [4]. A developmental validation for the PowerPlex® Fusion 6C System was conducted by a multi-laboratory group consisting of scientists from the National Institute of Toxicology and Forensic Science, Madrid, Spain; Connecticut Department of Emergency Services and Public Protection, DNA Unit; Los Angeles County Sheriff's Department Crime Laboratory; Minnesota Bureau of Criminal Apprehension; Erie County Central Police Services Forensic Laboratory; North Dakota Office of Attorney General, Crime Laboratory Division; National Institute of Standards and Technology (NIST), Applied Genetics Group; and Promega Corporation [5]. This cohort conducted a species specificity study, a human DNA sensitivity study to test low level samples, a stability study, a precision study, a reproducibility and repeatability study, case-type samples, characterization of

genetic markers and concordance, stutter study, mixture study, and various PCR-based studies. The values they obtained were used for comparison purposes against the data obtained in this validation.

Internal validations are done within the laboratory that will be implementing the new method or technology. Similar to the developmental validation, test data is acquired; however, the data is used to demonstrate the methods and procedures are working as expected. Internal validations must be performed before a new method or technology can be implemented in the laboratory. This type of validation is also used to establish quality assurance parameters and interpretation guidelines. Internal validations generally include, testing known and nonprobative evidence samples or mock evidence samples, sensitivity and stochastic studies, precision and accuracy studies, reproducibility and repeatability studies, mixture studies, a contamination assessment, and NGS-specific studies [4]. For the Promega PowerPlex® Fusion 6C Validation, a sensitivity study, an injection time study, an analysis threshold study, a stochastic study, a precision study, a reproducibility study, and a contamination study were conducted.

In recent years, automation has been increasingly used in forensic science laboratories. The Databank Unit currently has an automated extraction procedure and pre-amplification procedure on the Hamilton MICROLAB® STARlet Robotic System to increase throughput. This system allows for a higher throughput of samples compared to traditional extraction methods. Jaffredo *et al.* demonstrated how the Hamilton MICROLAB® STARlet workstation can be utilized in a DNA databasing laboratory [6]. The researchers were studying buccal samples on an automated system. Even though they were utilizing a different kit and different types of swabs instead of FTA cards, their study demonstrated the Hamilton MICROLAB® STARlet could be used for testing buccal samples. Lyons *et al.* demonstrated the Hamilton MICROLAB® STARlet

can be used for human mitochondrial genome sequencing [7]. All pipetting steps of their high-throughput sequencing were performed on the Hamilton MICROLAB® STARlet and the results demonstrated the system was efficient [7]. Marquardt *et al.* conducted a study titled, "Forensically Validated Automated Processing of FTA Cards for CODIS DNA Databasing", where he and his colleagues described the system as sensitive, robust, and reproducible [8]. Similar to this validation, Marquardt *et al.* tested both buccal and blood samples. Their study demonstrated how efficient this system can be in a forensic laboratory.

The DNA Databank Unit currently uses Qiagen's Investigator 24plex GO! Kit for amplification and capillary electrophoresis (CE) for sample processing. The Investigator 24plex GO! Kit is a 6-dye and includes the 20 core autosomal loci included in the expanded CODIS list, as well as, Amelogenin, SE33, DYS391, and a Quality Sensor. The Quality Sensor ensures PCR amplification was successful and provides a way to differentiate between failed PCR because of the absence of DNA and failed PCR due to inhibition from degraded DNA [9].

The DNA Databank wanted to validate the Promega PowerPlex® Fusion 6C as an alternative to the Investigator GO! Kit. This kit is a six-dye, 27-locus amplification kit that has been validated for use in forensic laboratories by numerous laboratories [5]. The kit includes Amelogenin, DYS391, Penta D, Penta E, SE33, DYS570, DYS576, and the 20 autosomal loci that are included in the expanded CODIS core loci. Amelogenin and DYS391 are used for gender discrimination in samples. DYS570 and DYS576 are also used for gender discrimination as they are rapidly mutating Y-STR specific loci [10]. The results of a multi-laboratory study demonstrated that even though the Promega PowerPlex® Fusion 6C has primers designed to amplify human DNA, some primers recognize sites within other non-human sources of DNA. However, since the samples used for this validation were known buccal samples and blood

samples, this was determined not to be a likely issue [5]. Multiple laboratories have successfully validated the sensitivity of the system when using one, two, or three 1.2 mm FTA punches [5]. Even though the system is sensitive enough to handle 1.2 mm FTA punches, 3.2 mm FTA punches were used for this validation. Multiple laboratories have also tested stability, precision, reproducibility, repeatability, case-type samples, characterization of genetic markers and concordance, stutter, mixtures, cycle-number, and other PCR-based procedures [5].

The first problem that was addressed was the DNA Databank Unit was only capable of processing a full plate (87 samples, reagent blank, and controls) on the Hamilton MICROLAB® STARlet Robotic System for extraction and pre-amplification setup of its samples before this validation. This was problematic in cases where running a full plate is not necessary; for example, when there are priority hit or rush verifications, or where the worklist total is low. In order to conserve reagents and time, it is beneficial to have an abbreviated method for the robotic system.

The second problem was that the QIAGEN Investigator GO! 24PLEX Kit currently used for offender profiling by the DNA Databank results in overlap in adjacent loci (i.e. allele 8 from D1S1656 appearing in locus DYS391) in some of the offender profiles. As it was the only kit validated for use in the laboratory before this validation was performed, a comparison with a different short tandem repeat (STR) amplification kit had to be performed to confirm this overlap. To confirm this overlap, this project involved the validation of Promega's PowerPlex® Fusion 6C Amplification Kit. The validation for the extraction and pre-amplification setup of quarter plates was run concurrently with the validation of the PowerPlex® Fusion 6C amplification Kit.

This project is significant because it allows the DNA Databank Unit to have alternative methods to process offender samples to fit their needs. It is helpful to have the PowerPlex® Fusion 6C Amplification Kit validated so it can be used to prove the overlap in offender samples when it occurs using the QIAGEN Investigator GO! 24PLEX Kit. This is significant because when overlap occurs, the allele that overlaps has to be deleted from the sample. This leads to the one allowed mismatch in CODIS; however, it would be beneficial if the PowerPlex® Fusion 6C Kit could be used so that allele would not need to be deleted. To clarify, when entering a sample into CODIS only one mismatch is allowed. For example, if a locus is supposed to be heterozygous but appears to be homozygous due to dropout, it can still be entered into CODIS as long as it only occurred once. If mismatches are observed at multiple loci, the sample cannot be entered into CODIS. Homozygous means an individual has two of the same allele at a locus; whereas heterozygous means an individual has two alternate alleles at the same locus. In addition to confirming the presence of the overlap, the Fusion 6C System can be used to test samples that had a low yield with the Investigator GO! Kit. Since there are more markers present in the Fusion 6C Kit, a more complete profile may be able to be obtained compared to the Investigator GO! Kit. As mentioned before, in cases where full plates are not necessary, it would be very beneficial for the laboratory to have a validated method for quarter plates so resources would not be wasted. An abbreviated method would also allow the laboratory to obtain results faster than the full plate method.

CHAPTER II

MATERIALS AND METHODS

For both validations, Promega's PowerPlex® Fusion 6C, the BSD600 Plus Semi-Automated Punch, Hamilton's MICROLAB® STARlet Robotic System, Applied Biosystems's Veriti® 96-well Thermal Cycler, and Applied Biosystems's 3500xL Genetic Analyzer were utilized. The samples for this project were offender samples from the DNA Databank. The offender samples used for this validation were buccal samples and blood samples that have been stored on FTA cards. The buccal samples were made using the Whatman EasiCollect™ System. For extraction and CE, the samples were prepared in MicroAmp™ Optical 96-well plates; whereas, the plates used for amplification were Axygen® 96-well Polypropylene PCR plates. This was because the Axygen® plates are thinner than the MicroAmp™ plates.

For the validation of the Hamilton MICROLAB STARlet Robotic System, reverse osmosis (RO) water was used in initial testing to avoid wasting reagents and samples. The new method was created by editing the DNA Databank's existing method for the Qiagen Investigator GO! Extraction and pre-amplification procedure, so it could fit the needs of the Quarter Plate Method and the Fusion 6C System. I first went through and removed any steps that were not necessary for the new method. I then went through and adjusted the volumes to fit the Fusion 6C specifications. I also had to adjust the order of the steps as the Investigator Go! Kit was set up to pipette Master Mix first, and samples second; where the Fusion 6C required the opposite. Figure 1 below provides the main steps of the method. There were some steps not included in Figure 1 as they are for maintenance purposes and to ensure the robot is counting tips properly. Most of the maintenance steps, including turning the heat block on and bringing it to temperature, were developed by engineers at Hamilton. These steps were not altered for this abbreviated method.

The purple boxes in Figure 1 denote where a loop is in the method. Loops allow a series of steps to be repeated without having to write the code repeatedly. The dark blue boxes denote steps that need to be performed manually.

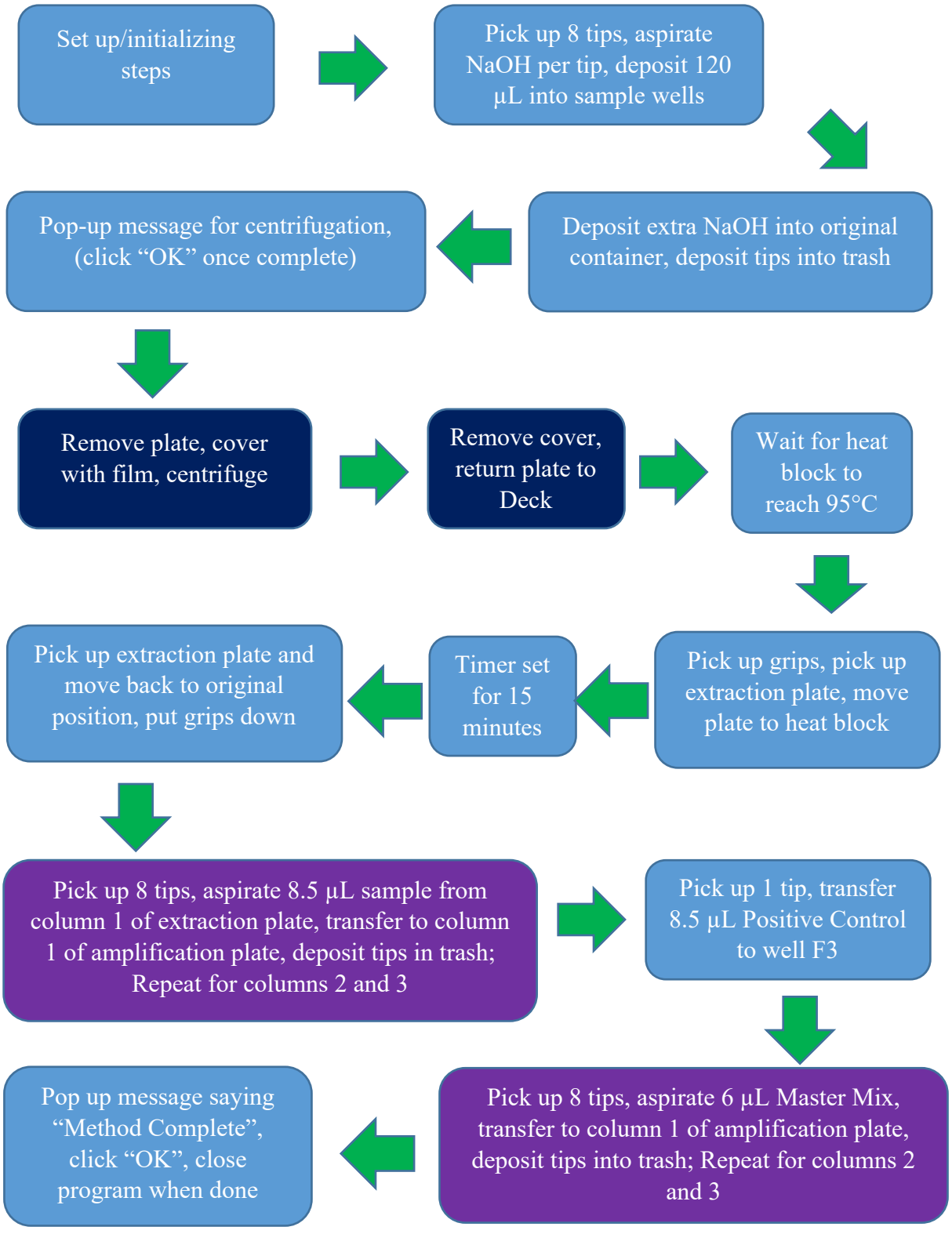

Figure 1: Quarter Plate Method for Fusion 6C on the Hamilton MICROLAB® STARlet

Before this procedure can be run, the amplification master mix and positive control must be prepared, and the deck must be loaded. Figure 2 provides the deck layout of the STARlet Robotics System, though it is not to scale. The black circle represents where the positive control is placed, the purple circle is where the Master Mix is placed, and the orange rectangle is where 5 µL of sodium hydroxide (NaOH) is placed. Preparation specifications for master mix and positive controls are discussed below.

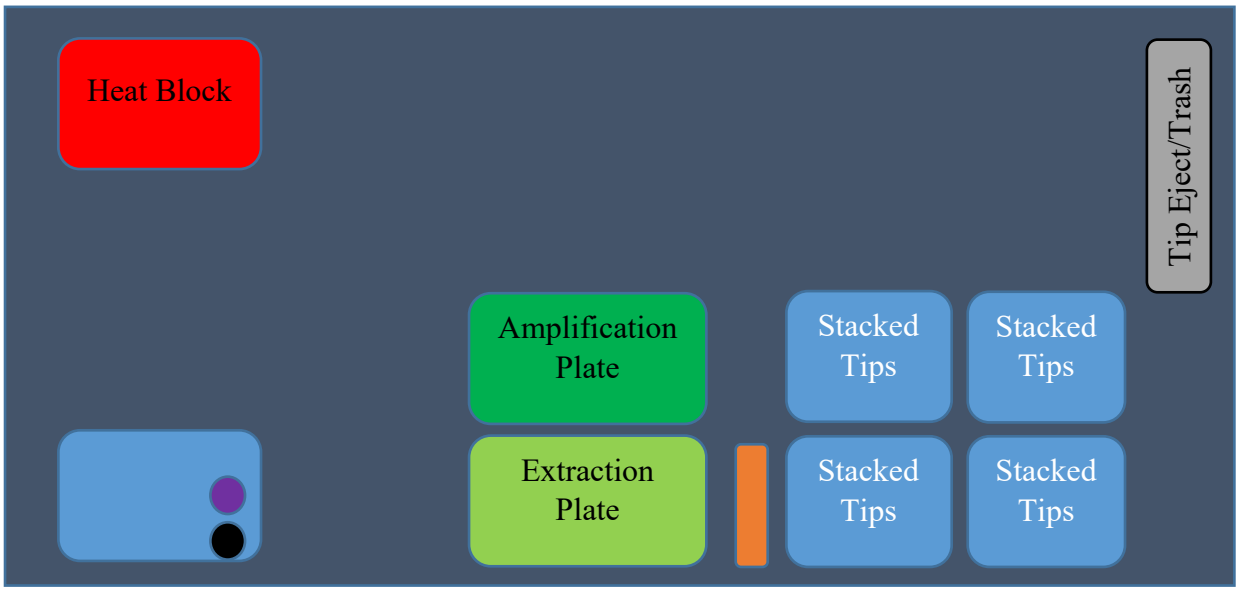

Figure 2: Deck layout of the STARlet used in the Databank Unit

Once the quarter-plate method worked properly while using water, I moved on to the Promega PowerPlex® Fusion 6C Validation. The first variable tested for the Promega PowerPlex® Fusion 6C Validation, was a phosphate buffered saline (PBS) wash. I tested this because in previous testing done by the Databank Unit using the Fusion 6C System, there was an issue with inhibition. Nine samples, which included eight convicted offender samples and my own sample were used for this test. The samples were prepared in duplicate in a 96-well plate. The BSD600 Plus Semi-Automated Punch was used to punch 3.2 mm punches from the FTA cards into the wells of a MicroAmp™ Optical 96-well plate. One set of the duplicates was

washed with PBS, while the other set was not (See Appendix A for plate map). For the PBS wash, 100 µL of PBS was pipetted into the chosen wells and the samples were left to soak for ten minutes. Once the ten minutes had passed, the PBS was removed from each sample individually using a pipette. For this procedure NaOH was used during extraction to lyse the cells. The NaOH lyses the cells while the heat block denatures the DNA. Once the ten minutes had elapsed, the PBS was removed from the samples using a pipette. The Master Mix and positive controls were then prepared, and the deck was loaded according to the layout provided in Figure 2. The extraction and pre-amplification procedures were performed using the Quarter Plate Method on the Hamilton MICROLAB® STARlet, which was previously discussed. For extraction, 120 µL of NaOH was pipetted into each of the wells containing sample and a well containing the reagent blank. Amplification was then performed using the specifications provided in Figure 3 on the Veriti® 96-well Thermal Cycler. Table 1 provides the amplification kit components and their reaction volumes. The specifications provided in Table 1 are different than Promega's suggested volumes. For direct amplification of DNA from storage card punches using a 12.5 µL, Promega suggests using 5 µL sample, 2.5 µL Master Mix, 2.5 µL Primer Pair Mix, and 2.5 µL 5X AmpSolution™ Reagent. It was decided not to use the 5X AmpSolution™ Reagent, and instead increase the sample volume to 7.5 µL, as the samples are washed with PBS and extracted using NaOH before amplification. The positive control was prepared by adding 2 µL 2800M Control DNA to 18 µL of Tris-EDTA. However, for all studies done after the injection time study, the positive control was prepared by adding 2 µL 2800M Control DNA to 13 µL of Tris-EDTA.

Table 1: PCR Amplification Mix for Direct Amplification of DNA from FTA Card Punches Using a 12.5 µL Reaction Volume from Promega's Technical Manual PowerPlex® Fusion 6C System for Use on the Applied Biosystems® Genetic Analyzers [*adapted from* 10]

PCR Amplification Mix Component	Volume Per Reaction	X	Number of Reactions	=	Final Volume
Sample/Positive Control	7.5 µL	X		=	
PowerPlex® Fusion 6C 5X Master Mix	2.5 µL	X		=	
PowerPlex® Fusion 6C 5X Primer Pair Mix	2.5 µL	X		=	
Total Reaction Volume	12.5 µL	X		=	

The volumes used to make the Master Mix were calculated as if there were 34 samples to account for any pipetting errors and to ensure the STARlet could aspirate the liquid 1 mm from the bottom of the tube without drawing up air.

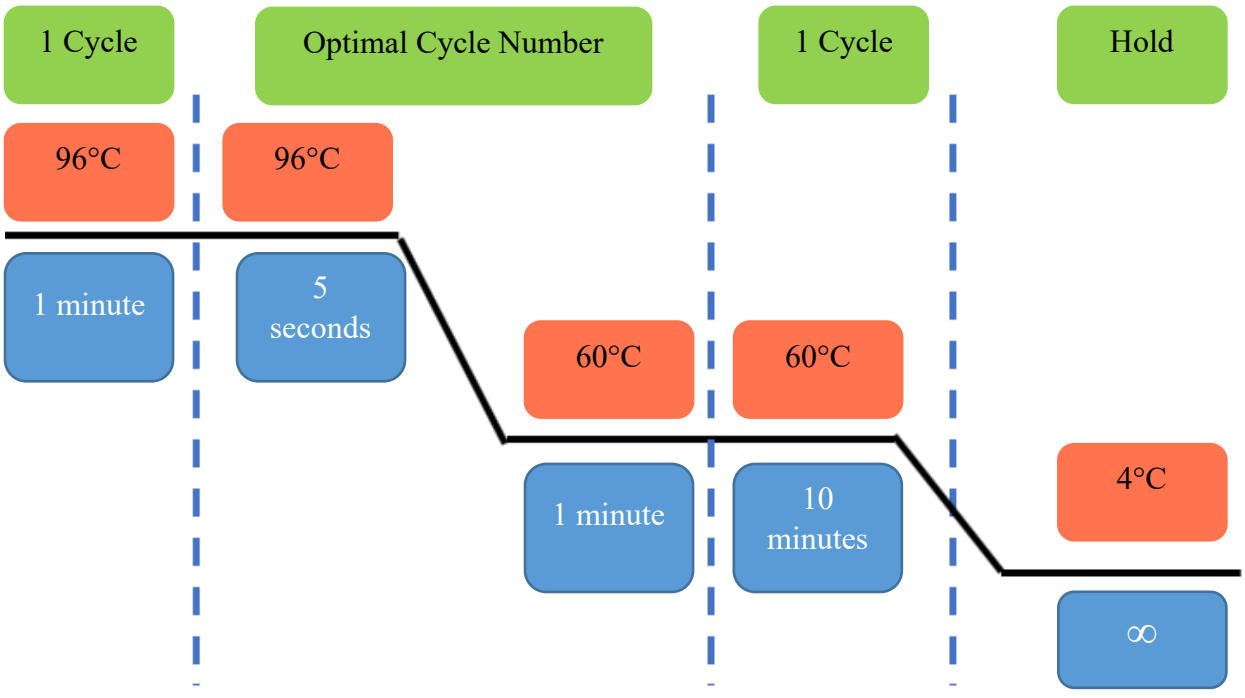

Figure 3: Thermal cycling protocol for the Veriti® 96-Well Thermal Cycler from Promega's Technical Manual PowerPlex® Fusion 6C System for Use on the Applied Biosystems® Genetic Analyzers [*adapted from* 10]

Upon completion of PCR, a plate was prepared for CE. The Master Mix was prepared according to the values provided in Table 2. Each well of the injection contained ten µL Master Mix and one µL of either sample, positive control, negative control, or ladder. For all quarter plate runs, the volumes were calculated using 25 as the number of reactions to account for error. A 3500xL was used for CE according to Promega's recommended settings, provided in Table 3.

Table 2: Calculations for volumes of CE plate components [*adapted from* 10]

CE Plate Components	Volume Per Reaction	X	Number of Reactions	=	Final Volume
Sample/Ladder/+/-	1	X		=	
WEN ILS 500	0.5	X		=	
HiDi Formamide	9.5	X		=	
Total Reaction Volume	11	X		=	

Table 3: Settings for 3500xL [*adapted from* 10]

Application Type:	HID
Capillary Length:	36 cm
Polymer:	POP-4
Dye Set:	Promega J6
Run Module:	HID36_POP4(xL)
Injection Time:	24 seconds
Injection Voltage:	1.2 kV
Run Voltage:	13 kV
Run Time:	1,500 seconds

Before the data from the PBS wash could be analyzed using GeneMapper ID-X, the size standard and analysis method had to be added into the software. The internal lane standard (ILS) used for this validation was WEN ILS 500. This ILS ranges from 60 bases to 500 bases. The analytical threshold was set to 175 relative fluorescence units (RFU) for initial testing.

For the sensitivity study, 20 samples were chosen from the convicted offender databank: five blood samples and 15 buccal samples. One of the blood samples was from 1995, while the other blood samples were from 2005. As for the buccal samples, one was from 2015 while the rest were from 2019. (See Appendix B for plate map). Again, the BSD 600 Plus punch was used to punch 3.2 mm punches from the FTA cards, and the Quarter Plate Method on the Hamilton MICROLAB STARlet was used for the extraction and pre-amplification of these samples. Three plates were created this way so that three different cycle numbers for PCR could be tested. For this study, 24 cycles, 25 cycles, and 26 cycles were chosen. This determined the ideal cycle numbers according to peak balance and allow for cycle number adjustments. The Veriti® 96-well Thermal Cycler was used to perform PCR and CE was performed using the Applied Biosystem's 3500xL with the recommended settings that were provided in Table 2 and Table 3. The same samples used in the sensitivity study were also used for the injection time, analytical threshold, and stochastic threshold studies.

Determining optimal injection time is important so that a complete, clean profile can be obtained. By a clean profile, I am referring to observing little, or no, stutter, pull-up or other artifacts in a sample. Injection time affects peak heights so increasing or decreasing the injection time can have a significant effect on DNA profiles. Therefore, two different injection times were compared. For the injection time study, the 25 Cycle plate and 26 Cycle plate were re-injected at 12 seconds. A 12 second injection time was tested because it may be used for overblown

samples. The resulting peak heights were then analyzed using GeneMapper ID-X and compared to the peak heights from 24 second injections of the 25 Cycle and 26 Cycle plates.

Analytical threshold studies are calculated so that true alleles can be differentiated from baseline noise, which can be caused by chemicals or the instrument. For the analytical threshold study, positive controls, negative controls, and reagent blanks were analyzed to obtain an optimal threshold. First, a plate was prepared for amplification manually using only positive controls. The positive control was prepared as if it would be put into 24 wells to account for 23 wells of the CE plate having positive control, as well as error. One well of the 24-well quarter plate for CE was reserved for the ladder. The positive control was prepared by adding 4 µL 2800M Control DNA to 26 µL of Tris-EDTA, TE^{-4}. The total volume of positive control prepared was 30 µL. After amplification using 25 cycles on the Veriti® 96-well Thermal Cycler, a plate was prepared for CE (See Appendix C for plate map). CE was performed and the results were analyzed using GeneMapper ID-X, as previously described.

In order for a homozygous peak to be considered a true homozygote, its peak height must be above the stochastic threshold. There are generally two alleles per locus, but tri-alleles may be observed in some cases. As stated previously, homozygous means an individual has two of the same allele at a locus; whereas heterozygous means an individual has two alternate alleles at the same locus. Therefore, in most cases, homozygous peaks are about double the height of heterozygous peaks at a given locus. If a peak is above the stochastic threshold in a single-source sample, it is reasonable to assume that no alleles have dropped out [11]. The stochastic threshold allows analysts to differentiate a true homozygous peak from a heterozygous peak where one allele is below threshold or is absent. Therefore, this threshold helps indicate if any data may be missing from a given sample. For the stochastic threshold study, a serial dilution was performed

using eight convicted offender samples. Table 4 outlines the dilution series and the plate map for this experiment can be found in the Appendix (D). To make the dilutions, 3.75 µL TE^{-4} were added to wells A2 through G9 µL, 7.5 µL undiluted sample were added to A1 through G1, and 3.75 µL undiluted sample were added to wells A2 through G2. Wells A2 through G2 were mixed thoroughly by pipetting, then 3.75 µL were transferred from each well to the corresponding wells in Column 3. For example, 3.75 µL from A2 was transferred to A3 and so on. After each transfer, the sample was pipetted up and down several times to mix before the next dilution. This process was then repeated for columns three through nine. In columns one and nine, 5 µL of Master Mix were added; while 2.5 µL of Master Mix were added to columns two through eight to keep the ratios correct. The positive control was prepared by adding 2 µL 2800M Control DNA to 13 µL of Tris-EDTA. The STARlet Robotic System was not used to create this plate, so a separate heat block was used for extraction. Amplification, CE, and analysis were performed according to the specifications previously discussed.

Table 4: Dilution series used for Stochastic Study

No dilution	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256

Allelic ladders are made up of common alleles found in the general population for the 27 loci included in the Fusion 6C Kit. Each of these alleles is associated with a certain size, so ladders are used as a size reference in analysis software, such as GeneMapper ID-X. The ladder was provided by Promega. Five ladders were chosen and analyzed for the precision study. The ladders were from the three plates from the sensitivity study, the analytical threshold study, and the stochastic study. The ladders were analyzed using GeneMapper ID-X with the specifications discussed previously in the first paragraph of page 14. A standard deviation <0.15 was expected

to be obtained and the average peak height RFU was calculated as a reference for future sensitivity tests. This value was chosen based on previous validation studies and guidelines from the DNA Databank Unit.

Reproducibility studies are used to demonstrate concordant results can be obtained using the same procedure on the same samples. For the reproducibility study, 20 convicted offender samples that have not been previously been used in the validation were chosen. Two plates of these 20 samples were prepared using the specifications established in the previous studies of this validation (See Appendix F for Plate Map).

Single source profiles with no resulting mixtures, negative controls, and reagent blanks with no profiles from plates used for other parts of the validation study served as the contamination study. For the negative controls and reagent blanks, there should be no peaks observed when they are analyzed using GeneMapper ID-X. The presence of peaks in either the negative controls or reagent blanks indicates contamination. When the single source profiles are analyzed using GeneMapper ID-X, only the peaks from a given sample should be observed. If other peaks are observed, it may indicate the presence of contamination.

It should be noted, since the DNA Databank only works with reference samples, it was decided quantitation and mixture studies would not be conducted during this validation.

CHAPTER III

RESULTS

Hamilton MICROLAB® STARlet Robotic System

Once the Quarter Plate Method was working properly and showed no error messages, I ran the method twice with water from start to finish. Each time water was transferred to the amplification plate, I paused the method on the instrument and used a pipette to manually check the volumes in each well. The results were concordant for both plates I tested with water. The results revealed that instead of transferring 7.5 µL of "sample" and 5 µL of "Master Mix", the system was transferring about 6 µL and 4 µL, respectively. At first, I thought this may have been due to viscosity of the water compared to the reagents; however, when I ran the PBS wash, the volumes transferred were still 6 µL for sample and positive control, and 4 µL for Master Mix. To correct this, the method was adjusted so that it was set to aspirate 8.5 µL for sample and positive control, and 6 µL for Master Mix. This error may be a calibration issue in the robotic system or in the pipette; however, without further testing I cannot determine where the problem is occurring. Since the robotic system is calibrated at least once a year, these values may need to be checked after each calibration.

It should be noted there are no allele call tables in this report since the samples used are from convicted offenders. Also, variability is expected since each punch is not guaranteed to have the same amount of DNA, and no quantitation or normalization was done. All calculations for peak heights, standard deviations, etc. were done in Excel.

PBS Wash

The average peak heights were compared to determine if it was beneficial to wash the samples with PBS. The average peak height for the samples that were washed with PBS was 5,133.092 RFU and the average peak height for the samples that were not washed with PBS was 5,697.065 RFU. The difference in average peak height was determined to be 563.9726 RFU. Even though this difference was not considered significant, my supervisor and I decided it would be beneficial to use the PBS wash in case there is inhibition in samples. Blood samples must be washed with PBS to remove inhibition caused by blood components, such as hemoglobin. Figure 4, below, provides the comparison of the results from the samples that were washed with PBS compared to the samples that were not washed with PBS.

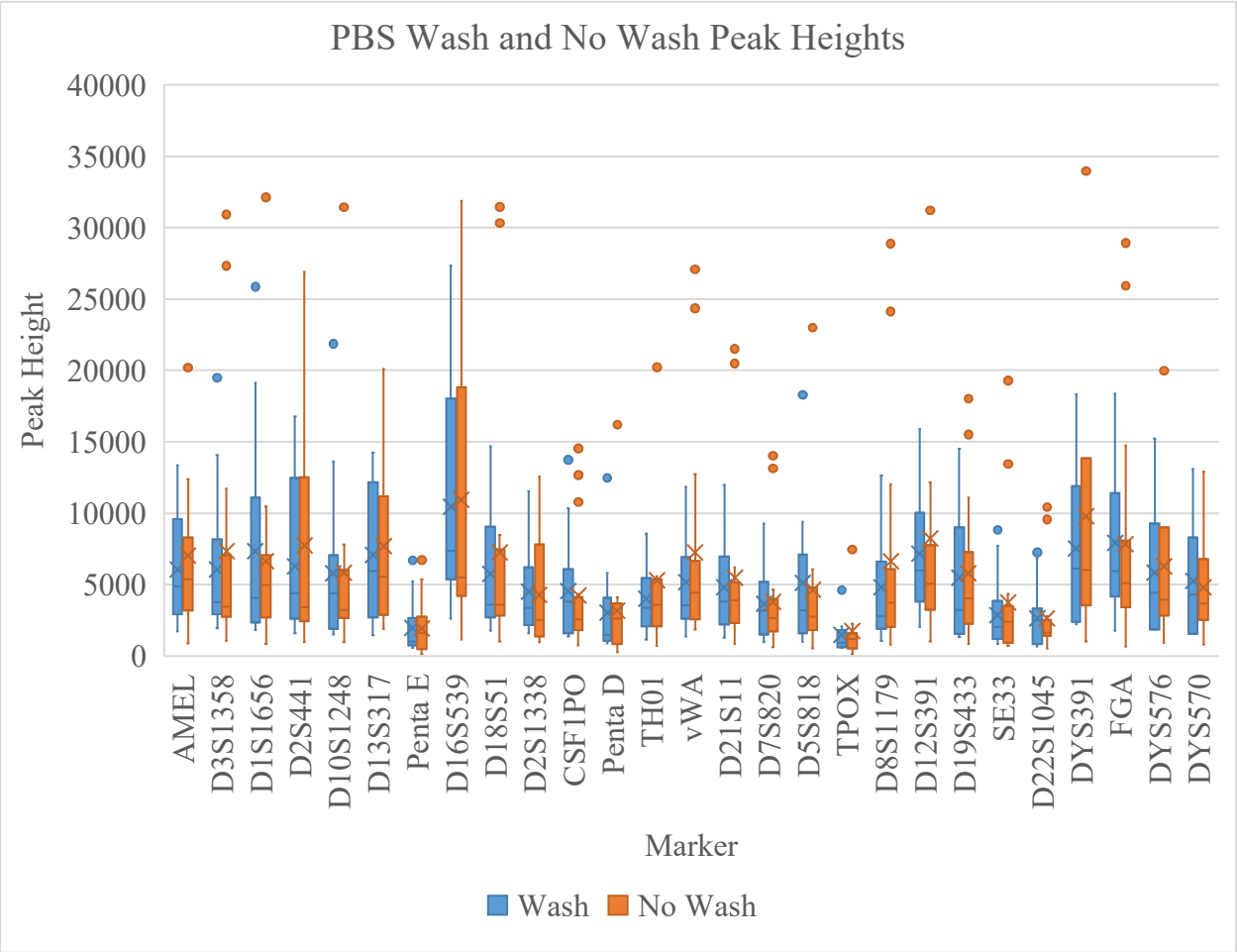

Figure 4: Box and whisker plot providing the peak heights for each marker from the samples that were washed compared to the samples that were not washed.

Sensitivity Study/Cycle Number

The peak heights for the three cycle numbers were compared to the positive control peak heights from the analytical threshold study. Figure 8 provides the peak heights for each marker of the positive controls from the analytical threshold study. The positive control for the analytical threshold study was prepared by adding 4 µL 2800M Control DNA to 26 µL of Tris-EDTA. The average peak height for 24, 25, and 26 cycles of PCR were 1,762.247 RFU, 4,617.092 RFU, and 9,335.264 RFU, respectively. The average peak height of the positive controls was 4,956.7 RFU. It was determined that 25 cycles of amplification would be optimal for the Databank Unit.

Figures 5 through 7 provide the peak heights for each marker for 24, 25, and 26 cycles of PCR amplification, respectively.

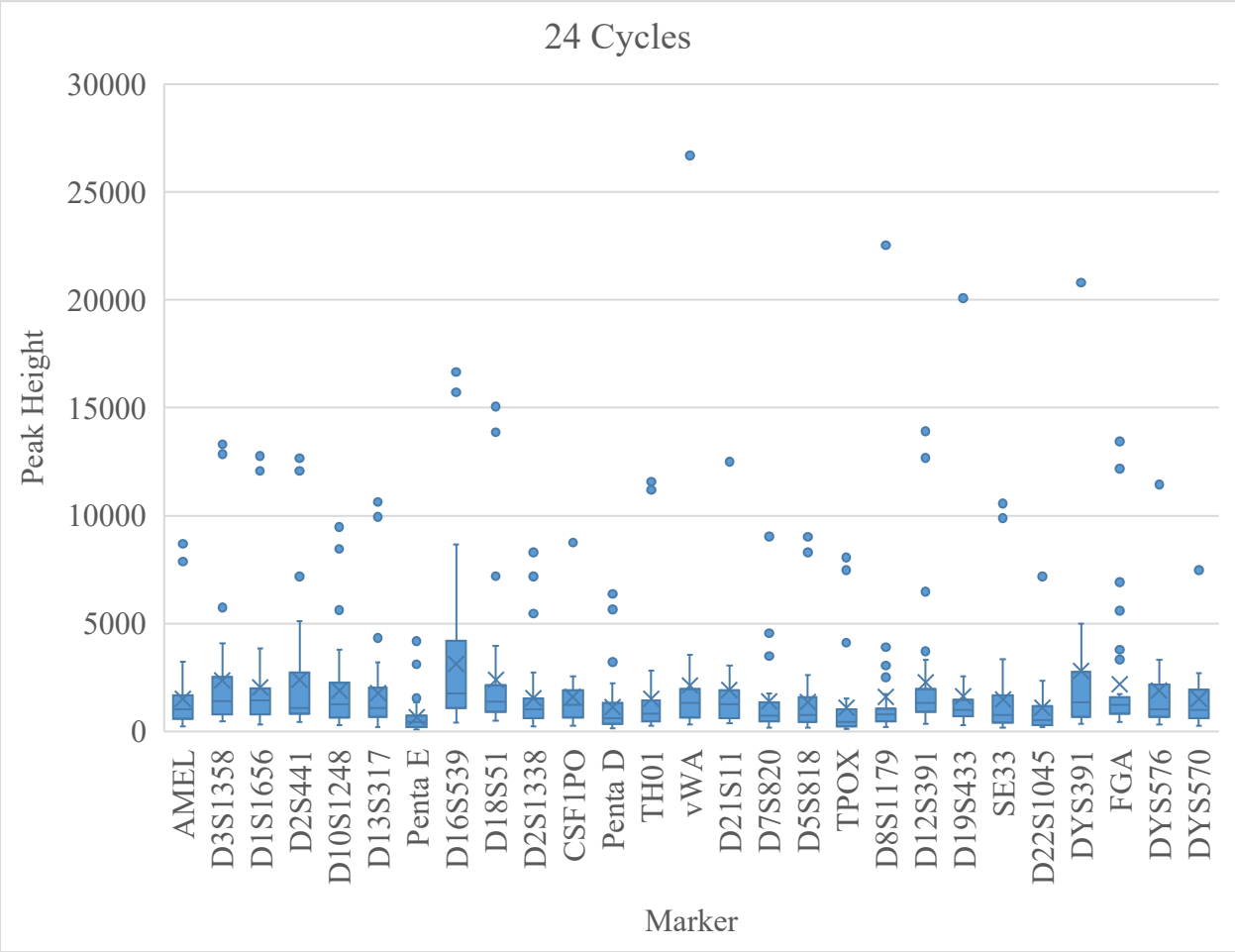

Figure 5: Box and whisker plot providing the peak heights for each marker for the samples tested with 24 cycles of PCR

Since the average peak height when using 24 Cycles was 1,762.247 RFU, it was decided 24 cycles would be excluded. This was because the average peak height was so much lower than the average peak height of the positive controls. This cycle number also could also lead to allelic dropout in low-level samples. Allelic dropout would be problematic for the Databank Unit because some samples would not be eligible to be included in CODIS if too many alleles are absent.

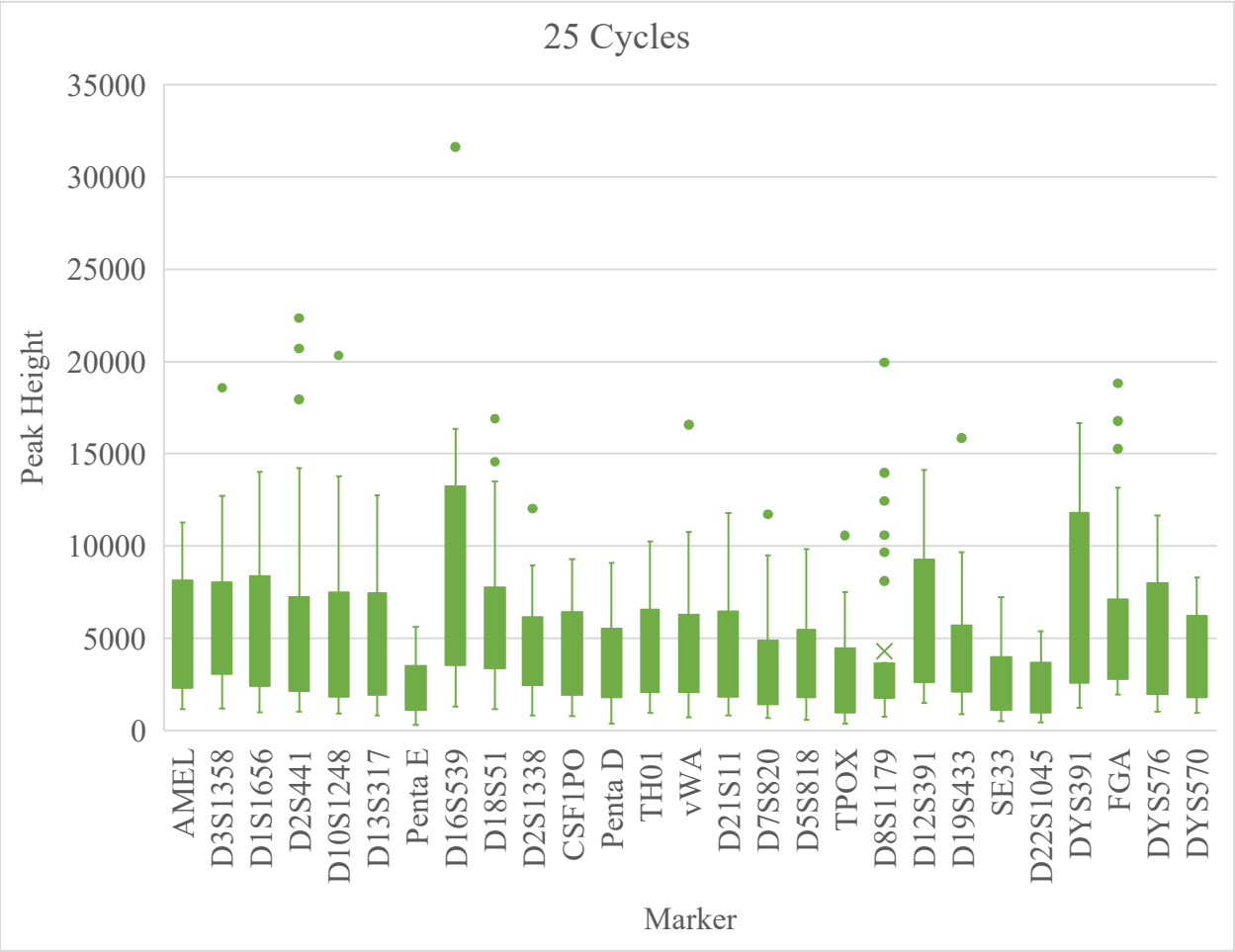

Figure 6: Box and whisker plot providing the peak heights for each marker for the samples tested with 25 cycles of PCR

For this validation, 25 cycles of PCR was decided to be the optimal number of cycles for the Databank Unit as the average peak height was 4,617.092 RFU. This cycle number was determined to be optimal because the average peak height was similar to the average peak height of the positive controls from the analytical threshold study. The average peak height of the positive controls was 4,956.7 RFU. This result is also concordant with the suggested cycle number in Promega's manual.

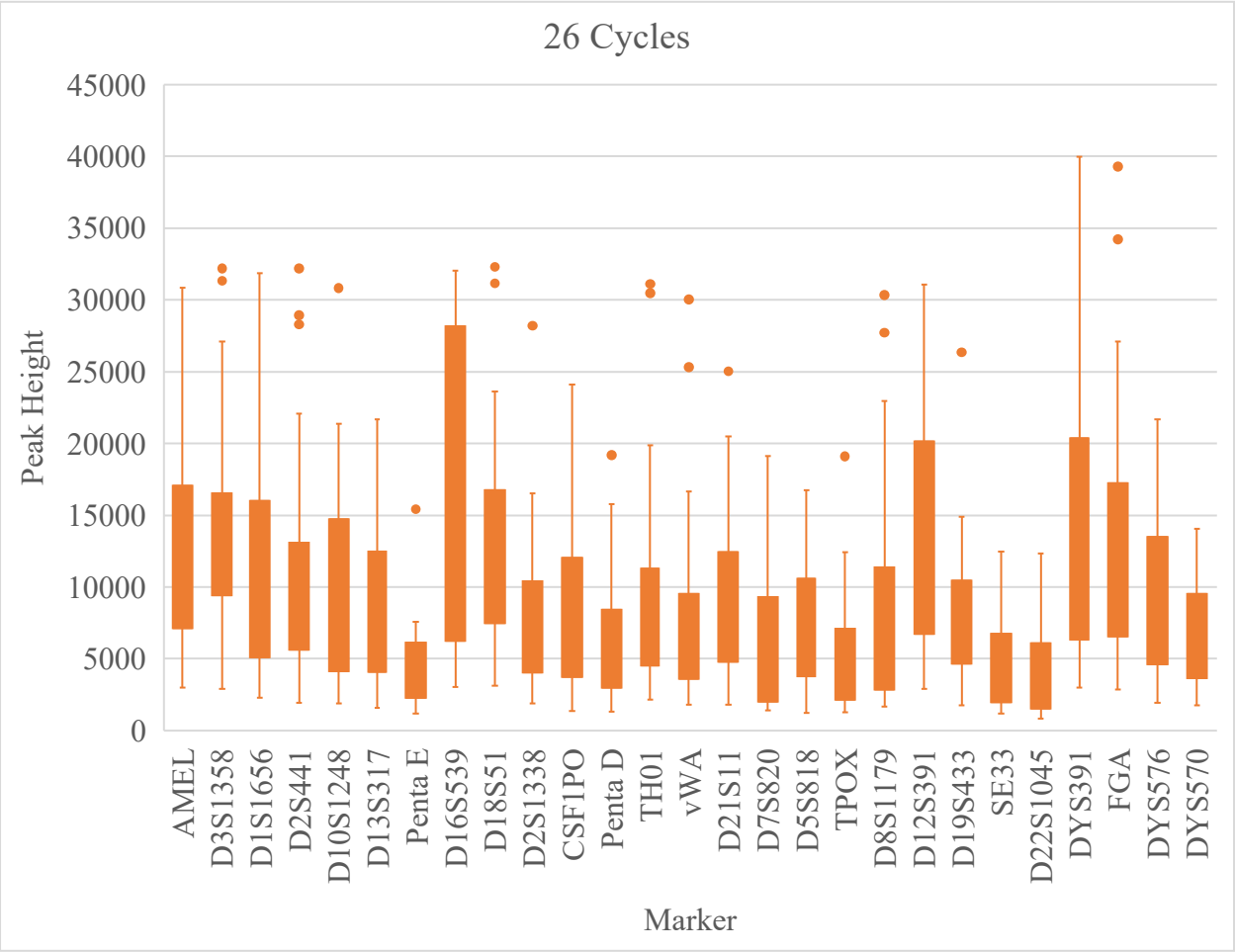

Figure 7: Box and whisker plot providing the peak heights for each marker for the samples tested with 26 cycles of PCR

Since the average peak height when using 26 Cycles was very high, at 9,335.264 RFU, it was decided 26 cycles would be excluded. This cycle number was excluded because the average peak height was so much higher than the average peak height of the positive controls from the analytical threshold study. The average peak height of the positive controls was 4,956.7 RFU. This cycle number was also excluded because several artifacts were observed in these samples. For example, pull up was observed in several loci. Pull up occurs when the signal from an overloaded sample overwhelms the charge-coupled device (CCD) camera in the CE and causes false peaks in other dye channels.

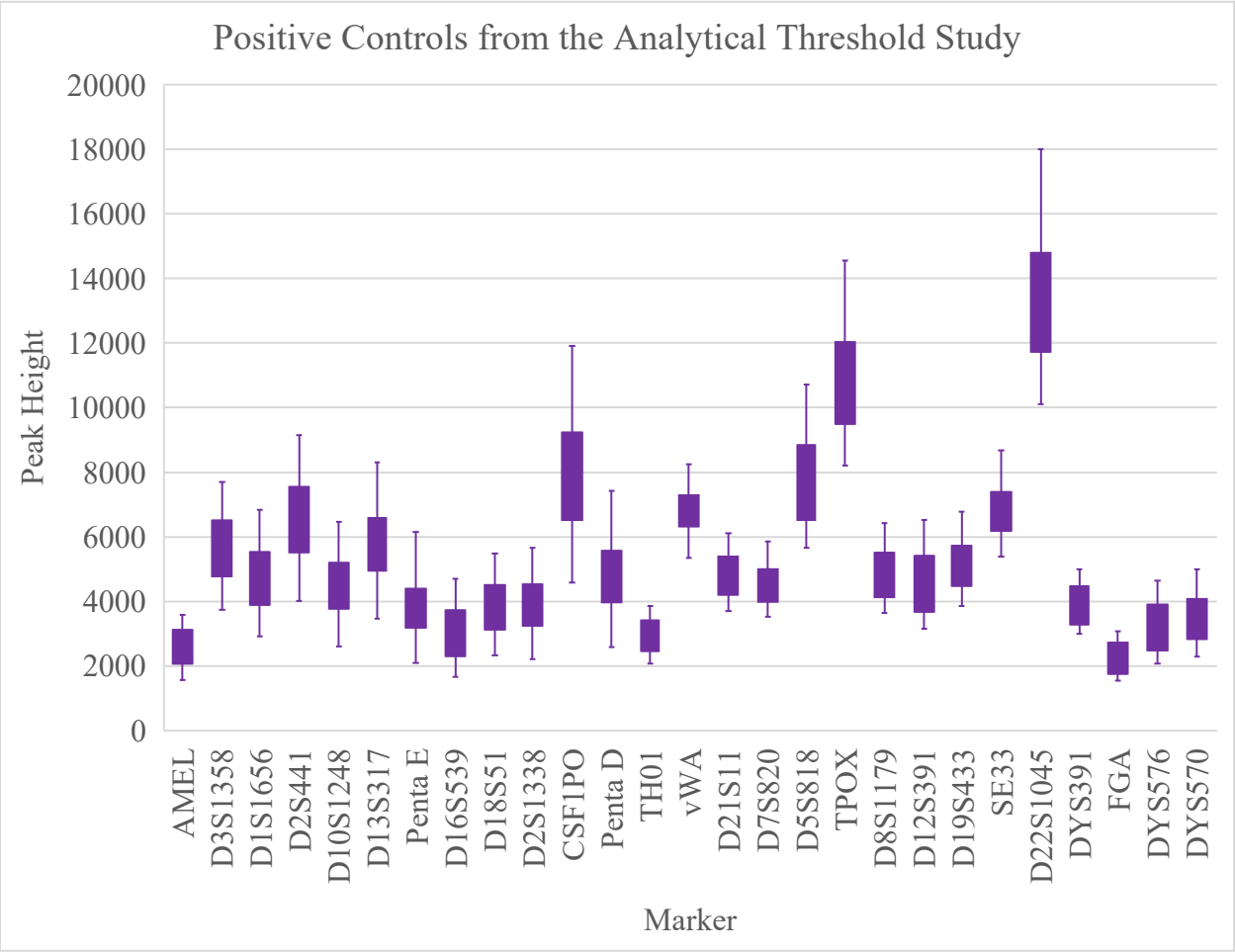

Figure 8: Box and whisker plot providing the peak heights for each marker for the positive controls from the analytical threshold study

Injection Time

Although 25 cycles of PCR was determined to be optimal, it was decided to also test 26 cycles at 12 seconds as well. This was done because in some cases it may be beneficial for the Databank Unit to amplify a sample for 26 cycles for a better yield in a low-level sample, while a decreased injection time could help prevent blowout. The peak heights for both 24 second and 12 second injection times for both 25 and 26 cycles are provided in Figures 9 and 10 below.

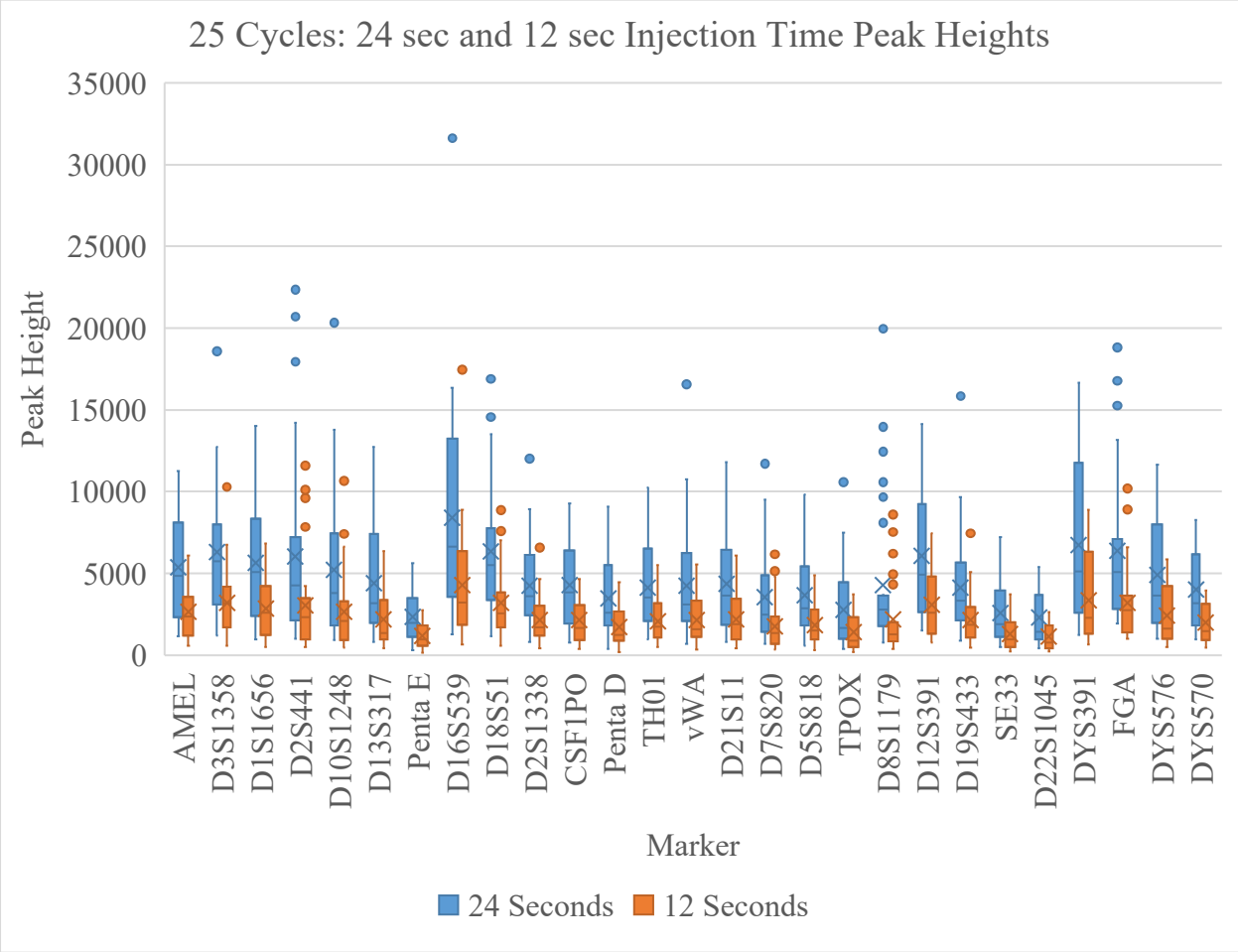

Figure 9: Box and whisker plot providing the comparison of the average peak heights of samples injected at 24 seconds or 12 seconds that were amplified for 25 cycles of PCR

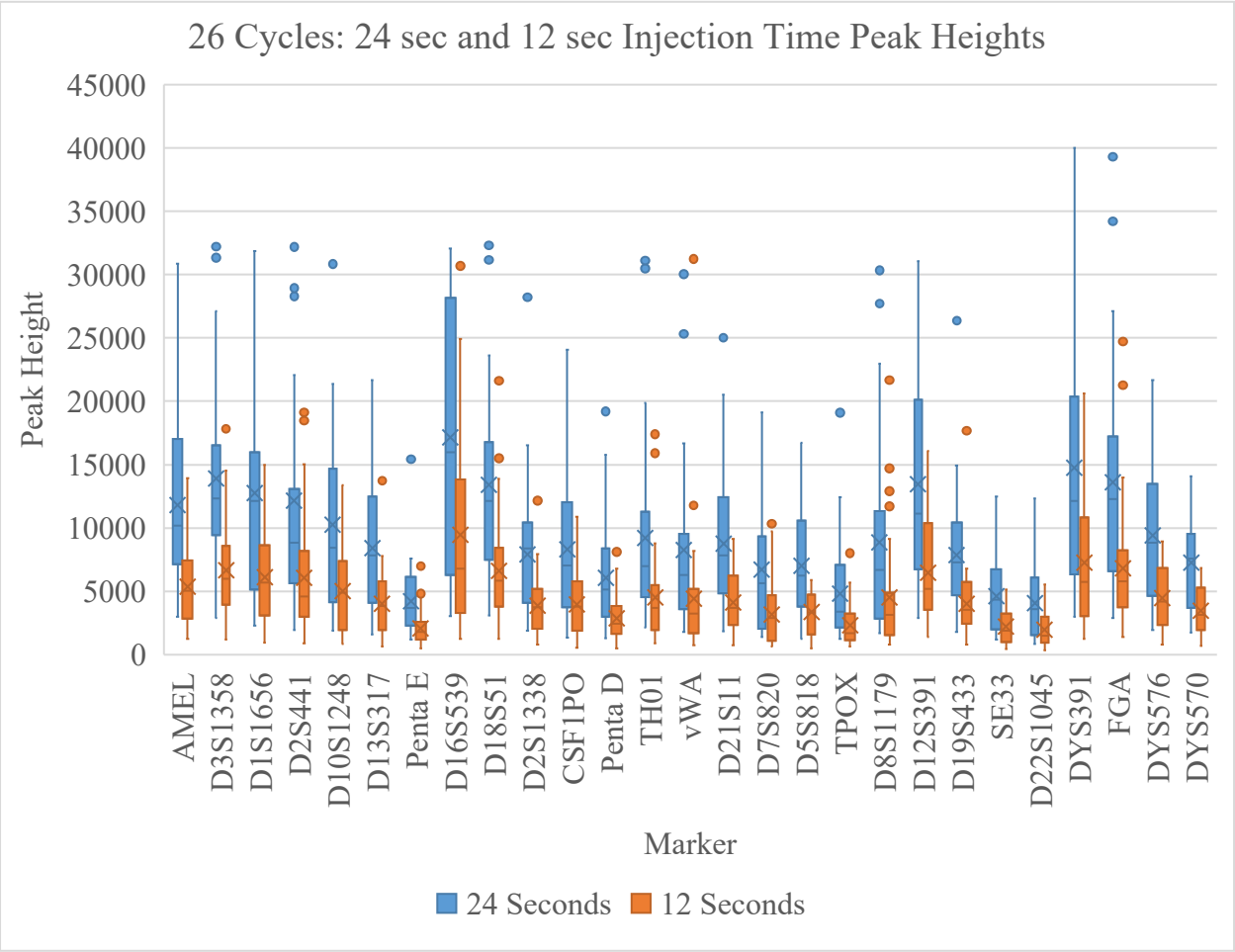

Figure 10: Box and whisker plot providing the comparison of the average peak heights of samples injected at 24 seconds or 12 seconds that were amplified for 26 cycles of PCR

The average peak heights for 25 cycles at 24 seconds and 12 seconds were 4,617.092 RFU and 2,325.676 RFU, respectively. The average peak heights for 26 cycles at 24 seconds and 12 seconds were 9,335.264 RFU and 4,583.696 RFU, respectively. Based on the results, it was determined the optimal injection time was 24 seconds with 25 cycles of PCR. These results are concordant with Promega's manual. If the Databank deems it necessary, 26 cycles of PCR can be used with a 12 second injection time since the average peak height of the samples injected for 12 seconds with 26 cycles of PCR was about the same as the average peak height of the samples injected for 24 seconds with 25 cycles of PCR.

Analytical Threshold

In order to calculate the analytical threshold, the limit of detection (LOD) and the limit of quantitation (LOQ) had to be calculated. The LOD was calculated by adding the average peak height of the dye channel to three times the standard deviation of the dye channel. The LOQ was calculated by adding the average peak height of the dye channel to 10 times the standard deviation of the dye channel. Due to either pipetting error or evaporation, there was only enough amplified positive control to fill 21 wells with positive control. The results of the positive control data are provided in Table 5 below. Stutter was not removed before the values shown in Table 5 were calculated, which lead to higher LOD and LOQ values compared to the expected values. The expected values should have been closer to the analytical threshold suggested by Promega's manual of 175 RFU. Since the values were so high, it was decided to use the negative control and reagent blank values from the 25 Cycle plate from the Sensitivity Study, Stochastic Threshold study, Reproducibility study, and any re-injections for those studies. Since the internal lane standard does not start until 60 base pairs in size, any data before 60 base pairs was deleted when analyzing the data from the negative controls and reagent blanks. The results from the negative controls are provided below in Table 6. It was decided to use Promega's recommended analytical threshold of 175 RFU. This was because the analytical threshold calculated using the negative controls was very low at 46.233309 RFU, and the analytical threshold using the positive controls would probably be closer to 175 RFU if all stutter was removed before the calculations were completed. Stutter is an artifact that is caused when the polymerase slips on the DNA strand during amplification. This slippage results in a minor allele peak that can either be shorter or longer in repeat units than the parent allele peak. It is generally observed one repeat unit, or four base pairs, shorter than the parent allele. In the future, the Databank may choose to delete

the stutter from the positive controls and re-calculate the analytical threshold. If the Databank chooses to do this, any data before 60 base pairs will also need to be deleted from the positive controls since those values were also not removed before the values in Table 5 were calculated.

Table 5: Positive Control data from Analytical Threshold Study

Dye	Average Peak Height	Standard Deviation	3X Standard Deviation	10X Standard Deviation	LOD	LOQ
Blue	18.324624	47.322328	141.96699	473.22328	160.29161	491.54791
Green	18.715844	43.848225	131.54468	438.48225	150.26052	457.19810
Yellow	20.061949	61.039462	183.11839	610.39462	203.18034	630.45657
Red	27.570340	94.200090	282.60027	942.00090	310.17061	969.57124
Purple	12.770003	24.631952	73.895857	246.31952	86.665860	259.08953
Orange	55.741125	915.98922	2747.96765	9159.89218	2803.70878	9215.63331

Table 6: Negative Control data from Analytical Threshold Study

Dye	Average Peak Height	Standard Deviation	3X Standard Deviation	10x Standard Deviation	LOD	LOQ
Blue	5.6532258	2.2847490	6.8542470	22.847490	12.507473	28.500716
Green	8.8979436	3.7335365	11.200610	37.335365	20.098553	46.233309
Yellow	5.2634033	2.2282688	6.6848063	22.282688	11.948210	27.546091
Red	8.8669355	2.9975358	8.9926075	29.975358	17.859543	38.842294
Purple	7.6817491	2.8553716	8.5661149	28.553716	16.247864	36.235465
Orange	2.48216767	1.22134694	3.66404082	12.2134694	6.1462085	14.695637

Stochastic Threshold

In order to determine the stochastic threshold, the peak heights for each marker for every dilution were compared to determine where sister alleles were either below threshold or absent. By sister alleles, I am referring to heterozygosity, where two alleles are observed at a locus. When dropout or below threshold peaks occur in sister alleles, the remaining peak may be falsely interpreted as a homozygous peak. A list was compiled of the peak heights of the 74 sister alleles that were still present after the second allele dropped out. In Table 7, the average peak height value was calculated by averaging the peak heights from the list of remaining sister alleles. The standard deviation was then calculated for the sister dropout allele peak heights. To calculate the stochastic threshold, the standard deviation was multiplied by three then added to the average peak height of the sister dropout alleles. Based on the values calculated, the stochastic threshold was determined to be 141.1393 RFU.

Table 7: Data based on sister dropout alleles from Stochastic Study

Average Peak Height	Standard Deviation	3X Standard Deviation	Standard Deviation
92.14864865	16.33022446	48.99067339	141.1393

Precision

There were numerous alleles in which the standard deviation was above 0.15; however, the results were deemed acceptable because there did not appear to be any problems in the ladders. For example, there were no off-ladder peaks. Generally speaking, the standard deviation should be as close to zero as possible. The closer the standard deviation is to zero, the more precise the ladders are. Precise ladders are important as they are used as a size reference for samples. The average standard deviation was 0.144973 for the blue dye channel, 0.11125 for the green dye channel, 0.120794 for the yellow dye channel, 0.09953 for the red dye channel, and 0.095144 for the purple dye channel. (See Appendix E for table of precision data)

Reproducibility

The results of the reproducibility study demonstrated concordance between the two plates. Figure 11 provides the peak heights from Plate 1 compared to Plate 2. The average peak height for Plate 1 was 2,211.103 RFU, while the average peak height for Plate 2 was 2,480.371 RFU. Some variation was expected because, as mentioned previously, even though two punches may come from the same sample, they may not have the same amount of DNA present.

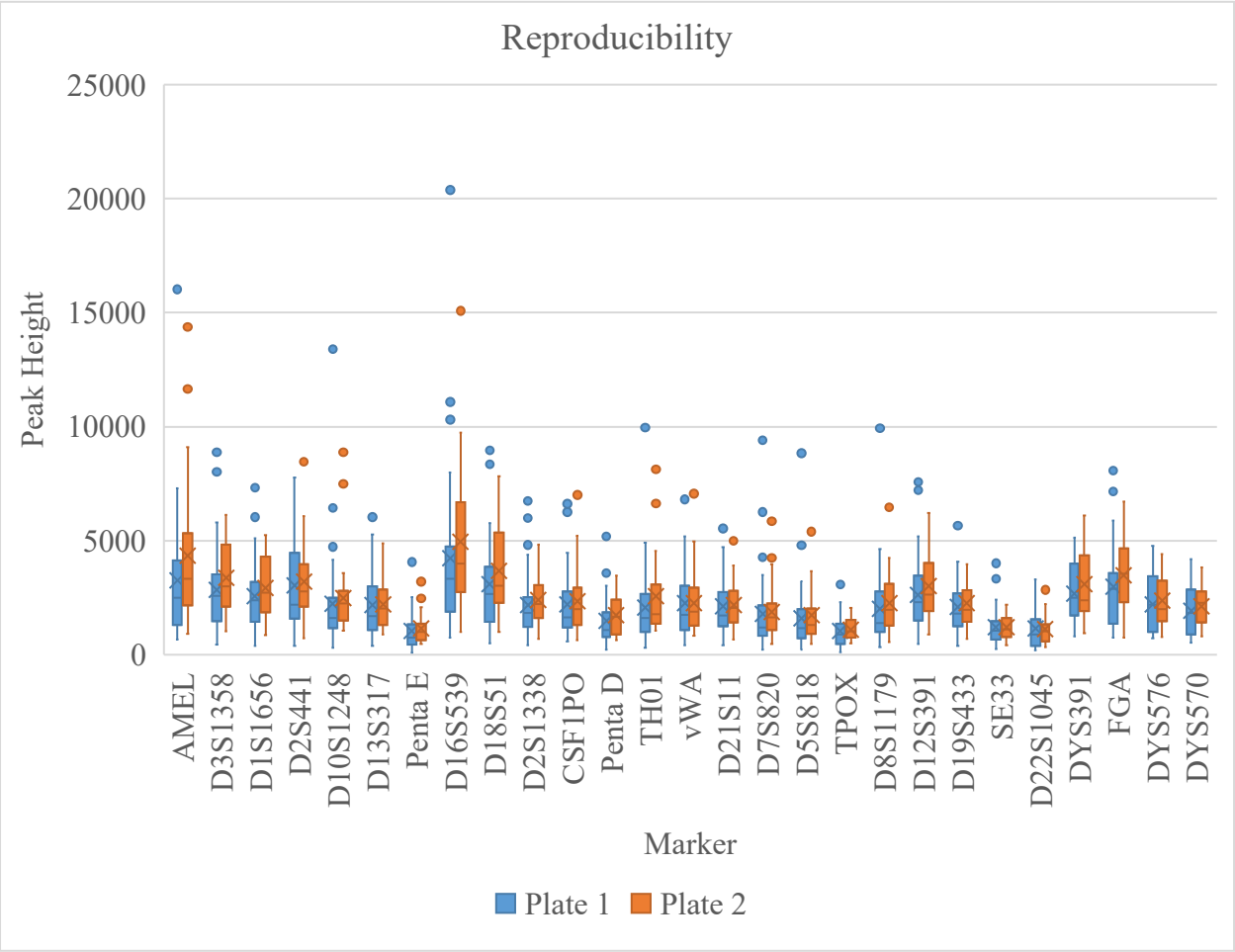

Figure 11: Box and whisker plot providing the peak heights for each marker of the two reproducibility plates

Contamination Study

There was no contamination observed in any of the negative controls, reagents blanks, or single source profiles in any section of this validation.

CHAPTER IV

CONCLUSION

This validation was completed to fit the needs of the DNA Databank Unit of the KBI. Currently the Databank utilizes Hamilton MICROLAB® STARlet Robotic System to automate their extraction and pre-amplification procedures. However, the unit only had a full-plate method validated for use in the laboratory. A quarter plate method is useful for the laboratory in circumstances where running a full plate is not necessary. Promega's PowerPlex® Fusion 6C Kit is also useful to have validated in the laboratory because there was only one amplification kit validated for use: QIAGEN's Investigator GO! 24PLEX Kit. The Fusion 6C Kit includes more loci than the Investigator GO! Kit and can also confirm overlap observed in the Investigator GO! Kit. Since only one mismatch is allowed in CODIS, it is beneficial to be able to confirm the overlap instead of excluding a locus.

The results indicated that the Quarter Plate Method developed for the Hamilton MICROLAB® STARlet Robotic System using Promega's PowerPlex® Fusion 6C Kit was successful. The results also demonstrate Promega's PowerPlex® Fusion 6C Kit was successfully validated. In the future, the Databank Unit will be able to use this kit for cases where they need to confirm overlap observed in samples tested with QIAGEN's Investigator GO! 24PLEX Kit, where they would like to use the kit with the higher number of loci, etc. These methods are also helpful because they have a shorter run time than the current methods used by the DNA Databank. Overall, these methods have been deemed reliable for use in the DNA Databank Unit.

APPENDIX

A. PBS Wash vs. No Wash Plate – Blue represents the washed samples; gray represents empty wells; columns 4-12 were not used so they are not represented

	1	2	3
A	201503813	201503813	BNS
B	201910346	201910346	BNS
C	201910390	201910390	ReagBlk
D	201910391	201910391	Negative Control
E	201910393	201910393	Positive Control
F	201910394	201910394	
G	201910400	201910400	
H	201910401	201910401	Ladder

B. Cycle Number and Injection Time Plate Map; columns 4-12 were not used so they are not represented

	1	2	3
A	201503396	201908732	200506353
B	201907569	201908764	200500683
C	201907577	201908817	200503716
D	201907615	201908849	200508429
E	201907695	201908930	Reagent Blank
F	201907834	201909017	Positive Control
G	201907924	201909051	Negative Control
H	201907927	199500424	Ladder

C. Analytical Threshold Plate Map for Positive Controls – gray represents empty wells; columns 4-12 were not used so they are not represented

	1	2	3
A	Positive Control	Positive Control	Positive Control
B	Positive Control	Positive Control	Positive Control
C	Positive Control	Positive Control	Positive Control
D	Positive Control	Positive Control	Positive Control
E	Positive Control	Positive Control	Positive Control
F	Positive Control	Positive Control	
G	Positive Control	Positive Control	
H	Positive Control	Positive Control	Ladder

D. Stochastic Threshold Plate Map – columns 10-12 were not used so they are not represented; gray represents empty wells

	None	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
	1	2	3	4	5	6	7	8	9
A	200503716								
B	201907577								
C	201907615								
D	201907695								
E	201907834								
F	201908732								
G	201908817								
H	Reagent Blank	Positive Control	Ladder	Negative Control		Ladder			Ladder

E. Ladder Precision Data

Marker	Allele	Average	STD DEV	3X STD DEV	Range	Min	Max
AMEL	X	80.836	0.2015688	0.60470654	0.5	80.48	80.98
	Y	87.028	0.1834939	0.550481607	0.43	86.71	87.14
D3S1358	9	97.232	0.1487279	0.446183819	0.36	96.97	97.33
	10	101.542	0.1648332	0.494499747	0.39	101.26	101.65
	11	105.896	0.1548548	0.464564312	0.37	105.63	106
	12	110.28	0.16	0.48	0.38	110.01	110.39
	13	114.632	0.1783816	0.53514484	0.43	114.32	114.75
	14	118.978	0.1497331	0.449199288	0.39	118.73	119.12
	15	123.244	0.1900789	0.570236793	0.46	122.92	123.38
	16	127.494	0.1782695	0.534808377	0.43	127.19	127.62
	17	131.73	0.1623268	0.486980492	0.41	131.45	131.86
	18	136.002	0.1630031	0.489009202	0.42	135.73	136.15
	19	140.174	0.1472413	0.441723896	0.34	139.92	140.26
	20	144.366	0.169204	0.507612057	0.42	144.07	144.49
D1S1656	9	157.972	0.1149783	0.344934776	0.31	157.78	158.09
	10	162.084	0.1425833	0.427749927	0.37	161.86	162.23
	11	166.188	0.1063955	0.319186466	0.26	166.01	166.27
	12	170.284	0.1275931	0.38277931	0.34	170.07	170.41
	13	174.42	0.1067708	0.320312348	0.25	174.23	174.48
	14	178.474	0.0981326	0.29439769	0.23	178.3	178.53
	14.3	181.586	0.1352036	0.405610651	0.32	181.36	181.68
	15	182.51	0.1238951	0.371685351	0.32	182.3	182.62
	15.3	185.638	0.1035374	0.310612299	0.26	185.46	185.72
	16	186.584	0.1071448	0.321434286	0.26	186.4	186.66
	16.3	189.702	0.1331165	0.399349471	0.34	189.48	189.82
	17	190.652	0.143248	0.42974411	0.36	190.43	190.79
	17.3	193.764	0.1490973	0.447291851	0.37	193.52	193.89
	18	194.718	0.1460137	0.438041094	0.38	194.47	194.85
	18.3	197.826	0.1509304	0.452791343	0.38	197.58	197.96
	19	198.78	0.1624808	0.487442304	0.4	198.53	198.93
	19.3	201.882	0.1490637	0.447191234	0.39	201.65	202.04
	20.3	205.938	0.1188276	0.356482819	0.29	205.75	206.04
D2S441	8	212.322	0.1651363	0.495408922	0.4	212.07	212.47
	9	216.404	0.1728583	0.51857497	0.45	216.12	216.57
	10	220.522	0.1648332	0.494499747	0.41	220.28	220.69
	11	224.62	0.141598	0.424794068	0.35	224.38	224.73

	11.3	227.648	0.1423728	0.427118251	0.36	227.41	227.77
	12	228.708	0.1346105	0.403831648	0.35	228.49	228.84
	13	232.848	0.1430734	0.429220223	0.35	232.61	232.96
	14	236.968	0.1298846	0.389653692	0.34	236.76	237.1
	15	241.084	0.1052616	0.315784737	0.26	240.91	241.17
	16	245.218	0.1370036	0.411010949	0.35	244.99	245.34
	17	249.33	0.141598	0.424794068	0.35	249.09	249.44
D10S1248	8	256.064	0.1680179	0.504053569	0.41	255.78	256.19
	9	260.12	0.1214496	0.364348734	0.31	259.92	260.23
	10	264.2	0.1372953	0.411885907	0.34	263.96	264.3
	11	268.246	0.1361249	0.408374828	0.33	268.01	268.34
	12	272.288	0.1423728	0.427118251	0.37	272.05	272.42
	13	276.298	0.1269646	0.380893686	0.32	276.09	276.41
	14	280.256	0.1608726	0.482617861	0.39	279.98	280.37
	15	284.226	0.1499333	0.449799956	0.38	283.97	284.35
	16	288.144	0.1651666	0.495499748	0.4	287.87	288.27
	17	292.034	0.1487616	0.446284663	0.37	291.79	292.16
	18	295.986	0.1289574	0.386872072	0.31	295.8	296.11
	19	299.888	0.1660422	0.49812649	0.37	299.63	300
D13S317	5	305.61	0.1332291	0.399687378	0.32	305.39	305.71
	6	309.642	0.1543373	0.463011879	0.38	309.4	309.78
	7	313.738	0.1357203	0.407160902	0.34	313.54	313.88
	8	317.792	0.1321741	0.396522383	0.32	317.59	317.91
	9	321.864	0.1277889	0.383366665	0.31	321.67	321.98
	10	325.964	0.1372224	0.411667341	0.32	325.77	326.09
	11	330.05	0.1053565	0.316069613	0.28	329.9	330.18
	12	334.146	0.1266096	0.379828909	0.33	333.94	334.27
	13	338.288	0.1196662	0.358998607	0.28	338.09	338.37
	14	342.356	0.1348332	0.404499691	0.32	342.15	342.47
	15	346.416	0.1123833	0.337149818	0.27	346.22	346.49
	16	350.488	0.1225561	0.367668329	0.3	350.29	350.59
	17	354.49	0.1256981	0.377094153	0.34	354.3	354.64
Penta E	5	368.484	0.1613382	0.484014463	0.39	368.23	368.62
	6	373.298	0.152217	0.456650851	0.35	373.06	373.41
	7	378.156	0.1705286	0.51158577	0.41	377.87	378.28
	8	383.082	0.1704993	0.511497801	0.39	382.83	383.22
	9	387.982	0.1696172	0.508851648	0.42	387.7	388.12
	10	392.854	0.1550161	0.465048385	0.37	392.59	392.96
	11	397.766	0.1659217	0.497765005	0.43	397.49	397.92

	12	402.564	0.1401071	0.420321306	0.35	402.35	402.7
	13	407.332	0.1535252	0.460575727	0.37	407.07	407.44
	14	412.144	0.1312631	0.393789284	0.34	411.92	412.26
	15	416.936	0.1434573	0.430371932	0.34	416.69	417.03
	16	421.738	0.1503995	0.451198404	0.39	421.49	421.88
	17	426.596	0.1532319	0.459695551	0.38	426.35	426.73
	18	431.586	0.133529	0.400587069	0.34	431.37	431.71
	19	436.534	0.146731	0.440193139	0.38	436.29	436.67
	20	441.524	0.114149	0.342447076	0.3	441.34	441.64
	21	446.512	0.1499	0.4496999	0.39	446.3	446.69
	22	451.454	0.1619568	0.485870353	0.38	451.23	451.61
	23	456.274	0.1627268	0.488180295	0.39	456.04	456.43
	24	461.09	0.1637071	0.491121166	0.41	460.86	461.27
	25	465.898	0.1843095	0.552928567	0.46	465.59	466.05
D16S539	4	75.748	0.232422	0.69726609	0.57	75.34	75.91
	5	80.134	0.2126735	0.638020376	0.49	79.76	80.25
	6	84.494	0.1721337	0.516401007	0.43	84.2	84.63
	7	88.876	0.1668233	0.500469779	0.4	88.58	88.98
	8	93.212	0.1435967	0.430789972	0.37	92.97	93.34
	9	97.564	0.1497665	0.449299455	0.36	97.3	97.66
	10	101.882	0.1706458	0.511937496	0.4	101.59	101.99
	11	106.308	0.1508973	0.452691948	0.38	106.05	106.43
	12	110.676	0.1420563	0.426168981	0.36	110.43	110.79
	13	115.062	0.139714	0.419141981	0.35	114.82	115.17
	14	119.392	0.1400714	0.420214231	0.33	119.15	119.48
	15	123.67	0.1509967	0.452990066	0.39	123.42	123.81
	16	127.922	0.1825377	0.547613002	0.44	127.61	128.05
D18S51	7	131.952	0.1382751	0.414825264	0.34	131.71	132.05
	8	136.19	0.1319091	0.395727179	0.34	135.98	136.32
	9	140.4	0.1336039	0.400811676	0.35	140.17	140.52
	10	144.592	0.1037786	0.311335832	0.26	144.41	144.67
	10.2	146.682	0.139714	0.419141981	0.38	146.45	146.83
	11	148.774	0.1199166	0.359749913	0.33	148.58	148.91
	12	152.98	0.0824621	0.247386338	0.22	152.84	153.06
	13	157.156	0.0856154	0.256846258	0.21	157.01	157.22
	13.2	159.202	0.0898332	0.269499536	0.25	159.06	159.31
	14	161.276	0.1028591	0.308577381	0.27	161.1	161.37
	15	165.398	0.091214	0.273642102	0.25	165.25	165.5
	16	169.506	0.0626897	0.188069136	0.15	169.4	169.55

	17	173.59	0.08	0.24	0.22	173.47	173.69
	18	177.678	0.08228	0.246840029	0.21	177.54	177.75
	19	181.76	0.0839643	0.251892834	0.21	181.62	181.83
	20	185.828	0.0872926	0.261877834	0.24	185.71	185.95
	21	189.914	0.1023719	0.307115613	0.26	189.74	190
	22	194.03	0.1022252	0.306675725	0.28	193.87	194.15
	23	198.088	0.123774	0.371321963	0.3	197.92	198.22
	24	202.164	0.1171751	0.351525248	0.3	202	202.3
	25	206.24	0.1122497	0.336749165	0.29	206.1	206.39
	26	210.368	0.1190378	0.357113427	0.28	210.22	210.5
	27	214.442	0.1171324	0.351397211	0.3	214.27	214.57
D2S1338	10	222.566	0.1085357	0.325607125	0.28	222.42	222.7
	12	230.666	0.1377679	0.413303762	0.36	230.46	230.82
	14	238.848	0.1207063	0.362118765	0.3	238.65	238.95
	15	242.936	0.1275931	0.38277931	0.32	242.72	243.04
	16	247.022	0.1219426	0.365827828	0.3	246.81	247.11
	17	251.128	0.1269646	0.380893686	0.32	250.92	251.24
	18	255.238	0.1190378	0.357113427	0.3	255.05	255.35
	19	259.368	0.1151955	0.345586458	0.3	259.18	259.48
	20	263.466	0.0850294	0.25508822	0.21	263.32	263.53
	21	267.548	0.1221475	0.366442356	0.3	267.36	267.66
	22	271.612	0.1175585	0.352675488	0.3	271.41	271.71
	23	275.646	0.1205404	0.361621349	0.3	275.45	275.75
	24	279.626	0.1167476	0.350242773	0.29	279.43	279.72
	25	283.61	0.124499	0.373496988	0.32	283.42	283.74
	26	287.606	0.1186592	0.355977527	0.28	287.42	287.7
	27	291.566	0.0937017	0.281104963	0.25	291.42	291.67
	28	295.48	0.1022252	0.306675725	0.27	295.34	295.61
CSF1PO	5	317.306	0.121573	0.36471907	0.3	317.12	317.42
	6	321.418	0.1366382	0.409914625	0.35	321.19	321.54
	7	325.474	0.133154	0.399462138	0.31	325.29	325.6
	8	329.578	0.112783	0.338348932	0.26	329.42	329.68
	9	333.692	0.0933809	0.280142821	0.21	333.56	333.77
	10	337.796	0.1121606	0.336481797	0.26	337.61	337.87
	11	341.862	0.1279453	0.383835902	0.3	341.67	341.97
	12	345.936	0.1277889	0.383366665	0.35	345.73	346.08
	13	350.002	0.1184061	0.355218243	0.29	349.81	350.1
	14	354.022	0.1281405	0.384421644	0.32	353.82	354.14
	15	358.03	0.1264911	0.379473319	0.29	357.84	358.13

	16	362.03	0.1153256	0.345976878	0.29	361.87	362.16
Penta D	2.2	378.822	0.120499	0.361496888	0.28	378.66	378.94
	3.2	383.932	0.0791833	0.237549995	0.19	383.82	384.01
	5	392.122	0.0892749	0.267824569	0.25	391.99	392.24
	6	397.23	0.0951315	0.285394464	0.25	397.09	397.34
	7	402.286	0.0726636	0.217990825	0.18	402.16	402.34
	8	407.248	0.0614003	0.184200977	0.15	407.17	407.32
	9	412.264	0.0320936	0.096280839	0.09	412.22	412.31
	10	417.284	0.0665582	0.199674736	0.15	417.19	417.34
	11	422.292	0.0614003	0.184200977	0.15	422.19	422.34
	12	427.364	0.0550454	0.165136307	0.15	427.29	427.44
	13	432.548	0.0238747	0.071624018	0.06	432.52	432.58
	14	437.758	0.0389872	0.116961532	0.11	437.7	437.81
	15	442.926	0.0384708	0.115412304	0.09	442.88	442.97
	16	448.134	0.0320936	0.096280839	0.08	448.09	448.17
	17	453.218	0.0370135	0.111040533	0.09	453.17	453.26
TH01	3	66.402	0.2288449	0.686534777	0.54	66	66.54
	4	70.87	0.2398958	0.719687432	0.56	70.46	71.02
	5	75.272	0.1904468	0.571340529	0.47	74.94	75.41
	6	79.548	0.1986706	0.596011745	0.48	79.2	79.68
	7	83.82	0.1544345	0.463303356	0.38	83.55	83.93
	8	88.1	0.1467992	0.440397548	0.36	87.85	88.21
	9	92.348	0.1575436	0.472630934	0.38	92.07	92.45
	9.3	95.502	0.1448102	0.434430662	0.36	95.25	95.61
	10	96.6	0.1174734	0.352420204	0.3	96.4	96.7
	11	100.856	0.1545316	0.463594651	0.36	100.59	100.95
	13.3	112.882	0.1490637	0.447191234	0.36	112.62	112.98
vWA	10	124.986	0.1745852	0.523755668	0.43	124.68	125.11
	11	129.324	0.1668233	0.500469779	0.41	129.03	129.44
	12	133.47	0.1570032	0.471009554	0.38	133.21	133.59
	13	137.624	0.1348332	0.404499691	0.35	137.4	137.75
	14	141.798	0.1567482	0.470244617	0.39	141.53	141.92
	15	145.99	0.1278671	0.383601356	0.33	145.77	146.1
	16	150.158	0.1308434	0.392530254	0.35	149.94	150.29
	17	154.332	0.135536	0.406607919	0.34	154.11	154.45
	18	158.44	0.1330413	0.399124041	0.32	158.21	158.53
	19	162.568	0.1143241	0.342972302	0.29	162.37	162.66
	20	166.62	0.1129159	0.338747694	0.28	166.43	166.71
	21	170.682	0.1075639	0.322691803	0.26	170.5	170.76

	22	174.732	0.0965401	0.289620441	0.24	174.57	174.81
	23	178.802	0.0957601	0.287280351	0.23	178.64	178.87
	24	182.842	0.1158447	0.347534171	0.29	182.64	182.93
D21S11	24	203.462	0.1468673	0.440601861	0.38	203.22	203.6
	24.2	205.528	0.1380942	0.414282512	0.37	205.31	205.68
	25	207.528	0.1384558	0.415367307	0.34	207.33	207.67
	25.2	209.564	0.1439792	0.431937495	0.36	209.34	209.7
	26	211.586	0.1451895	0.435568594	0.39	211.36	211.75
	27	215.7	0.1305756	0.391726946	0.34	215.5	215.84
	28	219.726	0.1487616	0.446284663	0.37	219.49	219.86
	28.2	221.754	0.1353883	0.406164991	0.35	221.53	221.88
	29	223.766	0.12502	0.375059995	0.33	223.57	223.9
	29.2	225.804	0.1217785	0.365335462	0.29	225.63	225.92
	30	227.846	0.1057355	0.317206557	0.27	227.68	227.95
	30.2	229.878	0.1317953	0.395385887	0.34	229.65	229.99
	31	231.95	0.0930054	0.279016129	0.23	231.81	232.04
	31.2	233.912	0.0816701	0.245010204	0.2	233.78	233.98
	32	236.028	0.1054514	0.316354232	0.27	235.85	236.12
	32.2	238.074	0.0896103	0.268830802	0.24	237.93	238.17
	33	240.18	0.0969536	0.290860791	0.23	240.01	240.24
	33.2	242.158	0.1032957	0.309887076	0.28	242	242.28
	34	244.236	0.0861974	0.258592343	0.23	244.09	244.32
	34.2	246.258	0.1116692	0.335007463	0.29	246.08	246.37
	35	248.306	0.1293445	0.388033504	0.32	248.09	248.41
	35.2	250.356	0.1064425	0.319327418	0.29	250.18	250.47
	36	252.476	0.1099091	0.32972716	0.28	252.29	252.57
	37	256.514	0.1033441	0.310032256	0.24	256.33	256.57
	38	260.61	0.0919239	0.275771645	0.23	260.47	260.7
D7S820	5	269.278	0.1003494	0.301048169	0.25	269.11	269.36
	6	273.382	0.1317953	0.395385887	0.33	273.16	273.49
	7	277.424	0.1054988	0.316496445	0.27	277.26	277.53
	8	281.442	0.1173456	0.35203693	0.32	281.25	281.57
	9	285.45	0.1270827	0.381247951	0.32	285.24	285.56
	10	289.428	0.1194571	0.358371316	0.32	289.24	289.56
	11	293.45	0.1345362	0.403608721	0.32	293.24	293.56
	12	297.46	0.1204159	0.361247837	0.31	297.26	297.57
	13	301.48	0.117686	0.353058069	0.31	301.3	301.61
	14	305.532	0.0923038	0.276911538	0.22	305.39	305.61
	15	309.604	0.1252198	0.37565942	0.32	309.4	309.72

	16	313.7	0.0948683	0.284604989	0.24	313.54	313.78
D5S818	6	322.506	0.1123833	0.337149818	0.3	322.33	322.63
	7	326.59	0.0972111	0.291633331	0.25	326.44	326.69
	8	330.7	0.0868907	0.260672208	0.21	330.57	330.78
	9	334.778	0.1075639	0.322691803	0.25	334.62	334.87
	10	338.9	0.0877496	0.263248932	0.2	338.77	338.97
	11	342.952	0.0683374	0.205012195	0.16	342.83	342.99
	12	347.028	0.0775887	0.232765977	0.19	346.9	347.09
	13	351.092	0.0980816	0.294244796	0.24	350.95	351.19
	14	355.078	0.0715542	0.214662526	0.18	354.97	355.15
	15	359.07	0.1072381	0.321714159	0.28	358.9	359.18
	16	363.072	0.0903881	0.271164157	0.22	362.93	363.15
	17	367.044	0.0950263	0.285078936	0.24	366.88	367.12
	18	371.048	0.0923038	0.276911538	0.21	370.93	371.14
TPOX	4	394.488	0.121326	0.363978021	0.3	394.29	394.59
	5	398.524	0.1522498	0.456749384	0.36	398.29	398.65
	6	402.544	0.1217785	0.365335462	0.29	402.35	402.64
	7	406.506	0.1293445	0.388033504	0.33	406.29	406.62
	8	410.462	0.1317953	0.395385887	0.33	410.24	410.57
	9	414.434	0.0870632	0.261189586	0.22	414.3	414.52
	10	418.428	0.0779102	0.233730614	0.19	418.29	418.48
	11	422.396	0.119708	0.359123934	0.31	422.19	422.5
	12	426.448	0.1114451	0.334335161	0.26	426.25	426.51
	13	430.56	0.0874643	0.262392835	0.21	430.42	430.63
	14	434.67	0.0964365	0.289309523	0.25	434.51	434.76
	15	438.832	0.0739594	0.221878345	0.19	438.71	438.9
	16	442.948	0.0980816	0.294244796	0.26	442.81	443.07
D8S1179	7	71.63	0.2483948	0.745184541	0.57	71.19	71.76
	8	76.056	0.2232263	0.669679028	0.53	75.66	76.19
	9	80.394	0.1792484	0.537745293	0.42	80.08	80.5
	10	84.722	0.1611521	0.483456306	0.39	84.44	84.83
	11	89.024	0.1604057	0.481217207	0.38	88.74	89.12
	12	93.326	0.156301	0.468902975	0.37	93.05	93.42
	13	97.782	0.1384558	0.415367307	0.35	97.54	97.89
	14	102.106	0.1530686	0.459205836	0.39	101.84	102.23
	15	106.46	0.1469694	0.440908154	0.39	106.21	106.6
	16	110.834	0.1329662	0.398898483	0.32	110.6	110.92
	17	115.304	0.1275931	0.38277931	0.32	115.08	115.4
	18	119.516	0.1588395	0.476518625	0.41	119.24	119.65

	19	123.908	0.1325519	0.39765563	0.33	123.68	124.01
D12S391	14	134.686	0.1745852	0.523755668	0.43	134.38	134.81
	15	138.854	0.1637987	0.491395971	0.4	138.57	138.97
	16	143.04	0.1443953	0.433185872	0.34	142.8	143.14
	17	147.182	0.1247798	0.374339418	0.3	146.96	147.26
	17.3	150.384	0.1543697	0.463109058	0.36	150.11	150.47
	18	151.354	0.128569	0.385707143	0.3	151.13	151.43
	18.3	154.522	0.1317953	0.395385887	0.33	154.29	154.62
	19	155.492	0.1049762	0.314928563	0.27	155.31	155.58
	20	159.618	0.1279453	0.383835902	0.34	159.4	159.74
	21	163.708	0.0931128	0.279338504	0.23	163.55	163.78
	22	167.76	0.087178	0.261533937	0.22	167.62	167.84
	23	171.772	0.1003494	0.301048169	0.26	171.6	171.86
	24	175.822	0.0858487	0.257546112	0.21	175.67	175.88
	25	179.844	0.1085357	0.325607125	0.25	179.66	179.91
	26	183.908	0.0941807	0.282542032	0.24	183.75	183.99
	27	187.982	0.1239758	0.371927412	0.31	187.77	188.08
D19S433	5.2	193.978	0.1221475	0.366442356	0.29	193.78	194.07
	6.2	198	0.1532971	0.459891292	0.38	197.75	198.13
	8	203.996	0.1537205	0.461161577	0.4	203.74	204.14
	9	207.99	0.1412445	0.423733407	0.36	207.76	208.12
	10	212.034	0.1490973	0.447291851	0.4	211.8	212.2
	11	216.044	0.1687602	0.506280555	0.44	215.77	216.21
	12	220.05	0.1423025	0.426907484	0.39	219.84	220.23
	12.2	222.096	0.1386723	0.416016827	0.36	221.88	222.24
	13	224.072	0.1373681	0.412104356	0.34	223.84	224.18
	13.2	226.096	0.1248199	0.374459611	0.31	225.89	226.2
	14	228.106	0.1465606	0.439681703	0.37	227.86	228.23
	14.2	230.134	0.1346477	0.403943065	0.35	229.92	230.27
	15	232.132	0.0954987	0.286496073	0.24	231.99	232.23
	15.2	234.172	0.1235718	0.370715524	0.3	233.96	234.26
	16	236.176	0.1451895	0.435568594	0.37	235.94	236.31
	16.2	238.24	0.1296148	0.388844442	0.34	238.02	238.36
	17	240.218	0.1317953	0.395385887	0.33	240.01	240.34
	17.2	242.27	0.1197915	0.359374457	0.29	242.09	242.38
	18	244.254	0.1006479	0.301943703	0.26	244.09	244.35
	18.2	246.296	0.130499	0.391497126	0.34	246.08	246.42
SE33	4.2	274.642	0.0742967	0.222890107	0.18	274.54	274.72
	6.3	283.722	0.0756307	0.226892045	0.2	283.6	283.8

	8	288.702	0.0785493	0.235648043	0.21	288.6	288.81
	9	292.686	0.0782943	0.23488295	0.2	292.61	292.81
	10	296.73	0.0781025	0.23430749	0.2	296.62	296.82
	11	300.74	0.0812404	0.243721152	0.2	300.65	300.85
	12	304.81	0.0430116	0.129034879	0.11	304.74	304.85
	13	308.858	0.0794984	0.238495283	0.21	308.75	308.96
	14	312.948	0.0486826	0.146047937	0.12	312.88	313
	15	316.996	0.0811172	0.243351597	0.2	316.93	317.13
	16	321.104	0.0766812	0.230043474	0.2	321	321.2
	17	325.236	0.0541295	0.162388423	0.11	325.19	325.3
	18	329.34	0.0533854	0.160156174	0.13	329.28	329.41
	19	333.456	0.0581378	0.174413302	0.16	333.37	333.53
	20	337.576	0.0536656	0.160996894	0.15	337.51	337.66
	20.2	339.59	0.0430116	0.129034879	0.1	339.54	339.64
	21	341.684	0.0194936	0.058480766	0.04	341.67	341.71
	21.2	343.762	0.0349285	0.104785495	0.08	343.7	343.78
	22	345.798	0.051672	0.155016128	0.12	345.73	345.85
	22.2	347.82	0.0458258	0.137477271	0.12	347.77	347.89
	23.2	351.89	0.0570088	0.171026314	0.14	351.81	351.95
	24.2	355.916	0.0522494	0.156748206	0.14	355.83	355.97
	25.2	359.93	0.0543139	0.162941707	0.13	359.86	359.99
	26.2	363.952	0.0576194	0.172858323	0.14	363.89	364.03
	27.2	367.99	0.0578792	0.173637554	0.14	367.93	368.07
	28.2	371.974	0.0502991	0.150897316	0.13	371.9	372.03
	29.2	376.032	0.0460435	0.138130373	0.12	375.99	376.11
	30.2	380.098	0.0460435	0.138130373	0.1	380.05	380.15
	31.2	384.198	0.0725948	0.217784297	0.19	384.12	384.31
	32.2	388.266	0.0661816	0.198544705	0.17	388.2	388.37
	33.2	392.342	0.0580517	0.174155103	0.15	392.29	392.44
	34.2	396.412	0.0645755	0.193726611	0.16	396.36	396.52
	35	398.484	0.0531977	0.159593233	0.13	398.44	398.57
	36	402.484	0.070214	0.210641876	0.17	402.43	402.6
	37	406.482	0.0432435	0.12973049	0.11	406.41	406.52
	39	414.474	0.0343511	0.103053384	0.08	414.42	414.5
D22S1045	7	434.862	0.0828855	0.248656389	0.21	434.72	434.93
	8	437.97	0.0674537	0.202361063	0.17	437.86	438.03
	9	441.112	0.0690652	0.20719556	0.18	441.02	441.2
	10	444.24	0.0524404	0.157321327	0.12	444.18	444.3
	11	447.31	0.0452769	0.135830777	0.11	447.25	447.36

	12	450.444	0.0541295	0.162388423	0.13	450.41	450.54
	13	453.47	0.0667083	0.200124961	0.17	453.37	453.54
	14	456.484	0.0820366	0.246109732	0.2	456.34	456.54
	15	459.48	0.0514782	0.154434452	0.13	459.42	459.55
	16	462.506	0.0676757	0.203027092	0.17	462.4	462.57
	17	465.494	0.0766812	0.230043474	0.2	465.39	465.59
	18	468.506	0.0823408	0.247022266	0.23	468.38	468.61
	19	471.506	0.0947629	0.284288586	0.26	471.38	471.64
	20	474.532	0.1105441	0.331632327	0.29	474.38	474.67
DYS391	5	80.95	0.1270827	0.381247951	0.33	80.73	81.06
	6	85.282	0.1578607	0.473582094	0.4	85.01	85.41
	7	89.6	0.1208305	0.362491379	0.3	89.39	89.69
	8	93.904	0.117601	0.352803061	0.3	93.7	94
	9	98.164	0.1232071	0.369621428	0.3	97.95	98.25
	10	102.534	0.1040673	0.312201858	0.25	102.35	102.6
	11	106.876	0.132778	0.398334031	0.31	106.64	106.95
	12	111.244	0.128569	0.385707143	0.32	111.02	111.34
	13	115.56	0.1313393	0.394017766	0.33	115.33	115.66
	14	119.896	0.1393915	0.418174605	0.34	119.66	120
	15	124.166	0.1368576	0.410572771	0.34	123.93	124.27
	16	128.382	0.153688	0.461063987	0.37	128.11	128.48
FGA	14	142.78	0.1319091	0.395727179	0.32	142.55	142.87
	15	146.976	0.1169615	0.350884596	0.3	146.79	147.09
	16	151.178	0.1380942	0.414282512	0.37	150.96	151.33
	17	155.352	0.0962808	0.288842518	0.27	155.22	155.49
	18	159.53	0.0966954	0.290086194	0.25	159.4	159.65
	18.2	161.586	0.1021274	0.306382114	0.28	161.44	161.72
	19	163.656	0.114149	0.342447076	0.31	163.47	163.78
	19.2	165.706	0.0779744	0.233923064	0.18	165.58	165.76
	20	167.744	0.0753658	0.226097324	0.2	167.62	167.82
	20.2	169.8	0.0911043	0.273313007	0.24	169.65	169.89
	21	171.84	0.1029563	0.308868904	0.28	171.68	171.96
	21.2	173.898	0.0637966	0.191389655	0.15	173.8	173.95
	22	175.944	0.0658027	0.197408207	0.18	175.84	176.02
	22.2	177.972	0.054037	0.162111073	0.13	177.88	178.01
	23	180.002	0.0601664	0.180499307	0.17	179.92	180.09
	23.2	182.076	0.0673053	0.201915824	0.17	181.96	182.13
	24	184.1	0.110227	0.330681115	0.28	183.92	184.2
	24.2	186.144	0.1011435	0.303430387	0.25	185.97	186.22

	25	188.178	0.0903881	0.271164157	0.23	188.03	188.26
	25.2	190.246	0.1057355	0.317206557	0.27	190.08	190.35
	26	192.268	0.0816701	0.245010204	0.23	192.15	192.38
	27	196.34	0.0961769	0.288530761	0.25	196.2	196.45
	28	200.39	0.1031988	0.309596512	0.27	200.26	200.53
	29	204.474	0.1186592	0.355977527	0.26	204.35	204.61
	30	208.562	0.1291124	0.387337063	0.33	208.38	208.71
	31.2	214.514	0.0978775	0.293632423	0.26	214.36	214.62
	32.2	218.638	0.0936483	0.280944834	0.25	218.51	218.76
	33.2	222.714	0.1064425	0.319327418	0.24	222.59	222.83
	42.2	260.1	0.0815475	0.244642596	0.22	260.01	260.23
	43.2	264.294	0.0522494	0.156748206	0.13	264.24	264.37
	44.2	268.374	0.0870632	0.261189586	0.23	268.28	268.51
	45.2	272.532	0.0834865	0.250459578	0.23	272.42	272.65
	46.2	276.574	0.060663	0.181989011	0.16	276.5	276.66
	48.2	284.52	0.0880341	0.264102253	0.21	284.42	284.63
	50.2	292.59	0.08	0.24	0.2	292.52	292.72
DYS576	11	309.892	0.0722496	0.216748702	0.19	309.78	309.97
	12	313.792	0.0970567	0.291170053	0.25	313.63	313.88
	13	317.732	0.0881476	0.26444281	0.22	317.59	317.81
	14	321.71	0.0845577	0.253673018	0.22	321.57	321.79
	15	325.69	0.1029563	0.308868904	0.22	325.58	325.8
	16	329.638	0.0742967	0.222890107	0.18	329.52	329.7
	17	333.612	0.0872926	0.261877834	0.21	333.46	333.67
	18	337.616	0.0726636	0.217990825	0.16	337.51	337.67
	19	341.604	0.0817313	0.245193801	0.2	341.47	341.67
	20	345.54	0.0578792	0.173637554	0.14	345.44	345.58
	21	349.508	0.1143241	0.342972302	0.28	349.32	349.6
	22	353.394	0.0850294	0.25508822	0.21	353.25	353.46
	23	357.266	0.1066771	0.320031248	0.26	357.08	357.34
DYS570	10	392.774	0.1132696	0.33980877	0.27	392.59	392.86
	11	396.74	0.0927362	0.278208555	0.24	396.59	396.83
	12	400.642	0.0967988	0.290396281	0.22	400.49	400.71
	13	404.494	0.1028591	0.308577381	0.26	404.32	404.58
	14	408.298	0.078867	0.23660093	0.2	408.16	408.36
	15	412.164	0.0896103	0.268830802	0.24	412.02	412.26
	16	416.042	0.0936483	0.280944834	0.23	415.89	416.12
	17	419.876	0.0502991	0.150897316	0.13	419.79	419.92
	18	423.726	0.0766812	0.230043474	0.18	423.59	423.77

	19	427.702	0.0597495	0.179248431	0.15	427.61	427.76
	20	431.668	0.0563028	0.168908259	0.14	431.57	431.71
	21	435.676	0.0723187	0.216956217	0.18	435.56	435.74
	22	439.67	0.0863134	0.258940148	0.23	439.55	439.78
	23	443.678	0.0804363	0.241308931	0.2	443.55	443.75
	24	447.678	0.0875785	0.262735609	0.23	447.56	447.79
	25	451.658	0.0732803	0.219840852	0.18	451.53	451.71

F. Reproducibility Plate Map – columns 4-12 were not used so they are not represented

	1	2	3
A	201510450	201510873	201910467
B	201510515	201510888	201910518
C	201509970	201910215	201910529
D	201509976	201910241	201910556
E	201510584	201910289	Reagent Blank
F	201510662	201910332	Positive Control
G	201510681	201910358	Negative Control
H	201510859	201910444	Ladder

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