Internal Validation of the Biology and DNA Section of the Dubai Police Headquarters' Science Research Laboratory

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Abstract

This project aimed to validate the Quantifiler® Trio DNA Quantification Kit, GlobalFiler™ PCR Amplification Kit, GlobalFiler™ Express PCR Amplification Kit, Yfiler™ Plus PCR Amplification Kit, PrepFiler Express™ Forensic DNA Extraction Kit, and PrepFiler Express BTA™ Forensic DNA Extraction Kit within the Biology and DNA section of the Science Research Laboratory at the Dubai Police Headquarters. All quantification was performed on the Applied Biosystems® 7500 Real-Time PCR System with the HID Real-Time PCR Analysis Software v1.3, thermal cycling on the Applied Biosystems® Veriti™ Thermal Cycler, and capillary electrophoresis on the Applied Biosystems™ 3500 Genetic Analyzer with the Applied Biosystems® GeneMapper® *ID-X* Software v1.5. Studies were performed in compliance with ISO/IEC 17025:2005 guidelines, while also incorporating some FBI Quality Assurance Standards for Forensic DNA Testing Laboratories (September 1, 2011) guidelines and SWGDAM (2010) recommendations. Studies revealed that Quantifiler® Trio was both repeatable and reproducible, with the standards having a maximum shelf life of one week. In addition, the true zero value for Quantifiler® Trio was determined to be ≤0.007 ng/µL. Mixture study results when using this kit revealed that it was acceptable in screening for mixture samples, but that ratios were more accurate when the female served as the major contributor. Prior to validation of the amplification kits, the variances in sizing (bp) and quantity (RFU) of the capillary array were evaluated. Results demonstrated that the array within the lab could reproduce low variance in both base pair sizing (±0.5 bp) and quantity (≤25 %CV); therefore, future capillary arrays used within this lab that produce similar variances can be considered reliable. Upon optimization of the GlobalFiler™ parameters, usable profiles could be obtained with 3.0 – 0.2 ng of input DNA and optimal profiles could be obtained with an input of 1.5 – 0.2 ng of DNA when using 29 PCR cycles, a 15-second injection time, an analytical threshold of 60 RFU, and a stochastic threshold of 250 RFU. In addition, two-person mixture ratios up to 14:1/1:14 could be reliably deconvoluted when using the GlobalFiler™ kit. For Yfiler™ Plus, usable profiles could be obtained with 6.0 – 0.2 ng of input DNA and optimal profiles could be obtained with an input of 1.5 – 0.35 ng of DNA when implementing 30 PCR cycles, a 1.5kV/16-second injection, and an analytical threshold of 75 RFU. Two-person mixture ratios of up to 10:1/1:10 could be reliably distinguished when using optimal PCR conditions for Yfiler™ Plus. In addition, buccal samples and blood samples on FTA were validated for direct amplification with the use of the GlobalFiler™ Express kit; buccal samples produced optimal results with 26 PCR cycles and a 15-second injection time, while FTA samples produced optimal results with 25 PCR cycles and a 20-second injection time. An adjusted analytical threshold of 200 RFU and an adjusted stochastic threshold of 300 RFU for buccal samples were implemented; however, the original analytical threshold of 55 RFU was used with FTA samples, along with a stochastic threshold of 110 RFU. Overall, lancet-induced and whole blood FTA samples showed inhibition and more variance when compared to buccal samples. All extracts from the PrepFiler Express™ and PrepFiler Express BTA™ kits contained sufficient DNA and were deemed acceptable for use within the lab. Future projects should incorporate contamination and concordance studies, three-person mixture studies, and internal stutter percentages; reduced volume reaction and differential extraction procedures (using PrepFiler Express™) could also be examined. Additionally, this project demonstrates adherence to necessary criteria for ISO/IEC 17025:2005 accreditation and serves as a helpful resource for new and developing forensic biology laboratories.

Introduction

The internal validation of all instruments, techniques, and kits to be utilized within forensic laboratories is paramount for many reasons. While serving as a means of ensuring the results obtained are reliable, robust, and reproducible, internal validations also properly equip forensic labs for accreditation. Further, accreditation not only demonstrates that a lab meets certain standards when processing casework, but it also ensures the results between labs are comparable. Thus, this project aimed to validate several processes within the Biology and DNA section of the Science Research Laboratory at the Dubai Police Headquarters for the purposes of obtaining adequate and correct results, meeting ISO/IEC 17025:2005 accreditation requirements, and enabling the forensic science community to become more unified internationally. The validations of the Quantifiler® Trio DNA Quantification Kit (Quantifiler® Trio; Applied Biosystems, Foster City, CA), GlobalFiler™ PCR Amplification Kit (GlobalFiler™; Applied Biosystems), GlobalFiler™ Express PCR Amplification Kit (GlobalFiler™ Express; Applied Biosystems), Yfiler™ Plus PCR Amplification Kit (Yfiler™ Plus; Applied Biosystems), PrepFiler Express™ Forensic DNA Extraction Kit (PrepFiler Express™; Applied Biosystems), and PrepFiler Express BTA™ Forensic DNA Extraction Kit (PrepFiler Express BTA™; Applied Biosystems) were performed during this project. Further, this report was organized and compiled in such a manner that it serves as a helpful resource for new and developing forensic biology laboratories, especially those adhering to ISO/IEC 17025:2005 guidelines.

Although validation and accreditation of forensic laboratories within the United States follow the Quality Assurance Standards set forth by the FBI, labs outside of the country must meet ISO/IEC 17025:2005 guidelines. The differences between these two guidelines, compounded with the short timeframe for this project, meant certain commonly employed tests

were not performed; these tests included contamination studies, concordance studies, and species specificity. Future studies should aim to incorporate these tests. Per ISO 17025:2005 Standard 5.4.5.3, detection limit, dynamic range, repeatability, precision, and sensitivity studies were performed (1,2). Where applicable, certain QAS and SWGDAM requirements were also met so that interoperability between U.S. labs and the Science Research Laboratory within the Dubai Police Headquarters could be achieved (3,4). This was viewed as especially important considering the recent expansion of the core CODIS loci to incorporate the European markers.

Materials and Methods

Balance Verification

The analytical balance within the lab was evaluated to determine the accuracy and precision of its calibration. A set of KERN traceable weights were assessed on the analytical balance, all observed weights were recorded, and the data was then analyzed to ensure the observed measurements were within ±0.0005 g (for weights of ten grams and below) or within ±5% CV (for weights above ten grams).

Pipette Verification

Once the analytical balance was determined to fall within the acceptable tolerance range, it was used to verify the pipettes within the lab were properly calibrated. Molecular Biology Grade (MBG) water was used for this assessment. For all pipettes, maximum (100%), 50%, and 20% volume were evaluated; on some pipettes, 80%, 40%, and 10% volumes were also evaluated. Special attention was given to ensure necessary volumes, such as two microliters for quantification and one microliter for capillary electrophoresis, were assessed. Five replicates of

each respective volume were dispensed onto the analytical balance, and the observed weight of each was recorded. The mean, standard deviation, relative standard deviation (RSD)/CV, and percent error of the recorded weights were then calculated and evaluated to ensure the pipettes were accurate and precise. Any insufficient measurements were repeated before considering the pipette as faulty to ensure that human error was not a factor.

Quantifiler® Trio Validation

Repeatability Study

Ten sets of Quantifiler® Trio standards were prepared on a single 96-well plate and analyzed on the Applied Biosystems® 7500 Real-Time PCR System with the HID Real-Time PCR Analysis Software v1.3 to determine the repeatability of the quantification method. All standard slopes and R^2 values were evaluated and compared to the criteria within the user guide (5). After determining whether or not each individual curve passed, two curves were set as standards and the rest as unknown samples to quantify the standards; this was repeated using various curve combinations so all curves were used and an average quantity for each standard could be obtained. A single factor ANOVA was then performed using four randomly selected sets of standards to determine if the results were statistically significant.

Reproducibility Study

Three sets of Quantifiler® Trio standards were prepared on three different days and analyzed on the 7500 Real-Time PCR System to determine the reproducibility of the quantification method. For each data series, the slopes and R^2 values were evaluated according to the criteria within the user guide. Then, the quantity of DNA within each standard was

determined by setting one series as standards and the other two as unknowns; this was repeated using various curve combinations so all curves were used and an average quantity for each standard could be obtained. The average, standard deviation, and CV were calculated for each standard with each target (Small Autosomal, Large Autosomal, and Y). A single factor ANOVA was performed to compare all three runs and evaluate their statistical significance.

Life of Standards Study

A set of Quantifiler® Trio standards was prepared and evaluated fresh, after one week, and after two weeks on the 7500 Real-Time PCR System to determine the shelf life of the standards. The slopes and R^2 values were evaluated and compared to the acceptance criteria within the user guide to assess the quality of the standards and denote them as passing or failing.

Sensitivity Study

A dilution series was prepared from the NIST SRM 2372 male DNA standard; the series included targeted DNA quantities of 0.00391 - 4.0 ng/μL in two-fold increments for a total of 11 dilutions. Each dilution was then quantified in triplicate using Quantifiler® Trio on the 7500 Real-Time PCR System to determine if the kit could detect the expected quantities of DNA. After quantification of each dilution, the series was amplified in triplicate using both GlobalFiler™ and Yfiler™ Plus on the Applied Biosystems Veriti™ Thermal Cycler. The amplified samples were then evaluated on the Applied Biosystems™ 3500 Genetic Analyzer using GeneMapper® *ID*-X Software v1.5 to establish at what concentration usable profiles could no longer be obtained (i.e. "true zero").

Mixture Study

One male and one female buccal sample were extracted using PrepFiler Express™ and quantified in triplicate using Quantifiler® Trio. Dilutions were then prepared based on the average quant results to cover 0.05 ng/µl, 0.0625 ng/µl, 0.0833 ng/µl, 0.125 ng/µl, 0.25 ng/µl, 0.50 ng/µl, and 1.0 ng/µl for each sample. To evaluate the ability of the kit to deduce mixture samples prior to amplification, mixtures were then prepared from the dilutions directly in the 96-well plate and quantified in triplicate following the table below (Table 1).

Table 1. Quantifiler® Trio Mixture Sample Preparation

Male:Female Ratio	Male DNA Added (ng)	Female DNA Added (ng)
20:1	1.0	0.05
16:1	1.0	0.0625
12:1	1.0	0.0833
8:1	1.0	0.125
4:1	1.0	0.25
2:1	1.0	0.50
1:1	1.0	1.0
1:2	0.50	1.0
1:4	0.25	1.0
1:8	0.125	1.0
1:12	0.0833	1.0
1:16	0.0625	1.0
1:20	0.05	1.0

Table 1. The samples for the mixture study were prepared directly in the 96-well plate by adding 1 µL of each respective dilution in the ratios above. Each ratio was prepared and quantified in triplicate.

3500 Genetic Analyzer Capillary Array Evaluation

Repeatability/Precision Study

Twenty-four allelic ladders were run during three different injections using GlobalFiler™ under manufacturer recommendations. Each capillary, as well as all three injections, were assessed using Applied Biosystems® GeneMapper® *ID-X* Software v1.5. For each ladder, an

allele in each dye channel was chosen and evaluated for base pair size and relative fluorescence units (RFUs); the mean, standard deviation, and coefficient of variation (CV) were calculated for both the base pair size and quantity (RFU) per dye channel. The results were analyzed to ensure the base pair sizing fell within ±0.5 base pairs with a standard deviation less than 0.15 (6). The data was also reviewed to verify that the overall capillary array variance was below 25%.

Reproducibility Study

Three allelic ladders were randomly selected from different runs and analyzed using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 to determine the reproducibility of the capillary array. The base pair and RFU values were evaluated for each dye channel on the subsequent runs to ensure all sizing met the requirements previously discussed regarding capillary array reproducibility/precision and consistently produced CV below 25%.

GlobalFiler™, GlobalFiler™ Express, and Yfiler™ Plus PCR Amplification Kits

Analytical Threshold Study

The analytical threshold study was performed in accordance with the guidelines suggested by Life Technologies (7). Sixteen amplification negative controls were prepared using GlobalFiler™ and Yfiler™ Plus per manufacturer recommendations. Since GlobalFiler™ Express would be evaluated using 25, 26, and 27 PCR cycles, three sets of sixteen amplification negative controls were prepared and evaluated at those cycles. All samples were run on the 3500 Genetic Analyzer and evaluated using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 at a minimum peak height of 1 RFU; the starting point for analysis was around 3400 data points, as that fell outside the range of the first ILS peak and the first locus in each dye channel. Peaks that

could be identified as pull-up from the ILS and peaks greater than 100 RFU were removed. For each dye color, the average of the peak heights, standard deviation of the peak heights, maximum peak height, and minimum peak height were calculated. Two equations were then used to determine the analytical threshold, and the larger threshold value was chosen.

Equation 1 (from Scientific Working Group for DNA Analysis Methods [SWGDAM])[4]:

Analytical Threshold = 2(Maximum peak height – Minimum peak height)

Equation 2 (Limit of Detection)[8]:

Analytical Threshold = Average peak height + (3 x *Standard deviation of peak height)*

In order to determine the most appropriate analytical threshold, the values obtained from each dye channel were considered. First, the analytical threshold was determined for the dye set as a whole by taking the larger of the calculated values for all dyes and rounding to the nearest five. Then, the calculated values for each dye channel were considered and the threshold was set accordingly (rounding to the nearest five when applicable). The results from each method were then compared to determine the best analytical threshold.

Sensitivity Study

The sensitivity series prepared from the NIST SRM 2372 male DNA standard for determining the sensitivity of Quantifiler® Trio was also used for the validation of GlobalFiler™ and Yfiler™ Plus; GlobalFiler™ Express will be discussed separately. The series was amplified in triplicate and evaluated on the 3500 Genetic Analyzer using Applied Biosystems® GeneMapper®

ID-X Software v1.5 to determine at what concentration usable profiles could not be obtained, as well as the optimal input range for the kits. Various reference and forensic samples (buccal and semen) were subsequently assessed to solidify the results. All profiles obtained were evaluated for percent alleles detected, percent allelic dropout, average peak height, average intra-locus balance (peak height ratio), percent samples exhibiting artifacts, and percent loci exhibiting artifacts. In addition, the average inter-locus balance (CV) was calculated following the method by Connon et. al (9); the peak height(s) for each locus was determined and divided by the sum of the profile's total peak heights to obtain the locus peak height to total peak height ratio (LPH:TPH). Then the CV was calculated for each sample. To demonstrate low variance and good inter-locus balance for the profiles, a CV of ≤0.350 was considered ideal (9).

In order to determine the DNA concentration at which viable profiles could no longer be obtained using GlobalFiler™, all electropherograms were also evaluated for the presence of the 13 original CODIS core loci (Table 2). Although the core loci have been recently expanded from 13 to 20, NDIS guidelines still only require the 13 original loci for a sample to be uploaded. According to the FBI NDIS Operational Procedures Manual, partial profiles may still be uploaded as long as they contain at least eight of the original loci and amount to a random match probability (RMP) of at least one in ten million (10). Thus, the average core loci were determined for each concentration in the study and the point at which these requirements could not be met was considered the true zero value.

Table 2. Original CODIS Core Loci

TPOX
D3S1358
FGA
D5S818
CSF1PO
D7S820
D8S1179
TH01
vWA
D13S317
D16S539
D18S51
D21S11

When determining the true zero value for Yfiler™ Plus, the electropherograms were evaluated for the presence of the minimal haplotype plus the two SWGDAM recommended markers (11 markers in total). Currently, no widely accepted minimum marker requirement for Y-STRs has been identified. After considering two separate sources, the 11 markers listed in the table below (Table 3) were considered as the "minimum" for this validation (11,12). The average "core" markers were determined for each concentration and the point at which the 11 markers could no longer be obtained was considered the true zero.

Table 3. SWGDAM Y-STR Markers

DYS19
DYS385a
DYS385b
DYS389I
DYS389II
DYS390
DYS391
DYS392
DYS393
DYS438
DYS439

GlobalFiler™ Express PCR Parameters Evaluation

Because GlobalFiler™ Express is a direct amplification kit, no sensitivity study could be performed. Instead, 13 buccal swabs were collected from two individuals to determine the optimal thermal cycling parameters (13). Following manufacturer recommendations, the 26 total swabs were processed using the kit at room temperature; the lysed samples were then amplified with 25, 26, and 27 cycles. Upon completion of amplification, the samples were analyzed on the 3500 Genetic Analyzer using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 with a 10% global cut-off filter (13). All profiles obtained were evaluated for percent alleles detected, percent allelic dropout, average peak height, average peak height ratio, CV, percent samples exhibiting artifacts, and percent loci exhibiting artifacts. When assessing the CV values for this kit, the Y Indel and DYS391 loci were excluded because the samples were contributed from females; including these loci would have unfairly increased the CVs.

To verify blood on FTA cards could also be used with this kit, a mini study was performed. Seven FTA cards were collected from the same individuals, five using blood from a collection tube (1-5) and two using blood from a finger prick (6-7). A single 1.2 mm punch was taken from FTA cards 1-5, while two punches were taken from FTA cards 6 and 7; this made for a total of nine samples per individual. All samples were then directly amplified per manufacturer recommendations as with the formerly mentioned buccal sample lysates. Data analysis was also performed in the same manner as previously described.

Stochastic Threshold Study

After determining the appropriate thermal cycling parameters for GlobalFiler™ (which were the manufacturer recommended settings), the sensitivity study samples amplified under

those parameters were also used to determine the appropriate stochastic threshold. All false homozygotes were identified and their RFUs recorded. Then, the mean and standard deviation of these values were calculated and Equation 3 was used to compute the stochastic threshold.

Equation 3:

$$Stochastic\ Threshold = Average\ PH + (3 \times Standard\ Deviation\ PH)$$

Since there was no sensitivity study performed with GlobalFiler™ Express, the above method could not be used to determine the stochastic threshold. Instead, the average and standard deviation of the peak height ratios obtained from the samples for each cycle were determined, and the equation below was used to calculate the stochastic thresholds.

Equation 4[14]:

$$Stochastic\ Threshold = Analytical\ Threshold \left(\frac{1}{Average\ PHR - 3 \times Standard\ Deviation\ PHR}\right)$$

After calculating the stochastic thresholds per kit, the data was evaluated to determine whether or not these thresholds were appropriate. The electropherograms from the samples were assessed for the highest false homozygote peak in each dye; the peak height of this false homozygote was expected to be lower than the threshold calculated. If the peak height of a false homozygote was above the calculated stochastic threshold, its height was rounded to the nearest five and the stochastic threshold was adjusted to incorporate the peak.

Injection Parameters Study

Although manufacturer recommendations for injection time and voltage (1.2 kV/15 seconds for GlobalFiler™ and 1.2 kV/16 seconds for Yfiler™ Plus) were initially used, additional injection parameters were evaluated to optimize the analysis methods. Keeping all other parameters the same, the sensitivity series samples for GlobalFiler™ were analyzed using 5 and 10 second injection times, and those for Yfiler™ Plus were evaluated using a 1.5 kV/16 seconds injection. For GlobalFiler™ Express, two samples from each donor and all positive controls were chosen and analyzed at two additional injection times: buccal samples from each PCR cycle evaluated (25, 26, and 27) were analyzed using 5 and 10 second injection times, and FTA samples from each PCR cycle evaluated (25, 26, and 27) were analyzed using 10 and 20 second injection times. All profiles obtained were then evaluated for percent alleles detected, percent allelic dropout, average peak height, average peak height ratio, CV, percent samples containing artifacts, and percent loci exhibiting artifacts.

Mixture Study

Two female buccal samples and two male samples (one buccal, one semen) were extracted with PrepFiler Express™ and used to prepare male:male, male:female, and female:female mixtures. Mixtures were created directly within the amplification tubes at ratios of 20:1 down to 1:20 by adding 1µL of each respective sample dilution (Table 4), using 1.0 ng as the maximum contributor amount (6). In addition to the mixture samples, each individual sample was amplified at an input of 1.0 ng to determine the expected profiles. All mixture samples were then amplified using GlobalFiler™ with the previously determined parameters, while only the male:male mixtures were amplified using Yfiler™ Plus.

The samples were run on the 3500 Genetic Analyzer in triplicate, and the electropherograms were analyzed and evaluated using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 to determine at which point major and minor contributors could no longer reliably be distinguished. For GlobalFiler™, this was performed by assessing the ratio at which alleles dropped out at the loci expected to have four peaks (i.e. two heterozygotes). In addition, the actual ratio for each mixture was determined by considering loci with four peaks, with three peaks where there was no sharing of alleles, and with two peaks (known homozygotes) to calculate the contribution from each individual. The same loci, excluding the known homozygotes, were used to determine the percentage of alleles from the minor contributor above stochastic threshold. Percent dropout for the minor contributor was also calculated using loci with four alleles. For Yfiler™ Plus, all loci with two or more alleles were used to calculate the observed ratios, as well as the percent allelic dropout for the minor contributor.

Table 4. Targeted Mixture Ratio Preparation

Ratio	Major Contributor (ng)	Minor Contributor (ng)
20:1, 1:20	1.0	0.05
16:1, 1:16	1.0	0.0625
12:1, 1:12	1.0	0.0833
8:1, 1:8	1.0	0.125
4:1, 1:4	1.0	0.25
2:1, 1:2	1.0	0.5
1:1	1.0	1.0

Table 4. The samples for the mixture study were prepared directly in the amplification tubes by adding 1 µL of each respective dilution to the PCR tube in the ratios above. This was done for male:male, male:female, and female:female mixtures using GlobalFiler™, as well as for male:male mixtures using Yfiler™ Plus.

PrepFiler Express™ and PrepFiler Express BTA™ Verification

Various reference and casework samples were extracted per manufacturer recommendations using both PrepFiler Express™ and PrepFiler Express BTA™ kits on the Applied Biosystems AutoMate Express™ DNA Extraction System. DNA from four buccal samples and two semen samples (i.e. reference samples) were extracted using PrepFiler Express™; two cigarette butt, two chewing gum, and two adhesive tape samples (i.e. casework/forensic samples) were extracted using PrepFiler Express BTA™. Each extracted sample was then quantified in triplicate using Quantifiler® Trio to ensure that the kits could obtain a sufficient amount of human DNA for downstream testing; this sufficient quantity of DNA was determined during the sensitivity study for the Quantifiler® Trio quantification kit and the GlobalFiler™ and Yfiler™ Plus amplification kits.

Results and Discussion

Balance Verification

The results from the balance verification using the KERN traceable weights can be found below (Table 5); only those weights applicable to forensic DNA analysis are shown. All measurements fell within the acceptance criteria of ±0.0005 g, revealing the balance could be reliably used for the lab and the pipette evaluation.

Table 5. Balance Verification Results

Actual Weight (g)	Measured Weight (g)	Conclusion (Pass/Fail)
5	5.0003	Pass
2	2.0002	Pass
1	1.0004	Pass
0.5	0.5003	Pass
0.2	0.2003	Pass
0.1	0.0999	Pass
0.05	0.0500	Pass
0.02	0.0201	Pass
0.01	0.0100	Pass
0.005	0.0050	Pass
0.002	0.0020	Pass
0.001	0.0011	Pass

Table 5. Results from the balance verification test are displayed above for weights applicable to forensic DNA analysis. All measurements were evaluated based on an acceptance criteria of ±0.0005 g.

Pipette Verification

Evaluation of the pipettes was performed to ensure that they were accurate and precise before any validation studies were conducted. Table 6 displays the average results for pipettes at volumes covering one and two microliters, which are crucial for most DNA analysis methods. All pipettes produced RSD/CV and percent error values less than 20%, signifying they were properly calibrated and could be considered both precise and accurate.

Table 6. Pipette Evaluation Results

Pipette ID	Volume Range (µL)	Target Volume (µL)	Actual Volume (µL)	RSD/CV (%)	Percent Error (%)	Conclusion (Pass/Fail)
SRL-MLPB-20	0.1 – 2.5	1.00	1.00	14.58	4.00	Pass
		2.00	2.00	4.14	1.00	Pass
SRL-MLPB-21	0.5 – 10	1.00	1.00	8.20	2.00	Pass
		2.00	1.80	5.08	12.00	Pass
SRL-MLPB-25	2 - 20	2.00	2.00	10.15	1.00	Pass
SRL-MLPB-29	0.1 – 2.5	1.00	1.00	8.54	2.00	Pass
		2.50	2.40	4.67	2.40	Pass
SRL-MLPB-16	0.1 – 2.5	1.00	1.00	14.54	2.00	Pass
		2.50	2.50	5.89	0.80	Pass
SRL-MLPB-13	0.5 – 10	1.00	0.90	17.63	14.00	Pass
		2.00	1.90	4.61	3.00	Pass
SRL-MLPB-17	0.5 – 10	1.00	0.90	13.25	14.00	Pass
		2.00	1.90	9.52	6.00	Pass
SRL-MLPB-24	2 - 20	2.00	2.00	9.35	0.00	Pass

Table 6. Results from the pipette verification test are displayed above for volumes routinely used in the forensic DNA analysis workflow. The observed volumes for each setting and pipette were averaged and displayed as the actual volumes. The relative standard deviation (RSD) and percent error for each pipette at these volumes were evaluated to assess the precision and accuracy, respectively.

Quantifiler® Trio Validation

Repeatability Study

All standard curve slopes and R^2 values from the repeatability study were evaluated and compared to the criteria within the user guide (5). The slopes when using both duplicate curves (Table 7) and individual curves (Table 8) all fell within the acceptable range. In addition, all R^2 values were ≥0.99. Together, these results signified the instrument and kit could produce passing curves within a single run.

Table 7. Quantifiler® Trio Repeatability Slopes (Duplicate Curves)

Standard Curve	Slope		
	Small Autosomal	Large Autosomal	Male (Y)
1	-3.313	-3.471	-3.438
2	-3.330	-3.444	-3.342
3	-3.400	-3.514	-3.518
4	-3.413	-3.452	-3.381
5	-3.309	-3.457	-3.390

Table 7. The ten sets of standards were evaluated in duplicate and their slopes were recorded for the small autosomal, large autosomal, and male targets. The curves were grouped in order from the individual curves 1-10. For example, Curve 1 equals the individual curves 1 and 2.

Table 8. Quantifiler® Trio Repeatability Slopes (Individual Curves)

Standard Curve	Slope		
	Small Autosomal	Large Autosomal	Male (Y)
1	-3.286	-3.442	-3.365
2	-3.341	-3.500	-3.512
3	-3.322	-3.441	-3.334
4	-3.339	-3.447	-3.350
5	-3.350	-3.489	-3.476
6	-3.449	-3.539	-3.560
7	-3.435	-3.521	-3.359
8	-3.391	-3.382	-3.404
9	-3.338	-3.470	-3.489
10	-3.280	-3.444	-3.290

Table 8. The ten sets of standards were evaluated individually and their slopes were recorded for the small autosomal, large autosomal, and male targets.

Various duplicate curve combinations were used to obtain an average quantity for each standard to assess the repeatability of the standards themselves (Table 9). The average and standard deviation of these quantities were then used to determine the quantification range for each standard (Table 10). As shown within these tables, all standards were close to their expected values with low standard deviations. To determine if the results were statistically significant, a single factor ANOVA was performed using four randomly selected sets of standards (Table 11). When assessing the small autosomal results with an alpha value of 0.05, a statistically significant difference was observed for all standards except for the two largest quantities. This assessment for the large autosomal and male results revealed a statistically significant difference for all standards except for the largest quantity. Although the data revealed a statistically significant difference measurements, this did not necessarily reflect the quality of the results. The expected quantities of the smaller standards (0.5, 0.05, and 0.005 ng) are so low, any slight difference could appear statistically significant; even a small standard deviation could cause the variation and differences in means to appear high. This data reveals that smaller

quantities of DNA should be considered as more of an estimate because the standards vary more at this lower concentration range. Since a lower alpha value reduces the chance of rejecting a true null hypothesis, the small autosomal dataset was also evaluated using a single factor ANOVA with an alpha value of 0.01. No statistically significant difference was observed for all standards during this evaluation; therefore, these findings coincide with the previous conclusion that the smaller standards are more likely to show a statistically significant difference due to their lower expected quantities.

Table 9. Quantifiler® Trio Repeatability Study Results (Duplicate Curves)

Standard Curve Series		Quantity (ng)				
		Standard 1 (50 ng)	Standard 2 (5 ng)	Standard 3 (0.5 ng)	Standard 4 (0.05 ng)	Standard 5 (0.005ng)
Small Autosomal	1	53.3617	5.6598	0.4967	0.0553	0.0071
	2	53.8410	5.0882	0.5663	0.0553	0.0056
	3	52.0158	5.0891	0.5012	0.0579	0.0056
	4	50.9814	5.0513	0.5417	0.0488	0.0056
	5	48.0776	4.8078	0.4565	0.0516	0.0045
	6	50.9437	5.2029	0.4295	0.0415	0.0040
	7	46.7117	4.8696	0.4154	0.0421	0.0036
	8	46.5556	4.6245	0.4248	0.0464	0.0039
	9	51.3280	5.1013	0.4727	0.0482	0.0059
	10	54.2158	5.3556	0.5294	0.0463	0.0078
Large Autosomal	1	44.3042	4.9746	0.4770	0.0519	0.0047
	2	47.6377	4.9829	0.4667	0.0524	0.0042
	3	47.9798	5.2402	0.4794	0.0492	0.0056
	4	48.4880	5.1071	0.5627	0.0524	0.0053
	5	48.9508	5.1495	0.5004	0.0526	0.0043
	6	48.8576	5.3188	0.4859	0.0473	0.0039
	7	47.5451	5.1566	0.4710	0.0585	0.0038
	8	47.4361	5.0778	0.5091	0.0552	0.0061
	9	48.6709	5.5032	0.5227	0.0598	0.0047
	10	49.3194	5.3426	0.5553	0.0525	0.0054
Male (Y)	1	55.2047	5.8870	0.5225	0.0682	0.0060
	2	50.7017	5.3927	0.5450	0.0542	0.0036
	3	50.8337	5.2473	0.5007	0.0538	0.0070
	4	48.4790	4.6428	0.5452	0.0484	0.0063
	5	48.6160	4.6474	0.4562	0.0440	0.0037
	6	50.7040	5.2362	0.4187	0.0419	0.0032
	7	47.4816	4.8100	0.4380	0.0577	0.0054
	8	46.9494	4.8994	0.4608	0.0606	0.0045
	9	47.8362	5.1650	0.4612	0.0523	0.0038
	10	49.1644	5.1366	0.5162	0.0533	0.0075

Table 9. Ten sets of standards were run and their quantities were determined using various duplicate curve combinations.

Table 10. Quantifiler® Trio Repeatability Quantification (Duplicate Curves)

Standard	Quantification Range (ng)		
	Small Autosomal	Large Autosomal	Male (Y)
1	50.803 ± 2.795	47.919 ± 1.246	49.597 ± 2.130
2	5.085 ± 0.237	5.185 ± 0.163	5.106 ± 0.317
3	0.483 ± 0.049	0.503 ± 0.027	0.486 ± 0.043
4	0.049 ± 0.005	0.053 ± 0.003	0.053 ± 0.007
5	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001

Table 10. The average and standard deviation of the quantities obtained when using duplicate curves were calculated to determine the quantification range for each standard.

Table 11. Quantifiler® Trio Repeatability Statistical Evaluation (Duplicate Curves)

Standard	F_{calc}		
	Small Autosomal	Large Autosomal	Male (Y)
1 (50 ng)	0.6058	0.2985	0.9727
2 (5 ng)	0.2023	6.0109	5.6723
3 (0.5 ng)	4.4163	20.8031	11.2932
4 (0.05 ng)	4.5133	21.3849	4.3647
5 (0.005 ng)	3.9944	19.9165	23.7738

Table 11. A single factor ANOVA was performed ($\alpha = 0.05$, $F_{crit} = 3.4903$) to compare the quantities obtained for each standard when using duplicate curves.

An average quantity for each standard was also obtained using the ten individual curves (Table 12). The average and standard deviation of these quantities were then used to determine the quantification range for each standard (Table 13). As shown within these tables, all standards were close to their expected values with low standard deviations. To determine if the results were statistically significant, a single factor ANOVA was performed using four randomly selected sets of standards (Table 14). When assessing the small autosomal results with an alpha value of 0.05, a statistically significant difference was observed for all standards except for the two largest quantities. This assessment for the large autosomal and male results revealed a statistically significant difference for all standards except for the largest quantity. Although the

data revealed a statistically significant difference in measurements, this did not necessarily reflect the quality of the results, as previously discussed with the data from the duplicate curves.

When comparing the results from the duplicate and individual curves, the results were similar; however, the standard deviations and F_{calc} values for the data when using individual curves were less favorable than when using duplicate curves. These findings signify standard curves should be run in duplicate during future applications to ensure the most accurate results are obtained.

Table 12. Quantifiler® Trio Repeatability Study Results (Individual Curves)

Standard Curve Series		Quantity (ng)				
		Standard 1 (50 ng)	Standard 2 (5 ng)	Standard 3 (0.5 ng)	Standard 4 (0.05 ng)	Standard 5 (0.005ng)
Small Autosomal	1	53.0701	5.6182	0.4922	0.0547	0.0070
	2	53.7590	5.0488	0.5590	0.0543	0.0055
	3	51.9832	5.0761	0.4991	0.0575	0.0055
	4	50.9475	5.0319	0.5382	0.0484	0.0055
	5	47.8692	4.8245	0.4621	0.0527	0.0047
	6	51.1654	5.2283	0.4319	0.0417	0.0040
	7	47.0280	4.9217	0.4218	0.0430	0.0037
	8	46.7010	4.6728	0.4328	0.0477	0.0041
	9	51.3647	5.0700	0.4667	0.0473	0.0057
	10	54.3529	5.3555	0.5282	0.0460	0.0078
Large Autosomal	1	44.3657	4.9911	0.4798	0.0524	0.0048
	2	48.0554	5.0148	0.4688	0.0525	0.0042
	3	47.9832	5.2276	0.4773	0.0489	0.0055
	4	48.7051	5.1145	0.5622	0.0522	0.0052
	5	48.7087	5.1505	0.5036	0.0533	0.0044
	6	48.7442	5.3156	0.4868	0.0475	0.0040
	7	47.8973	5.1592	0.4679	0.0577	0.0037
	8	47.3230	5.0821	0.5113	0.0557	0.0062
	9	48.5759	5.4797	0.5195	0.0593	0.0046
	10	49.1127	5.3170	0.5527	0.0523	0.0054
Male (Y)	1	54.5563	5.8521	0.5235	0.0689	0.0061
	2	50.2167	5.3211	0.5362	0.0533	0.0035
	3	51.3438	5.2609	0.4991	0.0534	0.0069
	4	48.7319	4.6270	0.5401	0.0477	0.0062
	5	48.2458	4.6664	0.4647	0.0456	0.0039
	6	50.7643	5.2732	0.4253	0.0429	0.0033
	7	47.7476	4.8261	0.4391	0.0578	0.0054
	8	47.4712	4.9252	0.4612	0.0605	0.0045
	9	48.2675	5.1586	0.4562	0.0513	0.0037
	10	49.0442	5.1486	0.5203	0.0540	0.0077

Table 12. Ten sets of standards were run and their quantities were determined using the individual curves.

Table 13. Quantifiler® Trio Repeatability Quantification (Individual Curves)

Standard	Quantification Range (ng)		
	Small Autosomal	Large Autosomal	Male (Y)
1	50.824 ± 2.749	47.947 ± 1.365	49.639 ± 2.159
2	5.085 ± 0.268	5.185 ± 0.152	5.106 ± 0.364
3	0.483 ± 0.048	0.503 ± 0.034	0.487 ± 0.042
4	0.049 ± 0.005	0.053 ± 0.004	0.054 ± 0.008
5	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.002

Table 13. The average and standard deviation of the quantities obtained when using individual curves were calculated to determine the quantification range for each standard.

Table 14. Quantifiler® Trio Repeatability Statistical Evaluation (Individual Curves)

Standard	F_{calc}		
	Small Autosomal	Large Autosomal	Male (Y)
1 (50 ng)	0.7017	0.3070	1.8856
2 (5 ng)	0.9502	11.7550	13.6300
3 (0.5 ng)	10.2117	30.8768	12.9508
4 (0.05 ng)	10.1871	20.3822	4.0029
5 (0.005 ng)	7.6178	18.0371	28.2417

Table 14. A single factor ANOVA was performed ($\alpha = 0.05$, $F_{crit} = 2.9011$) to compare the quantities obtained for each standard when using the ten individual curves.

Reproducibility Study

Three standard curves were run on three separate days and their average quantities were compared to determine the reproducibility of Quantifiler® Trio kit between runs. Prior to any data analysis, all slopes and R^2 values were assessed to ensure they fell within the acceptable ranges (Table 15). The average small autosomal quantification results were compared (Table 16) and a single factor ANOVA was performed. The F_{calc} values for Standard 1 (0.094), Standard 2 (0.158), Standard 3 (0.556), Standard 4 (0.428), and Standard 5 (0.109) were all less than the F_{crit} value of 5.143; therefore, no statistically significant difference was observed for the small autosomal quantification results. Based on this evaluation, the small autosomal results were determined to be reproducible. The findings of each standard were also graphically displayed below for visual comparison (Figure 1).

Table 15. Quantifiler® Trio Slope and R^2 Reproducibility

Run		Sample	Slope	Average Slope	R^2 Value	Average R^2
Small Autosomal	Run 1	Curve 1	-3.322	-3.337 ± 0.014	1.000	1.000 ± 0.000
		Curve 2	-3.339		1.000	
		Curve 3	-3.350		1.000	
	Run 2	Curve 1	-3.340	-3.346 ± 0.014	1.000	1.000 ± 0.001
		Curve 2	-3.362		0.999	
		Curve 3	-3.337		1.000	
	Run 3	Curve 1	-3.313	-3.349 ± 0.043	0.999	1.000 ± 0.001
		Curve 2	-3.338		1.000	
		Curve 3	-3.397		1.000	
Large Autosomal	Run 1	Curve 1	-3.441	-3.459 ± 0.026	1.000	1.000 ± 0.000
		Curve 2	-3.447		1.000	
		Curve 3	-3.489		1.000	
	Run 2	Curve 1	-3.430	-3.407 ± 0.061	1.000	1.000 ± 0.000
		Curve 2	-3.453		1.000	
		Curve 3	-3.338		1.000	
	Run 3	Curve 1	-3.395	-3.485 ± 0.093	1.000	1.000 ± 0.001
		Curve 2	-3.580		0.999	
		Curve 3	-3.481		1.000	
Male (Y)	Run 1	Curve 1	-3.334	-3.387 ± 0.078	0.999	0.999 ± 0.001
		Curve 2	-3.350		0.999	
		Curve 3	-3.476		1.000	
	Run 2	Curve 1	-3.433	-3.350 ± 0.094	1.000	0.998 ± 0.002
		Curve 2	-3.370		0.999	
		Curve 3	-3.248		0.996	
	Run 3	Curve 1	-3.322	-3.352 ± 0.051	0.999	0.999 ± 0.001
		Curve 2	-3.323		0.998	
		Curve 3	-3.411		1.000	

Table 15. Quantifiler® Trio slopes and R^2 values were compared for all targets between the three separate runs to ensure that the acceptance criteria were met.

Table 16. Reproducibility Study Results (Small Autosomal)

Run	Sample	Average Quant (ng/µL)	Target (ng/µL)	Std Dev.	%CV
Run 1	Standard 1	50.2666	50	2.1398	4.2569
	Standard 2	4.9775	5	0.1343	2.6982
	Standard 3	0.4998	0.5	0.0380	7.6108
	Standard 4	0.0529	0.05	0.0046	8.6501
	Standard 5	0.0053	0.005	0.0005	9.5208
Run 2	Standard 1	53.3124	50	14.6163	27.4163
	Standard 2	4.7962	5	1.4658	30.5617
	Standard 3	0.5852	0.5	0.1963	33.5544
	Standard 4	0.0516	0.05	0.0165	32.0308
	Standard 5	0.0051	0.005	0.0012	24.1073
Run 3	Standard 1	50.9289	50	5.6209	11.0368
	Standard 2	5.1993	5	0.3901	7.5023
	Standard 3	0.4896	0.5	0.0675	13.7960
	Standard 4	0.0457	0.05	0.0040	8.8343
	Standard 5	0.0054	0.005	0.0007	12.3965

Table 16. Quantifiler® Trio standards were prepared and run in triplicate on three different days. The average small autosomal quantity for each standard was determined, along with the standard deviation and CV.

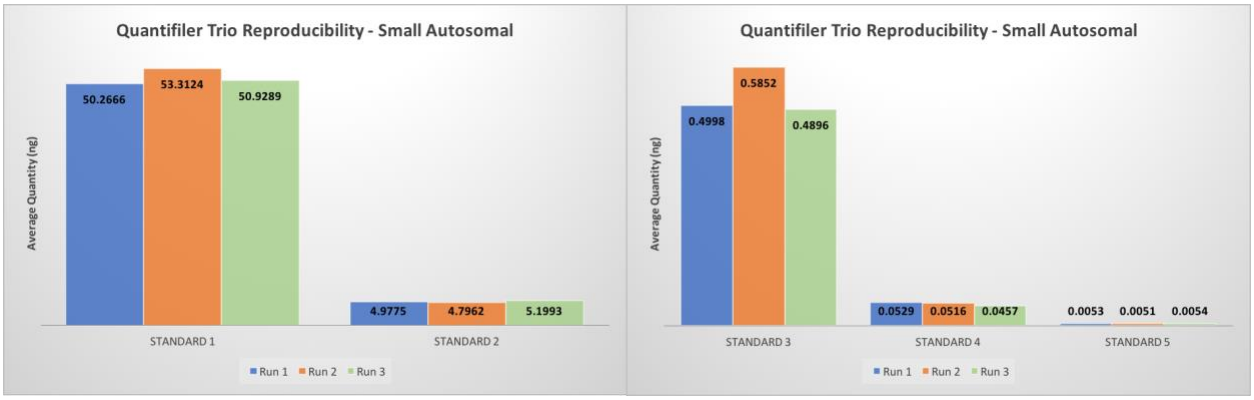


Figure 1: Quantifiler® Trio Small Autosomal Reproducibility.

The average large autosomal quantification results were compared (Table 17) and a single factor ANOVA was performed. The F_{calc} values for Standard 1 (0.352), Standard 2 (0.140), Standard 3 (0.030), Standard 4 (0.189), and Standard 5 (0.002) were all less than the F_{crit}

value of 5.143; therefore, no statistically significant difference was observed for the large autosomal quantification results. Based on this evaluation, the large autosomal results were determined to be reproducible. The findings of each standard were also graphically displayed below for visual comparison (Figure 2).

Table 17. Reproducibility Study Results (Large Autosomal)

Run	Sample	Average Quant (ng/µL)	Target (ng/µL)	Std Dev.	%CV
Run 1	Standard 1	48.4657	50	0.4178	0.8621
	Standard 2	5.1642	5	0.0578	1.1184
	Standard 3	0.5144	0.5	0.0435	8.4478
	Standard 4	0.0515	0.05	0.0023	4.3934
	Standard 5	0.0051	0.005	0.0005	10.8517
Run 2	Standard 1	51.1075	50	8.2123	16.0687
	Standard 2	5.0893	5	0.9860	19.3741
	Standard 3	0.5049	0.5	0.0667	13.2028
	Standard 4	0.0493	0.05	0.0049	9.9123
	Standard 5	0.0051	0.005	0.0006	11.2672
Run 3	Standard 1	47.4235	50	4.9693	10.4787
	Standard 2	5.3564	5	0.4909	9.1638
	Standard 3	0.5118	0.5	0.0296	5.7876
	Standard 4	0.0506	0.05	0.0050	9.9149
	Standard 5	0.0050	0.005	0.0016	31.7259

Table 17. Quantifiler® Trio standards were prepared and run in triplicate on three different days. The average large autosomal quantity for each standard was determined, along with the standard deviation and CV.

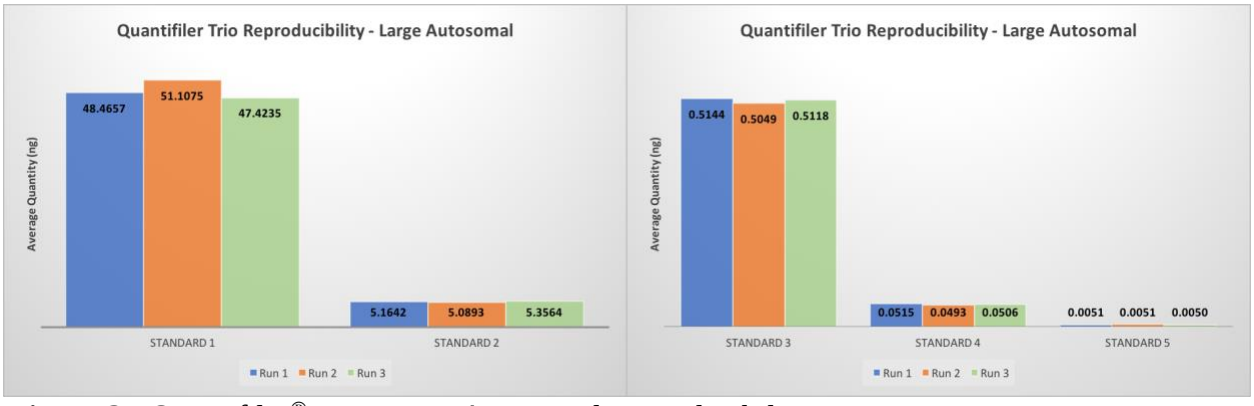


Figure 2: Quantifiler® Trio Large Autosomal Reproducibility.

The average male target quantification results were compared (Table 18) and a single factor ANOVA was performed. The F_{calc} values for Standard 1 (0.608), Standard 2 (0.172), Standard 3 (0.155), Standard 4 (0.448), and Standard 5 (0.001) were all less than the F_{crit} value of 5.143; therefore, no statistically significant difference was observed for the male quantification results. Based on this evaluation, the male results were determined to be reproducible. The findings of each standard were also graphically displayed below for visual comparison (Figure 3).

Table 18. Reproducibility Study Results (Male Target)

Run	Sample	Average Quant (ng/μL)	Target (ng/μL)	Std Dev.	%CV
Run 1	Standard 1	49.4405	50	1.6661	3.3699
	Standard 2	4.8514	5	0.3551	7.3202
	Standard 3	0.5013	0.5	0.0378	7.5336
	Standard 4	0.0489	0.05	0.0040	8.2421
	Standard 5	0.0057	0.005	0.0016	27.6394
Run 2	Standard 1	56.7186	50	14.9790	26.4094
	Standard 2	5.0792	5	1.8712	36.8404
	Standard 3	0.5182	0.5	0.1460	28.1679
	Standard 4	0.0448	0.05	0.0103	23.0137
	Standard 5	0.0056	0.005	0.0008	14.1740
Run 3	Standard 1	50.1257	50	3.4411	6.8650
	Standard 2	5.3806	5	0.2339	4.3477
	Standard 3	0.4777	0.5	0.0363	7.6085
	Standard 4	0.0444	0.05	0.0016	3.5101
	Standard 5	0.0056	0.005	0.0015	25.9520

Table 18. Quantifiler® Trio standards were prepared and run in triplicate on three different days. The average male quantity for each standard was determined, along with the standard deviation and CV.

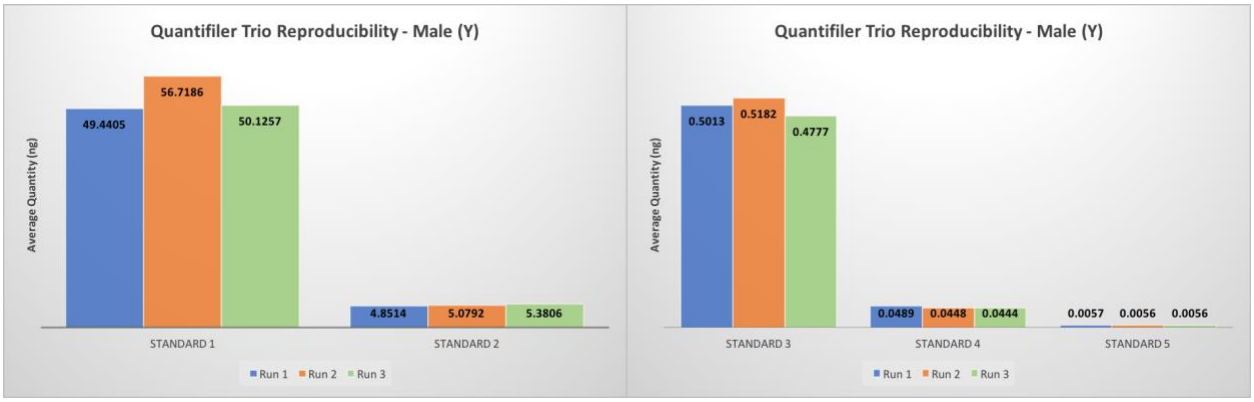


Figure 3: Quantifiler® Trio Male Target Reproducibility.

Life of Standards Study

In order to determine the shelf life of the Quantifiler® Trio standards, one set was prepared and evaluated fresh, after one week, and after two weeks. For each run, the standards were run in triplicate, and the resulting slopes and R^2 values were assessed (Table 19). As seen in Table 19 below, all R^2 values were ≥0.99 and therefore met the acceptance criteria. When assessing the slopes, all values for the small autosomal, large autosomal, and male targets fell within the acceptable range for the fresh and one-week-old standards; however, with two-week-old standards, slopes for the small autosomal and male targets were borderline while the slopes for the large autosomal target exceeded the range of acceptable values. Thus, the maximum shelf life for the Quantifiler® Trio standards was determined to be one week to ensure reliable results.

Table 19. Quantifiler® Trio Life of Standards

Run		Sample	Slope	Average Slope	R^2 Value	Average R^2
Small Autosomal	Fresh (31/05/2017)	Curve 1	-3.285	-3.274 ± 0.013	0.999	0.999 ± 0.001
		Curve 2	-3.260		1.000	
		Curve 3	-3.277		0.999	
	Week 1 (07/06/2017)	Curve 1	-3.562	-3.549 ± 0.049	0.996	0.997 ± 0.001
		Curve 2	-3.494		0.997	
		Curve 3	-3.590		0.998	
	Week 2 (14/06/2017)	Curve 1	-3.549	-3.599 ± 0.046	0.994	0.995 ± 0.001
		Curve 2	-3.609		0.995	
		Curve 3	-3.640		0.996	
Large Autosomal	Fresh (31/05/2017)	Curve 1	-3.387	-3.38 ± 0.019	0.999	0.999 ± 0.001
		Curve 2	-3.358		1.000	
		Curve 3	-3.395		0.999	
	Week 1 (07/06/2017)	Curve 1	-3.654	-3.646 ± 0.009	0.999	0.999 ± 0.001
		Curve 2	-3.637		0.999	
		Curve 3	-3.647		0.998	
	Week 2 (14/06/2017)	Curve 1	-3.800	-3.769 ± 0.040	0.997	0.996 ± 0.001
		Curve 2	-3.784		0.997	
		Curve 3	-3.724		0.995	
Male (Y)	Fresh (31/05/2017)	Curve 1	-3.333	-3.326 ± 0.017	1.000	1.000 ± 0.000
		Curve 2	-3.338		1.000	
		Curve 3	-3.306		1.000	
	Week 1 (07/06/2017)	Curve 1	-3.614	-3.525 ± 0.077	0.998	0.996 ± 0.002
		Curve 2	-3.490		0.996	
		Curve 3	-3.472		0.994	
	Week 2 (14/06/2017)	Curve 1	-3.594	-3.598 ± 0.007	0.997	0.997 ± 0.000
		Curve 2	-3.595		0.997	
		Curve 3	-3.606		0.997	

Table 19. Quantifiler® Trio standards were prepared and evaluated fresh, after one week, and after two weeks. The slopes and R^2 values were then evaluated after each run for all targets to determine if the values met the acceptance criteria.

Sensitivity Study

The two sets of dilution series prepared from the NIST SRM 2372 male DNA standard were quantified in triplicate using Quantifiler® Trio to determine if the kit could detect specified quantities of DNA. The theoretical DNA concentration and the average results for each target can be found in Table 20. When comparing the small autosomal and male quantities, the male results tended to be higher. The small autosomal quantity is typically considered when determining the amount of DNA within a sample, but the male quantity could be used for potential male:female mixtures. Considering these findings, if the male results were to be used for predicting mixtures within this lab, then quantities could be potentially overestimated.

Although the actual quantities for each dilution differed from the expected quantities, the series could still be used for subsequent validation studies because they covered the necessary DNA concentration range. This variation did not reflect the ability of Quantifiler® Trio to accurately quantify this DNA concentration range because many sources of variation and error were expected. The stock concentration of the NIST SRM 2372A standard was advertised as 57 ng/μL and was therefore initially treated as such, but variation from lot to lot could still exist. The concentration of SRM 2372A was initially determined by absorbance, while quantification uses fluorescence, so this value was not taken as absolute. In addition, pipetting error and instrument variation come into play when preparing dilution series. Furthermore, the kit was considered capable of detecting DNA quantities as low as 0.007 ng/μL (Table 20), and the results obtained were carried forward as the true values for the amplification kit studies. The true zero value for Quantifiler® Trio was subsequently determined using data from the amplification kit sensitivity studies discussed later.

Table 20. Sensitivity Series Quantification Results

Sample		Theoretical DNA (ng/μL)	Small Autosomal (ng/μL)	Large Autosomal (ng/μL)	Male (Y) (ng/μL)
Sensitivity Series 1	X	1.000	1.383	1.530	1.752
	X/2	0.500	0.608	0.757	0.788
	X/4	0.250	0.289	0.390	0.380
	X/8	0.125	0.108	0.162	0.155
	X/16	0.0625	0.054	0.087	0.077
	X/32	0.03125	0.027	0.045	0.033
	X/64	0.015625	0.013	0.023	0.017
	X/128	0.0078125	0.007	0.010	0.008
	X/256	0.00390625	0.004	0.005	0.005
Sensitivity Series 2	4X	4.000	6.491	7.495	8.720
	2X	2.000	3.114	3.615	4.109
	X	1.000	1.504	1.885	1.960
	X/2	0.500	0.690	0.949	0.932
	X/4	0.250	0.351	0.491	0.469
	X/8	0.125	0.168	0.252	0.235
	X/16	0.0625	0.088	0.123	0.117
	X/32	0.03125	0.046	0.064	0.058
	X/64	0.015625	0.023	0.031	0.033
	X/128	0.0078125	0.012	0.013	0.019
	X/256	0.00390625	0.007	0.008	0.011

Table 20. Two sensitivity series were prepared using the NIST SRM 2372 Component A (male), covering targeted concentrations of 4.0 – 0.00391 ng/μL. Each dilution was then quantified in triplicate using Quantifiler® Trio, and the results for each target were averaged.

Mixture Study

Male-to-female mixtures covering a range of 20:1 to 1:20 were prepared by adding one microliter of targeted concentrations directly into the 96-well plate. The samples were quantified in triplicate, and the average results for each target were displayed below (Table 21). The actual male:female ratios were then calculated and compared to the theoretical ratios. As seen within the table, the observed ratios when the male donor was the major contributor were not close to their expected ratios; however, the results for the ratios when the female donor was the major contributor produced more accurate observed ratios. These results, albeit inconsistent with the

expected ratios, revealed the ability of Quantifiler® Trio to detect male:female mixture samples. Furthermore, although the results were not ideal when the major contributor was male, the ability of the kit to more accurately quantify mixture samples when the major contributor was female suits the analysis of commonly encountered sexual assault samples.

Table 21. Mixture Study Quantifiler® Trio Results

Sample Name	Quantifiler Trio Results						
	Male:Female Theoretical Ratio	Large Autosomal (ng/μL)	Small Autosomal (ng/μL)	Average Autosomal (ng/μL)	Male (Y) (ng/μL)	Female (ng/μL)	Male:Female Observed Ratio
1.2	20:1	0.5258	0.3677	0.4467	0.2602	0.1865	1.4 : 1
2.2	16:1	0.5505	0.4581	0.5043	0.2871	0.2172	1.3 : 1
3.2	12:1	0.5191	0.4103	0.4647	0.2624	0.2023	1.3 : 1
4.2	8:1	0.5520	0.4328	0.4924	0.2623	0.2301	1.1 : 1
5.2	4:1	0.6323	0.4652	0.5487	0.2682	0.2805	1 : 1
6.2	2:1	0.7757	0.6528	0.7142	0.2655	0.4487	0.6 : 1
7.2	1:1	1.0335	0.8604	0.9470	0.2687	0.6783	0.4 : 1
8.2	1:2	0.7745	0.5610	0.6678	0.1345	0.5333	1 : 4
9.2	1:4	0.3571	0.2132	0.2852	0.0273	0.2579	1 : 9.4
10.2	1:8	0.4783	0.3026	0.3905	0.0236	0.3668	1 : 15.5
11.2	1:12	0.4604	0.2881	0.3743	0.0162	0.3580	1 : 22.1
12.2	1:16	0.5557	0.4495	0.5026	0.0182	0.4844	1 : 26.6
13.2	1:20	0.5736	0.4660	0.5198	0.0143	0.5055	1 : 35.3

Table 21. Male to female mixtures were prepared in various ratios within the 96-well plate in triplicate. The resulting values were averaged and displayed above.

Applied Biosystems™ 3500 Genetic Analyzer Capillary Array Evaluation

Repeatability/Precision Study

Twenty-four allelic ladders were evaluated during a single run using GlobalFiler™ to determine the repeatability and precision of the capillary array. One allele was chosen at a single locus in each dye channel and the variance in both quantity (RFU) and base pair sizing was calculated. As seen in Table 22, the capillary array produced variance in RFU less than 25% for all dyes. The ILS produced the lowest variance, which could have been attributed to pipetting since 10 μL of master mix was added to the sample wells while only 1 μL of allelic ladder was

added; there is naturally more variance when pipetting smaller volumes. In addition, all dye channels produced a standard deviation for base pair sizing well below the requirement of 0.15 bp (Table 22). The results were graphically displayed in Figure 4 to show the overall variance of the capillary array. Furthermore, the capillary array was able to produce low variance in both RFU and base pair sizing within a single run.

Table 22. Capillary Array Variance and Sizing Precision

Locus (Allele)	Array Variance (RFU)	Base Pair Sizing (Std. Dev.)
D16S539 (12)	22.6%	0.04
D18S51 (10.2)	20.8%	0.04
THO1 (9.3)	20.8%	0.05
D7S820 (6)	20.5%	0.04
D12S391 (21)	20.1%	0.05
LIZ 600 (240)	8.2%	N/A

Table 22. The variance (RFU) and sizing (base pairs) of the capillary array were evaluated using 24 allelic ladders within a single run.

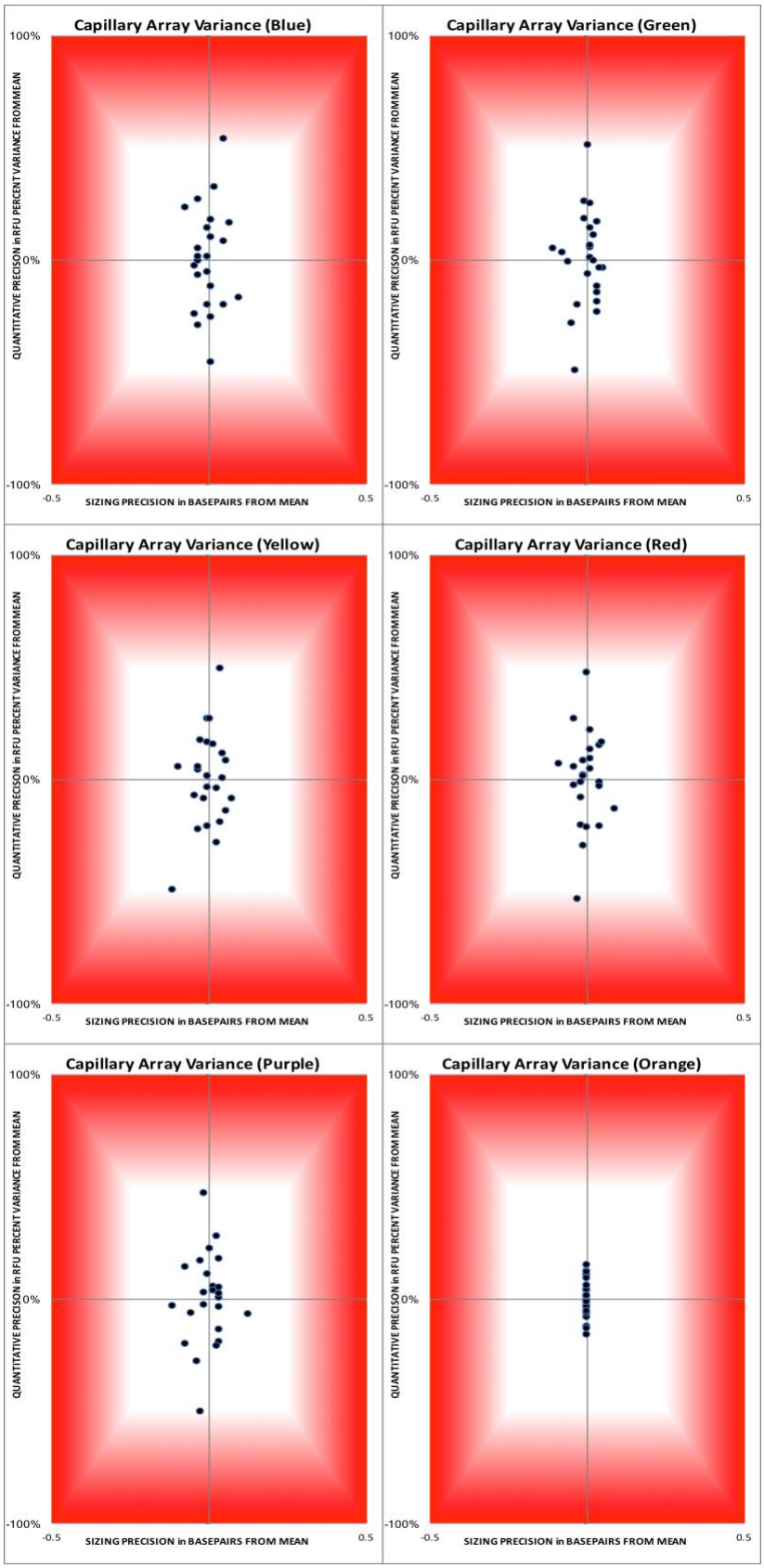


Figure 4: Capillary Array Variance and Sizing Precision Per Dye

Reproducibility Study

Three allelic ladders were randomly selected from three different runs and analyzed in the same manner as above to determine the reproducibility of the capillary array. The same loci and alleles as those chosen for the repeatability study were also evaluated for two of the three runs because GlobalFiler™ was used; however, the third data set was taken from a Yfiler™ Plus run, so different loci and alleles were chosen. As seen in Table 23, the capillary array consistently produced variance below 25%, as well as a standard deviation in base pair sizing below the 0.15 bp requirement; therefore, it was deemed reliable for producing low variance in both RFU and base pair sizing from run to run.

Table 23. Capillary Array Variance and Sizing Precision Reproducibility

Run	Locus (Allele)	Array Variance (RFU)	Base Pair Sizing (Std. Dev.)
1	D16S539 (12)	15%	0.03
	D18S51 (10.2)	13%	0.02
	THO1 (9.3)	15%	0.06
	D7S820 (6)	14%	0.04
	D12S391 (21)	14%	0.03
2	DYS635 (20)	11%	0.03
	YGATAH4 (12)	11%	0.06
	DYS438 (10)	11%	0.05
	DYS385 (23)	21%	0.04
	DYS439 (8)	17%	0.04
3	D16S539 (12)	11%	0.05
	D18S51 (10.2)	14%	0.05
	THO1 (9.3)	9%	0.01
	D7S820 (6)	15%	0.02
	D12S391(21)	13%	0.06

Table 23. The variance (RFU) and sizing (base pairs) of the capillary array were evaluated for three different runs to determine the reproducibility of the capillary array.

GlobalFiler™, GlobalFiler™ Express, and Yfiler™ Plus PCR Amplification Kits

Analytical Threshold Study

Applying Equations 1 and 2 to the results from the negative controls amplified with each kit, the analytical thresholds were calculated both per dye color and overall for GlobalFiler™ (Table 24), Yfiler™ Plus (Table 25), and GlobalFiler™ Express (Table 26). These thresholds were compared during the analysis of the samples for each amplification kit to determine if setting the analytical threshold per dye channel was more efficient than one overall value. Based on the results, it was determined that an overall threshold was more effective; this determination will be discussed in further detail with the sensitivity study results.

Table 24. GlobalFiler™ Analytical Threshold

Dye Channel	Calculated AT	AT (Per Dye)	AT (Overall)
Blue	30	30	60
Green	54	55	
Yellow	46	50	
Red	38	40	
Purple	56	60	
Orange	100	100	

Table 24. Two equations were used to calculate the analytical threshold for GlobalFiler™, and the largest value was chosen. For data analysis, the analytical threshold was then set both per dye channel and overall to determine the best analysis method for the kit.

Table 25. Yfiler™ Plus Analytical Threshold

Dye Channel	Calculated AT	AT (Per Dye)	AT (Overall)
Blue	48	50	75
Green	68	70	
Yellow	28	30	
Red	58	60	
Purple	54	55	
Orange	101	100	

Table 25. Two equations were used to calculate the analytical threshold for Yfiler™ Plus, and the largest value was chosen. Although 70 RFU was the largest value, this was rounded up to 75 because of the large standard deviation observed. For data analysis, the analytical threshold was then set both per dye channel and overall to determine the optimal analysis

Table 26. GlobalFiler™ Express Analytical Threshold

Dye Channel	Calculated AT			AT (Per Dye)	AT (Overall)
	25 Cycles	26 Cycles	27 Cycles		
Blue	32	34	38	40	55
Green	52	50	48	50	
Yellow	46	44	42	45	
Red	54	48	48	50	
Purple	48	40	38	50	
Orange	98	98	99	100	

Table 26. Two equations were used to calculate the analytical threshold for GlobalFiler™ Express at all three PCR cycles, and the largest value was chosen. For data analysis, the analytical threshold was then set both per dye channel and overall to determine the optimal analysis method for the kit.

Sensitivity Study

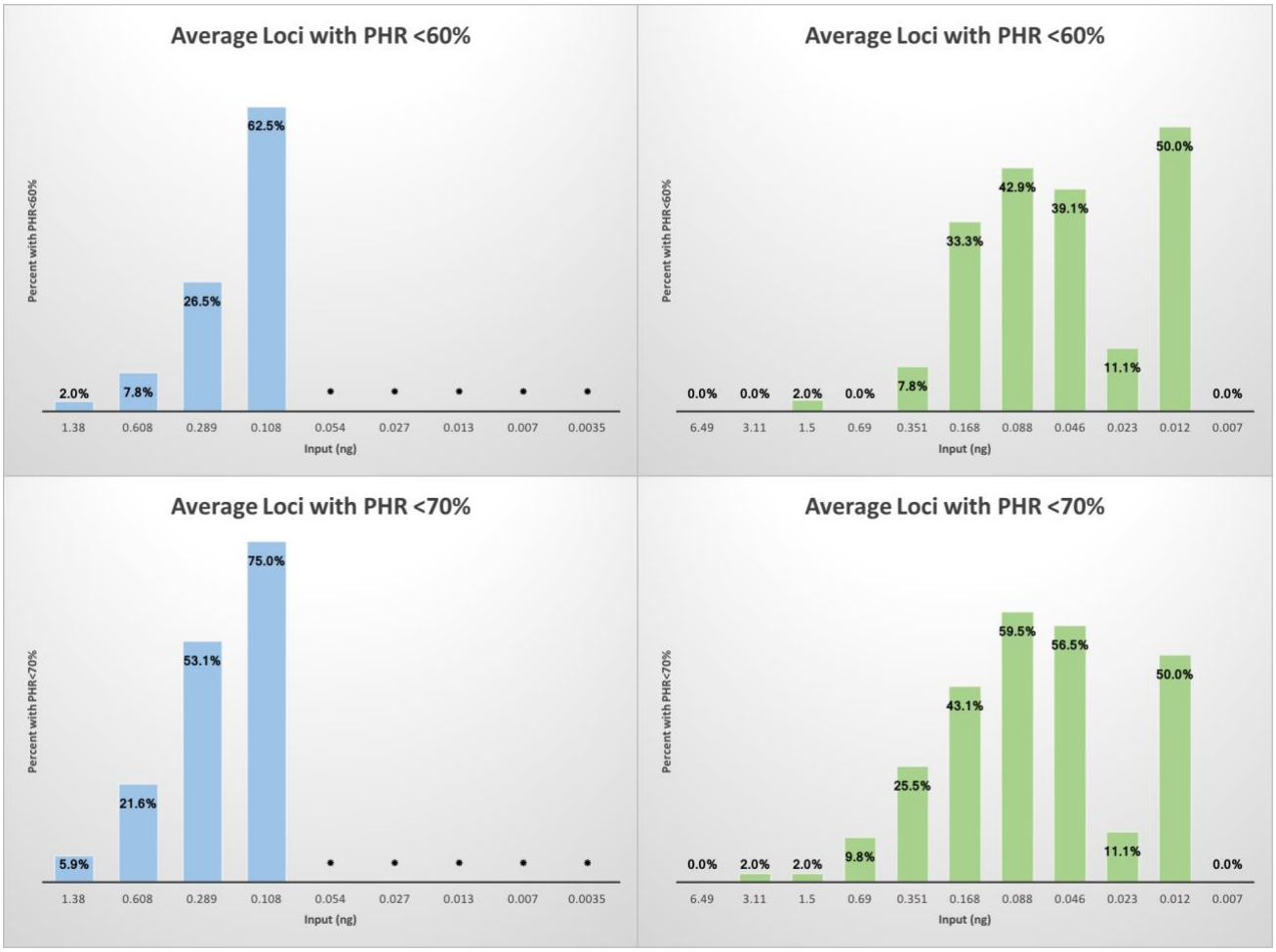
The two sensitivity series were amplified using GlobalFiler™ and evaluated on the 3500 Genetic Analyzer with an overall analytical threshold of 60 RFU; the data was also evaluated with the threshold set per dye channel, which will be discussed later. Analysis of the average percent alleles detected, average peak height, average PHR, and average CV was performed and graphically displayed below (Figure 5). Overall, the more recently prepared sensitivity series produced better results, which was expected. Although the older series was taken into consideration, the newer series was relied upon more heavily for the remainder of the study. Using the overall analytical threshold of 60 RFU, all alleles were detected down to 0.168 ng of input DNA, while 67.5% of alleles could still be detected at 0.046 ng of DNA (Figure 5). The average PHR was above 70% for DNA inputs as low as 0.168 ng (Figure 5). At 0.168 ng, less than half of the loci exhibited ratios below 60% and 70% (Figure 6). Although a PHR greater than 70% is preferred, these results were deemed acceptable as some labs accept PHRs as low as 50% for reference samples. When looking at inter-locus balance (CV), the maximum acceptable value of 0.350 was considered. All profiles for DNA input as low as 0.168 ng met this requirement (Figure 5).

In addition to the above criteria, the average number of the original CODIS core loci present within the profiles was taken into consideration (Table 27). According to SWGDAM, at least eight of the original 13 loci must be present to upload a partial DNA profile. In addition, profiles containing fewer than the 13 original loci must amount to a random match probability (RMP) of greater than one in ten million (10). DNA inputs of 6.49 – 0.088 ng generated profiles with all 13 loci. With a DNA input of 0.046 ng, ten of the 13 loci were observed and demonstrated an RMP of greater than one in ten million. With an input of 0.023 ng, eight of the 13 loci were observed and an RMP of less than one in ten million was shown.

The percentage of samples (Table 28) and average percentage of loci (Table 29) affected by artifacts were also evaluated. Samples with 6.49 – 0.351 ng of input DNA exhibited some form of stutter (Table 28), with the highest percentage of loci being affected in the 6.49 and 3.11 ng inputs (Table 29). DNA inputs of 1.50 – 0.351 ng had less than 6% of loci impacted by stutter. Baseline noise was only observed in the 6.49 ng samples (Table 28), with approximately 21% of the loci being affected; this was expected due to the overload of DNA. In addition, most of the samples with 6.49 – 1.50 ng of DNA had pull-up, while only a third of samples with 0.351, 0.168, and 0.046 ng of DNA had pull-up (Table 28). In the 1.50 – 0.046 ng samples, less than 3% of loci exhibited pull-up (Table 29). Furthermore, when considering percent alleles detected, peak height, PHR, CV, SWGDAM criteria, and artifacts, DNA inputs of 3.11 – 0.168 ng produced usable profiles. In addition, the optimal DNA input range using GlobalFiler™ was determined to be 1.5 – 0.20 ng to ensure all alleles would be detected.

*Calculations could not be performed.

Figure 5: GlobalFiler™ Sensitivity Series Results (60 RFU Threshold). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.



*Calculations could not be performed.

Figure 6. GlobalFiler™ Sensitivity Series PHR Evaluation (60 RFU Threshold). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.

Table 27. GlobalFiler™ True Zero Determination (Overall Threshold)

Sample	Theoretical [Autosomal]	Average [Small]	Average [Male]	Average Dropout	Average CODIS Core Loci	Average Random Match Probability
X	1.0000	1.3830	1.7524	0/41	13/13	*
X/2	0.5000	0.6079	0.7881	0/41	13/13	*
X/4	0.2500	0.2891	0.3800	-1/41	13/13	*
X/8	0.1250	0.1081	0.1545	-20/41	9/13	>1 in 10 million
X/16	0.0625	0.0544	0.0773	-36/41	3/13	*
X/32	0.0313	0.0270	0.0330	-40/41	1/13	*
X/64	0.0156	0.0133	0.0171	-40/41	0/13	*
X/128	0.0078	0.0072	0.0075	-40/41	0/13	*
X/256	0.0039	0.0035	0.0051	-40/41	0/13	*
4X	4.0000	6.4906	8.7201	0/41	13/13	*
2X	2.0000	3.1141	4.1089	0/41	13/13	*
X	1.0000	1.5042	1.9597	0/41	13/13	*
X/2	0.5000	0.6900	0.9321	0/41	13/13	*
X/4	0.2500	0.3509	0.4688	0/41	13/13	*
X/8	0.1250	0.1678	0.2350	0/41	13/13	*
X/16	0.0625	0.0878	0.1167	-3/41	13/13	*
X/32	0.0313	0.0463	0.0581	-13/41	10/13	>1 in 10 million
X/64	0.0156	0.0227	0.0331	-25/41	8/13	<1 in 10 million
X/128	0.0078	0.0120	0.0193	-34/41	4/13	*
X/256	0.0039	0.0069	0.0109	-38/41	1/13	*

* RMP not calculated

Table 27. Both sensitivity series prepared for the true zero determination were quantified in triplicate; the average results are displayed above. The electropherograms for each DNA input were then evaluated (using an analytical threshold of 60 RFU across all dye channels) for average allelic dropout and average core CODIS loci present. Of the samples displaying 8-12 core CODIS loci, the average random match probability was calculated using the Caucasian allele frequencies to determine if NDIS upload guidelines for partial profiles were met.

Table 28. Percent Samples Exhibiting Artifacts using GlobalFiler™ (Overall Threshold)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	100%	*	100%	*	*
4 - 6	0.6079	*	33%	*	33%	*	*
7 - 9	0.2891	*	*	*	*	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	33%	*
28 - 30	6.4906	*	100%	*	100%	100%	100%
31 - 33	3.1141	*	100%	*	100%	*	100%
34 - 36	1.5042	*	100%	*	100%	*	67%
37 - 39	0.6900	33%	33%	*	33%	*	*
40 - 42	0.3509	33%	67%	33%	100%	*	33%
43 - 45	0.1678	*	*	*	*	*	33%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	33%
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 28. The percentage of samples at each DNA input exhibiting artifacts was calculated (n = 3).

Table 29. Average Percent Loci with Artifacts using GlobalFiler™ (Overall Threshold)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	5.56%	*	5.56%	*	*
4 - 6	0.6079	*	1.39%	*	1.39%	*	*
7 - 9	0.2891	*	*	*	*	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	1.39%	*
28 - 30	6.4906	*	31.94%	*	31.94%	20.83%	63.89%
31 - 33	3.1141	*	13.89%	*	13.89%	*	25%
34 - 36	1.5042	*	1.39%	*	1.39%	*	2.78%
37 - 39	0.6900	1.39%	1.39%	*	2.78%	*	*
40 - 42	0.3509	1.39%	2.78%	1.39%	5.56%	*	1.39%
43 - 45	0.1678	*	*	*	*	*	1.39%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	1.39%
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 29. The percentage of loci at each DNA input exhibiting artifacts was calculated (n = 24).

The same GlobalFiler™ data was also evaluated with the analytical threshold set per dye channel to determine the optimal setting. Analysis of the average percent alleles detected, average peak height, average PHR, and average CV was performed and graphically displayed below (Figure 7). As previously mentioned, the newer series was relied upon more heavily for the remainder of the study. Using the analytical thresholds per dye, all alleles were detected down to 0.168 ng of input DNA, while 78% of alleles could still be detected at 0.046 ng of DNA (Figure 7). The average PHR was above 70% for DNA inputs as low as 0.168 ng (Figure 7). At

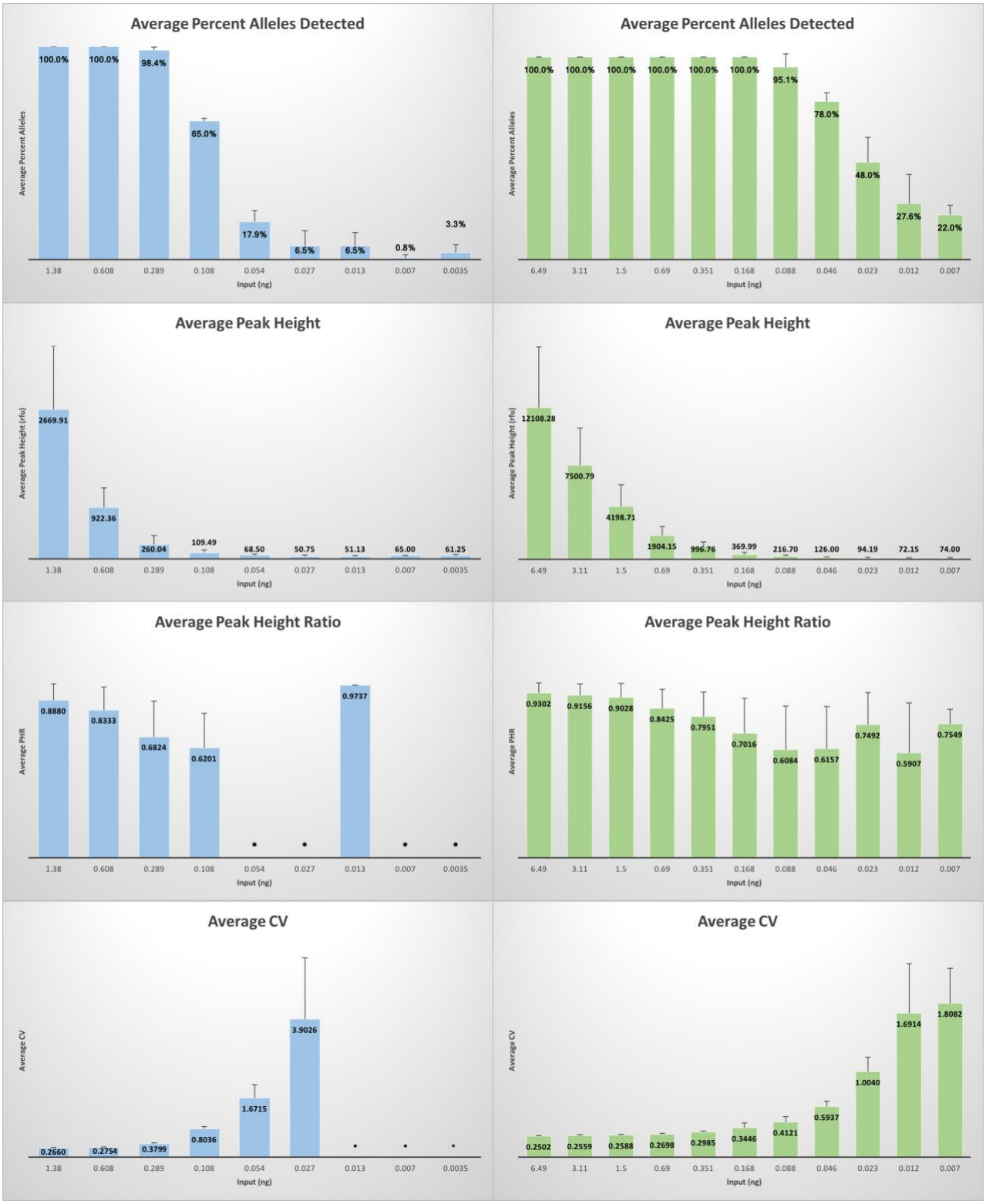
0.168 ng, less than half of the loci exhibited ratios below 60% and 70% (Figure 8). Although a PHR greater than 70% is preferred, these results were deemed acceptable as some labs accept PHRs as low as 50% for reference samples. When looking at inter-locus balance (CV), all profiles for DNA input as low as 0.168 ng met the requirement of ≤0.350 (Figure 7).

In addition to the above criteria, the average number of the original CODIS core loci present within the profiles was taken into consideration (Table 30). According to SWGDAM, at least eight of the original 13 loci must be present to upload a partial DNA profile. In addition, profiles containing fewer than the 13 original loci must amount to a random match probability (RMP) of greater than one in ten million (10). DNA inputs of 6.49 – 0.088 ng generated profiles with all 13 loci. With a DNA input of 0.046 ng, 12 of the 13 loci were observed and demonstrated an RMP of greater than one in ten million. With an input of 0.023 ng, 11 of the 13 loci were observed and an RMP of greater than one in ten million was shown. When setting a separate analytical threshold per dye color, an input of 0.012 ng represented the point at which the SWGDAM requirements could no longer be met.

The percentage of samples (Table 31) and average percentage of loci (Table 32) affected by artifacts were also evaluated. Samples with 6.49 – 0.168 ng of input DNA exhibited some form of stutter (Table 31), with the highest percentage of loci being affected in the 6.49 and 3.11 ng inputs (Table 32). DNA inputs of 1.50 – 0.351 ng had less than 7% of loci impacted by stutter. Baseline noise was observed in the 6.49 – 1.50 ng samples (Table 31), which was more than previously observed with the overall threshold of 60 RFU. In addition, most of the samples with 6.49 – 1.50 ng of DNA had pull-up, while only a third of samples with 0.690, 0.351, 0.168, and 0.046 ng of DNA had pull-up (Table 31). In the 0.690 – 0.046 ng samples, less than 2% of loci exhibited pull-up (Table 32). In addition to the artifacts listed in Tables 31-32, allelic drop-

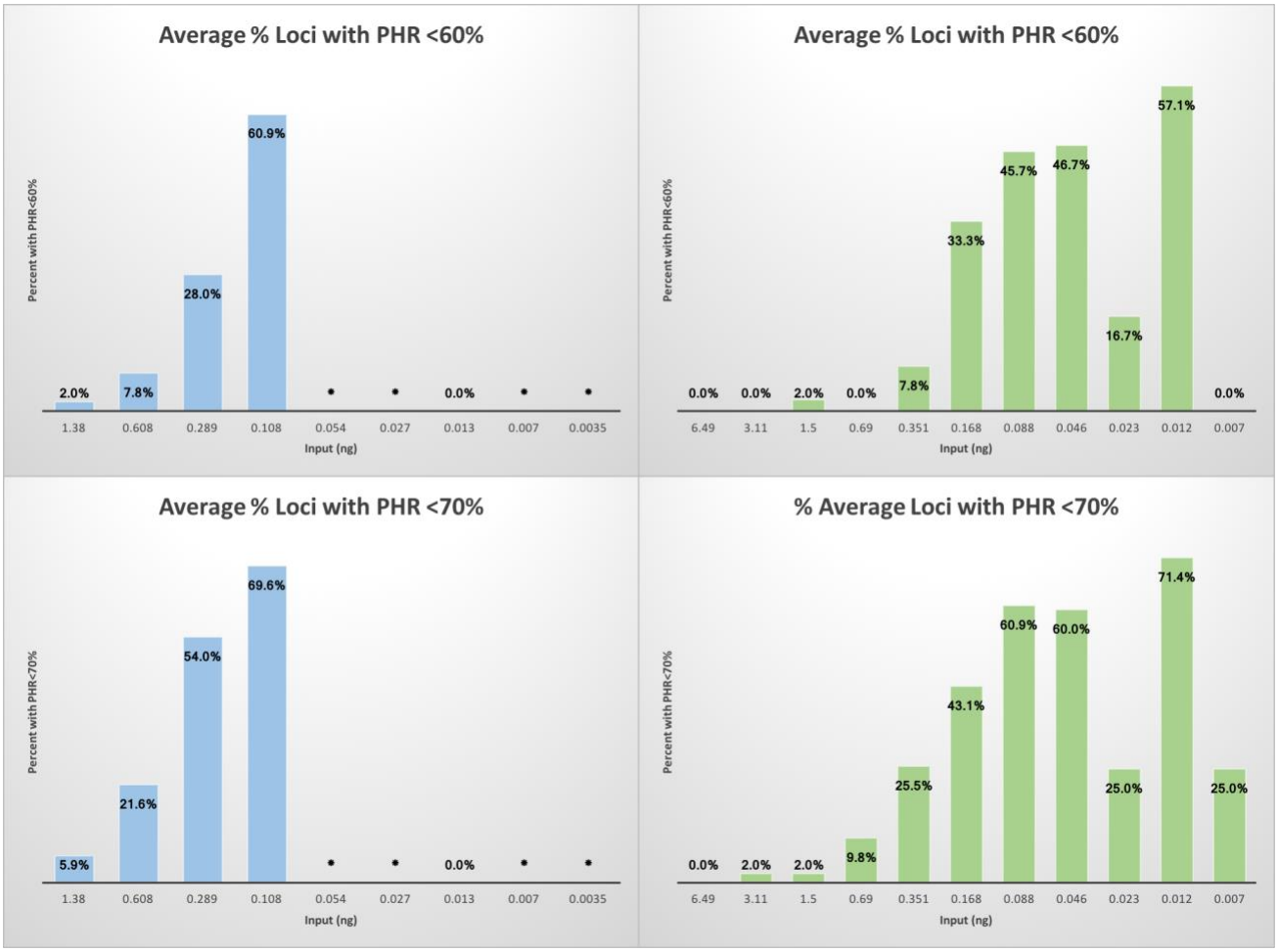
in was observed in one third of the 0.046 and 0.023 ng samples and only 1.39% of loci; this artifact was expected in such low template samples (6).

Furthermore, when considering percent alleles detected, peak height, PHR, CV, SWGDAM criteria, and artifacts, DNA inputs of 3.11 – 0.168 ng produced usable profiles. In addition, the optimal DNA input range using GlobalFiler™ was determined to be 1.5 – 0.20 ng to ensure all alleles would be detected. When comparing these results to those obtained from using an overall threshold, the only major differences were in the percentage of alleles detected and the number of artifacts observed; the same input range for usable profiles and optimal results was concluded. Thus, it was deemed more time-consuming than beneficial to set the analytical threshold per dye channel.



*Could not be calculated.

Figure 7: GlobalFiler™ Sensitivity Series Results (Threshold Per Dye). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.



*Calculations could not be performed.

Figure 8: GlobalFiler™ Sensitivity Series PHR Evaluation (Threshold Per Dye). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.

Table 30. GlobalFiler™ True Zero Determination (Threshold Per Dye)

Sample	Theoretical [Autosomal]	Average [Small]	Average [Male]	Average Dropout	Average CODIS Core Loci	Average Random Match Probability
X	1.0000	1.3830	1.7524	0/41	13/13	*
X/2	0.5000	0.6079	0.7881	0/41	13/13	*
X/4	0.2500	0.2891	0.3800	-1/41	13/13	*
X/8	0.1250	0.1081	0.1545	-14/41	12/13	>1 in 10 million
X/16	0.0625	0.0544	0.0773	-34/41	5/13	*
X/32	0.0313	0.0270	0.0330	-38/41	3/13	*
X/64	0.0156	0.0133	0.0171	-38/41	2/13	*
X/128	0.0078	0.0072	0.0075	-41/41	0/13	*
X/256	0.0039	0.0035	0.0051	-40/41	1/13	*
4X	4.0000	6.4906	8.7201	0/41	13/13	*
2X	2.0000	3.1141	4.1089	0/41	13/13	*
X	1.0000	1.5042	1.9597	0/41	13/13	*
X/2	0.5000	0.6900	0.9321	0/41	13/13	*
X/4	0.2500	0.3509	0.4688	0/41	13/13	*
X/8	0.1250	0.1678	0.2350	0/41	13/13	*
X/16	0.0625	0.0878	0.1167	-2/41	13/13	*
X/32	0.0313	0.0463	0.0581	-9/41	12/13	>1 in 10 million
X/64	0.0156	0.0227	0.0331	-21/41	11/13	>1 in 10 million
X/128	0.0078	0.0120	0.0193	-29/41	7/13	*
X/256	0.0039	0.0069	0.0109	-32/41	5/13	*

* RMP not calculated

Table 30. Both sensitivity series prepared for the true zero determination were quantified in triplicate; the average results are displayed above. The electropherograms for each DNA input were then evaluated (using analytical thresholds per dye channel) for average allelic dropout and average core CODIS loci present. Of the samples displaying 8-12 core CODIS loci, the average random match probability was calculated using the Caucasian allele frequencies to determine if NDIS upload guidelines for partial profiles were met.

Table 31. Percent Samples Exhibiting Artifacts using GlobalFiler™ (Threshold Per Dye)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	33%	100%	*	100%	*	67%
4 - 6	0.6079	33%	67%	*	67%	*	*
7 - 9	0.2891	*	*	*	*	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	100%	*	100%	100%	100%
31 - 33	3.1141	*	100%	*	100%	67%	100%
34 - 36	1.5042	*	100%	*	100%	33%	100%
37 - 39	0.6900	33%	67%	*	67%	*	33%
40 - 42	0.3509	67%	100%	33%	100%	*	33%
43 - 45	0.1678	*	33%	*	33%	*	33%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	33%
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 31. The percentage of samples at each DNA input exhibiting artifacts was calculated (n = 3).

Table 32. Average Percent Loci with Artifacts using GlobalFiler™ (Threshold Per Dye)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	1.39%	6.94%	*	8.33%	*	2.78%
4 - 6	0.6079	1.39%	2.78%	*	4.17%	*	*
7 - 9	0.2891	*	*	*	*	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	47.22%	*	47.22%	27.78%	75.00%
31 - 33	3.1141	*	23.61%	*	23.61%	4.17%	55.60%
34 - 36	1.5042	*	6.94%	*	6.94%	1.39%	13.89%
37 - 39	0.6900	1.39%	4.17%	*	5.56%	*	1.39%
40 - 42	0.3509	2.78%	4.17%	1.39%	5.56%	*	1.39%
43 - 45	0.1678	*	1.39%	*	1.39%	*	1.39%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	1.39%
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

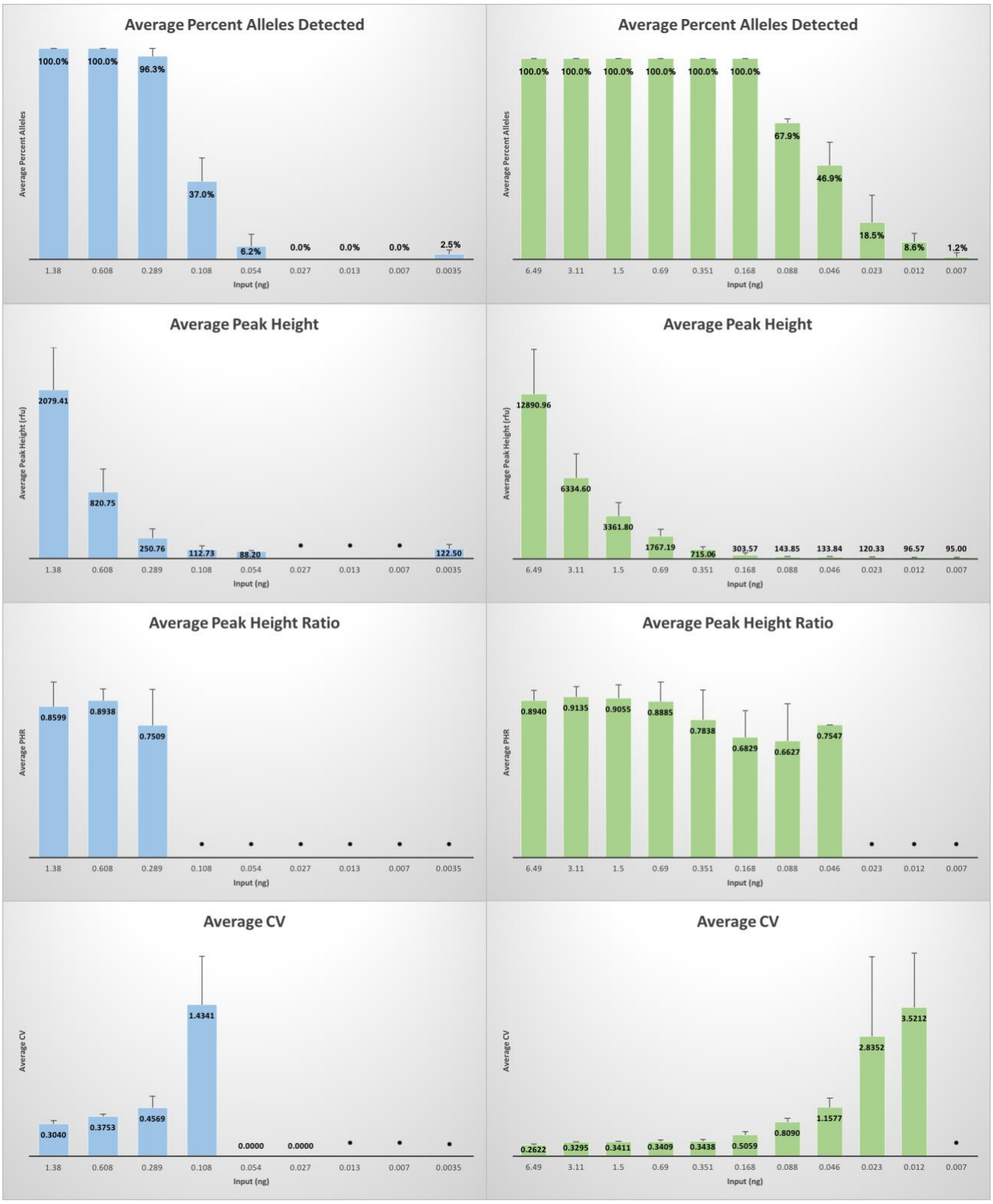
Table 32. The percentage of loci at each DNA input exhibiting artifacts was calculated (n = 24).

The two sensitivity series were also amplified using Yfiler™ Plus and evaluated on the 3500 Genetic Analyzer with an overall analytical threshold of 75 RFU; the data was also evaluated with the threshold set per dye channel, which will be discussed later. Analysis of the average percent alleles detected, average peak height, average PHR, and average CV was performed and graphically displayed below (Figure 9). The more recently prepared sensitivity series still produced better results, so it was relied upon more heavily. Using the overall analytical threshold of 75 RFU, all alleles were detected down to 0.168 ng of input DNA, while 67.9% of alleles could still be detected at 0.088 ng of DNA (Figure 9); these findings were similar to those from the developmental validation study (15). The average PHR was above 70% for DNA inputs as low as 0.351 ng (Figure 9). At 0.351 ng, less than 20% of the loci exhibited PHRs below 60% and 70% (Figure 10). Although PHR was considered, this was not as crucial for the validation of Yfiler™ Plus since it contains only two possible heterozygous loci. When looking at inter-locus balance (CV), the maximum acceptable value of 0.350 was considered. All profiles for DNA input as low as 0.351 ng met this requirement (Figure 9).

In addition to the above criteria, the average number of the SWGDAM recommended markers within the profiles was taken into consideration (Table 33). Since Yfiler™ Plus will be used as a secondary test, the presence of all 11 SWGDAM markers was the minimum requirement for a usable profile. DNA inputs of 6.49 – 0.168 ng generated profiles with all 11 markers.

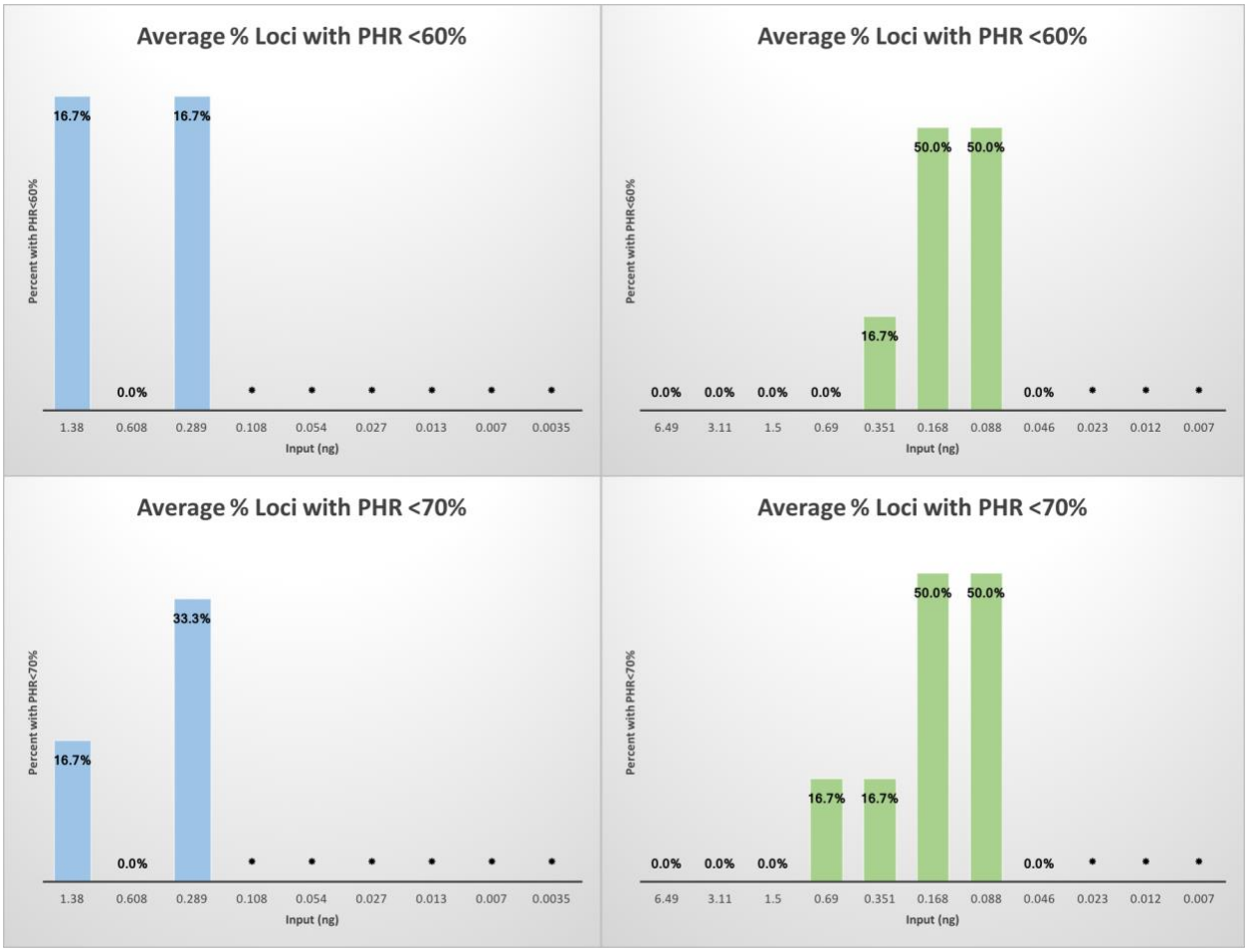
The percentage of samples (Table 34) and average percentage of loci (Table 35) affected by artifacts were also evaluated. Only the samples with 6.49 ng of input DNA exhibited stutter (Table 34), with only 1.33% of loci affected (Table 35). Only the 6.49 ng samples presented nontemplate addition (-A); all of the replicates exhibited -A, with only 9% of loci being affected.

Baseline noise was only observed in the 6.49 ng and 3.11 ng samples (Table 34), with approximately 15% of the loci being affected in the 6.49 ng samples; this was expected due to the overload of DNA. In addition, all of the samples with 6.49 – 1.50 ng of DNA had pull-up, while only a third of samples with 0.690 ng and 0.351 ng of DNA had pull-up (Table 34). In the 3.11 – 0.351 ng samples, less than 15% of loci exhibited pull-up (Table 35). Furthermore, when considering percent alleles detected, peak height, PHR, CV, SWGDAM markers, and artifacts, DNA inputs of 6.49 – 0.168 ng produced usable profiles. In addition, the optimal DNA input range using Yfiler™ Plus was determined to be 1.5 – 0.35 ng to ensure all alleles would be detected and profiles would be balanced.



*Calculations could not be performed.

Figure 9: Yfiler™ Plus Sensitivity Series PHR Evaluation (75 RFU). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.



*Calculations could not be performed.

Figure 10: Yfiler™ Sensitivity Series PHR Evaluation (75 RFU). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.

Table 33. Yfiler™ Plus True Zero Determination (Overall Threshold)

Sample	Theoretical [Autosomal]	Average [Small]	Average [Male]	Average Dropout	Average SWGDAM Markers
X	1.0000	1.3830	1.7524	0/27	11/11
X/2	0.5000	0.6079	0.7881	0/27	11/11
X/4	0.2500	0.2891	0.3800	-1/27	11/11
X/8	0.1250	0.1081	0.1545	-17/27	4/11
X/16	0.0625	0.0544	0.0773	-25/27	0/11
X/32	0.0313	0.0270	0.0330	-27/27	0/11
X/64	0.0156	0.0133	0.0171	-27/27	0/11
X/128	0.0078	0.0072	0.0075	-27/27	0/11
X/256	0.0039	0.0035	0.0051	-26/27	1/11
4X	4.0000	6.4906	8.7201	0/27	11/11
2X	2.0000	3.1141	4.1089	0/27	11/11
X	1.0000	1.5042	1.9597	0/27	11/11
X/2	0.5000	0.6900	0.9321	0/27	11/11
X/4	0.2500	0.3509	0.4688	0/27	11/11
X/8	0.1250	0.1678	0.2350	0/27	11/11
X/16	0.0625	0.0878	0.1167	-9/27	7/11
X/32	0.0313	0.0463	0.0581	-14/27	4/11
X/64	0.0156	0.0227	0.0331	-22/27	1/11
X/128	0.0078	0.0120	0.0193	-25/27	2/11
X/256	0.0039	0.0069	0.0109	-27/27	0/11

Table 33. Both sensitivity series were also used for the determination of the true zero value using Yfiler™ Plus. The electropherograms for each DNA input were evaluated (using an analytical threshold of 75 RFU across all dye channels) for average allelic dropout and average SWGDAM recommended markers present.

Table 34. Percent Samples Exhibiting Artifacts using Yfiler™ Plus (Overall Threshold)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	33%	*	33%	*	*
4 - 6	0.6079	33%	33%	*	67%	*	*
7 - 9	0.2891	33%	*	*	33%	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	33%	*	33%	100%	100%
31 - 33	3.1141	*	*	*	*	33%	100%
34 - 36	1.5042	*	*	*	*	*	100%
37 - 39	0.6900	*	*	*	*	*	33%
40 - 42	0.3509	*	*	*	*	*	33%
43 - 45	0.1678	*	*	*	*	*	*
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	*
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 34. The percentage of samples at each DNA input exhibiting artifacts was calculated (n = 3).

Table 35. Average Percent Loci with Artifacts using Yfiler™ Plus (Overall Threshold)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	1.33%	*	1.33%	*	*
4 - 6	0.6079	1.33%	1.33%	*	1.33%	*	*
7 - 9	0.2891	1.33%	*	*	*	*	*
10 - 12	0.1081	*	*	*	*	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	1.33%	*	1.33%	14.67%	41.33%
31 - 33	3.1141	*	*	*	*	1.33%	12.00%
34 - 36	1.5042	*	*	*	*	*	4.00%
37 - 39	0.6900	*	*	*	*	*	1.33%
40 - 42	0.3509	*	*	*	*	*	1.33%
43 - 45	0.1678	*	*	*	*	*	*
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	*
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 35. The percentage of loci at each DNA input exhibiting artifacts was calculated (n = 25).

After assessing the Yfiler™ Plus data using an overall analytical threshold (above), analysis was also performed with the analytical threshold set per dye channel. Analysis of the average percent alleles detected, average peak height, average PHR, and average CV was performed and graphically displayed below (Figure 11). As previously mentioned, the newer series was relied upon more heavily. Using the analytical thresholds per dye, all alleles were detected down to 0.168 ng of input DNA, while 67.9% of alleles could still be detected at 0.046 ng of DNA (Figure 11). The average PHR was above 70% for DNA inputs as low as 0.351 ng (Figure 11). At 0.351 ng, less than 20% of the loci exhibited PHRs below 60% and 70% (Figure

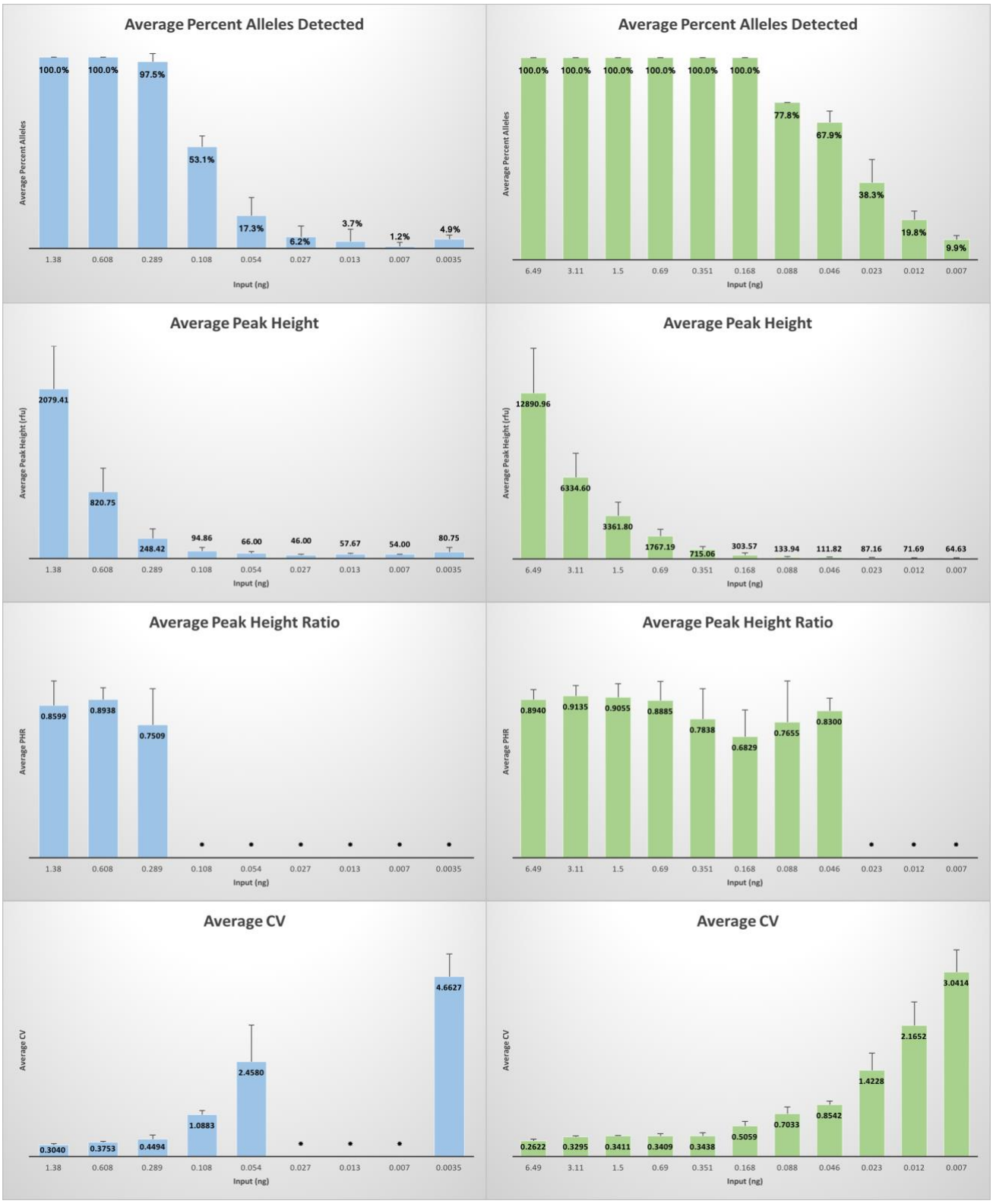
12). Although PHR was considered, this was not as crucial for the validation of Yfiler™ Plus because it contains only two possible heterozygous loci. When looking at inter-locus balance (CV), the maximum acceptable value of 0.350 was considered. All profiles for DNA input as low as 0.351 ng met this requirement (Figure 11).

In addition to the above criteria, the average number of the SWGDAM recommended markers within the profiles was taken into consideration (Table 36). Since Yfiler™ Plus will be used as a secondary test, the presence of all 11 SWGDAM markers was the minimum requirement for a usable profile. As with the data from the overall analytical threshold of 75 RFU, DNA inputs of 6.49 – 0.168 ng generated profiles with all 11 markers.

The percentage of samples (Table 37) and average percentage of loci (Table 38) affected by artifacts were also evaluated. The samples with 6.49 ng and 0.168 ng of input DNA exhibited stutter (Table 37), with only 1.33% of loci being affected (Table 38). The 6.49 ng and 1.50 ng samples were the only ones to have nontemplate addition (-A); all of the 6.49 ng samples and one-third of the 1.50 ng samples exhibited -A, with only 9.33% and 1.33% of loci being affected, respectively. Baseline noise was only observed in the 6.49 – 1.50 ng samples (Table 37), with approximately 22% of the loci being affected in the 6.49 ng samples; this was expected due to the overload of DNA. In the 3.11 ng and 1.50 ng samples, less than 10% of the loci were affected by baseline noise. In addition, all of the samples with 6.49 – 0.69 ng of DNA had pull-up, while only one-third of samples with 0.690 ng and two-thirds of samples with 0.351 ng of DNA had pull-up (Table 37). In the 3.11 – 0.168 ng samples, less than 18% of loci exhibited pull-up (Table 38).

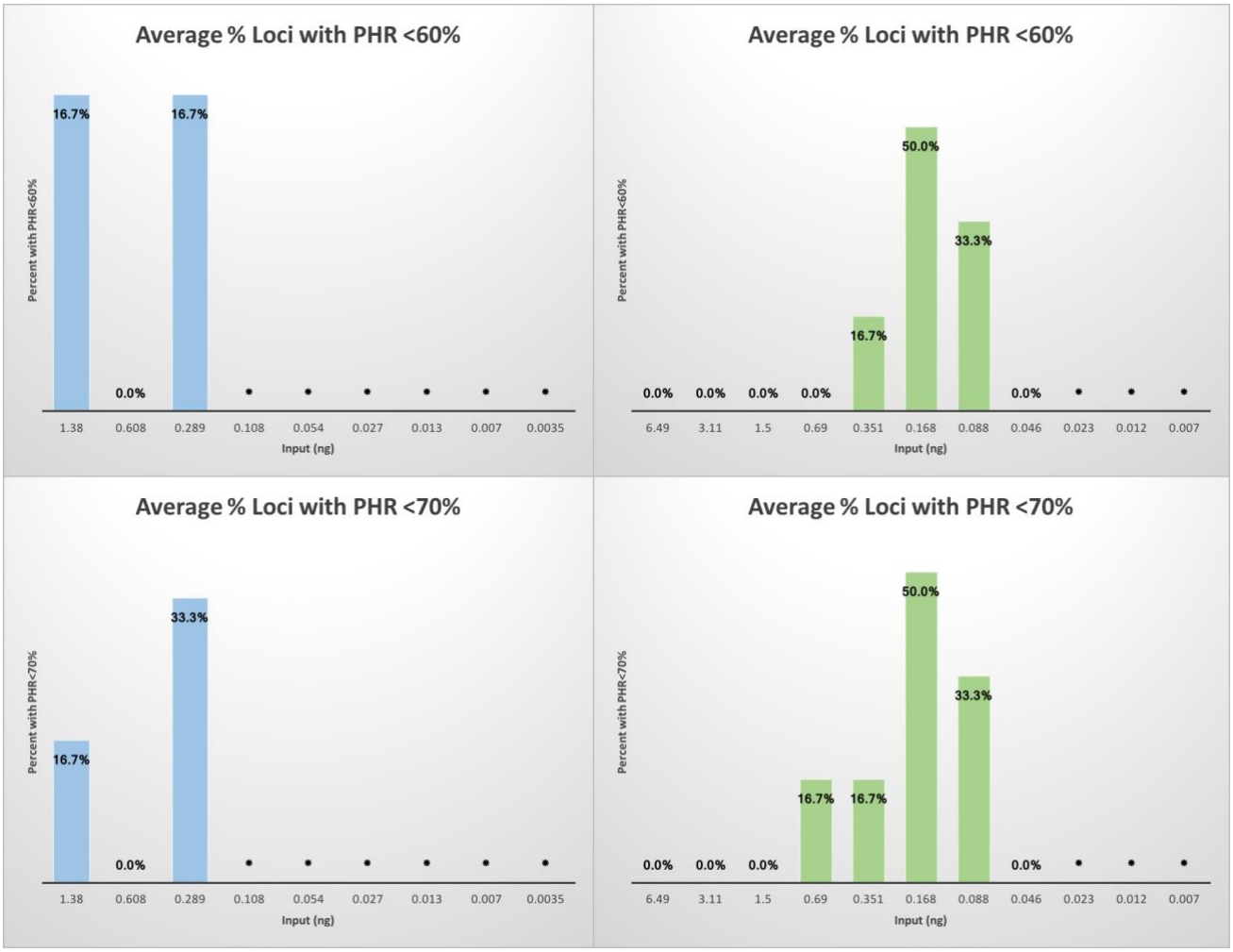
Furthermore, when considering percent alleles detected, peak height, PHR, CV, SWGDAM markers, and artifacts, DNA inputs of 6.49 – 0.168 ng produced usable profiles. In

addition, the optimal DNA input range using Yfiler™ Plus was determined to be 1.5 – 0.35 ng to ensure all alleles would be detected and profiles would be balanced. When comparing these results to those obtained from using an overall threshold, the only major differences were in the percentage of alleles detected and the number of artifacts observed; the same input range for usable profiles and optimal results was concluded. Thus, it was deemed more time-consuming than beneficial to set the analytical threshold per dye channel.



*Calculations could not be performed.

Figure 11: Yfiler™ Plus Sensitivity Series PHR Evaluation (Threshold Per Dye). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.



*Calculations could not be performed.

Figure 12: Yfiler™ Sensitivity Series PHR Evaluation (Threshold Per Dye). To the left (blue) are the results for the first sensitivity series and to the right (green) are the results for the second series.

Table 36. Yfiler™ Plus True Zero Determination (Threshold Per Dye)

Sample	Theoretical [Autosomal]	Average [Small]	Average [Male]	Average Dropout	Average SWGDAM Markers
X	1.0000	1.3830	1.7524	0/27	11/11
X/2	0.5000	0.6079	0.7881	0/27	11/11
X/4	0.2500	0.2891	0.3800	-1/27	11/11
X/8	0.1250	0.1081	0.1545	-13/27	6/11
X/16	0.0625	0.0544	0.0773	-22/27	2/11
X/32	0.0313	0.0270	0.0330	-25/27	0/11
X/64	0.0156	0.0133	0.0171	-26/27	0/11
X/128	0.0078	0.0072	0.0075	-27/27	0/11
X/256	0.0039	0.0035	0.0051	-26/27	1/11
4X	4.0000	6.4906	8.7201	0/27	11/11
2X	2.0000	3.1141	4.1089	0/27	11/11
X	1.0000	1.5042	1.9597	0/27	11/11
X/2	0.5000	0.6900	0.9321	0/27	11/11
X/4	0.2500	0.3509	0.4688	0/27	11/11
X/8	0.1250	0.1678	0.2350	0/27	11/11
X/16	0.0625	0.0878	0.1167	-6/27	8/11
X/32	0.0313	0.0463	0.0581	-9/27	6/11
X/64	0.0156	0.0227	0.0331	-17/27	4/11
X/128	0.0078	0.0120	0.0193	-22/27	3/11
X/256	0.0039	0.0069	0.0109	-24/27	2/11

Table 36. Both sensitivity series were also used for the determination of the true zero value with Yfiler™ Plus. The electropherograms for each DNA input were evaluated (using analytical thresholds per dye channel) for average allelic dropout and average SWGDAM recommended markers present.

Table 37. Percent Samples Exhibiting Artifacts using Yfiler™ Plus (Threshold Per Dye)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	33%	*	33%	*	100%
4 - 6	0.6079	33%	*	*	33%	*	33%
7 - 9	0.2891	67%	*	*	67%	*	*
10 - 12	0.1081	33%	*	*	33%	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	33%	*	33%	100%	100%
31 - 33	3.1141	*	*	*	*	100%	100%
34 - 36	1.5042	*	*	*	*	67%	100%
37 - 39	0.6900	*	*	*	*	*	100%
40 - 42	0.3509	*	*	*	*	*	33%
43 - 45	0.1678	*	33%	*	33%	*	67%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	*
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 37. The percentage of samples at each DNA input exhibiting artifacts was calculated (n = 3).

Table 38. Average Percent Loci with Artifacts using Yfiler™ Plus (Threshold Per Dye)

Sample	DNA Input (ng)	Stutter (N-4)	Stutter (N+4)	Stutter (N-2)	Stutter (Total)	Baseline Noise	Pull-up
1 - 3	1.3830	*	1.33%	*	1.33%	*	4.00%
4 - 6	0.6079	1.33%	*	*	1.33%	*	1.33%
7 - 9	0.2891	4.00%	*	*	4.00%	*	*
10 - 12	0.1081	1.33%	*	*	1.33%	*	*
13 - 15	0.0544	*	*	*	*	*	*
16 - 18	0.0270	*	*	*	*	*	*
19 - 21	0.0133	*	*	*	*	*	*
22 - 24	0.0072	*	*	*	*	*	*
25 - 27	0.0035	*	*	*	*	*	*
28 - 30	6.4906	*	1.33%	*	1.33%	21.33%	65.33%
31 - 33	3.1141	*	*	*	*	9.33%	17.33%
34 - 36	1.5042	*	*	*	*	2.67%	4.00%
37 - 39	0.6900	*	*	*	*	*	4.00%
40 - 42	0.3509	*	*	*	*	*	1.33%
43 - 45	0.1678	*	1.33%	*	1.33%	*	2.67%
46 - 48	0.0878	*	*	*	*	*	*
49 - 51	0.0463	*	*	*	*	*	*
52 - 54	0.0227	*	*	*	*	*	*
55 - 57	0.0120	*	*	*	*	*	*
58 - 60	0.0069	*	*	*	*	*	*

*No artifacts

Table 38. The percentage of loci at each DNA input exhibiting artifacts was calculated (n = 25).

GlobalFiler™ Express PCR Parameters Evaluation

The 26 buccal swabs were amplified with 25, 26, and 27 PCR cycles and analyzed on the 3500 Genetic Analyzer using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 with a 10% global cut-off filter. The global cut-off filter was implemented since only reference samples would be used with this kit. Analysis of the average percent alleles detected, average peak height, average PHR, and average CV was performed and graphically displayed below (Figure 13). All alleles were detected in every sample, which was expected. The average peak height increased with an increase in PCR cycles, which was also expected. According to the GlobalFiler™ Express user guide, the optimum PCR cycle number should generate profiles with peak heights of 3,000 – 12,000 RFU on the 3500 Genetic Analyzer (13); therefore, the use of 26 and 27 cycles met this requirement for all buccal samples (Figure 13). All PCR cycles produced profiles with average PHRs of 89-91% and fewer than 3% of loci exhibiting PHRs less than 70% (Figure 13-14). No profiles had loci with PHRs less than 60%, and no allelic dropout was observed. As the number of PCR cycles increased, the average CV decreased, but all were less than the maximum acceptable value of 0.350 (Figure 13).

The percentage of samples and average percentage of loci affected by artifacts were also evaluated (Table 39). As expected, the samples amplified with 25 PCR cycles exhibited the least artifacts and the fewest average loci affected (Table 39). In addition, the 27 PCR cycles produced the most artifacts and most average loci affected (Table 39). Half of the buccal samples were affected by pull-up when amplified with 27 PCR cycles, showing that over-amplification was occurring. In addition, more baseline noise was observed in the 27 cycle data, which could be attributed to an excess of products.

Furthermore, when considering percent alleles detected, peak height, PHR, CV, and artifacts, buccal samples directly amplified with 25 PCR cycles using GlobalFiler™ Express produced usable profiles with very few artifacts, but did not meet the recommended peak height. In addition, buccal samples directly amplified with 27 PCR cycles using GlobalFiler™ Express produced usable profiles, but had an excessive number of artifacts. The noted presence of off-scale peaks within the 27 cycle data was concordant with results from the kit's developmental validation (16). Thus, with the production of peak heights >3,000 RFU, PHRs ≥90%, CV <0.270, and minimal artifacts, the optimal thermal cycling parameters when using GlobalFiler™ Express with buccal samples was determined to be 26 cycles.

After assessing the data and determining the best thermal cycling parameters for buccal swabs, the minimum peak height and maximum artifact peak height were also evaluated. This was performed to determine if the analytical threshold could be raised from 55 RFU to prevent excessive artifacts and aide in data analysis. For the 26 cycle data, the minimum peak height was 954 RFU and the maximum artifact peak height was 367 RFU; therefore, it was decided to raise the analytical threshold to 200 RFU since real peaks would not be affected and most artifacts would be removed.

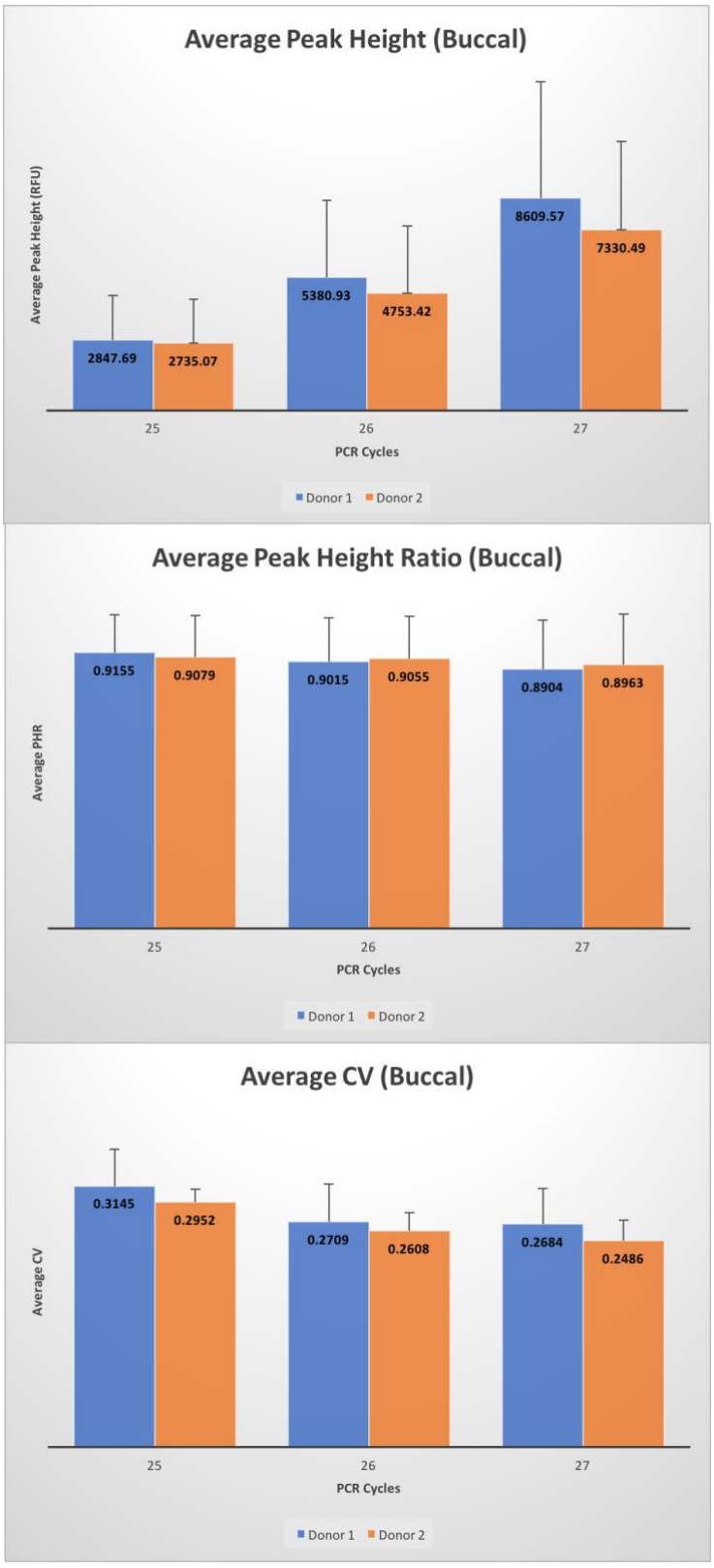


Figure 13: GlobalFiler™ Express Buccal Sample Results.

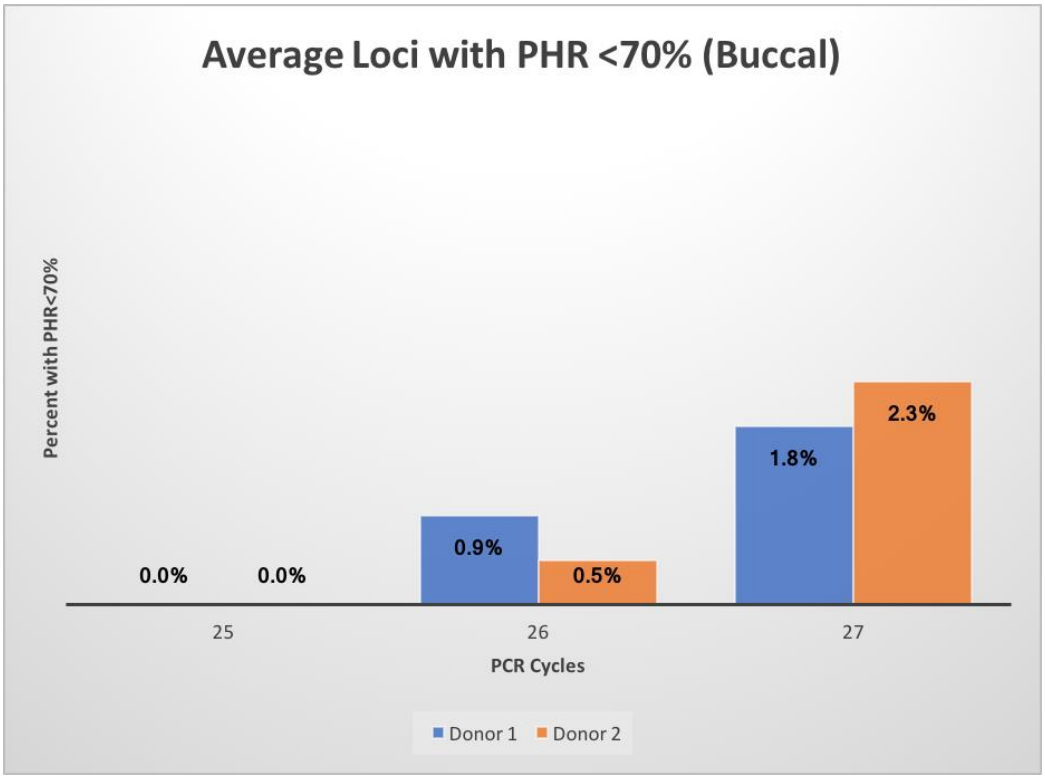


Figure 14: Electropherograms from the 26 buccal samples that were amplified using GlobalFiler™ Express were evaluated for average percent loci with peak height ratios less than 0.7.

Table 39. GlobalFiler™ Express Artifacts (Buccal Samples)

Artifact	% Samples			Average % Loci		
	25 Cycles	26 Cycles	27 Cycles	25 Cycles	26 Cycles	27 Cycles
Stutter (N-4)	*	7.69%	3.85%	*	0.32%	0.16%
Stutter (N+4)	*	*	*	*	*	*
Stutter (N-2)	*	*	*	*	*	*
Stutter (Total)	*	7.69%	3.85%	*	0.32%	0.16%
Baseline Noise	3.85%	11.54%	34.62%	0.64%	0.64%	1.76%
Pull-up	7.69%	26.92%	50.00%	0.48%	1.60%	4.00%
-A	*	*	*	*	*	*

*No artifacts

Table 39. Artifacts from the GlobalFiler™ Express buccal sample electropherograms were analyzed to calculate the percent of samples with each artifact and the average percent of loci that included these artifacts. This was done for samples amplified using 25, 26, and 27 cycles.

To verify blood on FTA cards could also be used with this kit, a mini study was performed using blood from two donors. Five FTA cards (from each donor) with whole blood spotted from a collection tube were directly amplified per manufacturer recommendations using 25, 26, and 27 PCR cycles and evaluated on the 3500 Genetic Analyzer using Applied Biosystems® GeneMapper® *ID-X* Software v1.5 with a 10% global cut-off filter. Data analysis was performed in the same manner as with the buccal samples and the results were graphically displayed (Figure 15). All alleles were detected in every sample from one donor, while samples from another donor showed allelic dropout with 25 and 26 cycles (Table 40). The samples showing allelic dropout were from one FTA card (different punches). The average peak height increased with an increase in PCR cycles, which was expected. According to the GlobalFiler™ Express user guide, the optimum PCR cycle number should generate profiles with peak heights of 3,000 – 12,000 RFU on the 3500 Genetic Analyzer (13); therefore, the use of 26 and 27 cycles met this requirement for all FTA samples (Figure 15). The use of 25 cycles produced profiles with average PHRs of 82-87%, 26 cycles produced profiles with average PHRs of 79-82%, and 27 cycles produced profiles with average PHRs of 77-80% (Figure 15). All data had ≤ 25% of

loci with PHRs less than 70% and ≤ 15% of loci with PHRs less than 60% (Figure 16). None of the samples produced CV values ≤0.350, showing poor inter-locus balance. This poor inter-locus balance was attributed to inhibition from Heme within the blood samples because the FTA punches were added directly to the amplification tubes without any incubation or washes (17). As the number of PCR cycles increased, the average CV mostly increased, which supported the conclusion that an inhibitor was present (Figure 15).

The percentage of samples and average percentage of loci affected by artifacts were also evaluated (Table 41). The samples amplified with 25 PCR cycles exhibited no artifacts. As expected, the 27 PCR cycles produced the most artifacts and most average loci affected (Table 41). Pull-up affected 46% of the FTA samples and baseline noise affected 76% of the FTA samples when they were amplified with 27 PCR cycles, showing that over-amplification was occurring and an excess of products was present (Table 41).

Furthermore, when considering percent alleles detected, peak height, PHR, CV, and artifacts, FTA samples directly amplified with 25 PCR cycles using GlobalFiler™ Express produced usable profiles with no artifacts, but did not meet the recommended peak height or CV requirements. In addition, FTA samples directly amplified with 27 PCR cycles using GlobalFiler™ Express produced mostly usable profiles, but had an excessive number of artifacts and very poor inter-locus balance. Because samples used with this procedure will be reference samples, CV was not as important when determining the optimum PCR parameters; there is much less uncertainty as to the true alleles with reference samples compared to forensic and mixture samples, so profile balance is not as crucial. Thus, with the production of peak heights >3,000 RFU, PHRs ≥79%, and minimal artifacts, the optimal thermal cycling parameters when using GlobalFiler™ Express with FTA samples was initially determined to be 26 cycles.

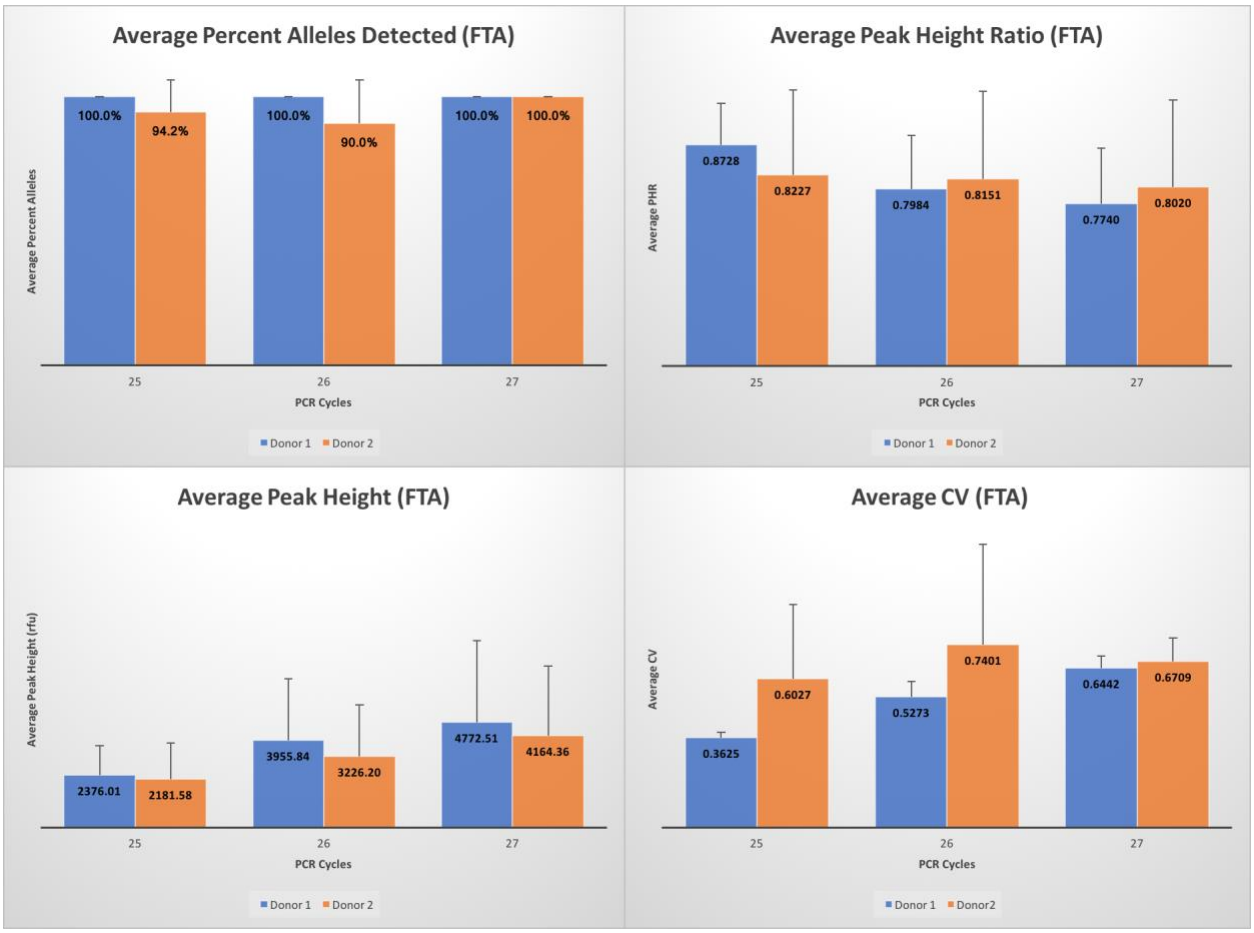


Figure 15: GlobalFiler™ Express FTA Whole Blood Sample Results.

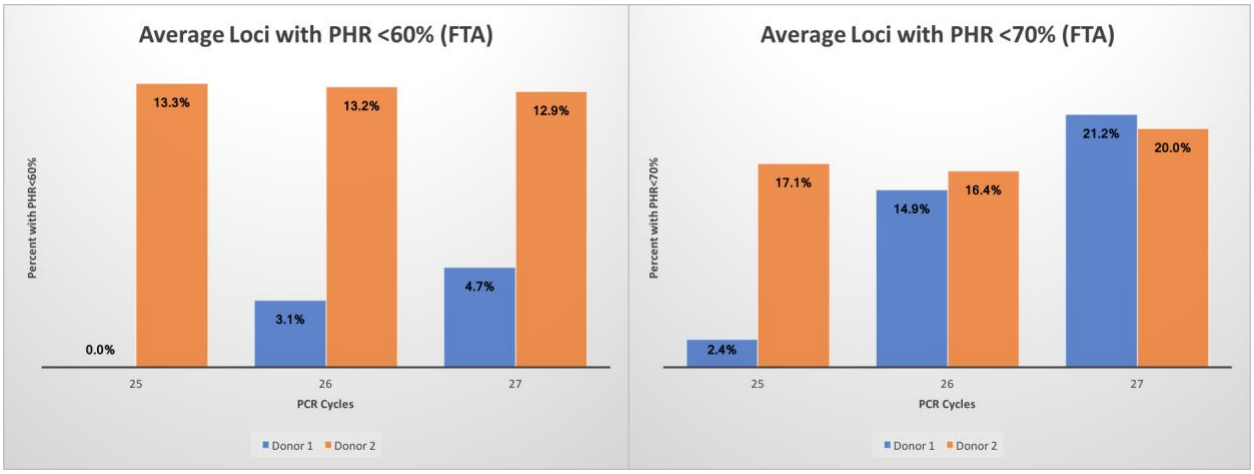


Figure 16: Electropherograms from the ten FTA samples (whole blood) that were amplified using GlobalFiler™ Express were evaluated for average percent loci with peak height ratios less than 0.6 and 0.7.

Table 40. GlobalFiler™ Express Allelic Dropout (FTA Whole Blood Samples)

Samples	% Samples			Average % Alleles		
	25 Cycles	26 Cycles	27 Cycles	25 Cycles	26 Cycles	27 Cycles
B1-B5	*	*	*	*	*	*
T1-T5	20.00%	30.00%	*	5.80%	10.00%	*

*No allelic dropout

Table 40. The whole blood FTA samples evaluated with a 15 second injection time were assessed for allelic dropout. The percentage of samples exhibiting dropout and the percentage of alleles that dropped out were calculated.

Table 41. GlobalFiler™ Express Artifacts (FTA Whole Blood Samples)

Artifact	% Samples		Average % Loci	
	26 Cycles	27 Cycles	26 Cycles	27 Cycles
Stutter (N-4)	*	*	*	*
Stutter (N+4)	*	*	*	*
Stutter (N-2)	*	*	*	*
Stutter (Total)	*	*	*	*
Baseline Noise	24.00%	76.67%	1.00%	3.33%
Pull-up	36.00%	46.67%	1.67%	3.06%
-A	*	*	*	*

*No artifacts

Table 41. Artifacts from the GlobalFiler™ Express FTA (whole blood) sample electropherograms were analyzed to the percentage of samples with each artifact and the average percent of loci that included these artifacts. This was done for samples amplified using 25, 26, and 27 cycles. No artifacts were observed for the samples amplified with 25 PCR cycles.

Because the blood on FTA cards received by the lab could be from a finger prick rather than a collection tube, those samples were also evaluated during this project. The four FTA cards (two from each donor) containing blood from a finger prick were evaluated the same way as the other FTA samples. Data analysis was also performed in the same manner as with the other samples, and the results were graphically displayed (Figure 17). Unlike with the whole blood samples, all alleles were detected in every sample. The average peak height tended to increase with an increase in PCR cycles, which was expected; however, just like the whole blood samples, the peak heights varied between samples and donors (Figure 17). Although the average

peak height varied greatly between the two donors, the average peak height for all finger prick samples at every PCR cycle evaluated during this study was greater than 3,000 RFU; therefore, all peak heights met the recommended value within the GlobalFiler™ Express user guide.

The use of 25 cycles produced profiles with average PHRs of 86-88%, 26 cycles produced profiles with average PHRs of 80-82%, and 27 cycles produced profiles with average PHRs of 77-84% (Figure 17). All data had ≤ 26% of loci with PHRs less than 70% and ≤ 12% of loci with PHRs less than 60% (Figure 18). Only the samples amplified with 25 PCR cycles produced CV values ≤0.350, whereas all other samples had much greater CVs (Figure 17). This demonstrated the finger prick samples had poor inter-locus balance, but not as poor as the whole blood samples. This poor inter-locus balance was attributed to inhibition from Heme within the blood samples (17). The slightly better CVs at all PCR cycles in comparison to the whole blood samples could have been the result of the different sources of blood and the presence of fewer red blood cells (i.e. less Heme) (17). As with the whole blood samples, the increase in number of PCR cycles led to an increase in the average CV, which supported the conclusion that an inhibitor was present (Figure 17).

The percentage of samples and average percentage of loci affected by artifacts were also evaluated (Table 42). The samples amplified with 25 PCR cycles exhibited no artifacts. As expected, the 27 PCR cycles produced the most artifacts and most average loci affected (Table 42). Pull-up affected 33% of the FTA samples and baseline noise affected 54% of the FTA samples when they were amplified with 27 PCR cycles, showing that over-amplification was occurring and an excess of products was present (Table 42). In comparison to the whole blood FTA samples, the finger prick samples contained fewer artifacts and fewer loci exhibiting these artifacts.

Furthermore, when considering percent alleles detected, peak height, PHR, CV, and artifacts, finger prick samples directly amplified with 25 PCR cycles using GlobalFiler™ Express produced usable profiles with no artifacts, but barely met the recommended peak height and CV requirements (Figure 17). In addition, finger prick samples directly amplified with 27 PCR cycles using GlobalFiler™ Express produced mostly usable profiles, but had an excessive number of artifacts and very poor inter-locus balance. Because samples used with this procedure will be reference samples, CV was not as important when determining the optimum PCR parameters. Thus, with the production of peak heights >3,000 RFU, PHRs ≥80%, and minimal artifacts, the optimal thermal cycling parameters when using GlobalFiler™ Express with FTA samples was initially determined to be 26 cycles. In addition, the finger prick FTA samples appeared to produce slightly better results than the whole blood FTA samples, especially when considering intra-locus and inter-locus balance. This could have resulted from the differing sources of the blood.

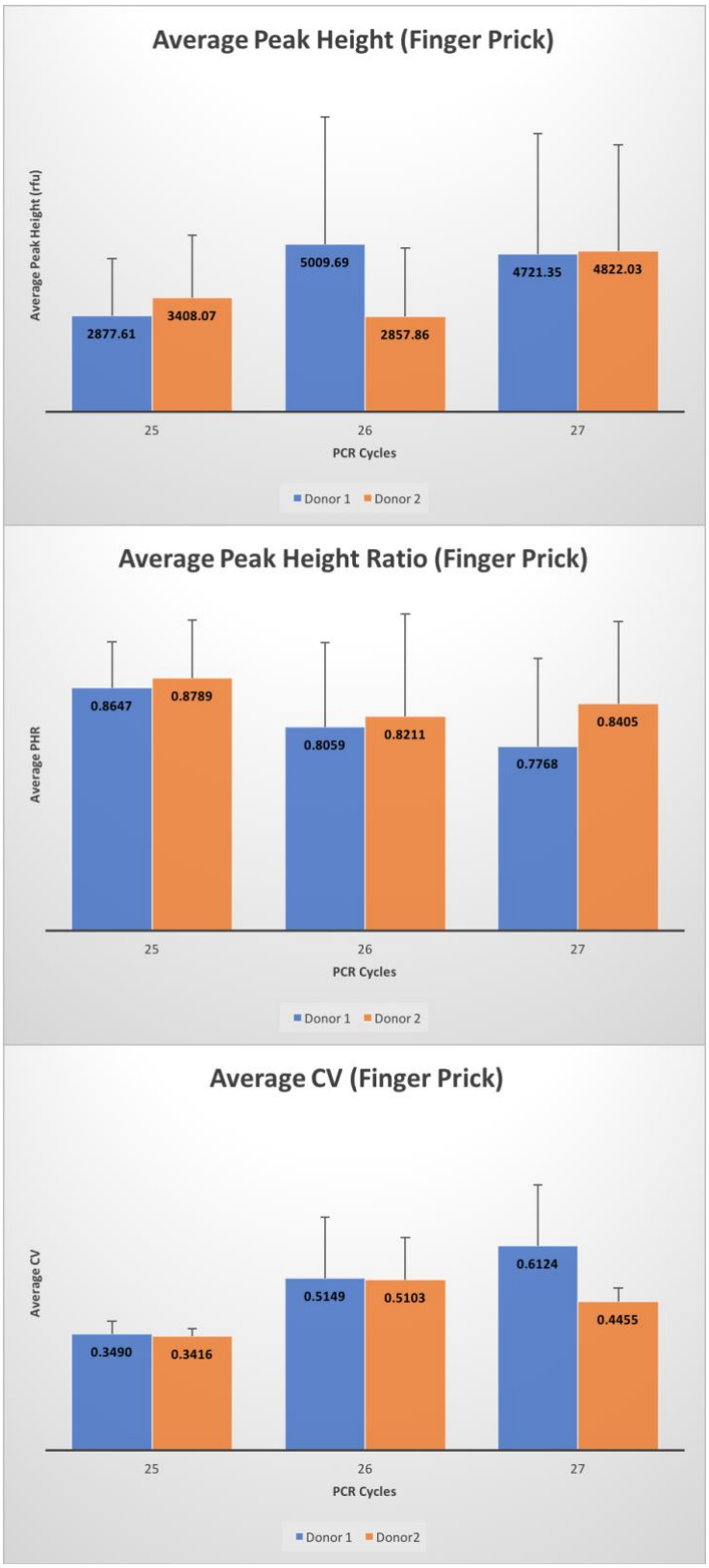


Figure 17: GlobalFiler™ Express FTA Finger Prick Sample Results.

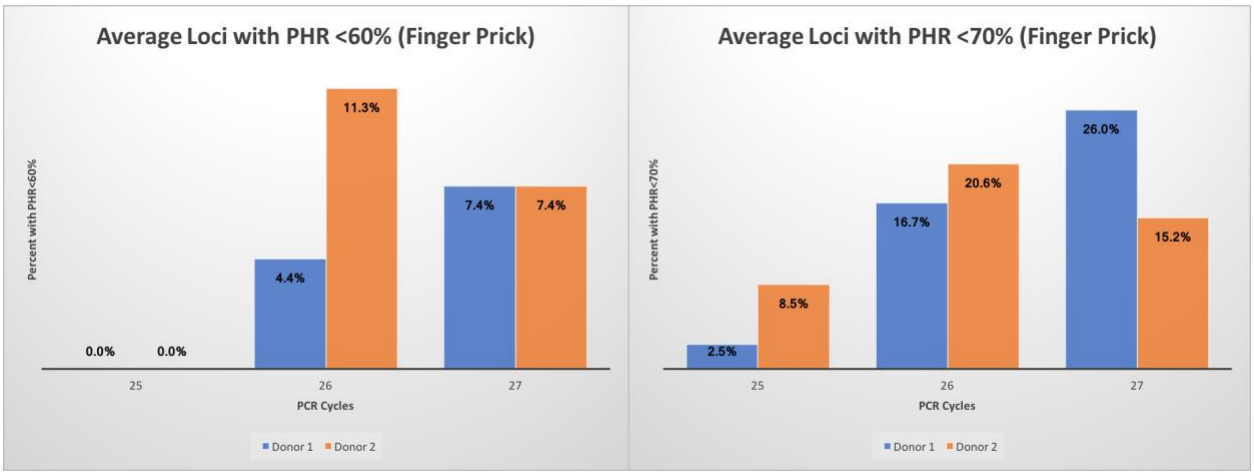


Figure 18: Electropherograms from the eight FTA samples (finger prick) that were amplified using GlobalFiler™ Express were evaluated for average percent loci with peak height ratios less than 0.6 and 0.7.

Table 42. GlobalFiler™ Express Artifacts (FTA Whole Blood Samples)

Artifact	% Samples		Average % Loci	
	26 Cycles	27 Cycles	26 Cycles	27 Cycles
Stutter (N-4)	*	*	*	*
Stutter (N+4)	*	*	*	*
Stutter (N-2)	*	*	*	*
Stutter (Total)	*	*	*	*
Baseline Noise	33.00%	54.20%	1.39%	2.26%
Pull-up	25.00%	33.00%	1.39%	1.74%
-A	*	*	*	*

*No artifacts

Table 42. Artifacts from the GlobalFiler™ Express FTA (finger prick) sample electropherograms were analyzed to calculate the percentage of samples with each artifact and the average percent of loci that included these artifacts. This was done for samples amplified using 25, 26, and 27 cycles. No artifacts were observed for the samples amplified with 25 PCR cycles.

Stochastic Threshold Study

The GlobalFiler™ and GlobalFiler™ Express data was also used to determine the appropriate stochastic threshold for each kit, using Equations 3 and 4. No stochastic threshold was calculated for the Yfiler™ Plus kit because Y-STRs give haplotypes and only two loci with potential heterozygosity. The stochastic thresholds were calculated using Equations 3 and 4 (see methods) and can be found in Table 43.

Table 43. Stochastic Threshold Calculations

Amplification Kit	PCR Cycles	Calculated Stochastic Threshold (RFU)	Highest False Homozygote (RFU)	Final Stochastic Threshold (RFU)
GlobalFiler™	29	250	299	250
GlobalFiler™ Express (Buccal)	25	75	*	75
	26	78	*	80
	27	82	*	85
GlobalFiler™ Express (FTA)	25	109	**	110
	26	120	203	205
	27	132	*	135

*No allelic dropout
**No false homozygote observed
Table 43. The stochastic threshold was calculated for each amplification kit evaluated. For GlobalFiler™ Express, the stochastic threshold was calculated for each PCR cycle and sample type assessed. False homozygote peaks were then identified, and the stochastic threshold was adjusted (when deemed necessary) to incorporate those peaks.

After calculating the stochastic thresholds, the electropherograms were assessed for the highest false homozygote peak. If the peak height of the false homozygote was above the calculated stochastic threshold, its height was rounded to the nearest five and the stochastic threshold was adjusted to incorporate the peak. The final stochastic threshold for each kit after any adjustments can also be found in Table 43. Although the highest false homozygote observed within the GlobalFiler™ data was 299 RFU, the stochastic threshold was not adjusted because it was believed to have been inflated by pull-up.

When determining the final stochastic threshold for GlobalFiler™ Express with buccal samples, the previously discussed adjustment for the analytical threshold was considered. The analytical threshold when using 26 PCR cycles was adjusted to 200 RFU to eliminate many artifacts seen within the data; therefore, an adjustment of equal magnitude was performed on the calculated stochastic threshold to raise it to 300 RFU. Since no allelic dropout was observed and reference samples will be used with this kit, this adjustment should not negatively impact results.

Injection Parameters Study

Additional injection parameters were evaluated for all amplification kits to optimize the results obtained. The sensitivity series samples for GlobalFiler™ were analyzed using 5 and 10 second injection times and all profiles obtained were then evaluated for percent alleles detected, percent allelic dropout, average peak height, average PHR, CV, percent samples affected by artifacts, and percent loci exhibiting artifacts. The results from the injection time study were compared to the initial 15 second results and all findings were graphically displayed for the DNA input range that would generate usable profiles, which was previously discussed in the GlobalFiler™ sensitivity series section (Figure 19). When comparing the different injection times, the profiles had more alleles detected, higher peaks and PHRs, and lower CVs when the samples were injected for 15 seconds (Figure 19). For the DNA input range expected to generate usable profiles (3.11 – 0.168 ng), the 15 second injection data had ≤ 25% of loci with PHRs less than 70% and ≤ 18% of loci with PHRs less than 60% (Figure 20). In addition, less allelic dropout was observed at all but one concentration when the highest injection time was used (Table 44). When taking all of this into consideration, the 15 second injection time should be used within the lab because it provided more favorable results than the lower injection times.

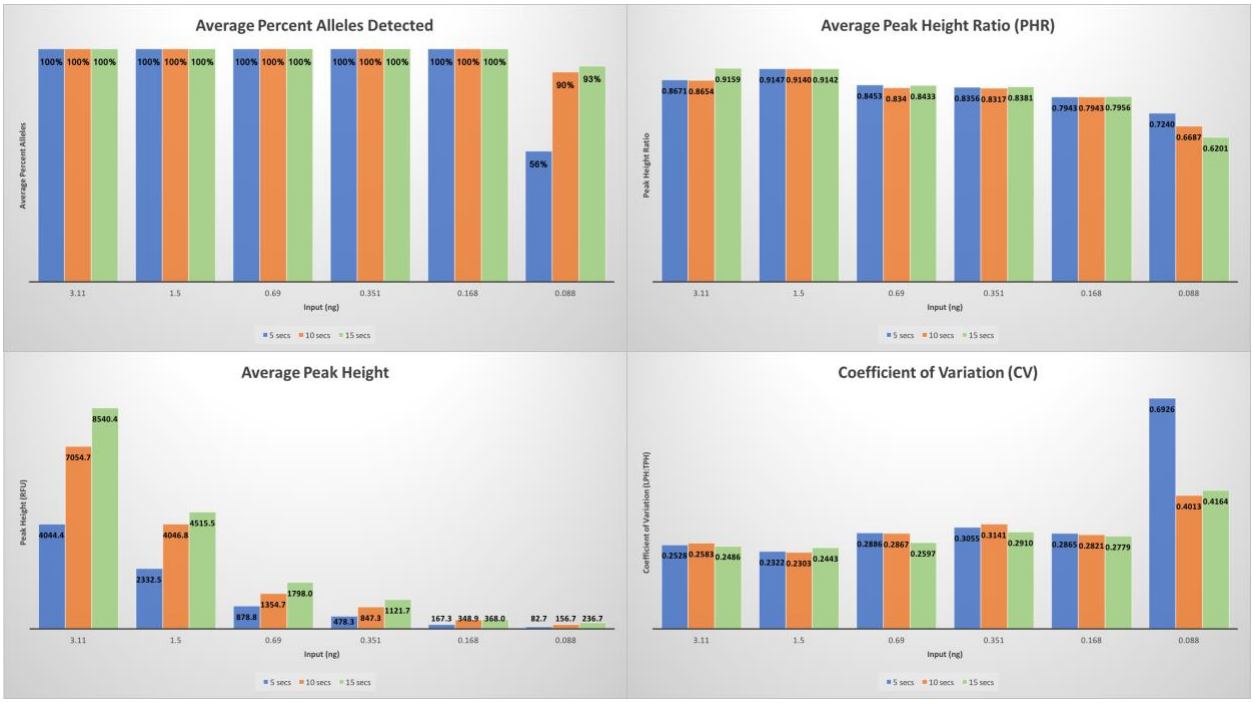


Figure 19: GlobalFiler™ Injection Study Results.

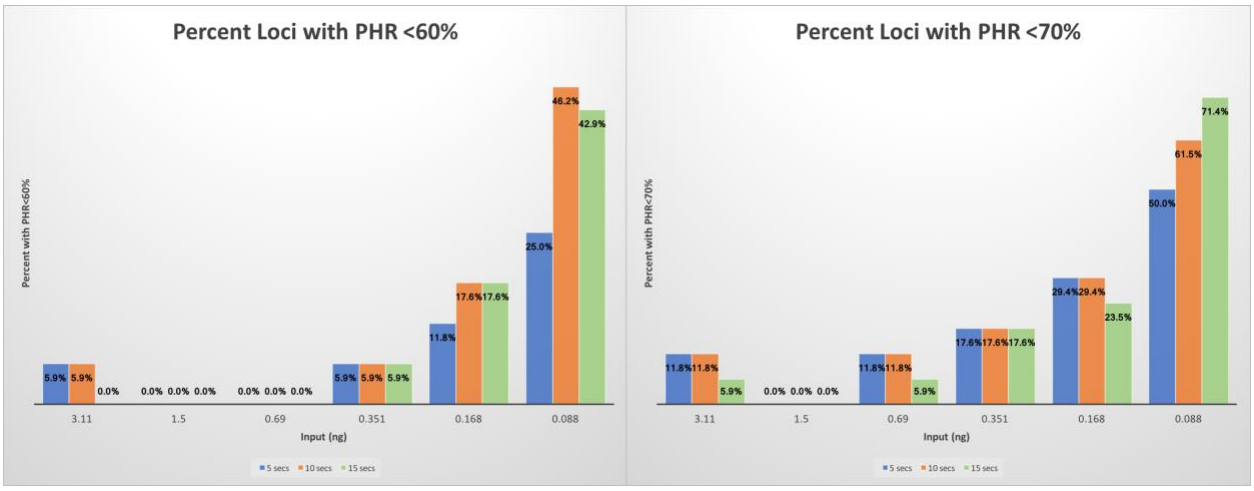


Figure 20: GlobalFiler™ Injection Study PHR Comparison.

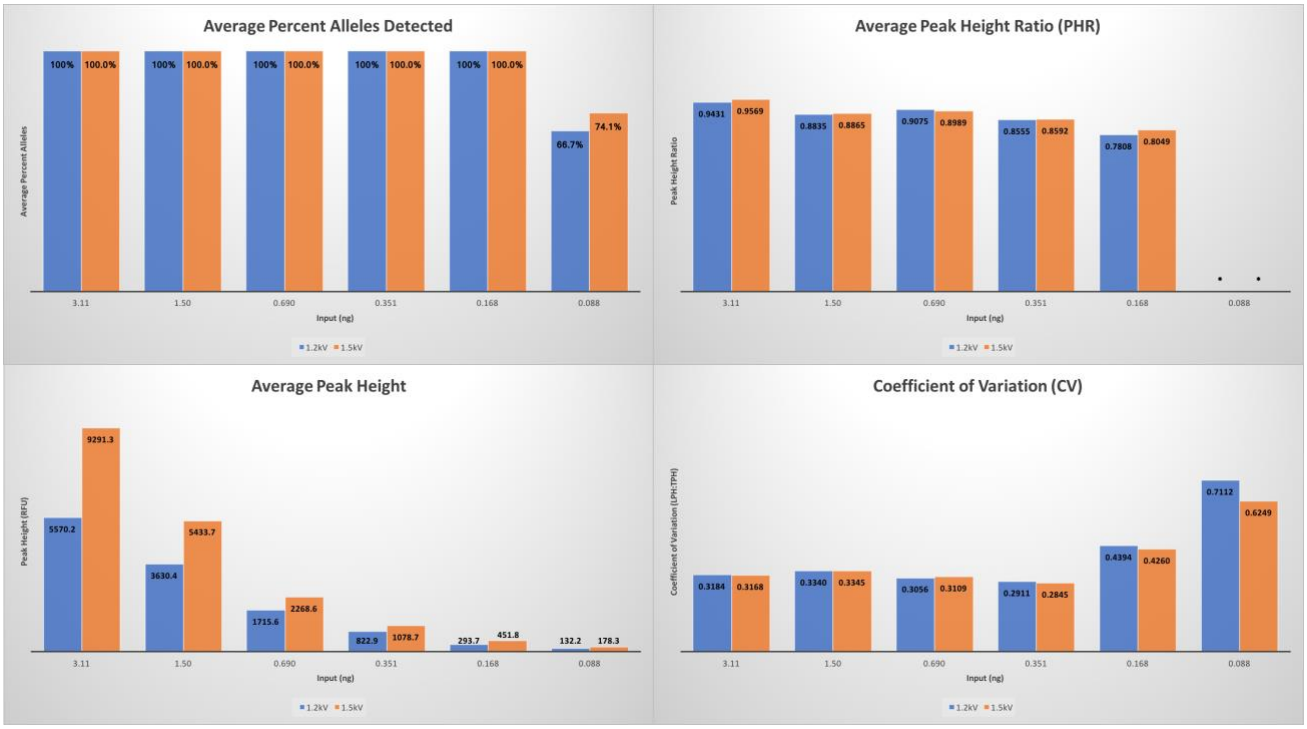
Table 44. GlobalFiler™ Injection Study Allelic Dropout

Sample (ng)	Injection Time		
	5 sec	10 sec	15 sec
2 (1.38)	0.0%	0.0%	0.0%
6 (0.608)	0.0%	0.0%	0.0%
7 (0.289)	7.3%	0.0%	0.0%
10 (0.108)	80.5%	48.8%	43.9%
14 (0.054)	100.0%	92.7%	82.9%
17 (0.027)	100.0%	97.6%	97.6%
20 (0.013)	100.0%	97.6%	97.6%
22 (0.007)	100.0%	100.0%	100%
27 (0.0035)	97.6%	100.0%	97.6%
28 (6.49)	0.0%	0.0%	0.0%
33 (3.11)	0.0%	0.0%	0.0%
34 (1.50)	0.0%	0.0%	0.0%
39 (0.690)	0.0%	0.0%	0.0%
42 (0.351)	0.0%	0.0%	0.0%
45 (0.168)	0.0%	0.0%	0.0%
46 (0.088)	43.9%	9.8%	7.32%
50 (0.046)	63.4%	31.7%	36.6%
53 (0.023)	97.6%	53.7%	43.9%
57 (0.012)	97.6%	90.2%	87.8%
59 (0.007)	100.0%	95.1%	90.2%

Table 44. The allelic dropout within the samples for GlobalFiler™ that were evaluated at 5, 10, and 15 second injection times were compared

The sensitivity series samples for Yfiler™ Plus were analyzed using a 1.5kV/16 seconds injection. All profiles obtained were then evaluated in the same manner as the GlobalFiler™ samples, and those results were graphically displayed for the DNA input range previously determined to generate usable profiles (Figure 21). When comparing the different injection voltages, the profiles had more alleles detected, higher peaks, generally higher PHRs, and generally lower CVs when the samples were injected using 1.5kV (Figure 21). The percent loci with PHRs less than 0.7 and 0.6 were not evaluated for the Yfiler™ Plus injection study results

because only two loci could potentially have PHRs. In addition, less allelic dropout was observed at all concentrations when the higher injection voltage was used (Table 45). When taking all of this into consideration, the 1.5kV injection should be used within the lab as it generated more favorable results than the 1.2kV injection.



*Calculations could not be performed.

Figure 21: Yfiler™ Plus Injection Study Results.

Table 45. Yfiler™ Plus Injection Study Allelic Dropout

Sample (ng)	Injection Voltage	
	1.2 kV	1.5 kV
3 (1.38)	0.0%	0.0%
6 (0.608)	0.0%	0.0%
8 (0.289)	0.0%	0.0%
12 (0.108)	51.9%	48.0%
14 (0.054)	88.9%	63.0%
17 (0.027)	100%	93%
21 (0.013)	100%	100%
24 (0.007)	100%	93%
26 (0.0035)	96.3%	96.0%
28 (6.49)	0.0%	0.0%
31 (3.11)	0.0%	0.0%
34 (1.50)	0.0%	0.0%
39 (0.690)	0.0%	0.0%
42 (0.351)	0.0%	0.0%
45 (0.168)	0.0%	0.0%
47 (0.088)	33.3%	26.0%
49 (0.046)	40.7%	37.0%
54 (0.023)	77.8%	70.0%
57 (0.012)	88.9%	81.0%
60 (0.007)	100%	85.0%

Table 45. The allelic dropout within the samples for Yfiler™ Plus that were evaluated at 1.2kV and 1.5 kV injections were compared

Four buccal samples and the positive control for GlobalFiler™ Express were additionally analyzed using 5 and 10 second injection times; the data was compared to the initial 15 second injection results, and all findings obtained were then evaluated for percent alleles detected, average peak height, average PHR, and CV (Figure 22). All alleles were detected in every sample. The average peak height increased with an increase in both PCR cycles and injection time, which was expected (Figure 22). When considering the suggested peak height range from the GlobalFiler™ Express user guide, samples amplified with 26 PCR cycles only met this recommendation when injected for 15 seconds. Samples amplified with 27 PCR cycles fell

within this range when injected for 10 and 15 seconds. On the other hand, no samples amplified with 25 PCR cycles met this peak height recommendation.

All PCR cycles and injection times produced profiles with average PHRs of 89-91% and ≤2% of loci exhibiting PHRs less than 70% (Figure 22-23). The 10 second injection time produced no loci with PHRs less than 70%, regardless of cycle number (Figure 23). No profiles had loci with PHRs less than 60%, and no allelic dropout was observed. When considering inter-locus balance, the 15 second injection time for the 26 and 27 cycle data produced the lowest CV values, but all CV values were less than the maximum acceptable value of 0.350 (Figure 22).

Furthermore, when comparing all of the results, buccal samples directly amplified with 26 PCR cycles and injected for 15 seconds using GlobalFiler™ Express produced usable profiles with peak heights >3,000 RFU, PHRs ≥90%, CV <0.270, and minimal artifacts. Thus, these parameters were considered optimal when using GlobalFiler™ Express with buccal samples. Although 27 PCR cycles injected for both 10 and 15 seconds also gave acceptable results, over-amplification was apparent and led to increased amplification artifacts, prolonging data analysis.

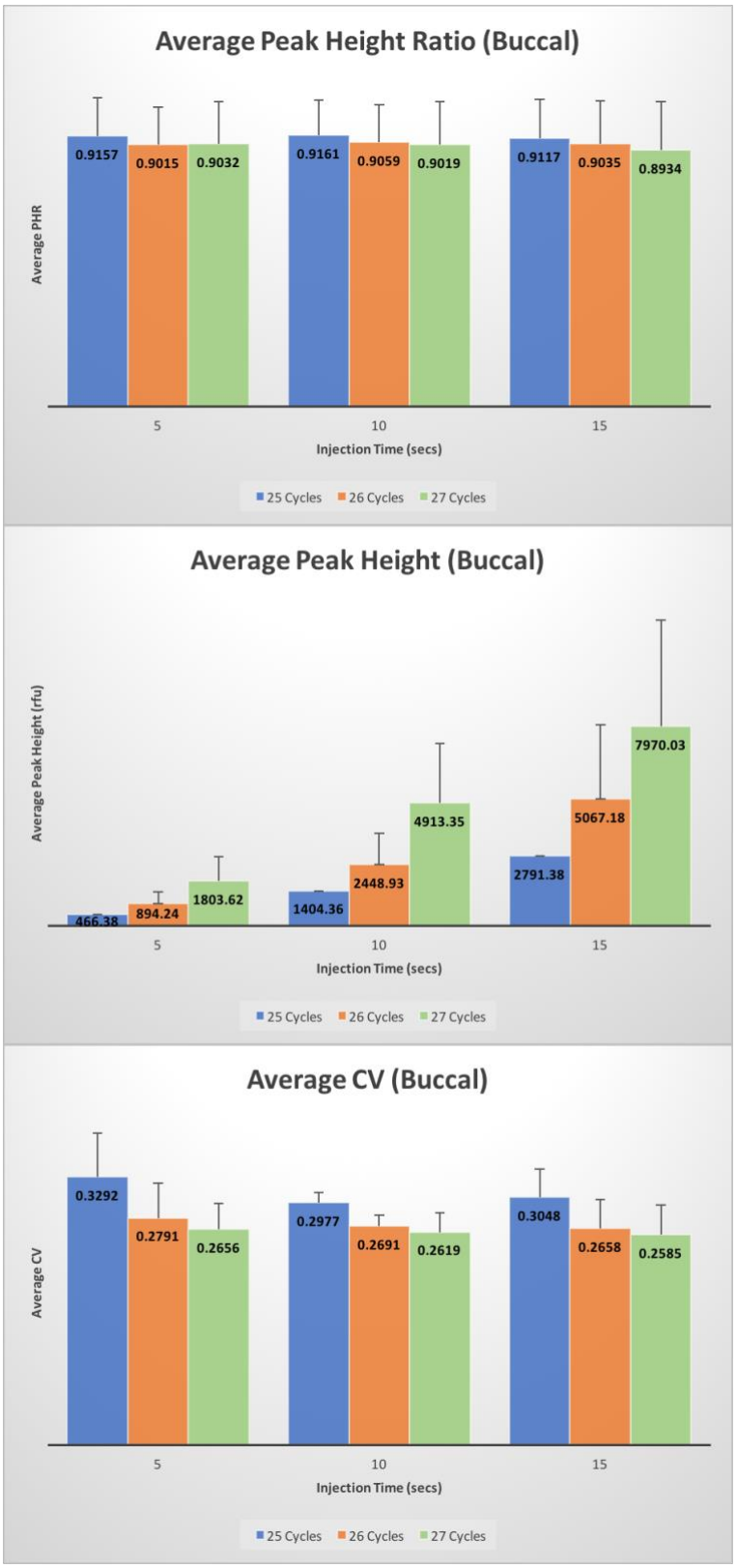


Figure 22: GlobalFiler™ Express Injection Study Results (Buccal Samples)

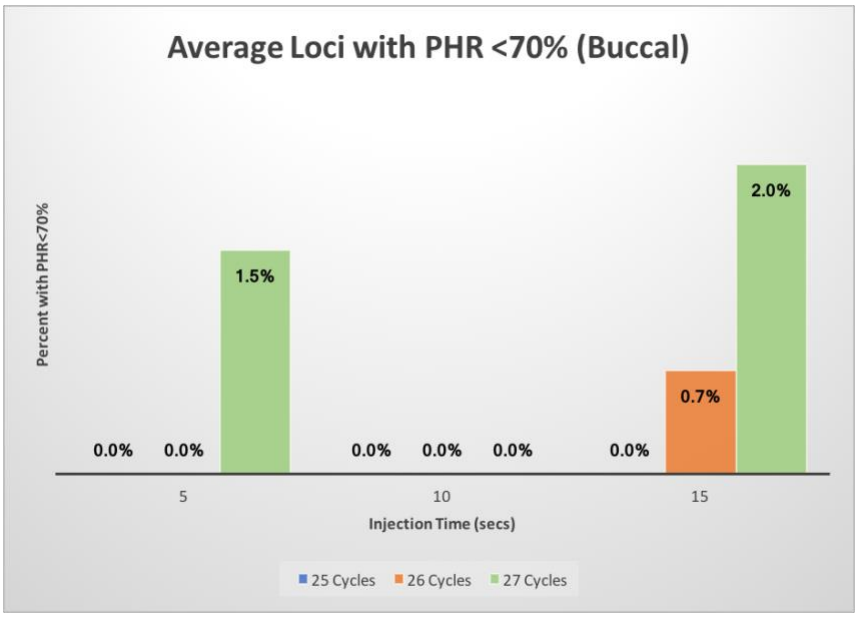


Figure 23: GlobalFiler™ Express Injection Study PHRs (Buccal Samples)

Four whole blood FTA samples and the positive control for GlobalFiler™ Express were analyzed using 10 and 20 second injection times and all profiles obtained were then evaluated for percent alleles detected, average peak height, average PHR, and CV (Figure 24). These injection times were chosen to see if a longer injection would improve results and remove allelic dropout. All alleles were detected in every sample, apart from the original 15 second injection samples; however, these findings could not be directly compared due to the difference in sample size. The average peak height increased with an increase in both PCR cycles and injection time, which was expected (Figure 24). When considering the suggested peak height range from the GlobalFiler™ Express user guide, samples amplified with 25 PCR cycles only met this recommendation when injected for 20 seconds. Samples amplified with 26 PCR cycles fell within this range when injected for 15 and 20 seconds. On the other hand, all samples amplified with 27 PCR cycles met this peak height recommendation, regardless of injection time.

All PCR cycles and injection times produced profiles with average PHRs of 78-87% (Figure 25). The samples amplified with 27 PCR cycles produced the greatest percentage of loci with PHRs less than 70% and 60%, while the samples amplified with 25 PCR cycles produced the smallest percentage (Figure 25). When considering inter-locus balance, no samples met the acceptance criteria of ≤0.350, regardless of cycle number or injection time (Figure 24). The poor inter-locus balance could be attributed to inhibition from the Heme within the samples; this coincided with the finding that samples amplified with 27 PCR cycles had the worst CV values, showing that more amplification led to increased inhibition. Because these samples will be reference samples, the inter-locus balance was not as crucial when optimizing parameters.

Furthermore, when comparing all of the results, whole blood FTA samples directly amplified with 25 PCR cycles and injected for 20 seconds using GlobalFiler™ Express produced usable profiles with all alleles detected, peak heights >3,000 RFU, PHRs ≥85%, and CV <0.395. In addition, no artifacts were encountered under these conditions. Thus, these parameters were considered optimal when using GlobalFiler™ Express with FTA samples. Although finger prick FTA samples were not evaluated during the injection time study, it was previously determined that these samples produce similar results to the whole blood samples; therefore, the same parameters could be used with both sample types.

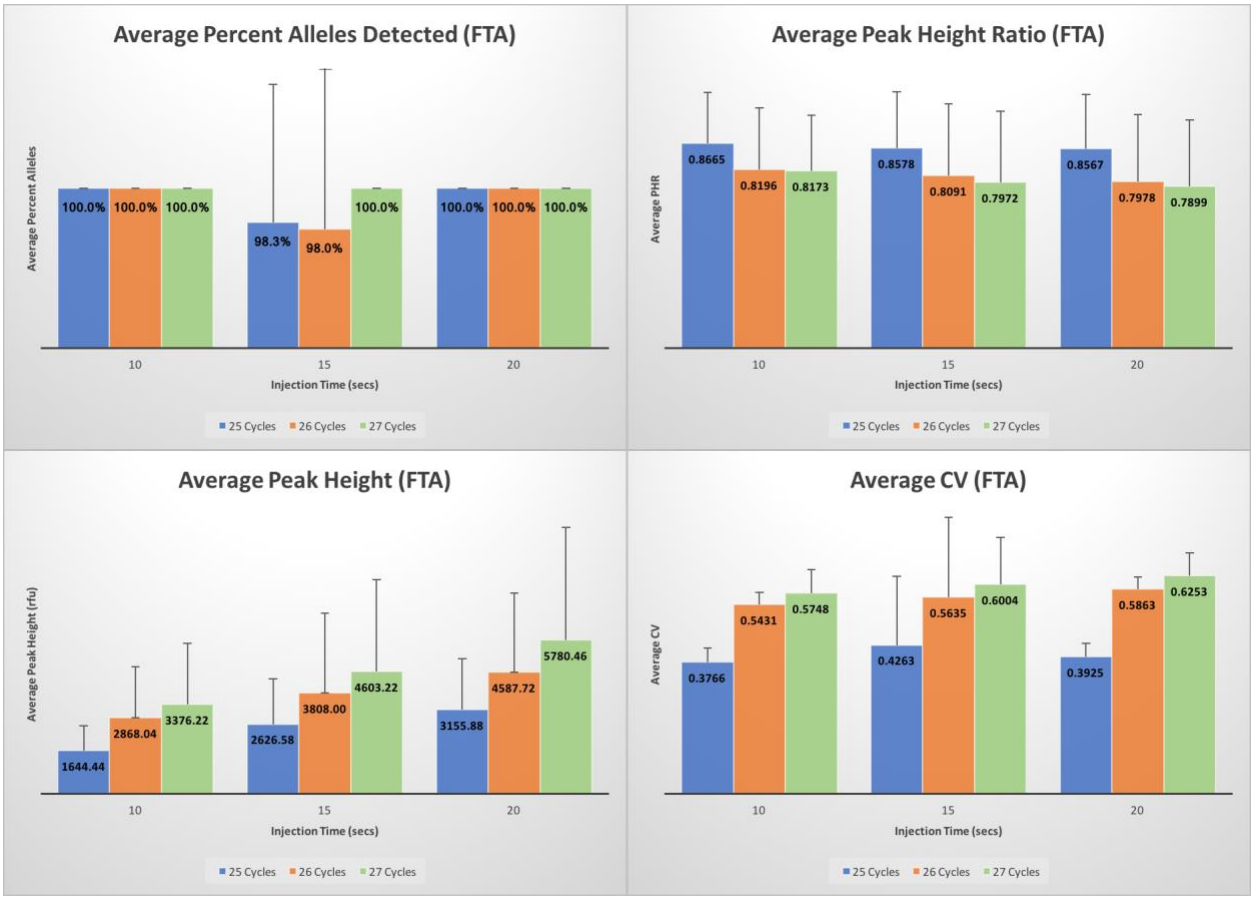


Figure 24: GlobalFiler™ Express Injection Study Results (FTA Whole Blood Samples)

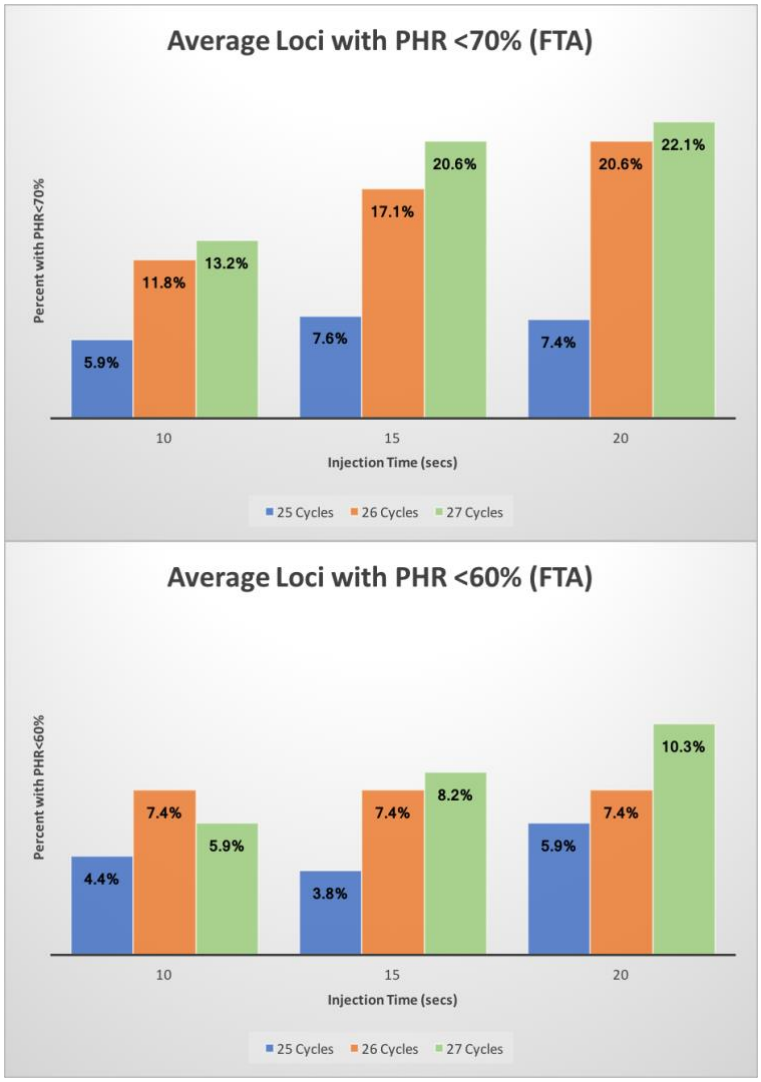


Figure 25: GlobalFiler™ Express Injection Study PHRs (FTA Whole Blood Samples)

Mixture Study

All mixture samples amplified using GlobalFiler™ with the previously determined parameters were run on the 3500 Genetic Analyzer in triplicate, and the electropherograms were analyzed and evaluated to determine the actual mixture ratios. These were calculated by considering loci with four peaks, with three peaks where there was no sharing of alleles, and with two peaks (known homozygotes) (Table 46). All calculated ratios for the male:male,

male:female, and female:female mixtures were close to their expected ratios, apart from 20:1 and 1:20. These calculations revealed better results for the male:female mixtures in comparison to the estimated ratios from the Quantifiler Trio mixture study, especially when the male was the major contributor. This further supports the previous conclusion that Quantifiler® Trio is an adequate presumptive tool for detecting the presence of male:female mixtures when the female is the major contributor, but the potential presence of a mixture should not be dismissed when Quantifiler® Trio does not detect one.

Additionally, the electropherograms were evaluated for percent dropout of the minor contributor and percent alleles of the minor contributor above stochastic threshold to determine at which point major and minor contributors could no longer be distinguished. The results were assessed for allelic dropout of the minor contributor at the loci expected to have four peaks. No dropout of the minor contributor was observed between 11.9:1 and 1:22.3 for the male:male samples, between 11.0:1 and 1:9.6 for the male:female samples, and between 17.1:1 and 1:12.8 for the female:female samples (Table 46). The percentage of alleles from the minor contributor above stochastic threshold was also evaluated using the loci with three peaks (one homozygote and one heterozygote) and four peaks (two heterozygotes). All alleles from the minor contributor were above stochastic threshold for ratios from 1.8:1 to 1:4.4 for the male:male samples, 1.9:1 to 1:2 for the male:female samples, and 3.7:1 to 1:6.2 for the female:female samples (Table 46). Taking both of these observations into consideration and averaging the results for all samples, the ability to distinguish major and minor contributors becomes unreliable at ratios approximately above 14:1/1:14.

Table 46. GlobalFiler™ Mixture Study Results

Target Ratio	Male to Male			Male to Female			Female to Female		
	Actual Ratio	Percent Dropout	Percent Above ST	Actual Ratio	Percent Dropout	Percent Above ST	Actual Ratio	Percent Dropout	Percent Above ST
20:1	77.3 : 1	50.0	0.0	46.8 : 1	43.3	9.3	22.5 : 1	8.3	14.8
16:1	17.1 : 1	100.0	22.2	22.5 :1	26.7	7.0	17.1 : 1	*	33.3
12:1	16.0 : 1	16.7	18.5	17.4 : 1	26.7	11.5	11.0 : 1	*	47.2
8:1	11.9 : 1	*	37.0	11.0 :1	*	61.1	7.2 : 1	*	72.2
4:1	5.3 : 1	*	96.3	4.9 : 1	*	88.9	3.7 : 1	*	100
2:1	1.8 : 1	*	100	1.9 : 1	*	100	1.6 : 1	*	100
1:1	1.2 : 1	*	100	1.1 : 1	*	100	1.3 : 1	*	100
1:2	1 : 2.1	*	100	1 : 2.0	*	100	1 : 2.7	*	100
1:4	1 : 4.4	*	100	1 : 4.8	*	98.0	1 : 6.2	*	100
1:8	1 : 11.2	*	94.4	1 : 9.6	*	70.6	1 : 12.8	*	74.4
1:12	1 : 14.1	*	57.1	1 : 13.9	10.0	37.8	1 : 26.9	12.5	5.5
1:16	1 : 22.3	*	38.1	1 : 18.6	20.0	46.7	1 : 28.8	20.8	13.0
1:20	1 : 32.3	100.0	33.3	1 : 25.4	36.7	24.7	1 : 45.8	62.5	4.2

*No allelic dropout

Table 46. The mixture samples amplified using GlobalFiler™ were analyzed and their actual ratios were determined. Loci containing four alleles, three alleles (one known homozygote and one known heterozygote), and two alleles (two known homozygotes) were used to calculate the observed mixture ratios. The same loci, excluding the known homozygote loci, were used to determine the percentage of alleles from the minor contributor that were above stochastic threshold. Percent dropout for the minor contributor was also calculated using loci with four alleles.

The male:male mixtures amplified using Yfiler™ Plus with the previously determined parameters were also run on the 3500 Genetic Analyzer in triplicate. The electropherograms were then analyzed and evaluated to determine the actual mixture ratios. These were calculated by considering all loci with four alleles, three alleles (one known homozygote and one known heterozygote), and two alleles (two known homozygotes). As shown below, the actual ratios were close to their theoretical values (Table 47).

Additionally, the electropherograms were evaluated for percent dropout of the minor contributor to determine at which point major and minor contributors could no longer be distinguished. The maximum ratio at which no allelic dropout of the minor contributor occurred was approximately 10:1/1:10 (Table 47). Thus, the point at which major and minor contributors

can no longer be reliably differentiated when using Yfiler™ Plus is around this ratio, which was also similar to findings from the developmental validation of the kit (15).

Table 47. Yfiler™ Plus Mixture Study Results

Targeted Ratio	Actual Ratio	Percent Dropout
20:1	15.4 : 1	85.2941
16:1	18.0 : 1	50.0000
12:1	15.9 : 1	44.1176
8:1	13.0 : 1	2.9412
4:1	4.8 : 1	*
2:1	1.8 : 1	*
1:1	1 : 1.3	*
1:2	1 : 2.3	*
1:4	1 : 4.7	*
1:8	1 : 9.9	*
1:12	1 : 16.0	2.9412
1:16	1 : 18.8	6.8627
1:20	1 : 21.8	24.4902

*No allelic dropout

Table 47. The mixture samples amplified using Yfiler™ Plus were analyzed and their actual ratios were determined. All loci containing two or more (different) alleles were used to calculate the observed mixture ratios, as well as the percent allelic dropout for the minor contributor.

PrepFiler Express™ and PrepFiler Express BTA™ Verification

PrepFiler Express™

Seven buccal swabs and two semen samples were extracted per manufacturer recommendations using PrepFiler Express™. All extracts were then quantified in triplicate using Quantifiler® Trio, and the results for the small autosomal target were averaged (Table 48). As shown below, all extracts had sufficient DNA when considering the true zero value previously

determined for Quantifiler® Trio. The use of half and whole swab cuttings was also evaluated during this study, and both methods provided sufficient DNA.

Table 48. PrepFiler Express™ Quantification Results

Sample	Quantity (ng/μL)			Average Small Autosomal (ng/μL)
	Small Autosomal	Large Autosomal	Male (Y)	
Buccal Swab 1 (Whole)	15.1770	16.0464	10.3198	17.2395
	17.5086	18.9411	11.8002	
	19.0330	19.3936	11.7821	
Buccal Swab 1 (Half)	6.4685	6.8306	4.3460	6.8986
	7.3316	7.6178	5.1687	
	6.8957	7.2327	4.6812	
Buccal Swab 2 (Whole)	28.1410	26.5203	*	27.8438
	27.2319	26.3666		
	28.1583	27.8122		
Buccal Swab 2 (Half)	27.1227	25.8387	*	27.7432
	27.3645	25.7355		
	28.7424	27.9842		
Buccal Swab 3 (Whole)	32.0920	36.8350	*	34.0386
	35.7110	38.3262		
	34.3129	39.3213		
Buccal Swab 3 (Half)	25.7997	29.9362	*	25.4084
	25.1841	31.2842		
	25.2413	30.5266		
Semen 1	31.6240	46.9574	31.8055	32.6037
	32.5277	49.7516	32.0458	
	33.6594	49.4118	32.6189	
Semen 2	31.6506	46.0634	29.5505	33.2085
	34.3091	48.4292	30.2857	
	33.6656	48.1531	30.4622	
Extraction Positive	14.9475	15.7899	*	14.9475

* Female Donor

Table 48. Seven buccal swabs and two semen samples were extracted using PrepFiler Express™. All extracts were then quantified in triplicate using Quantifiler® Trio, and the results for the small autosomal target were averaged.

PrepFiler Express BTA™

Two cigarette butt, chewing gum, and adhesive tape samples (i.e. casework/forensic samples) were extracted using PrepFiler Express BTA™ under manufacturer recommendations. Each extracted sample was then quantified in triplicate using Quantifiler® Trio, and the small autosomal quantities were averaged (Table 49). The kit obtained an average of 0.92 ng/μL DNA when extracting cigarette butts, an average of 0.75 ng/μL DNA when extracting chewing gum, and an average of 0.12 ng/μL DNA when extracting adhesive tape. Thus, all forensic sample types evaluated using PrepFiler Express BTA™ provided sufficient DNA for downstream testing since up to 10-15 μL of sample can be added during amplification (depending on the kit).

Table 49. PrepFiler Express BTA™ Quantification Results

Sample	Quantity (ng/μL)		Average Small Autosomal (ng/μL)
	Small Autosomal	Large Autosomal	
Cigarette Butt 1	0.8713	0.7134	0.8824
	0.9019	0.7698	
	0.8741	0.7723	
Cigarette Butt 2	0.9724	0.7730	0.9658
	0.9474	0.8442	
	0.9775	0.8363	
Chewing Gum 1	1.2560	0.9952	1.3223
	1.3295	1.0468	
	1.3813	1.0536	
Chewing Gum 2	0.1684	0.1652	0.1822
	0.1918	0.1658	
	0.1864	0.1759	
Tape 1	0.2084	0.1775	0.2208
	0.2076	0.1798	
	0.2465	0.1794	
Tape 2	0.0091	0.0080	0.0093
	0.0092	0.0109	
	0.0098	0.0098	

Table 49. Two cigarette butts, two chewing gums, and two tape samples were extracted using PrepFiler Express BTA™. All extracts were then quantified in triplicate using Quantifiler® Trio, and the results for the small autosomal target were averaged. No male quantification results were observed because all donors were female.

Conclusions

The validation of new instruments and kits is a crucial task within the forensic science community. Although manufacturers developmentally validate these components before they reach the forensic community at large, kits and instruments must also be internally validated at each individual lab before they can be utilized; this ensures that the kit or instrument will perform optimally and as expected within that particular lab. Furthermore, this project aimed to validate all aforementioned kits for the purposes of future accreditation and reliability of results. Not only did this project validate many processes within the Biology and DNA sections of the Science Research Laboratory at the Dubai Police Headquarters, but it also provided a roadmap for other developing labs seeking accreditation and pushed the forensic science community one step closer to international congruency.

Overall, the results showed that Quantifiler® Trio could provide repeatable and reproducible results. In addition, the true zero value was determined to be 0.007 ng/μL, meaning usable profiles could not be obtained at or below this quantity. When considering the shelf life of the standards, it was decided they should only be used up to one week after preparation to ensure reliability of quantification results. Quantifiler® Trio has the ability to serve as a screening tool for male:female mixtures, and this project ultimately revealed that mixture samples where the female is the major contributor can be more reliably detected than samples where the male is the major contributor.

Evaluation of the capillary array revealed that the lab could reproduce low variance in both base pair sizing (±0.5 bp with a standard deviation of ≤0.15 bp) and RFU (≤25 %CV). Prior to the use of any new capillary array, similar tests should be conducted to show that sizing remains within ±0.5 bp and ≤30 %CV. After demonstrating the reliability of the array, the

validation of the GlobalFiler™, GlobalFiler™ Express, and Yfiler™ Plus amplification kits was accomplished. Upon optimization of the GlobalFiler™ parameters, it was determined usable profiles (following SWGDAM recommendations) could be obtained with 3.0 – 0.2 ng of input DNA and optimal profiles could be obtained with an input of 1.5 – 0.2 ng of DNA. In addition, mixture ratios up to 14:1/1:14 could be reliably discerned when using the GlobalFiler™ kit. For Yfiler™ Plus, usable profiles (per SWGDAM recommendations) could be obtained with 6.0 – 0.2 ng of input DNA and optimal profiles could be obtained with an input of 1.5 – 0.35 ng of DNA. Mixture ratios of up to 10:1/1:10 when using optimal PCR conditions for Yfiler™ Plus could be reliably distinguished. Both buccal samples and blood samples on FTA were validated for direct amplification with the use of the GlobalFiler™ Express kit, with buccal samples producing better profiles. The optimal parameters for each amplification kit were displayed in Table 50 below.

Table 50. Amplification Kit Parameters

Amplification Kit	Analytical Threshold (RFU)	Stochastic Threshold (RFU)	PCR Cycles	Injection Time (s)	Injection Voltage (kV)	DNA Input (ng)	
						Usable Profile	Optimal Profile
GlobalFiler™	60	250	29	15	1.2	3.0 – 0.2	1.5 – 0.2
GlobalFiler™ Express (Buccal)	200	300	26	15	1.2	N/A	N/A
GlobalFiler™ Express (FTA)	55	110	25	20	1.2	N/A	N/A
Yfiler™ Plus	75	N/A	30	16	1.5	6.0 – 0.2	1.5 – 0.35

Various reference samples were extracted per manufacturer recommendations using PrepFiler Express™, while various forensic samples (cigarette butt, chewing gum, and adhesive tape) were extracted with PrepFiler Express BTA™. Upon quantification, all extracts contained sufficient DNA when considering the true zero value previously determined for Quantifiler® Trio; therefore, both extraction kits and protocols were deemed acceptable for use within the lab.

While this project met the ISO/IEC 17025:2005 requirements for validation, it did not encompass other commonly performed tests. These tests, such as the contamination and concordance studies required by the FBI's QAS guidelines, should be explored in future projects. In addition, data related to internal stutter percentages for this lab should be compiled from the results within this project to serve as a quick reference during data analysis. Although the mixture studies within this project evaluated two-person mixtures, future studies should assess the ability of each amplification kit to decipher three-person mixture samples. Development of a differential separation procedure when using the PrepFiler Express™ kit, as well as an evaluation of the ability of Quantifiler® Trio to detect degraded samples, could also be performed. Finally, with the increasing sensitivity of kits and instruments, as well as the rising use of touch DNA within the forensic community, future studies should also aim to validate smaller volume amplification reactions to target these sample types and improve results.

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