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It is common in the forensic science community to have standardization and uniformity in all laboratory processes. The method for the determination of a minimum detection threshold or synonymously an analytical threshold for genetic analysis is not uniform across forensic labs. Variation amongst the methods in DNA testing by forensic laboratories leads to variations in the results of the DNA testing.

The results of this study show a method using DNA sample types versus non-DNA sample types will better reflect the effects of baseline noise that may be encountered in forensic casework samples. In addition, there is a need for a calculation method to be designated as an appropriate tool in determining analytical thresholds. More studies on baseline noise and methods in distinguishing analytical thresholds will help in the determination of the most appropriate calculation method to be used across all forensic laboratories.

EVALUATING NOISE AND THE IMPLICATIONS OF METHODOLOGY
IN THE ANALYTICAL THRESHOLD DESIGNATION
FOR FORENSIC GENETIC ANALYSIS

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# EVALUATING NOISE AND THE IMPLICATIONS OF METHODOLOGY IN THE ANALYTICAL THRESHOLD DESIGNATION FOR FORENSIC GENETIC ANALYSIS

THESIS

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By

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# CHAPTER I

## INTRODUCTION

Forensic DNA (deoxyribonucleic acid) analysis is considered to be the "gold standard" of forensic testing. Its level of objectivity, scientific integrity, and value in the forensic community is the reason for its high standing. This technique has been utilized in law enforcement and the legal system to make human identifications and connecting individuals to a crime or crime scene. Forensic DNA typing is one of many disciplines in forensic science, which include areas such as ballistics, toxicology, and anthropology just to name a few. While forensic DNA analysis is considered to be the "gold standard" in forensic testing, it is simply a valuable tool amongst others that can be utilized together in the investigation and building of a case by the legal system (1-3).

It was Alec Jeffries in 1984 that first described the technique of DNA typing and its ability to distinguish between individuals barring identical twins. Over the years forensic DNA typing techniques have developed with the advancement of scientific research and technology. The process of forensic DNA typing includes the identification of a biological sample and extraction of the DNA, followed by quantification and amplification of the sample, and then separation and detection of DNA fragments for analysis in generating a DNA profile. The genetic markers utilized for generating a profile are short tandem repeats (STRs), which are genetic sequences containing a variable number of tandemly repeated short sequences. There are

two types of STRs utilized in forensics, autosomal STRs and Y-STRs. Autosomal STRs are inherited equally from both parents of an individual and Y-STRs are inherited strictly from the paternal line. It is the variability of autosomal STRs that gives a high discrimination power for individualization. While Y-STRs are not individualizing they are useful in the realm of forensics for determining paternal lineages and specifically distinguishing male DNA (2-4).

While STRs are the predominant genetic testing markers for forensic DNA analysis, there are other genetic markers that are being studied and developed for use in forensic genetic analysis. Indels are considered one of these markers. Indels are bi-allelic markers that are simply defined as an insertion or deletion of a segment of DNA. The purpose of these markers is to be a helping tool for challenging forensic samples, such as degraded DNA samples. Like STRs, Indels are a length polymorphisms which allows them to be tested and typed in the same manner as STRs. It is because of this that the development of new forensic DNA testing kits are able to incorporate indels into their STR based systems (4, 5).

Targeting these DNA fragments to generate a DNA profile requires the testing procedure of DNA typing. The first step in this procedure is the identification of a biological fluid. The purpose of this step is to make sure that the unknown samples that are being tested are actually of biological material. The common biological samples tested in a forensic laboratory can include blood, saliva, and semen. Once the sample has been identified as biological then the extraction process is performed. Extraction is the process of separating the nuclear material from the remaining components of the cell, in order to isolate the DNA. There is a secondary extraction procedure known as differential extraction that can be utilized if necessary. Differential extraction is used primarily in sexual assault cases, and the testing procedure allows for the separation of sperm cells from non-sperm cells. This allows for the separation of male and

female DNA in a mixed sample. After extraction forensic laboratories proceed to a quantification step which determines the amount of amplifiable target DNA. The quantification step is useful in determining the quality and quantity of the DNA sample that the lab is testing. This knowledge allows the lab to optimize the amplification reaction by normalizing the DNA sample to the target concentration for amplification. Amplification is a process that utilizes polymerase chain reaction (PCR) technology to amplify target DNA regions for forensic testing. PCR is a process of multiple heating and cooling cycles that uses a polymerase enzyme to replicate and make copies of the target DNA regions. Multiple DNA regions can be targeted in a single reaction by using commercially available multiplexing kits. The kits utilize a variety of oligonucleotide primer pairs which are specific to a region of interest. The primer pairs frame the target region to be amplified by the polymerase. After multiple cycles millions of copies, or synonymously amplicons or fragments, have been generated. Due to the magnitude of copies, the DNA regions can easily be detected and measured (2, 4, 6).

Separation and detection by capillary electrophoresis (CE) is the current technology performed in forensic labs. The genetic analyzers that utilize CE technology separate and detect the target DNA regions by using a fluorescence-based detection system. During PCR each primer pair is labeled with a fluorescent dye that is associated with its specific target DNA region. During capillary electrophoresis the fluorescently labeled amplicons travel through the capillary by charge, with the smallest amplicons passing through the capillary followed by amplicons increasing in size. The size of the amplicon travelling is mediated by the polymer sieving medium, which allows the smaller amplicons to travel easier through the capillary to the detection window than the larger amplicons. This allows the genetic analyzer to separate the DNA target regions by size. Detection of the amplicon occurs when the fluorescent dye reaches

the detection window and is excited by an argon laser which causes the dye to fluoresce. The light intensity emitted by the fluorescent dye is detected and measured by the charge-coupled device (CCD). The measured signal is then recorded as an arbitrary unit of measurement known as a relative fluorescence unit (RFU). There are different fluorescent dyes that are used to label the amplicons, and the different dyes emit light at specific wavelengths that correspond to visible color. The genetic analyzer uses time, size and dye color to identify and size the amplicons by comparing the amplicon with the known internal size standard and associating it to the allelic ladder (4, 6).

It is during this process when the genetic analyzer also detects signal that is not associated with the target DNA regions, and this signal is classified as background or baseline noise. The genetic analyzer reports detected signal as RFUs and visually displays the data as an electropherogram. The target DNA regions from the DNA typing process are STR alleles, and in the electropherogram the STR alleles are visualized as peaks. These peaks are what make up an individual's genetic profile. It is from the electropherograms that a DNA analyst evaluates and interprets the genetic data to ensure that the science and technology is accurately representing the genetic profile. Electropherograms can contain peaks that are not the targeted DNA regions. The source of the peaks can occur due to a variety of factors and have been well characterized by scientific research and defined as artifacts. Some examples of artifacts include stutter, pull-up, and dye blobs to name a few. Experience and knowledge of artifacts allow DNA analysts to interpret the profile to determine accuracy. Interpretation is based upon thresholds and guidelines that are set forth by a laboratory (4, 6).

The purpose of setting thresholds and determining limits is to obtain objective measures of detection and quantification for analytical processes. This concept has been reviewed in the

world of analytical chemistry for years, and it has long been a topic of discussion due to the widespread terminology and methodology of threshold determination. Terms such as limit of detection, limit of quantification, minimum distinguishable signal, and limit of purity all have been vaguely defined and thus used inconsistently. The need for a uniform concept in threshold determination was important and recognized by the International Union of Pure and Applied Chemistry (IUPAC) and International Organization for Standardization (ISO). IUPAC termed the limit of detection as the "smallest measure that can be detected with reasonable certainty for a given analytical procedure". The limit of detection should be based on signal-to-noise analyses. In forensic DNA analysis the concept of a detection threshold has been adopted for the interpretation of genetic data by defining an analytical threshold (7-9).

The initial threshold that must be determined for interpretation purposes in forensics is the analytical threshold. As defined by the SWGDAM Interpretation Guidelines the analytical threshold is "the minimum height requirement at and above which detected peaks can be reliably distinguished from background noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles." (10) Background or baseline noise is characterized as signal that is not associated with amplified DNA. Baseline noise can be affected by a variety of factors such as impure reagents, temperature fluctuations, instrument alignment, polymer condition, fluorescent dye, and etcetera. Due to the plethora of factors that can influence the baseline noise, the establishment of an analytical threshold is usually run specific. During detection by CE, baseline signal is detected and generally has low RFU values compared to alleles which have substantially higher RFU values (8, 11, 12). The difference in RFU values between baseline noise and true peaks allow for a threshold to be set at a certain RFU value that anything at or above that value can be deemed a true peak (6). The method for determining an

analytical threshold for forensic DNA analysis varies amongst laboratories but according to SWGDAM guideline 1.1 the threshold should be established based on signal-to-noise analyses (10). There are a variety of methods that have the ability to assess signal-to-noise, which have been discussed and reviewed in the realm of analytical chemistry (7-9). In the forensic community it has been deemed appropriate to establish an analytical threshold based on the assessment of negative or blank samples, and it has recently been argued that this might not be the most appropriate assessment of noise in forensic DNA analysis. There is evidence that the analyte or DNA of a sample affects the baseline noise. Methods comparing the use of DNA and non-DNA methods are being studied to determine which method type is the most appropriate for forensic casework. Bregu et al. outlines six different methods for calculating an analytical threshold, four utilizing the non-DNA method and two utilizing the DNA method. Grgicak outlines five different methods, three utilizing the non-DNA method and two utilizing the DNA method, including the method proposed by the SWGDAM guidelines (12, 13). Gilder et al. approaches the analytical threshold by determining whether the limit of detection (LOD) or the limit of quantification (LOQ) is an appropriate method in forensic casework for the establishment of the analytical threshold. The findings of these studies suggest that baseline noise is affected by run-specific procedures and an analytical threshold should be determined for each procedure in a lab. Gilder et al. defines LOD as the average amount of baseline noise ($\mu_b$) plus three standard deviations ($\sigma_b$)

$$LOD = \mu_b + 3\sigma_b$$

where this equation was presented by Kaiser for the detection limit . The LOQ is expressed as the average amount of baseline noise ($\mu_b$) plus ten standard deviations ($\sigma_b$)

$$LOQ = \mu_b + 10\sigma_b$$

where this equation was presented by IUPAC for the quantification limit (7, 8, 11). The LOD is a calculation that was also tested by Bregu et al. and Grgicak as a non-DNA method (12, 13). These studies demonstrate that the method for calculating an analytical threshold can have a wide range of threshold settings depending on the laboratory procedure and calculation method. In the Gilder et al. study the LOD, analytical thresholds ranged from 10.9-53.0 RFUs (11). Grgicak demonstrates a range of 7.0-39.0 RFUs using the same procedural process but utilizing different calculation methods (13). The calculation method can impact the threshold level, Bregu et al. reports threshold calculations ranging from 3-199 RFUs based on differing calculation methods for the same data (12). There are three calculation methods that both Grgicak presents and Bregu et al. studied which include Kaiser's LOD non-DNA method, IUPAC's critical value non-DNA method, and IUPAC's DNA method. IUPAC's non-DNA method is "the minimum significant value of an estimated net signal or concentration, applied as a discriminator against background noise" and is represented as

$$AT = \overline{Y}_{bl} + t_{1-\alpha,\nu}\frac{s_{bl}}{\sqrt{n}}$$

where the average amount of baseline noise ($\overline{Y}$) plus the critical value ($t_{1-\alpha,\nu}$) multiplied by the estimated standard deviation over the number of replicates determines the analytical threshold (AT). IUPAC's DNA method utilizes the "relationship between RFU and input DNA" over a DNA input dilution series and is represented as

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

where the y-intercept of the regression (b) plus the critical value ($t_{n-1,\alpha}$) multiplied by the standard error of regression ($S_y$) determines the AT (7, 12, 13). This equation assumes that the y-intercept of the linear regression of DNA is not different than the average baseline noise.

The SWGDAM guidelines state that the "analytical threshold may be based on two times the intensity difference the highest peak and the lowest trough within the instrumental noise data" and can be represented as

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

where the highest peak ($Y_{max}$) minus the lowest trough ($Y_{min}$) multiplied by two determines the AT (10, 13).

There are a variety of methods to calculate an analytical threshold for forensic laboratories and depending on the method chosen the interpretation of genetic data can differ. The SWGDAM Interpretation Guidelines define what an analytical threshold is, but it leaves the methodology in establishment up to the laboratory by stating "other scientific methods may be used" (10). The guidelines push for a standard operating procedure for the interpretation of genetic data in order to create greater consistency and accuracy in interpretations by DNA analysts within a lab. Following the SWGDAM guidelines pushes for standardization not only within labs but across labs. Forensic DNA laboratories are mandated by congress to follow the FBI Quality Assurance Standards. Standard 8.3.2 states that "internal validation shall define quality assurance parameters and interpretation guidelines" (14) and guidelines set forth by organizations such as SWGDAM play a role in standardization across the forensic DNA community (4). Standardization across forensic laboratories allows for the field to be less subjective and more objective.

It is common in the forensic science community for there to be standardization and uniformity in all laboratory processes. While most laboratory procedures are standardized, the method for the determination of a minimum detection threshold or synonymously an analytical

threshold for genetic analysis is not uniform across forensic labs. The analytical threshold is defined by the Scientific Working Group on DNA Analysis Methods (SWGDAM) as "the minimum height requirement at and above which detected peaks can be reliably distinguished from background noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles."(10) Forensic laboratories can have a range of analytical thresholds anywhere from 50-200 relative fluorescence units (RFUs) (11), and based on the procedure and method chosen to determine an analytical threshold an individual laboratory can have a threshold ranging between 3-199 RFUs (12). Changes in procedural conditions can affect the baseline noise associated with genetic analysis, ultimately effecting the determination of an analytical threshold for a particular lab. The SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories states in guideline 1.1 that "the laboratory should establish an analytical threshold based on signal-to-noise analyses" and gives an example of a method, but also states that other methods can be used (10). This approach allows for the determination of an analytical threshold to become subjective, and the goal of forensic DNA analysis is to be as objective as possible.

There are three aims of this study. The first aim is to determine the effects of specific parameters and variables of a DNA typing procedure, utilizing the Applied Biosystems GlobalFiler® Express PCR Amplification Kit, on baseline noise. The second aim is to demonstrate the variability of results associated with differing calculation methods in the determination of an analytical threshold. The third aim is to determine the most appropriate method in distinguishing an analytical threshold as it relates to forensic DNA analysis and interpretation. The hypothesis of this study is that baseline noise is affected by forensic DNA

typing procedure variables, and the most reliable method of distinguishing a true analytical threshold is to utilize DNA based calculation methods.

# CHAPTER II

# MATERIALS AND METHODS

## Sample Collection

Buccal swabs were obtained from three consenting participants, two female and one male. Samples were labeled with a de-identifier, Table 1. Each subject donated two buccal swabs, one swab was used to make a sample dilution series and the second swab was used to perform a direct amplification procedure. Once the samples were collected a DNA extraction procedure was performed on one of the buccal swabs from each subject and the second buccal swab was stored until direct amplification.

| Subject | Sample Type | Sample ID | Male/Female |
|---|---|---|---|
| 1 | Buccal Swab | 050815AM01 | Female |
| 2 | Buccal Swab | 050815AM02 | Female |
| 3 | Buccal Swab | 050815AM03 | Male |

**Table 1: Buccal swabs utilized in this study.** Swabs were collected and assigned a de-identifying alphanumeric sequence and designated as male or female for analysis purposes.

## DNA Extraction

DNA extraction was performed following the University of North Texas Center for Human Identification (UNTCHI) procedure "Organic DNA Extraction: Blood, Tissue and Other Biological Material" (15). The organic extraction procedure was performed on one swab from each subject. All tools and equipment were properly cleaned with a bleach solution to prevent

any contamination. The buccal swabs were cut in half and placed in individual microcentrifuge tubes. Half of the swab was consumed in the extraction process, and a reagent blank was processed with each sample. There are three parts to the organic extraction procedure. The first part of the process was a cell lysis and protein digestion step, followed by the Phenol-Chloroform-Isoamyl Alcohol (PCIA) Extraction, and finished with an ethanol precipitation step. Cell lysis and protein digestion involved adding Stain Extraction Buffer Working Solution and Proteinase K to each sample and samples to be processed as reagent blanks. The samples were then vortexed and centrifuged before being placed on a heat block set at 56°C for a 24 hour incubation period. After the incubation period the substrate was removed from the tubes and the samples went through the PCIA process. Equal volume of PCIA was added to each tube and were then vortexed and centrifuged to create an organic and non-organic layer. The organic layer was discarded from the tubes leaving the extracted DNA. The ethanol precipitation step involves a two-step process of adding different concentrations of ethanol to the tubes followed by centrifugation and then discarding of the ethanol. After the ethanol process, resolubilization of the DNA was done with molecular grade water and an incubation at 56°C for two hours. The volume recovered after the final step was 100µl for each of the six samples, which includes the reagent blanks. After the final incubation step samples were stored at 4°C until quantification was performed.

Quantification and Sample Dilution Series Preparation

Quantification was performed following the UNTCHI procedure "Human and Male DNA Quantification using Applied Biosystems Quantifiler® Duo Kit" (16). All tools and equipment were properly cleaned with a bleach solution to prevent contamination. Extracted samples were removed from the storage at 4°C to be quantified using Applied Biosystems

Quantifiler® Duo DNA Quantification Kit. The first part of the quantification process was to prepare the DNA quantification standards. Eight standards were prepared ranging in concentration from 50ng/µl to 0.023ng/µl by adding the appropriate amounts of DNA Dilution Buffer and Human Male DNA Standard. A stock solution of 50ng/µl was made for Standard 1 and a dilution series was performed to make standards 2-8. Once the standards were prepared, the master mix for the optical reaction plate was prepared for 30 reactions. Each reaction in the master mix includes 10.5µl of Primer Mix and 12.5µl of Reaction Mix. Then 23µl of the master mix was dispensed into each reaction well on the plate. Once the master mix was plated, 2µl of standard, sample, and controls were plated into the appropriate wells, and then the plate was sealed and centrifuged to be run on the 7500 SDS instrument. A plate document was created and the appropriate instrument parameters were set, and the reaction plate was processed on the 7500 SDS instrument. After the run was complete the results were analyzed. The standard curves for both male and human were evaluated and the slope, Y-intercept and $R^2$ values fell into the correct ranges without omitting any of the standards, allowing for a successful quantification of the samples. The quantification values of the samples were then utilized to prepare a dilution series for the amplification process. Each of the three samples were prepared as a dilution series to yield final total input DNA of 2ng, 1ng, 0.5ng, 0.25ng, 0.125ng, and 0.0625ng.

Amplification

Applied Biosystems GlobalFiler® Express PCR Amplification Kit was utilized for the amplification process, and the Applied Biosystems "GlobalFiler® Express PCR Amplification Kit User Guide" (17) was followed in performing the amplification procedure. The second buccal swab that was collected from each of the subjects during sample collection was used to perform direct amplification. Each swab head was detached and placed in individual tubes with

Prep-N-Go™ Buffer and incubated at room temperature for 20 minutes for lysis. After incubation the lysate was transferred to new individual tubes for the next step of the amplification process. During incubation the master mix with additive was prepared for the amplification reaction plates. The master mix with additive included master mix, master mix additive and primer set for 126.5 reactions, with 6µl of master mix/master mix additive and 6µl of primer set in each reaction. Master mix with additive was dispensed into the appropriate wells of the reaction plates, 12µl in each well. Then the appropriate volumes of Prep-N-Go™ Buffer, direct samples, extracted samples, controls and reagent blanks were plated for a final volume of 15µl per well. Each extracted sample dilution series and the direct samples were run in quadruplicate along with the reagent blanks, there were 2 DNA control 007 positive controls, and 17 Prep-N-Go™ negative controls for a total of 115 samples. The reaction plates were prepared for placement on the thermal cycler and the parameters were set for an initial incubation at 95°C for 1 minute, 28 cycles of 94°C for 3 seconds and 60°C for 30 seconds, a final extension at 60°C for 8 minutes and then to hold at 4°C until electrophoresis.

Capillary Electrophoresis and Genetic Analysis

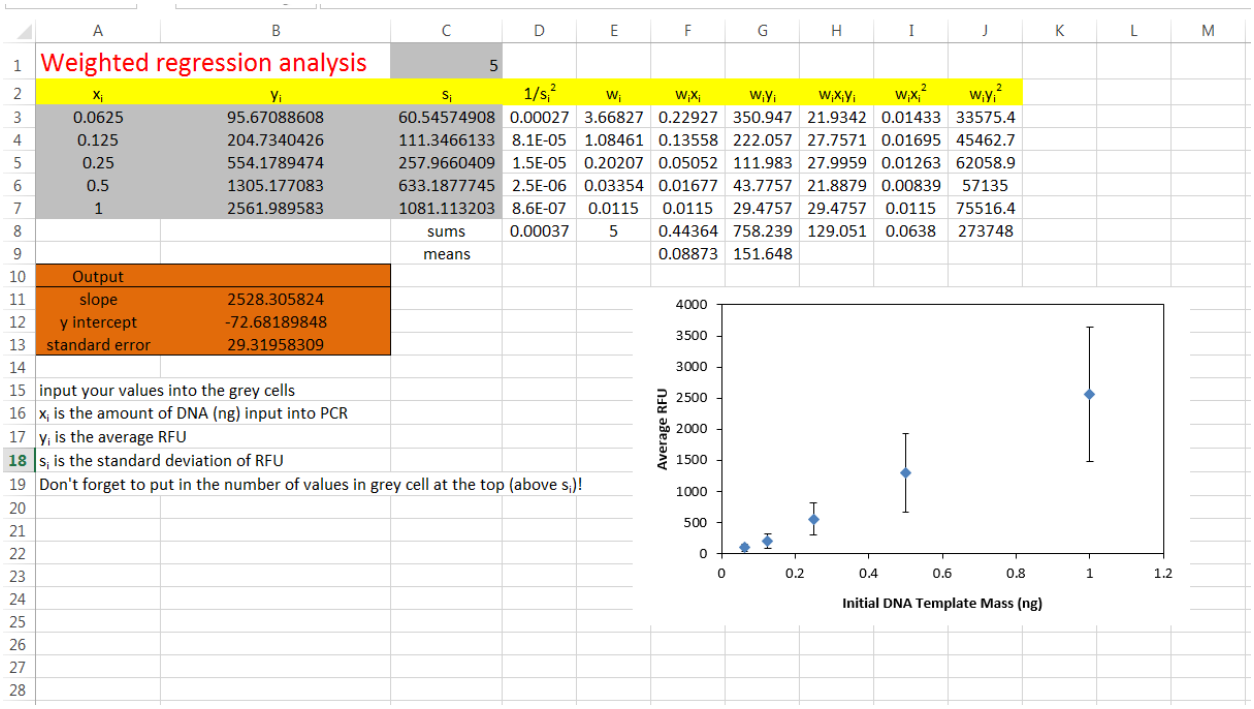
Electrophoresis and genetic analysis were performed following the Applied Biosystems "GlobalFiler® Express PCR Amplification Kit User Guide" (17) and the "Internal validation of the GlobalFiler™ Express PCR Amplification Kit for the direct amplification of reference DNA samples on a high-throughput automated workflow" (18) by Flores et al. Capillary electrophoresis (CE) plates were set up by preparing the master mix for 132 reactions, each reaction included 0.5µl of GeneScan™ 600 LIZ® Size Standard v2.0 and 9.5µl of Hi-Di™ Formamide. Each well was dispensed 10µl of the master mix and then 1µl of PCR product or Allelic ladder was added to the appropriate wells. The plate was sealed with a septa, vortexed

and centrifuged, heated on a thermal cycler for 3 minutes at 95°C, and then snap cooled on ice for 3 minutes. Once cooled the plate was placed on the 3500xL genetic analyzer and run under the conditions of HID36_POP4 run module, 1.2 kV/12 sec injection conditions, 13 kV/1550 sec run conditions, and Dye Set J6. The 12 second injection time was a modification from the user guide based on the internal validation by Flores et al. Once the run was complete the data was collected from the software and imported to GeneMapper® ID-X v1.4 for further analysis.

Data Analysis

All samples were analyzed with an analysis method with a peak detection threshold set at 1 relative fluorescence unit (RFU). The results were exported to Microsoft® Excel 2013 for evaluations and calculations of baseline noise and analytical thresholds. The evaluations and calculations were made based on calls between 60-400 base pair sizes for each sample and dye channel due to the expected size range of the GlobalFiler® Express PCR Amplification Kit. The average peak height and standard deviation was calculated for non-DNA samples, negative controls and reagent blanks, for each of the 5 dye channels. The same was done for the DNA samples per total DNA input, with peaks from alleles and PCR artifacts (forward/reverse stutter, pull-up, etcetera) removed before the average and standard deviation peak heights were calculated. The average peak heights and standard deviations of the true alleles were calculated for each dye channel per total DNA inputs ranging from 0.0625ng-1ng, any homozygous peaks were divided in two for this calculation. The averages and standard deviations were inputted into the weighted regression template (13) shown in figure to determine the y-intercept and standard error for the calculation of equation 6 in Table 2, with a t-stat of 3.75 (n-1=4, alpha 99%). The results from the average and standard deviation calculations as well as the weighted regression

analysis were used to determine analytical threshold values based on the calculation methods summarized in Table 2.



| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Weighted regression analysis | | 5 | | | | | | | |
| 2 | $x_i$ | $y_i$ | $s_i$ | $1/s_i^2$ | $w_i$ | $w_ix_i$ | $w_iy_i$ | $w_ix_iy_i$ | $w_ix_i^2$ | $w_iy_i^2$ |
| 3 | 0.0625 | 95.67088608 | 60.54574908 | 0.00027 | 3.66827 | 0.22927 | 350.947 | 21.9342 | 0.01433 | 33575.4 |
| 4 | 0.125 | 204.7340426 | 111.3466133 | 8.1E-05 | 1.08461 | 0.13558 | 222.057 | 27.7571 | 0.01695 | 45462.7 |
| 5 | 0.25 | 554.1789474 | 257.9660409 | 1.5E-05 | 0.20207 | 0.05052 | 111.983 | 27.9959 | 0.01263 | 62058.9 |
| 6 | 0.5 | 1305.177083 | 633.1877745 | 2.5E-06 | 0.03354 | 0.01677 | 43.7757 | 21.8879 | 0.00839 | 57135 |
| 7 | 1 | 2561.989583 | 1081.113203 | 8.6E-07 | 0.0115 | 0.0115 | 29.4757 | 29.4757 | 0.0115 | 75516.4 |
| 8 | | | sums | 0.00037 | 5 | 0.44364 | 758.239 | 129.051 | 0.0638 | 273748 |
| 9 | | | means | | | 0.08873 | 151.648 | | | |
| 10 | Output | | | | | | | | | |
| 11 | slope | 2528.305824 | | | | | | | | |
| 12 | y intercept | -72.68189848 | | | | | | | | |
| 13 | standard error | 29.31958309 | | | | | | | | |
| 14 | | | | | | | | | | |
| 15 | input your values into the grey cells | | | | | | | | | |
| 16 | $x_i$ is the amount of DNA (ng) input into PCR | | | | | | | | | |
| 17 | $y_i$ is the average RFU | | | | | | | | | |
| 18 | $s_i$ is the standard deviation of RFU | | | | | | | | | |
| 19 | Don't forget to put in the number of values in grey cell at the top (above $s_i$)! | | | | | | | | | |



**Figure 1: Image of weighted regression template for Microsoft Excel.** The weighted regression template is made available by Boston University through the ISHI 2010 Mixture Workshop presentation by Catherine M. Grgicak.

| **Non- DNA Calculation Methods** | | **DNA Calculation Methods** | |
|---|---|---|---|
| 1 | $LOD = \mu_b + 3\sigma_b$ | 4 | $LOD = \mu_b + 3\sigma_b$ |
| 2 | $LOQ = \mu_b + 10\sigma_b$ | 5 | $LOQ = \mu_b + 10\sigma_b$ |
| 3 | $AT^1 = 2(Y_{max} - Y_{min})$ | 6 | $AT^2 = b + t_{n-1,\alpha}S_y$ |

**Table 2: Summarized Table of Analytical Threshold Calculation Methods used in this study.** The limit of detection (LOD) and limit of quantification (LOQ) are used for both methods by utilizing the mean and standard deviation from the appropriate data sets. Calculation method 3 is the SWGDAM recommended calculation method. Calculation method 6 is the analysis based on a weighted regression of allele peak heights.

# CHAPTER III

## RESULTS

Figures 2-6 are visual representations of the detected baseline peak heights for each peak per dye channel used to calculate the average and standard deviation for the non-DNA samples. For all dye channels the peak heights seem to be equally distributed between the 60-460 base pairs.

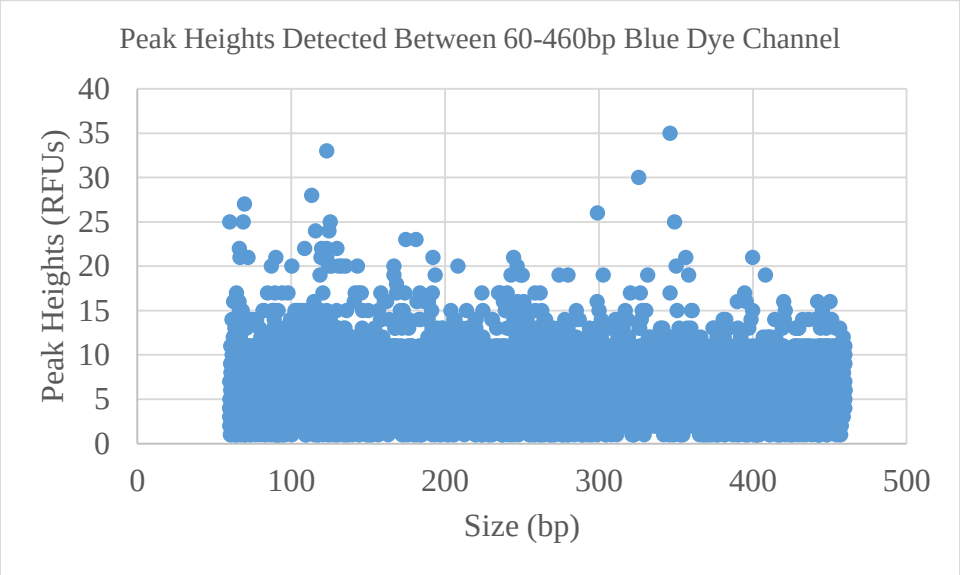



**Figure 2: Peak Heights Detected Between 60-460bp Blue Dye Channel.** All peak heights detected above the 1 RFU threshold for the blue dye channel of the non-DNA samples between 60-460 base pairs (bp).

In Figure 2, the baseline noise peak heights for the blue dye channel of the non-DNA samples do not exceed 40 RFUs. The green dye channel peak heights in Figure 3 do not exceed 70 RfUs. The peak heights in the yellow dye channel do not exceed 90 RFUs (Figure 4), the red dye channel peaks do not exceed 50 RFUs (Figure 5), and the purple dye channel's peaks do not exceed 80 RFUs (Figure 6).

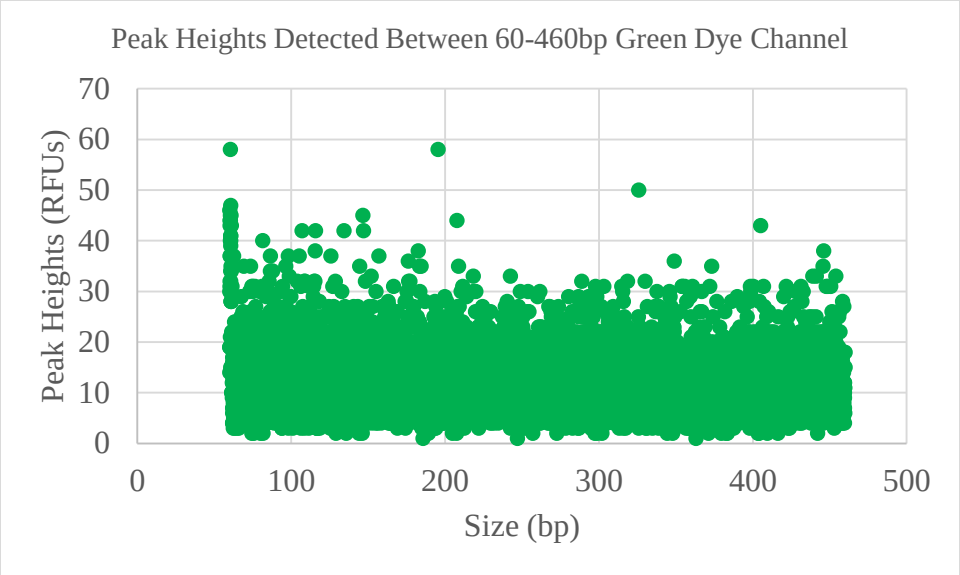



**Figure 3: Peak Heights Detected Between 60-460bp Green Dye Channel.** All peak heights detected above the 1 RFU threshold for the green dye channel of the non-DNA samples between 60-460 base pairs (bp).

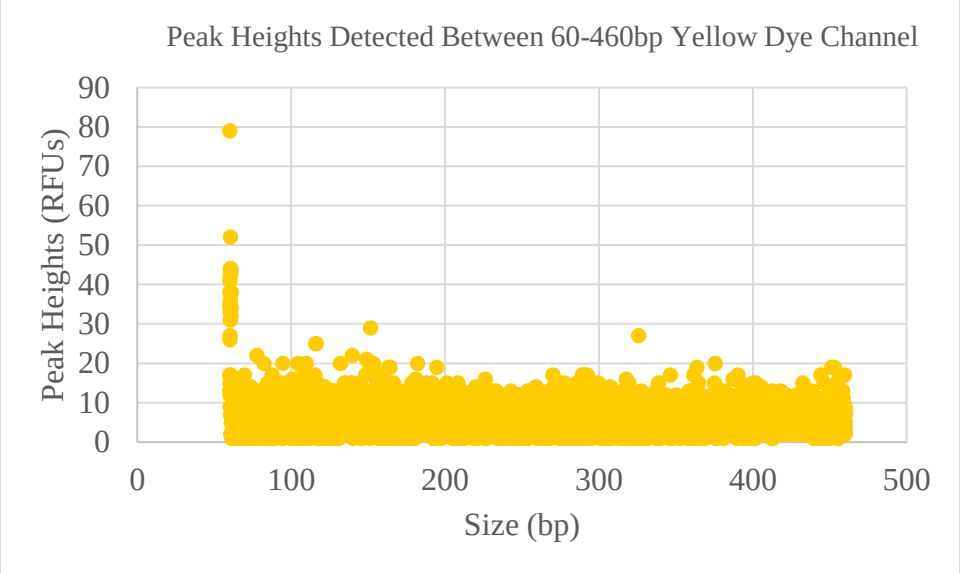



**Figure 4: Peak Heights Detected Between 60-460bp Yellow Dye Channel.** All peak heights detected above the 1 RFU threshold for the yellow dye channel of the non-DNA samples between 60-460 base pairs (bp).

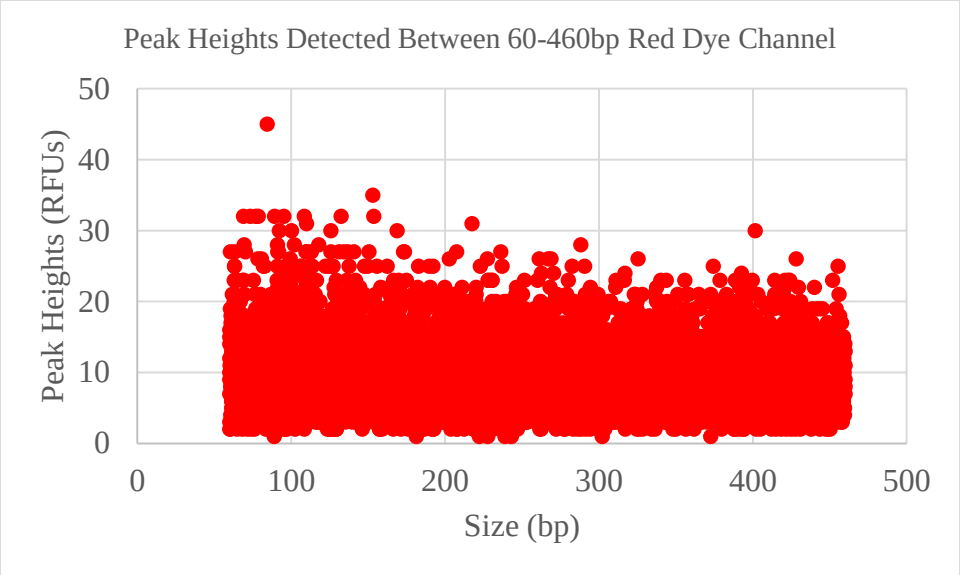



**Figure 5: Peak Heights Detected Between 60-460bp Red Dye Channel.** All peak heights detected above the 1 RFU threshold for the red dye channel of the non-DNA samples between 60-460 base pairs (bp).

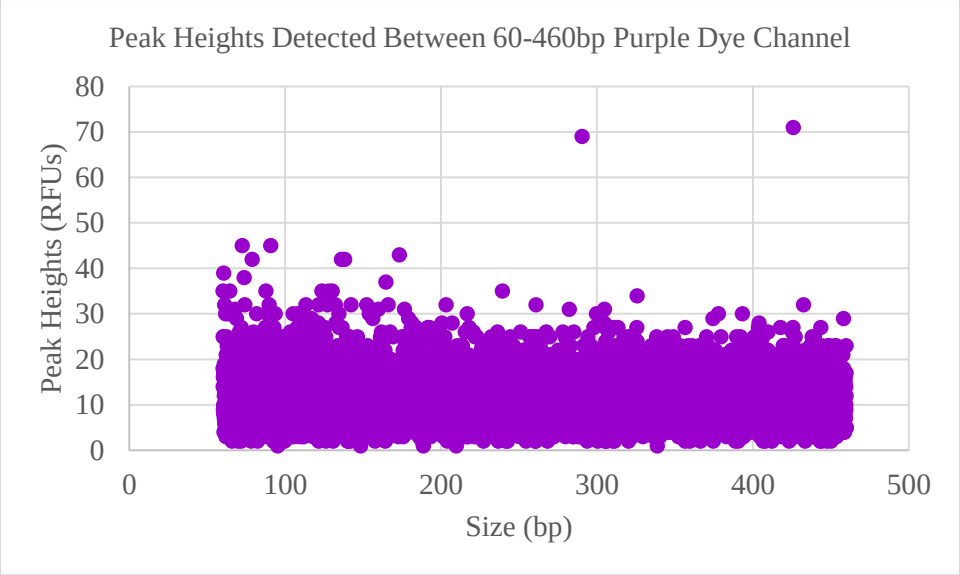



**Figure 6: Peak Heights Detected Between 60-460bp Purple Dye Channel.** All peak heights detected above the 1 RFU threshold for the purple dye channel of the non-DNA samples between 60-460 base pairs (bp).

The calculations of the average (mean) and standard deviation for each dye channel of the non-DNA samples are summarized in Table 3.

| Analysis | Dye Channel | Blue | Green | Yellow | Red | Purple |
|---|---|---|---|---|---|---|
| **AVG** | | 6.08406 | 12.46705 | 5.826512 | 9.815289 | 11.13017 |
| **STD** | | 3.082767 | 5.5444 | 3.264239 | 4.365599 | 5.056398 |

**Table 3: Average and Standard Deviation of non-DNA sample peak heights in RFUs.** The average (AVG) and standard deviation (STD) were calculated for each dye channel of the non-DNA samples.

The peak height average values for the non-DNA samples range between 5.826512 RFUs and 12.46705 RFUs across all the dye channels. The green dye channel is considered to be the noisiest dye channel because it has the greatest peak height average.

The calculations for average (mean) and standard deviation were calculated for each dye channel of each total input DNA of the DNA samples and are summarized in Table 4. The peak height average values for the DNA samples range between 5.445352 RFUs and 13.17742 RFUs across all the dye channels and total DNA input. The green dye channel is consistent with the non-DNA method as the noisiest dye channel. There are slight differences in the peak height averages between dye channels where the average RFU values differences range between 0.257549 RFUs and 6.640543 RFUs. While the differences in the peak height averages amongst the dye channels does not exceed 2 RFUs.

| ***Dye Channel*** | | ***Blue*** | ***Green*** | ***Yellow*** | ***Red*** | ***Purple*** |
|---|---|---|---|---|---|---|
| ***Total DNA Input*** | ***Analysis*** | | | | | |
| **Direct Reference** | **AVG** | 7.165673 | 13.17742 | 7.212259 | 10.72986 | 11.93672 |
| | **STD** | 4.878961 | 6.640434 | 5.684439 | 6.051617 | 5.869475 |
| **2 ng** | **AVG** | 5.699133 | 12.00589 | 5.99845 | 9.801605 | 11.14221 |
| | **STD** | 3.744448 | 5.904846 | 3.620998 | 4.890956 | 5.244241 |
| **1 ng** | **AVG** | 5.445352 | 11.59509 | 5.69994 | 9.42446 | 10.6278 |
| | **STD** | 2.750631 | 4.735798 | 2.983054 | 4.313983 | 4.659971 |
| **0.5 ng** | **AVG** | 5.590622 | 11.62912 | 5.687769 | 9.125513 | 10.61423 |
| | **STD** | 2.863524 | 4.720202 | 2.873337 | 3.936958 | 4.520876 |
| **0.25 ng** | **AVG** | 5.899251 | 12.3254 | 6.025627 | 9.857381 | 11.55909 |
| | **STD** | 2.558284 | 4.384768 | 2.618662 | 3.816666 | 4.196701 |
| **0.125 ng** | **AVG** | 6.495781 | 13.53386 | 6.383202 | 10.55879 | 12.05077 |
| | **STD** | 2.829729 | 5.483936 | 2.894782 | 4.545253 | 4.995499 |
| **0.0625 ng** | **AVG** | 5.874612 | 12.47564 | 5.78062 | 9.630474 | 10.93825 |
| | **STD** | 3.474246 | 6.613753 | 3.294565 | 5.301605 | 6.128935 |

**Table 4: Average and Standard Deviation of DNA sample peak heights in RFUs.** The average (AVG) and standard deviation (STD) were calculated for each dye channel and total DNA input of the DNA samples.

The total DNA input varied slightly amongst the dilutions for each dye channel, with the higher and lower end of the DNA inputs increasing in peak height average. Electropherograms generated with the GeneMapper® ID-X software visually represents the data generated by the 3500xL genetic analyzer. Figure 7 is a visual of the electropherograms for the baseline noise of a DNA sample and a non-DNA sample. The non-DNA sample is the top image and the DNA sample is the bottom image. Both images have a y-axis of RFUs with scale increments of 10 RFUs. It can be seen that the baseline noise between the samples is slightly different, with the baseline noise heights increased in the DNA sample compared to the non-DNA sample.

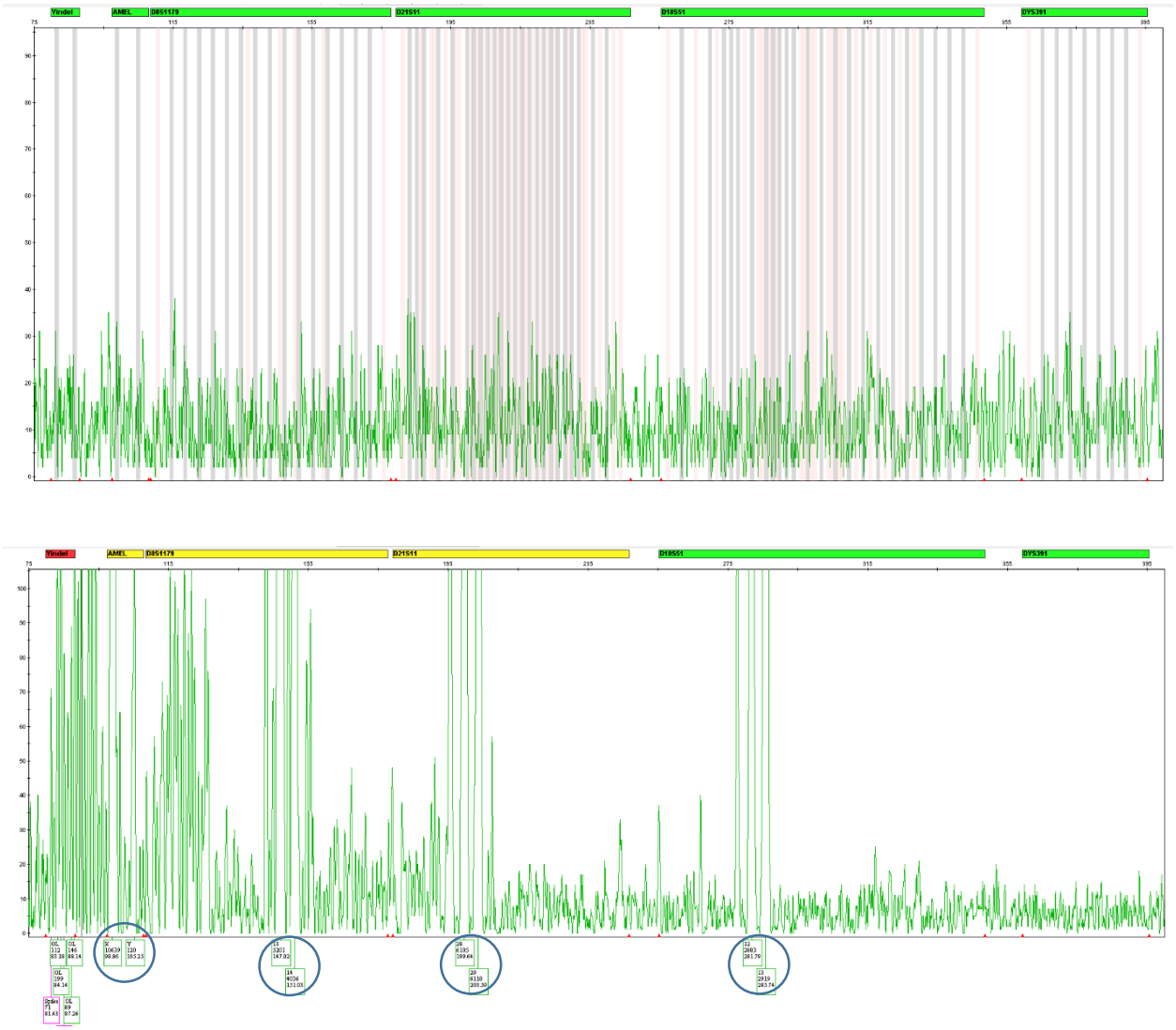


**Figure 7: Electropherograms of the baseline noise in the green dye channel for a DNA and non-DNA sample.** The electropherogram on the top is a negative control non-DNA sample and the electropherogram on the bottom is a direct reference DNA sample. The blue circles mark the true allele calls of the DNA sample.

With the averages and standard deviations determined, Analytical Thresholds (AT) were calculated using the calculation methods from Table 2. The results of non-DNA calculation methods are summarized in Table 5.

| Analysis | Dye Channel | Blue | Green | Yellow | Red | Purple |
|---|---|---|---|---|---|---|
| **AVG** | | 6.08406 | 12.46705 | 5.826512 | 9.815289 | 11.13017 |
| **STD** | | 3.082767 | 5.5444 | 3.264239 | 4.365599 | 5.056398 |
| **MAX/MIN HEIGHT** | | 35/0 | 58/0 | 79/0 | 45/0 | 71/0 |
| **AT Calculations** | | | | | | |
| **LOD** | | 15.33236 | 29.10025 | 15.61923 | 22.91209 | 26.29936 |
| **LOQ** | | 36.91174 | 67.91106 | 38.4689 | 53.47128 | 61.69415 |
| **$AT^1$** | | 70 | 116 | 158 | 90 | 142 |

**Table 5: Analytical Threshold calculations using non-DNA calculation methods.** AT determined for each dye channel using the non-DNA samples peak heights in RFUs. The calculation equations and explanations can be found in the appendix.

The calculated analytical thresholds range from as low as 15.33236 RFUs to as high as 158 RFUs across all dye channels and calculation methods. The threshold range of the LOD calculation method across all dye channels is 15.33236 RFUs to 29.10025 RFUs, the range of the LOQ calculation method is 38.4689 RFUs to 67.91106 RFUs, and for the $AT^1$ calculation method the range is 70 RFUs to 158 RFUs. Among non-DNA calculation methods there is a wide variation in ATs that differ between calculation methods as well as dye channels.

Utilizing the averages and standard deviations of the DNA samples data sets, analytical thresholds were calculated using the LOD and LOQ DNA calculation methods. The results of the LOD and LOQ calculations are summarized in Table 6. The weighted regression analysis for each dye channel of the DNA samples with total DNA inputs of 0.0625ng-1ng are summarized in Figure 8-12. The results of the $AT^2$ DNA calculation method using equation 6 in Table 2 and results from the weighted regression analysis are summarized in Table 7.

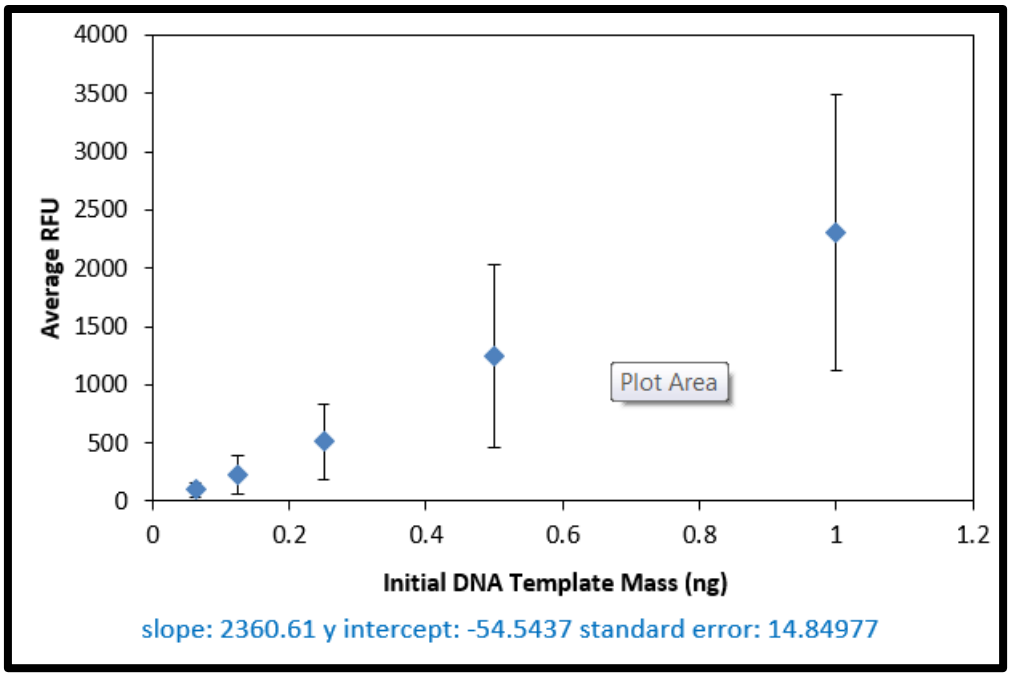



**Figure 8: Weighted regression analysis for the blue dye channel.** Average and standard deviation of allele peak heights (RFUs) were calculated for the blue dye channel at DNA inputs of 0.0625ng, 0.125ng, 0.25ng, 0.5ng, and 1ng to determine y-intercept and standard error for the data.

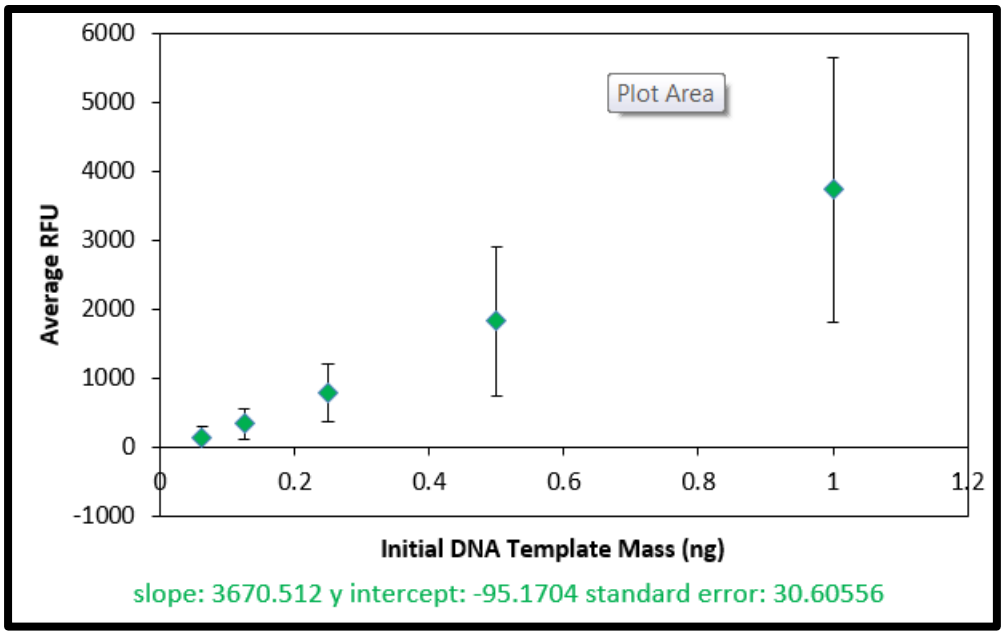



**Figure 9: Weighted regression analysis for the green dye channel.** Average and standard deviation of allele peak heights (RFUs) were calculated for the green dye channel at DNA inputs of 0.0625ng, 0.125ng, 0.25ng, 0.5ng, and 1ng to determine y-intercept and standard error for the data.

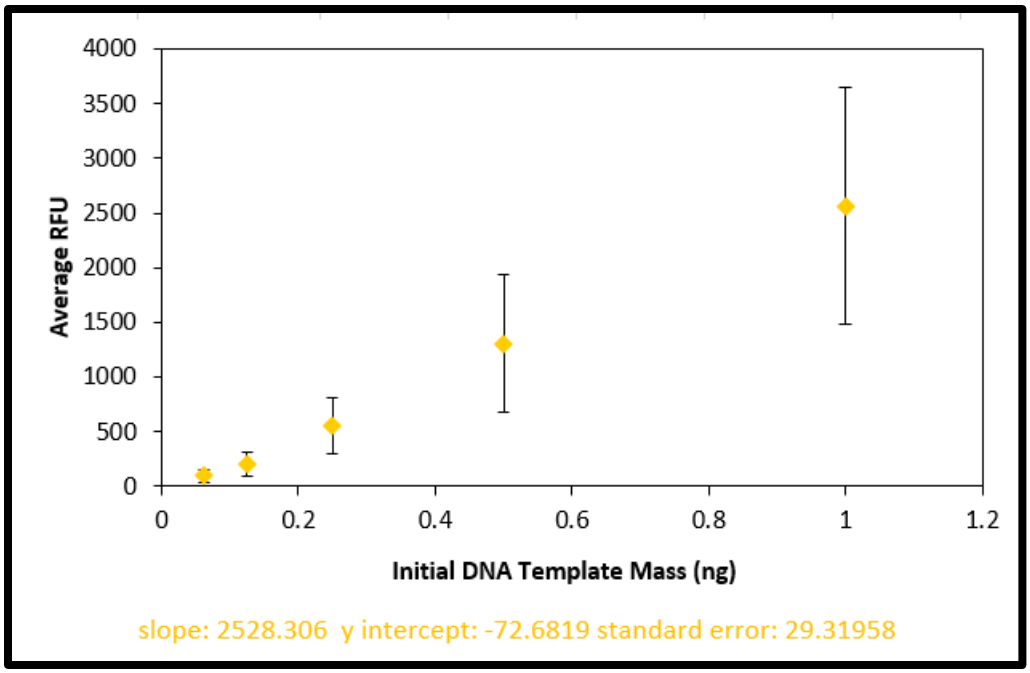



**Figure 10: Weighted regression analysis for the yellow dye channel.** Average and standard deviation of allele peak heights (RFUs) were calculated for the yellow dye channel at DNA inputs of 0.0625ng, 0.125ng, 0.25ng, 0.5ng, and 1ng to determine y-intercept and standard error for the data.

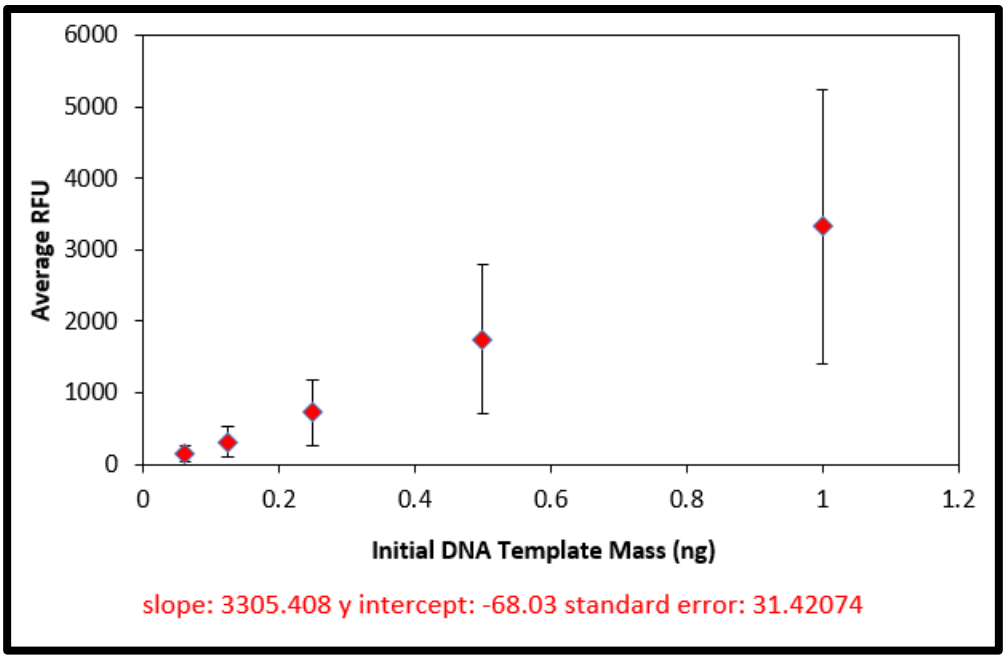



**Figure 11: Weighted regression analysis for the red dye channel.** Average and standard deviation of allele peak heights (RFUs) were calculated for the red dye channel at DNA inputs of 0.0625ng, 0.125ng, 0.25ng, 0.5ng, and 1ng to determine y-intercept and standard error for the data.

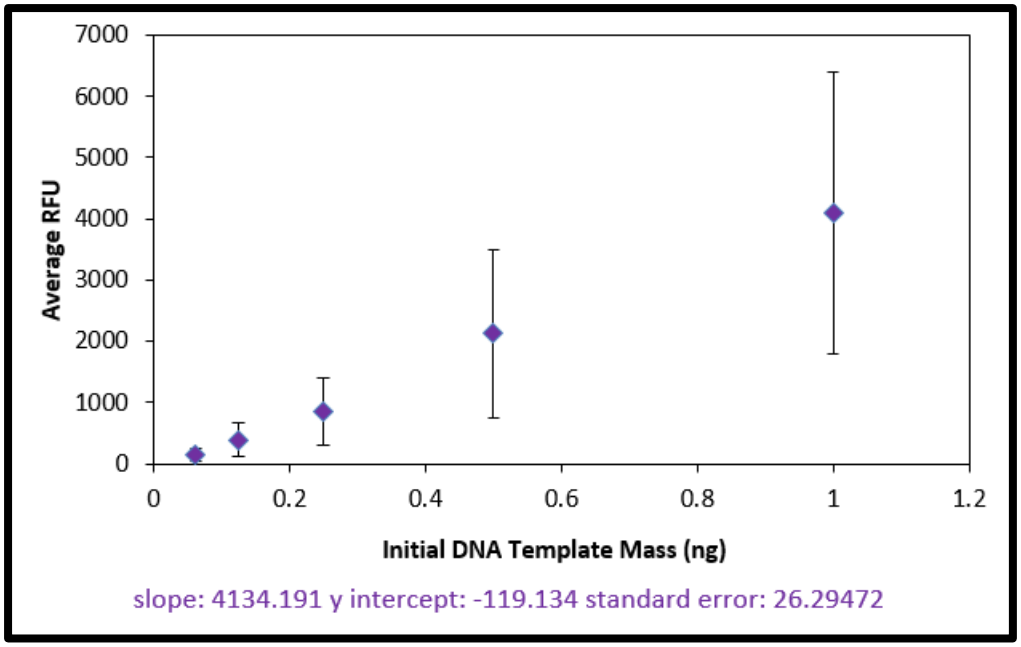



**Figure 12: Weighted regression analysis for the purple dye channel.** Average and standard deviation of allele peak heights (RFUs) were calculated for the purple dye channel at DNA inputs of 0.0625ng, 0.125ng, 0.25ng, 0.5ng, and 1ng to determine y-intercept and standard error for the data.

| ***Dye Channel*** | | ***Blue*** | ***Green*** | ***Yellow*** | ***Red*** | ***Purple*** |
|---|---|---|---|---|---|---|
| ***Total DNA Input*** | ***Analysis*** | | | | | |
| **Direct Reference** | **LOD** | 21.80256 | 33.09872 | 24.26558 | 28.88471 | 29.54515 |
| | **LOQ** | 55.95528 | 79.58176 | 64.05665 | 71.24603 | 70.63148 |
| **2 ng** | **LOD** | 16.93248 | 29.72042 | 16.86144 | 24.47447 | 26.87493 |
| | **LOQ** | 43.14362 | 71.05434 | 42.20843 | 58.71116 | 63.58462 |
| **1 ng** | **LOD** | 13.69725 | 25.80248 | 14.6491 | 22.36641 | 24.60771 |
| | **LOQ** | 32.95167 | 58.95307 | 35.53048 | 52.56429 | 57.2275 |
| **0.5 ng** | **LOD** | 14.18119 | 25.78973 | 14.30778 | 20.93639 | 24.17686 |
| | **LOQ** | 34.22586 | 58.83115 | 34.42114 | 48.4951 | 55.82299 |
| **0.25 ng** | **LOD** | 13.5741 | 25.4797 | 13.88161 | 21.30738 | 24.1492 |
| | **LOQ** | 31.48209 | 56.17307 | 32.21225 | 48.02404 | 53.5261 |
| **0.125 ng** | **LOD** | 14.98497 | 29.98567 | 15.06755 | 24.19455 | 27.03727 |
| | **LOQ** | 34.79307 | 68.37323 | 35.33103 | 56.01132 | 62.00576 |
| **0.0625 ng** | **LOD** | 16.29735 | 32.3169 | 29.32506 | 25.53529 | 15.66432 |
| | **LOQ** | 40.61707 | 78.61317 | 72.22761 | 62.64652 | 38.72627 |

**Table 6: Analytical Threshold calculations using DNA calculation methods LOD and LOQ.** AT determined for each dye channel using the DNA samples peak heights in RFUs. The calculation equations and explanations can be found in the appendix.

| **Dye Channel** | | **Blue** | **Green** | **Yellow** | **Red** | **Purple** |
|---|---|---|---|---|---|---|
| **Analysis** | **$AT^2$** | 1.142902 | 19.60045 | -20.5291 | 49.79779 | 37.26654 |

**Table 7: Analytical Threshold calculation using DNA calculation method $AT^2$.** AT determined for each dye channel using a weighted regression analysis of the DNA allele peak heights in RFUs. The calculation equation and explanation can be found in the appendix.

The results from the DNA calculation methods show variation in Analytical Threshold determination based on the calculation method used. The results using the LOD calculation method range from 13.5741 RFUs to 33.09872 RFUs, the LOQ calculation method ranged from 31.48209 RFUs to 79.58176 RFUs, and the $AT^2$ calculation method ranged from -20.5291 RFUs to 49.79779 RFUs. A -20.5291 RFUs is not a possible value for setting an analytical threshold, the next lowest analytical threshold value for the $AT^2$ calculation method is 1.142902 RFUs.

# CHAPTER IV

# CONCLUSION

The initial threshold that must be determined for interpretation purposes in forensic DNA analysis is the analytical threshold. The purpose of setting thresholds and determining limits is to obtain objective measures of detection and quantification for analytical processes. Analytical threshold is defined as "the minimum height requirement at and above which detected peaks can be reliably distinguished from background noise" (10).

The baseline noise detected during capillary electrophoresis can be challenging when performing DNA analysis and interpretation in a forensic setting. The determination of an analytical threshold is based upon the evaluation of the baseline noise of the system. The procedural elements associated with the forensic workflow potentially affect the baseline noise of the system, as well as the manufacturing of the amplification kits.

Baseline noise can be effected by procedural elements, such as amount of input DNA to the amplification reaction. The results from this study show that amount of total input DNA into the amplification reaction does have an effect on baseline noise. Total input DNA at the higher (2ng and DR) and lower ends (0.125ng and 0.0625ng) of the dilution series increased slightly compared to the middle range (0.25 ng to 1ng). The baseline noise of the non-DNA samples are similar to the baseline noise for the DNA dilution series 0.0625ng to 2ng, where there was a slight increase in baseline noise with the direct reference DNA samples compared to the non-DNA samples.

The green dye channel was consistent as the noisiest dye channel across the samples. Figure 13 is an electropherogram of all dye channels in a sample from this study. There is variation in the baseline noise across the dye channels, with the green dye channel having the highest baseline noise.

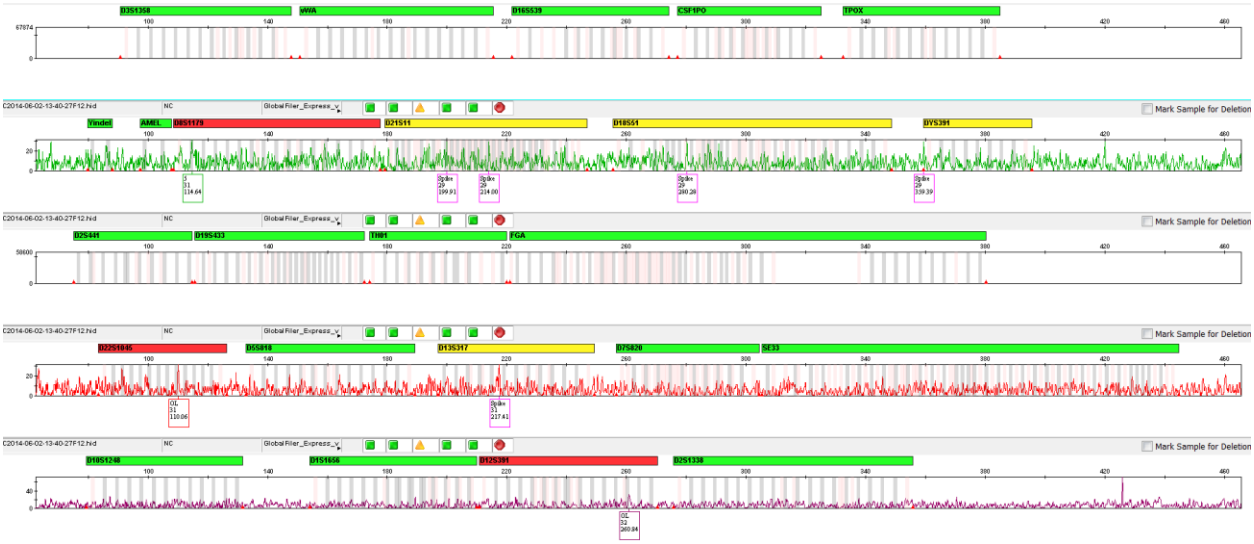


**Figure 13: Electropherogram of a negative control sample baseline noise.** The first row is the blue dye channel, second row is the green dye channel, third row is the yellow dye channel, fourth row is the red dye channel, and the fifth row is the purple dye channel. The baseline noise is prominent in the green dye channel.

The procedural process of adding DNA to the amplification reaction can increase baseline noise depending on the amount of total DNA added. Variation in baseline noise between dye channels and total DNA input, leads to variation in determining analytical thresholds.

Analytical threshold determination can be calculated in a multitude of ways. Four calculation methods were examined in this study. The LOD and LOQ were calculated for both the non-DNA samples and the DNA samples, and the $AT^1$ was calculated for the non-DNA samples and the $AT^2$ was calculated for the DNA samples. There was a wide range in threshold determinations across all calculation methods. Thresholds as low as -20.5291 RFUs to as high as 158 RFUs. A -20.5291 RFUs is an impossible value in setting an analytical threshold. This result

for the yellow dye channel demonstrates that the assumptions of the $AT^2$ calculation method, the y-intercept of the linear regression of DNA does not differ from the average baseline noise, are not met with the yellow dye channel of the DNA samples. If this value is discarded, the next lowest analytical threshold value for the calculation method is 1.142902 RFUs in the blue dye channel.

Most of the analytical thresholds will not filter out all baseline noise, with noise peaks in the non-DNA samples as high as 79 RFUs. There are analytical thresholds that will filter out noise but also filter out true alleles as well. The DNA samples had allele peak heights as low as 7 RFUs for the lowest total DNA input amount. The effects of total input DNA on baseline noise as well as the calculation methods in determining analytical thresholds has shown that variation does occur depending on the methods employed.

Variation amongst the methods in DNA testing by forensic laboratories leads to variations in the results of the DNA testing. This study has shown that evaluating baseline noise by procedure and using samples that contain DNA will more closely reflect the baseline noise encountered in forensic casework samples. There is an assortment of calculation methods and procedures used by forensic laboratories to set analytical thresholds, resulting in a wide range of potential analytical threshold values. This study demonstrated a range of analytical thresholds from -20.5291 RFUs to 158 RFUs based on two sample types (non-DNA and DNA) and four calculation methods (LOD, LOQ, $AT^1$, and $AT^2$). Distinguishing an appropriate method for the determination of analytical thresholds will help in minimizing the variation observed.

It is common in the forensic science community for there to be standardization and uniformity in all laboratory processes. There is currently no standardized method for determining an analytical threshold in the forensic science community. The importance of standardization

allows for the field to be as objective as possible. This study characterized baseline noise by the effects of the Applied Biosystems GlobalFiler® Express PCR amplification kit and DNA and non-DNA sample types to assist in determining the most appropriate method for establishing an analytical threshold. A method using DNA sample types versus non-DNA sample types will better encompass the effects of baseline noise that may be encountered in forensic casework samples. There is a need for a calculation method to be designated as an appropriate tool in determining analytical thresholds, which will allow for standardization across forensic laboratories and to reduce the variation amongst calculated analytical thresholds. Future studies should further characterize baseline noise to better understand how it behaves in forensic casework studies. Elements to take into consideration would be a broader range of total DNA input and sample types, degraded DNA samples, PCR cycle number, capillary electrophoresis sample reinjection, and other amplification kits to name a few. More studies on baseline noise and methods in determining analytical thresholds will help in the determination of the most appropriate calculation method to be used across all forensic laboratories.

# APPENDIX

## Equations

- Limit or detection (LOD) is the average amount of baseline noise ($\mu_b$) plus three standard deviations ($\sigma_b$) → $LOD = \mu_b + 3\sigma_b$
- Limit of quantification (LOQ) is the average amount of baseline noise ($\mu_b$) plus ten standard deviations ($\sigma_b$) → $LOQ = \mu_b + 10\sigma_b$
- Analytical Threshold equation 3 ($AT^1$) is the highest peak ($Y_{max}$) minus the lowest trough ($Y_{min}$) multiplied by two → $AT^1 = 2(Y_{max} - Y_{min})$
- Analytical Threshold equation 6 ($AT^2$) is the y-intercept of the regression (b) plus the critical value ($t_{n-1,\alpha}$) multiplied by the standard error of regression ($S_y$) of the relationship between RFU and input DNA" over a DNA input dilution series →

  $AT^2 = b + t_{n-1,\alpha}S_y$

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