INVESTIGATION OF BASELINE NOISE: ESTABLISHING AN RFU THRESHOLD FOR FORENSIC DNA ANALYSIS

by

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INVESTIGATION OF BASELINE NOISE: ESTABLISHING AN RFU THRESHOLD FOR FORENSIC DNA ANALYSIS

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ABSTRACT
Two methods to determine analytical thresholds for forensic DNA analyses were examined. These methods rely on analysis of the baseline of a number of amplification negative and run blank samples. Results showed that when a DNA mass larger than 1 ng was amplified the baseline noise increased, suggesting analytical thresholds derived from the two methods should not be utilized for these samples. These methods are only suitable for amplifications with low DNA inputs where such thresholds minimized the false labeling of noise, while maximizing the number of true alleles detected.
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Height of the noise peaks greater than the analytical threshold derived via Equation 1 from amplification negative samples and other non labeled peaks which could not be characterized as known artifacts plotted against the average peak height of the known alleles. Graphs include all data obtained from a total of 112 single-source samples of known genotype amplified at 0.06, 0.125, 0.25, 0.5, 1, 2 and 4 ng and run at 2 sec injection time. Loci with off-scale data are not included.

Height of the noise peaks greater than the analytical threshold derived via Equation 1 from amplification negative samples and other non labeled peaks which could not be characterized as known artifacts plotted against the average peak height of the known alleles. Graphs include all data obtained from a total of 112 single-source samples of known genotype amplified at 0.06, 0.125, 0.25, 0.5, 1, 2 and 4 ng and run at 5 sec injection time. Loci with off-scale data are not included.

Height of the noise peaks greater than the analytical threshold derived via Equation 1 from amplification negative samples and other non labeled peaks which could not be characterized as known artifacts plotted against the average peak height of the known alleles. Graphs include all data obtained from a total of 112 single-source samples of known genotype amplified at 0.06, 0.125, 0.25, 0.5, 1, 2 and 4 ng and run at 10 sec injection time. Loci with off-scale data are not included.

The number of noise peak heights above the AT calculated via Equation 1 utilizing amplification negatives, organized by injection time color channel and DNA input target.

The AT of at least 99.9 percentile rank against DNA target for 2s, 5s and 10s injection time. The horizontal black bar represents the highest AT calculated using amplification negatives at 99.9 percentile rank.
The number of noise peak heights above the AT calculated via Equation 1 utilizing amplification negatives, organized by association with allele peak height, injection time and color channel.
List of Abbreviations

µL - Microliter
ACS - American Chemical Society
AT - Analytical Threshold
°C - Degrees Celsius
CCD - Charge-coupled device
CE - Capillary electrophoresis
DNA - Deoxyribonucleic acid
dNTP - Deoxynucleotide triphosphate
EDTA - Ethylenediaminetetraacetic acid
GC - Guanine Cytosine
HiDi - Highly demonized
IUPAC - International Union of Pure and Applied Chemists
kV - Kilo Volt
LOD - Limit of Detection
M - Molar
MDS - Minimal Distinguishable Signal
mg - Milligram
ml - Milliliter
ng - Nanogram
PCR - Polymerase chain reaction
pH - Potential of hydrogen
RFLP – Restriction Fragment Length Polymorphism
RFU - Relative fluorescence unit
SDS - Sodium dodecyl sulfate

SSC - Saline sodium citrate

STR - Short tandem repeat

SWGDAM - Scientific Working Group on DNA Analysis Method

TE – Tris - Ethylenediaminetetraacetic acid

VNTR – Variable Number Tandem Repeats
Introduction

Forensic DNA typing using short tandem repeats (STRs) is one of the most powerful techniques for identity testing. The workflow for such a process involves a number of steps such as DNA extraction, quantification, amplification and fragment analysis using electrophoresis. Specifically, fragment analysis with capillary electrophoresis (CE) in conjunction with fluorescent detection is widely used in the field of human identity and forensic testing due to its low limit of detection, broad dynamic range and capability for multi-color analysis which enhances the simultaneous multi-locus analyses of DNA samples.

Alec Jeffreys was the first to describe DNA typing in 1985 by discovering the VNTR regions of the DNA sequence that were repeated over and over and could differentiate one individual to another. The technique used to examine such regions involved a restriction enzyme and was known as restriction fragment length polymorphism (RFLP). This technique which utilized single-locus or multi-locus probes has been replaced with the polymerase chain reaction (PCR) which uses STR regions instead of VNTRs in a multiplex-PCR technology.

After extraction, the DNA is measured to evaluate the quantity and amplified to the desired level through PCR during which specific regions of DNA containing the STR regions of interest are replicated over and over again. This process involves heating and cooling the sample in cycles. During each cycle a copy of the targeted DNA region is produced for every molecule of DNA that contains that region with boundaries defined by oligonucleotide primers complimentary to the 3’-end of the region. These primers
identify the region that needs to be copied and drive the PCR reaction using heat stable polymerases such as the AmpliTaq Gold® DNA polymerase. The PCR reaction allows more than one region to be copied simultaneously by introducing more than one set of primers into the reaction. This simultaneous amplification is known as multiplex PCR. The introduced primers are designed by taking into consideration various parameters such as primer length, GC content, melting temperature, primer self-complementarity, oligonucleotide sequence, distance between two primers on targeted sequence, difference between forward and reverse primers, etc. Primers are labeled by fluorescent dyes which emit light of varying wavelength and are attached on their 5’-end by non-nucleotide linkers which may serve the purpose of mobility modification to allow inter-locus spacing within the various color channels (1). Labeling primers with various fluorophores results in the ability to analyze DNA fragments of similar sizes simultaneously, making it possible to maintain small amplicon sizes beneficial to forensic DNA analyses.

After the DNA amplification process, the resultant fragments of varying lengths are separated via capillary electrophoresis fluorescent detection. The detection of DNA fragments via fluorescence involves the absorption of a photon by a molecule capable of fluorescence (i.e., fluorescent dye attached to the primer incorporated in the amplified DNA region) via a laser and the emission of a photon after the return to a ‘relaxed’ electronic state. The photon emitted from the fluorophore is of a lower energy and hence longer wavelength than the excitation light, resulting in a ‘Stokes’ shift which permits optical filters to separate the photons associated with excitation and emission (1).
on their chemical structure, fluorophores have characteristic absorption and emission patterns.

During electrophoresis, the fluorophore-labeled STR amplification products are introduced into the capillary by electrokinetic injection and consecutively detected as they move past a laser detection window located near the capillary end (2) where the laser is placed. It is the charged coupled device (CCD) that detects the laser-induced fluorescence and its intensity. The CCD consists of a two-dimensional matrix of detectors; one needed to achieve wavelength resolution and the other to record the development of an electropherogram as a function of time (3). The action of the photons striking the detector is converted into an electrical signal the strength of which is proportional to the intensity of the light striking the detector (1). The more amplified the target regions, the more fluorescent dye molecules will emit photons, increasing the intensity of the signal which will reflect the quantity of the amplified product. These signals are measured in relative fluorescence units (RFU) and make up the peaks that are visualized in the electropherograms. There is also signal that is not associated with amplified DNA known as baseline noise. Several factors such as changes in the optical alignment of the set-up, variations in composition of the CE buffer, air bubbles and the presence of other contaminants can influence both the analyte and background signal (3).

Different dyes emit fluorescence at different wavelengths which are captured from filters that make possible their separation. As the DNA amplicons are separated based on their size, they are associated with the appropriate color, sized in comparison to the internal size standard and correlated to an allelic ladder that has been sized in a
similar fashion with the internal standard. The result is a genetic profile consisting of peaks that correspond to each DNA fragment on the horizontal axis where amplitude reflects the number of DNA amplicons and is quantitatively represented on the vertical axis as Relative Fluorescence Units (RFU).

Examination of the profile data by a forensic DNA analyst is the next step in the process and depending on the number of individuals, the amount of the template and the ratio of contributors, may be considered the most difficult aspect of DNA evidence processing. The first task in DNA data interpretation is to assess the presence of an allele or peak. This is typically accomplished by utilizing an analytical threshold (AT), which excludes most of the baseline noise from analysis. This threshold has a large impact on DNA fragment analyses and interpretation especially when dealing with degraded samples, samples containing low-levels of DNA and complex mixtures. In such circumstances, if the AT is arbitrarily high, true signal will be incorrectly left unlabeled and will be lost during the analysis. On the other hand an AT which is too low will not exclude considerable background noise from analyses, increasing the probability that randomly high baseline noise will be incorrectly detected and labeled as true alleles.

To insure the maximum number of true peaks is labeled while minimizing the false labeling of noise, the process of establishing an analytical threshold requires a scientific and analytical approach. This can be accomplished by determining the minimal distinguishable signal (MDS) which is the signal at which a peak can reliably be detected and distinguished from noise. The MDS may be represented by the analytical threshold (AT) expressed in RFU.
The SWGDAM Interpretation Guideline for Autosomal STR Typing 3.1.1.1 states that dye artifacts may be distinguished from allelic peaks based on morphology and/or reproducibility while stutter and non-template dependent nucleotide addition peaks may be characterized based on size relative to an allelic peak amplitude (4). Additionally SWGDAM Interpretation Guideline for Autosomal STR Typing 3.1.1.2. states that the AT should be established on signal-to-noise considerations and should not be used for purposes of avoiding artifact labeling, which may result in loss of data (4).

Artifacts are extra peaks beside the alleles of interest that result due to the chemistry of the PCR reaction and the technology of the equipment. The most common artifacts are stutter and incomplete 3’ (A) nucleotide addition. Stutters are typically one repeat shorter than the corresponding allele peak. For interpretation, an upper-limit of percentage stutter is established. Incomplete 3’ (A) nucleotide known as +/- A artifacts result in split peaks that are one base pair different. Technology related artifacts include pull, up, dye blobs, voltage spikes, air bubbles, urea crystal peaks, etc. The ‘pull up’ results from the inability of the instrument to properly resolve dye colors due to spectral overlap of the fluorophores. A peak of another color ‘bleeds through’ to the other color channel and is observed as a smaller peak in an adjacent color. Dye blobs result when dyes detach from their primers and move independently through the capillary. These peaks are fairly broad and small. Air bubbles, urea crystals and voltage spikes, usually result in sharp peaks that appear throughout the four color channels and are not reproducible in the case of sample re-injection into the CE.
Various forensic laboratories use different ATs that can range from the very low to extremely high values (ie 10-200 RFU). Many laboratories rely on conservative ATs which are established during the validation process to minimize the effect of baseline noise and artifacts. A threshold of 150 RFU was advised by the Applied Biosystems (1998) AmpFISTR Profiler Plus Amplification Kit Users Manual to avoid typing low amounts of input DNA (5). The PCR cycle number and amplification conditions in the manual had been specified to produce peak heights of <150 RFU for a sample containing 35 pg human genomic DNA and for such reason they suggested to interpret these peaks with caution, due to the propensity of the sister alleles to ‘drop out’. However, instrumentation, chemistry and analyses have changed since 1998. With the advert of more interest in low copy number and complex mixture DNA analyses, a clear definitive threshold to determine the presence or absence of peaks is necessary. Such value should be one that maximizes allele detection while at the same time minimizing the false labeling of noise.

Numerous terminologies and definitions in the forensic and analytical literature such as lower limit of detection, limit of detection, minimum distinguishable signal, minimum discernable signal, detection sensitivity, sensitivity, limit of determination, limit of purity and limit of quantification (6-13) are used with significant inconsistency and ambiguity. To some, ‘detection limit’ means the critical concentration above which the detection decision is made and to others it indicates the ‘true’ detection capability (10). In the IUPAC documents ‘detection limit’ is derived using hypothesis testing theories of false positive probabilities \( \alpha \) and false negative probabilities \( \beta \) where
quantification limits have default values of \( \alpha \) and \( \beta \) set at 0.05 and relative standard deviation (RSD) of 10\% (10).

Brown (6) raises the concern of the proper way of reporting values that fall below an established limit of detection. He gives certain options of reporting these measurements such as not detected, zero, less than LOD, a fraction of the LOD or the actual value accompanied by a statement of uncertainty with the later concluded to be the best option because it provides the most information and does not bias descriptive statistics.

In 1975 IUPAC defined LOD in terms of concentration \((C_L)\) and signal \((X_L)\) expressing \((X_L)\) in terms of blank signal \((\mu_B)\) and standard deviation \((S_B)\) in the following equation where \((k)\) is numerically chosen factor based on the desired confidence level.

\[
X_L = \mu_B + kS_B \quad \text{(Equation 1)}
\]

The signal value \(X_L\) must be related to a non-zero analyte concentration and must reflect a true value. Equation 1 is expressed in terms of population statistics based on Gaussian distribution. When a \(k=3\) is utilized the type I error is 0.135\% and the probability that the blank signal will not exceed \(X_L\) is 99.865\%.

Winefordner and Long (13) further link \((C_L)\) to \((X_L)\) by the following equation, where \((m)\) is defined as the analytical sensitivity expressed as the slope of the calibration curve line that is obtained from the linear regression analyses.

\[
C_L = (X_L - \mu_B) / m \quad \text{(Equation 2)}
\]

Substituting \((X_L)\) from Equation 1 to Equation 2 gives:
\[ C_L = \frac{(k \cdot S_B)}{m} \]  \hspace{1cm} (Equation 3)

The SWGDAM Interpretation Guideline for Autosomal STR Typing 1.1 states that the Laboratory should establish an analytical threshold based on signal-to-noise analyses derived from empirical data giving an example where the analytical threshold may be based on two times the intensity difference between the highest and the lowest peak within the instrumental noise data. This recommendation can be expressed using the following equation where \((X_{\text{max}})\) represent the maximal observed noise peak and \((X_{\text{min}})\) represents the lowest observed noise peak.

\[ AT = 2 \cdot (X_{\text{max}} - X_{\text{min}}) \]  \hspace{1cm} (Equation 4)

Other methods that attempt to determine the point at which signal can reliably be distinguished from baseline noise have been proposed. One such method was adopted by IUPAC in 1995 (14) and is shown in the following equation:

\[ AT = \mu_B + t_{\alpha,v} \left( \frac{S_B}{\sqrt{n_b}} \right) \]  \hspace{1cm} (Equation 5)

The other is described in detail by Mocak et al (12)

\[ AT = \mu_B + t_{\alpha,v} \left( \frac{1 + 1/n_b}{n_b} \right)^{1/2} S_B \]  \hspace{1cm} (Equation 6)

In both of the above equations \(\mu_B\) and \(S_B\) and have the same definitions as in Equation 1, \(t_{\alpha,v}\) is the critical value obtained from the \(t\)-distribution for a given one-sided confidence interval and \(n_b\) is the number of blank samples. In both cases confidence intervals are calculated. Equation 6 takes into account the variance of the difference between average mean and individual blank signals where the term \((1 + 1/n_b)\) expresses the correction for the uncertainty of the true and calculated mean blank signal. Therefore as the number
of blank samples increases (ie $n_b$) the term $(1 + 1/n_b)$ approaches 1. These equations also assume a normal distribution.

Typically, the normal Gaussian distribution is assumed when establishing limits of detection. Such distribution is well defined and can be easily applied while possessing a large number of formulas and tables. However, the normal distribution may not always be the case in chemical analysis when technical limitations of the equipment or software may select data resulting in observed asymmetric distributions. As Kaiser states it is valuable to be aware of the power of pre-information in practically all analytical procedures because uncontrolled pre-information may lead to wrong or obsolete conventional ideas (15).

Chemical or biological samples containing the analyte of interest are not free of other chemicals or contaminants whose impact on the ability of an instrument or method to appropriately detect or quantify the analyte needs to be taken into consideration. This being said the samples used to evaluate different parameters or methods involved in sample analyses ideally need to include all the analytical procedures up to the step where such parameters and sample analyses methods are to be tested. For example, MDS derived from blank samples may be different from those derived from negative sample containing extra chemicals, which on the other hand may be different from samples that contain DNA. The fact that forensic DNA analysis utilizes an intermediary amplification step complicates the situation even more since non-specific amplification may take place in very concentrated samples. Such non-specific amplified product may contribute to
extra noise and the MDS calculated from blanks may not be suitable for such concentrated samples since it may be too low.

The work represented here studies methods of determining MDSs and the assumptions that come with such methods. Concerns regarding the false labeling of noise and non-labeling of true alleles are also discussed. Evaluation of the signal-to-noise determined whether MDSs derived from negatives are representative for samples amplified with common DNA input levels, or whether they need be derived from other methods that utilize samples which contain DNA.
Materials and Methods

Extraction

Phenol/Chloroform and alcohol precipitation were used to extract high molecular weight DNA from the whole blood of four different individuals. A volume of 750 μl of 1x SSC (0.15 M NaCl, 0.015 M tri-sodium citrate) was added to each 750 μl whole blood sample tube, bringing the volume to 1.5 ml. First the sample was mixed and centrifuged and then 1.0 ml of supernatant was removed while the remaining pellet was re-suspended in 1.0 ml of 1x SSC. After a second centrifugation and removal of 1.4 ml of supernatant, 375 μl of 0.2 M sodium acetate (pH 7.0), 25 μl of 10% SDS and 3.2 μl of proteinase K (31.5 mg/ml) were added to the sample to re-suspend the pellet and lyse the cells following an overnight incubation period at 56°C.

An equal volume of phenol/chloroform was added to the samples which were then gently mixed, centrifuged and the organic phase was discarded. Such a process was repeated with the addition of chloroform. To precipitate the DNA, 2 M sodium acetate, 0.8 μl of 20 mg/ml glycogen and an equal volume of isopropanol were added to the sample. A second overnight incubation period was allowed at -20°C.

The samples were centrifuged for thirty minutes at maximum angular velocity after which the supernatant was removed and discarded. After the addition of 1 ml of 80%, the tubes were once again centrifuged followed by another supernatant removal. The sample was allowed to air-dry and then the pellet was dissolved in 50 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) at 56°C
Quantification with UV-VIS Spectrophotometry

Every DNA sample concentration was determined using Visible spectroscopy (Genesys 10S, ThermoScientific, single beam instrument) making the assumption that one absorbance unit corresponded to 50 ng/μl of double stranded DNA. Minimal protein contamination was achieved by assessment of A260:A280 while a sample containing the dilution buffer was run prior to the samples and the background was subtracted. The scan range was from 400 to 200 nm. Each sample was run in triplicate using the mean concentrations to prepare them for amplification where the average and two standard deviation values of A260:A280 over all four samples was 1.8 and +/-0.2 respectively.

Amplification – AmpF™ STR® Identifiler®

Applied Biosystems AmpF™ STR® Identifiler® PCR Amplification Kit (16) was used for the amplification process. Each specimen coming from a different individual was amplified in quadruplicate for each of 7 different DNA targets (4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 ng) using TE Buffer (Tris-EDTA, 10−4 M) to make the dilutions. After combining the appropriate amount of master mix and DNA, a volume of 25 μl of the master mix/DNA mixture was aliquoted into four different wells on the same plate proceeded by PCR amplification in accordance with the manufacturer’s protocol, with an additional 60 minute extension period during thermalcycling which was performed on Applied Biosystems GeneAmp® PCR System 9700 using 9600 emulation mode. Positive and negative amplification controls were also run with the samples showing expected results.
Capillary Electrophoresis

A results group and plate map were created using Applied Biosystems 3130 collection software (v. 3.0), selecting the appropriate instrument protocol to be used with the AmpFLSTR® Identifiler® PCR Amplification Kit. Fragment separation was achieved using Applied Biosystems 3130 Genetic Analyzer and a mixture containing HiDi (highly-deionized) formamide (8.3 μl/sample) (Applied Biosystems, Foster City, CA) and GeneScan™-600 LIZ™ Size Standard (0.7 μL/sample) (Applied Biosystems, Foster City, CA).

A volume of 9 μL of the mixture described above and 1 μL of sample, negative or ladder were added to the appropriate wells. A total of 31 amplification negatives (extraction or amplification controls containing no DNA) and 31 run blanks (formamide and LIZ) were analyzed in addition to the 112 positive DNA samples (samples containing 4 to 0.0625 ng). The samples were heated at 95°C for 3 minutes, and snap-cooled at -20°C for 3 minutes. Two, five, and ten second injections at 3 kV were performed on each sample and run in accordance with the manufacturers recommended protocol (16).

Data Analysis

During Analysis with GeneMapper® ID v 3.1 (Applied Biosystems, Foster City, CA) an RFU threshold of 1 was used for the amplification negatives and run blanks and the labeled peaks were filtered such that those which were +/- 2 bases from a LIZ® peak were removed. All positive DNA samples were analyzed using the thresholds calculated form the amplification negative samples as shown in Table 2 of the Results and Discussion section and artifacts were removed. After analysis with with GeneMapper®
ID v 3.1 the sample name, allele, peak size and peak height for each sample were exported into Microsoft® Excel 2007 for further analysis.
Results and Discussion

Amplification Negative and Run Blank samples

DNA analyses as well as other chemical analyses are often challenged by the presence of very small quantity of analyte. Due to the limitation of samples recovered from crime scenes forensic DNA analyses face such phenomenon quite often. In order to accurately detect and report the presence of very small quantities of analyte the MDS must be determined. This would theoretically ensure that the labeled signal represents true signal and not just a randomly high level of baseline noise.

To determine the MDS one may choose to apply the classical approach and definitions proposed by the IUPAC (International Union of Pure and Applied Chemists) and ACS (American Chemical Society). The adaptation of this definition by the IUPAC and ACS were a response to Kaiser’s discussion on Quantitation in Elemental Analysis (17). This approach is one that focuses on the analysis of the baseline signal. To accomplish this, a number of blanks or negative samples (i.e., ~30) are run using the method of interest and the resultant signal analyzed. The mean and standard deviation of the baseline noise are subsequently used to determine its fluctuations on each analytical procedure by using the following equation where \( \mu \) represents the mean, \( \sigma \) represents the standard deviation and \( k \) represents the applied constant which determines the level of significance chosen. The AT represents the minimum value above which the signal is considered to be reliable (Equation 1).
If a normal distribution of baseline signals is assumed, a $k=3$ results in a 99.87% confidence (one sided) that noise peaks will be below the determined AT. However it should be noted that such a level of confidence may be associated to a $k=3$ only when there is a strictly normal distribution of baseline signal. Miller and Miller states that “although it can not be proven that repeated measurements of a single analytical quantity are always normally distributed, there is considerable evidence that this assumption is generally at least approximately true and any departure of the population from normality is not usually important on the statistical tests that are used most frequently” (18).

Gilder et al. give a representative histogram of distribution of measured RFU noise levels at all data collection points in their first 50 negative control samples for the blue color channel. They state that the distribution is ‘generally Gaussian’ but their representative histogram is truncated with the left side not being well represented (11). This results in an asymmetric distribution. Because of the asymmetry in the distribution, application of Equation 1 to obtain an AT would result in less than 99.86% confidence interval.

Figure 1. shows representative electropherograms of the green channel from run blank samples injected at 2, 5 and 10 s. Peaks which were +/- 2 bases from a LIZ® were not labeled and they were seen to increase with increase in injection time. Qualitatively the baseline signal seemed to remain constant regardless of injection time.
Figure 1. Green channel electropherograms from a run blank consisting of 9.3 μl formamide and 0.7 μl LIZ® 600 size standard with 2, 5 and 10 sec injection times.

Figure 2. shows the baseline noise peak distribution for both run blank and amplification negative samples. Here the run blanks represent samples that contain only formamide and internal lane standard, while amplification negatives represent samples with no DNA that are either extraction negatives or amplification controls. After being exported, the noise peaks of each color channel for each injection time were organized separately, counted based on their height and plotted in the following histograms.
Figure 2b. Histograms of signals obtained from the green channel of 31 run blanks and 31 amplification negatives run on a 3130 Genetic Analyzer using 2, 5 and 10 injections times.
Figure 2c. Histograms of signals obtained from the yellow channel of 31 run blanks and 31 amplification negatives run on a 3130 Genetic Analyzer using 2, 5 and 10 injections times.
Figure 2d. Histograms of signals obtained from the red channel of 31 run blanks and 31 amplification negatives run on a 3130 Genetic Analyzer using 2, 5 and 10 injections times.
Due to the threshold set to 1 RFU peaks smaller than 1 RFU were not labeled. Despite this, the left side of the histograms are represented well suggesting the mean and standard deviation may result in an AT where a significant number of baseline peaks fall below. On the other hand the right sides of the histograms raise some important concern in this regard because they consistently show tails which make the histograms asymmetrical. Due to this asymmetry, the apex is not in the middle of the histograms but is slightly shifted to the right side.

The histograms within color channels are similar in shape and breadth regardless of injection time. This typical shape of the histograms for each color channel appears to be relatively conserved between the run blanks and amplification negatives with a small consistent difference in which the spread of the data in the amplification negative samples seems to be slightly larger. There is an approximate difference of 2-3 RFU between the maximal peaks observed in blanks and negatives with the later showing the largest maximal peaks. On the other hand all the apexes are located at constant RFUs within a color channel and sample type regardless of injection time, suggesting there is little difference in the data spread for different injection times, indicating that injection time has an minimal effect on noise derived only from the instrument and run set-up.

To further analyze the data distribution, percentage cumulative frequencies for each observed level of RFU were also calculated separately for each color and injection time. Table 1. shows the percentage cumulative frequencies of the baseline noise obtained from the 5 sec injection of the blue channel for the amplification negative samples. Here, the percentage cumulative frequency represents the percentage of all noise
peaks below the peak corresponding to that particular percentage cumulative frequency.

The same process was repeated for each color channel in each injection time for both amplification negative and run blank samples.

Table 1. An example of the percentage cumulative frequency of the baseline noise also known percentile rank calculated from the 5 sec injection of the Amplification negative samples for the Blue channel.

<table>
<thead>
<tr>
<th>RFU Signal of 5 sec injection Amplification negative samples (Blue Channel)</th>
<th>Frequency</th>
<th>Relative Frequency (%)</th>
<th>Cumulative frequency (%)</th>
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<td>10.90655</td>
</tr>
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<td>43.83594</td>
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<td>99.93009</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>0.069914</td>
<td>100</td>
</tr>
</tbody>
</table>

Figure 3. shows the graphs of the percentage cumulative frequency plotted against each labeled peak. Each graph organizes the cumulative percentages based on sample type and injection time. In the Gaussian distribution the percentage cumulative frequency plotted against the labeled peaks is expected to result in an S-shaped curve. As clearly seen in Figure 3., all the obtained curves are not similar to an S-shaped curve. Instead they are more similar to the shape expected from a truncated normal or log-normal distribution which is also supported by the histograms in Figure 2.
The observation that noise may not be normally distributed raises some concern as to whether Equation 1 should be used to determine the AT for forensic purposes.
Table 2 shows the ATs calculated for each injection time organized for each color channel for both amplification negatives and run blanks using Equation 1 and utilizing $k = 3$. Next to each of the AT values calculated using Equation 1, the percentage of noise peaks below that AT value is also given in Table 2. This percentage of noise is the percentage cumulative frequency also known as percentile rank as described above. At the same time ATs using a 99 and 99.9 percentile rank are included in the table for both blanks and negatives. The chosen percentile ranks were the closest values to 99 and 99.9 with the condition that they would rather exceed these values than be smaller.
Table 2. ATs calculated using Equation 1 utilizing \( k = 3 \) and their corresponding percentile ranks as well as ATs calculated using the percentile rank method for the 99 and 99.9 percentile rank.

<table>
<thead>
<tr>
<th>Color and Injection Time in sec.</th>
<th>AT of Blanks using Equation 1 (Percentile rank)</th>
<th>AT of Negatives using Equation 1 (Percentile rank)</th>
<th>AT Blanks in RFU using ~99 Percentile rank</th>
<th>AT of Negatives in RFU using ~99 Percentile rank</th>
<th>AT Blanks in RFU using ~99.9 Percentile rank</th>
<th>AT of Negatives in RFU using ~99.9 Percentile rank</th>
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<tr>
<td>BLUE</td>
<td>2</td>
<td>5 (99.91)</td>
<td>7 (99.90)</td>
<td>4 (99.21)</td>
<td>6 (99.52)</td>
<td>5 (99.91)</td>
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<tr>
<td></td>
<td>5</td>
<td>6 (99.93)</td>
<td>7 (99.72)</td>
<td>5 (99.67)</td>
<td>6 (99.28)</td>
<td>6 (99.93)</td>
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<tr>
<td></td>
<td>10</td>
<td>6 (99.32)</td>
<td>8 (99.50)</td>
<td>6 (99.32)</td>
<td>8 (99.50)</td>
<td>9 (99.92)</td>
</tr>
<tr>
<td>GREEN</td>
<td>2</td>
<td>7 (99.92)</td>
<td>9 (99.82)</td>
<td>6 (99.60)</td>
<td>8 (99.22)</td>
<td>7 (99.92)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7 (99.94)</td>
<td>9 (99.57)</td>
<td>6 (99.40)</td>
<td>9 (99.57)</td>
<td>7 (99.94)</td>
</tr>
<tr>
<td></td>
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<td>10 (99.57)</td>
<td>6 (99.32)</td>
<td>9 (99.24)</td>
<td>7 (99.91)</td>
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<td>12 (99.56)</td>
<td>10 (99.01)</td>
<td>12 (99.56)</td>
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</tr>
<tr>
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<td>12 (99.08)</td>
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<td>16 (99.34)</td>
<td>11 (99.41)</td>
<td>13 (99.12)</td>
<td>14 (99.96)</td>
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<tr>
<td>RED</td>
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<td>12 (99.64)</td>
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<td>11 (99.24)</td>
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<td>13 (99.74)</td>
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<td>12 (99.17)</td>
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<td>15 (99.28)</td>
<td>11 (99.22)</td>
<td>15 (99.28)</td>
<td>14 (99.97)</td>
</tr>
</tbody>
</table>

As shown, if used with the currently discussed samples all the ATs calculated using Equation 1 would eliminate at least 99.28% of the background noise from analyses with the maximal noise elimination of 99.96% as shown by the lower and higher percentile rank for the green channel of the 10 sec injection for the negatives and the yellow channel of the 2 sec injection for the run blanks respectively. Generally it is observed that the AT from negatives is always larger than the ATs derived from blanks. Additionally the percentage rank using approximately 99.9 gives values which are larger...
than any of the other ATs. For example the 10 sec injection yellow channel of the negatives exhibits an AT of 47 RFU when the percentile rank of 99.9 is used while the AT calculated using Equation 1 is 16 RFU. This suggests that if an AT using Equation 1 with a $k=3$ was utilized, the confidence interval is closer to 99 percentile rank as opposed to 99.9 percentile rank. Since an electropherogram is expected to contain approximately 150 baseline points greater than 1 RFU, a confidence level of approximately 99% would suggest that two of every three electropherograms are expected to have baseline that is labeled. This may be considered too high for some forensic applications. In contrast a confidence level of at least 99.9% suggests that approximately one of every seven electropherograms will have a randomly high noise peak above AT. Due to the number of baseline peaks this may be considered a more appropriate interval for DNA analyses.

When the distribution of baseline noise is not certain, the percentile rank method may be an alternative method to establishing the AT since such a method is not dependent upon the type of distribution. To apply this method, a laboratory can determine the percentile ranks as shown on Table 1 and then choose an RFU level based on the desired level of the confidence. The application of such a method is very convenient since it does not require sophisticated software or high expertise in statistical analyses.

The ATs calculated from the run blanks are derived from electrophoresis of samples containing only formamide and LIZ. As such, the results showing that the thresholds were not significantly different between injection times were expected since this noise is the result of the electrophoretic run influenced by factors such as electronic noise, temperature fluctuation, laser source noise etc.
The realistic situation involves the performance of several steps prior to DNA electrophoresis where DNA will be extracted, quantified and amplified, introducing primers, dNTPs and other compounds into the electrophoretic run. Even though the extraction clean up methods should be able to remove the interfering components and contaminants while primers and dNTPs should be quickly electrophoresed prior to any DNA fragment without any effect on the baseline noise around the alleles, total elimination of interfering components can not be assumed. For this reason it was of interest to determine the ATs obtained from the amplification negatives did not differ substantially from those obtained from run blanks as summarized in Table 2.

Generally ATs obtained for run blank samples exhibited slightly lower MDSs than those obtained from the amplification negative samples. Even though injection time did not have significant effect on ATs within a color channel ATs were different between color channels. The highest AT was obtained on the yellow channel of the 10 sec injection of the amplification negative sample and the lower AT was obtained on the blue channel of the 2 sec injection of the run blank samples. Even though these differences do not appear to be drastic, the amplification negative samples are recommended for MDS calculations as they are a better representation of forensic samples. This is even more convenient because in order to calculate these ATs, data that are already present in the laboratory can be used. Twenty to thirty extraction or amplification negatives that have been run with casework samples with no indication of contamination may be utilized saving time and reagents that the laboratories would have otherwise used to do a new
validation experiment. In addition to that, this does not require purchase of expensive and sophisticated statistical software.

As shown in Table 2 the ATs derived for each color channel are different from one another. The forensic analysis software allows the separate input of the RFU for each color channel without significantly increasing the time required to do so. As such, the use of color specific thresholds is recommended.

The ATs obtained using Equation 1 should reflect the value of the “true signal”. The true signal is the signal obtained from the analyte and not from randomly high level baseline noise. If the error of the signal also follows a normal distribution and is equal to those of the blank than the distribution curve will resemble to the distribution obtained from the noise signal of the blanks.

Figure 4. shows each type of error and illustrates a portion of the discussion presented by Winefordner et al (12) as follows:

The probability that the smallest true signal can be distinguished from baseline signal will depend on the number of standard deviation units that \( \mu_0 \) is from \( \mu_1 \). If \( \mu_0 \) is 3 standard deviations away form \( \mu_1 \) the area to the right of the AT calculated using \( k = 3 \) will be no less than 0.0013 meaning that \( \alpha \) error will reflect a 0.13% chance that a signal measured would originate from a randomly high blank signal. On the other hand, half of the time, true signal measurement would fall below that value and would not be considered a signal. To avoid this high probability of a Type II error (\( \beta \) error), the limit may be set at 3 standard deviations away from \( \mu_1 \).
or 6 standard deviations away $\mu_0$. In this case, the areas of $\alpha$ and $\beta$ are equal and the chance for a Type II error is reduced to 0.13%.

![Diagram of normal distribution](image)

Figure 4. General plots of frequency of signal versus signal for normally distributed data where the first curve represents the blank signal, the second and third represent sample signal. $\alpha$ represents the risk that a randomly high blank peak will be greater than AT calculated using $k=3$ and therefore be erroneously labeled while $\beta$ represents the risk that the true signal will not be labeled.

Alternative values of k have been proposed in the literature. Gilder et al. go as far as saying a $k=10$ could be adopted to determine an AT for purposes of detection. However choosing an arbitrarily large k-value is not recommended for the purpose of peak detection, since the impact of high RFU thresholds on the ability to interpret data is significant.

Others have proposed k-values from 6 to 10 with the various thresholds being referred to as limit of purity, limit of quantification and identification limit (10-12, 19). Signals greater than $\mu_0 + 9\sigma$, are described as being in the quantification range (13). Samples with signal between $\mu_0 + 3\sigma$ and $\mu_0 + 9\sigma$ are in the limit of detection range which
is the range where a decision regarding presence of the analyte can be made reliably (11). This means that the concentration of the analyte is high enough to determine its presence but not high enough to guarantee a reliable quantification. Choosing a $k$ higher than 9 will further decrease Type I error (false positive) but this will also decrease the probability of not making a Type II error.

It is clear that high $k$ values used for the purpose of avoiding false negatives are not appropriate for forensic DNA peak detection as the forensic analyst is concerned with peak detection - not peak quantification. Therefore even though using STR peak height/area information is helpful in determining the contributor ratios, it is not recommended that a high $k$ is chosen for such purposes since this will decrease the chances of detection. The interpretation of mixtures requires analyses of the probability of allelic drop-out and peak height ratio discrepancies at small input levels (20-23). For example, Gill et al. (21) examine experimental dataset that exhibit extreme drop-out using logistic regression with the derived probabilities employed in a graphical model to determine the relative risks of wrongful designations.
Positive Amplification Samples

The analytical thresholds calculated and described above were performed on samples containing no DNA. Even though samples containing no analyte are commonly used to determine thresholds, in environmental, analytical and physical chemistry it needs to be examined whether such samples are appropriate for use in forensic casework. (6, 8, 14, 24). The AT derived from negative samples should be used only if the baseline noise obtained form such samples is similar to the baseline noise obtained from samples that contain DNA.

Figure 5. shows the baselines of three types of single source samples taken from the green channel. Panel a) represents a sample with a high DNA input of 2 ng, panel b) shows an amplification negative sample and panel c) represents a sample with a low DNA input of 0.06 ng sample. Samples were prepared using 1 μl for 5 sec injection time.

Figure 5. Electropherograms (green channel) for DNA samples amplified at a) 2 ng of DNA, b) 0 ng of DNA (amplification negative samples) and c) 0.06 ng DNA utilizing 5 s injection time.
As observed in Figure 5, the baselines are different between samples with panel a) containing the highest input of DNA appearing to be the noisiest.

Figures 6a, 6b and 6c show the average RFU signal at a given locus plotted against all baseline noise peaks associated with that locus which were greater than the analytical threshold calculated from the amplification negative samples using Equation 1 (as shown in Table 2). A total of 112 single-source samples were run using 2, 5 and 10 s injection times. The samples came from 4 different individuals with known genetic profiles and were run in quadruplicate for each of the DNA input targets of 0.06, 0.125, 0.25, 0.5, 1, 2 and 4 ng. The known genetic profiles of all of the four individual samples were used to distinguish the alleles from all the other noise peaks. Additionally artifacts such as bleed through between colors, stutters, spikes, etc. were removed. Therefore the peaks in the figure represent noise peaks which could not be characterized as any other artifact or peak.
Figure 6a. Height of the noise peaks greater than the analytical threshold derived via Equation 1 from amplification negative samples and other non labeled peaks which could not be characterized as known artifacts plotted against the average peak height of the known alleles. Graphs include all data obtained from a total of 112 single-source samples of known genotype amplified at 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 ng and run at 2 sec injection time. Loci with off-scale data are not included.
Figure 6b. Height of the noise peaks greater than the analytical threshold derived via Equation 1 from amplification negative samples, and other non labeled peaks which could not be characterized as known artifacts plotted against the average peak height of the known alleles. Graphs include all data obtained from a total of 112 single-source samples of known genotype amplified at 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 ng and run at 5 sec injection time. Loci with off-scale data were not included.
The average allele height for each locus in the x-axis represents the average of both allele heights for the heterozygous condition, and half of the allele height for the homozygous condition. In the rare occasions where there was a drop out of one of the known alleles, the peak height of the present allele was used to represent the mean allele height. The loci that did not show any noise peaks after being analyzed using the AT...
calculated from amplification negative samples using Equation 1, were associated with noise peaks whose height was 1 RFU unit below the used AT. Those peaks are located in the ‘flat’ area of the scatter graph on the far left and all the points above that ‘flat’ part show the peaks whose height is larger than the used AT.

Figure 6. shows that with an increase in allele peak height, comes a considerable level of increase in the baseline noise RFU levels. This increase leads to a higher risk of false labeling of noise. It is also noticed that the highest noise peaks originate from samples containing 2 and especially 4 ng of DNA suggesting that high input of DNA target is the cause of such noise. On the other hand also noticed is an increase in noise peak height as the injection time increases from 2 to 5 and 10 sec for the samples containing 2 and 4 ng of DNA. As a result of this increase, if an analyst were to utilize ATs derived from amplification negative baseline signals where the ATs are shown to be in the range of 5-16 RFU, a significant amount of noise signal would be labeled as a true signal. Figure 6. also shows that the blue and yellow channel seem to consistently have the highest noise peaks heights while the red channel seems to consistently have the lowest noise peak heights.

Figure 7 shows the number of noise peak heights above the AT calculated from negatives for each DNA input for each injection time and color channel.
As observed, injection time has no considerable impact on the total amount of noise peak heights that are above AT for all the DNA input targets up to 1 ng. On the other hand, there is an obvious increase in the number of peak heights above AT for the DNA inputs of 2 and especially 4 ng. There is also a noticeable increase in the amount of these noise peaks for the 1 ng sample injected for 10 sec. All this is important because it shows the increase of probability of labeling noise peaks when samples of 2 ng and higher are used utilizing the AT calculated from negatives using Equation 1. For these high template samples, this risk increases with the increase in injection time as well.

Figure 7 showing the number of noise peak heights above the AT calculated via Equation 1 utilizing amplification negatives, organized by injection time color channel and DNA input target.
Therefore, determination of ATs based on negatives may not be suitable for casework samples with high amounts of DNA input and another method may need to be considered.

Figure 8. shows the AT calculated using the percentile rank method on the positive samples calculated separately for each DNA target utilizing the 99.9 percentile rank.
Figure 8. shows the AT of at least 99.9 percentile rank against DNA target for 2s, 5s and 10s injection time. The horizontal black bar represents the highest AT calculated using amplification negatives at 99.9 percentile rank.
As observed the ATs seem to remain fairly constant up to the 2 ng range within the 2 and 5 sec injection times while showing a considerable increase as well as more variability between color channels for the 4 ng samples. The ATs of the 10 sec injection time samples show even higher increase in RFU level and variability between color channels with a drastic increase starting at the 2 ng range.

Also, Figure 8. illustrates that if a significant mass of DNA is amplified, ATs derived from methods which utilize analyses of blanks are not sufficient and new methods are required. Specifically the samples amplified with 2 ng and 10 sec injection require ATs of 40-120 RFU – a significant difference from those derived from Equation 1 (8-16 RFU) or the percentile rank method (16-47 RFU).

Table 3. compares the ATs of positive samples for each DNA target as well as ATs of the negative samples. All the ATs were calculated using the percentile rank method and utilizing the 99.9 percentile rank.
Table 3. ATs calculated using the percentile rank method at the 99.9 percentile rank for negatives and positive samples

<table>
<thead>
<tr>
<th>DNA target (ng)</th>
<th>Blue</th>
<th>Green</th>
<th>Yellow</th>
<th>Red</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 sec</td>
<td>2 sec</td>
<td>2 sec</td>
<td>2 sec</td>
</tr>
<tr>
<td>Negatives</td>
<td>7</td>
<td>10</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>0.0625</td>
<td>7</td>
<td>10</td>
<td>11</td>
<td>12</td>
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<tr>
<td>0.125</td>
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<td>12</td>
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<td>0.25</td>
<td>12</td>
<td>8</td>
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<td>11</td>
</tr>
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</tr>
<tr>
<td>1</td>
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As suggested by Table 3., if the ATs obtained from the negative samples were to be used on the 2 ng samples for all injection times a lot of baseline noise would be allowed in the DNA analysis increasing the risk of false allele labeling. For the 10 sec
injection time ATs calculated from negatives seem to be unsuitable for the 4 ng samples as well for the same reason mentioned above.

The mean ATs calculated by Gilder et al. (11) using Equation 1 are at the 17 RFU range for all types of samples including DNA containing samples which are positive controls provided by the manufacturer of the test kit. The issue with this type of analyses is that the DNA target is not taken in consideration when analyzing baseline noise making the wrong assumption that noise behaves the same regardless of DNA target.

Figure 6. indicates the blue channel has among the highest noise peaks for the 2 and 4 ng samples. Figure 7. shows the highest number of noise peaks above the utilized AT for the 2 and 4 ng samples. Such observations may imply that the loci associated with this channel could be affected the most by erroneous noise labeling. This can further be exacerbated with increased injection time. The red channel also showed the smallest number of noise peaks above the utilized AT implying that the loci associated with the red channel could be the ones that are the least affected by erroneous noise labeling. The difference in baseline noise between color channels may be explained by the fact that the choice of the detection wavelength can have a major influence on precision where as a general rule, less baseline noise is obtained at longer wavelengths (25). As stated by Kok et al (3) in small misalignments of the excitation laser beam the extra stray light in the spectrograph results in high signals on the short-wavelength side of the emission spectrum. Figure 9. is a histogram that shows the number of noise peak heights above the AT calculated via Equation 1 utilizing amplification negatives, organized by association with allele peak height, injection time and color channel.
As expected, with an increase in injection time comes an increase in allele peak height and an increase in the total number of noise peak heights above the used AT.

Fig 9. shows the number of noise peak heights above the AT calculated via Equation 1 utilizing amplification negatives, organized by association with allele peak height, injection time and color channel.
These histograms include the data obtained for all DNA targets (0.06, 0.125, 0.25, 0.5, 1, 2 and 4 ng) and do not give any target specific information as do Figure 6a., 6b., 6c., Figure 7. and Figure 8. As peak height can be a good representation of target, Figure 9. may be used to determine at which point ATs derived from positives should be used over these derived from the negatives. As observed in the histograms for the 2 sec injection time, a significant increase in the number of noise peaks above AT starts when allele peak heights are >1000 RFU. For the 5 and 10 sec injection this significant increase is seen when allele peaks height is >1500 RFU and >2000 RFU respectively indicating that amplification noise is expected after a certain input mass of DNA and AT should be determined via this criteria. As mentioned above the ATs derived from negatives can be used on samples with low input DNA. To examine such methodology the largest peaks of the profile may be taken into consideration. If such peaks are < 1000 RFU, ATs derived from negatives may be suitable. It should be stated that for mixtures with a minor and a major contributor ATs derived from negatives may not be suitable if the major contributor’s peak heights are > 1000 RFU, even if those of the small contributor are <1000 RFU. The amplification of non-specific product is a known phenomenon especially when the DNA target is high and it contributes to what is called amplification noise. Because of this, as the signal from the major contributor increases, interpretation of low-level minor contributor becomes even more difficult.

As the results above show with over-amplification (amplification of > 2 ng), a significant increase in baseline noise is observed. Even though the current recommended amplification targets range from 0.5 to 1.5 ng, the amount of DNA input into the PCR
reaction is strongly affected by the ability of the laboratory to accurately quantify the amount of DNA. Due to qPCR irreproducibility and the difficulties of producing accurate calibration standards during qPCR set-up (26-28), there is the risk of sub-optimal DNA input level which may be larger or smaller than the aimed one therefore risking to surpass the 2 ng range. This emphasizes the need for laboratories to establish ATs that are representative of samples and signals as they are generally observed in real casework. Therefore, the use of ATs derived from amplification negatives is not appropriate for samples that exhibit significantly high signal because the baselines of negatives are not representative of the baselines of such samples. However, the baselines of negatives are representative of baselines of low-level DNA amplifications, and therefore thresholds derived from negatives are suitable for use with these types of samples.

As mentioned above over-amplification of samples without analyst’s knowledge is possible and may be suspected if there are a significant number of small off-ladder peaks or peak imbalances within color channels. In this case the signal assessment should be performed to decide if re-amplification using a smaller volume is necessary instead of just re-injecting the sample.

Regardless of the method used to determine analytical thresholds, they still need to be re-evaluated when there is a QC failure and/or significant instrument modification. New instruments, can have an up to fivefold signal-to-noise ratio when compared to instruments that have been in use (25), therefore parameters such as sensitivity and MDS should be evaluated on a regular bases, particularly after detector or laser replacement/alignment.
Conclusion

If widely implemented, the determination of ATs based on the MDS needs to be a relatively simple technique. The method utilized by the laboratory to calculate the AT, needs to be chosen in regards to the baselines that are typically observed. The calculation of the MDS from amplification negatives using Equation 1 (utilizing a k of 3 or 4) or the Percentile Rank method (using a rank of 99% or higher) is recommended only when applied to samples whose baseline noise appears the same as that of the amplification negative samples. The ATs calculated from such methods are not recommended to be used on samples with medium to high signal because these thresholds disregard the extra amplification noise which might occur with significantly high DNA inputs. These kinds of samples require an AT calculated using methods that are based on samples containing DNA. Further studies of such methods are suggested. If excessive noise is suspected to have resulted due to non-specific amplified product as a result of over-amplification, re-amplification of the sample using less input of DNA is necessary.
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- DNA Analysis using GeneMapper® ID v 3.1
- Statistical techniques to determine best practices in establishing an Analytical Threshold for DNA Analysis

Degrees Awarded

- MS, Biomedical Forensic Sciences, Boston University, MA (September 2011)
  Concentration: Forensic Biology and DNA Analysis
  Thesis: Investigation of Baseline Noise: Establishing an Analytical Threshold for Forensic DNA Analysis
- BS, Boston University, MA (January 2009)
  Major: Biology

Honors

First Prize in the Faculty of Medicine University of Tirana entrance competition, Albania (2003)