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BCI Validation: YFiler Plus Kit Validation

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BCI VALIDATION STUDY:
YFILER PLUS KIT VALIDATION

CHLOE KOON

HONORS PROJECT

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BCI Validation Study:
YFiler Plus Kit Validation

Introduction

Validation Purpose and Utility

Validation studies are common practice of the forensic scientific community allowing scientists and laboratories to have scientifically sound work that provides factual information about questioned evidence in a case. By doing so, laboratories are reassured that they are providing quality products and clients, such as law enforcement officers and the general public, are reassured that they are receiving quality work and accurate results. Protocols within laboratories are also set and maintained by validation studies. Upon external accreditation, any dubious protocols may be questioned, and the acceptable standards to be followed by all personnel are set by the results of a validation as well. The wide variety of benefits of validating anything before it is used in a laboratory has resulted in many laboratories upholding their lab work with this scientific quality assurance.

Some forensic disciplines don't necessarily have to run validation studies because they are not required by any authoritative entity, just suggested, however forensic DNA analysis does require validation studies. The Federal Bureau of Investigation (FBI) holds its own Quality Assurance Standards (FBI-QAS) that require numerous standards and regulations of laboratories and their personnel in order for scientists to have access to the Combined DNA Index System (CODIS) database. These standards range from how the laboratory space is to be set up to the educational classes that a forensic DNA analyst must have taken, but most importantly, this is where validation studies for the equipment, procedures, and materials used in the laboratories are stated as being required¹. It is important to have access to this database because it allows

forensic DNA analysts to compare their DNA results to this database of collected DNA profiles for casework. Further, more guidance has been given about the nature of the required standards and how to directly apply them by the Scientific Working Group on DNA Analysis Methods (SWGDM) so that laboratories are correctly completing these standards and will be able to use the CODIS database². The group also has published documentation on the validation aspect of the FBI-QAS so that a proper validation study is conducted that meets the FBI standards³.

Validation Methods

There are multiple types of validations that exist including external and internal validation studies in forensics. Internal studies are done by a laboratory before introducing a new method, technology, or material into the space while external studies are done outside of a laboratory itself, such as a developmental validation study done by a manufacturer before selling and distributing a product⁴. The Ohio Bureau of Criminal Investigation (Ohio BCI) is a laboratory system that performs forensic DNA analysis, and therefore, must complete internal validation studies of their own. The Ohio Attorney General's Center (the Center) for the Future of Forensic Science at Bowling Green State University often works with the Ohio BCI to further research and help with projects if needed. The Center was asked to help complete further validation studies into the YFiler Plus amplification kit currently being used in the Ohio BCI laboratory system. Previous validation has been done, but upon external accreditation, it was decided that areas of the original study needed to be developed in order to create a more solid validation in argument of continued use of the kit.

An original developmental validation was performed by the YFiler Plus amplification kit developers that illustrated that the kit could be successfully used and made suggestions as to what limitations the kit might have⁵. This does not however fulfill the FBI-QAS requirement for

internal validation which was the reason for the Ohio BCI performing their first validation study. It was determined by the outside accreditors that the male-to-male mixture study and the male-mixed-female study both were lacking in enough data for the laboratory system to accurately set protocols that allowed use of the kit without more cautionary measures of its use. The manufacturer, Applied Biosystems, also discussed their validation studies into the kit's use along with potential issues that might arise in the future within the user guide they distributed for the kit⁷. This includes possible artifacts that they noticed appeared often and what level DNA concentrations might result in incomplete or inconclusive results.

DNA Typing

Autosomal DNA typing in forensic science has been used for several years and most of its uses are widely accepted in the scientific community. This type of DNA analysis includes the chromosomes in both females and males and can be used to help distinguish an individual, or even multiple individuals in a mixture sample. Mixture samples can have their drawbacks, however, when analyzing the data due to the ability to have multiple alleles, short repeated sequences of DNA that can differ by person, at one locus, specified location in DNA. One person could have up to two different alleles at one locus while two people could have up to four alleles at one locus and so forth⁴. This makes typical autosomal typing more difficult and some level of uncertainty is added to the DNA results, so more caution is taken into how reliable the profiles are. Y-chromosome short tandem repeat (Y-STR) typing can be used in male-female mixtures to help obtain more confident male DNA profiles. Since the Y-chromosome is only found in biological males, areas of the chromosome can be amplified and run through the capillary electrophoresis so that the DNA profile(s) should only contain male DNA even if mixed in with female DNA.

Y-Chromosome Typing

The Y-chromosome is very useful in forensics due to some of its unique qualities in a variety of casework including sexual assault cases and lineage studies. There are several Y-STRs that can be chosen which makes the chromosome easy to study and amplify along with the inheritance factor of the Y-chromosome through the male line that contribute to some of its usefulness ⁶. Between different lineages, males have been shown to be significantly unique in their allele combinations between investigated loci and identification is much more reliable and easier to distinguish between males. However, obtaining individual profiles from a mixture of related males proves much more difficult due to the similarity of their profiles from inheriting the Y-chromosome in the family lineage ⁸. There has been study into the level of contrast needed to distinguish between a father and his son, but the most useful solution found has been to use more loci to increase the chance that the two profiles are different enough to be significant ⁹.

The YFiler Plus kit in this validation study is an example of this solution. The YFiler Plus kit has 10 more loci than the previous YFiler kit in hopes of increasing the distinguishability of the resulting DNA profiles. Some of the new loci are high-mutating STRs which are thought to be better used in identity testing because the chance of related males having different repeats at a given locus increases while slow-mutating STRs are still preferred in lineage casework ¹⁰. Other improvements were made to this new kit including lowering the PCR cycle run times, creating greater inhibitor tolerance, and most importantly, the kit is advertised as having more sensitivity to male DNA in the presence of high female DNA ⁵. This sensitivity aspect is important especially in sexual assault cases to ensure that the female DNA doesn't overwhelm the primers included in the amplification kit and the male DNA still receives primer binding to allow the amplification process to work.

Current Validation Development

This study was originally modeled after other developmental validation studies that were reviewed along with a testing scheme and validation report previously done by the Center. The testing scheme for the 3500 Genetic Analyzer and its accompanying analysis software served as an example as to how to determine the layout of the remaining studies that should be done ¹¹. Following some of these ideas ensured that proper validation techniques were being performed and enough testing would take place that would fulfill the lacking requirements. The validation report from the Center also served the purpose of ensuring that the proper techniques and procedures were taking place as well as the methods for setting thresholds and other protocols for use in the laboratory ¹². A development and validation study on a previous Y-STR kit (AmpFISTR YFiler PCR Amplification Kit) showed how developers validated for the AmpFISTR kit with 17 loci in comparison to the original having 11 loci ¹³. This is applicable to the current validation study since the YFiler Plus kit is an increase in the number of loci from the previous kit, so this validation's methods can be used as a comparison of how to do the validation in this study.

DNA Profile Editing, Interpretation, and Data Analysis

To determine the true extent that YFiler Plus should be used in the BCI's laboratory system, the DNA profiles must be analyzed and interpreted so that statistical testing can occur. While software, such as GeneMapper ID-X, can provide preliminary calls based off pre-set thresholds as to what peaks, indicating the amplified repeats at a locus, may be true DNA, it is the role of the designated forensic DNA analyst to decide what peaks are truly DNA and a part of the DNA profile(s). Other peaks are considered various types of artifacts, and an analyst must eliminate artifacts such as spikes that occur during high fluorescence, pull-up which involves

excess fluorescence flowing into other dye channels, and other common artifacts according to the protocols set by their laboratory ¹⁴. This becomes harder to do in autosomal DNA typing of mixtures since each locus can include multiple alleles, and it can be difficult to determine if a peak is true DNA or not. Y-STR typing helps this issue with typically having less alleles at each locus, aside from a select few loci, so separating a mixture into multiple profiles is generally easier. SWGDAM has published guidelines to help laboratories set their protocols for both autosomal and Y-STR typing with those for Y-STRs having emphasis on appropriately separating DNA profiles in a mixture ¹⁵.

After obtaining the Y-STR DNA profiles from the mixture, analysis of data can occur. There are three main studies being focused on in this overarching validation study all of which use various analyses to draw final conclusions. The first of which is the male sensitivity study which uses several male samples in a concentration dilution series. This is meant to show when alleles begin to drop out and can be used to determine where to set the analytical threshold, the minimum height a peak must be to at least be considered DNA, for the laboratory protocols ¹³. The next study involves constant male DNA in a concentration dilution series of female DNA. Data from this will allow the BCI to know if there is a possibility of allele drop out in samples with extremely high amounts of female DNA and what the male DNA profile may appear if the female DNA overwhelms it. The final study involves male-to-male mixtures from 2-person to 4-person mixtures. This study will illustrate when DNA profiles start to become difficult to interpret as well as the likelihood of allele dropout and encountering incomplete profiles. These mixtures also feature pairings of profiles that are known to be similar or dissimilar. Since close allele combinations can be challenging to obtain individual profiles, it is important to demonstrate what those mixture profiles could look like ¹⁶. The intended purpose of this

validation study is to set appropriate protocols and recommendations for the Ohio Bureau of Criminal Investigation laboratory system's use of the YFiler Plus amplification kit. This will be done through three appropriate studies: a male sensitivity study, a male-female sensitivity study, and a male-to-male mixtures study.

Materials and Methods

The first part of this validation study was done in Fall of 2021. This portion involved obtaining the eight male DNA sample sources (DR, LOM, LS, MML, RHE, SAT, SW and TA) from the BCI and mixing the different sources into the appropriate combinations and concentrations for the various studies. Each individual sample extract was prepared with a total volume of 40 µl, enough to amplify each sample in triplicate. Once samples were mixed, they were returned to the BCI where staff scientists amplified all samples in triplicate using their predefined protocols for the YFiler Plus PCR kit. Following amplification, all sample replicates were electrophoresed on a 3500xL genetic analyzer with a 1.2 kV injection for 24-seconds following the BCI's predetermined protocols. Amplification and capillary electrophoresis were performed along with negative and positive controls and allelic ladders. The electronic data were received at the Ohio Attorney General's Center (the Center) for the Future of Forensic Science at Bowling Green State University after the beginning of 2022 and editing of the DNA using GeneMapper ID-X (v1.6) software began shortly thereafter.

Analytical Thresholds

The first step to completing the validation study was to reevaluate the previously set analytical threshold for using the YFiler Plus amplification kit. This was done by editing both the negative controls and the male sensitivity at low RFUs (relative fluorescence units). Then

analysis was performed to determine an appropriate level of RFU that wouldn't allow too much baseline noise peaks but avoided not calling true DNA.

Male Sensitivity Study

Using the chosen analytical threshold (100 RFU), the male sensitivity samples were reanalyzed and edited again. These were samples ranging in DNA concentration from 2ng to 0.00391 ng for each of the eight males. These could then be interpreted and analyzed for allele dropout and a level of confidence can be determined for what concentration of DNA could still obtain a reasonable DNA profile.

Male with Excess Female Study

The samples used for this study were a mixture of one sample consisting of an excessive amount of female DNA and another sample of male RHE's DNA concentration was held constant at 1ng while the female DNA increased from 1ng to 999ng. Each sample was analyzed, edited, and then interpreted to look for any allele dropout or cross-reactivity that occurred with the increasing amounts of female DNA.

Male: Male Mixtures Study

Mixtures samples were created in combinations of 2, 3, or 4 males at varying amounts of DNA (1ng, 0.25ng, 0.125ng, and 0.062ng). Each combination was also performed at different ratios between individuals at every DNA concentration level. 2-person combinations (TA:LOM and SW:ML) had ratios at 20:1, 10:1, 5:1, and 2:1. 3-person combinations (TA:LOM:LS and SW:ML:DR) had ratios at 1:1:1, 2:1:1, 5:1:1, and 10:1:1. 4-person combinations (TA:LOM:LS:RHE and SW:ML:DR:ST) had ratios of 1:1:1:1, 3:1:1:1, 3:2:1:0.5, 4:3:2:1, and 5:5:1:1. Each sample was analyzed, edited, and then interpreted to look for any allele dropout,

overlap, major-minor profile, or mixture ratio information that may help in interpreting male-male mixture samples.

Results

Analytical Thresholds

Analysis of the negative controls yielded differing suggested minimum analytical thresholds for each of the dye channels between 25 RFU and 50 RFU. The blue channel had a minimum analytical threshold of 30 RFU, the green channel had 45 RFU, the yellow channel had 25 RFU, the red channel had 35 RFU, the purple channel had 45 RFU, and the orange channel had 50 RFU. The average suggested a 45 RFU level for analytical threshold overall between all six dye channels. The overall average RFU baseline peaks seen were 7.03 RFU with a standard deviation of 3.54 RFU. The overall Limit of Detection (average RFU plus four standard deviations) was determined as 21.21 RFU, and the Limit of Quantitation (average RFU plus ten standard deviations) was 42.47 RFU.

Looking at the male sensitivity samples at low RFUs yielded different results for the minimum analytical threshold. Each locus was analyzed for their average peak height and standard deviation (in RFUs) for every input DNA level (assuming data was available at that input level). Loci *DYS385* and *DYF387S1* were both omitted from this part of the study due to their ability to have multiple peaks at one locus, unlike the rest of the studied loci. The average peak height and standard deviation were then determined by input level for each of the five standard dye channels and for the overall loci included between dye channels. The average peak heights were seen to decrease as the level of DNA input decreased. A weighted regression analysis was performed for each dye channel and for all loci together at both an alpha value of 0.05 and 0.01. The minimum analytical threshold for each dye channel, at an alpha value of 0.05

and 0.01 respectively, was 75 RFU and 140 RFU for blue, 85 RFU and 160 RFU for green, 40 RFU and 75 RFU for yellow, 75 RFU and 135 RFU, and 75 RFU and 165 RFU for purple. The overall minimum analytical thresholds were suggested to be at 75 RFU at an alpha value of 0.05 and 140 RFU at 0.01.

Male Sensitivity Study

Editing of the male sensitivity samples occurred again at the decided upon analytical threshold (100 RFU) before the samples' peak heights were used for the actual sensitivity study purposes. As with the analytical thresholds study, the loci DYS385 and DYF387S1 were both omitted due to known duplication events that occur at those loci. The basic process of finding the average peak heights and standard deviations for the loci at all input levels, then by dye channels and by overall between all the loci at all the input levels, remained the same as what was previously done for the analytical threshold study. However, there were many more peaks in the study analyzed at low RFUs in comparison to that done at the 100 RFU analytical threshold. As seen in **Figure 1**, there was a typically a decrease of roughly half between each DNA concentration that was halved. One exception was the decrease from 2 ng of input DNA to 1 ng of input DNA that had a decrease of roughly over half. Also, once the concentrations reach about 0.0625 ng, the decrease no longer resembled a decrease of a half, and they start to level out.

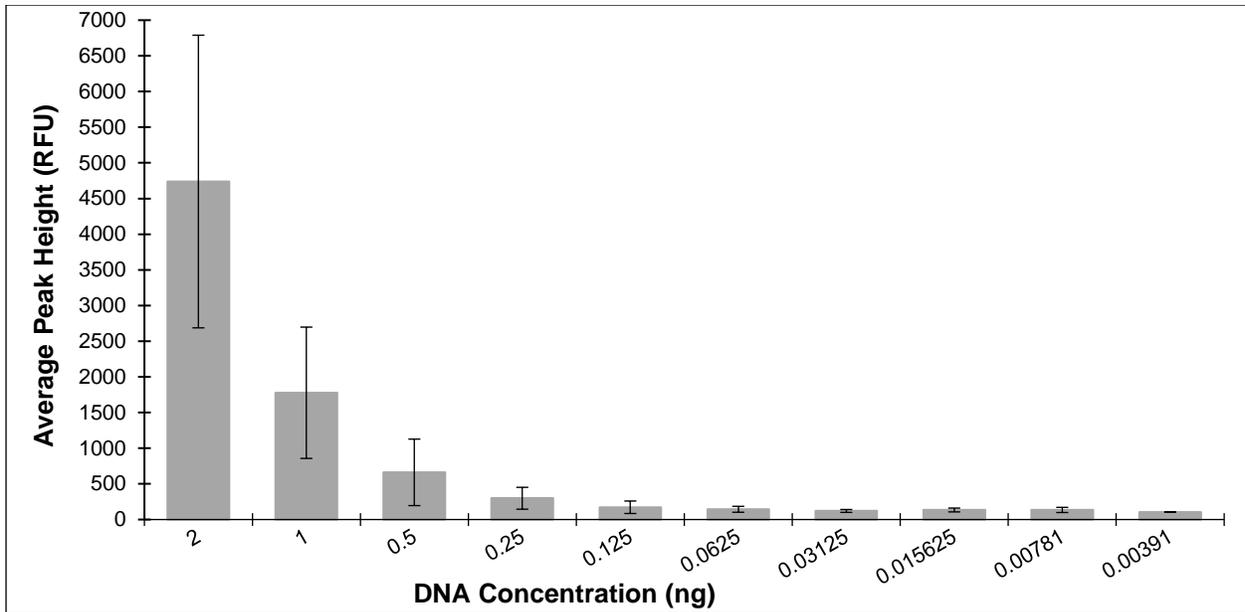


Figure 1. Graph depicting the average peak height (RFU) of all the loci data at a given DNA input concentration (ng).

Each dye channel appeared to perform about the same as the others. As seen in **Figure 2**, there was some slight differences at 2 ng of input DNA, but as the concentration decreases, the dye channels become more tightly packed in average peak height. The biggest difference was seen between the yellow dye channel and the green dye channel at 2 ng and 1 ng of input DNA.

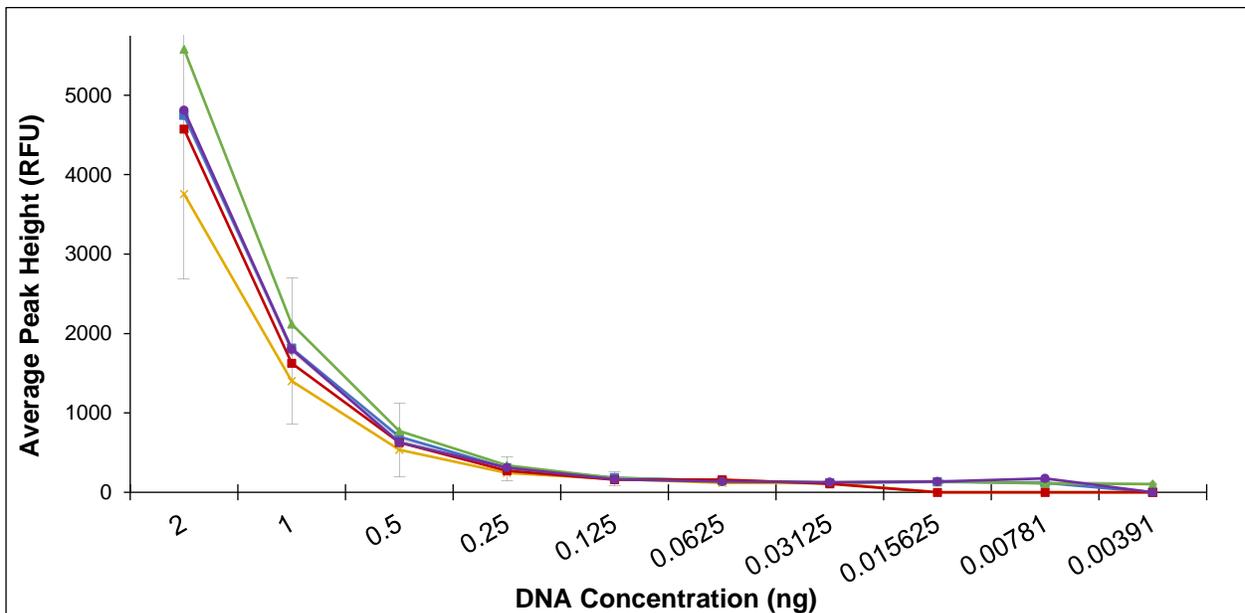


Figure 2. Graph depicting each dye channel’s average peak height (RFU) at each given DNA concentration (ng).

Some loci, such as DYS389II and DYS449, showed more dropout than other loci at all input levels as shown in **Figure 3**. It can be seen within the figure that there are a few loci that have, on average, much lower peak heights throughout the various concentration levels.

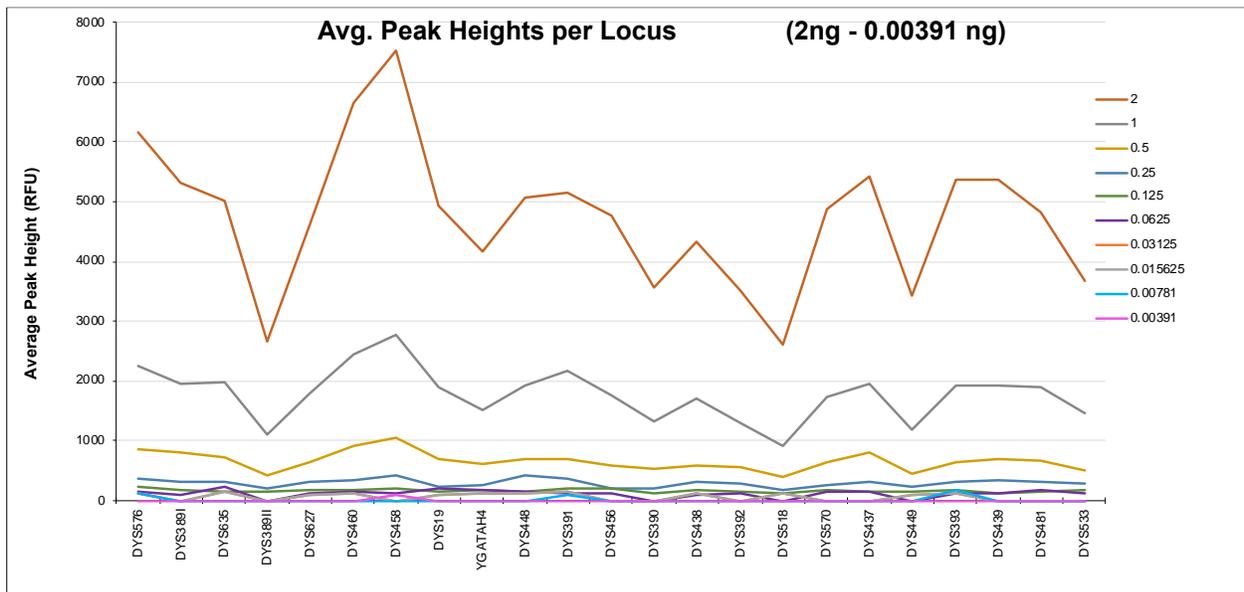


Figure 3. Graph depicting each loci’s average peak height (RFU) at each given DNA concentration (ng) for all concentrations in the sensitivity study.

As the DNA input decreased to lower concentrations at 0.125 ng and below, there was significantly more dropout of the intended alleles even in those that were shown to readily appear in high DNA concentration levels. This can be seen in **Figure 4** where is also indication of dropout of certain loci at the lowest concentrations. There is much more variation seen of peak heights across all the loci than at the concentrations 0.125 ng and above.

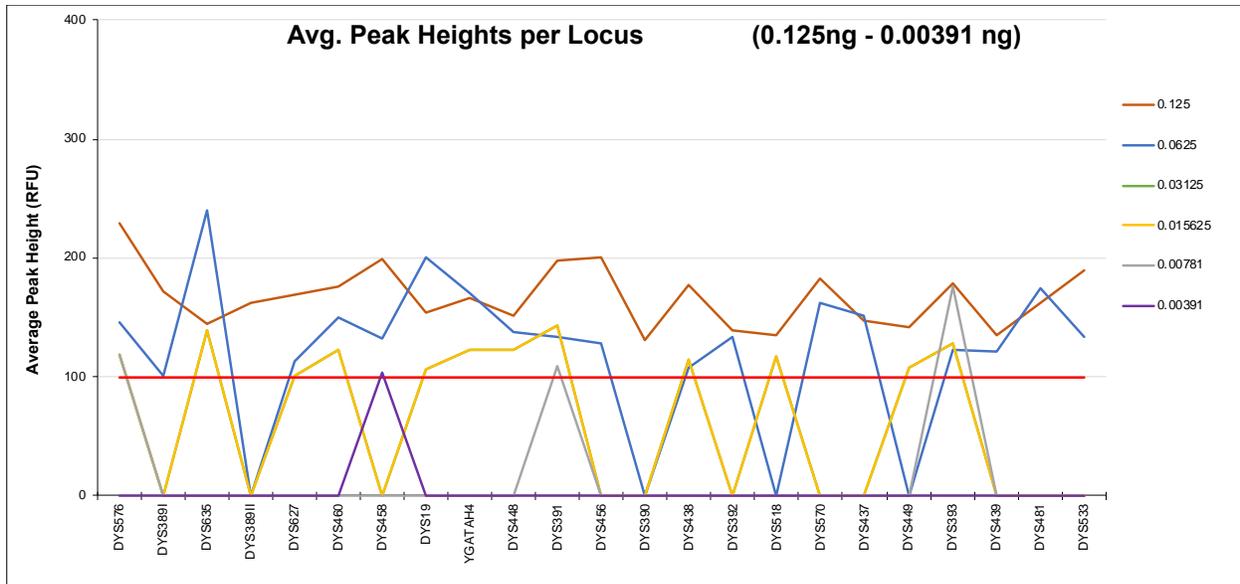


Figure 4. Graph depicting each loci's average peak height (RFU) at each given DNA concentration (ng) from 0.125 ng down to 0.00391 ng.

Setting the stochastic threshold value required examination of the two loci with multiple possible peak heights or loci duplications, DYS385 and DYF387S1. These loci showed dropout of one of two possible peak heights at a locus around the 0.25 ng input. The tallest of the peak heights that was remaining after dropout was seen in its counterpart was 365 RFU.

Male with Excess Female Study

Once again, the average peak heights and standard deviations were found across all the loci, except DYS385 and DYF387S1, but now at the varying ratios of male to female DNA from 1:0 to 1:1000 (male: female). The average peak heights and standard deviations were then determined for each dye channel and for the overall between all loci for each ratio as well. As seen in **Figure 5**, there does not appear to be much difference as the female contribution increases. An ANOVA test gave a p-value of 0.0006 indicating that there is a difference between at least one average peak height for a given ratio in comparison to another. Further pairwise analysis testing determined that there were six instances of statistically different results of one ratio being different than another individual ratio. These were 1:2 and 1:100, 1:2 and 1:500, 1:5

and 1:100, 1:20 and 1:100, 1:20 and 1:500, and 1:100 and 1:750. However, those differences don't appear to be truly meaningful or appreciable, according to **Figure 5**. Average peak height each different dye channels and by locus did not yield any significant dropout or results.

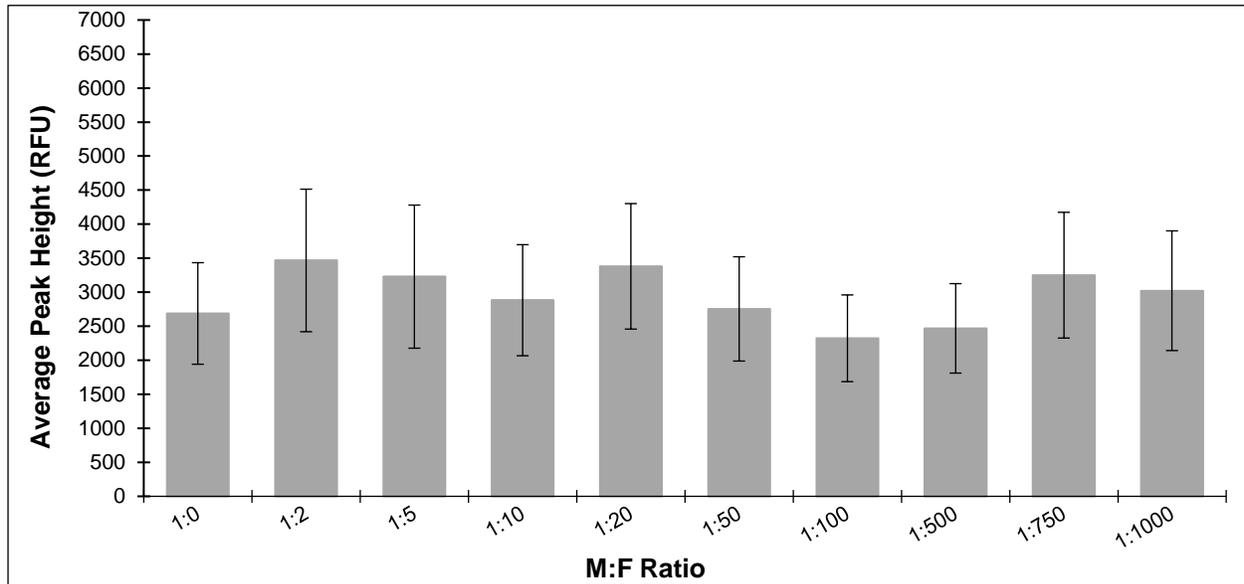


Figure 5. Graph depicting the average peak height (RFU) for each given male to female ratio.

Male: Male Mixtures Study

Complete editing was performed on the raw data for all mixture samples. The analysis process was not complete in time to write this paper, but it is in progress and will be completed in a timely manner to relay any important findings to the Ohio Bureau of Criminal Investigation.

Discussion and Conclusions

While the negative control method suggested that the minimum analytical threshold should be at least 45 RFUs based off the Limit of Detection and Limit of Quantitation, the analysis of the male sensitivity samples at low RFUs suggested that the minimum be at least 75 RFUs if not 140 RFUs. There was also the notion that each dye channel may have a separate analytical threshold value since there was great variance in the suggested values between each channel in both the negative controls method and the male sensitivity method. Ultimately, it was

decided in consultation with the Ohio Bureau of Criminal Investigation that the dye channels would remain constant between their analytical thresholds. Additionally, the Ohio Bureau of Criminal Investigation determined that they would proceed with an analytical threshold value of 100 RFU as a middle ground to the suggested levels, which would correspond to an alpha of less than 0.05 but greater than 0.01 confidence.

Examination of the single source male sensitivity samples at the new analytical thresholds gave indications of important boundaries to set when looking at the peaks of most of the studied loci. At low levels of input DNA, it is known that there may be detection of alleles that are above the analytical threshold. However, based on the data having increased dropout under 0.125 ng of input DNA, it is suggested that the dynamic range of when to expect significant DNA profiles is between 2 ng and 0.125 ng of input DNA. This Limit of Linearity's upper limit is the peak heights of 2 ng samples due to having large error indicated on **Figure 1** and the increasing number of artifacts present at this level. The lower limit is the analytical threshold (100 RFUs) since no peak can be called as true DNA below it. Outside of the Limit of Linearity indicates that the appropriate relationship between the peak heights and DNA concentrations exists (halving the concentration causing halving of the peak heights). The ideal target of DNA was determined to be between 0.5 ng and 1 ng, approximately 0.75 ng of input DNA. At this level, there is the most confidence that there will be full profiles obtained, and there will be consistent peak heights across all the loci within the same dye channel with perhaps some variance in heights between the dye channels.

Determining the stochastic threshold can help set the guidelines as to when to carefully use the peaks in a DNA profile at the loci with duplications of intended peak heights, DYS385 and DYS387S1. The stochastic threshold can be set to determine at what peak height can one be

most confident that the loci is truly only supposed to have one peak. Since the tallest remaining peak when dropout occurred was 365 RFU, a suggested stochastic threshold is 400 RFU. This means if a single peak is at one of the two loci, DYS385 or DYS387S1, it would need to be at least 400 RFU before it could be considered as a homozygous locus and not to have possible dropout of its counterpart duplication peak.

The study of the male DNA mixed with increasing amounts of female DNA was intended to indicate at what concentration female DNA interfered with the male DNA profile obtained in doing Y-STR typing. Due to limited amounts of male DNA, this was only completed at the 1 ng level for the male contribution while the female DNA was mixed in at ratios up to 1:1000. Although the ANOVA and pairwise test indicated that there was difference between some individual ratios, it altogether does not have significance to this study. Normal amounts of male DNA were used, and full DNA profiles were still being obtained at the various levels of female DNA. The female DNA did not seem to have an adverse effect on the profiles being obtained. This means that if enough male DNA is present in the mixture, then there is confidence that a full male DNA profile can be obtained up to a 1:1000 ratio with female DNA present.

Despite the male-to-male mixtures analysis not being completed yet, several aspects of the finished studies should prove useful to the Ohio Bureau of Criminal Investigation. There was an analytical threshold of 100 RFU decided upon and a suggested stochastic threshold of 400 RFU that can be investigated as a possible new guideline in the laboratory system. It was also important to show the boundaries within the male sensitivity study, so it can provide solid foundation to setting the guidelines for the mixture study. Having the range of 2 ng down to 0.125 ng can help determine whether a sample should be sent for further DNA analysis and profiling. It was also important to set an ideal target of about 0.75 ng of input DNA for yielding

the best DNA profile. While it was shown that the excess female DNA in a mixture didn't have any effect on the quality of a male DNA profile at normal analysis levels, future study could be done with larger amounts of male DNA with the increasing female DNA and with low amounts of male DNA to see if good quality DNA profiles are still obtained. Overall, these studies made excellent progress in progressing the protocols for the Ohio Bureau of Criminal Investigation.

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