The Internal Validation of Life Technologies® Quantifiler® Trio and Qiagen® Investigator® Quantiplex HYres DNA Quantification Kits, and a Comparative Analysis to Life Technologies® Quantifiler® Duo

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Table of Contents *Page*

Abstract

The Federal Bureau of Investigation's (FBI) Quality Assurance Standards (QAS) specify that when a kit is implemented into a laboratory it must undergo an internal validation to ensure it performs as expected in that specific laboratory [1]. In order to meet the FBI QAS, the following studies were performed with the Life Technologies® Quantifiler® Trio DNA Quantification Kit and the Qiagen® Investigator® Quantiplex HYres kit: standard curve quality metrics, accuracy, precision, sensitivity, repeatability, stochastic, known/non-probative, mixture, and contamination. This project focused on the validation of the Quantifiler® Trio and Investigator® Quantiplex HYres DNA quantification kits, as well as comparing these kits with the Life Technologies® Quantifiler® Duo DNA Quantification kit, which is the quantification kit currently implemented by the West Virginia State Police Forensic Laboratory's (WVSPFL) Biochemistry Section [2].

While the Quantifiler® Duo kit had been previously validated by the laboratory, the studies were performed with this kit as well in order to produce current data under the same parameters for comparison purposes. The current validated procedures used by the WVSPFL Biochemistry Section were used for the Quantifiler® Duo quantifications, while the manufacturers' protocols were used for the Quantifiler® Trio and Investigator® Quantiplex HYres quantifications [3, 4]. Statistical calculations were then performed on the concentration and threshold cycle (C_T) results of each sample in order to evaluate the kits' performances and allow for comparisons between the kits.

The ultimate goal was to identify which of the three kits would best suit the needs of the laboratory by looking at the statistical results and overall outcomes of the previously

mentioned studies. In order to determine this, it was imperative to show that each of the kits were robust and reliable, and would therefore have the potential to be implemented for casework. After this was determined, the kits could then be compared in the areas thought to be of high importance to the WVSPFL Biochemistry Section. It is vital that a quantification kit be able to indicate a male contributor in a mixture with a high concentration of female DNA present; this ability was evaluated for all kits. Each kit was in fact able to detect mixtures at numerous concentrations; however, the ratios produced by each kit varied from one another. By comparing the multiple aspects studied, while focusing on those deemed most important, a decision was able to be made by the WVSPFL Biochemistry Section regarding which quantification kit was the best fit for their laboratory.

Section 1: Introduction

Standard 9.4 of the Quality Assurance Standards (QAS) set forth by the Federal Bureau of Investigation (FBI) states that any accredited forensic DNA laboratory "shall quantify the amount of human DNA in forensic samples prior to nuclear DNA amplification" [1]. Therefore, quantification becomes a vital step in the DNA analysis process. Not only is it required for forensic samples in accredited laboratories, quantification also ensures the DNA is from a human source, assists downstream processes by optimizing the reactions, and allows for the normalization of samples. After quantification, real-time polymerase chain reaction (PCR) is performed to amplify the desired segments of DNA. This reaction typically requires between 0.5 and 1.0 ng of DNA to achieve optimal results [5]. By quantifying the DNA in an unknown sample prior to this process, the analyst is able to dilute or concentrate the sample as needed in order to reach this ideal concentration range.

Advances in human-specific real-time PCR have allowed for the quantification process to become highly sensitive and provide ample information about the samples being tested [6]. Not only do the current kits involved with the forensic science community provide human and male DNA quantities, but also information about the presence of PCR inhibitors, and in some cases, indicate potential degradation [4, 7]. With these improvements comes a growing necessity to test the kits' capabilities and demonstrate their use within a forensic setting.

The Applied Biosystems® Quantifiler® Duo (Life Technologies®, Foster City, CA) is a target-specific assay that uses Ribonuclease PRNA Component H1 (RPPH1) as the human target, the Sex-determining Region of the Y chromosome (SRY) and an internal positive control (IPC), which is a synthetic sequence not found in nature [8, 9]. This kit utilizes a TaqMan® Minor Groove Binding (MGB) probe which consists of a reporter dye (either FAM™, VIC®, or NED™ depending on the target sequence), a MGB, and a nonfluorescent quencher (NFQ). This probe binds to a complimentary sequence found between the forward and reverse primer sequences. When it is intact, the quencher dye suppresses the reporter dye's fluorescent signal. However, when the DNA polymerase begins extending the new DNA strand, the probe is cleaved, thereby releasing the reporter dye from the quencher and allowing a fluorescent signal to be emitted. Therefore, an increase in fluorescence indicates an increase in amplified product [9]. This fluorescence is then detected by the instrument and used to quantify the sample through the use of computer software. Figure 1 demonstrates the probe binding, strand extension, cleavage of the probe, and the resulting fluorescence emission process.

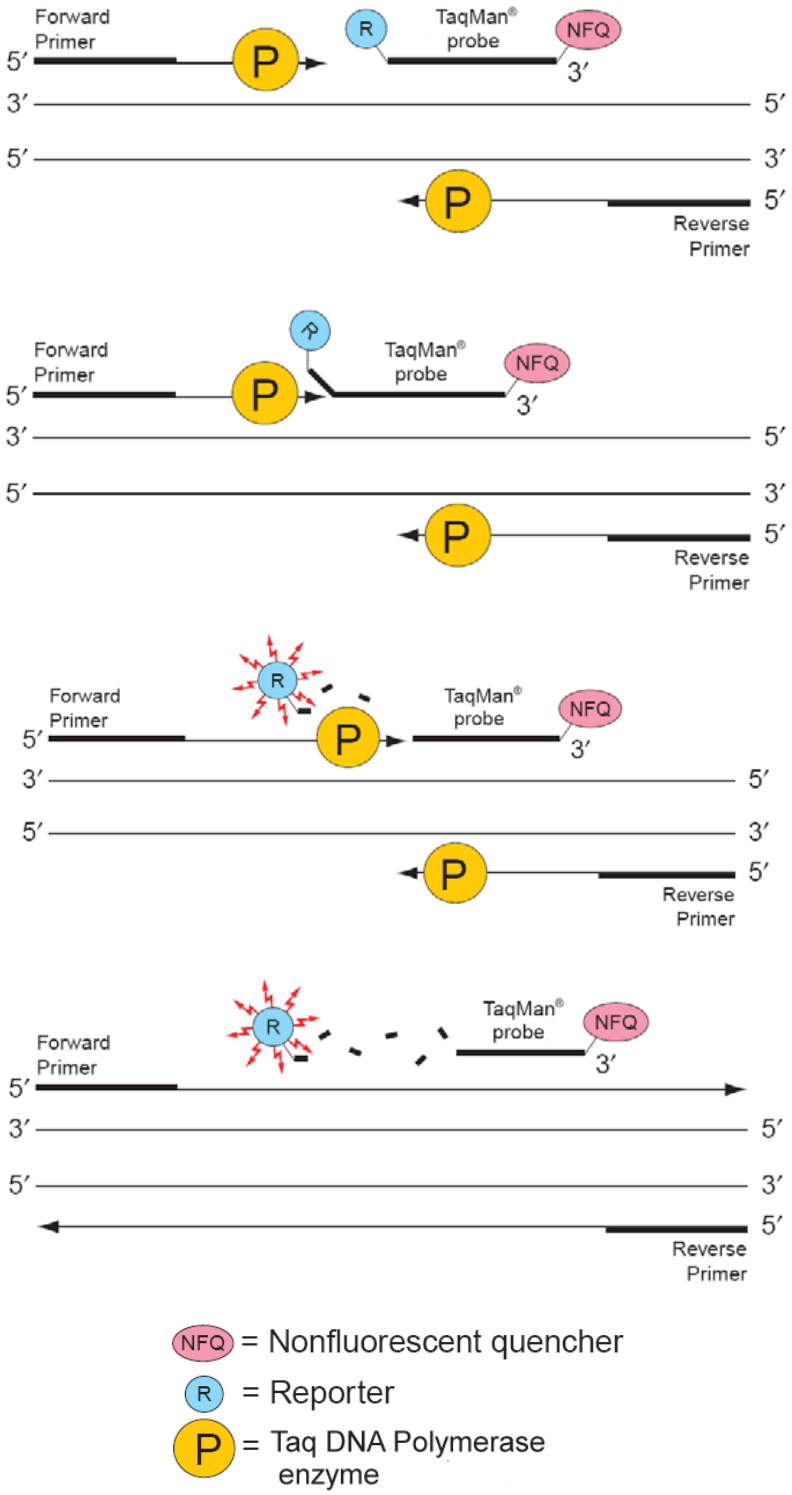


Figure 1: TaqMan® Probe [9]

The Applied Biosystems® Quantifiler® Trio DNA Quantification Kit (Life Technologies®, Foster City, CA) also relies on the TaqMan® probe as a means of detecting the amount of amplicons generated during PCR. The main difference between Quantifiler® Trio and Quantifiler® Duo is that Quantifiler® Trio implements two human autosomal targets, as opposed to just the one found in Quantifiler® Duo. One of these amplicons is 80 bases in length and deemed the "small autosomal", while the other is 214 bases and called the "large autosomal". The larger autosomal target would be more susceptible to degradation, therefore when its concentration is compared to that of the small autosomal target, the analyst is able to determine if the sample is exhibiting degradation and take additional steps to attempt to compensate for the issue. By including these two sizes, the corresponding software is able to determine if any degradation may be present in the sample [4].

The Qiagen® Investigator® Quantiplex HYres (Qiagen®, Hilden, Germany) kit functions very similarly as Quantifiler® Duo in that it is also a target-specific assay which targets one human autosomal and one male Y target, as well as contains an internal positive control. Unlike the Quantifiler® kits, the Investigator® Quantiplex HYres Quantification Kit uses Scorpions® Primers, which are bi-functional molecules containing a PCR primer covalently linked to a probe made up of a fluorophore and a quencher. During PCR, the probe binds to the products, the fluorophore and quencher are separated, and fluorescence results. Therefore, increased fluorescence is indicative of increased PCR product [3]. Figure 2 demonstrates how these Scorpions® Primers function during PCR.

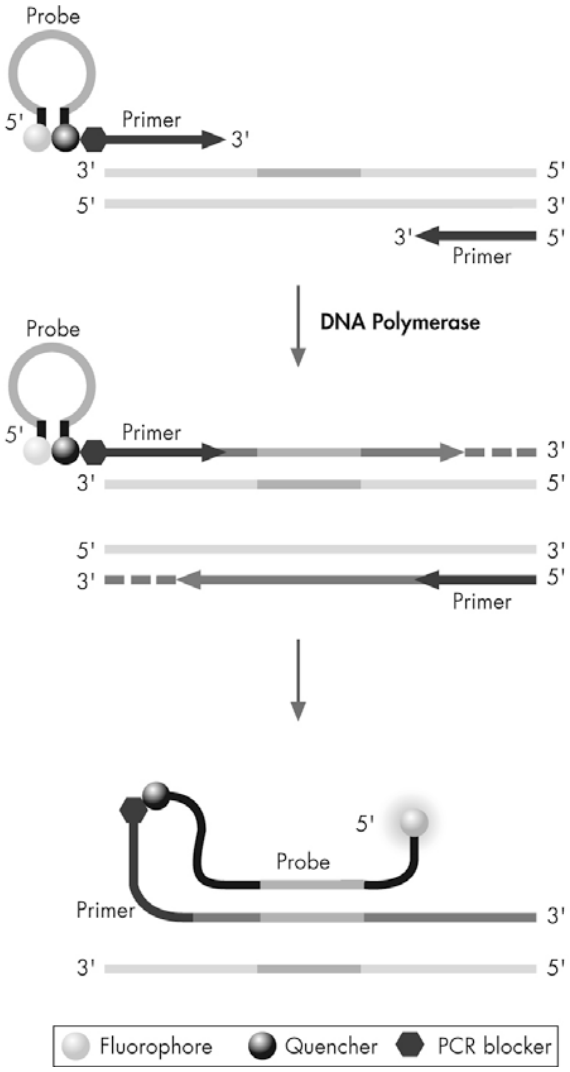


Figure 2: Scorpions® Primers [3]

In concordance with the FBI QAS, the Scientific Working Group of DNA Analysis Methods (SWGDAM) specifies that when a kit is implemented into a laboratory it must undergo an internal validation to ensure it performs as expected in that specific laboratory [10]. In concordance with these guidelines, the following studies were performed for all three kits: standard curve quality metrics, sensitivity, accuracy, precision, repeatability, stochastic, known and non-probative, mixture, and contamination. Quantifiler® Duo had previously been internally validated within the West Virginia State Police Forensic Laboratory (WVSPFL)

Biochemistry Section and used for casework; the same studies were run with this kit in order to provide current data for comparisons. The results of these studies were then assessed, compared to one another, and used to determine which kit best suits the needs of the WVSPFL Biochemistry Section.

Section 2: Methods

2.1 *Standard Curve Quality Metrics*

A standard curve quality metrics study was performed for each of the three kits in order to verify that the standard curves generated consistently fell within the manufacturer's guidelines. In order to evaluate this, twelve Quantiplex HYres, twelve Quantifiler® Trio, and ten Quantifiler® Duo sets of standards were made on three respective 96-well plates. Due to simultaneous tests running on the Quantifiler® Duo plate, only ten sets were able to be run.

The curves were generated using the suggested volumes of DNA standard and dilution buffer as suggested by the kit's respective manufacturers, with the exception of the Quantifiler® Duo kit [3, 4]. Since the Quantifiler® Duo procedure was written into the current West Virginia State Police DNA Analysis Procedures Manual, that protocol was followed when creating the standards and plate for that kit [2].The curves were generated by analyzing two sets (two columns) of adjacent standards each. For example, columns 1 and 2 were paired, 3 and 4 were paired, and so on. Following the West Virginia State Police DNA Analysis Procedures Manual, either of the two data points used to generate the curve at any one concentration may be omitted to meet the required values [2].

2.2 *Accuracy and Precision*

An extracted buccal swab was selected to be used for the accuracy and precision studies. After extraction and quantification using the Quantifiler® Duo kit, serial dilutions were made and each of these dilutions were quantified using the three kits. Since Quantifiler® Duo is the validated kit currently in use by the WVSPFL Biochemistry Section, its values were used as the standard (expected value) with which to compare the other two kits (unknown value). The average concentration for each sample, as well as the difference between the obtained average and the target concentration was calculated in order to demonstrate the accuracy of the kits.

The samples used for the accuracy study were also utilized for the precision study. However, instead of looking at how far the reported quantities deviated from the true values, the standard deviation of the three values given for each of the concentrations was taken for each kit to determine how closely related the quantified values were to each other. Essentially, this provides an indication of how consistent each kit is when determining the quantity of DNA in a sample multiple times; however, this does not indicate how close the value given is to the actual or true value. The precision, when combined with the accuracy, can provide an indication of how reliable a quantification kit is. Ideally, one would want a kit with both high accuracy and precision to obtain the best and most reliable results.

The relative standard deviation (RSD) was also calculated by dividing the standard deviation by the average concentration and multiplying by 100, resulting in a percentage. The closer to zero the percentage is, the less variation in the measurements when compared to the overall average. This statistic provides another view on how precise the kits are at each concentration.

The C_T value of a sample is the cycle at which it crossed a defined threshold. This verifies for the analyst that DNA amplification did occur, and gives an approximate indication of how much DNA might be present in the sample, or if the sample contains potential inhibitors. As the concentration of DNA decreases, it requires more cycles for the threshold to be met, and therefore increases the C_T value. These C_T values were also evaluated statistically by calculating the average, standard deviation and RSD.

In order to be able to sufficiently compare the kits, a chi square statistic was also calculated to determine if there was a statistical difference between the desired values (those produced by Quantifiler® Duo) and the obtained values (those produced by Quantifiler® Trio and Quantiplex HYres). This calculation is able to determine if two values are significantly different from one another, from a statistical standpoint.

2.3 *Sensitivity and Stochastic*

The sensitivity and stochastic studies were conducted by taking a serial dilution of the 2800M amplification control beyond the concentration ranges of the kits to determine if they were able to detect DNA at extremely low quantities. The values chosen were 0.005, 0.0025, 0.0005 and 0.00025 ng/µL. While 0.005 ng/µL is still included in the range for the Quantifiler® Trio and Quantiplex HYres kits, it was believed that the value would still be a good indication of the kits abilities to detect low copy number DNA and test any limitations of the kits. All four of the dilutions were quantified in triplicate, with the exception of the 0.00025 ng/µL sample using the Quantiplex HYres kit. Due to the sensitivity samples being amplified prior to the Quantiplex HYres kit quantification, there was not adequate sample left for the quantification. Therefore,

only two quantifications were made of the 0.00025 ng/µL sample for Quantiplex HYres, as opposed to three replicates with the other two quantification kits.

A low quantity of DNA may induce stochastic effects, which may indicate the resulting electropherogram may not be a full profile [10]. By studying the concentration levels at which various results, such as full or partial profiles, are produced, analysts are thereby able to anticipate these stochastic effects and make informed decisions regarding future steps for their casework during DNA analysis. Therefore, ensuring that they are able to obtain the most complete and informative profile possible. The ultimate goal of this study was to determine if a 0.000 ng/µL or "Undetermined" result obtained for a forensic sample truly indicates that no DNA is present. This was tested by amplifying the maximum volume of DNA allowed by the WVSPFL Biochemistry Section protocol and performing capillary electrophoresis to determine if a full or partial DNA profile could be obtained from these low copy number samples [2]. This step has the potential to greatly affect casework and streamline the DNA analysis process. If these quantification kits can be trusted to reliably indicate that no DNA is present, reagents, time, and money can be saved by not using these resources on samples that will most likely not produce a DNA profile that can be used for comparison purposes.

2.4 *Repeatability*

The idea that a method demonstrates repeatability relies on one analyst being able to perform a method several times and yield similar results each time. This aspect of the quantification procedures was tested by running each study, when adequate sample was available, in triplicate on each quantification plate. Not only did this provide more statistical

data for the study overall, but it also helped to demonstrate whether or not variation existed when the same operator performed the quantification.

***2.5** Known and Non-probative*

Known and non-probative samples were created to simulate casework samples that would most likely be encountered by the WVSPFL Biochemistry Section. The study focused on sample types that are frequently seen by the analysts to ensure results are produced as expected by the kits undergoing the internal validation. The known samples included a buccal swab and a FTA blood card cutting, while the questioned unknown samples consisted of a hair, cigarette filter, swab from a bottle, and swab with several drops of blood.

The knowns were extracted first, using the WVSPFL Biochemistry Section EZ1® (Qiagen®, Hilden, Germany) protocol for stains on solid material and purified using the EZ1® Trace protocol on an EZ1® Advanced XL instrument. The questioned samples were then extracted using the respective protocol for the type of sample involved, and all were purified using the EZ1® Trace protocol [2]. After purification, the samples were quantified with each of the three kits. The results were then evaluated and compiled.

Inhibited and degraded samples were also included in the known/non-probative study in order to account for these possible scenarios arising during casework and evaluate how the quantification kits handle these issues. A control system designed to alert the analyst to possible inhibition within the sample is set in place for each of the three kits. Each company has designed their own, yet the methodology is essentially the same: Internal PCR control (IPC) system for Quantifiler® Duo and Quantifiler® Trio, and internal control (IC) system for Quantiplex HYres. The control systems consist of a synthetic DNA template, corresponding

primers, and fluorescent dye [3, 4, 9]. Each of these components is added in a set amount to each well and amplified with each sample, control, and standard.

This control is expected to result in an expected C_T value: between 28 and 31, with a variation of 1 C_T value across the standard curve samples for Quantifiler® Duo and Quantifiler® Trio, and approximately 31 for Quantiplex HYres [3, 4, 9]. While these values should be validated for each specific laboratory, the WVSPFL Biochemistry Section follows the manufacturer's recommendations for Quantifiler® Duo and it was decided that the same would be done for the other kits as well [2]. Keeping these guidelines in mind, the analyst is able to determine if inhibition may be occurring in the sample if the IPC's C_T value is not within the expected range. Table 1 is an example of how IPC values can assist the analyst with interpretations and indicate that inhibition has taken place [9].

Table 1: IPC Interpretation Guidelines for Quantifiler® Duo [9]

Duo Human (VIC® dye) and/or Duo Male (FAM™ Dye)	**Duo IPC (NED™ Dye)**	**Interpretation**
No amplification	Amplification	Negative result - no human DNA detected
No amplification	No amplification	Invalid result
Amplification (low C_T and high ΔR_n)	No amplification or C_T higher than 31	IPC result inconclusive
Amplification (high C_T and low ΔR_n)	No amplification or C_T higher than 31	PCR inhibition

The inhibited samples were created by adding inhibitors directly to buccal swabs, as well as to extracts of purified DNA. Since it was expected that the extraction process would most likely remove most of the inhibitor from the samples where it was added prior to extraction,

the process of adding inhibitors to the extracts allowed for the inhibitors to be represented in a sample. Since casework samples would go through extraction in the DNA analysis process, samples were designed to mimic this work-flow as closely as possible. However, it was also necessary to test the quantification kits with truly inhibited samples. Therefore, samples with inhibitors added before and after the extraction process were created.

The inhibitors selected were ones that the WVSPFL Biochemistry Section might encounter on a regular basis. They included indigo dye, soil, and river water. Indigo dye is often used to dye blue jeans, and soil contains humic acid, both of which can inhibit the PCR process. River water may also contain humic acid from the river bed, polysaccharides from plant material, and bacteria; all of which can also interfere with DNA replication [11].

The degradation agents were added to the buccal swabs prior to extraction since the extraction process should not affect the level of degradation present and this is how the samples would most likely be encountered while processing casework. The degradation agents were also selected based on their commonality to the casework, as well as the laboratory; they included bleach, Eliminase®, and Ultra-violet (UV) radiation. For the liquid degradation agents, two samples were created—one with neat, undiluted reagent and one with a 1:20 dilution. For the UV'd samples, one swab was placed under a dead air space with a UV light for 10 minutes. Then a second swab was added and both were exposed to UV radiation for an additional 5 minutes.

As mentioned previously, Quantifiler® Trio is able to generate a degradation index to indicate how potentially degraded a sample may be, based on the presence of large autosomal targets versus small autosomal targets [4]. The other kits do not possess this ability; however,

this study aimed to see how Quantifiler® Duo and Quantiplex HYres handled and presented these types of samples, as well as how accurate the degradation index appeared to be for Quantifiler® Trio.

Six buccal swabs were taken from three male volunteers with known profiles. One person was chosen for each inhibitor and each degradation agent and used for the both the neat and diluted for degradation, or addition of the inhibitor before or after extraction. For example, volunteer HBM was used for all of the Eliminase® samples and all of the samples containing soil. This was done in order to obtain a clearer understanding of how much inhibition or degradation was taking place, as the swabs from the same individual most likely have similar concentrations. In addition, one swab was extracted from each individual to act as a control; therefore no additional agents were added at any time and they were extracted as known samples.

Once the samples had been extracted using the WVSPFL Biochemistry Section protocol for buccal swabs and the EZ1 Trace Protocol, the inhibitors were added to their corresponding extracts and all extracts were quantified in triplicate with each of the three quantification kits [2]. This was performed in order to ensure that he inhibitors were not removed during the extraction process. Based on the results obtained, dilutions were made at 1:10, 1:50, and 1:100 ratios for samples which demonstrated inhibition. These ratios were then quantified with all three kits in triplicate to determine if the inhibition had been overcome. All inhibition and degradation samples, including any dilutions made, continued on through the DNA analysis process to amplification and capillary electrophoresis in order to determine if those results yielded any indication of inhibition or degradation.

***2.6** Mixture*

The ability to detect the presence of a mixture is vital in a criminal casework environment. Each of these kits must be able to reliably indicate that a mixture is present, specifically a small amount of male DNA in the presence of a high amount of female DNA. To test whether or not each of the kits demonstrated this ability, mixtures were simulated and quantified at varying male to female ratios.

Samples taken from a male and a female were extracted separately then quantified using Quantifiler® Duo. The male sample, a cutting from a NIST-traceable blood card, quantified at 0.521 ng/µL, while the buccal swab from a female was quantified at 5.75 ng/µL. Before utilizing the samples to create the mixtures, each was normalized to 0.1 ng/µL. The mixtures were created by adding set amounts of each dilution to create specific ratios of male to female DNA. The ratios were as follows (Male:Female): 8:1, 4:1, 2:1, 1:1, 1:2, 1:4, and 1:8. The mixtures were then quantified in triplicate using each of the three kits. One of the main goals of this study was to determine if the quantification kits could be used to obtain an accurate representation of the male to female ratio of DNA, or if amplification and capillary electrophoresis must be performed to determine this.

The samples were all amplified using the Promega® PowerPlex® 16 System (Promega® Corporation, Madison, WI) and taken through capillary electrophoresis in order to obtain the relative fluorescent units (RFU) of the alleles. By comparing the RFU's of the male and female alleles, the analyst is able to determine the ratio of male to female DNA present in the sample. These ratios were then compared to the ratios calculated from the quantification values for

human and male DNA to determine which method yielded the most accurate and reliable ratios.

***2.7** Contamination*

Contamination was evaluated with the use of Known Reagent Controls (KRC's). These KRC's consisted of all reagents included in a reaction; however no sample or DNA was added. KRC's were made during the extraction and amplification. If no DNA was found in the quantification or capillary electrophoresis results, it could be said that contamination was not present and the results obtained for the other samples are considered valid, provided all other controls met their requirements as well.

Section 3: Results

***3.1** Standard Curve Quality Metrics*

The West Virginia State Police DNA Analysis Procedures Manual was used to determine the requirements for Quantifiler® Duo's slope and R-squared (R^2) values, while the manufacturer's manuals were used for the two kits not yet internally validated [2, 3, 4]. The slope and R^2 value was documented for each of the standard curve sets, which were made up of 2 columns of standards each. The maximum and minimum values, as well as the average, were documented for each kit and target. Tables 2 and 3 show the results for the autosomal (small autosomal for Quantifiler® Trio) and male targets, respectively, for all three kits. Table 4 depicts the results for the large autosomal target in the Quantifiler® Trio kit.

Table 2: Requirements and results of the autosomal target

Standard Curve Quality Metrics- Autosomal Target						
	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Slope	R^2	Slope	R^2	Slope	R^2
Requirements	-3.0 to -3.6	≥0.98	-3.0 to -3.6	≥0.99	-3.0 to -3.6	≥0.99
Max.	-3.13276	0.996829	-3.176	1	-3.291	0.999
Min.	-3.35792	0.991192	-3.521	0.999	-3.48	0.998
Average	-3.2249842	0.995	-3.25767	0.999	-3.388	0.999

Table 3: Requirements and results of the male target

Standard Curve Quality Metrics- Male Target						
	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Slope	R^2	Slope	R^2	Slope	R^2
Requirements	-3.0 to -3.6	≥0.98	-3.0 to -3.6	≥0.99	-3.0 to -3.6	≥0.99
Max.	-3.151307	0.997825	-3.2	0.999	-3.229	0.999
Min.	-3.35792	0.987206	-3.358	0.996	-3.362	0.998
Average	-3.2368554	0.994	-3.28333	0.999	-3.284	0.999

Table 4: Requirements and results of the Quantifiler® Trio large autosomal target

Standard Curve Quality Metrics- Large Autosomal Target		
	Quantifiler® Trio	
	Slope	R^2
Requirements	−3.1 to −3.7	≥0.99
Max.	-3.301	1
Min.	-3.405	0.998
Average	-3.3495	0.999

There were not any wells that needed to be omitted in order to fit the requirements for the Quantifiler® Trio or Quantiplex HYres kits. Two wells did need to be omitted for the Quantifiler® Duo kit (H9 and H12) during analysis. These two wells were omitted in two out of the five standard curves used for the study; however, all standard curves did meet the requirements after these omissions were made.

3.2 *Accuracy and Precision*

The concentration of each well, as well as the corresponding C_T value, were exported and documented to be used in the statistical analysis for the precision study. In regards to the Quantifiler® Trio kit, only the small autosomal target was used for these calculations, as this is

the target that would be used in casework to estimate the amount of DNA present in the sample [4]. The average of all of the values obtained was calculated and difference between the average and target concentration was determined. These values helped in determining the accuracy of each of the kits, or how close their measured values were to the actual concentration of the sample, and are shown in Table 5 for the human target.

Table 5: Human Target Accuracy Statistics

Accuracy-Human Target Average Concentration and Difference between Desired and Actual Concentration						
Desired Concentration (ng/µL)	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average (ng/µL)	Difference (ng/µL)	Average (ng/µL)	Difference (ng/µL)	Average (ng/µL)	Difference (ng/µL)
14.643	14.323	0.320	11.053	3.590	12.679	1.964
9.000	8.790	0.210	6.970	2.030	8.230	0.770
3.000	2.633	0.367	2.545	0.455	2.559	0.441
1.000	0.722	0.278	0.837	0.163	0.690	0.310
0.050	0.0390	0.011	0.0434	0.00660	0.030	0.020

The standard deviation of the concentrations obtained was calculated in order to demonstrate how much variation exists for that set of values. This statistic indicates the precision of each kit at that particular concentration. As stated previously, this calculation did not take into account the expected or targeted concentration. Instead, it looked at the reported values to determine how consistent each kit is at varying concentrations. Table 6 shows the average of the reported concentrations, standard deviation, and RSD for the human target of each kit.

Table 6: Human Target Precision Statistics

Precision- Human Target Average, Standard Deviation, and Relative Standard Deviation of Concetrations									
Desired Concentration (ng/µL)	Quantifiler® Duo			Quantifiler® Trio			Investigator® Quantiplex Hyres		
	Average (ng/µL)	Std. Dev.	RSD (%)	Average (ng/µL)	Std. Dev.	RSD (%)	Average (ng/µL)	Std. Dev.	RSD (%)
14.643	14.323	1.662	11.604	11.053	0.981	8.878	12.679	1.973	15.559
9.000	8.790	0.503	5.725	6.970	0.233	3.346	8.230	0.331	4.027
3.000	2.633	0.400	15.191	2.545	0.166	6.534	2.559	0.131	5.112
1.000	0.7223	0.0449	6.210	0.8367	0.0356	4.260	0.6897	0.0619	8.976
0.050	0.03900	0.00964	24.727	0.04340	0.00282	6.489	0.03000	0.00265	8.819

These calculations were then repeated for the C_T values. However, C_T values do not have a specific target value, so the difference between the desired and actual values could not be calculated. The statistics for the C_T values obtained are shown in Table 7 for the human target.

Table 7: Human Target C_T Value Averages, Standard Deviations, and Relative Standard Deviations

Accuracy and Precision- Human Target Average, Standard Deviation, and Relative Standard Deviation of C_T Values									
Sample	Quantifiler® Duo			Quantifiler® Trio			Investigator® Quantiplex Hyres		
	Average	Std. Dev.	RSD (%)	Average	Std. Dev.	RSD (%)	Average	Std. Dev.	RSD (%)
K_CGW	25.45	0.166	0.652	23.178	0.133	0.572	19.203	0.216	1.122
CGW2	26.11	0.08	0.306	23.850	0.049	0.206	19.789	0.055	0.278
CGW3	27.78	0.211	0.760	25.329	0.097	0.384	21.401	0.072	0.335
CGW4	29.55	0.087	0.294	26.959	0.062	0.230	23.212	0.121	0.520
CGW5	33.59	0.36	1.072	31.298	0.094	0.300	27.538	0.118	0.428

The results of the chi-squared calculations can be seen in Tables 8 and 9 comparing Quantifiler® Duo to the other two quantification kits.

Table 8: Chi-Squared Results, Comparing Quantifiler® Duo and Quantifiler® Trio

Average Concentrations and Chi-Squared Results				
Sample	Quantifiler® Duo	Quantifiler® Trio	Chi Sq.	Significant?
	Average (ng/µL)	Average (ng/µL)		
K_CGW	14.323	11.053	0.7468	No
CGW2	8.790	6.970	0.3768	No
CGW3	2.633	2.545	0.00294	No
CGW4	0.722	0.837	0.0181	No
CGW5	0.0390	0.0434	0.000496	No

Table 9: Chi-Squared Results, Comparing Quantifiler® Duo and Quantiplex HYres

Average Concentrations and Chi-Squared Results				
Sample	Quantifiler® Duo	Investigator® Quantiplex Hyres	Chi Sq.	Significant?
	Average (ng/µL)	Average (ng/µL)		
K_CGW	14.323	12.679	0.1888	No
CGW2	8.790	8.230	0.0356	No
CGW3	2.633	2.559	0.00210	No
CGW4	0.722	0.690	0.00148	No
CGW5	0.0390	0.0300	0.002077	No

3.3 *Sensitivity and Stochastic*

Quantifiler® Duo only resulted in one value for the first two dilutions in the series, for both targets. Quantiplex HYres only produced two of the three values possible for the 0.0025 ng/µL sample human target, but all three for the male target. Quantifiler® Trio was able to produce three values for the human and male targets for the first two samples in the dilution series. The quantification statistics for the human and male targets can be found in Tables 10 and 11, respectively, and the C_T statistics can be seen in Tables 12 through 14.

Table 10: Human Target Concentration (ng/µL) Results

Sensitivity- Human Target Average Concentration and Standard Deviation						
Targeted	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
Concentration	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation
0.005	0.00209	N/A	0.00113	0.0000535	---	N/A
0.0025	0.00326	N/A	0.000946	0.000321	0.000243	0.0000216
0.0005	---	N/A	---	N/A	---	N/A
0.00025	---	N/A	---	N/A	---	N/A

Table 11: Male Target Concentration (ng/µL) Results

Sensitivity- Male Target Average Concentration and Standard Deviation						
Targeted	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
Concentration	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation
0.005	0.0069	N/A	0.000715	0.000695	---	N/A
0.0025	0.00263	N/A	0.000573	0.000276	0.000459	0.000304
0.0005	---	N/A	---	N/A	---	N/A
0.00025	---	N/A	---	N/A	---	N/A

Table 12: Human Target C_T Results

Sensitivity- Human Target Average and Standard Deviation of C_T Values						
Targeted	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
Concentration	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
0.005	38.05	N/A	36.48	0.0677	Undetermined	N/A
0.0025	37.39	N/A	36.79	0.455	34.706	0.125
0.0005	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
0.00025	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A

Table 13: Male Target C_T Results

Sensitivity- Male Target Average and Standard Deviation of C_T Values						
Targeted	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
Concentration	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
0.005	37.25	N/A	36.845	1.490	Undetermined	N/A
0.0025	38.73	N/A	36.793	0.809	35.289	1.436
0.0005	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
0.00025	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A

Table 14: IPC Target C_T Results

Sensitivity- IPC Target Average and Standard Deviation of C_T Values						
Targeted Concentration	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
0.005	28.300	0.0656	27.366	0.1647	29.843	0.1604
0.0025	27.920	0.4915	27.209	0.1205	30.229	0.1848
0.0005	28.233	0.2495	27.109	0.1292	30.174	0.1602
0.00025	28.367	0.1563	27.184	0.2165	29.726	0.6582

The sensitivity samples were amplified using the PowerPlex® 16 System in triplicate for the 0.0025 and 0.0005 ng/µL samples and duplicate for the 0.005 and 0.00025 ng/µL samples; a summary of their capillary electrophoresis results can be seen in Table 15. Examples of each sensitivity sample's electropherograms are shown in Figures 3 through 6.

Table 15: Summary of Capillary Electrophoresis Results for Sensitivity Samples

Targeted Concentration (ng/µL)	CE Results
0.005	Partial profile-4 loci with peaks.
	Partial profile-1 loci with 1 peak.
0.0025	Partial profile- 10 loci with peaks.
	Partial profile- 13 loci with peaks.
	Partial profile- 4 loci with peaks.
0.0005	No peaks called.
	No peaks called.
	No peaks called.
0.00025	No peaks called.
	No peaks called.

Figure 3: Electropherogram of 0.005 ng/µL Sample

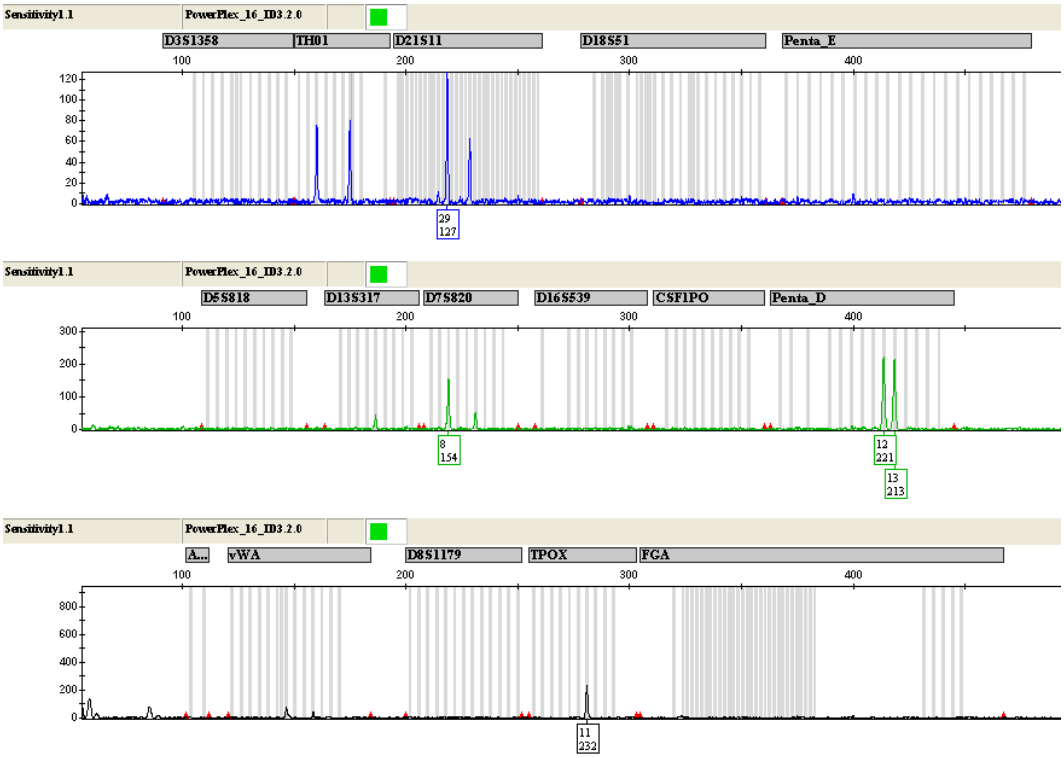


Figure 4: Electropherogram of 0.0025 ng/µL Sample

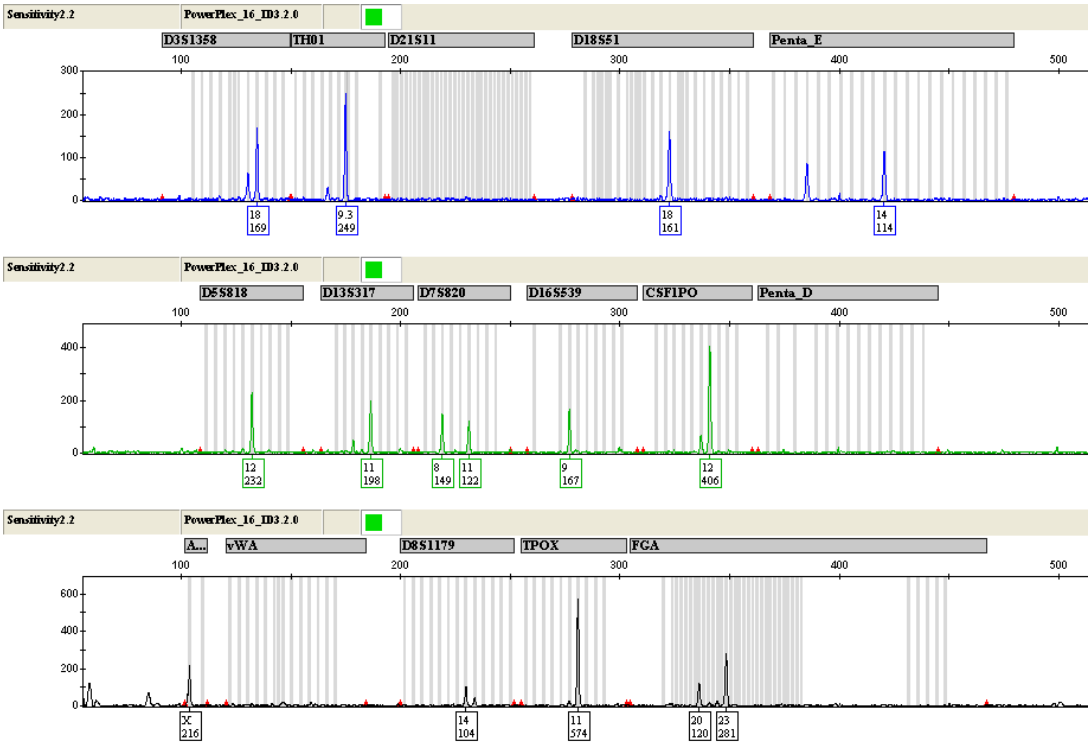


Figure 5: Electropherogram of 0.0005 ng/µL Sample

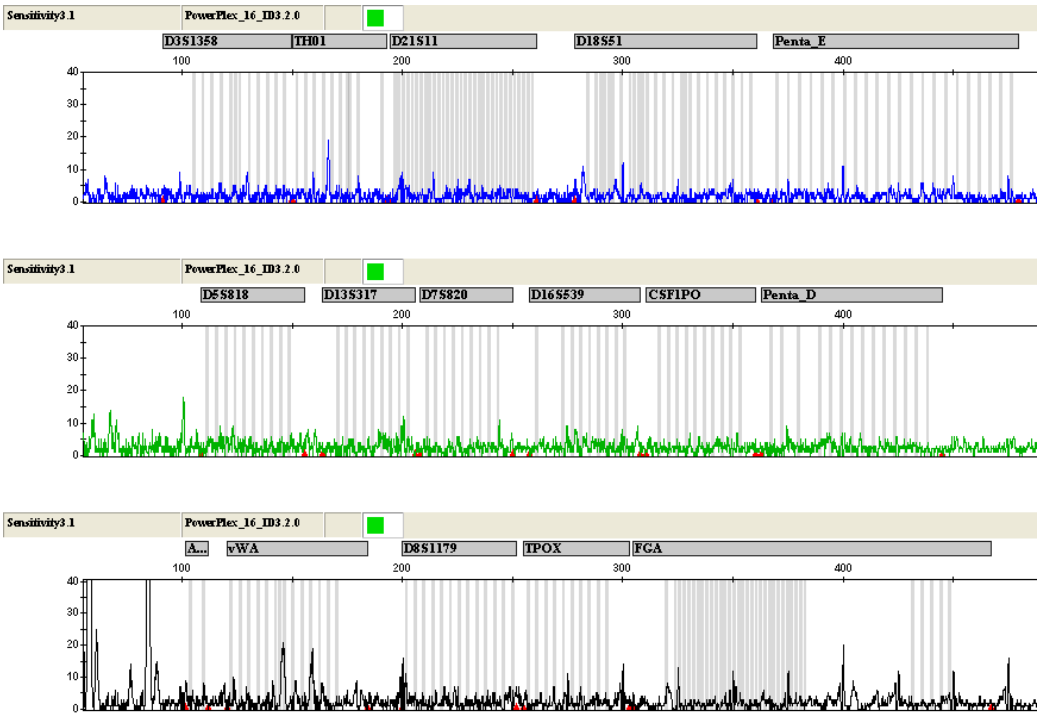
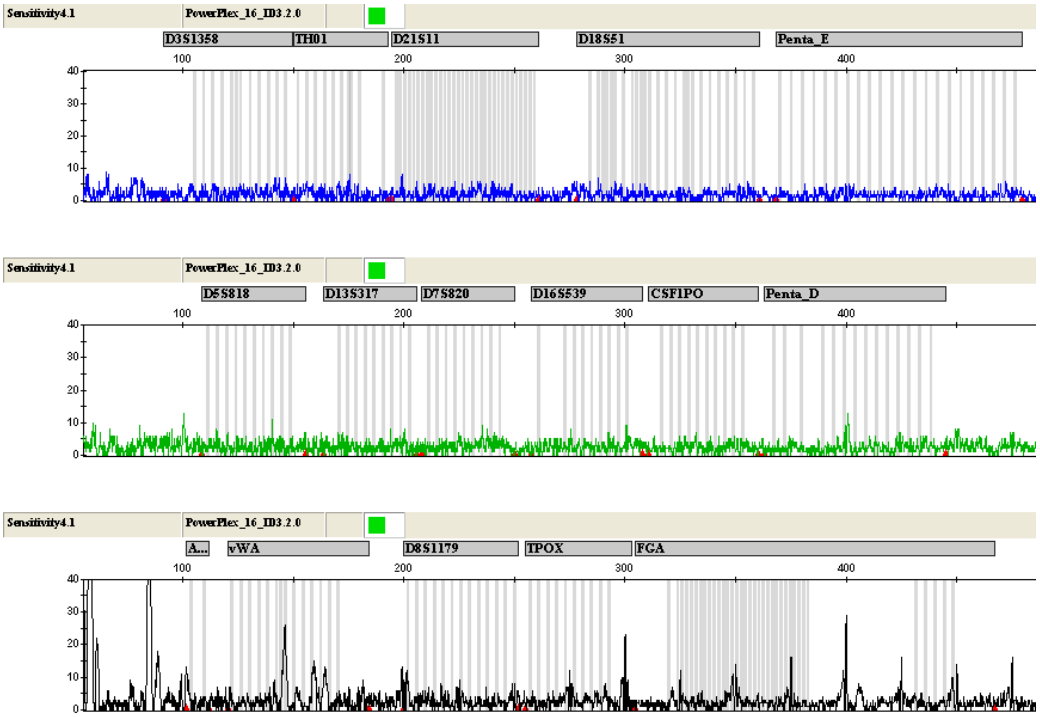


Figure 6: Electropherogram of 0.00025 ng/µL Sample



3.4 *Repeatability*

A set of the samples utilized during the course of the validations was selected to demonstrate the repeatability of the three kits. The three concentrations obtained for each Known/Non Probative sample are shown in Table 16, as well as their respective standard deviations. Any standard deviations above the set limit of 0.15 are highlighted in red.

Table 16: Quantification Values and Standard Deviations of Known/Non Probative Samples

Known/Non Probative--Quantification Concentrations and Standard Deviations												
Sample	Quantifiler® Duo				Quantifiler® Trio				Investigator® Quantiplex Hyres			
	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)	Std. Dev.	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)	Std. Dev.	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)	Std. Dev.
K_JCH	1.040	0.968	1.210	0.124	0.677	0.772	0.784	0.059	1.256	0.896	0.893	0.209
K_SK	4.470	4.060	6.660	1.398	5.374	5.619	5.103	0.258	6.714	5.136	5.364	0.853
Q_CigFilter	0.151	0.166	0.179	0.014	0.093	0.108	0.119	0.013	0.148	0.167	0.165	0.010
Q_MAChair	1.110	1.380	1.470	0.187	0.757	0.762	0.676	0.048	0.811	0.626	0.855	0.122
Q_SM-blood	0.084	0.084	0.083	0.001	0.047	0.058	0.059	0.006	0.069	0.060	0.080	0.010
Q_SM-saliva	0.265	0.355	0.387	0.063	0.246	0.296	0.298	0.029	0.283	0.324	0.345	0.032

3.5 *Known and Non-probative*

Table 17 shows the quantification results of the known and non-probative samples from each of the three kits.

Table 17: Quantification Results of Known/Non-Probative Study

Known/Non Probative--Quantification Concentrations									
Sample	Quantifiler® Duo			Quantifiler® Trio			Investigator® Quantiplex Hyres		
	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)	Set 1 (ng/µL)	Set 2 (ng/µL)	Set 3 (ng/µL)
K_JCH	1.040	0.968	1.210	0.677	0.772	0.784	1.256	0.896	0.893
K_SK	4.470	4.060	6.660	5.374	5.619	5.103	6.714	5.136	5.364
K_KRC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Q_CigFilter	0.151	0.166	0.179	0.093	0.108	0.119	0.148	0.167	0.165
Q_MAChair	1.110	1.380	1.470	0.757	0.762	0.676	0.811	0.626	0.855
Q_SM-blood	0.084	0.084	0.083	0.047	0.058	0.059	0.069	0.060	0.080
Q_SM-saliva	0.265	0.355	0.387	0.246	0.296	0.298	0.283	0.324	0.345
Q_KRC	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

The C_T averages and standard deviations can be seen in Tables 18 through 20 for the human, male, and IPC targets.

Table 18: Human Target C_T Averages and Standard Deviations

Inhibition/Degradation- Human Target Average C_T Values and Standard Deviation of C_T Values						
Sample	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
ID_Awcontrol	26.350	0.114	23.966	0.117	21.142	0.136
Inhib_BeforeDyeAW	26.980	0.217	24.525	0.076	21.451	0.078
Inhib_AfterDyeAW	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
Deg_NeatBleachAW	27.583	0.107	25.081	0.025	22.212	0.120
Deg_DilBleachAW	27.433	0.159	24.930	0.042	22.055	0.041
ID_HBMcontrol	25.507	0.112	23.633	0.131	21.188	0.029
Inhib_BeforeSoilHBM	25.733	0.126	23.306	0.041	20.753	0.025
Inhib_AfterSoilHBM	28.147	0.155	24.076	0.081	22.148	0.015
Deg_NeatElimHBM	25.200	0.066	23.351	0.035	20.703	0.120
Deg_DilElimHBM	25.413	0.114	23.325	0.068	20.785	0.062
ID_JTHcontrol	25.890	0.193	22.897	0.127	20.538	0.018
Inhib_BeforeRiverJTH	26.063	0.046	23.053	0.078	20.513	0.041
Inhib_AfterRiverJTH	26.573	0.085	23.637	0.016	21.121	0.057
Deg_15UVJTH	26.883	0.202	23.890	0.071	21.221	0.052
Deg_5UVJTH	26.540	0.114	23.564	0.040	20.963	0.018
Deg_KRC	Undetermined	N/A	39.499	0.163	Undetermined	N/A
Inhib_KRC	Undetermined	N/A	39.526	N/A	Undetermined	N/A

Table 19: Male Target C_T Averages and Standard Deviations

Inhibition/Degradation- Male Target Average C_T Values and Standard Deviation of C_T Values						
Sample	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
ID_Awcontrol	26.410	0.053	23.577	0.074	21.842	0.214
Inhib_BeforeDyeAW	26.993	0.206	23.998	0.050	22.174	0.117
Inhib_AfterDyeAW	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
Deg_NeatBleachAW	27.610	0.030	24.691	0.039	22.936	0.080
Deg_DilBleachAW	27.610	0.046	24.603	0.048	22.780	0.157
ID_HBMcontrol	26.373	0.110	22.968	0.061	22.254	0.154
Inhib_BeforeSoilHBM	26.347	0.049	22.611	0.018	21.858	0.066
Inhib_AfterSoilHBM	28.940	0.156	23.351	0.040	23.249	0.107
Deg_NeatElimHBM	26.213	0.021	22.634	0.021	21.778	0.127
Deg_DilElimHBM	26.130	0.066	22.630	0.054	21.691	0.199
ID_JTHcontrol	25.923	0.137	22.409	0.033	21.205	0.160
Inhib_BeforeRiverJTH	26.040	0.036	22.518	0.045	21.104	0.130
Inhib_AfterRiverJTH	26.523	0.015	23.103	0.038	21.784	0.143
Deg_15UVJTH	27.033	0.090	23.285	0.069	22.092	0.116
Deg_5UVJTH	26.557	0.059	23.003	0.040	21.780	0.152
Deg_KRC	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
Inhib_KRC	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A

Table 20: IPC Target C_T Averages and Standard Deviations

Inhibition/Degradation- IPC Average C_T Values and Standard Deviation of C_T Values						
Sample	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
ID_Awcontrol	28.407	0.023	27.567	0.361	32.408	2.749
Inhib_BeforeDyeAW	28.523	0.081	27.982	0.125	30.371	0.476
Inhib_AfterDyeAW	Undetermined	N/A	Undetermined	N/A	Undetermined	N/A
Deg_NeatBleachAW	28.430	0.098	27.764	0.232	30.211	1.157
Deg_DilBleachAW	28.227	0.107	27.199	0.837	32.199	2.850
ID_HBMcontrol	28.387	0.245	27.769	0.445	32.564	2.646
Inhib_BeforeSoilHBM	28.563	0.309	27.945	0.298	33.400	1.170
Inhib_AfterSoilHBM	27.303	0.180	26.257	0.213	32.267	2.026
Deg_NeatElimHBM	28.623	0.182	27.904	0.131	33.017	2.012
Deg_DilElimHBM	28.733	0.065	27.750	0.289	33.277	2.884
ID_JTHcontrol	28.347	0.286	27.723	0.198	33.262	3.455
Inhib_BeforeRiverJTH	28.267	0.582	27.608	0.603	32.520	0.980
Inhib_AfterRiverJTH	28.483	0.358	27.593	0.169	32.813	1.107
Deg_15UVJTH	28.497	0.179	27.555	0.035	33.627	2.913
Deg_5UVJTH	28.580	0.053	27.578	0.218	33.409	2.768
Deg_KRC	28.463	0.142	26.967	0.459	29.632	0.175
Inhib_KRC	28.533	0.076	26.796	0.684	29.478	0.319

The average and standard deviation of the concentrations can be seen in Table 21 for the human target and Table 22 for the male target, while the degradation index for Quantifiler® Trio may be seen in Table 23.

Table 21: Human Target Concentration Averages and Standard Deviations

Inhibition/Degradation- Human Target Average Concentrations and Standard Deviation of Concentration						
Sample	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation
ID_Awcontrol	6.160	0.473	8.269	0.714	10.846	1.053
Inhib_BeforeDyeAW	3.963	0.629	5.515	0.300	8.725	0.478
Inhib_AfterDyeAW	Blank	N/A	Blank	N/A	Blank	N/A
Deg_NeatBleachAW	2.577	0.192	3.690	0.067	5.129	0.439
Deg_DilBleachAW	2.873	0.335	4.115	0.125	5.714	0.164
ID_HBMcontrol	11.177	0.931	10.527	0.976	10.473	0.212
Inhib_BeforeSoilHBM	9.520	0.871	13.293	0.387	14.197	0.243
Inhib_AfterSoilHBM	1.737	0.195	7.627	0.440	5.351	0.056
Deg_NeatElimHBM	13.870	0.632	12.864	0.324	14.742	1.237
Deg_DilElimHBM	11.957	0.928	13.119	0.653	13.890	0.609
ID_JTHcontrol	8.573	1.204	17.907	1.597	16.500	0.207
Inhib_BeforeRiverJTH	7.527	0.263	15.969	0.908	16.798	0.477
Inhib_AfterRiverJTH	5.243	0.305	10.467	0.121	10.983	0.431
Deg_15UVJTH	4.250	0.624	8.724	0.441	10.239	0.378
Deg_5UVJTH	5.377	0.439	11.034	0.319	12.261	0.152
Deg_KRC	Blank	N/A	0.000	0.000	Blank	N/A
Inhib_KRC	Blank	N/A	0.000	N/A	Blank	N/A

Table 22: Male Target Concentration Averages and Standard Deviations

Inhibition/Degradation- Male Target Average Concentrations and Standard Deviation of Concentration						
Sample	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation
ID_Awcontrol	9.020	0.346	6.816	0.365	6.702	1.053
Inhib_BeforeDyeAW	6.033	0.903	5.030	0.179	5.268	0.427
Inhib_AfterDyeAW	Blank	N/A	Blank	N/A	Blank	N/A
Deg_NeatBleachAW	3.883	0.090	3.053	0.086	3.069	0.175
Deg_DilBleachAW	3.887	0.117	3.252	0.111	3.438	0.390
ID_HBMcontrol	9.277	0.695	10.573	0.468	4.989	0.559
Inhib_BeforeSoilHBM	9.457	0.335	13.675	0.173	6.582	0.302
Inhib_AfterSoilHBM	1.530	0.173	8.018	0.232	2.462	0.182
Deg_NeatElimHBM	10.370	0.118	13.443	0.207	6.978	0.628
Deg_DilElimHBM	10.970	0.499	13.493	0.532	7.447	1.061
ID_JTHcontrol	12.750	1.220	15.814	0.382	10.483	1.219
Inhib_BeforeRiverJTH	11.690	0.301	14.624	0.476	11.244	1.009
Inhib_AfterRiverJTH	8.327	0.104	9.593	0.261	6.953	0.705
Deg_15UVJTH	5.820	0.373	8.417	0.422	5.583	0.449
Deg_5UVJTH	8.147	0.361	10.304	0.296	6.977	0.732
Deg_KRC	Blank	N/A	Blank	N/A	Blank	N/A
Inhib_KRC	Blank	N/A	Blank	N/A	Blank	N/A

Table 23: Quantifiler® Trio Degradation Indexes

Inhibition/Degradation- Average and Standard Deviation of Degradation Index		
Sample	Quantifiler® Trio	
	Average	Standard Deviation
ID_Awcontrol	0.822	0.113
Inhib_BeforeDyeAW	0.695	0.044
Inhib_AfterDyeAW	Blank	N/A
Deg_NeatBleachAW	0.737	0.049
Deg_DilBleachAW	0.935	0.080
ID_HBMcontrol	0.882	0.138
Inhib_BeforeSoilHBM	1.019	0.081
Inhib_AfterSoilHBM	0.825	0.094
Deg_NeatElimHBM	0.837	0.042
Deg_DilElimHBM	0.815	0.076
ID_JTHcontrol	1.007	0.139
Inhib_BeforeRiverJTH	0.886	0.073
Inhib_AfterRiverJTH	0.872	0.045
Deg_15UVJTH	1.178	0.106
Deg_5UVJTH	1.067	0.082
Deg_KRC	Blank	N/A
Inhib_KRC	Blank	N/A

Since the sample with indigo dye added after extraction, labeled Inhib_AfterDyeAW, was the only sample to demonstrate potential inhibition, it was the only one diluted down to

attempt to overcome this inhibition. The quantification and C_T results of this dilution series can be seen in Tables 24 through 26.

Table 24: Inhib_AfterDyeAW Dilution Series Quantification Results

Inhib_AfterDyeAW Dilutions- Human Target Average Concentrations and Standard Deviation of Concentration						
Dilution	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation	Average (ng/µL)	Standard Deviation
1:10	---	---	0.451	0.019	1.122	0.097
1:50	0.051	0.006	0.156	0.011	0.234	0.014
1:100	0.056	0.008	0.085	0.004	0.112	0.010

Table 25: Inhib_AfterDyeAw Dilution Series Human Target C_T Results

Inhib_AfterDyeAW Dilutions- Human Target Average and Standard Deviation of C_T Values						
Dilution	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
1:10	---	---	27.935	0.062	22.832	0.122
1:50	33.273	0.177	29.454	0.103	25.035	0.081
1:100	33.133	0.222	30.313	0.064	26.072	0.128

Table 26: Inhib_AfterDyeAw Dilution Series IPC C_T Results

Inhib_AfterDyeAW Dilutions- IPC Target Average and Standard Deviation of C_T Values						
Dilution	Quantifiler® Duo		Quantifiler® Trio		Investigator® Quantiplex Hyres	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
1:10	---	---	28.422	0.009	29.548	0.175
1:50	29.020	0.066	26.530	1.077	29.976	0.123
1:100	28.677	0.072	27.048	0.216	30.187	0.042

The capillary electrophoresis results of the Known/Non-Probative samples can be seen in Table 27 and the CE results of the Inhibition/Degradation samples, as well as the Inhib_AfterDyeAW dilutions, can be seen in Table 28. The Inhibition/Degradation samples were run in duplicate with varying amounts of DNA loaded into the amplification reactions; the most informative CE results were reported.

Table 27: Capillary Electrophoresis Results of Known/Non Probative Samples

Sample	CE Results
K_JCH	All peaks present.
K_SK	All peaks present.
K_KRC	No peaks called.
Q_CigFilter	Full profile, no idication of dropout.
Q_MAChair	Full profile, no indication of dropout.
Q_SM-blood	Full profile, no indication of dropout.
Q_SM-saliva	Full profile, no indication of dropout.
Q_KRC	No peaks called.

Table 28: Capillary Electrophoresis Results of Inhibition/Degradation and Inhib_AfterDyeAW Dilution Samples

Sample	CE Results
ID_Awcontrol	All peaks present.
Inhib_BeforeDyeAW	All peaks present, no indication of inhibition for any BeforeDye sample.
Inhib_AfterDyeAW	No peaks called.
Deg_NeatBleachAW	All peaks present, no indication of degradation for and NeatBleach sample.
Deg_DilBleachAW	All peaks present, no indication of degradation for any of the DilBleach samples.
ID_HBMcontrol	All peaks present.
Inhib_BeforeSoilHBM	All peaks present, no indication of inhibition.
Inhib_AfterSoilHBM	Partial profile-5 loci with peaks called.
Deg_NeatElimHBM	All peaks present, no indication of degradation for any NeatElim sample.
Deg_DilElimHBM	All peaks present, no indication of degradation for any of the DilElim samples.
ID_JTHcontrol	All peaks present.
Inhib_BeforeRiverJTH	All peaks present, no indication of inhibition for any BeforeRiver sample.
Inhib_AfterRiverJTH	All peaks present, no indication of inhibition.
Deg_15UVJTH	All peaks present. Blue and Yellow dye channels indicate potential degradation.
Deg_5UVJTH	All peaks present. Blue dye channel indicates potential degradation.
Inhib_KRC	No peaks called.
Deg_KRC	No peaks called.
Inhib_1:10	No peaks called.
Inhib_1:50	All peaks present.
Inhib_1:100	All peaks present.

Electropherograms of selected samples are shown in Figures 7 through 12 to further demonstrate the results of these studies.

Figure 7: Electropherogram of Inhib_BeforeDyeAW Sample

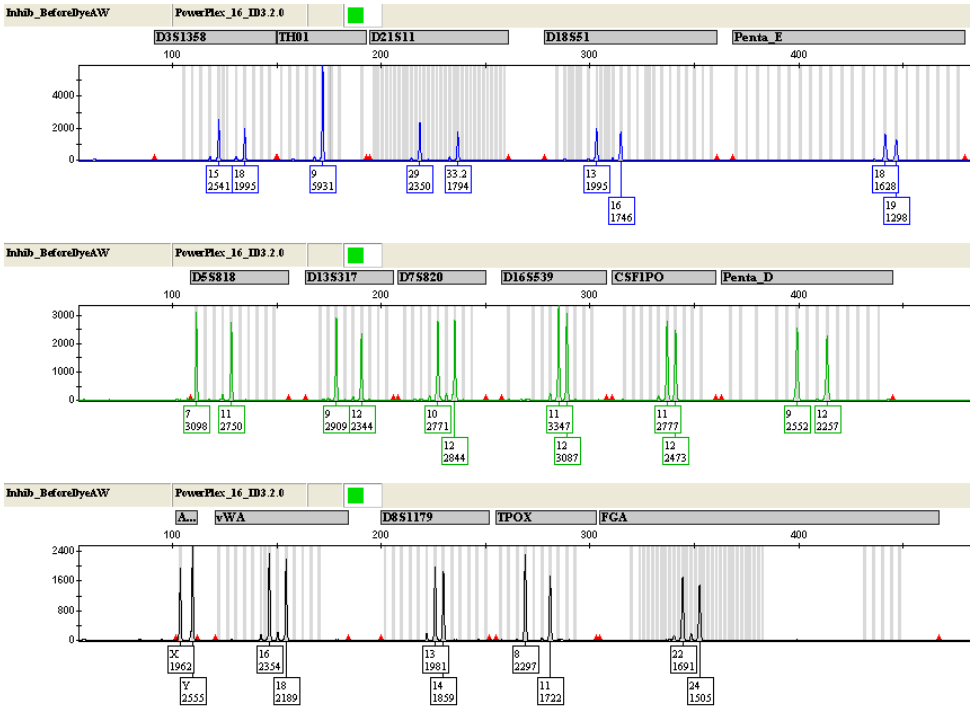


Figure 8: Electropherogram of Inhib_AfterDyeAW Sample

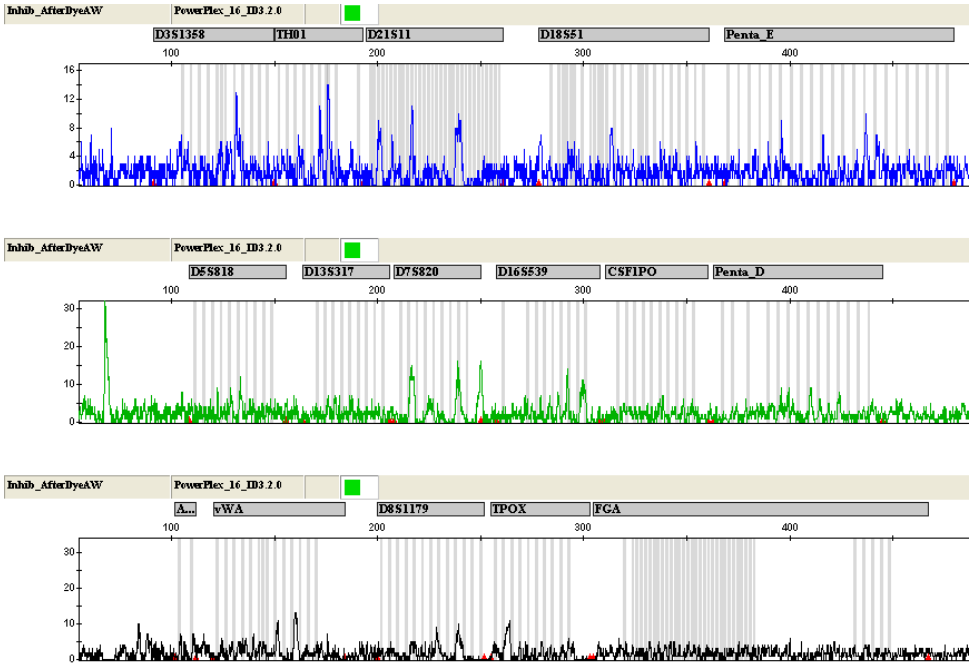


Figure 9: Electropherogram of Deg_15UVJTH Sample

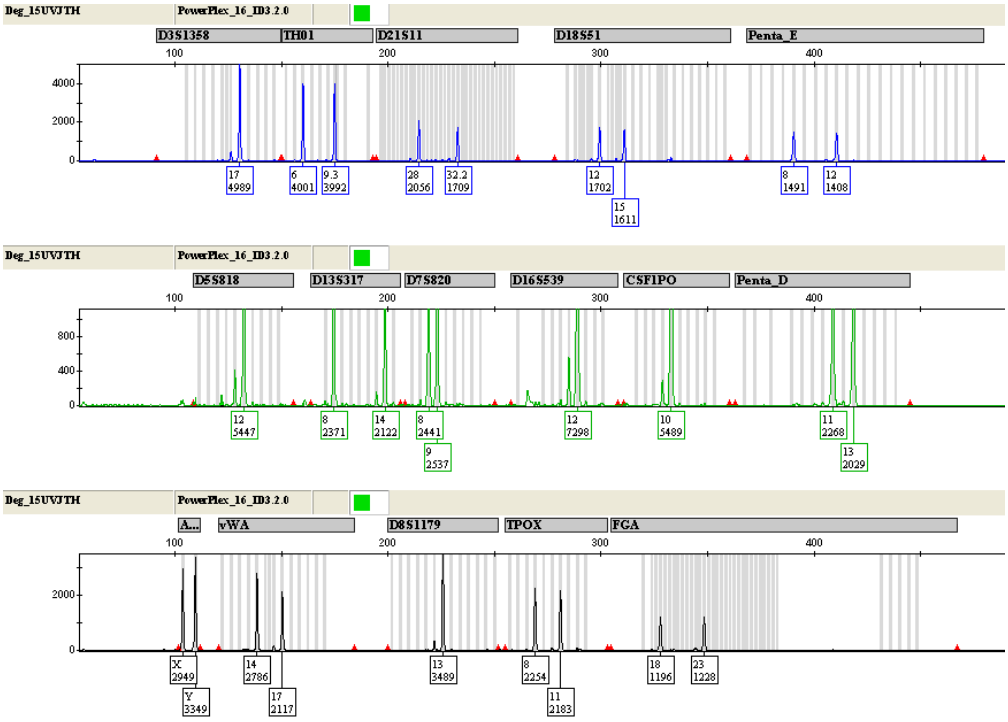


Figure 10: Electropherogram of Deg_5UVJTH Sample

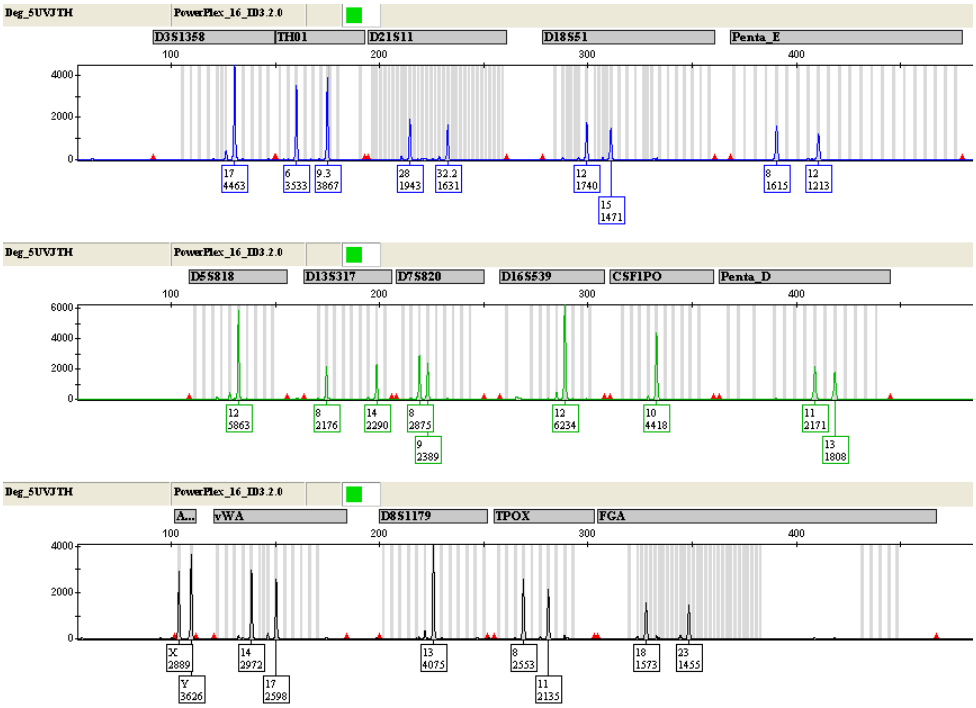


Figure 11: Electropherogram of Inhib_1:10 Sample

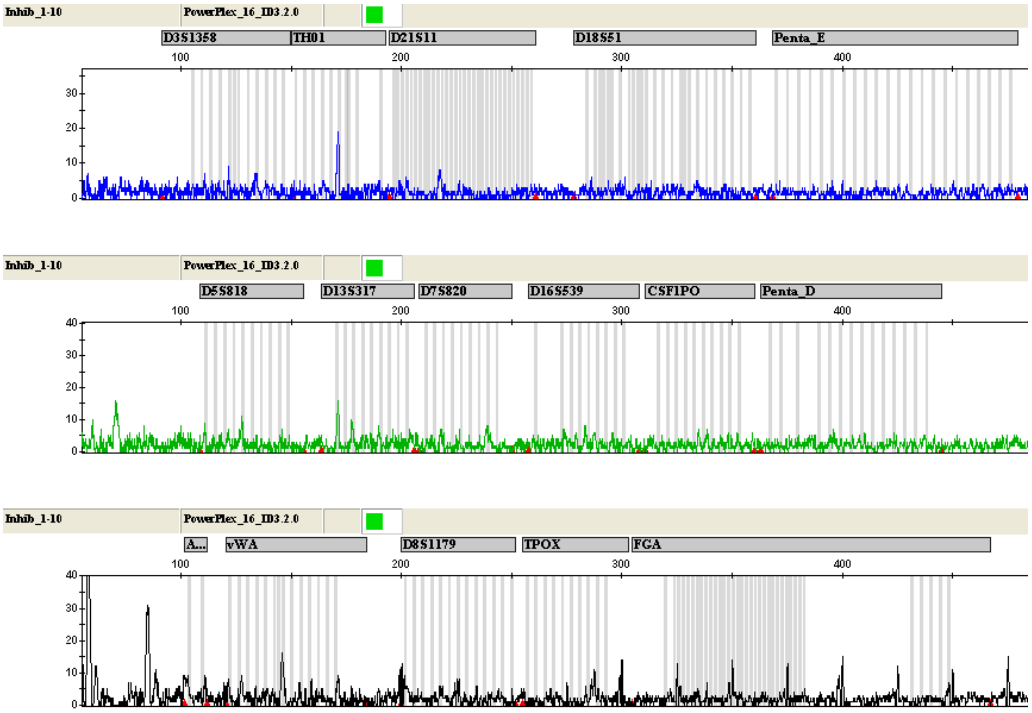
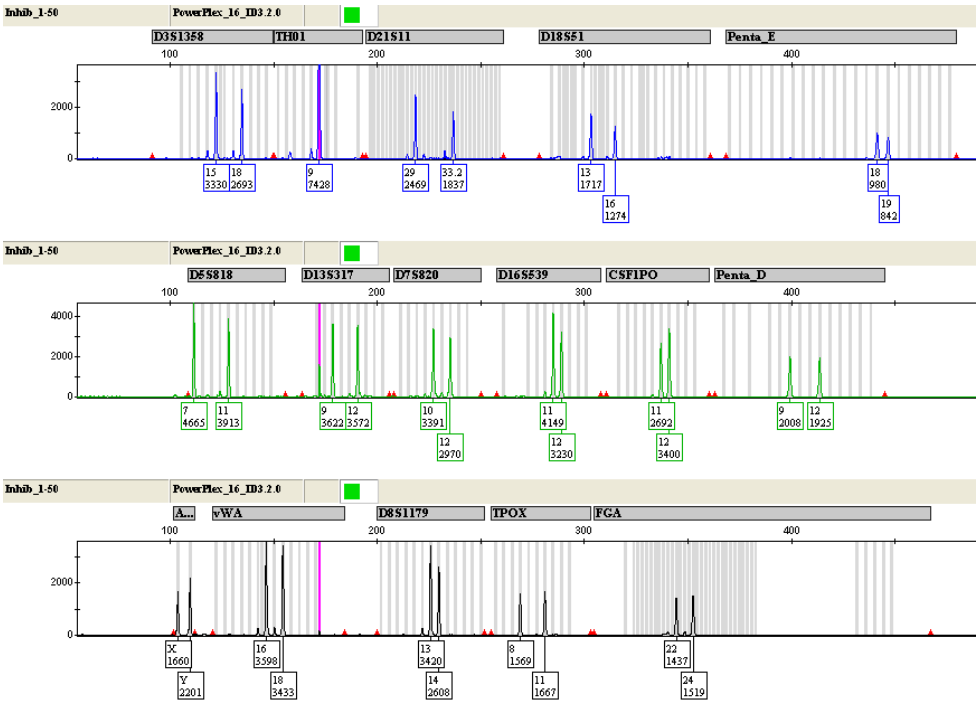


Figure 12: Electropherogram of Inhib_1:50 Sample



3.6 *Mixture*

The average concentrations (ng/µL) and calculated male-to-female ratios can be seen in Table 29.

Table 29: Mixture Concentration Averages (ng/µL) and Calculated Male-to-Female Ratios

Mixture- Average Concentrations and Calculated M:F Ratio				
Targeted Ratio	Quantifiler® Duo			
	Human (ng/µL)	Male (ng/µL)	Calculated Female (ng/µL)	Calculated Ratio
8:1	0.103	0.082	0.0210	3.9:1
4:1	0.094	0.077	0.0170	4.5:1
2:1	0.074	0.041	0.0327	1.3:1
1:1	0.061	0.033	0.0280	1.2:1
1:2	0.070	0.032	0.0373	1:1.2
1:4	0.061	0.015	0.0460	1:3.1
1:8	0.057	0.016	0.0413	1:2.6
Targeted Ratio	Quantifiler® Trio			
	Human (ng/µL)	Male (ng/µL)	Calculated Female (ng/µL)	Calculated Ratio
8:1	0.068	0.085	-0.0169	-5.0:1
4:1	0.057	0.060	-0.0031	-19.3:1
2:1	0.054	0.050	0.0045	11.0:1
1:1	0.052	0.035	0.0176	2.0:1
1:2	0.066	0.037	0.0290	1:0.8
1:4	0.046	0.021	0.0250	1:1.2
1:8	0.059	0.017	0.0414	1:2.4
Targeted Ratio	Investigator® Quantiplex Hyres			
	Human (ng/µL)	Male (ng/µL)	Calculated Female (ng/µL)	Calculated Ratio
8:1	0.086	0.051	0.0353	1.4:1
4:1	0.079	0.041	0.0381	1.1:1
2:1	0.079	0.038	0.0409	0.9:1
1:1	0.082	0.030	0.0513	0.6:1
1:2	0.081	0.023	0.0578	1:2.5
1:4	0.080	0.017	0.0625	1:3.6
1:8	0.087	0.009	0.0781	1:9.1

3.7 *Contamination*

All KRC's extracted with the samples quantified at 0.000 ng/µL, meaning they were either shown as blank quantities with "undetermined" C_T values in the exported data, or if a quantity was given for the concentration, it rounded to 0.000 ng/µL. The CE results also

indicated no DNA was present in these samples, as no peaks were called during analysis and each passed the requirements set forth in the WVSP DNA Analysis Procedures Manual [2].

Section 4: Discussion

4.1 *Standard Curve Quality Metrics*

Regarding the simultaneous testing performed with Quantifiler® Duo during this study, these tests did not interfere with the results and it was determined that sufficient data was collected from the ten sets of standards in order to draw accurate conclusions. While all three kits did meet the manufacturer's requirements with all of the standard curves generated, Quantifiler® Duo had a lower average R^2 value when compared to the Quantifiler® Trio and Quantiplex HYres; 0.995 versus 0.999 for both, respectively. While 0.995 is still greater than the 0.99 that is required for the standard curve to pass, an R^2 value of 1.0 would indicate the best closeness of fit between the standard curve regression line and the individual C_T data points [3, 4, 5]. Both Quantifiler® Trio and Quantiplex HYres came closer to that value than Quantifiler® Duo. Quantifiler® Trio was also the only kit to result in three standard curves with R^2 values of 1.0. Regarding the slope of the autosomal (small autosomal for Quantifiler® Trio) and the male targets, the acceptable range is -3.0 to -3.6, with -3.3 indicating 100% PCR amplification efficiency [3,4,5]. All three kits had an average slope near -3.3 and there did not appear to be any significant differences between the kits, in regards to slope.

4.2 *Accuracy and Precision*

Since the serial dilution was created based on Quantifiler® Duo's initial quantification, Quantifiler® Duo understandably had much higher precision than the other two kits. However, the values produced by Quantifiler® Trio and Quantiplex HYres are not statistically significantly

different from those produced by Quantifiler® Duo for the same sample. Therefore, it can be determined that all three kits performed with sufficient accuracy.

Regarding the precision of each quantification kit, Quantifiler® Trio resulted in the lowest standard deviation and RSD percentage at every concentration, with the exception of the sample with the 3.000 ng/µL concentration. With this sample, Quantiplex HYres produced the lowest value. These results are also reflected in the C_T values' calculations, further supporting the claim that Quantifiler® Trio was the most precise kit overall.

4.3 *Sensitivity and Stochastic*

One of the largest discrepancies between the three kits was demonstrated during the sensitivity study. As mentioned previously, Quantifiler® Duo was only able to produce one quantity for both the human and male targets out of the three times the 0.005 and 0.0025 ng/µL samples were quantified. Both of these samples resulted in partial profiles that could have been beneficial had they been samples involved in a case. If these samples had been quantified for casework with Quantifiler® Duo and happened to be one of the times resulting in "0.000 ng/µL" and not processed further, this additional information could have been overlooked.

The results from Quantiplex HYres were not as expected, since it was unable to generate quantities for the 0.005 ng/µL sample, yet produced two out of the potential three for the 0.0025 ng/µL sample. The CE results for the 0.0025 ng/µL sample were also more beneficial and yielded more peaks and more loci than the 0.005 ng/µL sample. This could indicate that the sample was not mixed properly prior to pipetting, even though all measures were taken to ensure an accurate sampling was taken.

In contrast, Quantifiler® Trio produced quantities for all three repeats of the first two dilutions in the series. This demonstrates that the kit is reliable when quantifying these low quantities, as well as extremely sensitive, since 0.0025 ng/µL is outside of the kit's concentration range. While it was not able to detect DNA in the last two dilutions, these samples did not result in any usable information from amplification and CE. Therefore, the results of this study indicate that a result of 0.000 ng/µL from the Quantifiler® Trio kit truly means there is no usable DNA present and that the DNA analysis process could be stopped at quantification for the sample types tested in this validation. However, additional studies would need to be performed in order for this hypothesis to be solidified for all sample types and any final conclusions to be drawn.

4.4 *Repeatability*

Overall, the standard deviations were low and demonstrated that the kits are generally reliable when quantifying samples often encountered in casework. Quantiplex HYres had the most standard deviations above the decided limit of 0.15 with three total, while Quantifiler® Duo had the highest standard deviation for one set of samples: 1.398 for one of the two buccal swabs tested. Quantifiler® Trio had the fewest number of high standard deviations: one of 0.258. The sample set selected for this study was rather small and additional testing could be performed in the future to ensure and further validate these findings.

4.5 *Known and Non-probative*

The quantification results of the known and questioned samples were as expected for their respective sample types and all kits quantified them similarly. The electropherograms for the known samples contained all expected alleles. The questioned samples' electropherograms

resulted in full profiles as well, with no indication of dropout. Both KRC's quantified at concentrations of 0.000 ng/µL and did not result in any called alleles in their electropherograms.

In comparison, not all of the inhibited and degraded samples quantified as expected. The inhibited samples' IPC results did not indicate inhibition occurred, with the exception of the sample with indigo dye added after extraction, labeled "Inhib_AfterDyeAW". This sample did not amplify due to the inhibition and was moved on to the next step for inhibited samples. After the Inhib_AfterDyeAW dilutions were created and quantified, Quantifiler® Trio and Quantiplex HYres were able to detect DNA in all three dilutions for both the human and male targets, while Quantifiler® Duo was not able to detect any DNA in the 1:10 dilution. However, no alleles were detected for the 1:10 dilution sample during capillary electrophoresis. This is most likely due to the coloration of the sample, as it was visibly darker than the other two dilutions. It's possible that the HID software used by Quantifiler Trio and Quantiplex HYres was able to interpret the fluorescence during quantification more easily than the CE instrument and its corresponding software. The sample with indigo dye added prior to extraction did not exhibit any coloration and produced the expected results for both quantification and CE, therefore this issue would most likely not be encountered during casework; however, it is something to be aware of since it could affect the outcome of the final DNA profile.

The inhibition sample with soil added after extraction also demonstrated unexpected results. The quantification values indicated an ample amount of DNA present, yet after it was diluted down to the target range for PCR and run through CE, it only yielded a partial profile. This might be explained by the nature of the sample. By adding soil after extraction, the extract

essentially became mud. There was not a great amount of liquid present in the sample after all quantifications had been completed for the three kits. This could have affected the DNA left in the liquid available for amplification, and therefore resulted in a partial profile. Due to the C_T values and IPC results of this sample during quantification, it is not believed that the CE results are an indication of inhibition, but simply a lack of DNA present in the sample.

The degraded samples with bleach and UV did quantify slightly lower than their respective controls, while the Eliminase® samples quantified higher than their control. The degradation indexes, however, were not significantly higher than their controls. Based on this information alone, it could be inferred that the samples simply had less DNA present, but were not degraded. The capillary electrophoresis results did indicate some potential degradation in certain dye channels; however it was not enough degradation to significantly interfere with interpretation of the profiles.

***4.6** Mixture*

The calculated mixtures did not reflect the expected results. While the Quantiplex HYres kit's ratios did follow the same general trend as the targeted ratios, the male components did not appear to be as prominent as was intended. However, when the mixtures were calculated based on the RFU values, they seemed to be relatively close to the targeted values, indicating the ratios were made correctly. It does not appear that the ratios would not be able to be reliably calculated based off the quantification values alone. However the WVSPFL Biochemistry Section does not rely on these values for such a calculation, therefore this was not deemed to be a major concern for their laboratory.

This study was able to indicate that all three kits are able to detect a male component with a high concentration of female DNA present. This is a key ability when dealing with sexual assault kits and other casework, and it has been demonstrated that each of these kits is able to perform in these situations. Increasing the amount of female DNA present in the samples could add to the data and further solidify the kits' abilities to detect male DNA in a mixture. Additional studies could also be performed using complex mixtures, samples including three or four individuals, to see the kits' capabilities in these circumstances.

4.7 *Contamination*

Due to all KRC's demonstrating a lack of DNA present, the results of all other samples could also be considered to be free of contaminating DNA. This not only validates the results from the quantifications and allows conclusions to be drawn from the data obtained, but also verifies that the reagents for quantification are free of contaminating agents.

Conclusion

Regarding the internal validation for Quantiplex HYres and Quantifiler® Trio, it was able to be demonstrated that both quantification kits are robust and reliable and have the potential to be implemented by the WVSPFL Biochemistry Section for casework. Throughout the process of comparative analysis, it became apparent that several studies would contribute key aspects and lead to a final conclusion regarding which quantification kit best serves the needs of the WVSPFL Biochemistry Section. The sensitivity and stochastic study highlighted Quantifiler® Trio's potential to detect extremely low concentrations of DNA that the other two quantification kits in this study could overlook. The precision and reproducibility studies also

demonstrated an aspect of Quantifiler® Trio's abilities, showing how reliable and precise the kit is when tested numerous times, compared to the other quantification kits involved.

While the results of these studies will play a large factor in helping the WVSPFL Biochemistry Section make a decision regarding quantification kit implementation in the future, many other aspects must also be considered. In addition, future studies, such as additional sensitivity and concordance studies, must be performed in order for the best decision for the laboratory to be made.

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