REPRODUCIBILITY OF QUANTIFILER® TRIO ASSAY-SPECIFIC STANDARD CURVES OVER A RANGE OF VARIABLES TO GENERATE GUIDELINES FOR CRIME LABORATORY WORKFLOWS

by

Stephanie Marie Betts

A Research Project
Submitted to the
Forensic Science Forensic Research Committee
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Forensic Science

Primary Research Advisor
Megan M. Foley, MSFS, F-ABC
Visiting Assistant Professor
The George Washington University

Secondary Research Advisor
Professor Kelly L. Knight
Associate Professor of Forensic Science
George Mason University

GMU Graduate Research Coordinator
Dr. Joseph A. DiZinno
Associate Professor
GMU Forensic Science Program

Fall Semester 2021
George Mason University
Fairfax, VA

Acknowledgements

First and foremost, I would like to thank my research advisors, Megan Foley, Professor Kelly Knight, and Dr. DiZinno for helping me throughout this tedious process and guiding me to produce an original research project that I am confident can be useful to the ever-expanding field of Forensic Biology, DNA Analysis. I would also like to take this opportunity to thank Thermo Fisher Scientific for the donation of a Quanifiler® Trio DNA Quantification kit for my research project, without this kit, this project would not have been possible.

A huge thank you to Carol Palmer, Forensic Biology Group Supervisor, with the Department of Forensic Science for her generous donation of extraction solutions. These supplies aided in both organic and differential DNA extractions.

Additionally, I would like to give a shout out to Bonnie Madden, Sr. Microbial Lab and Research Specialist with George Mason University's Biology Department, for allowing me to utilize her BSL-2 laboratory and safety hood to perform my DNA extractions. Furthermore, not only did Mrs. Madden allow me access to her laboratory, but she was generous enough to provide me with reagents and equipment essential to performing said extractions, so thank you so much Bonnie and the Biology Department.

Finally, I would like to thank all my friends and family for not only supporting me throughout this research journey, but for also being there for me during my disoriented and chaotic schedule, along with my overabundance of stress and anxiety, I could not have done this without you.

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List of Definitions/Acronyms/ Abbreviations

qPCR	Quantitative Polymerase Chain Reaction
Quantifier Trio	DNA Quantification kit produced by Thermofisher that contains all "essential reagents for amplification, detection and quantification of two human-specific DNA targets and a human male-specific DNA target" (Foster city, CA, 2018).
Standard curve	A calibration curve used in quantitative research
Organic Extraction	A chemical induced processes used by laboratories to isolate DNA from other cellular material
Differential Extraction	A chemical process used by laboratories to isolate male DNA (sperm fraction) from female DNA (non-sperm fraction)
PC or PCIA (Phenol–Chloroform)	A type of Liquid–Liquid Extraction technique widely used for isolation of DNA, RNA, and protein
DTT	Dithiothreitol
TNE	Tris:NaCl:EDTA buffer
RPM	Rotation per minute

DNA	Deoxyribonucleic Acid
STR	Short Tandem Repeat
ASC	Assay-Specific Standard Curve
VSC	Virtual Standard Curve
ng	Nano Gram
µL	microliter
STD	Standard
SWGDAM	Scientific Working Group on DNA Analysis Methods

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Abstract

Quantitative PCR (qPCR) is the preferred method of quantitation in forensic DNA analysis, used to determine the amount of amplifiable human DNA present in evidence or reference samples. A standard curve is created via quantitation of a serial dilution containing known DNA concentrations. Quantifiler® Trio manufacturer guidelines were followed for the creation of five concentrations varying from 50 ng/µL to 0.005 ng/µL. The primary goal of this research was to generate laboratory guidelines and recommendations for quantitation standards for forensic laboratories hoping to streamline their workflows, and to determine how long standards are valid to decrease the amount of time and money spent on assay-specific standard curves. Most often, a standard curve is generated every time an assay is performed. The research data was generated over a two-month period. Through multiples runs, including two analysts involved in plating of samples, standard curves were analyzed for variation in curve parameters, e.g., has the slope or quantitation range changed over time. Mock case samples were prepared and analyzed to check the efficacy of the assay-specific standards versus a virtual curve to validate the suitability in DNA crime laboratories. In order to illustrate the relevance of laboratory generated internal standards (assay standard curves). First, if the standard curve is slightly different every time a new curve is generated, how could it affect the laboratory DNA results? Secondly, how variable are these standard curves overtime when performed by the same or multiple individuals; this will be evaluated by a comparison of linear regression values calculated by the software for each standard curve, between analysts. Results indicated little to no difference in the values for T.Y, small, and large autosomal targets. Linearity remained consistent beyond recommended discard of 14-days. Whether a laboratory prefers ASC or VSC the data provided shows that there is little to no difference between the two curve methods prior to amplification. The use of a Quantifiler® Trio kit to generate laboratory standard curves remains effective for a maximum of 21-days, experimentation will prove that the length of laboratory generated standard curves will last well beyond the manufactured data.

Keywords: *Forensic Science, DNA Quantitation, Virtual standard curve, Quantifier Trio*

Introduction

Overview

In the field of Forensic Biology, DNA analysts use quantification, a process that determines the amount of human DNA present in a sample. Too little or too much DNA can pose a problem downstream. Knowing the amount of DNA present indicates to the analyst whether a dilution is required, because there is too much amplifiable DNA or if the entirety of the extract must be amplified, because there is not enough amplifiable DNA present. Too little DNA can cause stochastic fluctuation (allelic drop in/out, or peak height imbalance), while too much DNA can cause off-scale peaks, elevated stutter peaks, a raised baseline, and can generate profiles with uninterpretable data (Butler, 2009) (Rapley & Whitehouse, 2007). All steps of quantification are beneficial for the generation of optimal and useable results.

This research focuses on optimizing/enhancing the quantification step for the purposes of generating guidelines to be set in Forensic Biology laboratories. This research evaluates the use of a single standard dilution over a period of time and compares this to a virtual curve function of the software utilizing mock case work samples that mimic samples that crime laboratories would typically receive day to day.

There are three parameter values that are critical in the execution of this research project; slope, y-intercept and R^2 for determining whether a prepared standard curve is suitable for use. Slope is used to measure the efficiency of the PCR reaction. According to Thermofisher guidelines, a slope of -3.3 is indicative of a 100% efficient PCR reaction (*Poor PCR Efficiency - US*, n.d.), (Foster city, CA, 2015b), (*Using Standard Curve to Estimate DNA Quantity - Forensic Focus #4*, 2016). Additionally, efficiency of the PCR should be between 90–100% or 110%, meaning slope values should be in between -3.1 and not exceeding lower than −3.6.

"The Y-intercept value is the expected C_T value for a 1 ng/µL sample" (*Using Standard Curve to Estimate DNA Quantity - Forensic Focus #4*, 2016) (Foster city, CA, 2015b). Passing of this metric test permits comparison against unknown samples. The C_T value of unknowns can be measured and compared to that of the standard curve known concentrations, to approximate the DNA concentration of unknown samples. Furthermore, y-intercept denotes sensitivity of the assay specific curve by providing theoretical limit detection of the reaction, i.e., detection of low copy targets.

R^2, aka correlation coefficient or coefficient of determination, allows the analyst to measure the linearity of the standard curve. It answers the question of how proximal (close) does the data fit within the regression line. R^2 values equaling 1 are ideal but typically the R^2 value will equal 0.99. A 0.99 and higher R^2 value is suggestive of good precision (*Real-Time PCR Handbook,* 2016.).

Importance of Research

While there hasn't been any research published on this topic, this research is important to the Forensic Science community, more specifically, Forensic Biology, because it can save laboratories time and money by not having to regenerate standard curves as frequently. Moreover, any laboratory looking to move to the newer virtual curve method can see based on this research, that it is a feasible option. The primary goal of this research was to generate laboratory guidelines and recommendations for quantitation standards for forensic laboratories hoping to streamline their workflows, and to determine how long standards are valid for to decrease the amount of time and money spent on assay-specific standard curves. Most often, a standard curve is generated every time an assay is performed. By evaluating whether one dilution set could be used over a longer

period of time, laboratories can save time and money. The research data was generated over a two-month period, through multiples runs, standard curves were analyzed for variation in curve parameters, e.g., has the slope changed or has the quantitation range changed over time. One objective was to pinpoint at what frequency these changes occur, so that laboratories know at exactly what point a new standard should be generated.

The results will determine the frequency of curve and standard generation by evaluating the stability of one standard dilution over a given period of time. Additionally, the ability for a given standard to be repeated by multiple analysts was evaluated. The mock case samples were used to check the efficacy of the assay-specific standards versus the virtual curve to validate the suitability in DNA crime laboratories as an alternative to using a virtual curve. This is beneficial for evaluating how variations in standard curves with each generation could impact DNA laboratory results. Furthermore, if a laboratory chooses to use a virtual curve, potential positive controls were assessed, that could be used to satisfy the requirement by the FBI QAS (*Quality Assurance Standards for Forensic DNA Testing Laboratories*, 2016). This research is important because it provides a standard to be set for forensic laboratories that commonly utilize standard curves or are looking to move to a virtual curve to quantify the concentration of DNA in casework samples.

It is hypothesized that multiple standard quant curves produced do not compare significantly and one preparation of a standard curve can be utilized multiple times for case processing in laboratories. It will be beneficial for forensic laboratories to know at what point are their standard curves no longer valid and therefore new dilutions must be generated. Alternatively, it is hypothesized that multiple standard quant curves compare significantly and thus the preparation of one standard curve cannot be utilized for multiple runs when completing cases in a

crime laboratory, and thus the generation of a new dilution is required every run to adequately capture data in a crime laboratory.

Additionally, the use of a Quantifiler™ Trio kit to generate laboratory standard curves remains effective for a maximum of 14-days according to the manufacture guidelines. Experimentation will prove that the length of laboratory generated standard curves will last well beyond the manufactured data. This will set guidelines for laboratories to allow for more cost-effective DNA casework.

Background Information

Crime laboratories regularly receive an influx of evidence samples to be processed. There are numerous departments in crime laboratories, and each department is responsible for a different processing technique/specialty. For example, in the Forensic Biology field, these analysts specialize in crime samples containing biological materials, whether that is blood/ bloodstains, semen, saliva, and/or touch DNA swabs. These samples can contain a unique human DNA profile. To obtain DNA profiles, analysts in the field of Forensic Biology must first process each sample individually and extract the available DNA present from the samples by following various DNA extraction procedures (Becks, 2019; Butler, 2005, 2009, 2014). Each case is different, so the type of samples collected are different and must be processed accordingly.

Samples that contain a single source contributor are handled differently than samples that could contain multiple contributors, such as vaginal swabs from a sexual assault kit (SAK). “Typically, vaginal swabs from a SAK contains the female victim's epithelial cells and varying concentrations of sperm cells left by the perpetrator. The key to analyzing these types of samples

is to separate the mixture into male and female fractions" (Becks, 2019). This was accomplished for this research project through the use of DNA extraction.

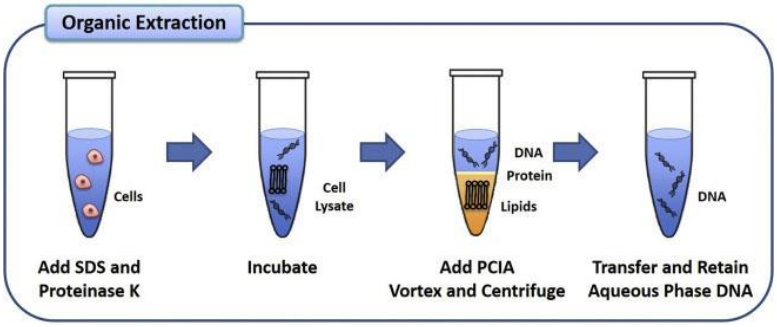

Figure 1. Organic extraction. A single source isolation steps.

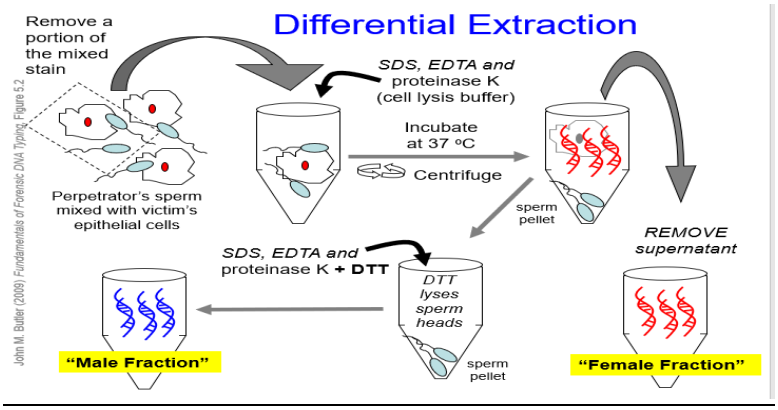

Figure 2. (Becks, 2019) "*Schematic of differential extraction process used to separate male sperm cells from female epithelial cells."* (Butler, 2005)

There are numerous types of DNA extraction methods for isolating DNA in both single and multiple contributor samples. This research utilized an Organic Extraction method for single source and a Differential Extraction for multi-source samples. DNA extraction is an important first step in the DNA analysis process. Extractions allow for the separation of cellular material from the DNA molecules (Butler, 2005), so that future analyses can be performed. Following DNA extraction, analysts use quantitation (quantity/concentration and quality of a sample)

(Becks, 2019; Butler, 2005) to determine the amount of amplifiable human DNA present in a given sample (Butler, 2009).

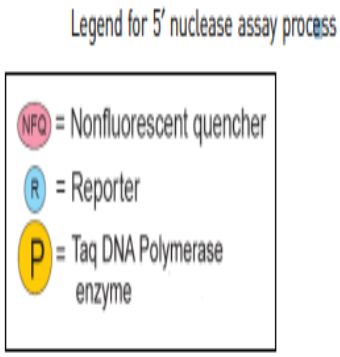

Figure 3A.

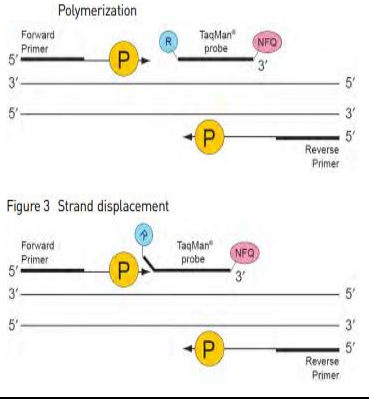

Figure 3B.

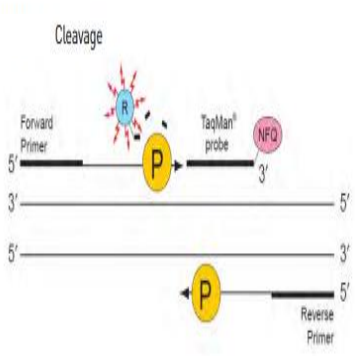

Figure 3C.

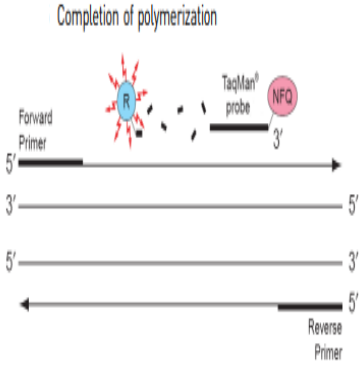

Figure 3D.

The most commonly utilized method for quantitation in Forensics is qPCR, quantitative Polymerase Chain Reaction, which uses a TaqMan®-based qPCR. This method was the foundation of this research. TaqMan®-based qPCR is human specific and works by utilizing fluorescent probes (Butler, 2009). The probes consist of a reporter and quencher dye. To start, no fluorescence is observed when the reporter and the quencher molecules are in proximity to each other (Butler, 2009). However, when Taq begins replicating the DNA starting at the primer, and reaches the probe during the extension phase, exonuclease activity will cleave the probe

nucleotide by nucleotide (Butler, 2009). This results in the release of the fluorescent molecules from the probe. The fluorescence will now occur since the reporter molecule is no longer near the quencher (quantity) (Butler, 2009). This process helps analysts make decisions about how to proceed, by providing quality and quantity information of the DNA present in an extract while displaying sensitivity and specificity to DNA quantitation. This process is required for question (unknown concentration) samples according to the FBI QAS guidelines (*Quality Assurance Standards for Forensic DNA Testing Laboratories*, 2016).

In order to perform quantitation, a quantitation kit with assay specific for quantification of human DNA is required for evidentiary items and necessary for short tandem repeat (STR) analysis (Becks, 2019; Rapley & Whitehouse, 2007).“This is the current gold standard for DNA profiling in forensic casework” (Rapley & Whitehouse, 2007). According to research performed by Grgicak et al. and Holt et al., the Thermofisher Quantifiler® Trio DNA Quantification kit enabled their research, because this kit was able to provide better performance for casework due to its sensitivity and robustness, compared to earlier quantitation kits. Moreover, Quantifiler™ Trio is able to use multicopy target loci for greater detection (Holt et al., 2016).

For qPCR, standard curves are generated, which is important for evaluating the accuracy, precision, reliability, and reproducibility of the data, which are all core values for validation, as listed by SWGDAM guidelines (*Ragsdale, R,* 2005.). Standard curves are generated during quantitation through dilution series for the purpose of optimization. This research utilizes laboratory generated standard curves (internal) as a reference, for comparison of unknown DNA concentrations samples to known DNA concentration samples. This is achieved through a calculation that depends on the correlation between cycle threshold (C_T) and the known DNA concentration. The threshold cycle allows the HID Real-Time PCR Analysis Software to define

the level of detectable fluorescence. The C_T value is based on the number of cycles required for that detectable fluorescence signal to surpass a specific value that is established arbitrarily by the instrument software. A comparison of unknown sample values and standard values can be achieved through the usage of a curve generated formula: $C_T = m\,[\log (\text{Qty})] + b$ (Foster city, CA, 2015b, 2018).

Previous Research

(Grgicak et al., 2010) evaluated the manufacturer guidelines to determine that it is always necessary to validate for specific laboratory parameters for a given set of standards based on variation seen between instruments. Like the research performed for this project, the authors prepared multiple dilution series with various concentrations. It was concluded that assay specific standard curves are not always the best method, because every laboratory setting is different, so parameters must be set individually for the laboratory in operation. The parameters that were used in the (Grgicak et al., 2010) research included running a standard curve on every plate, along with the amount of the serial dilutions. By doing so, the data indicated errors in preparation variability of the dilutions over numerous assays verses the instrument itself. Authors also concluded that reproducibility and quality increases over a longer period with external standard curves.

(Holt et al., 2016) conducted a study assessing the developmental validation of the Quantifiler™ HP and Quantifiler™ Trio DNA Quantification Kits. These kits were designed specifically to quantify human genomic DNA in forensic casework samples and were intended as a preliminary screening quantification step for performing analyses like STR genotyping with improved sensitivity and robustness compared to earlier DNA quantification kits. The newer

DNA quantification kit is able to concurrently quantify up to three human genomic targets, to include Y-chromosome (male contributor), while also indicating a sample's DNA degradation state. This is all made possible through the usage of the kit's new multiplex TaqMan™ assay-based fluorescent probe technology. As more and more forensic laboratories perform analyses on low concentration DNA samples, like 'touch' or 'trace' DNA, a need for sensitivity enhancement is vital to yield probative results. According to Holt's research, when compared to the Quantifiler™ Duo DNA Quantification Kit, the HP and Trio kits were able to detect less than a cell's-worth of DNA, extending detection into the sub-pg/µL range. This helps safeguard that low-grade DNA samples have a chance of providing beneficial genotype data.

(Cicero, 2014) conducted research that depicted reproducibility and errors as it relates to qPCR methodology in the development and analysis of an assay specific standard curve. In terms of laboratory workflow processing cost and errors, manufacturer's protocols for running assays are not the most efficient. The qPCR method from the manufacturer ensures more reproducibility errors, is more labor intensive for the laboratory personnel, along with being more time consuming, which leads to cost increases. (Cicero, 2014) devised two alternative methods which involved, using a validated standard curve, and using linear regression of efficacy, to compare the results of those protocols to accepted protocols. The results of Cicero's study revealed that concentrations were not consistent over an extended period of time and that calibrations verse different kits appeared to have a greater impact on the outcome of the data of standard curves. The use of a single curve method resulted in less DNA concentration variation error between runs. In conclusion, (Cicero, 2014) determined that instrument calibration played the largest role, and suggests the generation of a new external standard curve every time the

instrument is calibrated to ensure accuracy, as well as the curve should only be used on the kit that it was generated from.

Materials and Methods

Materials

1. Quantifiler® Trio DNA Quantification kit (Contains standard THP, standard dilution buffer, PCR reaction mix, and primer mix)
2. MicroAmp® Optical 96-Well Reaction Plate
3. MicroAmp® Optical Adhesive Film
4. MicroAmp® Adhesive Film Applicator
5. QuantStudio® 5 Real-Time PCR Instrument
6. HID Real-Time PCR Analysis Software v1.3
7. Microsoft Excel
8. Notebook
9. Pen and Pencil
10. Heat Block/Water bath
11. ThermoMixer
12. Microcentrifuge
13. Microcentrifuge tubes
14. Microcentrifuge tube rack
15. Vortex mixer
16. Refrigerator / freezer
17. Fume Hood

18. Tweezers
19. Scalpel
20. Pipettes (varying sizes)
21. Sterile pipette tips (varying sizes)
22. Spin-Ease Baskets
23. Kimwipes
24. Transfer pipettes
25. Microcon® 100 concentrator assembly
26. Gloves
27. Extraction solutions for organic and differential extractions (TNE, 20% Sarkosyl, UltraPure™ distilled water, Proteinase K, Dithiothreitol [DTT], Stain Extraction buffer, Phenol–Chloroform [PC or PCIA], 1X TE buffer)
28. 10% Bleach
29. 70% Ethanol
30. Mock case work samples (blood, semen, saliva, and touch DNA on a multitude of substrates)

Methods

In order to generate sufficient data, this research was supposed to be conducted over a six-month period (approximately), however, due to the Covid-19 pandemic, this research was conducted over a three-to-four-month period, in which the following occurred:

Sample Preparation

A George Mason IRB approval was obtained before research began. A Quantifiler® Trio DNA Quantification Kit was used to generate standard curves. According to the guidelines of the kit, the standard includes 100 nanograms (ng) per microliter (µL) stock solution of the Quantifiler® THP DNA Standard (Foster city, CA, 2018). The standard was first validated through quantification to obtain a recorded value as it pertains specifically to the kit. To quantitate the stock solution from the Quantifiler® Trio DNA Quantification Kit, two preparations occurred. First, a 1:1 serial dilution was created (10 µL DNA standard + 10 µL Buffer), to make standard one (STD 1) with a concentration of 50ng/ µL. From STD 1, a 1:9 serial dilution was created for the remainder of the standards (10 µL STD 1 + 90 µL buffer) to make STD 2, STD 3 was made using the same format, 10 µL STD 2 + 90 µL buffer. This process was continued until there was a serial dilution of five concentrations (50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and 0.005 ng/µL). These stock solution sample were all plated in duplicate at full scale reaction, 18 µL master mix plus 2 µL sample.

Secondly, two no template controls (NTC) were plated in duplicate, which contained master mix plus buffer. Additionally, STD zero was created by making a 1:10 dilution (1 µL DNA standard + 9 µL Buffer) and was plated in triplicate at full scale reaction. After the running the plate, it was determined through the averages of the plated stock solution standards, that the stock solution was sufficient to proceed with experimentation.

Upon validating the kit, calculations were performed for an appropriate serial dilution from the 100 ng/µL stock solution, concentrations varying from 50 ng/µL to 0.005 ng/µL, as per the recommendation of the manufacturer. This serial dilution was used to create a standard curve, in which each of the standards were run in duplicate time at full scale reaction each plating

period. Furthermore, DNA extractions were executed on mock casework samples to check the efficacy of the assay-specific standards versus a virtual curve to validate the suitability in DNA crime laboratories. See Table 1. for a detailed list of mock case type samples and the extraction method utilized.

Mock Case Work Samples for Extraction			
	Item name	Plate abbreviation	Extraction type
Sample 1	Drinking vessel	DV	Organic
Sample 2	Cigarette bud – 1	CB1	Organic
Sample 3	Cigarette bud – 2	CB2	Organic
Sample 4	V swab jeans	VSJ	Organic
Sample 5	V swab mask filter	VSMF	Organic
Sample 6	Blood on cotton denim	BLD – D	Organic
Sample 7	Blood on cotton	BLD – C	Organic
Sample 8	V swab – bra	VSB	Organic
Sample 9	Blood on FTA paper	BLD – FTA	Organic
Sample 10	Gun swab - magazine	GSM	Organic
Sample 11	Gun swab – trigger	GST	Organic
Sample 12	Gun swab – grip	GSG	Organic
Sample 13	Gun swab – slide serrations	GSSS	Organic
Sample 14	Cartridge cases	CC	Organic
Sample 15	Semen on bedsheet	SB – OD	Organic with DTT
Sample 16	Vaginal swab + semen (Sperm and non-sperm fractions)	VAG – NSP VAG – SP	Differential
Sample 17	Underwear + semen (Sperm and non-sperm fractions)	UND – NSP UND - SP	Differential
Sample 18	Reagent blank organic	RB - ORG	Organic
Sample 19	Reagent blank non-sperm fraction	RB - NSP	Organic
Sample 20	Reagent blank sperm fraction	RB - SP	Organic

Table 1. Mock case work samples, their abbreviations, and their extraction type.

This research relied on the organic and differential extraction methods to extract DNA from mock case samples. This step of the research is important because it will provide a standard to be set for forensic laboratories that commonly utilize standard curves or are looking to move to a virtual curve to quantify the concentration of DNA in casework samples.

Standard Curve Preparation

The standard curve preparation was done following Thermofisher guidelines (Foster city, CA, 2015a). Setup of the standard curve preparation can be seen below in Table 1. The creation of standard (STD) one required the calculation C1V1 = C2V2, where the starting concentration (C) and starting volume (V) were represented by C1V1. The final concentration amount and final volume were represented by C2V2. This formula provided the suitable amount of solution to be incorporate for standard curve generation. The calculation is as follows:

C1 = 106.5 ng/µL according to kit validation (a dilution was performed, so actual representing value is 10.65 ng/µL).

V2 = 60 µL

C2 = 50 ng/µL

So, then V1 = 28.1 µL …

Standard Curve Preparation		
Kit 1 – Analyst 1		
Standard (std.)	**Concentration (ng/µL)**	**Volumes**
Std. 1	50	28.1 µL (106.5 ng/µL stock) +31.9 µL Quantifiler® THP DNA dilution buffer

Std. 2	5	10 µL (std. 1) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 3	0.5	10 µL (std. 2) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 4	0.05	10 µL (std. 3) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 5	0.005	10 µL (std. 4) + 90 µL Quantifiler® THP DNA dilution buffer
Kits 2 – Analyst 2		
Standard (std.)	**Concentration (ng/µL)**	**Volumes**
Std. 1	50	10 µL (100 ng/µL stock) + 10 µL Quantifiler® THP DNA dilution buffer
Std. 2	5	10 µL (std. 1) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 3	0.5	10 µL (std. 2) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 4	0.05	10 µL (std. 3) + 90 µL Quantifiler® THP DNA dilution buffer
Std. 5	0.005	10 µL (std. 4) + 90 µL Quantifiler® THP DNA dilution buffer

Table 2. Preparation of standards for both analysts.

In order to determine the actual amount of the standard sample for the kit, that was used in this research project, the standard was first quantified based on the manufacturer's protocol for a dilution. A standard curve was generated to obtain a stock solution value, which aided in the appropriate calculation for all standards generated in the research's serial dilution. From the 100 ng/µL stock solution, varying concentrations were made per recommendations of the manufacturer: 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and to 0.005 ng/µL.

To obtain the amount of standard dilution buffer needed to be added, the initial volume, 28.1 µL, was subtracted from the total volume, 60 µL, to yield 31.9 µL. STD two was created by utilizing 10 µL of STD one and 90 µL standard dilution buffer. This process was continued until the fifth STD was created. Each sample plated used a full-scale reaction volume of 18 µL master

mix and 2 µL of sample. To create the master mix, a 'Quant Plate set-up' in excel was used to generate volume amounts of reaction mix and primer mix, based on sample size, using a calculated formula. Reagent amount (µL) multiplied by number of samples yields the total volume of reaction mix or primer, where the starting reagent amount was based on a full-scale reaction (18 µL total). The master mix ratios were in concordance with the manufacturer's recommendations.

Lastly, three positive control samples were created with a concentration of 5 ng/µL, 1 ng/µL, and 0.5 ng/µL. Positive control 5 ng/µL sample was STD two from the dilution series explained above, that was created day one for the kit validation; positive control 1 ng/µL sample was created by making a 1:10 dilution using standard two created day one for the kit validation, and the 0.5 ng/µL positive control sample was STD three from day one's validation plating.

Samples were run on a set schedule, checking for data deviation after each run. This included an assay on day 1, day 4, day 7, day 14, day 21, day 28, day 35, and day 42. The sample size was based on two key factors, one, how many times can the standards run before the data failed, and two, accessibility to 'case work' samples. The 'case work' samples represented common sample types that would be found in crime laboratories. In total, there were 17 'case work' samples, prior to the DNA extraction process. There were 22 samples following the DNA extraction process, which included reagent blanks and a two-part separation fraction that was yielded from the differential extractions (samples 16 and 17).

DNA Extraction

The DNA extraction procedures included: Differential Extraction and Organic Extraction protocols from the Department of Forensic Science with slight deviations based on the protocols

from The Center for Forensic Science Research and Education (CFSRE). For this research project, the extraction processes were completed over a two-day period. On day one, the differential extraction procedure was performed, and the organic extraction procedure was setup. To yield better results, the organic extraction called for an extended incubation period, overnight.

Differential Extraction Procedure

The differential extraction process followed this procedure: "DNA Extraction Method for Mixed Body Fluid Stains (Differential Procedure)" (*FBS09 - Differential Organic DNA Extraction, 2018).* A cutting of samples 16 and 17 were added to their individual labeled tubes. Two control tubes were also created. The following reagents were added individually to tubes 16 and 17: 400 µL TNE, 25 µL 20% Sarkosyl, 75 µL UltraPure™ distilled water, and 5 µL Proteinase K

After all of the reagents were added to each tube, the tubes were lightly vortexed to ensure that the cuttings were submerged into the liquid. Both tubes were then placed into a thermomixer to incubate for 40 minutes at 56º Celsius (C) while shaking at 850 rotations per minute (RPM). The tubes were then vortexed vigorously for 20-30 seconds and then pulse spun. Sample 16 and 17 cuttings were carefully removed from the liquid in their tubes and placed into a new unused Spin-Ease basket (CoStar). The baskets were then placed into their respectable tubes and the lids were closed. Each tube was place into the microcentrifuge to spin for 10 minutes at 10,000 RPM to remove the excess liquid from the cutting.

Each Spin-Ease basket holding the cuttings were remove from each tube and discarded. Using a pipette, almost all of the supernatant (liquid) was carefully transferred into a newly labeled microcentrifuge tube without disrupting the sperm pellets at the bottom of the tubes.

Supernatant should contain the non-sperm fraction (mostly female epithelial DNA) and was set aside until extraction (*FBS09 - Differential Organic DNA Extraction*, 2018). At the bottom of the tubes was a small pellet that contained the sperm fraction (male DNA). The pellet was then washed to remove inhibitory factors and left-over epithelial cells by being resuspended in 500 µL of UltraPure™ DNase/RNase-Free distilled water (ThermoFisher Scientific) and vortexed briefly. The tube was then placed in a microcentrifuge for 10 minutes at 10,000 RPM. Most of the supernatant was removed and discarded.

In the original DFS protocol (outlined in the Appendix), the pellet was to be washed three times, however, multiple washes significantly decrease the number of recoverable sperm cells. The recommendation to wash the pellet a single time before re-suspension was followed, as per the modification by the CFSRE protocol for a differential extraction. The sperm pellet was re-suspended individually in 400 µL of TNE, 25 µL of 20% Sarkosyl, 75 µL of UltraPure™ DNase/RNase-Free distilled water, 5 µL of Proteinase K, and 20 µL of 1Molar (M) Dithiothreitol (DTT). The samples and their controls were then incubated at 89 ºC for 40 minutes at 850 RPM in a thermomixer. After incubation, the tubes were pulse spun for 5 seconds to remove the liquid from the caps and stored in a secure refrigerator until the organic extraction could be performed to purify the DNA.

Organic Extraction Procedure

Samples 1-15 were prepped for the organic extraction using the following procedure: “Organic Extraction Method for Buccal Cell Type Samples, Bloodstains and Tissue Samples and Manual Purification of DNA” (*FBS08 - Organic DNA Extraction, 2018*).Tubes were pre-labeled with their designated sample abbreviation, and 400 µL of stain extraction buffer (a pre-mixed solution that was donated to this research by the DFS) and 10 µL of Proteinase K, Sigma Life

Science, was used to saturate each sample individually. Then the tubes were lightly vortexed, and pulse spun to ensure the samples were in the liquid. Samples were placed in a water bath to incubate over night at 56 ºC.

On day two of the extraction process, the organic extraction procedure was finished, along with the completion of the manual purification of each DNA sample. Samples were removed from incubation and vortexed vigorously for 20-30 second, along with being pulse spun at 10,000 RMP for a few minutes to ensure the entirety on the tube's content was at the bottom of each tube. The cuttings were carefully removed from the liquid and placed into a new unused Spin-Ease basket; the baskets were then placed into their respectable tubes and the lids were closed. Each tube was placed into the microcentrifuge to spin for 10 minutes at 10,000 RPM to remove the excess liquid from the cuttings. Spin-Ease baskets holding the cuttings were removed from each tube and discarded.

Manual Purification of DNA – a continuation of the organic extraction process

The differential extraction samples, from day one of the extraction processes were removed from the refrigerator, so that the manual purification of all DNA samples could begin. Microcon® 100 concentrators (Sigma Aldrich) were assembled prior to the start of the purification process. 500 µL of phenol-chloroform-isoamyl alcohol was added to each tube sample. All tubes were mixed thoroughly by hand until the solution of the tubes appeared milky in color. Each tube was then placed into a microcentrifuge for 3 minutes at 10,000 RPM to separate the two phases. Microcon® 100 concentrators were inserted into labeled filtrate vials (tubes) and 100 µL of UltraPure™ DNase/RNase-Free distilled water from Thermofisher Scientific, was added to each concentrator.

A visual inspection indicated two distinct layers in each sample tube. The top layer contained the DNA, while the bottom layer contained the organic solvent (*FBS08 - Organic DNA Extraction, 2018).* The aqueous top layer was transferred to the pre-assembled Microcon® concentrator and the filtrate vial was then capped. The Microcon® assemblies were placed in the microcentrifuge to spin for 10 minutes at approximately 5,000 RPMs or until the volumes were reduced. The concentrators were then carefully removed from each Microcon® assembly. The fluid was discarded from each filtrate vial. The concentrators were then returned to their vials, respectively.

UltraPure™ distilled water was added to each concentrator for washing purposes, at 200 µL per tube. The caps were replaced and the Microcon® assemblies were placed in the microcentrifuge to spin again for an additional 10 minutes at approximately 5,000 RPM or until the volume was reduced. Caps were removed from the concentrators and 30 µL of 1X TE^{-4} buffer was added to each tube. The concentrators were removed from the filtrate vials and the vials were discarded.

At this point, the concentrators were then carefully inverted and placed into newly labeled retentate vials. The Microcon® assemblies with the inverted concentrators were then placed into a microcentrifuge to spin for 5 to 10 minutes at 5,000 RPM to dislodge the DNA from the Microcon® membrane. Following spinning, the concentrator units were discarded, and the caps were secured on the retentate vials. All samples were frozen until quantitation could be performed.

Virtual Curve Preparation

To further explain the quantitation relationship between analysts, the 'Generation of Parameters for HID Virtual Standard Curve' software was used to select which analyst's data was to be set as the standard and which analyst's data would be omitted. To accomplish this, the virtual curve preparation was set-up following Thermofisher guidelines (Foster city, CA, 2015b). In order to prepare a virtual standard curve, first each standard curve that was generated and its corresponding data set was examined. From each curve, the computer software generates the slope and y-intercept for each assay run; the slope and y-intercepts from three main targets, large and small autosomal, and Y-target were collected in an excel sheet and their averages were determined. The averages were then plugged into the virtual function to generate a virtual curve.

Upon concluding data collection, the generation of standard cures were created using Microsoft Excel, and then the curves were evaluated over time to see if there were any visible changes that took place, if so where and how often, based on created data.

Data Analysis and Interpretation

Overall, this research data was generated with the use of the new HID Real-Time PCR Analysis Software v1.3 that is compatible with the QuantStudio® 5 Real-Time PCR Instrument. This software enabled data analysis with a virtual standard curve feature, to help minimize variations while also reducing the costs and time associated with preparing standards for each quantitation run. With the use of the standard curve slope and y-intercept values from preceding runs, a virtual curve was created. This function was used against the mock case work samples

and was able to detect an error with certain plates. The virtual curve preparation is outlined above.

From the software generated data, values (slope, Y-intercept, and R^2) were transcribed into excel worksheets to create charts that plotted each analyst averages over time. Moreover, to better analyze the data between analysts, tables were created for each target (T. small, T. large, and T.Y), outlining the average, maximum, minimum, and standard deviation for each analyst. To further highlight the significance of the research data, calculations were performed in excel to produce theoretical GlobalFiler™ data, comparing assay standard curve volume amounts to virtual standard curve volume amounts. All data was interpreted through manual comparison of the values to that of the recommendations outlined in the Quantifiler™ Trio user guide for a passing range, on top of charting the linearity of the values.

Results & Discussion

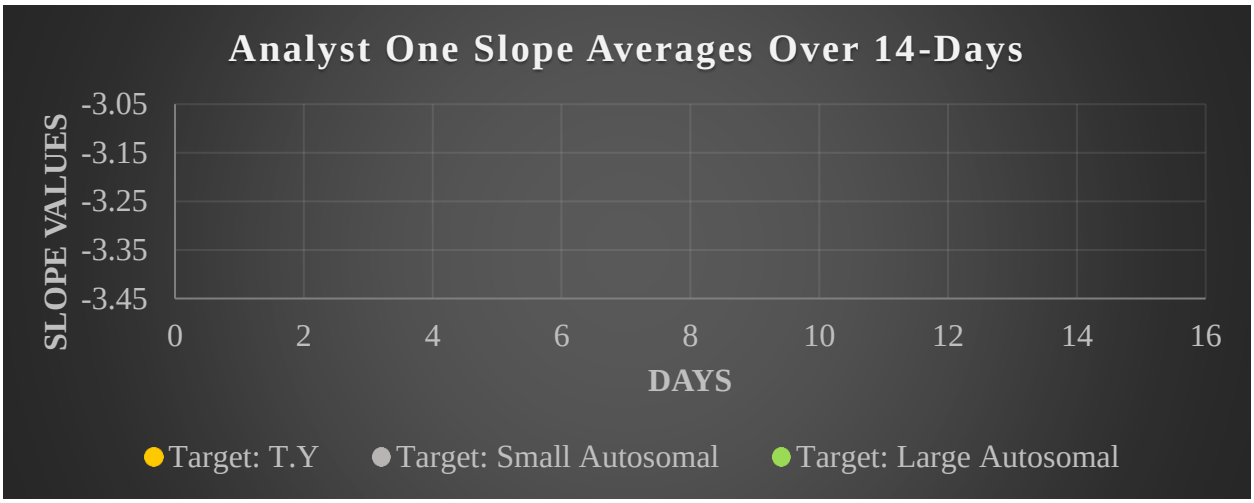

Figure 4. The average slope values for analyst one over a 14-day period. The chart plots slope averages over time.

In figure 4., the data represents the average slope values for analyst one over a 14-day period. The chart plots slope averages over time because time is a key component of this research project. Analyst one produced 4 curves in which slope values of the curves that were calculated. The purpose of plotting the analyst's data like this, is to see if there are any large deviation between day 0 and day 14, looking for degradation over time or looking for a non-linear pattern because this will indicate to the laboratory that a new standard curve should be generated. The spread looks large upon first glance, however, the increments are 0.05, so the spread is not as large as it may initially appear, in fact, the values fit perfectly within the recommended values range. According to Thermofisher's Quant Trio recommendations, slope values should range between -3.58 and -3.10.

Slope Averages with Analyst One as Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	-3.32	-3.22	-3.29

Table 3. The averages of the plotted slope values for analyst one over a 14-day period. The chart summarizes Figure 4.

The values listed in Table 3. indicate the average slopes values for analyst one over a 14-day period. The averages are that for each curve run by analyst one for each of the three isolated targets, T.Y, small, and large autosomal.

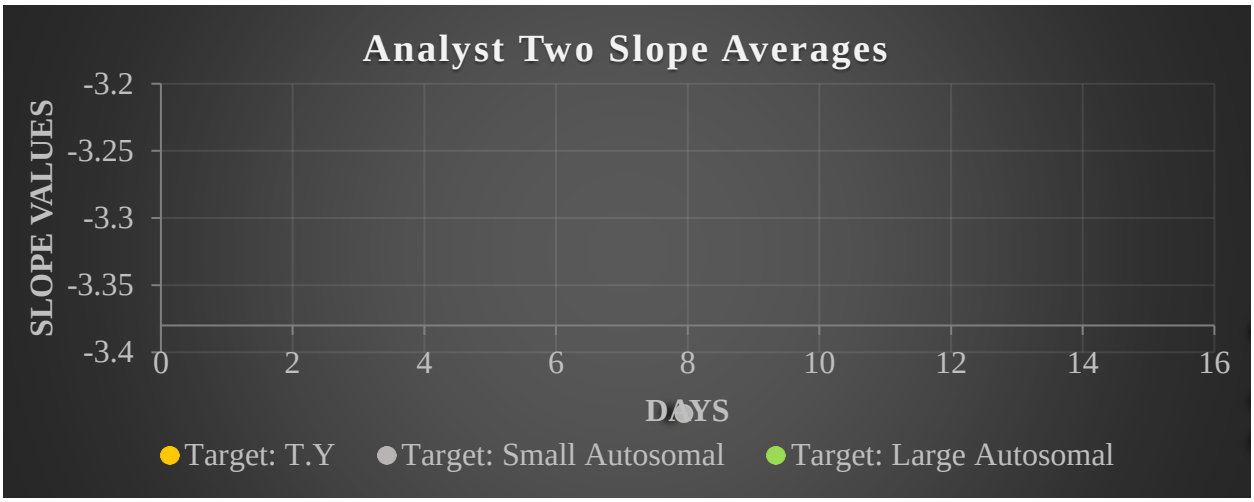

Figure 5. The average slope values of analyst two over a 14-day period. The chart plots slope averages over time.

The data presented in figure 5. represents the average slope values, however the data is that of analyst two over a 14-day period. The purpose of plotting each analyst set as the standard, allows for a comparison between the two analysts; each analyst has a unique pipetting style and values can differ based on a particular analyst. By changing which analyst is set as the standard, the values can be compared to see if there is a significant difference in the values, and if there is not, then the Quantifiler™ Trio data can be reproduced adequately.

Slope Averages with Analyst Two Set as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	-3.29	-3.24	-3.28

Table 4. The averages of the plotted slope values for analyst two over a 14-day period. The chart summarizes Figure 5.

The values listed in Table 4. indicate the average slopes values for analyst two over a 14-day period. The averages are that for each curve run by analyst two for each of the three isolated targets, T.Y, small, and large autosomal.

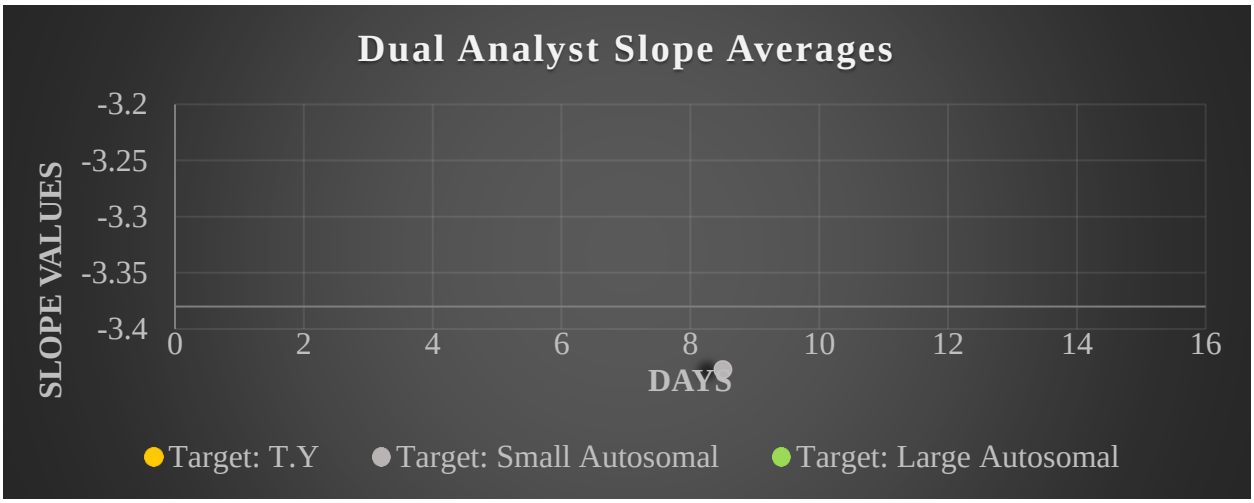

Figure 6. The average slope values for analyst one and analyst two over a 14-day period. The chart plots slope averages over time.

Setting both analysts as the standard allows for the software to average the two standards together for a combined slope for each curve run. By doing this, the data can be interpreted, to see if the combined value continues to remain within the recommended values rage of -3.10 to -3.6.

Slope Averages with Both Analysts as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	-3.30	-3.23	-3.28

Table 5. The average of the plotted slope values over a 14-day period. The chart summarizes Figure 6.

The values listed in Table 5. indicate the average slopes values for both analyst one and analyst two over a 14-day period. The averages are that for each curve run by both analysts for each of the three isolated targets, T.Y, small, and large autosomal.

Change of Slopes over 14-Days					
Standard Set	**Target**	**Average**	**Max**	**Min**	**Standard Deviation**

Analyst One	T. Small	**-3.22**	-3.19	-3.27	0.0349
	T. Large	-3.29	-3.19	-3.39	0.0847
	T. Y	-3.32	-3.24	-3.42	0.0753
Analyst Two	T. Small	**-3.24**	-3.22	-3.27	0.0189
	T. Large	-3.28	-3.24	-3.32	0.0324
	T. Y	-3.29	-3.24	-3.36	0.0563
Both Analysts	T. Small	-3.23	-3.22	-3.27	0.0237
	T. Large	-3.28	-3.21	-3.33	0.0495
	T. Y	-3.30	-3.25	-3.36	0.0583

Table 6. The average slope, maximum slope, minimum slope, and standard deviation for slope values for analyst one, analyst two and both analysts combined over a 14-day period.

Linear values allow analysts to see if there is a change in the data values as time progresses or with each assay that is performed. Table 6. displays slope averages from Figures 1., 2., and 3. for the slope values from the linear regression calculated by the software for each standard curve. The values in Table 6. do not vary by analyst for the different targets. For T. small, the value goes from -3.22 to -3.24 between the different analysts, as can be referenced by the bolded values in Table 6. This is a small step in quantitation. Remember that the manufacturer suggests that a curve between -3.1 to -3.6 is a passing curve. This is seen in the other two targets as well, where T. Large goes from a value of -3.29 to -3.28, and T.Y goes from -3.32 to -3.29.

In the last column of Table 6., standard deviation for each data points are provided. This calculation is a statistic for the purposes of quantifying how close the numerical values are in relation to one another (good precision) (Cooper, 2008.). Laboratories can use the information provided by the standard deviation as an estimate of the tests consistency for particular concentrations for the purpose of reproducibility (Cooper, 2008.). According to Thermofisher,

the classification of a high C_T outlier, slope values can increase or decrease based on C_T values that are above 35 cycles (*Poor PCR Efficiency - US*, n.d.). However, some laboratories state that the limit of quantitation has been reached when standard deviation of a standard makes greater than or equal (≥) to 0.5 (*Poor PCR Efficiency - US*, n.d.). Likewise, low C_T outliers can lead to over-efficient PCR, in which the slope may be greater than -3.3, for example a slope of -2.9. In this event the linear regression will appear normal minus that one point, and that value should be omitted and the curve reanalyzed (*Poor PCR Efficiency - US*, n.d.).

Change of Y-intercept Over 14-Days

Standard Set	Target	Average	Max	Min	Standard Deviation
Analyst One	T. Small	27.1	27.2	27.0	0.0426
	T. Large	25.2	25.3	25.1	0.0805
	T. Y	26.3	26.4	26.2	0.0858
Analyst Two	T. Small	27.1	27.1	27.0	0.0509
	T. Large	24.9	24.9	24.8	0.0194
	T. Y	26.2	26.3	26.1	0.0774
Both Analysts	T. Small	27.1	27.1	27.0	0.0305
	T. Large	25.0	25.1	25.0	0.0445
	T. Y	26.2	26.3	26.2	0.0759

Table 7. The average Y-intercept, maximum Y-intercept, minimum Y-intercept, and standard deviation for Y-intercept values for analyst one, analyst two and both analysts combined over a 14-day period.

The averages for the y-intercept values from the linear regression calculated by the software for each standard curve are presented in Table 7. When analyst one T. small is compared to analyst two T. small, the values are identical at 27.1, as seen bolded in Table 7. Furthermore, if the other two targets for the analysts are compared, the data shows that there is little difference in the values. For T. Large: 25.2 and 24.9, For T.Y: 26.3 and 26.2.

Change of R2 Over 14-Days					
Standard Set	**Target**	**Average**	**Max**	**Min**	**Standard Deviation**
Analyst One	T. Small	0.999	1	0.998	0.000957
	T. Large	0.998	1	0.997	0.00129
	T. Y	0.999	1	0.999	0.0005
Analyst Two	T. Small	0.999	0.999	0.999	0
	T. Large	0.999	1	0.999	0.0005
	T. Y	0.999	1	0.998	0.000816
Both Analysts	T. Small	0.999	1	0.998	0.000816
	T. Large	0.997	0.999	0.995	0.00171
	T. Y	0.999	0.999	0.999	0

Table 8. The average, maximum, minimum, and standard deviation for R^2 values for analyst one, analyst two, and both analysts combined over a 14-day period.

Table 8. displays the averages for the R^2 values from the linear regression calculated by the software for each standard curve. If the values from Table 8. are compared, there is no little to no difference in the values between the analysts. Moreover, manufacturer guidelines suggest that R^2 should be not less than 0.98 and if it is, the following should be reevaluated: “Quantity

values entered for quantification standards in the Plate Setup - Assign Targets to the Selected Wells during experiment setup, making of serial dilutions of quantification standards, loading of reactions for quantification standards, and Failure of reactions containing quantification standards"(Foster city, CA, 2018).

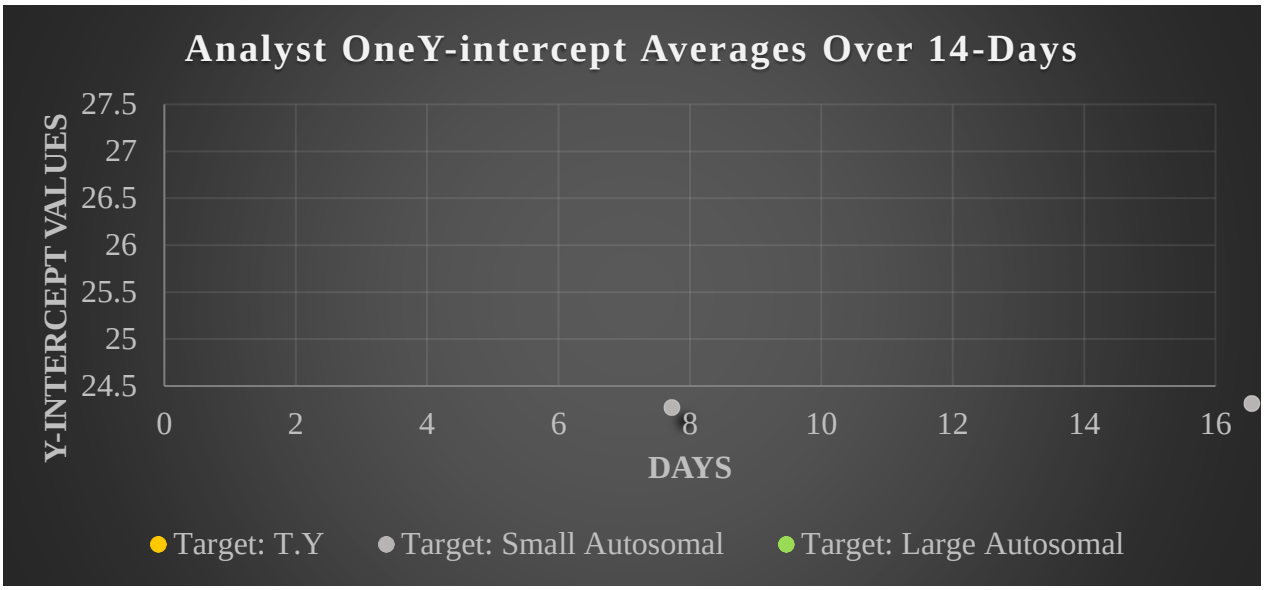

Figure 7. The average Y-intercept values of analyst one over a 14-day period. The chart plots Y-intercept averages over time.

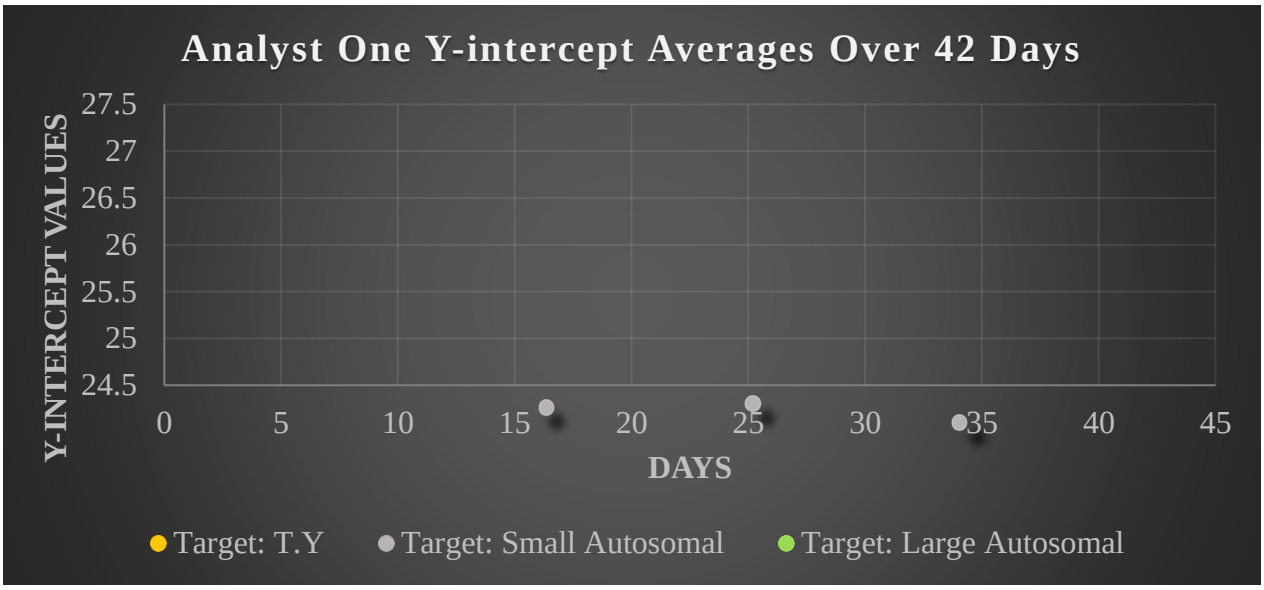

Figure 8. The data here represents the average Y-intercept values of analyst one over a 42-day period. The chart plots Y-intercept averages over time.

Y-intercept Averages with Analyst One Set as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	26.28425	27.105	25.21075
42	26.26385	27.06185714	25.17857143

Table 9. The averages of the plotted Y-intercept values over a 14-day period and over a 42-day period. The chart summarizes Figure 4. and Figure 5. for comparison.

When Figure 7. is compared to Figure 8., the assay continued to behave in a linear pattern even after the recommended discard of 14-days. The data above represents the longevity of the prepared standard dilution beyond the kit's recommendation. This is significant because this means that laboratories could save time and money by not having to regenerate dilutions as frequently.

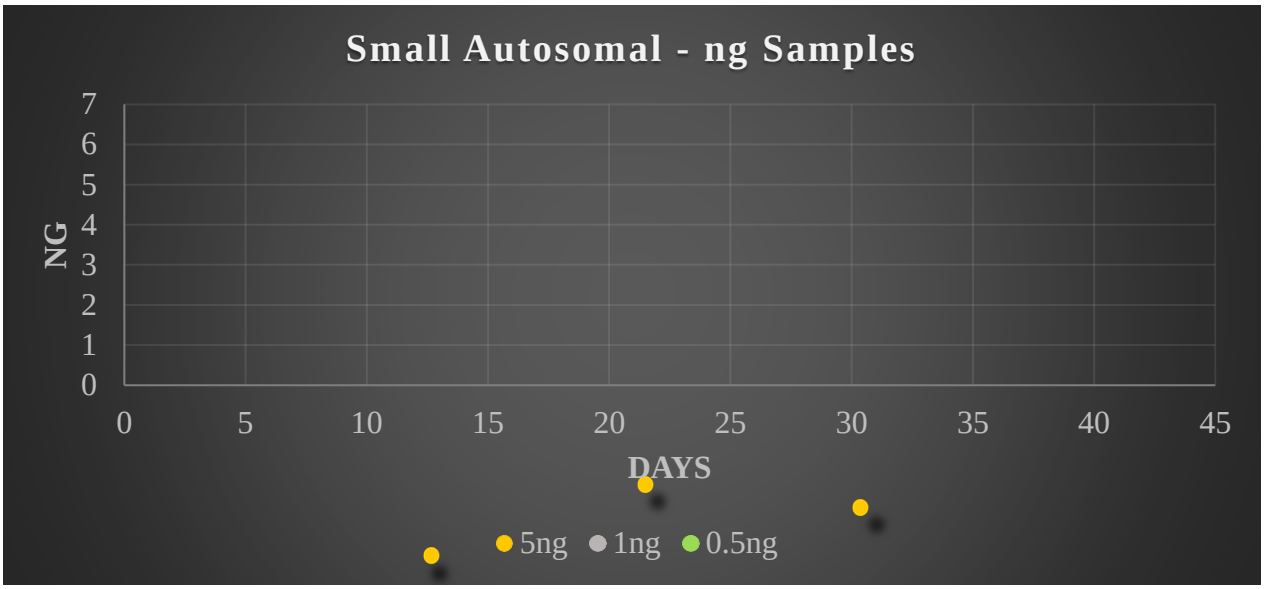

Figure 9. The positive control values for the Small autosomal target over a 42-day period. The chart plots positive control values over time.

Small Autosomal – Positive Control Sample Averages			
Days	5ng	1ng	0.5ng
1	4.82555	0.994	0.45405
4	5.4205	1.02415	0.49265
7	5.23185	1.00145	0.4909
14	4.93415	0.96015	0.47945
21	5.8949	1.0496	0.5136
35	5.0239	0.9579	0.50875
42	5.13565	1.0767	0.48145
Average	5.2095	1.01	0.49
% CV	7%	4%	4%

Table 10. The average positive control values for the Small autosomal target.

For this research, positive control samples were plated with the standards. The positive controls for this research included 5, 1, and 0.5 ng/µL that were prepared using the kits standard. Controls are stated as requirements according to the FBI quality assurance guidelines (9.5.2), that whenever quantification is used, so shall a quantification standard in reference to if laboratories decide to perform a virtual or external standard curve (*Quality Assurance Standards for Forensic DNA Testing Laboratories*, 2016). When performing an external or virtual standard curve, this data depicts how the values change over time for 5, 1, and 0.5 ng/µL. Data from this study can be used as a guide for laboratories to set what a passible range would be for utilizing one of these 3 concentrations as a positive control. The proceeding Figures (10 & 11) and Tables (11 & 12) represent the positive control averages for the other two critical target, T. Large autosomal and T.Y chromosome.

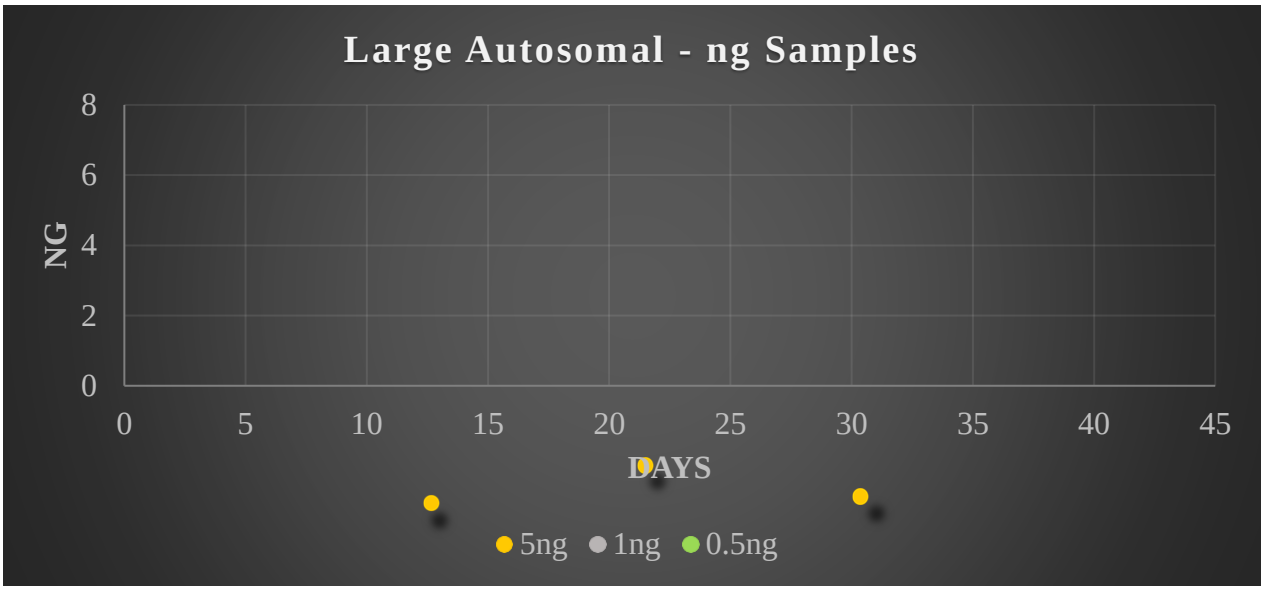

Figure 10. The positive control values for the Large autosomal target over a 42-day period. The chart plots positive control values over time.

Large Autosomal – Positive Control Sample Averages			
Days	5ng	1ng	0.5ng
1	6.02055	1.375	0.6651
4	6.38635	1.1988	0.54015
7	6.0844	1.16775	0.5965
14	5.80005	1.1445	0.60535
21	6.69015	1.27405	0.6467
35	5.6077	1.10655	0.54315
42	6.12195	1.2922	0.6127
Average	6.101592857	1.222693	0.601379
% CV	6%	8%	8%

Table 11. The average positive control values for the Large autosomal target.

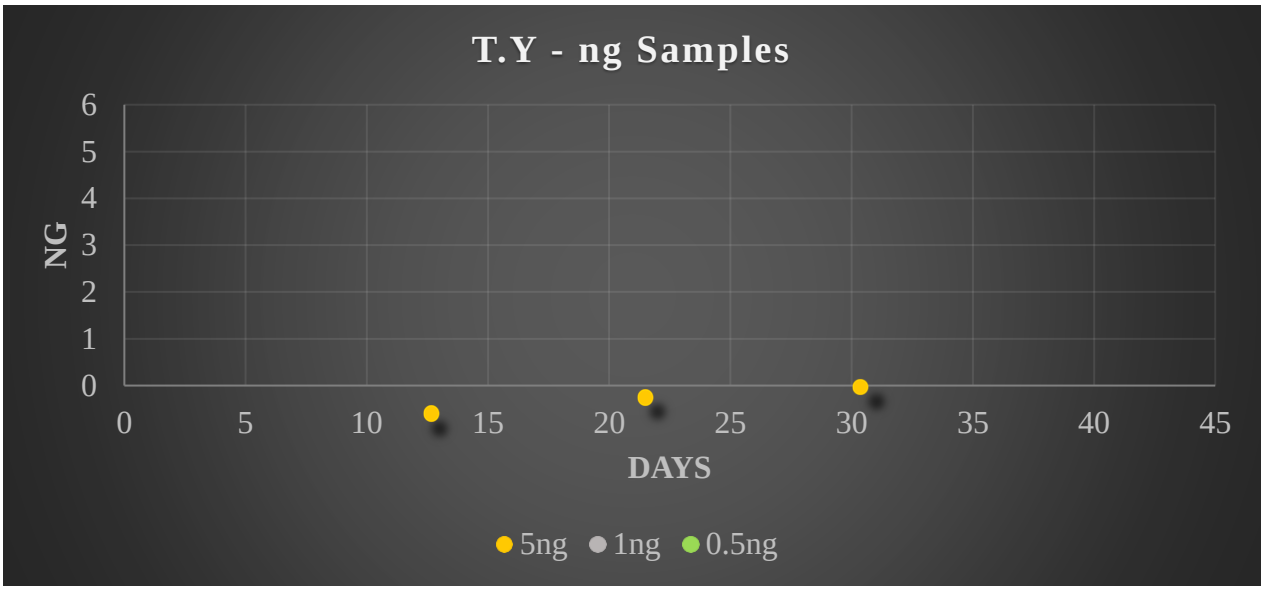

Figure 11. The positive control values for T.Y target over a 42-day period. The chart plots positive control values over time.

T. Y – Positive Control Sample Averages

Days	5ng	1ng	0.5ng
1	5.16295	1.1458	0.52715
4	5.27745	1.02	0.42985
7	5.3543	1.05085	0.5151
14	5.011	0.9247	0.4971
21	5.707	1.0181	0.49195
35	5.0344	0.99675	0.5131
42	5.097	1.0704	0.482
Average	5.23	1.03	0.494
% CV	5%	7%	6%

Table 12. The average positive control values for the T.Y target.

GlobalFiler™ Theoretical

Sample Name	DNA Concentration (ng/µL) from ASC	DNA Concentration (ng/µL) from VSC		DNA Amplified Dilution 1 ASC ng/µL	Dilution Type ASC	DNA Amplified Dilution 1 VSC	Dilution Type VSC	Volume Needed ASC	Volume Needed VSC	Actual Volume ASC	Actual Volume VSC
SB-OD	6.9607	6.8454	1 ng Total	0.69607	1:10	0.68454	1:10	1.43663712	1.460835013		
5NG	4.452533333	4.400633333	1 ng Total	0.445253333	1:10	0.440063333	1:10	2.245912439	2.272400185		
DV	2.8371	2.8184	1 ng Total	0.28371	1:10	0.28184	1:10	3.524725953	3.548112404		
BLD-FTA	0.0722	0.0747	1 ng Total					10	10	0.722	0.747
0.5NG	0.464766667	0.471133333	1 ng Total					10	10	4.647666667	4.711333333
CB1	0.12185	0.1254	1 ng Total					10	10	1.2185	1.254
GSM	0.0168	0.0176	1 ng Total					10	10	0.168	0.176
STD1 OG	12.22215	11.9451	1 ng Total	0.6111075	1:20	0.597255	1:20	1.636373306	1.674326711		
CB2	0.04325	0.045	1 ng Total					10	10	0.4325	0.45
GST	0.02285	0.02395	1 ng Total					10	10	0.2285	0.2395
STD5 OG	0.0011	0.0012	1 ng Total					10	10	0.011	0.012
VSJ	0.00195	0.00215	1 ng Total					10	10	0.0195	0.0215

GSG	0.02895	0.03025	1 ng Total					10	10	0.2895	0.3025
RB NSP	0.0008	0.00085	1 ng Total					10	10	0.008	0.0085
RB SP	0.0035	0.00375	1 ng Total					10	10	0.035	0.0375
BLD-D	2.35225	2.34165	1 ng Total	0.235225	1:10	0.234165	1:10	4.251248804	4.270493028		
GSSS	0.03865	0.04025	1 ng Total					10	10	0.3865	0.4025
VAG NSP	2.526	2.5126	1 ng Total	0.2526	1:10	0.25126	1:10	3.958828187	3.979941097		
VAG SP	15.79925	15.396	1 ng Total	0.7899625	1:20	0.7698	1:20	1.265882874	1.299038711		
BLD-C	0.5339	0.5404	1 ng Total					10	10	5.339	5.404
CC	0.0028	0.00295	1 ng Total					10	10	0.028	0.0295
UND NSP	2.0642	2.0579	1 ng Total	0.20642	1:10	0.20579	1:10	4.844491813	4.859322661		
UND SP	8.5609	8.40005	1 ng Total	0.85609	1:10	0.840005	1:10	1.168101485	1.190469104		
VS-B	0.06195	0.06425	1 ng Total					10	10	0.6195	0.6425
Sample 1		46.7219	1 ng Total			0.934438	1:50		1.070161958		
Sample 2		4.9435	1 ng Total			0.49435	1:10		2.022858299		
Sample 3		0.55175	1 ng Total						10		5.5175
Sample 4		0.0508	1 ng Total						10		0.508
Sample 5		0.00545	1 ng Total						10		0.0545

Table 13. Theoretical amplification scenario between assay specific curves and virtual specific curves.

To further emphasize the benefit of this research, Table 13. displays theoretical GolbalFiler^{TM} data generated in excel, that compares the values of assay standard curves (ASC) to that of a virtual standard curve (VSC) if these samples were to be taken through amplification. As seen with sample SB-OD, semen on a bedsheet, when volumes are compared, there is little to no difference in the values between ASC and VSC. The ASC volume for the SB-OD sample is 1.44 uL, whereas the VSC indicate a need for 1.46 uL.

This information guides analysts to know how much sample is needed for the best results during amplification. Ideally, analysts look to amplify 1000 picograms (pg), but some samples, as shown in Table 16. above, are less, as indicated by the actual volume columns. The sample where it is less than 1ng, this value is more critical for comparison of ASC and VSC, because

variable results could be shown. However, Globalfiler™ is sensitive and even a 0.0625 (or 62.5 pg) sample will still yield a profile.

Conclusion

It was hypothesized that multiple standard quantification curve dilutions produced do not compare significantly and one preparation of a standard curve can be utilized multiple times for case workflows processing in laboratories. It will be beneficial for forensic laboratories to know at what point are their standard curve dilutions are no longer valid and therefore new preparations must be generated. To determine this information, multiple standard curves were evaluated for variability overtime when performed by the same or multiple individuals. Additionally, the longevity of these standard dilution preparations was also assessed.

On the opposing argument, it was hypothesized that multiple standard quant curves compare significantly and thus the preparation of one standard curve dilution cannot be utilized for multiple runs when completing cases in a crime laboratory. The generation of a new curve is required every run to adequately capture data in a crime laboratory. According to the data presented in this research, the hypothesis can be accepted with valid supporting data.

While manufacturer guidelines are a great starting reference, using these guidelines and protocols introduces unnecessary variation when running a standard curve with each assay. As seen by this research, the linear regression parameter values (slope and y-intercept) remain constant over time. Additionally, labs can cut time and cost by extending the length of these standard curve dilutions beyond the manufacturer's recommendation of 14-day. Parameters between multiple analysts do not vary, increasing the reproducibility factor. Manufacturers recommend making a dilution series on a biweekly bases, but as proved by this research the

longevity of the dilution extends beyond the recommendation, as seen by the 42-day linear data. For the usage of external or virtual standard curves, a positive control must be in effect (*Quality Assurance Standards for Forensic DNA Testing Laboratories*, 2016). This research provides positive control parameters for values of 5, 1 and 0.5 ng/µL. Data from this study can be used as a guide for laboratories to set what a passible range would be for utilizing one of these three concentrations as a positive control. From the researcher's perspective, while all three concentrations are viable options, sample 1ng/µL positive control contains the most consistent results while also being the middle control range.

Mock case work samples were used to show the variability of quant values on multiple standard quant curves and compared to virtual curves for the purpose of setting new guidelines for crime laboratories. It is recommended that each lab evaluate the linearity of their curves, checking for deviations in the lab specific set parameters, and if the values fall outside the parameters, then the assay is no long valid. The usage of mock case work samples validated that assay standard curves and virtual standard curve are both feasible options for crime laboratories workflows.

Things to consider when using this research as a reference for setting laboratory guidelines; time was a limiting factor of this research, so future researchers should conduct experimentation over a longer period of time to facilitate better data collection. Additional future directions should include this research being conducted in half-scale reaction to compare the result to that of the full-scale reaction that was completed in this particular experimentation. This research utilized data collected from two different analysts to show reproducibility, so ideally more than two analysts worth of data should be generated. Furthermore, anyone else interested in the contribution this research has to the Forensic Biology community, should also

research/evaluate the effects of freeze thaw cycles of standards. Lastly, the next step of this research would be to amplify the samples.

In conclusion, manufacturer guidelines should be followed with caution because they could end up costing a lab more time and money. If the lab carefully monitors the linearity of their standard curves, then less time and money would be wasted on generating new curves bi-weekly. Additionally, the laboratory could adopt a virtual standard curve practice to further decrease the time and money spent on generating standards. As stated in the FBI quality assurance, positive controls must be used when quantification is performed using an external/virtual curve (*Quality Assurance Standards for Forensic DNA Testing Laboratories*, 2016), and this research provides consistent values for positive controls in the in the 5, 1 and 0.5 ng/µL. Whether a laboratory prefers assay-specific standard curve or virtual standard curve the data provided shows that there is no significant difference between the two curve methods prior to amplification.

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Appendix

	Item	Lot number / REF
1	Quantifiler® Trio DNA Quantification kit	LOT 2006085
2	Quantifiler™ Trio PCR Reaction Mix	LOT 2006078
3	Quantifiler™ THP DNA Standard	LOT 2006117
4	Standard Dilution Buffer	LOT 2002031
5	Quantifiler™ Trio Primer Mix	LOT 2005039
6	(AM) Quantifiler® Trio DNA Quantification kit	LOT 2007087
7	(AM) Quantifiler™ Trio PCR Reaction Mix	LOT 2007080
8	(AM) Quantifiler™ THP DNA Standard	LOT 2007120
9	(AM) Standard Dilution Buffer	Unknown
10	(AM) Quantifiler® Trio Primer Mix	Unknown
11	UltraPure™ Phenol: Chloroform: Isoamyl Alcohol	LOT 19K0456023
12	Proteinase	REF V302B 242471
13	Proteinase K from Tritirachium album	LOT 089M4034V
14	96-Well Region of Interest (ROI) and Background Plates	LOT 2006252
15	96-Well Spectral Calibration Plate with ABY™ Dye	LOT 2006085
16	96-Well Spectral Calibration Plate with JUN™ Dye	LOT 2006088
17	96-Well 0.2-mL Spectral Calibration Plate 3 (TAMRA™, NED™, and Cy® 5 dyes)	LOT 2006075
18	96-Well 0.2-mL Spectral Calibration Plate 2 (ABY®, JUN®, and MUSTANG PURPLUE® dyes)	LOT 2006075
19	96-Well 0.2-mL Spectral Calibration Plate 1 (FAM™, VIC™, ROX™ and SYBR® dyes)	LOT 2006084
20	Optical Adhesive Covers	LOT 201807084
21	MicroAmp® Optical 96-Well Reaction Plate	REF N8010560
22	MicroAmp® Optical 96-Well Reaction Plate with Barcode	REF 4306737
23	UltraPure™ Distilled Water	LOT 2147371

Table 14. Reagent LOT numbers/ REF numbers

Note: In Forensic DNA, it is customary to document every and all reagent LOT (batch of product) or REF (reference) number for trackability/traceability purposes.

Procedures that were followed:

Differential Extraction

Equipment

- heat blocks 37 ºC
- thermomixer 56 ºC
- microcentrifuge
- microcentrifuge tube rack
- vortex mixer
- Tweezers
- Pipettes – 10 µL, 100 µL, 200 µL, and 1000 µL

Materials

- Microcentrifuge tubes
- Sterile ART tips for pipettes – 10 µL, 100 µL, 200 µL, and 1000 µL
- Kimwipes
- Spin-Ease baskets
- Gloves

Reagents

- TNE
- 20% sarkosyl
- UltraPure™ distilled water
- Proteinase K
- PCR digest buffer

- 1.0 M DTT
- 10% bleach

Procedure

1. Add to the tube with cutting:

   400 µL TNE

   25 µL 20% Sarkosyl

   75 µL UltraPure™ distilled water

   5 µL Proteinase K
2. Mix by hand or light vortexing and then pulse spin to force the cutting into the liquid.
3. Place the tube in a thermomixer and incubate at 56 ºC for 40 minutes while shaking at 850 RPM.
4. Vortex vigorously for 20-30 seconds and pulse spin the tube. Remove the cutting for the liquid and place into a new unused spin-ease basket. Place the basket in the tube and close lid. Spin the tube for 10 minutes in a microcentrifuge at a minimum of 10,000 RPM to remove the excess liquid from the cutting.
5. Remove the spin-ease basket containing the cutting and discard.
6. Using a pipette, carefully transfer as much liquid (supernatant) as possible into a newly labeled microcentrifuge tube with a lid. Be care not to dislodge or disturb the pellet on the bottom of the tube. The supernatant removed from the pellet is the NON-SPERM FRACTION. The pellet on the bottom of the tube will become the SPERM FRACTION.

7. Set the non-sperm fraction tube aside.
8. Wash the pellet as follows: add 500 µL of UltraPure™ distilled water and resuspend the pellet by vortexing briefly. Spin the tube for 10 minutes in a microcentrifuge at a minimum of 10,000 RPM. Remove as much liquid as possible and discard.
9. Recreate the master mix from step one in the tube with the pellet, as well as adding 20 µL 1M DTT, to resuspend the pellet.
10. Incubate sample and control at 89 ºC for 40 minutes at 850 RPM. Remove the tube from the thermomixer and pulse spin for 5 seconds to remove the liquid from the cap.
11. Store both fractions in the fridge until the organic extraction.

Organic Extraction

Equipment

- Heat block or incubator, 56 °C
- Microcentrifuge
- Microcentrifuge tube rack
- Vortex mixer
- Tweezers
- Pipettes - Pipettes – 10 µL, 100 µL, 200 µL, and 1000 µL

Materials

- Microcentrifuge tubes, 1.5 mL
- Microcentrifuge tubes, 2.0 mL

- Transfer pipettes
- Sterile ART tips for pipettes – 10 µL, 100 µL, 200 µL, and 1000 µL
- Kimwipes
- Spin-Ease baskets
- Gloves

Reagents

- Stain Extraction Buffer
- Proteinase K – 20mg/mL (frozen when not in use)
- 10% bleach solution
- Isopropanol

Procedure

1. Add 400 µL of stain extraction buffer and 10 µL of Proteinase K to saturate the sample.
2. Mix by hand or light vortexing and pulse spin to force the sample into the liquid.
3. Place the tube into a 56ºC incubator or heat block and incubate overnight.
4. Vortex vigorously for 20-30 seconds and pulse spin the tube at 10,000 rpm.
5. Remove the cutting from the liquid and place into a new unused Spin-Ease basket.
6. Place the basket in the tube and close the lid.
7. Spin the tube for 5 minutes in a microcentrifuge at a minimum of 10,000 rpm to remove the excess liquid from the cutting.
8. Remove the Spin-Ease basket containing the cutting and discard.

Manual Purification of DNA

Equipment:

- Pipettes – 2 µL, 10 µL, 100 µL, 200 µL, and 1000 µL
- Heat block or incubator, 56 °C
- Microcentrifuge
- Microcentrifuge tube rack
- Vortex mixer

Materials:

- Microcentrifuge tubes, 1.5 mL
- Microcentrifuge tubes, 2.0 mL
- Transfer pipettes
- Sterile ART tips for pipettes – 10 µL, 100 µL, 200 µL, and 1000 µL
- Microcon® 100 Concentrator Assembly
- Kimwipes
- Gloves

Reagents:

- Phenol-choloroform-isoamyl alchohol (PCIAA), pre-warmed to room temperature
- 1X TE-4 buffer
- UltraPure™ distilled water

Procedures:

1. Add 500 µL phenol-cholorform-isoamyl alcohol to each tube.

2. Cap the tube tightly and mix thoroughly by hand or light vortexing until the solution has a milky appearance.
3. Spin the tube for 3 minutes at a minimum of 10,000 rpm to separate the two phases.
4. Insert a Microcon® 100 Concentrator into a labeled filtrate vial (microcentrifuge tube provided with the Microcon® assembly) and add 100 µL of UltraPure™ distilled water to the concentrator.
5. Transfer the aqueous phase (top layer containing DNA in Step 3) to the Microcon® concentrator, avoiding transfer of any of the organic solvent (bottom layer in Step 3) and place the cap from the filtrate vial on the concentrator.
6. Spin the Microcon® assembly in a microcentrifuge for 10 minutes at approximately 5,000 rpm until the volume is reduced.
7. Carefully remove the concentrator until from the Microcon® assembly and discard the fluid from the filtrate vial. Return the concentrator to the top of the filtrate vial.
8. Add 200 µL of UltraPure™ distilled water to the concentrator. Replace the cap and spin the Microcon® assembly in a microcentrifuge for 10 minutes at approximately 5,000 rpm until the volume is completely reduced.
9. Remove the cap from the concentrator and add 30 µL 1X TE-4 buffer.
10. Remove the concentrator from the filtrate vial and discard the vial. Carefully invert the concentrator and place into a new labeled retentate vial (same type of tube as the filtrate vial).
11. Spin the Microcon® assembly with the inverted concentrator in a microcentrifuge for 5 minutes at 5,000 rpm.
12. Discard the concentrator unit and place the cap on the retentate cup.

13. Freeze until Quant.

Remainder of Experimental Data Results (Figures and Tables)

Figure 12. The average slope values for analyst one over a 42-day period. The chart plots slope averages over time.

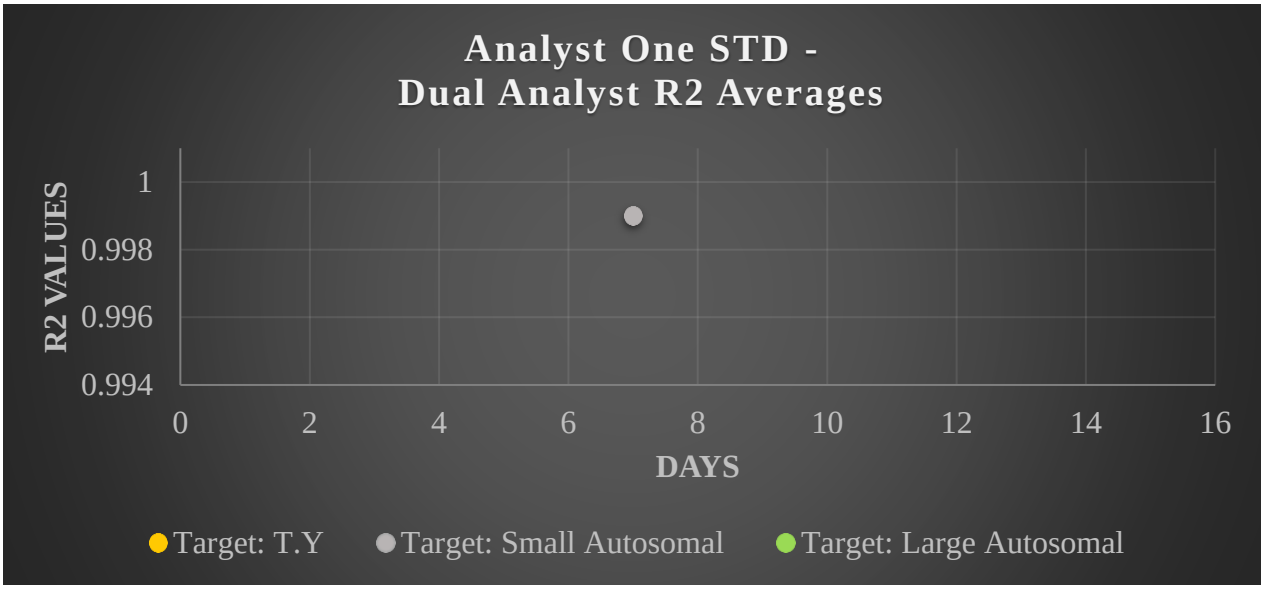

Figure 13. The average R2 values for analyst one over a 14-day period. The chart plots R2 averages over time.

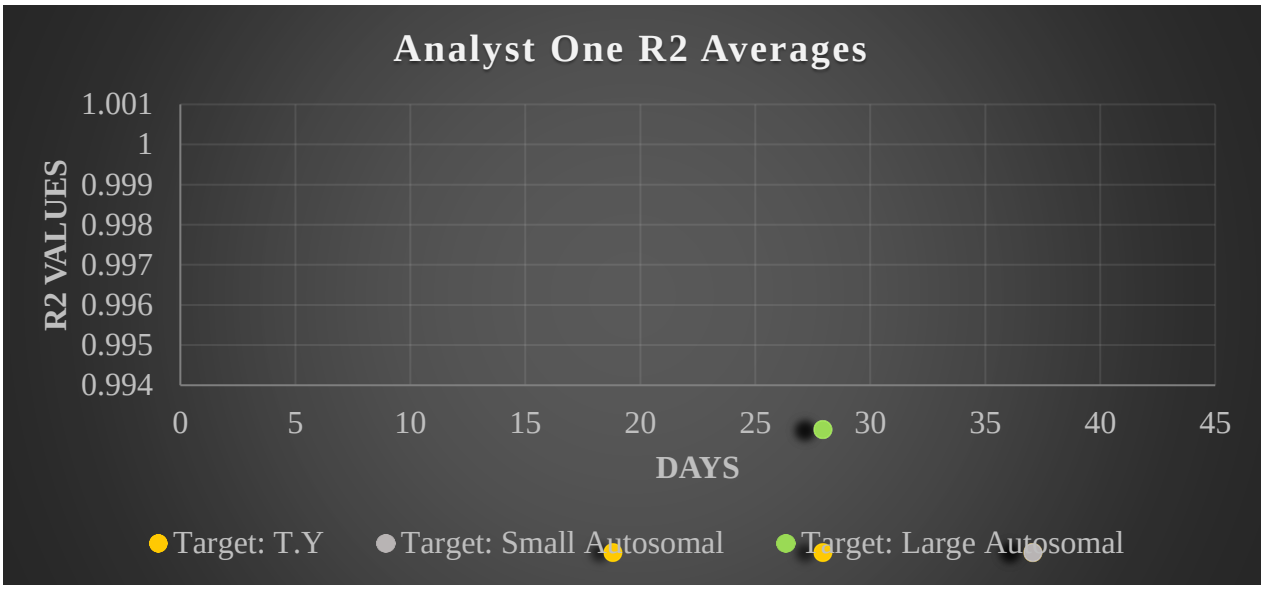

Figure 14. The average R2 values for analyst one over a 42-day period. The chart plots R2 averages over time.

R2 Averages with Analyst One Set as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	0.99925	0.99925	0.9985
42	0.9978571	0.999	0.998571429

Table 15. The average R2 values for analyst one as the standard over a 42-day period.

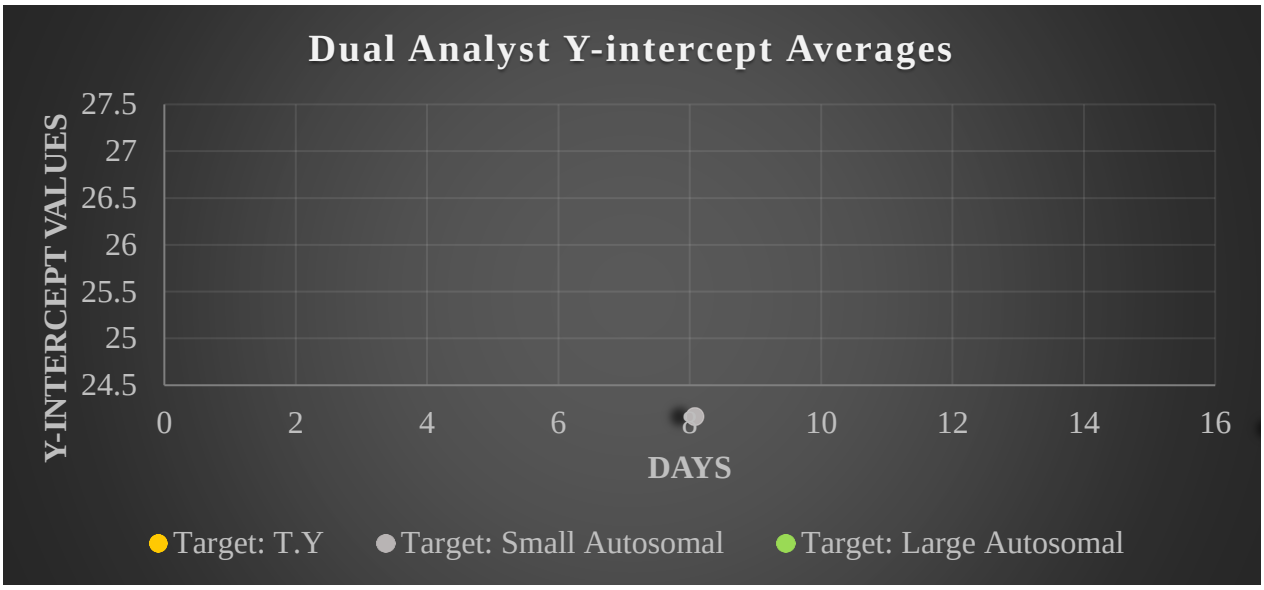

Figure 15. The average Y-intercept values for analyst two over a 14-day period. The chart plots Y-intercept averages over time.

Y-intercept Averages with Analyst Two Set as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	26.2115	27.061	24.87025

Table 16. The average y-intercept values for analyst two as the standard over a 14-day period.

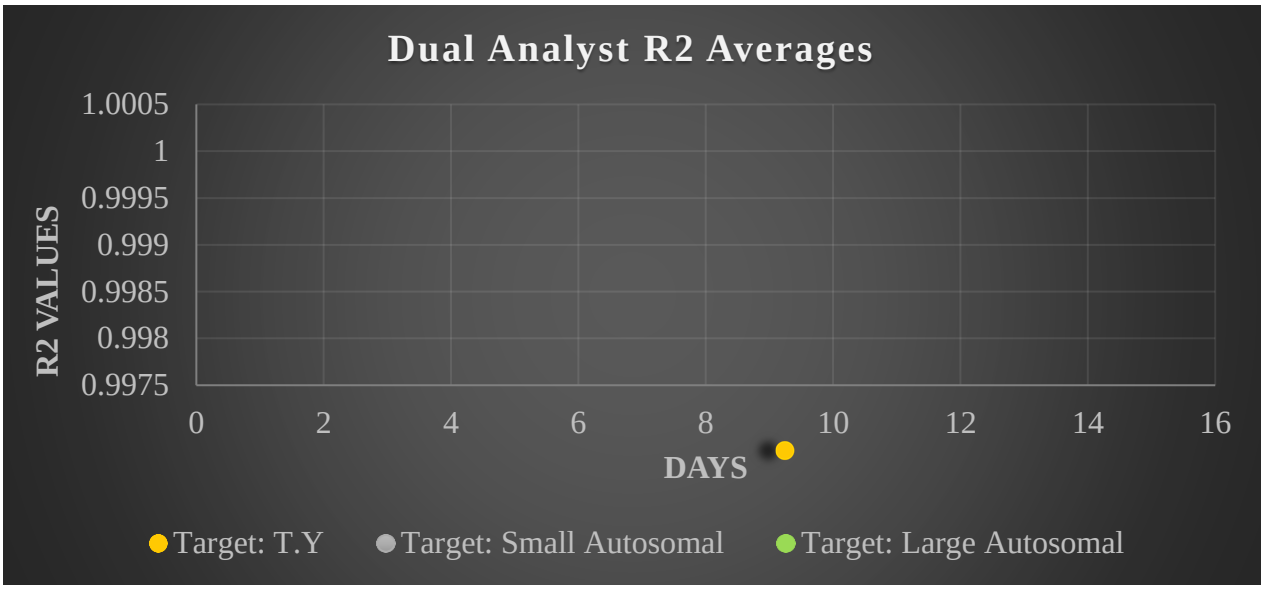

Figure 16. The average R2 values for analyst two over a 14-day period. The chart plots R2 averages over time.

R2 Averages with Analyst Two Set as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	0.999	0.999	0.99925

Table 17. The average R2 values for analyst two as the standard over a 14-day period.

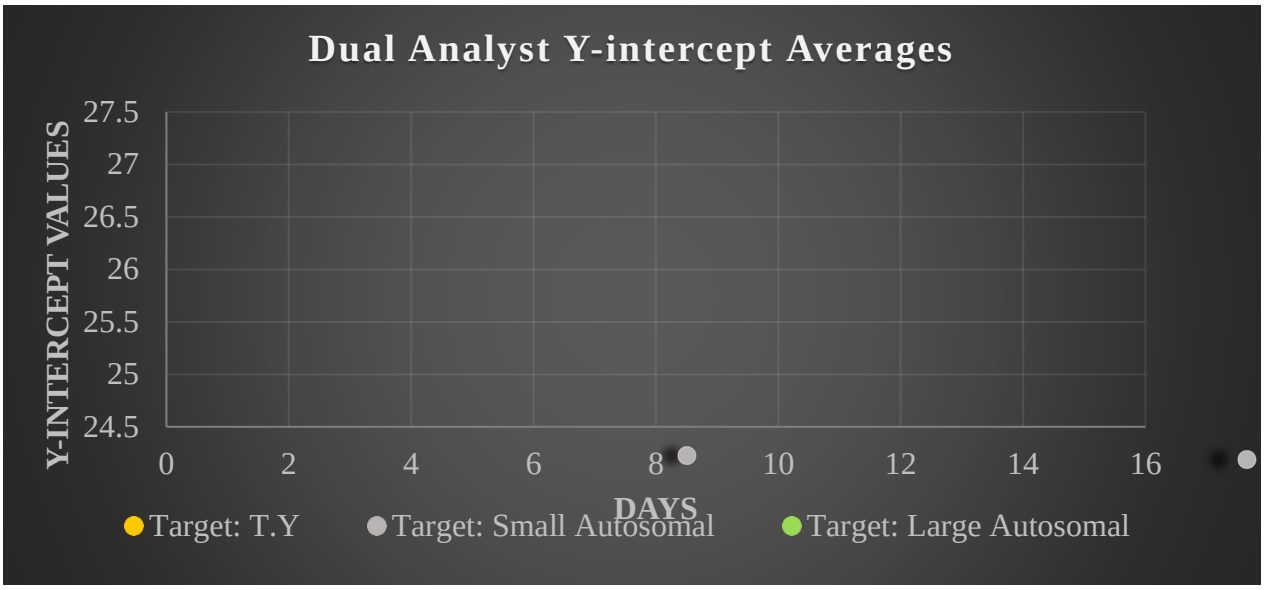

Figure 17. The average Y-intercept values for both analysts over a 14-day period. The chart plots Y-intercept averages over time.

Y-intercept Averages with Both Analysts as the Standard			
Days	T. Y	Small Autosomal	Large Autosomal
14	26.24775	27.083	25.0405

Table 18. The average Y-intercept values for both analysts as the standard over a 14-day period.

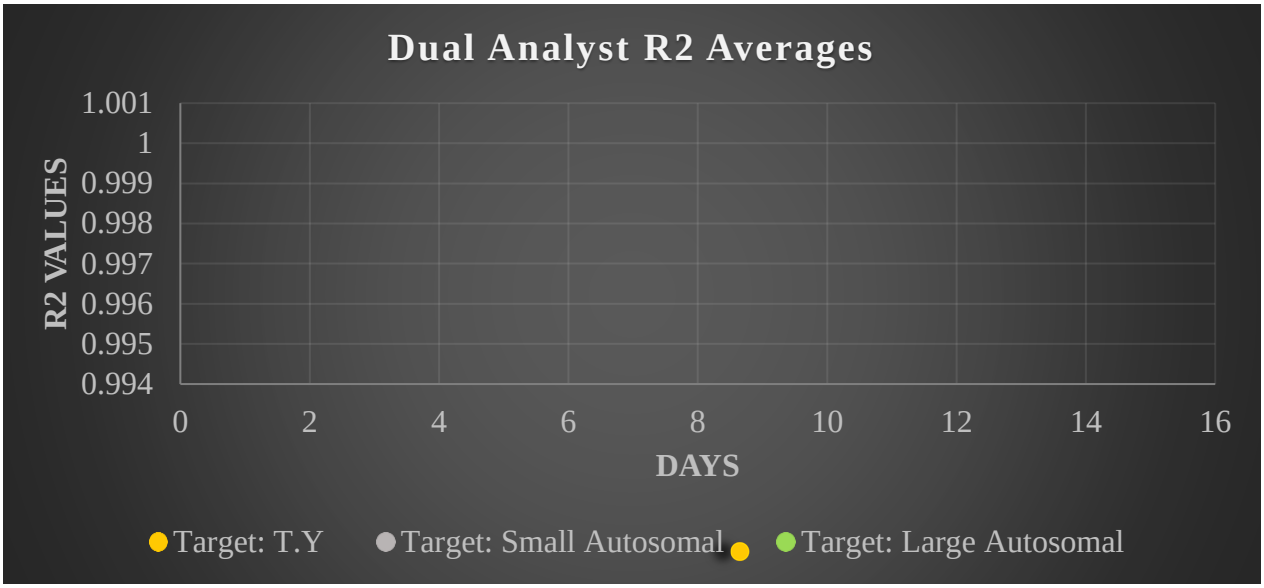

Figure 18. The average R2 values for both analysts over a 14-day period. The chart plots R2 averages over time.

R2 Averages with Both Analysts as the Standard

Days	T. Y	Small Autosomal	Large Autosomal
14	0.999	0.999	0.99725

Table 19. The average R2 values for both analysts as the standard over a 14-day period.