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Evaluation of error and reproducibility of qPCR for absolute quantification of DNA

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**EVALUATION OF ERROR AND REPRODUCIBILITY OF QPCR FOR
ABSOLUTE QUANTIFICATION OF DNA**

by

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ABSTRACT

Absolute quantitative PCR (qPCR) is a method that determines the concentration of DNA in a sample. Accurate, and reproducible quantification is required during forensic DNA processing since the results determine the volume of sample used during STR genotyping. If too little DNA is utilized allelic dropout can occur; if too much DNA is used an increase in the number of artifacts can result. In either case, sub-optimal DNA input-masses can lead to the misinterpretation of the evidentiary profile, by increasing the probability of drop in and/or drop out.

Generally, the qPCR method used during forensic DNA processing employs a set of standards, which are run with the questioned samples and used to generate a standard curve. These data are then used to establish a linear equation that is subsequently utilized to estimate the concentration of DNA in the unknown sample. However, standard curves have been shown to be prone to systematic and random error effects that impact the accuracy of the concentration estimate.

This study examines two alternative methods to determine the DNA concentration for unknown samples, and compares them to the currently accepted protocol of running new dilutions/standards with every assay. The two alternative methods are: 1) using a validated standard curve, and 2) using linear regression of efficiency.

To examine the feasibility of using these two methods for forensic purposes, two samples were quantified, using qPCR, in quadruplicate over the course of three years and concentrations were calculated using all three methods. Effects that time, kit lot, and instrument calibration had on the concentrations was examined for both total human and Y-DNA. Specifically, methods were compared by examining variances in concentration over the three-year period, and contrasting these results with the variances obtained within runs. The method which resulted in the smallest changes in concentration over time was regarded as the most stable.

Results show that of the three methods, the use of a validated curve resulted in less variation of DNA concentration between multiple runs. Further, the factor that had the largest impact on concentration variance was the calibration of the instrument. Based on these results, recommendations are provided.

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ABBREVIATIONS

CCD	Charge-coupled Device
C_t	Cycle Threshold
DNA	Deoxyribonucleic Acid
ΔR_n	Change in Fluorescence at Cycle n
E_C	Amplification Efficiency at cycle C
E_{max}	Maximum Efficiency of Amplification
F_C	Fluorescence at cycle C
F_0	Target Quantity
F_{max}	Maximum Fluorescence
IPC	Internal Positive Control
LRE	Linear Regression of Efficiency
MAK2	Mass Action Kinetic
MRP	Manufacturer's Recommended Protocol
μL	Microliters
ng	Nano grams
OCF	Optical Calibration Factor
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
ROI	Region of Interest

RPPH1	Ribonuclease P RNA Component H1
RSD	Relative Standard Deviation
SD	Standard Deviation
SRY	Sex-Determining Region Y
VC	Validated Curve

Introduction

Quantification of human DNA is an integral part of the forensic DNA processing scheme. Quantification of DNA via UV-Vis spectroscopy, and slot blot (1) are not optimal methods for forensic purposes because they are either not human specific or are associated with a large level of uncertainty. Therefore, a more accurate (2) way to determine the initial human DNA concentration of samples is required.

Quantitative PCR (qPCR) is a real-time PCR method used to quantify DNA in a sample. Accurate quantification allows the mass of DNA during STR typing to be controlled such that optimal genotyping results may be obtained (3).

There are generally two types of qPCR used in forensics: SYBR®-based qPCR (4), and TaqMan®-based qPCR (5). SYBR®-based qPCR employs a molecule that fluoresces when bound to double stranded DNA. Therefore, fluorescence increases with each PCR cycle as the DNA amplifies. TaqMan®-based qPCR utilizes probes that bind to specific regions of the DNA. These probes are attached to both a fluorophore and a quencher molecule. When the Taq polymerase reaches the probe during extension, its exonuclease activity degrades the probe, thereby separating the quencher from the reporter. Therefore, as the cycle number increases, the signal intensity of the reporter also increases. While it is possible for SYBR® green assays to be engineered to be human specific (6), TaqMan® assays have the capability of being both human specific (7), and multiplexed (8).

One commonly employed TaqMan® based kit, available commercially, is the Quantifiler Duo® kit. It is an AmpliTaq®-based assay (9) that has the capability of quantifying the amount of total human and male DNA present in a sample. Quantifiler Duo® is a multiplex qPCR assay that uses probes with different reporter dyes which target human-specific and Y-specific regions. The human-specific probe targets the gene Ribonuclease P RNA Component H1 (RPPH1), and the Y-specific probe targets the a region on the Y chromosome (SRY) (5). The kit also contains an internal PCR control (IPC), which is used to test for the presence of PCR inhibitors that could affect the amplification of the sample. The IPC is also used as a control to assess whether the assay/thermocycler is working as expected (5).

The Quantifiler Duo® kit has been previously validated for use in forensics (3,10,11). These studies concluded the assay was able to distinguish between total human and male DNA, and had the capability of detecting as little as 25 pg/μl of male DNA in a mixture of male and female DNA.

Accurate quantification of a sample is imperative during human identification testing. If the sample DNA is not quantified accurately, then an incorrect mass of DNA will be used during STR amplification. If too little DNA is used for STR analysis, allelic dropout may occur. If the rates of dropout are extreme, samples that contain a mixture of DNA from two contributors can incorrectly be considered single source. Further, the propensity to underestimate the number of contributors would increase as the actual number of contributors

increased. Alternatively, if too much DNA is used the profile would exhibit indications of imbalanced amplification, detectable forward stutter, and/or high baseline noise levels (12,13). Currently, the method commonly used to determine DNA quantity in forensic laboratories was recommended by the manufacturer (5). The manufacturer's recommended protocol (MRP) is based on the creation of a standard curve made up of 8 serial dilutions. The eight standards range from 0.023 to 50 ng/ μ l of DNA. It is recommended that these standards be run in duplicate with every plate or run. A standard curve is then generated via an ordinary least squares regression and a relationship between the cycle threshold value and DNA concentration is established. The resulting equation is used to determine the concentration of the unknown samples run on that plate. Further, the standard samples can act as a control for the assay; if the standard curve does not result in the expected slope or y-intercept, then this may indicate an issue with the sample set up, instrument, or run. It may however, also be a symptom of incorrect serial dilution generation.

While commonly employed and validated for forensic processing, using the linear parameters obtained from standards run on every plate may not be the optimal method to determine unknown DNA concentrations. For example, Grgicak *et al* (14) showed that 14 dilution sets which ranged in concentration from 50-0.023 ng/ μ l resulted in significant variation in the resultant slopes and Y-intercepts. This research also suggested that the variation in these standard curves was not dependent on stock DNAs, but rather the serial dilutions

themselves (14). It was suggested that error propagated over the course of the serial dilutions can have substantial impacts (15,16,17,18), and can lead to concentrations that vary by a factor of up to 3. As a result, it was recommended that a single standard curve be generated and used throughout a substantial period of time. That is, the authors argued that the instrument, kit lots, and pipettes were more stable over time than the dilution series. (14)

There are inherent benefits to using the method of a validated curve over the MRP. For one, it saves time and allows more samples to be run on each plate. Second, it was argued that it could also lead to less sample loss since intralaboratory reproducibility is expected to increase. (14) However, it should be noted that no stability study was conducted and no recommendations as to the length of time the validated curve should remain in use were given.

Directly utilizing the resultant PCR sample curve and fitting it to a kinetic-based PCR model has also been shown to be a viable option for determining concentration and has been presented as an alternative to the MRP. By using a standard curve, the MRP assumes the amplification efficiency of each sample is equal; but this may not necessarily be the case (19). In fact, each sample has its own amplification efficiency and the efficiencies are known to vary from cycle to cycle. The types of models used to determine the amplification efficiency and the concentration of samples can be divided into two categories: The first are models that determine the efficiency from the slope of the log-linear phase of amplification (20) and the second type are non-linear regression models. Boggy

et al (21) show that using a two-parameter mass action kinetic model of qPCR results in accurate quantification of samples without the need for a standard curve. This is based on a non-linear regression that examines the exponential phase of PCR and predicts the non-linear decline of amplification efficiency with every cycle. (21)

Amplification efficiency may also be modeled using a sigmoidal function (22-26). One type of sigmoidal model, called linear regression of efficiency (LRE) by Rutledge *et al.* (27-30) uses a kinetic-based sigmoidal model to determine the concentration of individual samples without the use of an external calibrator. This method takes the raw fluorescence data and estimates the amplification efficiency, which is then used to determine the copy number of the sample. In combination with the theory, Rutledge *et al.* also developed a software program (30) that performs the calculations and provides the user with the DNA copy number, which can then be converted into the concentration.

Given the recent advancements in qPCR analysis and the newly established methods to determine DNA concentrations, the goal of this research was to examine three different methods of generating concentrations from qPCR data for forensic purposes. These methods are the manufacturer's recommended protocol (MRP), which utilizes a standard curve run on every plate, the validated curve (VC) method, which utilizes a single validated standard curve over the course of multiple runs, and linear regression of efficiency (LRE) modeling, which uses a modeling program to determine the concentration. For this analysis, two

DNA samples run in quadruplicate over the course of 13 runs over 3 years, using four kit lots, and between three preventative maintenance calibrations were analyzed using all three quantification methods. The variations in concentrations over time, kit and calibration number were examined, and the three methods performance and reproducibility were compared.

Materials and Methods

Raw Data

The qPCR data (i.e. C_t values) were collected from two samples (Sample 1 and 2) which were run over 13 different assays, over a three-year period using the 7500 Sequence Detection System (Applied Biosystems, Foster City CA) and the Quantifiler® Duo quantification kit (Applied Biosystems, Foster City CA). Each sample was run in quadruplicate, resulting in a total of 52 runs per sample. During this period, the instrument was calibrated three times and four reagent kits lots were used. Table 1 shows the kit and calibration information for each run.

Table 1. Run Information for Samples

Run	Kit	Calibration
1	1	1
2	1	1
3	1	1
4	1	1
5	2	2
6	3	2
7	3	2
8	3	3
9	3	3
10	3	3
11	3	3
12	4	3
13	4	3

Since the Quantifiler® Duo kit tests for both total human and Y- DNA, both were analyzed during the course of this study. The concentrations for each assay and replicate were assessed using the three aforementioned methods; 1) MRP, 2) VC, and 3) LRE.

IPC Stability Study

The IPC cycle threshold from the standard curves, Sample 1 and Sample 2, were compared to each other to identify any variation in the IPC C_t's. The means and standard deviations of the C_t values were calculated for each set based on run date, calibration date, and the lot number of the kit. Plots exhibit C_t values for Samples 1 and 2 for each run, with error bars representing twice the standard deviation.

Manufacturer's Recommended Protocol (MRP)

The manufacturer's recommended protocol (5) (MRP) used a newly generated standard curve each time an assay was run. The standard curve was generated by comparing the cycle threshold to the logarithm of the concentration of a series of knowns varying in concentration from 50 to 0.023 ng/μl. All standards were made after an initial serial dilution from a 200 ng/μl stock solution. Each standard was run on the plate in duplicate. Ordinary least squares linear regression of the dilution series was performed to generate a standard curve that was ultimately used to determine the concentrations of the samples on the plate.

The data used for analysis were exported from the 7500 sequence detection software (SDS), and contained the following information: the concentration of total Human DNA of Sample 1 and 2, Y-DNA of Sample 1 and 2, and the IPC threshold values of all samples.

Validated Curve Protocol (VC)

The validated curve was generated following Grgicak *et al.*'s (14) recommendation of generating validated standard curves. Specifically, nine standard dilution series were generated using multiple pipettes, and Quantifiler Duo® kit lots. The dilution series were run on three separate plates. All of the C_t values were plotted against the log of the DNA concentration and an ordinary least squares linear regression resulted in one representative slope and Y-

intercept used as the parameters in the validated curve (VC). The same method was used for both total human and Y DNA.

The human DNA concentration was therefore calculated using the following validated curve (VC):

$$C_t = -3.311 \log[DNA] + 28.561 \quad (\text{Equation 1})$$

where C_t is the cycle threshold value and $[DNA]$ is the concentration of DNA.

Similarly, the Y DNA concentration was calculated using the following validated curve:

$$C_t = -3.395 \log [DNA] + 29.505 \quad (\text{Equation 2})$$

where C_t is the cycle threshold and $[DNA]$ is the initial DNA concentration of DNA in ng/ μ l.

These validated curves were used to calculate the DNA concentration for all 13 assays over the three year period.

Human Concentrations

The mean concentration and the standard deviation were calculated based on run date, calibration date, and lot number for MRP and VC. Scatter plots were also created. The information for each individual result was plotted along with the average for each run. Error bars represent two times the standard deviation. Dividing lines indicate the date of the last calibration and brackets show which kit lots were used. Scatter plots comparing calibration date to human concentration, and lot numbers to human concentration were also created.

Y Concentrations

Scatter plots of Y-DNA concentrations of each sample were created in a similar fashion as the Human DNA concentrations described above. The concentration for each sample calculated by MRP and VC was plotted along with the average. Error bars represent twice the standard deviation about the means. Lines demarcate the dates of calibration. Similarly plots, which compare calibration date to Y concentration, and lot number to Y concentration were also created.

MRP and VC Comparisons

A student's t-test was performed to compare the mean concentrations of each kit lot and calibration for MRP and VC. This was performed for both the total human and Y DNA concentrations of Sample 1 and Sample 2 using the *comparing means* function in AnalystSoft Inc., StatPlus:mac LE Version 2009.

An F-test, which compared the variances for each kit lot and calibration for MRP and VC, was performed with AnalystSoft Inc., StatPlus: mac LE Version 2009. This was completed for both the total human and Y DNA concentrations of Sample 1 and 2.

LRE Determination

Raw data (ΔR_n) for all runs were imported into the newest version of the LRE Analyzer (version 0.8.0) developed by Robert Rutledge (30). The copy number was calculated for each run in the software using the default settings. The Optical Calibration Factor (OCF) value was varied until the concentration was correct for as many of the expected values for the standard samples as possible. The Sample 1 data was then analyzed using the same calculated OCF value for each run. The E_{\max} value was changed to 100%. The concentration was calculated from the copy number using the assumption that a single cell contains approximately 7pg of DNA.

This same procedure was performed for Sample 2 for the RPPH1 and SRY loci.

LRE Comparisons to VC

To compare the LRE and VC data, the means and standard deviation were calculated based on run date, calibration date, and lot number. A scatter plot containing concentrations calculated by LRE and VC was created and compared run date to total human DNA concentration. The information for each sample was plotted along with the average for each run overlaying them. Error bars, representing two standard deviations, are also presented. Lines were placed on the graph demarcating the date of the last calibration. Brackets were added under the runs to indicate the kit lots used. Graphs were also generated

using the same method to compare both calibration date and lot number to human concentration.

The same procedure and scatter plots were made for each Sample for both the Human and Y concentrations.

A t-test was performed to compare the mean concentrations from each kit lot and calibration for LRE and VC. This was performed for both total human and Y DNA.

An F-test to compare the variances of concentration obtained for each kit lot and calibration for LRE and VC was performed. This analysis was repeated for all samples and loci.

Radar Plots

The overall average concentration of the human DNA for Sample 1 calculated by VC was used as the reference point and all subsequent concentrations were compared to this by examining the sum of squares over the course of 13 runs. The sum of squares was then calculated for each calibration. These results were plotted along with the results of the overall sum of squares on a radar graph.

Another radar graph compared the MRP, VC, and LRE data obtained after the last calibration. The overall average concentration calculated by MRP was subtracted from the averages of each run during the third calibration cycle. The

differences were squared and the results were plotted on a radar plot. This was repeated for both VC and LRE methods.

The final radar plot was generated using the relative standard deviation (RSD) calculated (for the third calibration only) using the following equation:

$$\text{RSD} = \left(\frac{\sigma}{\bar{x}}\right) \times 100\% \text{ (Equation 3)}$$

where σ , is standard deviation, and \bar{x} is average DNA concentration after the third calibration. The RSDs were calculated for MRP, VC and LRE, and plotted on the same radar plot for comparison.

Male to Female Ratios

The concentrations calculated by MRP for both total human and Y DNA were used to calculate the amount of female DNA by subtracting the amount of Y DNA from the total human DNA. Once the concentration of female DNA was determined, the average for each run was calculated for both male and female DNA. The male and female DNA concentrations were plotted on a bar graph, and sorted by run number. The error bars, representing twice the standard deviation, is also presented.

The male to female ratio was calculated by taking the Y-DNA concentration and dividing it by the female DNA concentration. The male to female ratios were plotted on a bar graph, sorted by run number, and an error

bar representing twice the standard deviation is presented. These same calculations and graphs are presented for both the VC and LRE methods.

Results and Discussion

IPC Data

An internal PCR control (IPC) is run with every sample and is expected to cross the fluorescence threshold at the same cycle each time. The IPC is an artificial template designed to be different from the target DNA, thus eliminating interaction between the control DNA and the probes designed for the total human and Y-DNA analysis (33). The fluorescence of the IPC is read using a unique reporter, thus allowing simultaneous analysis of the SRY, RPPH1, and IPC amplifications.

Since the samples used in this study originated from whole-frozen liquid sources, the IPC data for the 13 runs can also be used to assess long term stability of qPCR. Further IPC values for Sample 1, Sample 2 and the standard can be combined since the C_t values are expected to be the same, regardless of DNA source.

Figure 1 shows the C_t value of the IPC for the 13 runs. The C_t values for Calibration 1 remain constant ranging from 29.31 to 29.43. When runs 4 and 5 are compared, the C_t 's change from an average of 29.31 to 29.85, indicating a shift in average C_t 's of the IPC's when calibration occurred. However, it should be noted that, the kit was also changed at this time. More telling is the change in

IPC values between runs 7 and 8. Here both runs were completed using kit 3 and the average IPC's remain roughly the same. However, the variance within the run in run 8 is much larger than the variance in run 7 ($P = 0.4$), again suggesting the calibration, or corrective action taken during this calibration, significantly impacted the IPC signal across the plate.

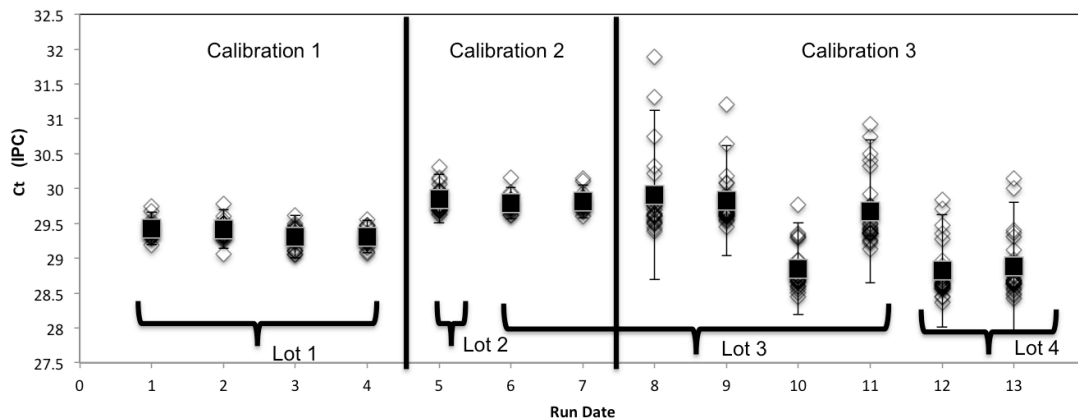


Figure 1. IPC C_t values over the course of 3 years with error bars representing 2SD of the mean. The black vertical lines after run 4 and run 7 indicate the point at which calibration took place.

In order to examine the confounding effect, if any, that calibration and kit lot has on the C_t 's of the IPC, the data was sorted by calibration (Figure 2). The results indicate that a significant amount of variation occurred, not necessarily because of the kit, but because of the calibration. For example, Kit 3 was used after Calibration 2 and Calibration 3, and there is a larger variation in the data from Calibration 3 than Calibration 2. If the change in the C_t was solely due to changes in kit, then it is expected that the same average C_t would be observed in both Calibration 2 and 3. The average C_t 's observed were not the same, however a t-test showed that the difference was not significant ($P = 0.07$). The variances

of the C_t 's are significantly affected by instrument calibration as indicated by the significant changes in variance of the IPC's C_t 's within Lot 3 and between Calibration 2 and 3. Although instrument calibration has a significant impact on the variance of the IPC, changes in kit lot may also impact these results as indicated by the drop in average C_t value from 29.60 to 29.05 for kit 3 and kit 4 within Calibration 3. However, the change in average C_t for the IPC between kits cannot explicitly be taken as evidence of run variation between kits, as drop in the IPC C_t 's between kit 3 and 4 cannot be isolated from the expected small changes in IPC concentrations expected between lots.

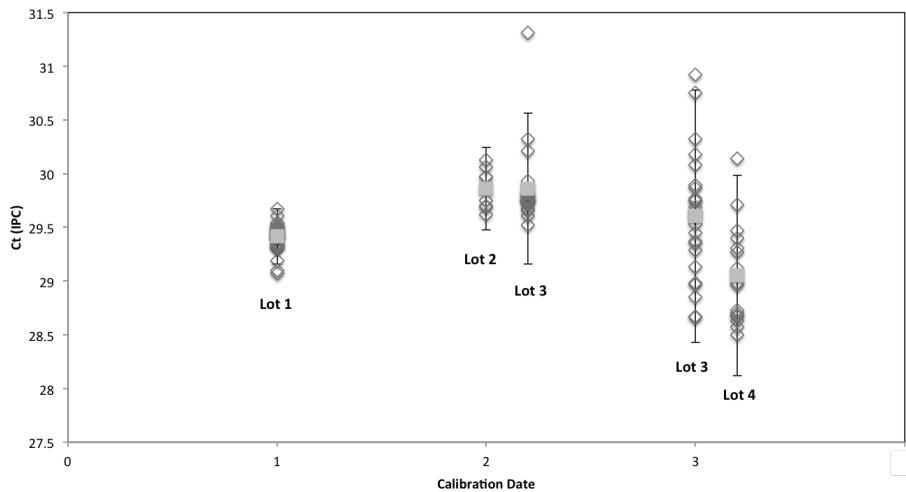


Figure 2. C_t of IPC for each kit, sorted by calibration #, with error bars representing 2SD of the mean.

Manufacturer's Recommended Protocol (MRP) vs. Validated Curve

According to the Applied Biosystems® Quantifiler Duo® manual (5), quantification of DNA should be based on the generation of a standard curve

calculated using a dilution series, run in duplicate on every assay/plate. This is the manufacturer's recommended protocol or MRP. The data generated from the standards run on the plate are used to generate a linear equation, and the concentration of the unknown samples are determined using that equation. According to the MRP, the dilution series should be regenerated on a bi-weekly basis, thereby requiring new DNA standards be created and used a minimum of 24 times per year.

Previous studies have shown that it is difficult to generate dilution series such that the linear parameters remain constant over time (14). Further, it was shown that small changes in y-intercept between curves can have a large impact on the DNA concentration obtained for an unknown. Therefore, to further test the stability (or lack thereof) of the MRP, two samples were run over a three-year period using four different kit lots and three different instrument calibrations.

In contrast to the MRP, a single validated curve may be utilized to determine DNA quantity. A validated curve (VC) is an equation for a standard curve that is used over a specified amount of time. Validated curves typically have a set number of runs for which they are valid, and that number is determined by the lab that is generating and using the curves. The VC is used instead of the MRP standards that are run with every assay, and is used the same way the MRP equation is applied. Here, the C_t value of each sample is substituted into the equation to solve for the initial DNA concentration.

$$y = m * \log(x) + b \text{ (Equation 4)}$$

where, y is the C_t value, m is the slope, x is the initial DNA concentration, and b is the y-intercept. In order to determine the concentration, Equation 4 is rearranged to,

$$x = 10^{\left(\frac{y-b}{m}\right)} \text{ (Equation 5)}$$

The slope and y-intercept obtained during validation are substituted into Equation 5 and used throughout the duration of the validated curve's lifetime so the only information needed from a single assay is the C_t values of the samples containing unknown DNA concentrations. As a result of using a validated curve laboratories would be able to run 16 additional samples per assay/plate (14).

The method for generating validated curves, introduced in Grgicak *et al.* (14), was used in this study. This method attempts to introduce as much variation as possible during the creation of the standards which are used to generate the VC parameters. Therefore, nine dilution series were made using multiple pipettes and kit lots. To introduce further variation, the nine standard curves were subdivided into three groups, and run on three separate plates. The parameters for both human and Y- DNA were determined in this manner.

For this experiment, the validated curve used to determine total human DNA concentration was:

$$x = 10^{\left(\frac{y-28.561}{-3.311}\right)} \text{ (Equation 6)}$$

The validated curve used to determine Y DNA concentration was,

$$x = 10^{\left(\frac{y-29.505}{-3.395}\right)} \quad (\text{Equation 7})$$

Figures 3 and 4 show a comparison of the total human DNA concentration for Samples 1 and 2 calculated by MRP and VC over the course of 13 runs.

Figure 3 shows that there is variation between runs when the total human DNA concentrations were calculated using MRP and VC. The overall mean of total human DNA concentration for Sample 1 when calculated by MRP was 4.33 ± 1.07 ng/ μ l, while the overall mean when calculated by VC was 3.91 ± 0.08 ng/ μ l. A t-test was performed in order to compare the means of each method, and showed that there was a significant difference between the means ($P = 0.025$). The largest within run variance of Sample 1 for MRP was seen in run 5 at 1.28. The largest within run variance for VC was observed in run 13 at 0.82. In contrast, the overall variance (i.e. variance obtained from all runs) for MRP was 1.15, while the overall variance for VC was 0.65. For the MRP method, the only run that had a within run variance higher than the overall variance was run 5. For the VC method, runs 9 and 13 had within run variances higher than the overall variance. The majority of runs for each method had within run variations smaller than the overall run variation suggesting reproducibility between runs is lower than the reproducibility within a run, which theoretically may be improved. Further, an F-test was performed to compare the overall variance of the two methods and it was found that there is a significant difference in the variance

between VC and MRP ($P = 0.044$). The variance is lower when calculated by VC, showing that VC is more reproducible than MRP.

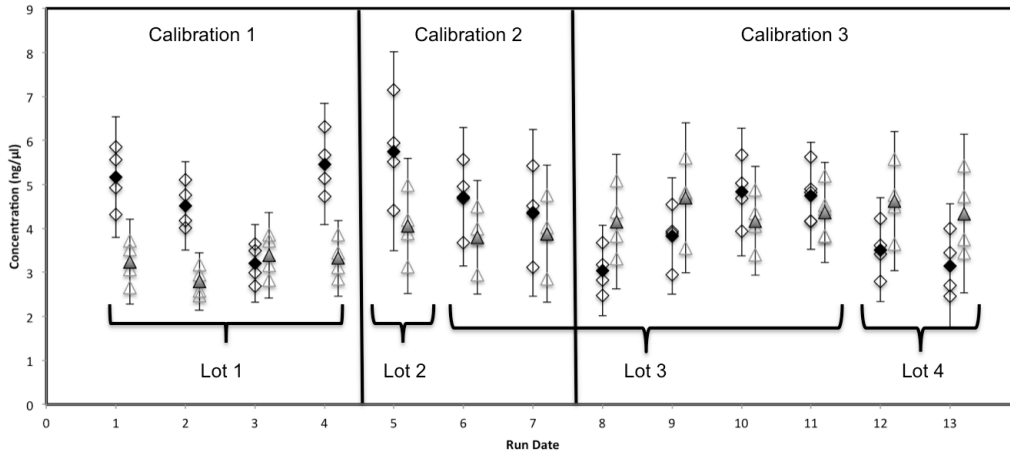


Figure 3. Total human DNA concentration for Sample 1 calculated using MRP (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Figure 4 shows the concentrations of DNA for Sample 2 and shows the same trend observed in Sample 1. The overall mean of the total human DNA when calculated by MRP was 1.64 ± 0.40 ng/ μ l. When calculated by VC, the mean concentration was 1.44 ± 0.21 ng/ μ l. A t-test to compare the means of the two methods was performed and showed there was a significant difference between the means ($P = .002$). The concentrations calculated using VC had an overall variance of 0.04, which is smaller than the variance of the concentrations of Sample 2 when calculated by MRP (0.16). The largest within run variance of Sample 2 for MRP was seen in run 3 at 0.19. The largest within run variance of

Sample 2 for VC was also observed in run 3 at 0.22. For MRP, the only run that had a within run variance higher than the overall variance was run 3. For VC runs, 3 and 13 had a within run variance higher than the overall variance. As with Sample 1, the majority of within run variances are lower than the overall variance for both methods, suggesting the reproducibility between runs is lower than the reproducibility within a run. An F-test comparing the overall variances obtained from VC and MRP was performed and showed there was a significant difference in the calculated variances ($P = 0.00001$).

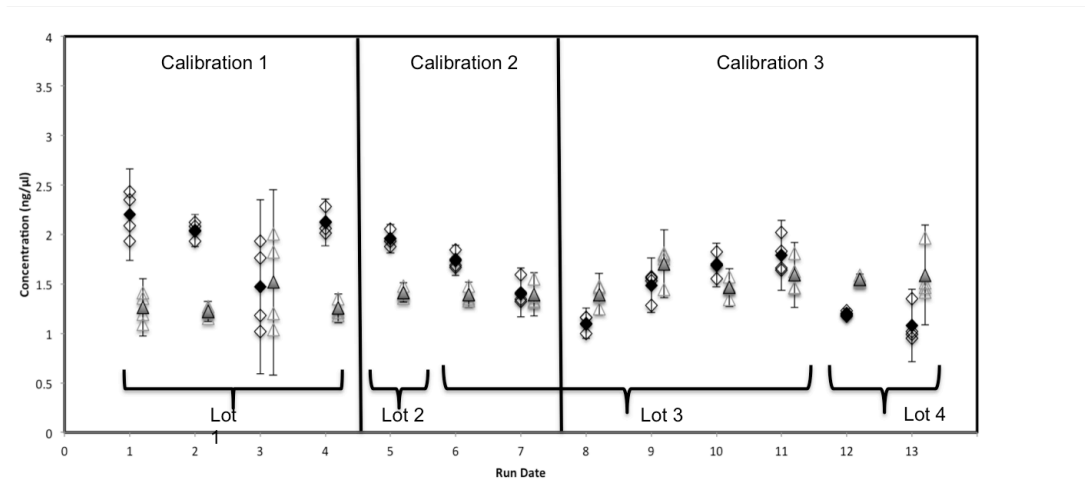


Figure 4. Total human DNA concentration for Sample 2 calculated using MRP (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

There were significant differences seen over time in variance between the MRP and VC for both Sample 1 and Sample 2. In both cases VC had the lower

variance. This suggests that for Sample 1 and Sample 2 calculating total human DNA is more reproducible with the VC method than with the MRP.

Similarly, Figures 5 and 6 show a comparison of the Y DNA concentrations for Samples 1 and 2, calculated by both MRP and VC over the course of 13 runs.

Figure 5 shows that, like with total human DNA concentrations, Y DNA concentrations also vary over time when calculated using the MRP and VC methods. The overall mean of Y DNA concentration when calculated by MRP was 0.31 ± 0.09 ng/ μ l. The overall mean when calculated by VC was 0.29 ± 0.07 ng/ μ l. A t-test was performed in order to compare the means of each method. The t-test showed that there was no significant difference between means of the two methods ($P = 0.28$). The largest within run variance of Sample 1 for MRP was seen in run 12 at 2.75×10^{-3} . The largest within run variance for VC was seen in run 12 at 5.5×10^{-3} . In contrast, the overall variance of all runs for MRP was 8.4×10^{-3} , while the overall variance for VC was 5.0×10^{-3} . For MRP all of the within run variances were less than the overall variance, while for VC the only within run variance greater than the overall variance was seen in run 12. An F-test was performed to compare the overall variances of the two methods and it was found that there was no significant difference between them ($P = 0.065$).

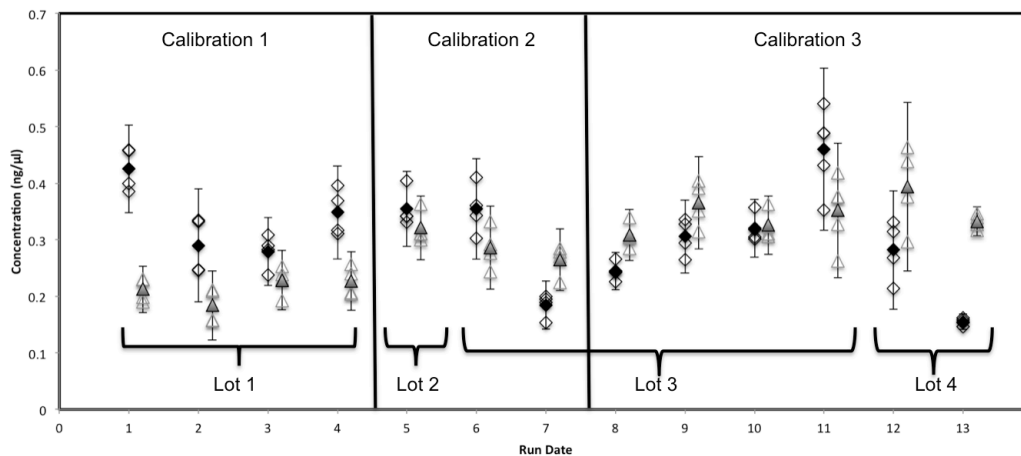


Figure 5. Y DNA concentration for Sample 1 calculated using MRP (◆) and VC(▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Figure 6 shows the same trend of variance observed with Sample 1, Sample 2 and Sample 1Y. This plot indicates that over time the concentration of Y DNA in Sample 2 does not remain the same. The overall mean concentration of Y DNA when calculated by MRP was 1.71 ± 0.39 ng/μl. The overall mean when calculated by VC was 1.63 ± 0.34 ng/μl. A t-test was performed in order to compare the means of each method, and showed there is no significant difference between the means ($P = 0.26$). The largest within run variance of Sample 2 for MRP was observed in run 3 at 3.6×10^{-1} . The largest within run variance for VC was also observed in run 3 at 3.4×10^{-1} . The overall variance for MRP was 0.15, while the overall variance of VC was 0.11. The within run variance for each method was smaller than the overall variance with the exception of run 3, suggesting that reproducibility between runs is worse than it is

within the same run. An F-test to compare the overall variances was also performed for this data and suggested that there was no significant difference in variance between the two methods ($P = 0.283$).

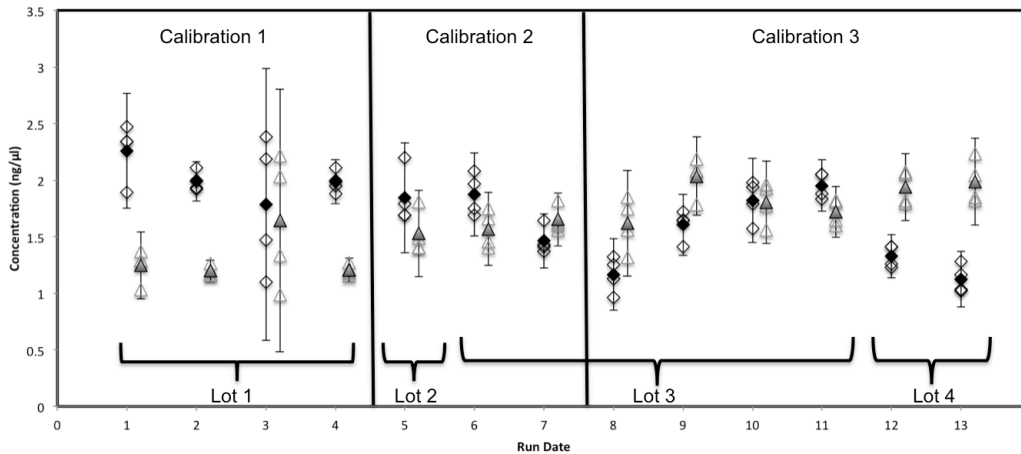


Figure 6. Y DNA concentration for Sample 2 calculated using MRP (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Even though there was only a significant difference in variance for Sample 1Y, in both cases the variance was lower when using the VC method to calculate Y DNA. This corroborated the findings obtained when the total Human DNA concentrations were determined and suggests that using the VC method is more reproducible than using the MRP method over extended periods of time.

The variation exhibited between runs can have a significant impact on downstream analysis. Since the concentration generated from qPCR is used to

determine the volume of DNA extract used during STR amplification, it is important to assure the quantity obtained between runs is reproducible.

Variables that could lead to this variation are the differences in DNA standard concentrations, component concentrations and/or efficiency of the polymerase between kit lots. To test this, four different kit lots were used during the course of this experiment. Kit lot 1 was used during runs 1-4, kit lot 2 was used to prepare run 5, kit lot 3 was used to prepare runs 6-11, and kit lot 4 was used to prepare the last two runs.

Figures 7 and 8 show the total human DNA concentrations for Samples 1 and 2 based on the kit lot. The 2SD spread of Sample 1, when calculated by MRP, for kits 1 through 4 was 1.41, 2.26, 1.81, and 1.26. The resultant Sample 1 DNA concentrations when calculated via the VC resulted in 2SD spreads of 0.90, 1.54, 1.39 and 1.60. T-tests were performed in order to compare the means of kit lot concentrations. For MRP, P values < 0.05 were obtained when comparing kit lots 1 and 4, 2 and 4, and kit lots 3 and 4. For VC, P values < 0.05 were obtained between kits 1 and 3, and kits 1 and 4. It is difficult to determine if kit lot is the origin of these differences since the differences could have also been the result of modifications to the instrument system during calibration. If the runs using kit lot 3 during calibration 2 for MRP are removed and a t-test performed to compare the means of the remaining kit lot 3 and kit lot 4 samples, then there is still a significant difference in mean concentration between kit lots 3 and 4. This suggests that kit lot may also have an impact on the quantitation of DNA when

using MRP. When comparing the variances between kit lots of Sample 1 via F-tests for MRP, there was no significant difference in variance. When comparing the variances between kit lots of Sample 1 via F-tests for VC, P values <0.05 were obtained between kits 1 and 3, and kits 1 and 4.

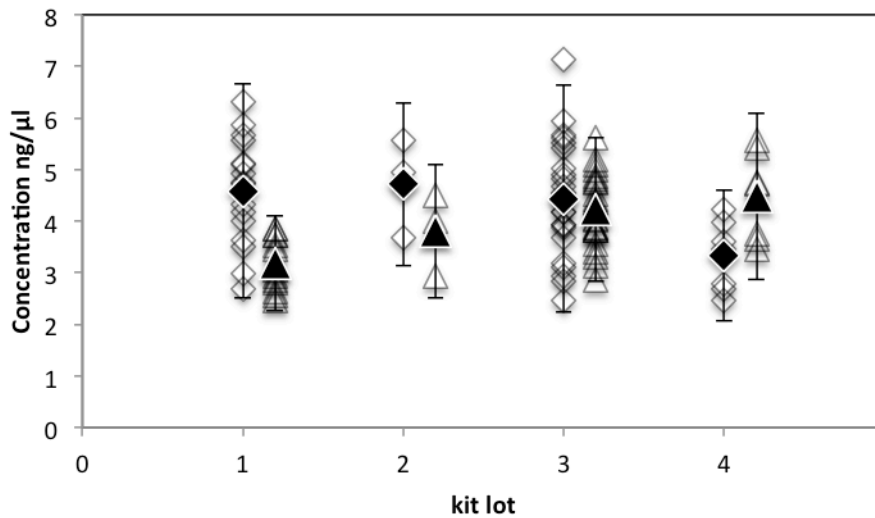


Figure 7. Total human DNA concentration for Sample 1 calculated by MRP (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

The 2SD spread of Sample 2 for kits 1 through 4 was 0.32, 0.14, 0.53, and 0.27, and of 0.18, 0.10, 0.33, and 0.34 for the MRP and VC methods respectively. When a t-test was performed on Sample 2 for MRP, P values < 0.05 were obtained when comparing all kit lots. T-tests were performed on the VC values for Sample 2 and P values < 0.05 were obtained between kits 1 and 4, 2 and 3, and kits 2 and 4. When comparing the variances between kit lots of Sample 2 via F-tests for MRP, P values <0.05 were obtained between kits 1 and

2, 1 and 4, 2 and 3 and kits 3 and 4. When comparing the variances between kit lots of Sample 2 via F-tests for VC, P values < 0.05 were obtained between kits 1 and 2, and kits 1 and 3. For Sample 2, it appears that VC yields more reproducible results because the means and variances are more similar.

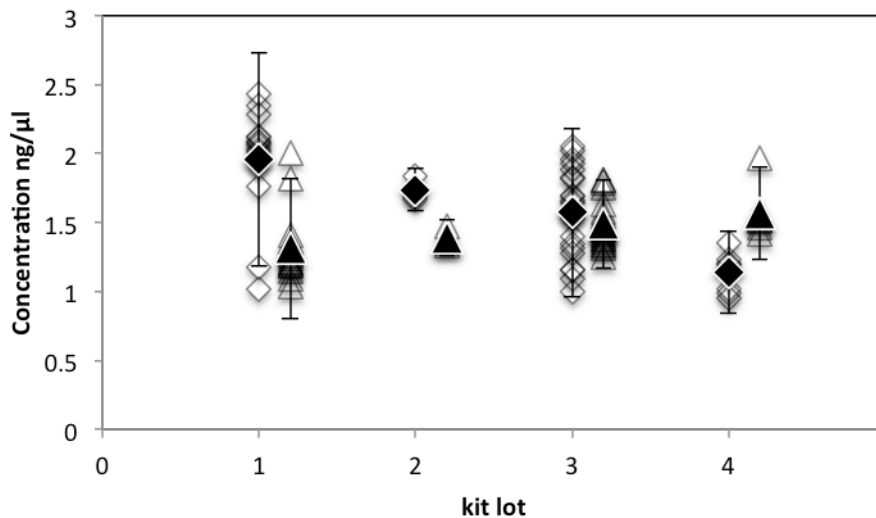


Figure 8. Total human DNA concentration for Sample 2 calculated by MRP (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

Figures 9 and 10 show the Y DNA concentrations for Samples 1 and 2 based on the kit lot used. The 2SD spread of Sample 1 when calculated by MRP for kits 1 through 4 was 0.14, 0.006, .197 and .152, while Sample 1, when calculated by VC, had 2SD spreads of 0.06, 0.057, 0.10, and 0.119. T-tests comparing the means of each kit lot for Sample 1 Y when calculated using MRP resulted in P values < 0.05 between kits 1 and 4, 2 and 4, and kits 3 and 4. T-tests comparing the means of each kit lot for Sample 1 Y when calculated using

VC resulted in P values < 0.05 between all kits except for between kits 2 and 3. When comparing the variances between kit lots of Sample 1Y via F-tests for MRP there was no significant difference seen. When comparing the variances between kit lots of Sample 1Y via F-tests for VC, P values <0.05 were obtained between kits 1 and 3, and kits 1 and 4.

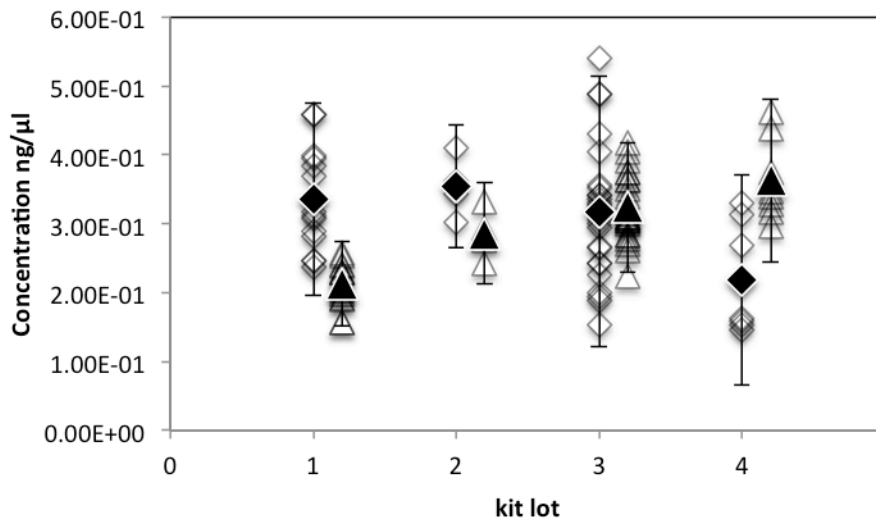


Figure 9. Y DNA concentration for Sample 1 calculated by MRP (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

The 2SD spread of Sample 2 for kits 1 through 4 was 0.40, 0.49, 0.62, and 0.30, and 0.18, 0.38, 0.43, and 0.32 for the MRP and VC methods respectively. T-tests comparing the means of each kit lot for Sample 2 Y, when calculated using MRP, resulted in P values <0.05 between kits 1 and 3, 1 and 4, 2 and 4, and kits 3 and 4. T-tests comparing the means of each kit lot for Sample 2 Y, when calculated using VC, resulted in P values <0.05 between kits 1 and 3, 1 and 4, 2 and 4, and kits 3 and 4. When comparing the variances between kit lots

of Sample 2 via F-tests for MRP, P values <0.05 were obtained between kits 1 and 4, and kits 3 and 4. When comparing the variances between kit lots of Sample 2 via F-tests for VC, P values <0.05 were obtained between kits 1 and 3, and kits 1 and 4.

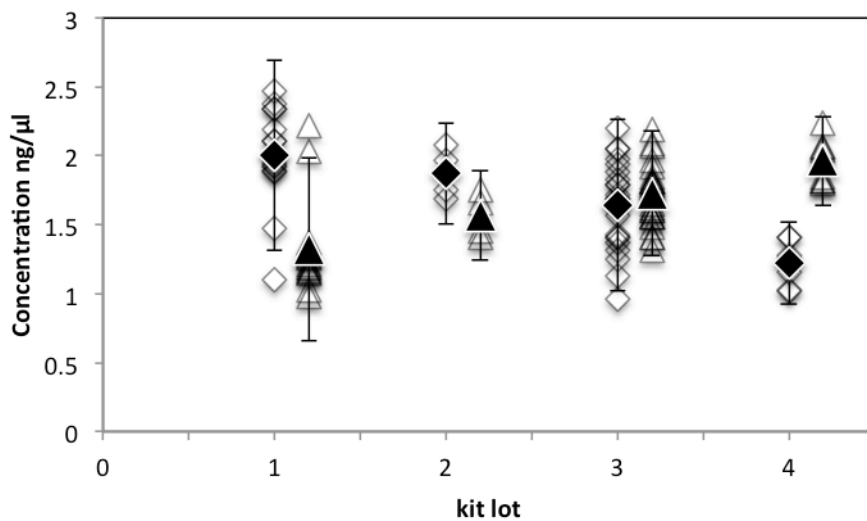


Figure 10. YDNA concentration for Sample 2 calculated by MRP (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

When calculating total human and Y DNA, variance is present between runs. This analysis shows that some of the variance is due to the kit lots. However the significant variance present between a majority of the kit may also be the result of normal instrument modification. As a result, the effects of calibrations were also examined.

One confounding parameter, not considered in the previous analysis, was the effect of instrument calibration of the DNA quantification results. During the

yearly calibration that takes place during preventative maintenance, a number of tests and modifications are made to the 7500 sequence detection system. Some major modifications include block lamp and detector replacement; while moderate adjustment includes, but is not limited to, filter wheel calibration, the heated cover and/or ROI alignment and/or CCD alignment. Each of these are expected to impact the fluorescent signal obtained after each PCR cycle. If the source/detection path has changed significantly between calibrations, a change in calculated concentration may be the result. By using the MRP, the signal of the standards should be affected in the same way as the signal of the samples, thereby rendering differences between calibrations minimal.

Figure 11 shows the total human DNA concentration calculated by MRP and VC for Sample 1, sorted by calibration. The mean total human DNA concentration when calculated by MRP was 4.59 ng/ μ l, 4.94 ng/ μ l, and 3.88 ng/ μ l. The mean total human DNA concentration when calculated by VC was 3.18 ng/ μ l, 3.91 ng/ μ l, and 4.39 ng/ μ l. T-tests were performed to compare the means of each calibration for MRP and VC to determine if the differences between them are significant. For MRP, P values <0.05 were obtained when comparing calibrations 1 and 3, and calibrations 2 and 3. For VC, P values <0.05 were obtained when comparing calibrations 1 and 2, and calibrations 1 and 3. When comparing the variances between calibrations of Sample 1 via F-tests for MRP, no significant difference was observed. When comparing the variances

between calibrations of Sample 1 via F-tests for VC, P values <0.05 were obtained between calibrations 1 and 3.

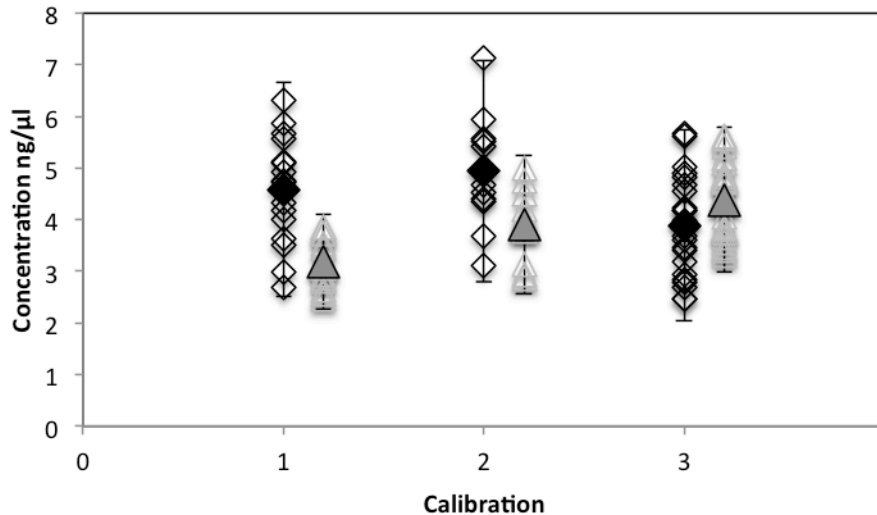


Figure 11. Total human DNA concentration for Sample 1 calculated by MRP (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 12 shows the total human DNA calculated by MRP and VC for Sample 2 sorted by calibration. The mean concentration for each calibration when calculated by MRP was 1.96 ng/μl, 1.70 ng/μl, and 1.39 ng/μl. The mean concentration when calculated by VC was 1.31 ng/μl, 1.40 ng/μl, and 1.54 ng/μl. T-tests performed to compare the means of each calibration for MRP and VC to determine if there were significant differences between the two were performed. For MRP, P values <0.05 were obtained when comparing all calibrations. For VC, P values <0.05 were obtained when comparing calibrations 1 and 3, and calibrations 2 and 3. When comparing the variances between calibrations of

Sample 2 via F-tests for MRP, no significant differences were found. When comparing the variance between calibrations of Sample 2 via F-tests for VC, P values <0.05 were obtained for calibrations 1 and 3, and calibrations 2 and 3.

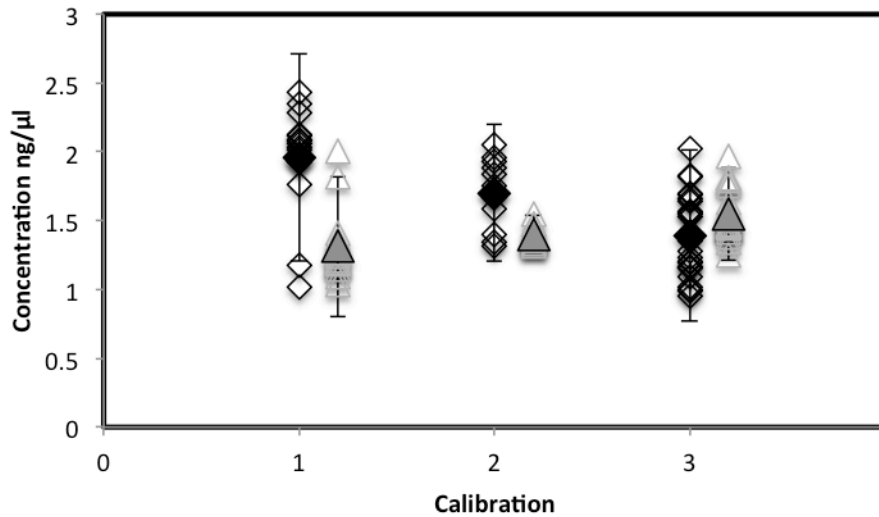


Figure 12. Total human DNA concentration for Sample 1 calculated by MRP (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 13 shows the Y DNA concentration calculated by MRP and VC for Sample 1 sorted by calibration. The mean Y DNA concentration when calculated by MRP was 0.336 ng/μl, 0.298 ng/μl, and 0.301 ng/μl. The mean Y DNA concentration when calculated by VC was 0.213 ng/μl, 0.290 ng/μl, 0.346 ng/μl. T-tests were performed to compare the means of each calibration for MRP and VC to determine if the differences are significant. The t-tests determined that there were no significant differences between calibrations for MRP. For VC, P values <0.05 were obtained when comparing calibrations 1 and 2, 1 and 3, and

calibrations 2 and 3. When comparing the variances between calibrations of Sample 1 via F-tests for MRP, no significant differences were found. When comparing the variances between calibrations of Sample 1 via F-tests for VC, P values <0.05 were obtained when comparing calibrations 1 and 3.

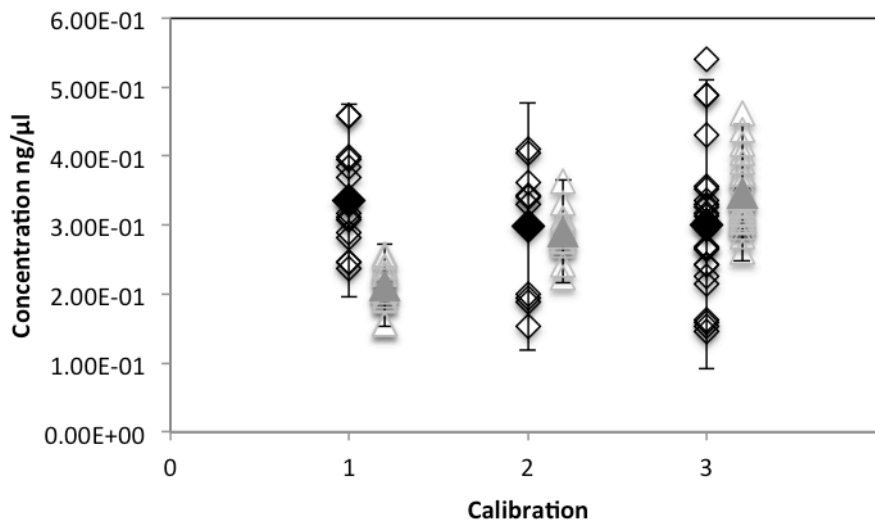


Figure 13. Y DNA concentration for Sample 1 calculated by MRP (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 14 shows the Y DNA concentration calculated by MRP and VC for Sample 2 sorted by calibration. The mean Y DNA concentration when calculated by MRP was 2.01ng/μl, 1.73ng/μl, and 1.50ng/μl. The mean Y DNA concentration when calculated by VC was 1.32ng/μl, 1.58ng/μl, and 1.85ng/μl. T-tests were performed to compare the means of each calibration for MRP and VC to determine if there was any significant difference. For MRP, P values <0.05 were obtained when comparing calibrations 1 and 2, 1 and 3, and calibrations 2 and 3.

For VC, P values <0.05 were obtained when comparing calibrations 1 and 2, 1 and 3, and calibrations 2 and 3. When comparing the variances between calibrations of Sample 2 via F-tests for MRP no significant differences were found. When comparing the variances between calibrations of Sample 2 via F-tests for VC, P values <0.05 were obtained between calibrations 1 and 2, and calibrations 1 and 3.

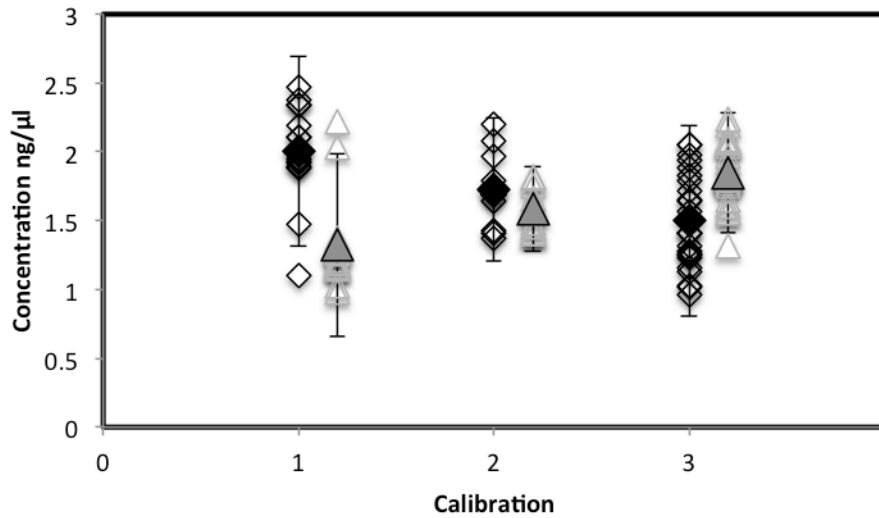


Figure 14. Total human DNA concentration for Sample 2 calculated by MRP (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Validated Curve Radar Plot for Calibrations Comparison

Figure 15 is a radar plot, which is used to display multivariate data in the form of a two-dimensional chart of three or more quantitative variables. In Figure 15, each number on the outside of the graph represents a single assay, and the y-values are the difference of squares between the mean concentration for a

particular run date and the mean concentration over all runs. Specifically, the plot exhibits the value calculated using:

$$y = (\bar{x}_n - \bar{x}_{all})^2 \text{ (Equation 8)}$$

where \bar{x}_n is mean concentration for run n, and \bar{x}_{all} is the mean concentration of all 13 runs.

Also displayed are the values calculated using:

$$y = (\bar{x}_n - \bar{x}_{cal_n})^2 \text{ (Equation 9)}$$

where \bar{x}_n is mean concentration for run n and \bar{x}_{cal_n} is the mean concentration of all of the runs that took place during calibration n.

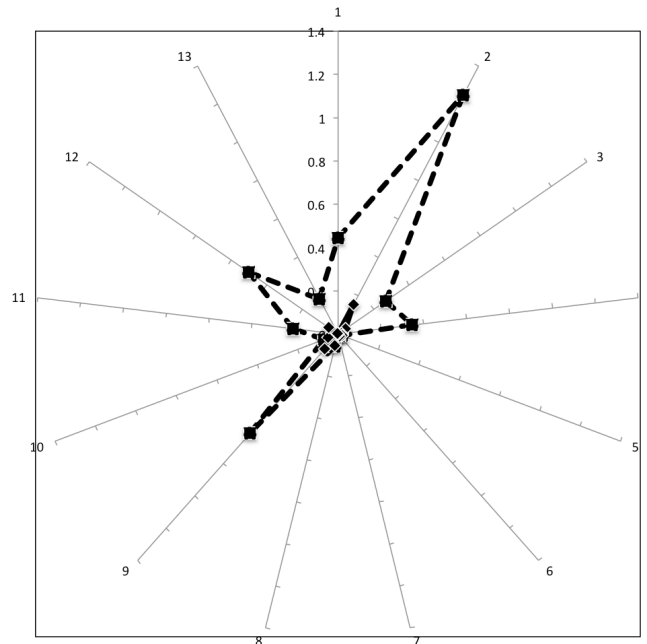


Figure 15. Difference of Means Squared comparing overall average concentration for all calibrations (■) to individual calibrations (◆)

The graph shows that there is a smaller sum of squares for the diamonds and solid line than the squares and dotted line, signifying that there is less concentration variation within a calibration than there is over multiple calibrations. The radar analysis suggests that if a new validated curve is generated each time the instrument is calibrated, a higher level of reproducibility between assays is expected. Figure 15 also demonstrates that if the same validated curve is used over the course of multiple calibrations, a larger difference in the spread of the concentrations is the expected result.

Linear Regression Efficiency (LRE) vs. Validated Curve (VC)

Linear Regression Efficiency (LRE) is a modeling method developed by Robert Rutledge (27-30) to determine sample concentration using qPCR and utilizes the ΔR_n information (i.e. fluorescence signal). This method allows for the calculation of sample concentration without the need to compare results to DNA standards. It has the potential to generate more accurate and reproducible concentrations because, unlike standard curve based methodologies, LRE modeling does not assume that the amplification efficiency of each sample is equivalent (35).

LRE is a kinetic-based sigmoidal model that determines a sample's concentration. The LRE Analyzer Software was developed (29-31) such that the fluorescent signal can be imported directly from the qPCR analysis system.

This software uses the fluorescence readings produced by single PCR reactions, and generates a linear representation of amplification from those values. This is accomplished by plotting cycle efficiency (E_c) versus reaction fluorescence (F_c). This graph is known as the LRE plot, and the correlating equation for the linear curve is

$$E_c = \Delta E \times F_c + E_{\max} \quad (\text{Equation 10})$$

where E_c is the amplification efficiency at cycle C (cycle efficiency), F_c is the fluorescence reading at cycle C, E_{\max} is the maximal amplification efficiency, ΔE is the rate of loss of cycle efficiency.

The amplification efficiency is empirically determined by evaluating the change in fluorescence with each PCR cycle as per the following equation

$$E_c = \frac{F_c}{F_{c-1}} - 1 \quad (\text{Equation 11})$$

where E_c is cycle efficiency, F_c is the fluorescence at cycle C and F_{c-1} is the fluorescence from cycle C-1. The linear equation is only used for the points that fall within the linear range, and are considered to be within the “LRE window” (27-31). The following equation,

$$F_0 = \frac{F_{\max}}{1 + \left(\frac{F_{\max}}{F_c} - 1\right)(E_{\max} + 1)^c} \quad (\text{Equation 12})$$

is used to convert the fluorescence signal present in the LRE window into the target quantity in fluorescence units. Where F_0 is target quantity expressed in fluorescence units, F_C is the fluorescence at cycle C, E_{\max} is the maximal amplification efficiency (determined by Equation 10), and F_{\max} is the maximum fluorescence signal.

The F_0 value is then converted into absolute copy number using the optical calibration factor or OCF. Typically the OCF is determined by amplifying a known quantity of standard and dividing the resulting F_0 value by the predicted quantity.

When utilizing the LRE method and corresponding software tool to analyze the DNA quantities of the samples for this work it was observed that the concentrations determined via LRE analysis were significantly different from the expected concentrations. As LRE was originally created for SYBR® Green assays (29), modifications to the software and corresponding OCF were required for data analysis of Taqman®-based assays.

The first modification included a change of E_{\max} (maximum amplification efficiency) to 100% as opposed to the E_{\max} calculated in Equation 10. Since Taqman® probes exhibit a decrease in fluorescence intensity over time, a lower F_{\max} value is detected. As a result, the E_{\max} determined via Equation 11 may be underestimated. If E_{\max} is underestimated then, as per Equation 12, the F_0 and corresponding concentrations of the samples would be overestimated.

Figures 16 and 17 show a comparison of the total human DNA concentration for Samples 1 and 2 calculated by both LRE and VC over the course of 13 runs. The solid lines in between runs four and five and runs seven and eight represent points at which the calibrations took place. The brackets underneath the data points represent different kit lots used.

As seen previously with the MRP and VC figures, Figure 16 shows that there is both within run and between run variation for LRE and VC. The overall mean of total human DNA concentration when calculated by LRE was 4.93 ± 1.21 ng/ μ l compared to the 3.91 ± 0.81 ng/ μ l concentration calculated by VC. A t-test was performed in order to compare the means of LRE and VC. The t-test showed there was a significant difference between the means of LRE and VC ($P = 6.12 \times 10^{-6}$). The largest within run variance of Sample 1 for LRE was seen in run 8 at 3.49. The overall variance of all runs for LRE was 1.46. Also the within run variance for the LRE method was larger and is particularly noticeable in runs 3 and 8.

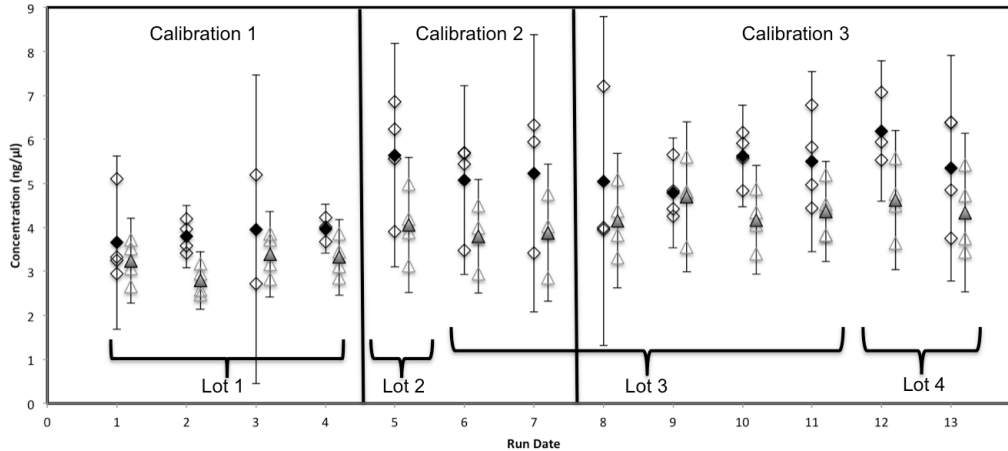


Figure 16. Total human DNA concentration for Sample 1 calculated using LRE (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Figure 17 shows the quantification results for Sample 2. The overall mean of total human DNA concentration when calculated via LRE was 2.15 ± 0.38 ng/ μ l compared to the 1.44 ± 0.21 ng/ μ l concentration calculated by VC. A t-test was performed in order to compare the means of LRE and VC. It showed that there was a significant difference between the means of LRE and VC ($P = 0$). The largest within run variance of Sample 2 for LRE was seen in run 3 at 0.551. The overall variance of all runs for LRE was 0.142. Unlike with Sample 1, for Sample 2 all of the within run variances are smaller than the overall variance. An F-test to compare the variance of LRE and VC was performed and showed that there was a significant difference in the variance ($P = 3.8 \times 10^{-5}$). The variance is lower for Sample 2 when calculated by VC, showing that VC is again more reproducible than the LRE quantification method.

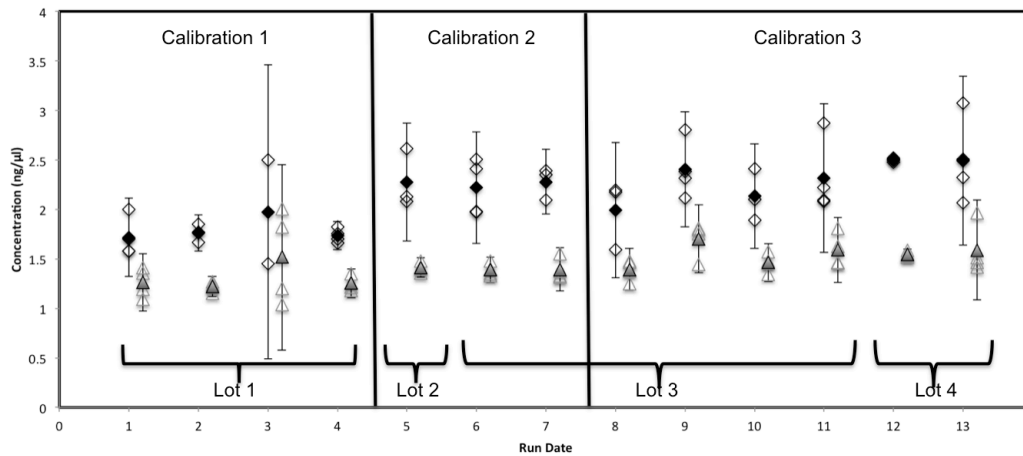


Figure 17. Total human DNA concentration for Sample 2 calculated using LRE (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Figure 18 shows the concentration of Y DNA in Sample 1 calculated by LRE and VC. The overall mean of Y DNA concentration when calculated by LRE was 0.16 ± 0.04 ng/ μ l, compared to the 0.29 ± 0.07 ng/ μ l concentration calculated by VC. A t-test was performed to compare the means of LRE and VC. The test showed that there was a significant difference between the means of LRE and VC ($P = 0$). The largest within run variance of Sample 1 for LRE was seen in run 12 at 1.5×10^{-3} . The overall variance of all runs for LRE was 1.5×10^{-3} , compared to the overall variance of all runs for VC, which was 5.0×10^{-3} . For Sample 1Y all of the within run variances are equal to or lower than the overall variance. An F-test was performed to compare the variance of LRE and VC and it was found that there is a significant difference in the variance ($P = 2.9 \times 10^{-5}$). This is the first case where VC did not have the lowest variance.

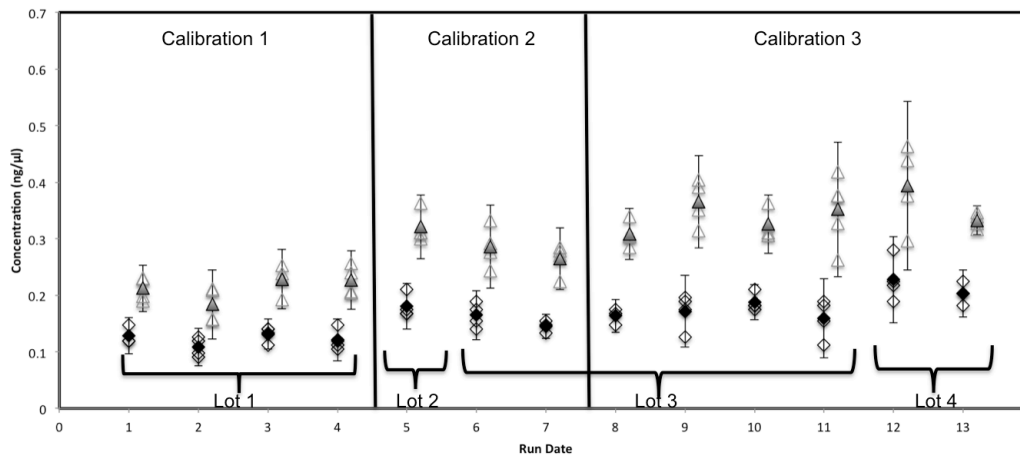


Figure 18. Y DNA concentration for Sample 1 calculated using LRE (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

Figure 19 shows the concentration of Y DNA in Sample 2 calculated by LRE and VC. The overall mean of Y DNA concentration when calculated by LRE was 1.42 ± 0.31 ng/ μ l, compared to the 1.63 ± 0.34 ng/ μ l concentration calculated by VC. A t-test was performed in order to compare the means of LRE and VC. The test showed that there was a significant difference between the means of LRE and VC ($P = 1.6 \times 10^{-3}$). The largest within run variance of Sample 2 for LRE was seen in run 8 at 0.114. The overall variance of all runs for LRE was 0.097, compared to the overall variance of all runs for VC, which was 0.11. For Sample 2Y all but one of the within run variances was lower than the overall variance. An F-test was performed to compare the variance of LRE and VC and it was found that there is no significant difference in the variance ($P = 0.30$).

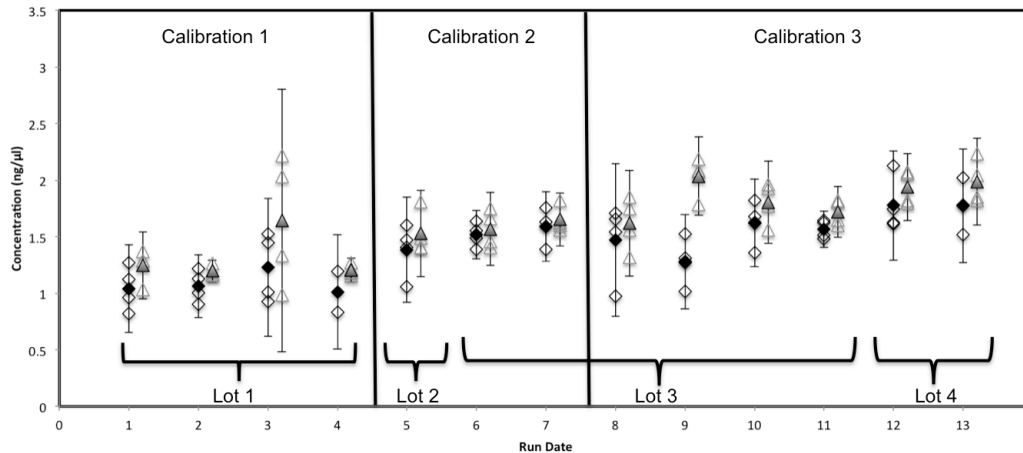


Figure 19. Y DNA concentration for Sample 2 calculated using LRE (◆) and VC (▲) over 3 years with error bars representing 2SD of the mean. The vertical black lines after runs 4 and 7 indicate point at which calibration took place. The brackets under the data points show which kit lots were used.

When calculating Y DNA for Sample 1 and Sample 2 the variance was smaller than the variance for VC, which shows that LRE has potential. However, there were some instances where the data input into the LRE program did not yield a result, and when calculating total human DNA VC still shows a lower variance. This suggests that VC is the more reliable method.

Figures 20 and 21 show the total human DNA concentrations for Samples 1 and 2 based on the kit lot used. The 2SD spread of Sample 1 when calculated by LRE for kits 1 through 4 was 1.20, 2.54, 2.08, and 2.23, while Sample 1 when calculated by VC had 2SD spreads of 0.90, 1.54, 1.39, and 1.60.

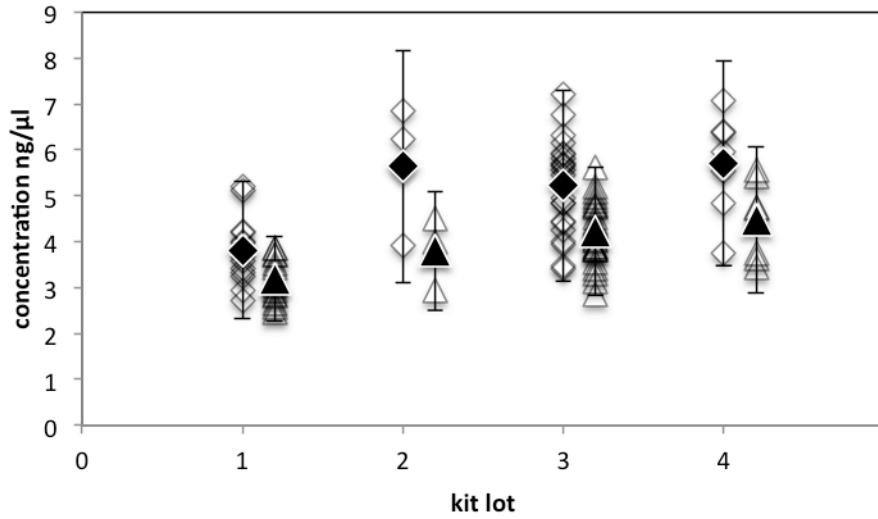


Figure 20. Total human DNA concentration for Sample 1 calculated by LRE (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

The 2SD spread of Sample 2 when calculated by LRE for kits 1 through 4 was 0.25, 0.60, 0.58, and 0.56, while Sample 2 when calculated by VC had 2SD spreads of 0.18, 0.10, 0.33, and 0.34.

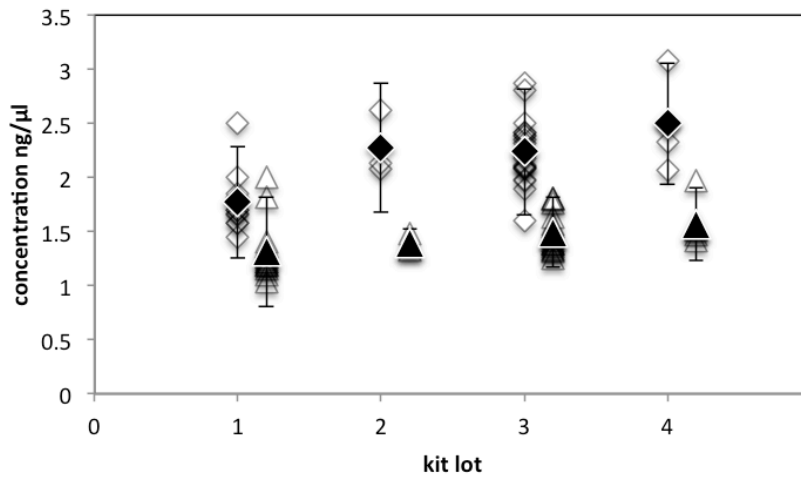


Figure 21. Total human DNA concentration for Sample 2 calculated by LRE (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

Figures 22 and 23 show the Y DNA concentrations for Samples 1 and 2 based on the kit lot used. The 2SD spread of Sample 1 when calculated by LRE for kits 1 through 4 was 0.04, 0.04, 0.05, and 0.08, while Sample 1 when calculated by VC has 2SD spreads of .06, .06, 0.01, and 0.12.

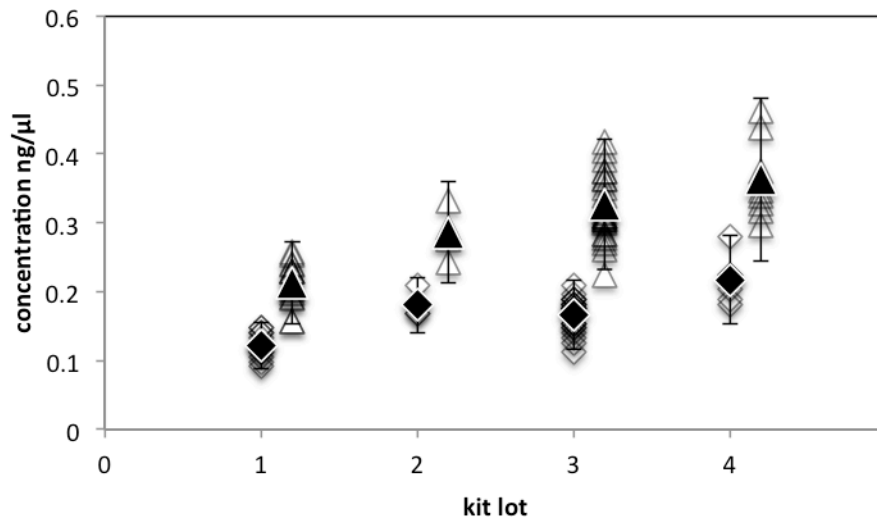


Figure 22. Y DNA concentration for Sample 1 calculated by LRE (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

The 2SD spread of Sample 2 when calculated by LRE for kits 1 through 4 was 0.33, 0.47, 0.42, and 0.45, The Y-DNA concentrations for Sample 2 when calculated by VC had 2SD spreads of 0.18, 0.38, 0.43, and 0.32.

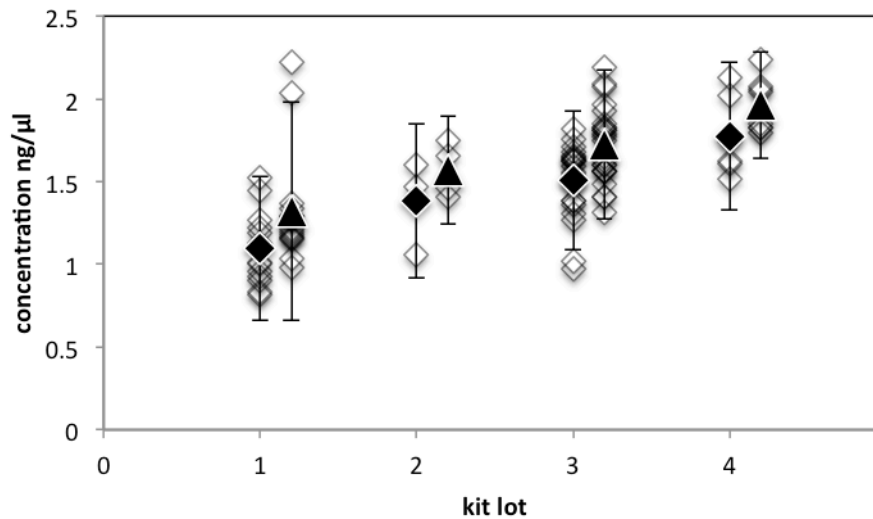


Figure 23. Y DNA concentration for Sample 2 calculated by LRE (◆) and VC (▲) sorted by kit lot, with error bars representing 2SD of the mean.

Figure 24 shows the total human DNA calculated by LRE and VC for Sample 1 sorted by calibration. The mean total human DNA concentration when calculated by LRE was 3.82 ± 0.75 ng/μl, 5.32 ± 1.18 ng/μl, and 5.40 ± 1.03 ng/μl. The mean total human DNA concentration when calculated by VC was 3.18 ± 0.46 ng/μl, 3.91 ± 0.67 ng/μl, and 4.39 ± 0.70 ng/μl.

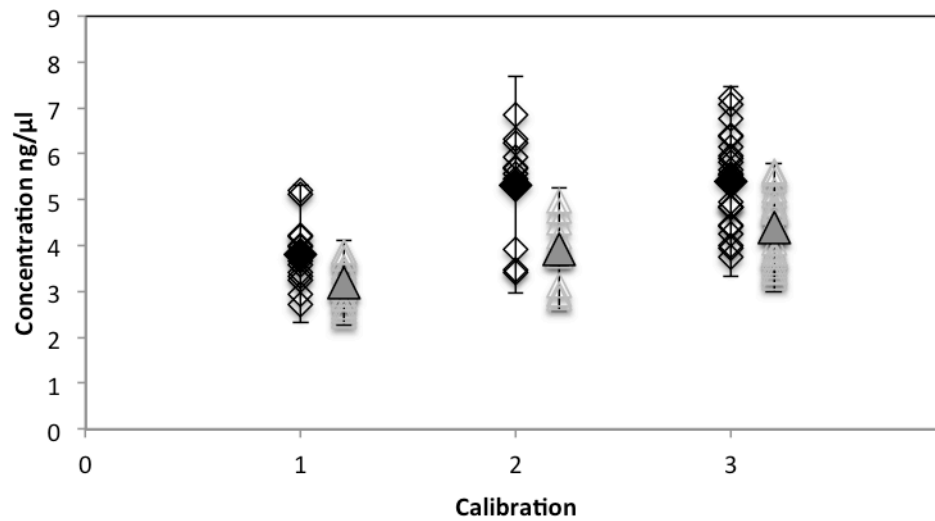


Figure 24. Total human DNA concentration for Sample 1 calculated by LRE (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 25 shows the total human DNA calculated by LRE and VC for Sample 2 sorted by calibration. The mean total human DNA concentration when calculated by LRE was 1.77 ± 0.23 ng/μl, 2.25 ± 0.23 ng/μl, and 2.33 ± 0.33 ng/μl. The mean total human DNA concentration when calculated by VC was 1.31 ± 0.25 ng/μl, 1.40 ± 0.07 ng/μl, and 1.55 ± 0.17 ng/μl.

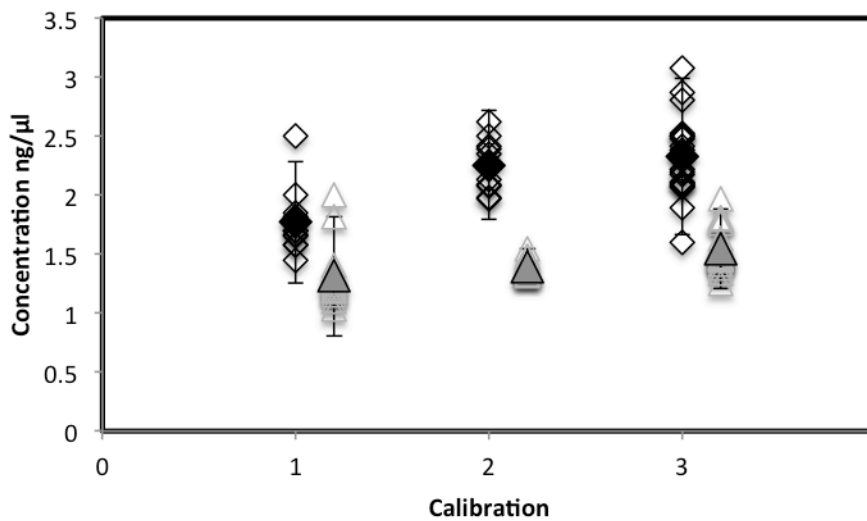


Figure 25. Total human DNA concentration for Sample 2 calculated by LRE (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 26 shows the Y DNA calculated by LRE and VC for Sample 1 sorted by calibration. The mean Y DNA concentration when calculated by LRE was 0.12 ± 0.02 ng/μl, 0.17 ± 0.02 ng/μl, and 0.19 ± 0.04 ng/μl. The mean Y DNA concentration when calculated by VC was 0.21 ± 0.03 ng/μl, 0.29 ± 0.04 ng/μl, and 0.35 ± 0.10 ng/μl.

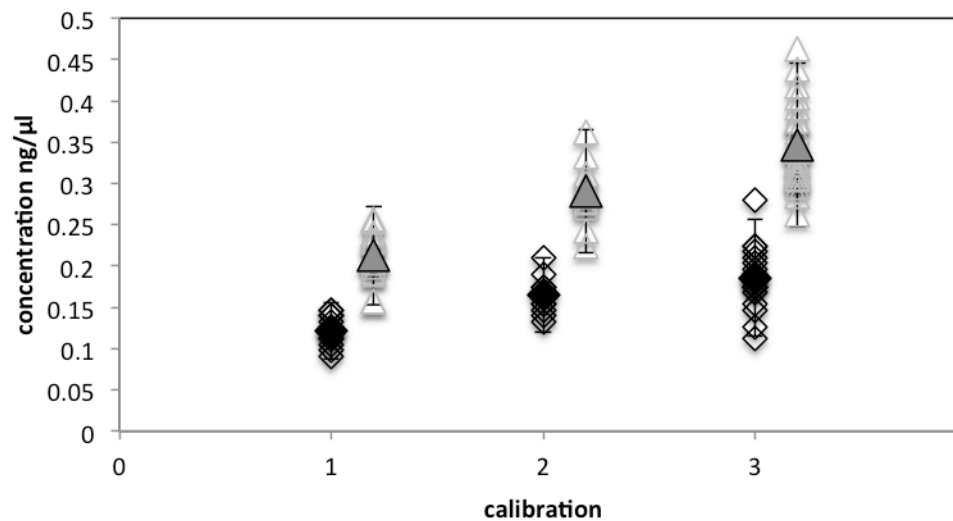


Figure 26. Y DNA concentration for Sample 1 calculated by LRE (◆) and VC (▲) sorted by calibration, with error bars representing 2SD of the mean.

Figure 27 shows the Y DNA concentration calculated by LRE and VC for Sample 2 sorted by calibration. The mean Y DNA concentration when calculated by LRE was 1.10 ± 0.22 ng/μl, 1.50 ± 0.18 ng/μl, and 1.57 ± 0.27 ng/μl. The mean Y DNA concentration when calculated by LRE was 1.32 ± 0.33 ng/μl, 1.58 ± 0.15 ng/μl, and 1.85 ± 0.22 ng/μl.

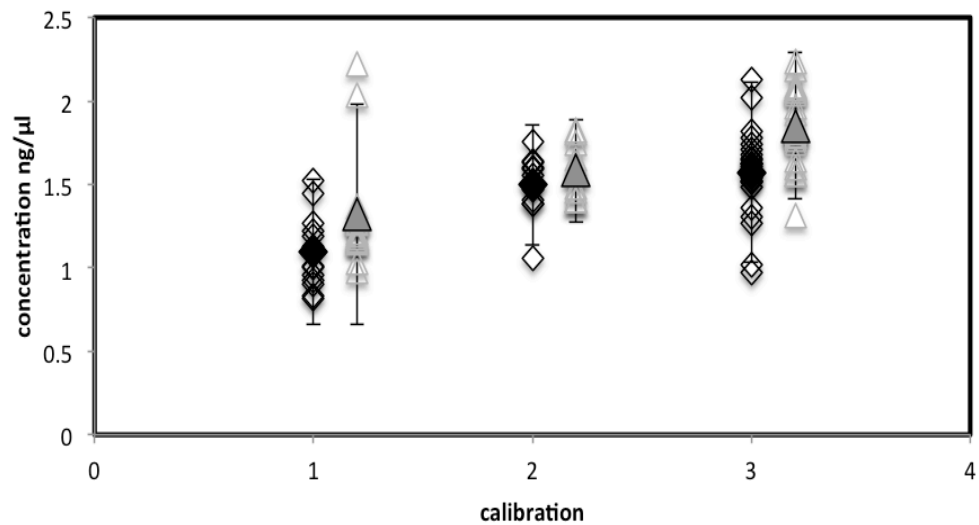


Figure 27. Y DNA concentration for Sample 2 calculated by LRE (◆) and VC(▲) sorted by calibration, with error bars representing 2SD of the mean.

One issue that did occur with the LRE software, was that not every sample input into the software gave a result. The software was not able to determine the amplification efficiency, which led to the samples being unable to be modeled and no concentrations were able to be determined. For total human DNA of Sample 1, 12% of the samples failed, for total human DNA of Sample 2, 13% of the samples failed, for Y DNA of Sample 1, 9% of the samples failed and for Y DNA of Sample 2, 6% of the samples failed. The samples that did not work showed extreme plateau drift or profile arching and it is hypothesized that this could have been caused by the TaqMan® probe chemistry. This indicates that modifications to the E_{max} value is not sufficient and LRE based qPCR analysis may not be a suitable analysis for TaqMan® based chemistries.

One issue with LRE modeling is that it assumes a symmetric relationship between the baseline and plateau phase of amplification. *Speiss et al (23)* have proposed a change to the sigmoidal model that adds another parameter to allow for asymmetry between the two stages. This could potentially lower the percentage of failed samples analysis as it may account for the plateau drift and profile arching.

Therefore, in order for this methodology to be an alternative to VC, further changes need to be implemented to lower the variances and to improve the accuracy of the quantification result.

Radar Graphs

Figure 28 shows a radar plot comparing the differences between the mean concentration and the actual concentrations during calibration 3 of Sample 1 for MRP, VC, and LRE.

Calibration 3 was used because it is the calibration that contained the most runs. The area under the lines of the graph representing MRP, VC, and LRE, were calculated to determine which one had the most desirable (smaller) area. The area for VC (4.3×10^{-3}) was lower than the area for MRP (5.3×10^{-1}) and LRE (3.3×10^{-2}); meaning that within the third calibration, the concentrations calculated by VC are more reproducible than those calculated by MRP or LRE.

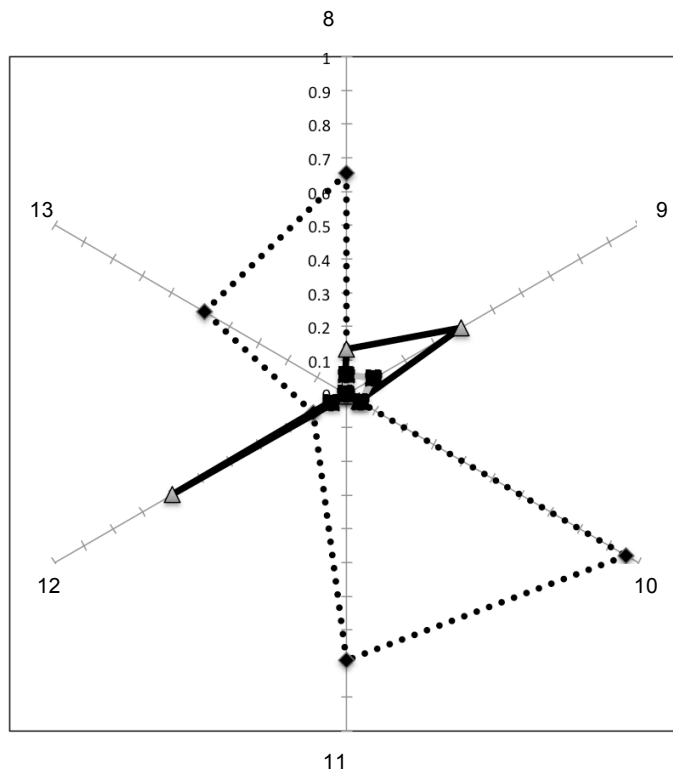


Figure 28. Difference of Mean Squared comparing MRP (◆), VC (■) and LRE (▲) during Calibration 3.

Figure 29 shows the coefficient of variation (CV) during calibration 3 for the concentrations calculated by MRP, VC, and LRE. The coefficient of variation is largest for LRE, which suggests that LRE has the largest spread of data points from the mean and is consistent with the larger standard deviations seen in Figures 16, 17, 18 and 19 for the LRE method. The areas under the lines of the graph for each method were calculated for MRP, VC, and LRE to determine which method had the smallest area. The area calculations showed that VC had

the smallest area (616) compared to MRP (699) and LRE (939), again suggesting that within a calibration the VC method results in greater quantification reproducibility.

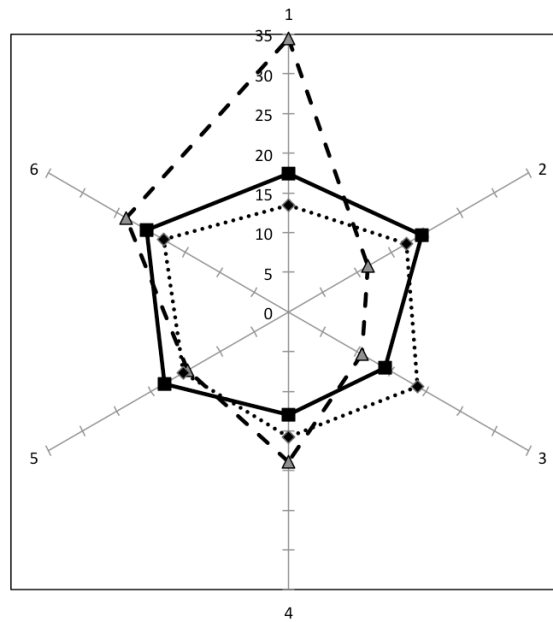


Figure 29. Relative Standard Deviation for MRP (◆), VC (■) and LRE (▲) during the six runs of Calibration 3.

Male to Female ratio

Mixtures are commonly encountered in forensics. The Quantifiler Duo® chemistry is designed such that both total human and Y-DNA can be detected and quantified. Since both are known, the amount of female DNA is discernable.

Figure 30 shows the male DNA concentrations and the female DNA concentrations in Sample 1 calculated by MRP.

Figure 31 shows the corresponding male: female ratios for Sample 1 calculated by MRP. The figure shows that the ratio is not consistent over time. This can be an issue because determining the wrong ratio has an effect on the data analysis portion of STR.

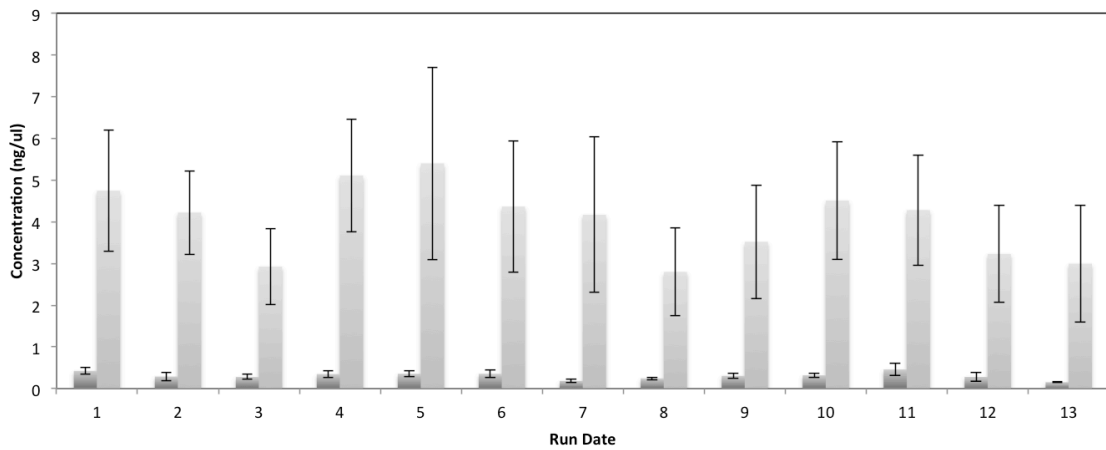


Figure 30. Male (Dark Grey) and Female (Light Grey) DNA concentrations calculated by MRP with error bars representing 2SD

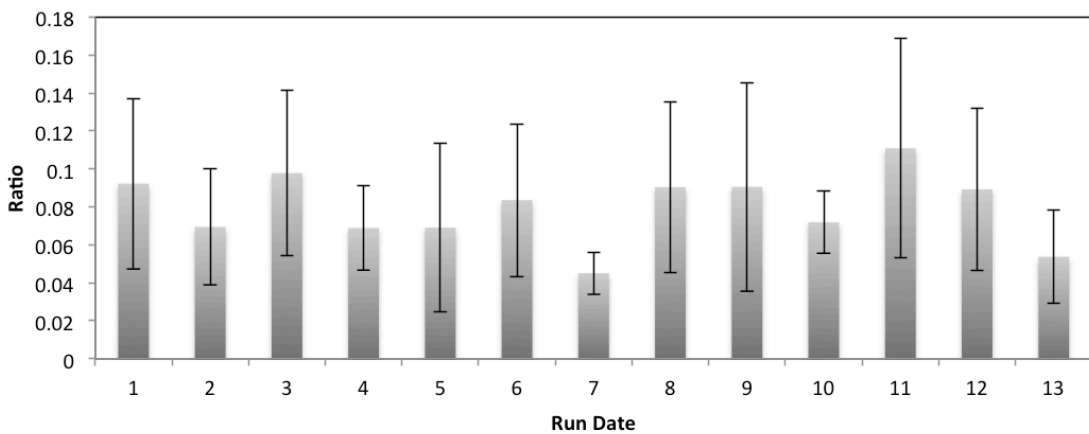


Figure 31. Male : Female mixture ratio determined by MRP with error bars representing 2SD of the mean

Figure 32 shows the male and female DNA concentrations in Sample 1 determined by VC. Both male and female DNA concentrations calculated via VC appear to be more consistent over time than those calculated by the MRP method. Figure 33 shows the male to female ratio calculated by VC, which is also more consistent over time than it was for MRP.

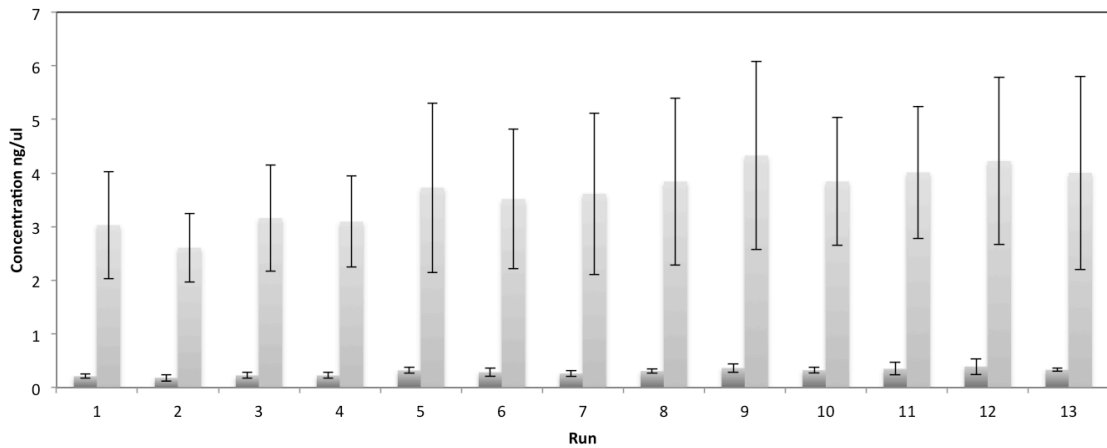


Figure 32. Male (Dark Grey) and Female (Light Grey) DNA concentrations calculated by VC with error bars representing 2 SD of the mean

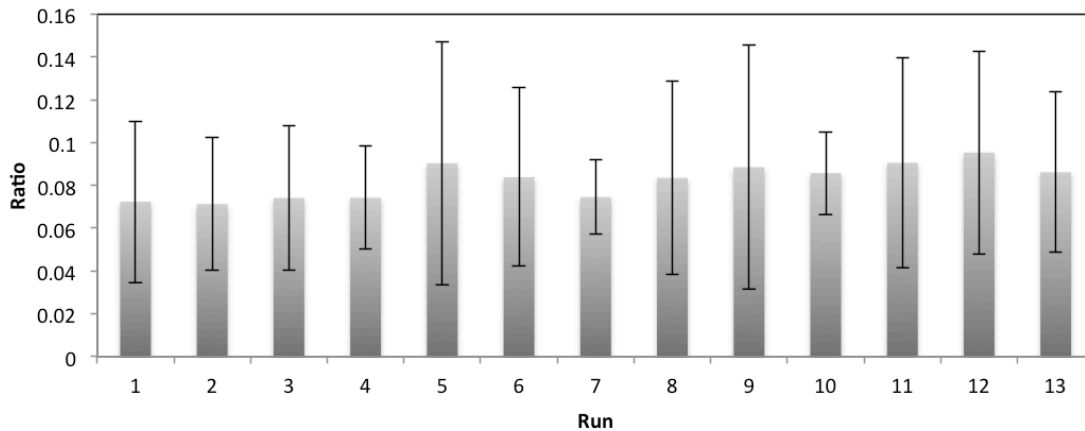


Figure 33. Male : Female mixture ratio determined by VC with error bars representing 2SD of the mean

Figure 34 shows the male and female DNA concentrations in Sample 1 determined by LRE. Figure 35 shows the male to female ratio calculated by LRE. LRE lacks the consistency of VC and MRP.

This suggests that the accuracy of the DNA concentrations for both the total human and Y DNA play a pertinent role in determining the male: female ratio and the female quantity of DNA. If the ratio is calculated incorrectly it may lead to the incorrect decision on whether to test the sample using Y-STR's. Therefore consistency in the absolute quantification of the human and male DNA is of importance.

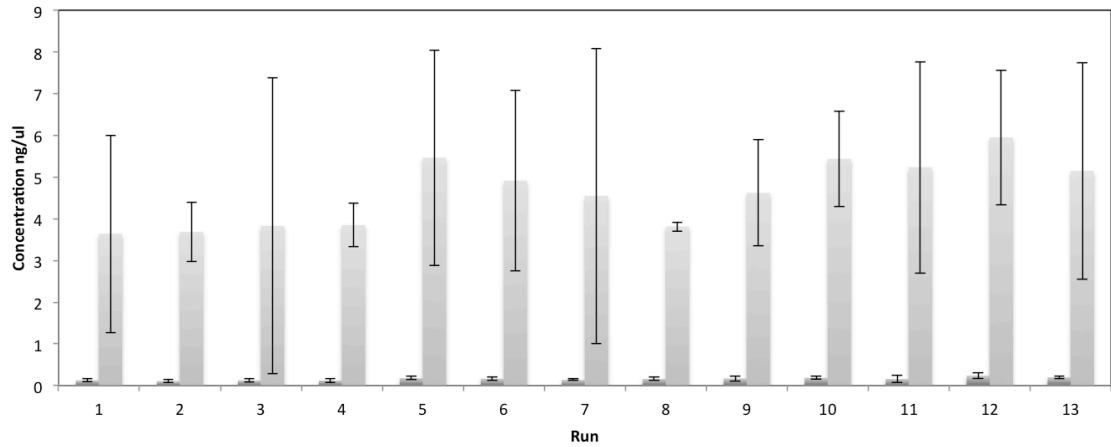


Figure 34. Male (Dark Grey) and Female (Light Grey) DNA concentrations calculated by LRE with error bars representing 2SD of the mean

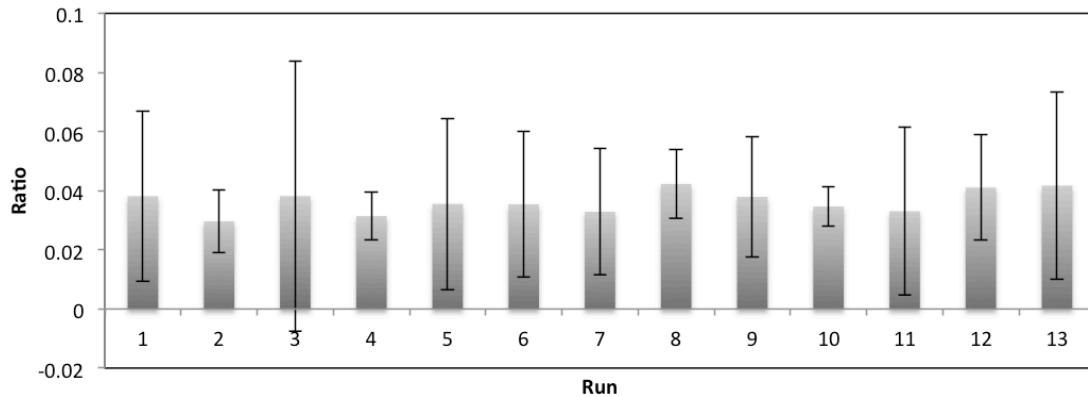


Figure 35. Male : Female mixture ratio determined by LRE with error bars representing 2SD of the mean

In summary, the male: female ratios calculated by MRP ranged from 1:9 to 1:22, the ratios calculated by VC ranged from 1:10 to 1:14, and the ratios calculated by LRE ranged from 1:24 to 1:34. The ratios calculated by LRE are higher than the other two methods because the concentrations determined by

LRE for the Y-DNA of Sample 1 are much lower than the values calculated by the other two methods. As previously stated, the LRE method is not a suitable method for forensic DNA quantification when used in conjunction with TaqMan® chemistries. Further, the smaller spread in mixture ratios between the MRP and VC methods suggest the VC method can improve the qPCR reproducibility over extended periods.

Conclusions

This experiment examined three methods of quantifying DNA for samples that were run in quadruplicate over the course of 26 months. Those three methods were MRP, VC, and LRE. The MRP and VC calculations are based on the use of standards of known concentration to calculate the DNA concentration of an unknown, while LRE is a modeling-based method that does not require a standard curve to quantify the DNA.

Currently, MRP is the standard method for quantification. There are however a few issues with this method. The first issue is reproducibility. Each time an assay is run, a new standard curve must be generated and simultaneously run with the questioned samples. This not only uses more time and reagents, but introduces the error associated with the procurement of the linear parameters. This work corroborates previous findings and shows the concentrations of the same samples run over a significant period of time does not remain the same. It also shows that there is variation both within and between

runs. This reproducibility problem can lead to issues during downstream PCR processing. Another issue is the cost; running sixteen standards on every plate takes up space that potentially could be used to run other samples. Using the MRP does allow for a control other than the IPC. It is possible to use the standard curve to judge the quality of a run; if the standard curve does not result in expected slopes and/or y-intercepts then there could have been an issue with the run. However, if the standards fail, it is impossible to determine the source of the error. That is, additional testing would be required to assess whether the failure was due to the reagents, instrument or the standards themselves.

Using modeling software like LRE that bases the concentration on the fluorescence of the individual sample may be considered an alternative to the standard curve based methods. Further, it has the potential to decrease cost and, like the VC method, allows for more samples to be run on a single plate. The LRE software is a freeware, easy to use, and it integrates well with the current analysis software. However, the software, in its current form, is not ready to be used as the main means of DNA concentration determination in a forensic setting, especially if the lab utilizes a TaqMan® probe based assay like Quantifiler® Duo.

Using a validated curve is a worthwhile alternative to determine DNA concentration. This method provides consistent results and shows less variation over time than both LRE and MRP. Furthermore, it is a cost effective method

since it allows for more samples to be run on an individual plate, and does not take any longer than the standard MRP method.

For the validated curve to be used effectively, certain factors need to be taken into consideration. Since detection systems are expected to differ between instruments. Therefore, if a lab has more than one qPCR instrument, then each machine may require its own validated curve. Further, a different validated curve should also be made for the different assays being used. For example, a validated curve for Quantifiler Duo® will only work for that kit.

This work also examined two possible causes of run variation, which are kit lot and calibration. While it appears that kit lot may cause some of the variation, data suggests that instrument calibration has the largest effect. The effect that calibration has seems to be larger for VC than the other methods. The variation is much smaller within a calibration than it was throughout all the runs combined. This leads to the conclusion that if a validated curve is going to be used, a new validated curve should be generated after each calibration in order to maintain the highest level of consistency and accuracy during DNA quantification.

References

LIST OF JOURNAL ABBREVIATIONS

J. Forensic Sci.	Journal of Forensic Sciences
Mol Vis	Molecular Vision

1. Walsh, P. Sean, Joseph Varlaro, and Rebecca Reynolds. "A Rapid Chemiluminescent Method for Quantitation of Human DNA." *Nucleic Acids Research* 20, no. 19 (1992): 5061–5065.
2. Nielsen, K., H.S. Mogensen, B. Eriksen, J. Hedman, W. Parson, and N. Morling. "Comparison of Six DNA Quantification Methods." *International Congress Series* 1288 (April 2006): 759–761.
doi:10.1016/j.ics.2005.09.095.
3. Barbisin, Maura, Rixun Fang, Manohar R Furtado, and Jaiprakash G Shewale. "Quantifiler® Duo DNA Quantification Kit: A Guiding Tool for Short Tandem Repeat Genotyping of Forensic Samples." *Journal of Forensic Research* 02, no. 02 (2011). doi:10.4172/2157-7145.1000118.
4. Wittwer, Carl T., Mark G. Herrmann, Alan A. Moss, and Randy P. Rasmussen. "Continuous Fluorescence Monitoring of Rapid Cycle DNA Amplification." *Biotechniques* 22, no. 1 (1997): 130–139.

5. Applied Biosystems®. Quantifiler Duo® DNA Quantification Kit User's Manual. Foster city, CA: 2008.
6. Shewale, Jaiprakash G., Elaine Schneida, Jonathan Wilson, Jerilyn A. Walker, Mark A. Batzer, and Sudhir K. Sinha. "Human Genomic DNA Quantitation System, H-Quant: Development and Validation for Use in Forensic Casework." *Journal of Forensic Sciences* 52, no. 2 (March 2007): 364–370. doi:10.1111/j.1556-4029.2006.00369.x.
7. Green, Robert L., Ines C. Roinestad, Cherisse Boland, and Lori K. Hennessy. "Developmental Validation of the Quantifiler™ Real-time PCR Kits for the Quantification of Human Nuclear DNA Samples." *J Forensic Sci* 50, no. 4 (2005): 809–25.
8. Horsman, Katie M., Jeffrey A. Hickey, Robin W. Cotton, James P. Landers, and Lewis O. Maddox. "Development of a Human-Specific Real-Time PCR Assay for the Simultaneous Quantitation of Total Genomic and Male DNA*." *Journal of Forensic Sciences* 51, no. 4 (July 2006): 758–765. doi:10.1111/j.1556-4029.2006.00183.x.
9. Moretti, Tamyra, Barbara Koons, and Bruce Budowle. "Enhancement of PCR Amplification Yield and Specificity Using AmpliTaq Gold DNA Polymerase." *BioTechniques* 25, no. 4 (1998): 716–722
10. Barbisin, Maura, Rixun Fang, Cristin E. O'Shea, Lisa M. Calandro, Manohar R. Furtado, and Jaiprakash G. Shewale. "Developmental

- Validation of the Quantifiler[®] Duo DNA Quantification Kit for Simultaneous Quantification of Total Human and Human Male DNA and Detection of PCR Inhibitors in Biological Samples.” *Journal of Forensic Sciences* 54, no. 2 (March 2009): 305–319. doi:10.1111/j.1556-4029.2008.00951.x.
11. Steadman, Shelly, and Sarah Geering. “Performance Verification of the Quantifiler[®] Duo DNA Quantification Kit and Implementation of Y-STR Typing: A Streamlined Approach to Co-Validation.” Accessed June 1, 2013. http://marketing.appliedbiosystems.com/images/Product_Microsites/QUANTDUO/pdf/March09_FN_CC_2_FINAL.pdf.
12. Kline, Margaret C., David L. Duewer, Janette W. Redman, and John M. Butler. “Results from the NIST 2004 DNA Quantitation Study.” *J. Forensic Sci* 50, no. 3 (2005): 571–578.
13. Westring, Christian G., Richard Kristinsson, Dustin M. Gilbert, and Phillip B. Danielson. “Validation of Reduced-Scale Reactions for the Quantifiler? Human DNA Kit.” *Journal of Forensic Sciences* 52, no. 5 (September 2007): 1035–1043. doi:10.1111/j.1556-4029.2007.00525.x.
14. Grgicak, Catherine M., Zena M. Urban, and Robin W. Cotton. “Investigation of Reproducibility and Error Associated with qPCR Methods Using Quantifiler[®] Duo DNA Quantification Kit*.” *Journal of Forensic Sciences* 55, no. 5 (September 2010): 1331–1339. doi:10.1111/j.1556-4029.2010.01460.x.

15. Higgins, Karen M., Marie Davidian, Ginger Chew, and Harriet Burge. "The Effect of Serial Dilution Error on Calibration Inference in Immunoassay." *Biometrics* (1998): 19–32.
16. A. Racine-Poon, C. Weihs, and A. F. M. Smith. "Estimation of Relative Potency with Sequential Dilution Errors in Radioimmunoassay." *International Biometric Society* 47, no. 4 (December 1991): 1235–1246.
17. Sivaganesan, Mano, Shawn Siefring, Manju Varma, Richard A Haugland, and Orin C Shanks. "A Bayesian Method for Calculating Real-time Quantitative PCR Calibration Curves Using Absolute DNA Standards." *BMC Bioinformatics* 9, no. 1 (2008): 120. doi:10.1186/1471-2105-9-120.
18. Nordgård, Oddmund, Jan Terje Kvaløy, Ragne Kristin Farmen, and Reino Heikkilä. "Error Propagation in Relative Real-time Reverse Transcription Polymerase Chain Reaction Quantification Models: The Balance Between Accuracy and Precision." *Analytical Biochemistry* 356, no. 2 (September 2006): 182–193. doi:10.1016/j.ab.2006.06.020.
19. Goll, Rasmus, Trine Olsen, Guanglin Cui, and Jon Florholmen. "Evaluation of Absolute Quantitation by Nonlinear Regression in Probe-based Real-time PCR." *BMC Bioinformatics* 7, no. 1 (2006): 107.
20. Tichopad, A. "Standardized Determination of Real-time PCR Efficiency from a Single Reaction Set-up." *Nucleic Acids Research* 31, no. 20 (October 15, 2003): 122e–122. doi:10.1093/nar/gng122.

21. Boggy, Gregory J., and Peter J. Woolf. "A Mechanistic Model of PCR for Accurate Quantification of Quantitative PCR Data." *PLoS One* 5, no. 8 (2010): e12355.
22. Alvarez, Mariano J, Guillermo J Vila-Ortiz, Mariano C Salibe, Osvaldo L Podhajcer, and Fernando J Pitossi. "Model Based Analysis of Real-time PCR Data from DNA Binding Dye Protocols." *BMC Bioinformatics* 8, no. 1 (2007): 85. doi:10.1186/1471-2105-8-85.
23. Spiess, Andrej-Nikolai, Caroline Feig, and Christian Ritz. "Highly Accurate Sigmoidal Fitting of Real-time PCR Data by Introducing a Parameter for Asymmetry." *BMC Bioinformatics* 9, no. 1 (2008): 221. doi:10.1186/1471-2105-9-221.
24. Ritz, C., and A.-N. Spiess. "qPCR: An R Package for Sigmoidal Model Selection in Quantitative Real-time Polymerase Chain Reaction Analysis." *Bioinformatics* 24, no. 13 (May 14, 2008): 1549–1551. doi:10.1093/bioinformatics/btn227.
25. Swillens, Stéphane, Barbara Dessars, and Hakim El Housni. "Revisiting the Sigmoidal Curve Fitting Applied to Quantitative Real-time PCR Data." *Analytical Biochemistry* 373, no. 2 (February 2008): 370–376. doi:10.1016/j.ab.2007.10.019.

26. Weihong Liu, and David A. Saint. "Validation of a Quantitative Method for Real Time PCR Kinetics." *Biochemical and Biophysical Research Communications* 294, no. 2002 (2002): 347–353.
27. Rutledge, R. G. "Mathematics of Quantitative Kinetic PCR and the Application of Standard Curves." *Nucleic Acids Research* 31, no. 16 (August 15, 2003): 93e–93. doi:10.1093/nar/gng093.
28. Rutledge, R. G. "Sigmoidal Curve-fitting Redefines Quantitative Real-time PCR with the Prospective of Developing Automated High-throughput Applications." *Nucleic Acids Research* 32, no. 22 (December 14, 2004): e178–e178. doi:10.1093/nar/gnh177.
29. Rutledge, Robert G., and Don Stewart. "Assessing the Performance Capabilities of LRE-Based Assays for Absolute Quantitative Real-Time PCR." Edited by Michael Polymenis. *PLoS ONE* 5, no. 3 (March 17, 2010): e9731. doi:10.1371/journal.pone.0009731.
30. Rutledge, Robert G. "A Java Program for LRE-Based Real-Time qPCR That Enables Large-Scale Absolute Quantification." Edited by Michael Polymenis. *PLoS ONE* 6, no. 3 (March 2, 2011): e17636. doi:10.1371/journal.pone.0017636.
31. Rutledge, Robert G. Enabling large-scale absolute quantification. Retrieved August 12, 2012, from sites.google.com/site/lreqpcr

32. Karlen, Yann, Alan McNair, Sébastien Perseguers, Christian Mazza, and Nicolas Mermoud. "Statistical Significance of Quantitative PCR." *BMC Bioinformatics* 8, no. 1 (2007): 131. doi:10.1186/1471-2105-8-131.
33. Hartman, Laurie J., Susan R. Coyne, and David A. Norwood. "Development of a Novel Internal Positive Control for Taqman® Based Assays." *Molecular and Cellular Probes* 19, no. 1 (February 2005): 51–59. doi:10.1016/j.mcp.2004.07.006.
34. Brigitte Baldi, and David S. Moore. *The Practice of Statistics in the Life Science*. Vol. 1. 1st ed. New York: W.H. Freeman and Company, 2009.
35. Ruijter, J. M., C. Ramakers, W. M. H. Hoogaars, Y. Karlen, O. Bakker, M. J. B. van den Hoff, and A. F. M. Moorman. "Amplification Efficiency: Linking Baseline and Bias in the Analysis of Quantitative PCR Data." *Nucleic Acids Research* 37, no. 6 (January 21, 2009): e45–e45. doi:10.1093/nar/gkp045.

VITA

