Emily Cropper. An assessment of qPCR assays for DNA concentration and degradation. Master of Science (Biomedical Sciences, Forensic Genetics). May 2019, 65 pages, 21 tables, 21 figures, 31 references.

Forensically challenged samples are often composed of degraded, damaged, or low template mitochondrial DNA (mtDNA). A real-time quantitative polymerase chain reaction (qPCR) assay can help determine if there is sufficient quantity and robust quality of mtDNA to move forward with downstream sequencing and analysis. The fundamental issue with qPCR is that the nominal quantity of the DNA calibrated along the commercial standard used for quantification can vary depending on the supplier and lot numbers. The National Institute of Standards and Technology (NIST) has developed a commercially available human DNA standard, Standard Reference Material (SRM) 2372a, which consists of nuclear DNA (nDNA) and mtDNA data on three well-characterized human genomic DNA preparations. The SRM 2372a was used to compare three qPCR assays: a non-commercial triplex assay, for mtDNA quantification, and two commercial assays, Quantifiler Trio (QFTrio) for nDNA quantification, and NovaQUANT for nDNA quantification and determination of the mtDNA/nDNA ratio. Quantification of the SRM uniformly across these three qPCR assays allowed for the conclusion that a robust, reproducible, accurate, and efficient qPCR assay is dependent on (1) the quality and reliability of the DNA standard, (2) the specificity of the qPCR chemistry, and (3) sound primers and probes, to name a few. The findings indicate that commercially available qPCR assays do not necessarily perform as marketed and should be re-verified by a validated DNA SRM.

AN ASSESSMENT OF QPCR ASSAYS FOR DNA CONCENTRATION AND DEGRADATION
THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Emily Cropper, B.S., B.A.

Fort Worth, TX

May 2019

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Michael Coble, for all of the assistance and guidance throughout my educational career at the University of North Texas Health Science Center. I would like to express my deepest thanks to my co-advisor and mentor Dr. Mark F. Kavlick at the Counterterrorism and Forensic Science Research Unit (CFSRU) of the FBI Laboratory for the opportunity to participate in the Visiting Scientist Program (VSP) while undergoing my thesis work. Also, I want to thank Gene Peters and Dawnele Holbrook at the CFSRU. I would like to thank my committee member Dr. Joseph Warren for believing in me and accepting me into the Forensic Genetics program and for all of the continued encouragement throughout these last two years. A very special thank you to my committee member Dr. Nicole Phillips for always taking the time to meet with me and provide feedback and encouragement. In addition, I would like to thank my University Member, Dr. Rebecca Cunningham for her knowledgeable insight. I am extremely grateful for guidance and friendship from Dr. Angie Ambers. I would especially like to acknowledge my laboratory mate at UNTHSC, Ms. Rachel McGehee for her continued support and friendship. I would also like to thank my lab colleagues at the VSP. In particular, I want to thank Jessica McLamb for her endless assistance, guidance, and friendship. I would also like to thank Dr. Kyleen Elwick for her suggestions and insight. Also, thank you to Dr. Natalie Damaso, Maria Lawas, and Luisa Forger for troubleshooting experiment issues. Lastly, I want to give thanks to my mom, Jill Goff, my sister, Claire Cropper, and my dad, Kenneth Cropper for their continued love and support over the years.

DEDICATION

I would like to dedicate this thesis to my dear friend Dr. Brian Molles, whose life was stolen from him July of 2018. His death has given me a newfound purpose of helping to seek justice in the name of science for humankind.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES CONT.

LIST OF FIGURES

LIST OF FIGURES CONT.

CHAPTER I

INTRODUCTION

Degraded, damaged, or low template DNA presents a major challenge for forensic DNA analysts because nuclear DNA (nDNA) may fail to amplify for short tandem repeat (STR) analysis. In these cases, mitochondrial DNA (mtDNA) is considered a potential alternative template for analysis [1]. Sufficient quantity and robust quality of nDNA and/or mtDNA are essential factors for obtaining reliable results [2-5]. A substantial amount of time, money, and resources may be committed to type or sequence a sample with low level and/or poor-quality DNA. A real-time quantitative polymerase chain reaction (qPCR) can be utilized to determine whether there is an adequate concentration and quality of nDNA or mtDNA to amplify. Thus, qPCR can better assist in determining the best DNA targets for downstream typing or sequencing, compared to the qualitative agarose gel approach this is common in many mtDNA laboratories [6]. As an example, by applying qPCR, the University of Innsbruck (Austria) reduced their re-amplifications from 18% down to 7% while examining 12,000 casework samples over a two year period [7]. Effective quantification of DNA requires an assay with a high degree of efficiency, sensitivity, specificity, accuracy, reproducibility, and precision [4,5,7-9]. To achieve these goals, the qPCR assay requires calibration to an external standard of a specific DNA concentration that can be expressed in ng/µL or copies/µL [2,10].

The fundamental issue with qPCR is that the nominal concentration of a DNA standard used for quantification can vary depending on the supplier and lot numbers [2,10,11]. Discrepancies in quantitative values can result from contamination of the standard due to continued laboratory

use, inappropriate shipping temperature when transporting the samples, DNA adhering to the inside of the storage tube, degradation of plasmid or synthetic oligomer standards, the use of DNA originating from cell lines which can contain variable concentrations of DNA from not accurately replicating the primary cells [12], and inappropriate specificity of the primers. In addition, the amplification of mitochondrial pseudogenes which are sequences of mtDNA integrated into the nuclear genome [13], may lead to an inaccurate mtDNA concentration for unknown samples [2,10,11] .

The National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2372a was developed after the depletion of its predecessor, SRM 2372 [3]. The SRM 2372a includes mtDNA and nDNA data while the SRM 2372 only included data for nDNA. This study utilizes the commercially available NIST SRM 2372a containing three human DNA calibration standards derived from (A) single male donor, (B) single female donor, and (C) 1:3 mixture of a male and a female donor, rather than originating from cell lines that are used in most qPCR assays. NIST recommended values for the three components include nDNA copy number (copies/nL), and ng/µL as well as a mtDNA/nDNA ratio [2,10] **Table 1**.

Table 1: *NIST recommended values to be used in the Certificate of Analysis and 95 % Uncertainties* [10]

Component	Units	Value†	±/U95(Value) ‡
A (nDNA Male)	Copies per nanoliter	15.1	1.5
B (nDNA Female)	Copies per nanoliter	17.5	1.8
C (nDNA 1:3 male/female)	Copies per nanoliter	14.5	1.5
A (nDNA)	ng/µL	49.8	5.0
B (nDNA)	ng/µL	57.8	5.8
C (nDNA)	ng/µL	47.9	4.8
A	mtDNA/nDNA	174	4
B	mtDNA/nDNA	206	5
C	mtDNA/nDNA	279	7

‡ Interval Value ± U95(Value) is believed, with 95 % confidence, to contain the true value of the measurand.
† NIST recommended (now will be referred to as *expected)* true value of the measured within 95% confidence

The NIST SRM 2372a can enable suppliers of secondary standards to more reliably assess their materials and forensic testing laboratories can use the NIST SRM 2372a to validate their own in-lab/commercial quantification procedures and materials [10]. The values in (**Table 1)** for the NIST SRM 2372a were obtained by evaluating eleven nDNA and three mtDNA qPCR assays using digital droplet PCR (ddPCR) (Kline, Duewer et al. 2009). In this qPCR assay examination, the NIST SRM 2372a allows for a uniform comparison of DNA concentration across three different qPCR assays. One of the assays examined was a non-commercial mtDNATriplex assay containing two mtDNA targets and an internal positive control, which was designed by Dr. Mark F. Kavlick at the Federal Bureau of Investigation, Counterterrorism and Forensic Science Research Unit, Laboratory Division, [9,14]. The other two qPCR assays evaluated were commercially available: the nDNA Quantifiler Trio (QFTrio) assay containing three nDNA targets (Thermo Fisher Scientific, Waltham, MA) and the NovaQUANT (NQ) Human

Mitochondrial to Nuclear DNA Ratio Kit (EMD Millipore Corporation, San Diego, CA) containing two nDNA targets and two mtDNA targets. The Duplex mtDNA assay (the precursor to the Triplex, also developed by Kavlick at the FBI) and the QFTrio assay were two of the assays used in establishing the recommended values for the NIST SRM 2372a. The SRM 2372a standard was not tested by NIST using the Triplex and NQ assays.

Mitochondrial DNA vs Nuclear DNA

In forensic DNA analysis, nDNA short tandem repeat (STR) analysis is the preferred method of human identification because it can uniquely identify the DNA contributor to an unknown sample. A unique DNA profile can be obtained because each nuclear cell contains one nDNA locus copy from the mother and one copy from the father, and on examination of thirteen or more loci, even siblings can be distinguished from each other, with the exception of identical twins. On the other hand, because mtDNA is transmitted from mother to child, the entire maternal lineage, which includes siblings who share the same mother, will have the same mtDNA haplotype, barring mutation. Thus, because more than one person can have the same mtDNA haplotype, mtDNA can only be used to include or exclude a person of interest rather than identify a unique individual [15].

There are other differences between nDNA and mtDNA. While there are two copies of nDNA per cell, there are hundreds to thousands of copies per cell of mtDNA [15]. Moreover, the nDNA and mtDNA molecules differ in the number of base pairs [bp]. Each diploid nDNA genome contains more than six billion bp whereas each mtDNA genome contains only 16,569 bp of DNA [16]. Even though there are many more copies of mtDNA per cell than nDNA, the vastly larger size of the nDNA molecule is why a human's DNA is virtually 100% nDNA. Despite this, mtDNA analysis may succeed where nDNA might fail because the greater number of copies

of mtDNA per cell allows its persistence in samples which contain little or degraded DNA, compared to nDNA.

Mitochondrial DNA in Forensic Investigations

Human mtDNA sequence analysis is used for a wide range of applications such as disease diagnosis, forensic investigations (including missing persons cases and human identification in natural or mass disasters), ancestry studies, studies of biological evolution, anthropology, and ecotoxicology [1,15,17-23]. Hypervariable regions 1 and 2 (HV1 and HV2) within the mtDNA molecule may be sequenced in cases where nDNA is degraded or is in low-to-undetectable concentrations. DNA degradation or low quantity DNA is often a result of environmental damage such as UV irradiation, acidic soil, heat, microbial growth, and humidity. In addition, these insults can also be sources of PCR inhibitors [17,19,21,22]. Most often, cases associated with mtDNA include cold cases, missing persons cases, and mass disasters. Commonly, the evidence submitted for analysis may include small quantities of biological material like skeletal remains of bones and teeth, naturally shed hairs (pubic and head), formalin fixed paraffin embedded (FFPE) tissue [24], and unidentified remains with environmental damage [1,14,17,20-22,25]. In degraded or low-level samples, mtDNA analysis can yield some genetic information due to the high copy number of mtDNA (hundreds or thousands per cell) compared to two copies of nDNA per cell. In addition, the circularity of the mtDNA may make it more resistant to exonuclease digestion and the organelle compartmentalization adds another physical barrier for protection and enhances mtDNA persistence [5,7,15]

qPCR

Absolution vs Relative

There are two types of qPCR: absolute and relative. Absolute quantification determines an absolute quantity, more specifically concentration, of DNA using a standard curve method that

quantifies unknowns through comparison to a known DNA standard quantity. Once a standard curve (**Figure 1)** is created then unknown samples can be compared to the standard curve and a value can be interpolated.

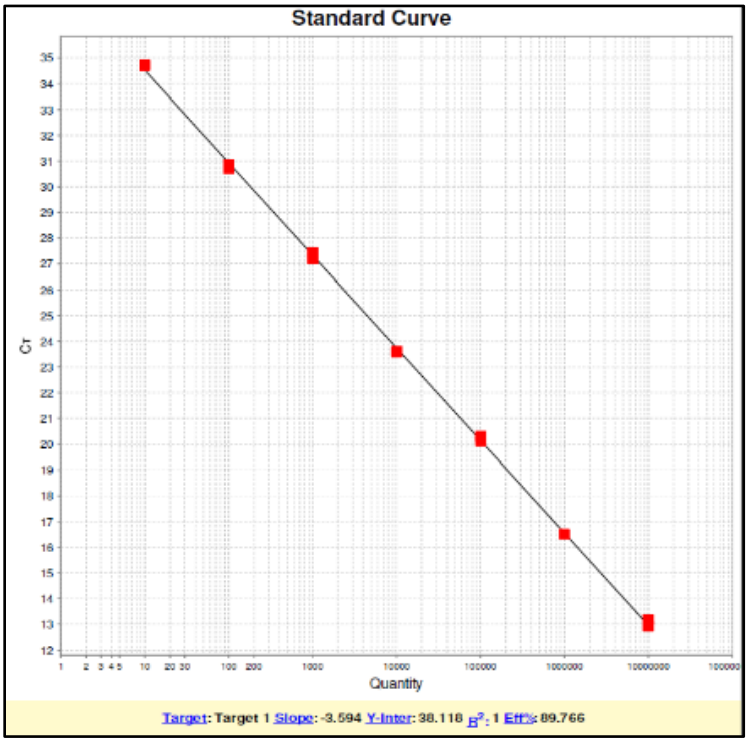


Figure 1: Example absolute qPCR standard curve (Ct vs quantity of DNA). *Shown is a standard curve which contains seven (7) 10-fold serial dilutions of a DNA standard of known quantities, i.e. concentrations. Ct is the cycle at which the normalized fluorescent signal after subtracting out instrument background noise (ΔRn) for a sample or standard crosses a defined threshold (cycle threshold)*[26].

QPCR and ddPCR are both absolute methods for quantification. While the NIST SRM 2372a was certified using ddPCR, qPCR was conducted in this study. They are both considered to be comparable methods for quantification, but there are some advantages and disadvantages to both. While qPCR uses a standard curve to interpolate a value, ddPCR does not require a standard curve and instead the absolute number or mass of molecules are calculated from the total measure of fluorescence from thousands of partitioned wells. They both deliver rapid results, but qPCR delivers data in real time while ddPCR delivers end point data. QPCR technology is more

established and widely used than ddPCR. They both have a wide dynamic range of DNA concentration quantification.

Relative qPCR determines quantity of a DNA target, e.g. mtDNA, relative to the quantity of another, e.g. nDNA. This is accomplished by using cycle threshold (Ct) values (Bustin, Benes et al. 2009). One example of relative qPCR is the NovaQUANT assay which determines mtDNA quantity relative to nDNA (**Table 2)**.

Table 2: *Relative Quantification using the NovaQUANT Assay (table reproduced from page 6 out of the NovaQUANT protocol [27])*

Target	**Ct**	**ΔCt**	**$2^{\Delta Ct}$**	**Avg mtDNA/nDNA**
ND1 (mtDNA)	18	ΔCt1 = 9.5	724	677
BECN1 (nDNA)	27.5			
ND6 (mtDNA)	17.6	ΔCt2 = 9.3	630	
NEB (nDNA)	26.9			

Table 2*: **Example relative quantitative method for NovaQUNANT mtDNA/nDNA.***
Shown is the calculation of mtDNA/nDNA. This is accomplished by averaging ND1/BECN1and ND6/NEB using their Ct values like in the table above.

Whether absolute or relative, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines [28] describe several variables to consider when analyzing a qPCR assay. Those variables are efficiency, analytical sensitivity, analytical specificity, accuracy, precision, reproducibility, repeatability, and linear dynamic range.

Efficiency

In absolute quantification, the standard curve is a graph of the Ct of quantification standard reactions plotted against the starting quantity of the standards. QPCR instrument software generates a regression line by calculating the best fit with the quantification standard data points. The regression line formula has the form: Ct = m [log (Qty)] + b where m is the slope, b is the y-

intercept, and Qty is the starting DNA quantity. The efficiency is based on the slope of this regression line: efficiency = [10(-1/m)]-1. An efficiency of 100% (m = -3.3219) indicates that the amount of amplification product doubles with each cycle. However, an efficiency within the range of 90 – 110% (-3.6 ≥ slope ≥ -3.3219) may be considered effectively synonymous with 100% efficiency and therefore acceptable. Efficiencies lower than this range can be due stochastic effects from low levels of DNA, poor primer design, poorly constituted serial dilutions, and pipetting errors. Efficiencies above this range could due to poorly prepared standard dilutions, polymerase inhibition, or assay chemistry characteristics.

Analytical Sensitivity

Analytical sensitivity refers to the minimum number of copies in a sample that can be measured accurately with an assay. A very sensitive assay can detect very low copy numbers of DNA. Since Ct values are inversely correlated with DNA detection of Ct values >35 also indicates a qPCR assay with high sensitivity. That is, the higher the Ct value, the lower the concentration of DNA.

Analytical Specificity

Analytical specificity refers to when a qPCR assay senses the precise target sequence rather than other, nonspecific targets also present in a sample.

Accuracy

Accuracy refers to the closeness of experimentally measured and actual concentrations presented as fold changes or copy number estimates. In the case of absolute qPCR, it depends on the accuracy of the standards. The SRM was used to check the accuracy of the nDNA standards. In

the case of the Triplex, the standards are accurate since they are synthetic, pure, and well-characterized.

Precision

Precision refers to the random variation of repeated measurements. Enhanced precision in a qPCR reaction enables one to discriminate smaller differences in nucleic acid copy numbers or fold changes. Precision is measured by the coefficient of variation (CV) or standard deviation (SD) of the DNA quantification of the measured sample duplicates. CVs below 10% show low variability therefore high repeatability of the data.

Repeatability

Repeatability or (short-term precision) refers to the precision and robustness of the assay with the duplicate samples analyzed in the same assay. Repeatability is measured by CV or SD of the DNA quantification of the measured sample duplicates. CVs below 10% show low variability therefore high repeatability of the data.

Reproducibility

Reproducibility or (long-term precision) refers to the variation in results between different runs of the assay, different operators, different days the assays is performed, different machines, or different laboratories. Reproducibility of an assay is expressed by either CV or SD of DNA quantification of the measured sample duplicates. CVs below 10% show low variability therefore high reproducibility of the data.

Linear Dynamic Range

The dynamic range over which a reaction is linear. The maximum to the minimum quantifiable DNA concentration is established by means of a calibration curve. When creating a serial dilution of a qPCR standard, it is suggested to have a range of three orders of magnitude which

contains least five dilutions [28]. The standard curve ideally should include the interval of the samples being quantified.

Ideal qPCR Standard

An ideal mtDNA quantitative standard is one which is highly sensitive, has high specificity, and yields reproducible results. A highly sensitive standard is one that can be used to detected to ≤ 1copies/μL, which is helpful in assessing low-level DNA forensic specimens, e.g. some calcified tissue and hair shafts. The specificity of the standard can be enhanced by using sequence-specific fluorogenic probes. Preferably the standard should contain a DNA sequence that is similar to, but distinguishable from the analyzed sequence, i.e. the HVR, allowing it to be identified as a contaminant [9].

Causes of qPCR Variation

There are a variety of sources that can contribute to qPCR variation such as pipetting errors, the standard used for the calibration curve, the type of instrument used, and the qPCR chemistry in the reaction. In this experiment, the qPCR assays Triplex and QFTrio use a hydrolysis probe technology known as TaqMan. The TaqMan approach monitors change in fluorescence due to hydrolysis of a dye-labeled probe. The TaqMan probes can be labeled with different, distinguishable reporter dyes which allows for the detection of specific amplification of products. In addition, each probe is labeled with a non-fluorescent quencher (NFQ) dye that quenches the fluorescence signal of the reporter until the target sequence is amplified. In contrast to the TaqMan chemistry, NQ is run with an intercalating dye, SYBR Green, which binds all double-stranded molecules, independent of sequence specificity. The SYBR Green assay will detect formation of any unintended PCR product including nonhuman (e.g. bacterial or fungal), which may cause an overestimation of the true DNA quantity.

qPCR Assays Compared in this Study

mtDNA Triplex

The Triplex mtDNA quantification assay utilizes a unique 115 bp ultramer synthetic standard dsT8sig [9]. The dsT8sig is comprised of all of the features required for a successful standard and is also highly reproducible. The d8T8sig ensures high quality control each time a new standard is prepared. Its signature synthetic sequence can be distinguished from naturally occurring sequences allowing it to be identified as a contaminant. In turn this produces greater accuracy in quantification [9]. It contains two complementary, PAGE-purified oligonucleotides (Ultramers; Integrated DNA Technologies)[14] listed in (**Table 3)** corresponding to positions 13,283-13,397 of the mtDNA revised Cambridge Reference Sequence (CRS: gi_115315570. This is the target sequence plus five additional base pairs at both the 5' and 3' ends [9]. The Triplex uses TaqMan chemistry for amplification of the targets.

Table 3: *Triplex dsT8sig standard forward and reverse sequence* [9]

qPCR standard (forward strand)
Tfor8sig:5'-CAA TCG GCA T CA ACC AAC CAC ACC TAG CAT TCC TGC ACA TCT GTA CCC ACG CCT TCT TCA AAT AAC GAC TAT TTA TGT GCT CCG GGT CCA TCA TCC ACA ACC TTA ACA ATG AAC A-3'
qPCR standard (reverse strand)
Treverse8sig:5'-TGT TCA TTG TTA AGG TTG TGG ATG ATG GAC CCG GAG CAC ATA AAT AGT CGT TAT TTG AAG AAG GCG TGG GTA CAG ATG TGC AGG AAT GCT AGG TGT GGT TGG TTG ATG CCG ATT G-3'

Quantifiler Trio (QFTrio)

The Quantifiler Trio DNA Quantification Kit (QFTrio)[26] enables forensic laboratories to simultaneously obtain quantitative and qualitative information for human DNA samples in a single, highly sensitive real-time qPCR reaction. QFTrio has three targets all amplified in a TaqMan assay. The first target is a small autosomal (SA) 80 [bp]. The second is a large autosomal (LA) target 214 [bp] with a longer amplicon (>200 bases) to determine if a DNA sample is degraded. In addition, QFTrio contains a Y component that signifies the presence or non-presence of male DNA. The Y component of QFTrio can also help to determine

contamination in the sample collected from a known female. QFTrio and the HID v1.2 software is designed for use with the 7500 Real-Time qPCR System (Applied Biosystems). This system is highly sensitive and has a limit of detection of <1 pg/µL of nDNA. It quantifies nDNA concentrations from 0.005 ng/µL to >50 ng/µL. The THP (Thymidine-3',5'-Diphosphate) standard is a high molecular weight pooled male genomic DNA isolated from The THP1 monocyte cell line.

NovaQUANT (NQ) Real-Time mtDNA:nDNA ratio qPCR Assay

The NovaQUANT Human Mitochondrial to Nuclear DNA Ratio Kit is a real-time qPCR assay that compares quantities of mtDNA to nDNA. NQ can be used for low level quantities of DNA and the manufacturers claim that the primers do not amplify mitochondrial pseudogenes. NovaQUANT amplifies four gene in singleplex SYBR Green qPCR assays. Each sample uses four wells with one gene target in each well. There are two mtDNA gene targets (ND6 and ND1) and two nDNA targets (BECN1 and NEB). The kit provides four qPCR plates with pre-loaded lyophilized primers and probes for each of the four genes. NQ highlights the use of the relative qPCR method in its protocol, however there is a standard curve option (with the 143B standard DNA) which is what was utilized in this study. No prior NQ research has used the standard curve method.

Overview of Study

This study evaluated the concentration of NIST SRM 2372a via the Triplex, QFTrio, and NQ assays. In addition, eighteen low quantity, degraded human DNA bone extracts produced by M.F. Kavlick (MK) in 2017 [14] were used to compare the quantification values between the three assays. Furthermore, the previous (MK) and new contemporaneous results produced by the author, E.R. Cropper (EC) in 2019 Triplex and QF (Quantifiler) data were tested for concordance of quantification and degradation indices. Furthermore, a degradation index assessment was

conducted. Utilizing the quantification and degradation data obtained by the Triplex, Virtual copy number (VCN) determinations for each bone [14] were calculated for mtDNA HVR amplification. Subsequently, post amplification quantification data from the bones was examined to determine whether the quantity of DNA is adequate for sequencing.

There are seven questions of primary relevance of forensic DNA analysis that this project addresses. First, how accurately did the SRM 2372a via the Triplex, QFTrio, and NQ assays quantify to the NIST expected values? Second, were the concentrations of the various qPCR quantification standards as determined using SRM 2372a concordant with what was stated in their documentation? Third, was there concordance of the bone extract quantifications among the three qPCR assays tested? Forth, referring to the MIQE guidelines[28] for the optimum qPCR assay, how did the commercial assays compare with the non-commercial? Fifth, how did the previous bone Triplex data compare to the new data? Fifth, for end users of qPCR assays, what were the lessoned learned on the advantages and limits to the assays evaluated in this study?

CHAPTER II

MATERIALS AND METHODS

Quantification

NIST SRM 2372a

The NIST SRM 2372a components A(male), B(female), and C (1:3 male/female) were supplied solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3 propanediol hydrochloride (Tris HCI) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) pH 8.0 buffer (TE-4). Each tube contained approximately 55 µL of DNA solution [10]. Each sample was prepared to yield an optical density (OD) of 1.0 on a spectrophotometer when examined using a 260nm wavelength of light, corresponding to 50 micrograms of dsDNA per milliliter of solution [3,10]. The SRM serial dilutions had to fit within the range listed above depending standard serial dilutions of the Triplex, QFTrio, and NQ assays. The dilutions were prepared from NIST-designated concentrations of SRM A (49.8 ng/µL), SRM B (57.8ng/µL) SRM C (47.9 ng/µL). The first dilution in the series was a **1:10** dilution followed by six **3-fold** serial dilutions (**Figure 2).**

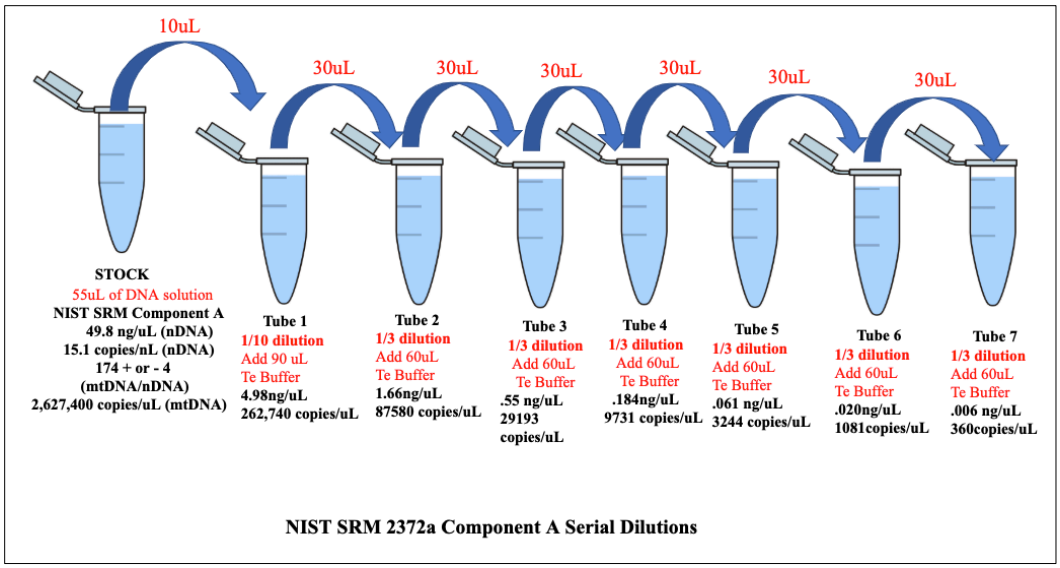


Figure 2: Example SRM A dilutions prepared from NIST-designated concentration (49.8ng/µL).
*The first dilution in the series was a **1:10** dilution followed by six **3-fold** serial dilutions.*

Three different dilution series (designated EC2, EC3, and MK) for each component were prepared. The three dilution series were separately assessed using the Triplex and QFTrio assays. Based on those results, the three dilution series were pooled (*SRM pooled*) prior to subsequent qPCR experiments on the bone extracts which were limited in volume.

Bone Extracts

Eighteen bone extracts were kindly provided by M.F. Kavlick who prepared them two years prior to this study according to an organic extraction method previously described [14]. Extracts were continuously stored at -20 °C in the interim.

mtDNA Triplex Real-Time qPCR assay

Paired forward and reverse oligonucleotides were separately reconstituted in tris EDTA (TE) buffer (10mM Tris-HCL, pH 8.0, 0.1 µM EDTA), quantified by absorbance at ~ 260nm and adjusted to 2µM [9,14]. The adjusted oligonucleotides were combined in equal proportions to generate a 1µM double-stranded, primary standard stock. The primary stock was diluted further

with TE to generate the first standard 10^7copies/µL, which was then serially diluted 10-fold to create the remaining standards of 10^6, 10^5, 10^4,10^3,10^2,10^1 copies/µL [14] (**Figure 3)**.

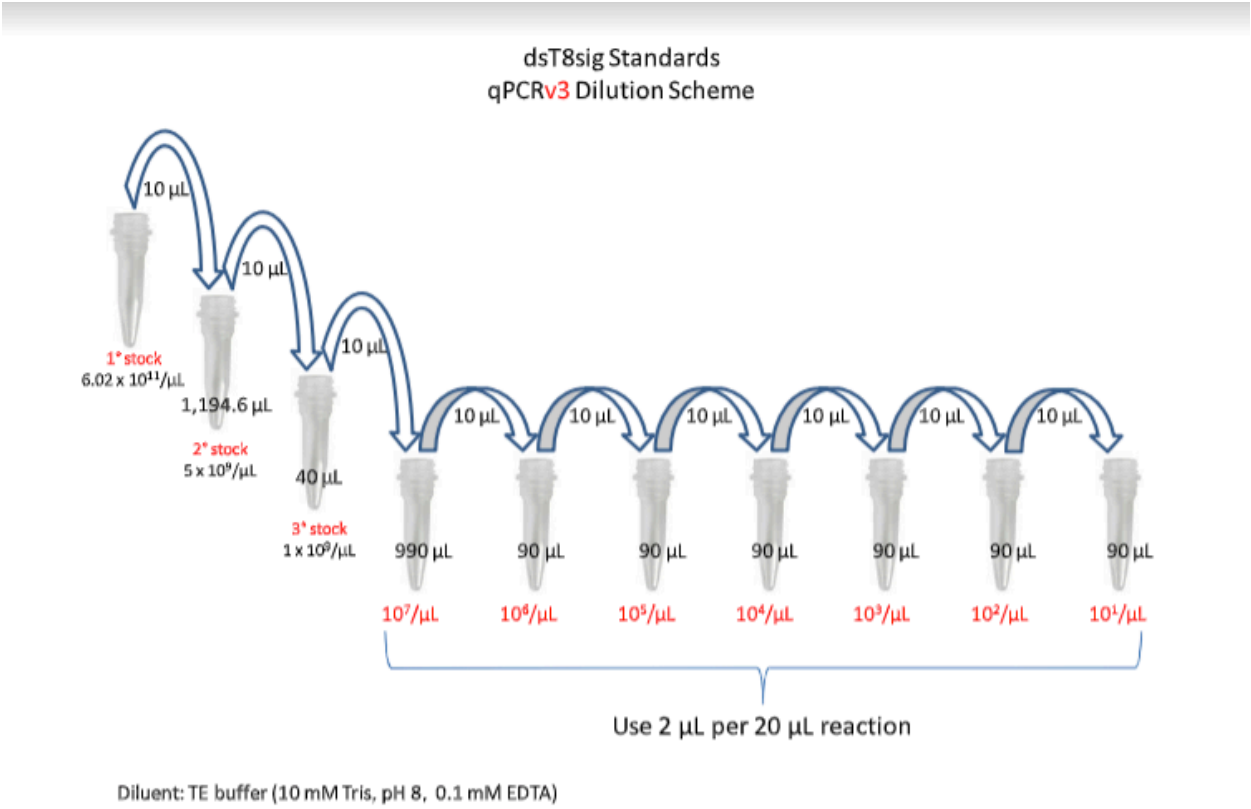


Figure 3: Triplex dilution series for dsT8sig standard.
Dilutions are mtDNA copies/µL ***10^7*** *(10,000,000 copies/µL)* ***10^6*** *(1,000,000 copies/µL)* ***10^5*** *(100,000 copies/µL)* ***10^4*** *(10,000 copies/µL)* ***10^3*** *(1000 copies/µL)* ***10^2*** *(100 copies/µL)* ***10^1*** *(10 copies/µL). Figure reproduced with permission by M.F. Kavlick.*

The assay combines absolute quantification of a short mtDNA target using the dsT8sig standard described above, relative quantification of a long mtDNA sequence target using the delta, delta Ct (ΔΔCt) method [need refs] for assessment of degradation, and qualitative assessment of a novel internal positive control [14].

Therefore, the Triplex assay is comprised of three simultaneous amplifications each containing HPLC purified primers and a 3'minor groove binder non-fluorescent quencher (MGB-NGQ). The first amplification is a FAM-labeled short (105 bp) target shown in (**Table 4).**

The region is the NADH dehydrogenase subunit 5 (ND5, positions 13,288~13,392 of the mtDNA revised Cambridge Reference Sequence (rCRS-GenBank:AC_000021 gi: 115315570).

Table 4: *Triplex Short (105bp) mtDNA target* [9]

Forward primer
Qfor8: GGC ATC AAC CAA CCA CAC CTA
Reverse primer
Qrev8: ATT GTT AAG GTT GTG GAT GAT GGA
Probe
QRL8: 6FAM CAT TCC TGC ACA TCT G MGBNFQ

The second amplification is a VIC-labeled long (316 bp) target in the 16S region listed in (**Table 5)** with nucleotide positions 2,332 ~ 2,647 of the mtDNA rCRS (GenBank accession no. NC_012920).

Table 5: *Triplex Long (316bp) mtDNA target in 16s rRNA Gene* [14]

Forward primer (5' - YGC ATA AGC CTG CGT CAG AT - 3')
Reverse primer (5' - CCC TCG TGG AGC CAT TCA TA - 3')
Probe (5' VIC - AAC ACA GGC ATG CYC - 3' MGB-NFQ)

Because the 16S target is 3 times longer (316bp) than the short 105 bp target, the amount of relative degradation can be determined by comparing the length of the long target to the short target. The delta, delta Ct (ΔΔCt) method [29]served as the degradation index (DI) as computed in the following equation[14]:

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator} = (Ct_{316} - Ct_{105})_{sample} - (Ct_{316} - Ct_{105})_{calibrator}$$

Briefly, the delta Ct (ΔCt) values were acquired by subtracting the short target Ct value from the long target Ct value for each sample. This was similarly executed for the HL60 calibrator (diluted to 20pg/μL) which is not degraded. This process compensates for potential differences in amplification efficiency of the two targets. The ΔCt value for the calibrator was subtracted from the ΔCt value of the sample which derives the ΔΔCt degradation index (DI) value. Samples with a ΔΔCt value of 0 was interpreted to have equal copies of both targets and therefore undegraded. Samples having ΔΔCt values of 1 were interpreted to have half as many large target

copies as the small target (minor degradation) and ΔΔCt values of 3 suggested 2^3 (8-fold) fewer large targets indicating a higher state of sample degradation [14].

The third amplification of the Triplex targets a synthetic double-stranded template not found in nature. The Internal Positive Control (IPC) (**Table 6)** was diluted in equimolar proportions to 1,250 copies/µL**.** The IPC may indicate the presence of inhibitors such as humic acid, tannic acid, melanin, and EDTA [8,9,14,30].

Table 6*:* Internal Positive Control (IPC) [14]

Forward primer (5'- CGC GAG ATA CAC TGC CAG AA - 3')
Reverse primer (5' - GAC CAC AGC CAG ATT AAA TTT ACC A - 3')
Probe (5' NED - TCC GCG TGA TTA CG - 3' MGB-NFQ)

Triplex qPCR reactions were performed using the components listed in (**Figure 4**) in a 20 µL reaction.

	Stock Concentrations	Volume Per Reaction	qPCR Reaction Final Concentrations
Short (105 bp) mtDNA Target in ND5 Gene [Kavlick 2011] †	(µM)	(µL)	(nM)
Forward primer (5' - GGC ATC AAC CAA CCA CAC CTA - 3')	1.25	0.8	50
Reverse primer (5' - ATT GTT AAG GTT GTG GAT GAT GGA - 3')	22.5	0.8	900
Probe (5' FAM - CAT TCC TGC ACA TCT G - 3' MGB-NFQ)	6.25	0.8	250
Long (316 bp) mtDNA Target in 16s rRNA Gene ††			
Forward primer (5' - YGC ATA AGC CTG CGT CAG AT - 3')	7.5	0.8	300
Reverse primer (5' - CCC TCG TGG AGC CAT TCA TA - 3')	22.5	0.8	900
Probe (5' VIC - AAC ACA GGC ATG CYC - 3' MGB-NFQ)	6.25	0.8	250
Internal Positive Control (IPC)			
Forward primer (5' - CGC GAG ATA CAC TGC CAG AA - 3')	1.25	0.8	50
Reverse primer (5' - GAC CAC AGC CAG ATT AAA TTT ACC A - 3')	7.5	0.8	300
Probe (5' NED - TCC GCG TGA TTA CG - 3' MGB-NFQ)	6.25	0.8	250
IPC ds template * (1,250 copies/µL)		0.8	1,000 copies/µL
Fast Advanced Master Mix, 2X (Applied Biosystems)		10	
qPCR Component Subtotal		18	
Standard [Kavlick 2011], samples, NTC, or Calibrator		2	
TOTAL Reaction		20	

† Corresponds to nucleotide positions 13,288 – 13,392 of the mtDNA rCRS (GenBank accession no. NC_012920).

†† Corresponds to nucleotide positions 2,332 – 2,647 of the mtDNA rCRS (GenBank accession no. NC_012920).

* Duplex of the following 65 bp sequence with its reverse complement in equimolar proportions

5' - CGC GAG ATA CAC TGC CAG AAA TCC GCG TGA TTA CGA GTC GTG GTA AAT TTA ATC TGG CTG TGG TC - 3'

Figure 4: Triplex qPCR assay Components and Reaction Set-up.
This includes primers, probes and IPC. 2µL of sample, standard, or calibrator were pipetted along with 18µL of master mix was pipetted into each well [14].Figure reproduced with permission from M.F. Kavlick.

The Triplex qPCR assay was performed on the dsT8sig standard, seven point standard dilution series ranging from 10^7 to 10^1 copies/µL, NIST SRM 2372a component serial dilutions A, B, and C, the calibrator HL60 (20 pg/µL), eighteen bone extracts, TE buffer as the no template control (NTC). Unless otherwise stated all qPCR reactions were amplified in duplicate on a 7500 Real-Time PCR System (Applied Biosystems) as a custom assay using the instrument's HID Real-Time PCR Software V1.2 with the following conditions: 2 minutes at 50 °C, 20 seconds at 95 °C followed by 40 cycles of 3 seconds at 95 °C and 30 seconds at 60 °C [8,14]. Analysis settings were set at 0.2 Ct threshold and a baseline of three.

Calculation of mtDNA target virtual copy numbers (VCNs)

The eighteen bone extracts are all in different states of degradation. Therefore, in order to determine their optimal mtDNA HVR amplification, their VCNs were calculated from the quantification and ΔΔCt values from the Triplex qPCR data. VCNs are derived from a calculation rather than absolute quantification or relative quantification hence their name of *virtual* copy numbers. The equation below shows how VCNs are calculated. (Kavlick,2018).

$$\text{copy number}_{n\ \text{bp}} = \text{copy number}_{105}/(2^{\text{DI}})^{((n\text{-}105)/(316\text{-}105))}$$

Once HVR targets were established for each of the eighteen bones, then HVR PCR amplification commenced.

HVR PCR Amplification

Eighteen bone extracts were diluted for optimal PCR amplification from among four mtDNA regions: whole control region (WCR) (1228 bp), hypervariable region 2 (HV2) (401 bp), hypervariable region B (HV2B) (276 bp), and the mini-primer 4 set (MP4) (108 bp). For each bone, the region for amplification was selected based on the qPCR data as described above. Each reaction contained molecular biology grade water, 10X PCR buffer containing magnesium ions (Applied Biosystems), bovine serum albumin (BSA), dNTP mix, forward and reverse primers, and AmpliTaq Gold (Applied Biosystems) following the DNA Casework Unit, FBI Laboratory protocol [31]. PCR reactions were amplified on a Proflex Thermal Cycler (Applied Biosystems, Foster City, CA) with the following conditions: (WCR) 9 minute hold at 95 °C; 36 cycles of 10 seconds at 95 °C, 30 seconds at 53°C, 30 seconds at 72°C; 10 minute hold at 72 °C then hold at 4°C indefinitely; (HV2 or HV2B) 9 minute hold at 95°C; (36 cycles) 10 seconds at 95°C, 30 seconds at 60°C, 30 seconds at 72°C; then hold at 4°C indefinitely; (MP4) 12 minutes hold at

95°C; (36 cycles) 15 seconds at 95°C, 30 seconds at 56°C, 45seconds at 72°C; then hold at 4°C. Amplicons were then purified by ExoSAP-IT with the following parameters: 15 minutes at 37 °C, 15 minutes at 80°C; then hold at 4°C indefinitely.

Post-PCR Quantification

PCR amplicons were sized and quantified using Agilent DNA 1000 and Agilent DNA 7500 kits on a 2100 Bioanalyzer using 2100 Expert software (Agilent Technologies, Inc., Waldbronn Germany).

nDNA QFTrio Real-Time qPCR assay

The QFTrio qPCR assay was performed according to the manufacturer's instructions on a 7500 Real-Time PCR System using HID Real-Time PCR Software V1.2 with the following conditions: denature at 95°C for 2 minutes, followed by 40 cycles of 9 seconds at 95°C, and 30 seconds at 60°C [26]. The following were assayed via QFTrio in duplicate: THP standard, a five point standard dilution series ranging from 50ng/µL to 0.005ng/µL; NIST SRM 2372a component serial dilutions A, B, and C; eighteen bone extracts; and NTCs. The reaction contained Quantifiler Trio Reaction Mix, Quantifiler Trio Primer Mix, and 10X buffer containing magnesium ions (Applied Biosystems).

NovaQUANT (NQ) Real-Time mtDNA:nDNA ratio qPCR Assay

Table 2 shows the relative method to calculate the mtDNA/nDNA copies using the Ct values [27]. **Table 7** shows the expected Ct values for the 143B DNA provided.

Table 7: *Expected Ct Values 143b DNA provided (from NQ instructions)* [27]

ND1	16.12 +/- 1.00
BECN1	25.12 +/- 1.00
ND6	16.76 +/- 1.00
NEB	25.01+/- 1.00

Rather than utilizing the relative method, for uniform comparisons of the SRM 2372a, absolute quantification using a standard curve was conducted with the NovaQUANT assay. The DNA

standard included with NQ was 143B DNA (10ng/µL), isolated from an osteosarcoma cell line. The first dilution of this standard series was a 1:5 dilution followed by four subsequent 8-fold dilutions, resulting in a dilution series which ranged from 10ng/µL to 0.5 pg/µL, alternatively, QFTrio THP DNA was also used for a standard dilution series which consisted of an initial 1:20 dilution followed by four 1:10 dilutions (**Figure 5).** The following were tested via NQ: 143B standard dilution series, THP standard dilution series, serial dilutions of NIST SRM 2372a components A, B, and C; fourteen bone extracts, and NTC (TE or QFTrio dilution buffer).

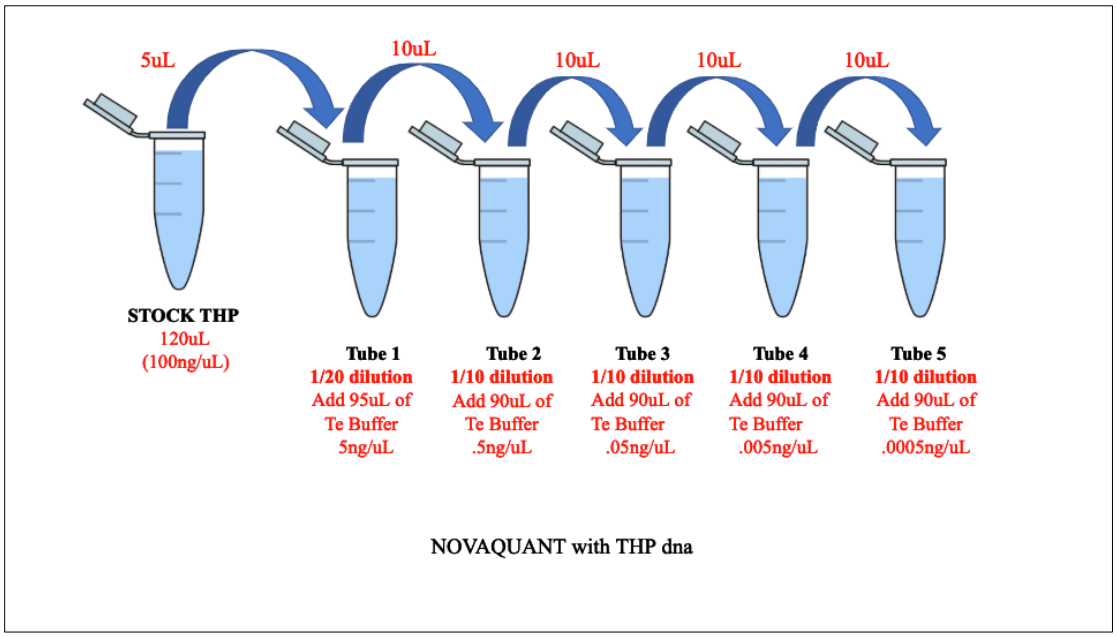


Figure 5: QFTrio THP for NQ dilutions prepared from starting concentration of (100ng/µL). *The initial 1:20 dilution followed by four 1:10 dilutions.*

The NQ assay was performed according to the manufacturer's instructions for each of the four targets in single reactions as described above. Briefly, NQ reactions contained 2 µL of standard, sample, or control, 8µL 10X dilution buffer (TE or QFTrio dilution buffer), and 10 µL Power SYBR Green PCR Mater Mix (Applied Biosystems).

QPCR reactions were amplified in duplicate on a 7500 Real-Time PCR System (Applied Biosystems) as a custom assay using HID Real-Time PCR Software V1.2 and the following

conditions: denature at 95 °C for 10 minutes, followed by 40 cycles of 15 seconds at 95 °C, and 1 minute at 64 °C [27].

CHAPTER III

RESULTS

Quantification

For the following results comparing qPCR assays, **Table 8** should be referenced.

Table 8: *NIST SRM2372a expected values +/- U95*

NIST EXPECTED VALUES +/- U95					
SRM 2372a component		**NIST Expected †**	**U95 +/- 2.5% ‡**	**NIST Lowest ^{L} interval value**	**NIST Highest ^{H} interval value**
A	mtDNA copies/µL	2,627,400	323,140	2,310,300	2,956,580
B	mtDNA copies/µL	3,605,000	448,000	3,165,750	4,061,750
C	mtDNA copies/µL	4,045,500	506,050	3,549,600	4,561,700
		NIST Expected †	**U95 +/- 10% ‡**	**NIST Lowest ^{L} interval value**	**NIST Highest ^{H} interval value**
A	nDNA copies/µL	15,100	1,510	13,590	16,610
B	nDNA copies/µL	17,500	1,750	15,750	19,250
C	nDNA copies/µL	14,500	1,450	13,050	15,950
		NIST Expected †	**U95 +/-10% ‡**	**NIST Lowest ^{L} interval value**	**NIST Highest ^{H} interval value**
A	ng/µL	49.8	5	44.8	54.8
B	ng/µL	57.8	5.8	52	63.6
C	ng/µL	47.9	4.8	43.1	52.7
		NIST Expected †	**U95 +/-2.5% ‡**	**NIST Lowest ^{L} interval value**	**NIST Highest ^{H} interval value**
A	mtDNA/nDNA	174	4	170	178
B	mtDNA/nDNA	206	5	201	211
C	mtDNA/nDNA	279	7	272	286

mtDNA copies/µL and corresponding U95 values were calculated by multiplying the nDNA copies/µL by the mtDNA/nDNA NIST expected values then multiplying the NIST expected mtDNA copies/µL by 2.5%. Lowest and Highest interval values were calculated by subtracting and adding the U95 value from the corresponding expected value, respectively. All remaining data were transcribed from Table 25 of the NIST publication [10]

† NIST expected values with 95% confidence to contain the true value of the measured
‡ Interval Value ± U95(Value) is believed, with 95 % confidence, to contain the true value of the measurand.
L Interval value low end of the 95% confidence interval.
H Interval value High end of the 95% confidence interval

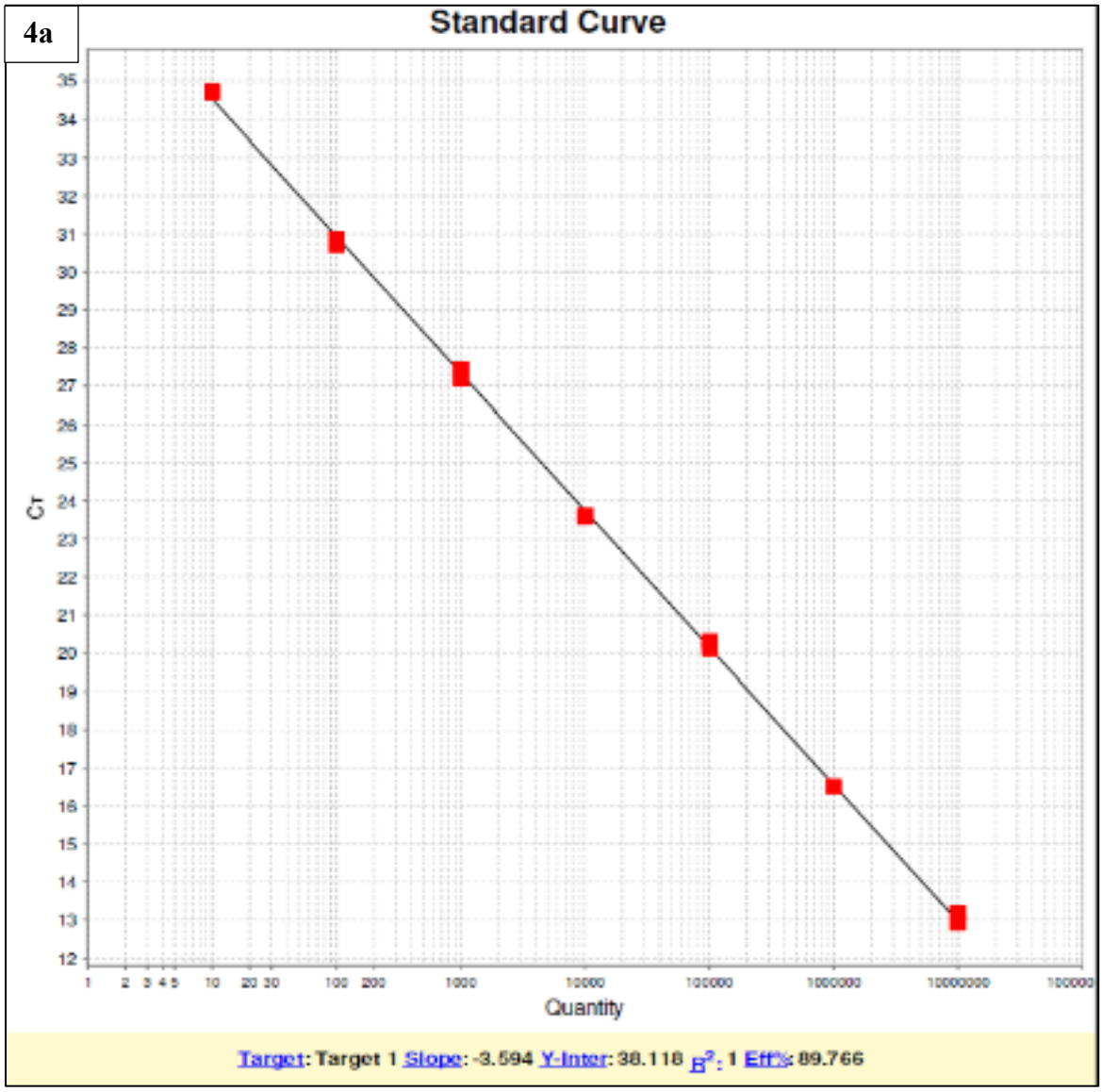
Triplex quantification of NIST SRM2372a EC2, EC3, and MK Dilutions (mtDNA copies/µL)

This set of experiments examined how accurately the three separate SRM dilution series (EC2, EC3, and MK) quantified with the Triplex. This tested intra-user variability and pipetting repeatability of the analyst. The null hypothesis stated that quantification results of the SRM were within the interval (U95 +/-2.5%) of the NIST expected values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. **Figure 6a** shows a representative Triplex standard curve from which the quantification values were determined. **Table 9** demonstrates that all EC2 values quantified within the expected interval. Therefore, the null hypothesis was accepted. With EC3, the SRM component B quantified lower than the expected interval, but components A and C quantified within the expected interval. The values for MK SRM component C quantified higher than the expected interval, but components A and B quantified within the expected interval. The CVs for EC2, EC3, and MK were all below 3%. This indicates high repeatability with little variation.

QFTrio quantification of NIST SRM2372a EC2, EC3, and MK Dilutions (nDNA ng/µL)

This experiment tested how accurately three separate SRM dilutions (EC2, EC3, and MK) quantified with the QFTrio SA. The null hypothesis stated that each SRM dilution (EC2, EC3, and MK) quantification results were within the interval (U95 +/-10%) of the NIST expected values. The alternative hypothesis stated that results were *not* within the interval. **Figure 6b** shows a representative QFTrio standard curve from which the quantification values were interpolated. The results (**Table 9)** demonstrate that the EC2 SRM components A and C quantified lower than the expected interval. Hence, the null hypothesis was rejected for A and C. With EC3, all three components quantified lower than the expected interval, therefore the null hypothesis was rejected. MK SRM components A and C quantified higher than the expected

interval, and B quantified within the expected interval. The CVs were 16.1% and below which indicates average assay repeatability with a moderate amount of variation.



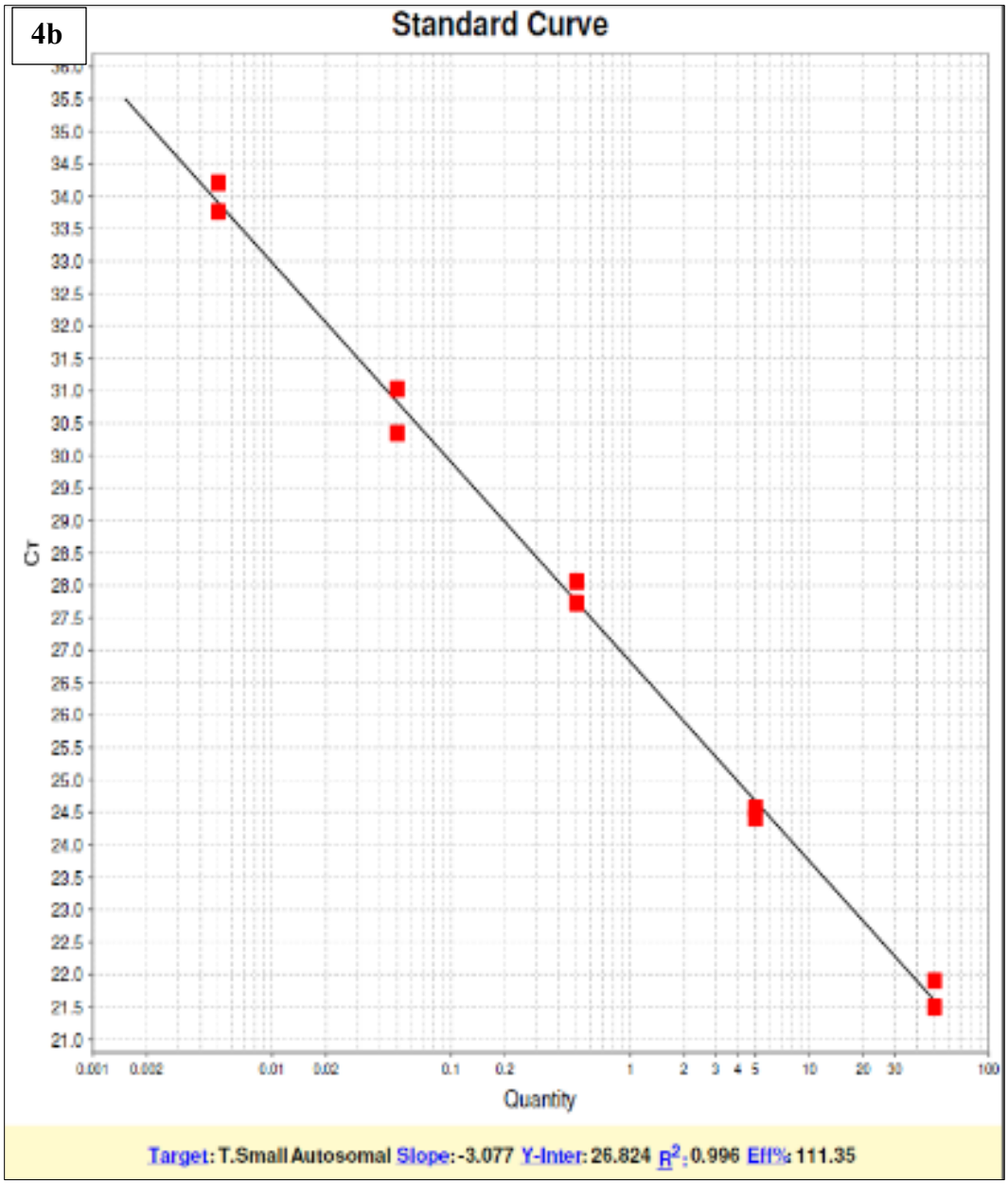


Figure 6. Representative standard curves for Triplex and QFTrio.
*The Triplex (**4a**) is amplified along a seven (7) point 10-fold serial dilution standard curve using a standard. QFTrio (**4b**) is amplified along a five (5) point 10-fold serial dilution standard curve using THP DNA. Ct is the amplification cycle at which the ΔRn crosses the predefined cycle threshold.*

Table 9: *Triplex & QFTrio SA quantification of NIST SRM2372a EC2, EC3, and MK Dilutions vs. NIST expected values +/- U95*

Triplex & QFTrio SA - EC2, EC3, MK SRM dilutions vs. NIST EXPECTED RECOMMENDED VALUES +/-U95 CV						
	Within NIST +/-U95					
	Lower than NIST +/-U95					
	Higher than NIST +/-U95					
SRM 2372a component	**Triplex EC2 copies/µL**	*Std dev*	*CV*	**NIST Expected † copies/µL**	**NIST Lowest ^{L} *I*nterval value U95 -2.5% copies/µL**	**NIST Highest ^{H} interval value U95 +2.5% copies/µL**
A	2,498,112	8,773	0.003	2,627,400	2,310,300	2,956,580
B	3,293,641	87,323	0.026	3,605,000	3,165,750	4,061,750
C	4,099,345	149,279	0.036	4,045,500	3,549,600	4,561,700
SRM 2372a component	**Triplex EC3 copies/µL**	*Std dev*	*CV*	**NIST Expected † copies/µL**	**NIST Lowest ^{L} interval value U95 -2.5% copies/µL**	**NIST Highest ^{H} interval value U95 +2.5% copies/µL**
A	2,467,099	35,484	0.014	2,627,400	2,310,300	2,956,580
B	2,955,571	2,881	0.000	3,605,000	3,165,750	4,061,750
C	4,326,486	163142	0.037	4,045,500	3,549,600	4,561,700
SRM 2372a component	**Triplex MK copies/µL**	*Std dev*	*CV*	**NIST Expected † copies/µL**	**NIST Lowest ^{L} interval value U95 -10% copies/µL**	**NIST Highest ^{H} interval value U95 +2.5% copies/µL**
A	2,788,921	29710	0.010	2,627,400	2,310,300	2,956,580
B	3,593,870	3054	0.000	3,605,000	3,165,750	4,061,750
C	4,884,907	87191	0.017	4,045,500	3,549,600	4,561,700
SRM 2372a component	**QFTrio SA EC2 ng/µL**	*Std dev*	*CV*	**NIST Expected † ng/µL**	**NIST Lowest ^{L} interval value U95 -10% ng/µL**	**NIST Highest ^{H} interval value U95 +10% ng/µL**
A	37.1	1.3	0.036	49.8	44.8	54.8
B	52.3	6.0	0.115	57.8	52.0	63.6
C	37.0	2.0	0.054	47.9	43.1	52.7
SRM 2372a component	**QFTrio SA EC3 ng/µL**	*Std dev*	*CV*	**NIST Expected † ng/µL**	**NIST Lowest ^{L} interval value U95 -10% ng/µL**	**NIST Highest ^{H} interval value U95 +10% ng/µL**
A	42.9	1.5	0.035	49.8	44.8	54.8
B	48.1	3.0	0.062	57.8	52.0	63.6
C	41.4	2.0	0.048	47.9	43.1	52.7
SRM 2372a component	**QFTrio SA MK ng/µL**	*Std dev*	*CV*	**NIST Expected † ng/µL**	**NIST Lowest ^{L} interval value U95 -10% ng/µL**	**NIST Highest ^{H} interval value U95 +10% ng/µL**
A	54.9	3.8	0.070	49.8	44.8	54.8
B	62.15	10.0	0.161	57.8	52	63.6
C	53	3.0	0.057	47.9	43.1	52.7
SA = Small Autosomal						
EC2 = SRM 2nd dilution series constituted by EC run in duplicate						
EC3 = SRM 3rd dilution series constituted by EC run in duplicate						
MK = SRM 3rd dilution series constituted by MK run in duplicate						
† NIST expected values with 95% confidence to contain the true value of the measured						
L Interval value low end of the 95% confidence interval.						
H Interval value High end of the 95% confidence interval						
CV coefficient of variation of the mean of all replicates of (EC2, EC3, MK SRM dilutions)						
Std dev standard deviation from the mean of all replicates of (EC2, EC3, MK SRM dilutions)						

Triplex quantification of NIST SRM2372a Pooled Dilutions (mtDNA copies/µL)

This experiment examined how accurately the NIST SRM 2372a (all three components) quantified with the Triplex. The null hypothesis stated that quantification results of the SRM were within the interval (U95 +/-2.5%) of the NIST expected values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. The results (**Table 10)** demonstrate that SRM component A and C were within the expected interval and therefore, the null hypothesis was accepted. The null hypothesis was rejected for SRM component B since it was not within the expected interval. The % dif values (deviation from NIST expected values) for SRM component A and C were within +/- 10% which indicates a high accuracy in measurement of quantification.

QFTrio SA quantification of NIST SRM 2372a Pooled Dilutions (nDNA copies/µL and ng/µL)

This experiment examined how accurately the SRM (all three components) quantified with QFTrio SA. The null hypothesis stated that quantification results of the SRM were within the interval (U95 +/-10%) of the NIST expected values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. The results in **Table 10** show all values were below the expected interval. Therefore, the null hypothesis was rejected. The % dif (deviation from the NIST expected values) were below 15% which indicates an average quantification measurement accuracy.

Triplex/QFTrio quantification of NIST SRM 2372a Pooled Dilutions (mtDNA/nDNA)

This experiment examined how accurately the SRM (all three components) quantified with the Triplex/QFTrio. The null hypothesis stated that the SRM quantification results were within the interval (U95 +/-2.5%) of the NIST expected values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. The results (**Table 10)** demonstrate that all values were above the expected interval. Therefore, the null hypothesis was rejected. The Triplex/QFTrio

ratio was a reflection of the individual quantifications of the Triplex and QFTrio. Because the QFTrio quantified lower, it is reasonable to expect a higher mtDNA/nDNA ratio.

Table 10: *Triplex & QFTrio quantification of NIST SRM2372a Pooled Dilutions vs NIST expected values +/- U95*

TRIPLEX & QFTRIO quantification of NIST SRM2372a Pooled Dilutions vs. NIST expected values					
		Within NIST +/-U95			
		Lower than NIST +/-U95			
		Higher than NIST +/-U95			
SRM 2372a component	**Triplex copies/µL Mean Pooled**	*% dif*	**NIST Expected † mtDNA copies/µL**	**NIST Lowest [L] interval value U95 -2.5% copies/µL**	**NIST Highest [H] interval value U95 +2.5% copies/µL**
A	2,376,544	-0.095	2,627,400	2,310,300	2,956,580
B	3,162,019	-0.123	3,605,000	3,165,750	4,061,750
C	4,443,067	0.098	4,045,500	3,549,600	4,561,700
SRM 2372a component	**QFTrio SA copies/µL Mean Pooled**	*% dif*	**NIST Expected † nDNA copies/µL**	**NIST Lowest [L] interval value U95 -10% ng/µL**	**NIST Highest [H] interval value U95 +10% ng/µL**
A	12678	-0.160	15,100	13,590	16610
B	13598	-0.223	17,500	15,750	19250
C	11239	-0.225	14,500	13,050	15950
SRM 2372a component	**QFTrio SA ng/µL Mean Pooled**	*% dif*	**NIST Expected † nDNA ng/µL**	**NIST Lowest [L] interval value U95 -10% ng/µL**	**NIST Highest [H] (h) interval value U95 +10% ng/µL**
A	41.8	-0.160	50	44.8	54.8
B	45	-0.223	58	52	63.6
C	37.1	-0.225	48	43.1	52.7
SRM 2372a component	***Triplex/QFTrio SA* Mean Pooled**	*% dif*	**NIST Expected † mtDNA/nDNA**	**NIST Lowest [L] interval value U95 -2.5% copies/µL**	**NIST Highest [H] interval value U95 +2.5% copies/µL**
A	187	0.077	174	170	178
B	233	0.129	206	201	211
C	395	0.417	279	272	286
SA = Small Autosomal					
pooled Combined SRM dilutions (EC2, EC3, MK) conducted in duplicate					
Mean pooled mean of pooled qPCR runs of the assay listed					
† NIST expected values with 95% confidence to contain the true value of the measured					
L Interval value low end of the 95% confidence interval.					
H Interval value High end of the 95% confidence interval					
% dif percent difference of the Mean of pooled vs NIST expected values					

NQ quantification of NIST SRM 2372a Pooled Dilutions (mtDNA/nDNA)

This experiment examined how accurately the SRM (all three components) quantified with NQ. The null hypothesis stated the SRM quantification results were within the interval (U95 +/-

10%) of the NIST expected values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. **Figure 7** shows a representative NQ standard curve from which the quantification values were determined. Several issues were initially experienced with the NQ assays. These included 1) the three SRM components quantified much lower than the NIST-recommended values, 2) the Ct values for the three NQ targets (ND6, NEB, and BECN1) were higher than expected, 3) the fourth target and one of the two mtDNA targets (ND1) did not amplify (**Figure 8),** and 4) ΔRn values for one of two nDNA targets (NEB) was low. These issues were reported to Millipore and a new kit with a different lot number was provided; however, those issues persisted. After troubleshooting, it was determined that the 143B DNA had a concentration much lower than the manufacturer reported which resulted in the first two issues described. Therefore, it was decided to use the THP DNA (utilized in the QFTrio assay) for the NQ standard curve instead of the 143B DNA supplied in the NQ kit. Issues 3 and 4 appear to be manufacturing defects. However, issue 4 was mitigated by lowering the cycle threshold from the manufacturer's recommendation of 0.2 to 0.02 and those results are reported here. Unfortunately, ND1 data (issue 3) could not be obtained and is not reported. The results for this are shown in **Table 11.** With ND6, SRM component A quantified lower than expected, while SRM components B and C quantified within the expected interval. Both nDNA targets, NEB and BECN1, quantified higher than expected while the ND6/NEB ratio quantified lower than expected. The CVs for ND6 ranged from 10-18% suggesting moderate amounts of variation while the CVs for NEB and BECN1 range from 14-29.8% suggesting moderate to high variation.

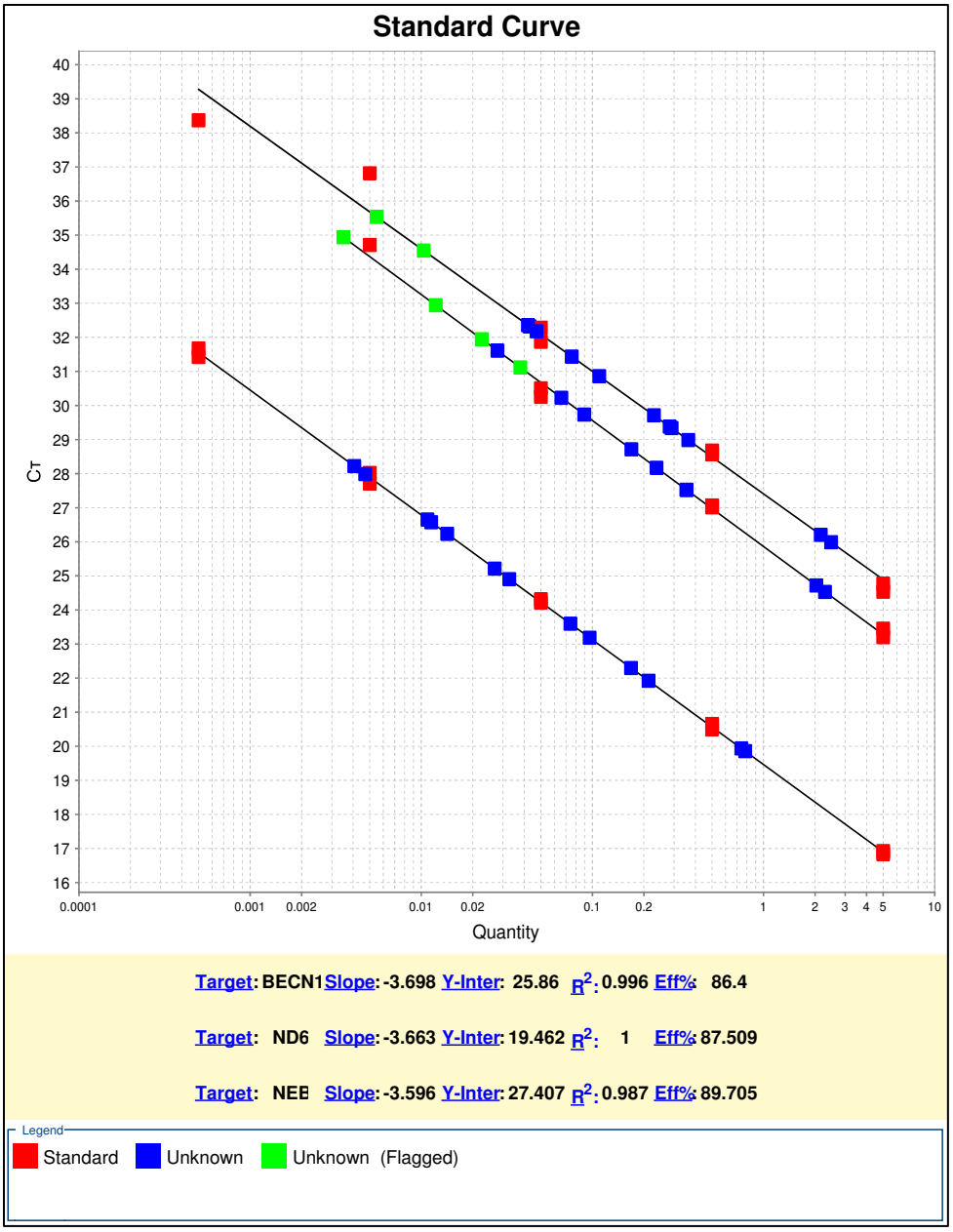


Figure 7: NQ Standard Curve with THP as standard (SRM A + Bone 08).
Three gene targets ND6, NEB, BECN1 (ND1 not shown because of amplification failure) amplified along a 5 point 10-fold serial dilution standard curve using THP DNA.

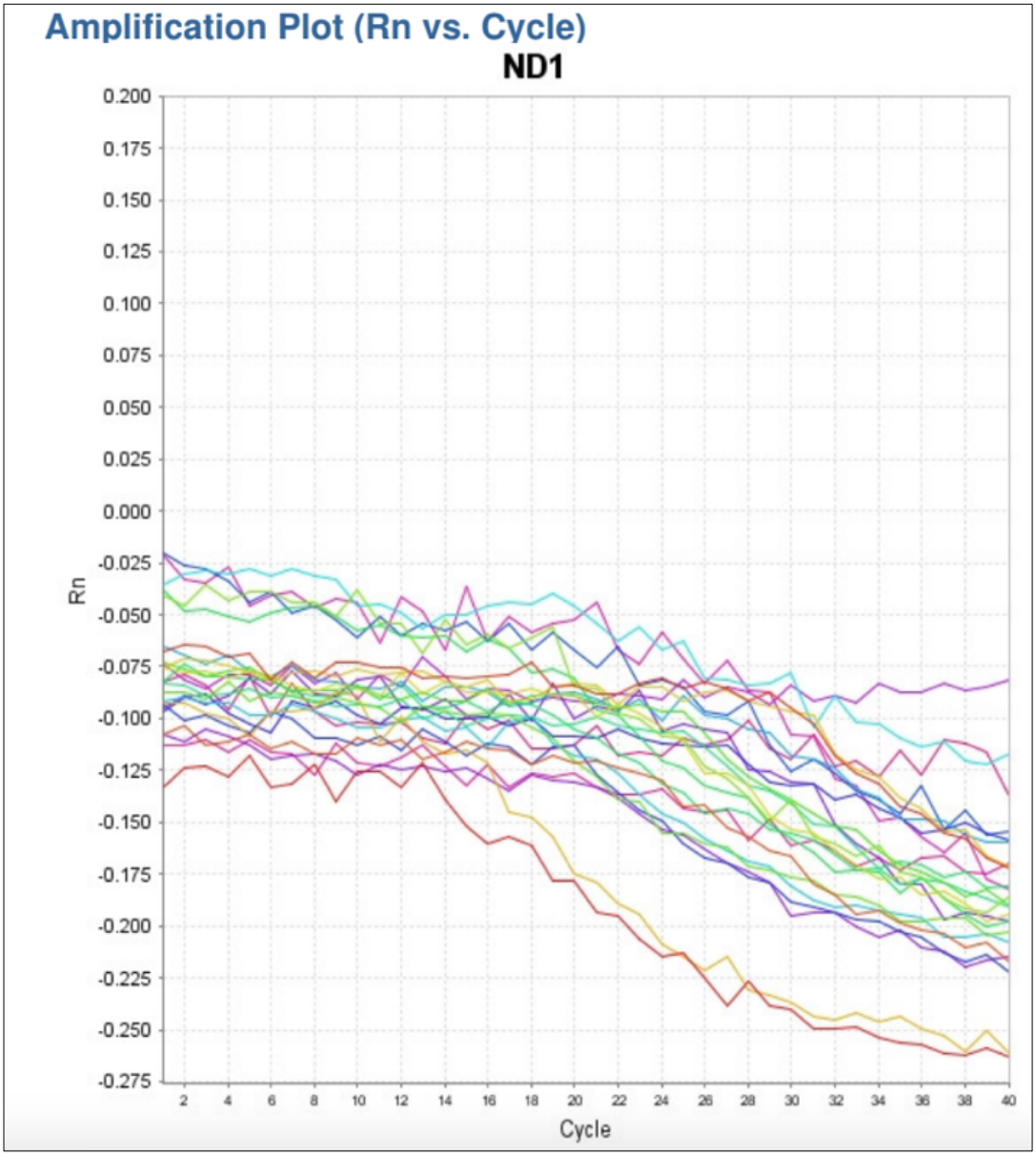


Figure 8: NQ ND1 Amplification Failure.
Shown is a representative qPCR in which ND1 failed to amplify for all standards and samples. All plots were similar exhibiting a flat Rn which was <0.0 over the 40 cycles.

Table 11: *NQ quantification of NIST SRM2372a Pooled Dilutions vs. NIST expected values +/- U95*

NQ vs. NIST Expected VALUES +/-U95							
	Within NIST +/-U95						
	Lower than NIST +/-U95						
	Higher than NIST +/-U95						
SRM 2372a component	**mtDNA (ND6) copies/µL Mean Pooled**	*Std dev*	*CV*	*% dif*	**NIST Expected † copies/µL**	**NIST Lowest [L] interval value U95 - 2.5% copies/µL**	**NIST Highest [H] interval value U95 +2.5% copies/µL**
A	1,628,990	265,527	0.163	0.380	2,627,400	2,310,300	2,956,580
B	3,708,244	681,130	0.184	0.029	3,605,000	3,165,750	4,061,750
C	3,885,718	405,181	0.104	0.039	4,045,500	3,549,600	4,561,700
SRM 2372a component	**nDNA (NEB) ng/µL Mean Pooled**	*Std dev*	*CV*	*% dif*	**NIST Expected † ng/µL**	**NIST Lowest [L] interval value U95 - 10% ng/µL**	**NIST Highest [H] interval value U95 +10% ng/µL**
A	68.2	25.6	0.375	0.369	50	44.8	54.8
B	117.9	28.8	0.244	1.040	58	52	63.6
C	105.1	29.8	0.284	1.194	48	43.1	52.7
SRM 2372a component	**nDNA (BECN1) ng/µL Mean Pooled**	*Std dev*	*CV*	*% dif*	**NIST Expected † ng/µL**	**NIST Lowest [L] interval value U95 - 10% ng/µL**	**NIST Highest [H] interval value U95 +10% ng/µL**
A	57.5	14.2	0.246	0.155	50	44.8	54.8
B	91.9	26.5	0.288	0.590	58	52	63.6
C	73.2	20.6	0.281	0.528	48	43.1	52.7
SRM 2372a component	**mtDNA (ND6)/nDNA (NEB) Mean Pooled**	*Std dev*	*CV*	*% dif*	**NIST Expected † mtDNA/nDNA**	**NIST Lowest [L] interval value U95 - 2.5%**	**NIST Highest [H] interval value U95 +2.5%**
A	88	34.5	0.392	0.494	174	170	178
B	108	36.4	0.336	0.474	206	201	211
C	131	20.3	0.155	0.530	279	272	286

NQ NovaQUANT
*ND6 mt*DNA gene in the NovaQUANT qPCR assay
NEB nDNA gene in the NovaQUANT qPCR assay
BECN1 nDNA gene in the NovaQUANT qPCR assay
ND1 mtDNA gene not listed due to qPCR amplification failure
pooled Combined SRM dilutions (EC2, EC3, MK) conducted in duplicate
Mean pooled mean of pooled qPCR runs of the assay listed
† NIST expected values with 95% confidence to contain the true value of the measured
L Interval value low end of the 95% confidence interval.
H Interval value High end of the 95% confidence interval
% dif percent difference of the Mean of pooled vs NIST expected values
CV coefficient of variation of the mean of all pooled replicates
Std dev standard deviation from the mean of all pooled replicates

QFTrio SA/LA quantification of NIST SRM 2372a Pooled Dilutions (nDNA ng/µL) – NIST Expected

This experiment examined how accurately the SRM quantified with the QFTrio SA and LA targets. The null hypothesis stated that quantification results of the SRM were within the interval

(U95+/-10%) of the *NIST-expected* values (**Table 8**). The alternative hypothesis stated that results were *not* within the interval. The results (**Table 12)** demonstrate that all three SRM components using the QFTrio SA quantified lower than expected. The QFTrio LA, SRM components A and B quantified within the interval, while component C quantified higher than the interval. There was a less of a % dif (compared to QFTrio SA) between the QFTrio LA and the NIST expected values, indicating a higher measurement of accuracy.

Table 12: *QFTrio SA/LA quantification of NIST SRM 2372a Pooled Dilutions vs. NIST expected values +/- U95 CV*

QFTrio SA/LA vs. NIST EXPECTED VALUES +/-U95	
	Within NIST +/-U95
	Lower than NIST +/-U95
	Higher than NIST +/-U95

SRM 2372a component	**QFTrio SA Mean Pooled runs ng/µL**	*% dif*	**NIST Expected** †	**NIST Lowest ^{L} interval value U95 - 10%**	**NIST Highest ^{H} interval value +10%**
A	41.8	-0.160	50	44.8	54.8
B	45	-0.223	58	52	63.6
C	37.1	-0.225	48	43.1	52.7

SRM 2372a component	**QFTrio LA Mean Pooled runs ng/µL**	*% dif*	**NIST Expected** †	**NIST Lowest ^{L} interval value U95 - 10%**	**NIST Highest ^{H} interval value +10%**
A	49.9	0.001	50	44.8	54.8
B	61.5	0.063	58	52	63.6
C	53.7	0.121	48	43.1	52.7

SA = Small Autosomal
LA = Large Autosomal
pooled Combined SRM dilutions (EC2, EC3, MK) run in duplicate
Mean pooled mean of pooled qPCR runs
† NIST expected values with 95% confidence to contain the true value of the measured
L Interval value low end of the 95% confidence interval.
H Interval value High end of the 95% confidence interval
% dif percent difference of the Mean of pooled vs NIST expected values

QFTrio SA/LA/Y quantification of NIST SRM 2372a Pooled Dilutions (nDNA ng/µL) NIST-Tested QFTrio

This experiment examined how accurately the SRM quantified with QFTrio SA, LA, and Y targets. The null hypothesis stated that quantification results of the SRM were within the interval (U95 +/-10%) of the *NIST-Tested* QFTrio SA, LA, and Y values. The alternative hypothesis

stated that results were *not* within the interval. The results (**Table 13)** demonstrate that all three components using the QFTrio SA quantified lower than the NIST-*tested* results. All three components using the QFTrio LA quantified higher than the NIST-*tested*. The QFTrio Y quantified SRM component A higher than the NIST tested and SRM component C lower than the NIST tested. The 3% difference demonstrates QFTrio Y quantified SRM component C most accurately compared to the NIST tested while the 42.9% difference between QFTrio LA component B and the NIST tested showed the least amount of accuracy. The consistency by which the QFTrio SRM components quantified lower compared to the NIST-*tested* and NIST-expected throughout this study is important to note.

Table 13: *QFTrio SA/LA/Y quantification of NIST SRM 2372a Pooled Dilutions vs NIST Tested QFTrio SA/LA/Y*

QFTri SA, LA, Y vs. NIST TESTED VALUES +/-U95					
	Within NIST +/-U95				
	Lower than NIST +/-U95				
	Higher than NIST +/-U95				
SRM 2372a component	**QFTrio SA Mean Pooled ng/µL**	*% dif*	**NIST [T] QFTrio SA**	**NIST Lowest [L] interval value U95 - 10%**	**NIST Highest [H] interval value +10%**
A	41.8	-0.106	46.8	42.1	51.5
B	44.9	-0.111	50.5	45.5	55.6
C	37.1	-0.291	52.3	47.1	57.5
SRM 2372a component	**QFTrio LA Mean Pooled ng/µL**	*% dif*	**NIST [T] QFTrio LA**	**NIST Lowest [L] Interval value U95 -10% ng/µL**	**NIST Highest [H] interval value U95 +10% ng/µL**
A	49.9	0.378	36.2	32.58	39.82
B	61.5	0.429	43	38.7	47.3
C	53.7	0.421	37.8	34.02	41.58
SRM 2372a component	**QFTrio Y Mean Pooled ng/µL**	*% dif*	**NIST [T] QFTrio Y**	**NIST Lowest [L] interval value U95 -10% ng/µL**	**NIST Highest [H] interval value U95 +10% ng/µL**
A	45.7	0.229	37.2	33.48	40.92
B	ND	ND	ND	ND	ND
C	11.4	0.035	11.0	9.9	12.1
SA = Small Autosomal					
LA = Large Autosomal					
Y=Male specific assay					
pooled Combined SRM dilutions (EC2, EC3, MK) run in duplicate					
Mean pooled mean of pooled qPCR runs of the assay listed					
T NIST tested values of QFTrio SA/LA/Y with 95% confidence to contain the true value of the measured					
L Interval value low end of the 95% confidence interval.					
H Interval value High end of the 95% confidence interval					
% dif percent difference of the Mean of pooled vs NIST tested values					
ND Not detected Component B was prepared using tissue from only female donors; it's not expected to respond to male-specific assays					

mtDNA Triplex vs NQ (ND6) quantification (mtDNA copies/µL)

This experiment (**Table 14)** examined concordance via correlation testing among DNA concentrations of fourteen bone extracts between the Triplex and the NQ (ND6). The null hypothesis stated that DNA concentration results of the Triplex were concordant with NQ (ND6). The alternative hypothesis stated that results were *not* concordant. There was a correlation of 81% (**Figure 9),** between the Triplex and NQ (ND6) DNA concentration. In addition, a t-test performed also confirmed they were not significantly different with a p-value of .783 so the null hypothesis was accepted that the two values were concordant. **Figure 10** directly compares Triplex and ND6 DNA concentration for each bone showing a low standard deviation

between the replicates. (**Table 14)** The Triplex CVs were ≤ 7.9% with an average of 3.6%, while the NQ (ND6) CVs ranged from 57.8% to 0.01% with an average of 16.9% showing a higher variation with NQ (ND6). In addition, on average, NQ (ND6) quantified higher than the Triplex by 62.6%, which may not be surprising given the variance in the data, especially for the NQ assay.

Table 14: *mtDNA Triplex vs NQ (ND6) quantification (copies/µL)*

mtDNA Quantification of Bone Extracts Triplex vs. NQ (ND6)								
	Sample Name	Triplex copies/µL	Triplex Std dev	Triplex CV	NQ (ND6) copies/µL	NQ (ND6) Std dev	NQ (ND6) CV	% dif Triplex vs NQ (ND6)
1	Bone8	2444	97	4.0%	969	560	57.8%	60%
2	Bone9	1025	18	1.8%	9077	497	5.5%	786%
3	Bone10	4259	311	7.3%	5873	3158	53.8%	38%
4	Bone11	2953	117	4.0%	2775	328	11.8%	6%
5	Bone15	2981	117	3.9%	4015	486	12.1%	35%
6	Bone16	523	33	6.2%	536	44	8.2%	3%
7	Bone19	1680	20	1.2%	277	89	32.1%	84%
8	Bone20	1605	84	5.2%	765	39	5.1%	52%
9	Bone25	797	2	0.2%	574	30	5.2%	28%
10	Bone31	183	1	0.8%	166	18	10.8%	9%
11	Bone33	480	6	1.2%	570	67	11.8%	19%
12	Bone37	2796	77	2.8%	5809	8	0.1%	108%
13	Bone41	952	40	4.2%	1584	86	5.4%	66%
14	Bone45	42990	3409	7.9%	50505	*	*	*
			Avg CV	**3.60%**		**Avg CV**	**16.90%**	**62.60%**
	ND neither replicate quantifed							
	* one replicate could not be quantified therefore Std dev and CV could not be calculated							
	ND1 mtDNA gene not listed due to qPCR amplification failure							
	SA small autosomal							
	CV coefficient of variation of the mean							
	Std dev standard deviation of the mean							
	pg/uL picograms per microliter							
	% dif percent difference between Triplex and NQ (ND60							

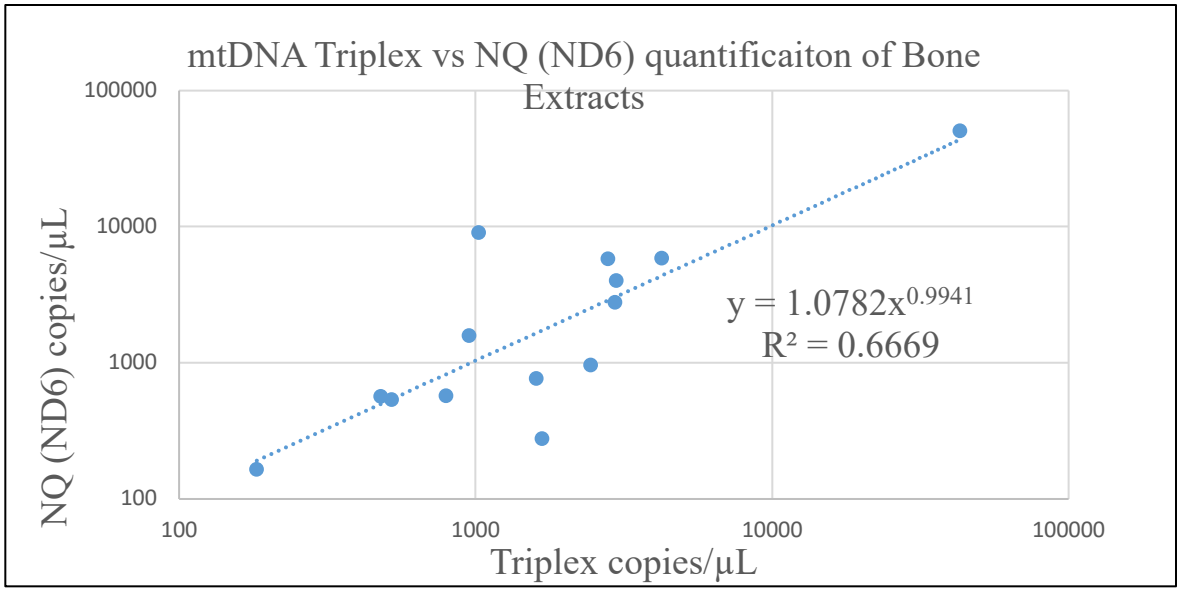


Figure 9: Triplex vs NQ (ND6) Quantification Linear Plot
A strong positive correlation was demonstrated between Triplex and NQ (ND6) quantification data from fourteen bone samples. The R^2 value of 0.6669 signifies the correlation of 81%. The Triplex and NQ (ND6) quantification results were not significantly different (F-test for variances, p= 0.277; t-test assuming equal variances, p=0.783)

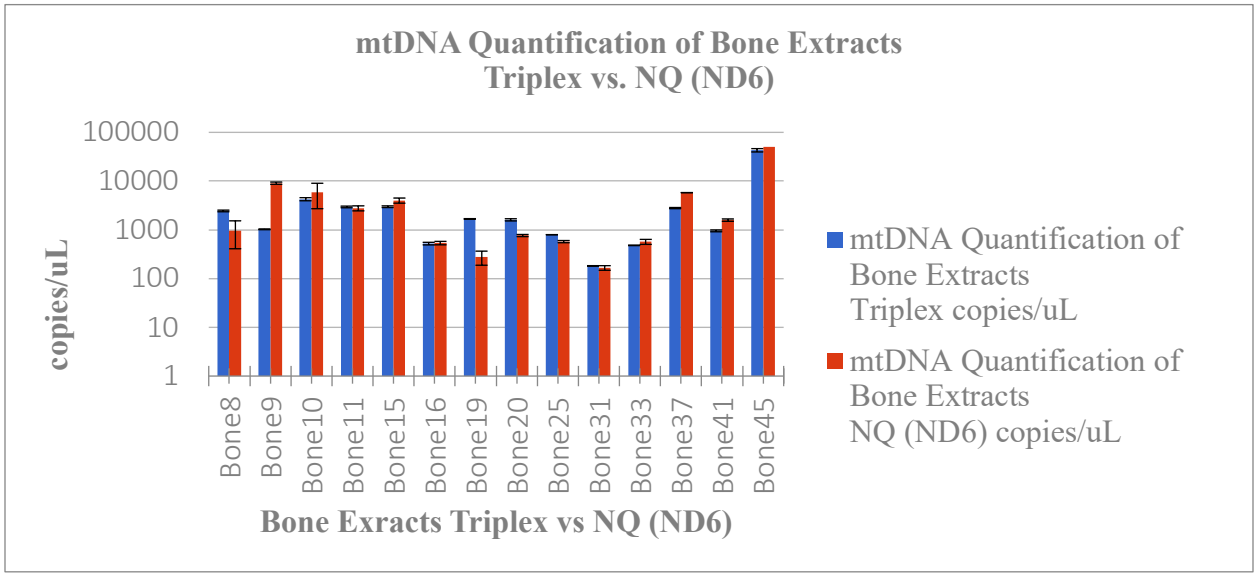


Figure 10: mtDNA Triplex vs NQ (ND6) (copies/µL)
Standard deviation is displayed as error bars.

nDNA QFTrio SA vs NQ (NEB) and NQ (BECN1) quantification (nDNA ng/µL)

This experiment (**Table 15)** examined concordance via correlation testing among DNA concentration of fourteen bone extracts between QFTrio SA and NQ (NEB), QFTrio SA and NQ (BECN1), and NQ (NEB) and NQ (BECN1). The null hypothesis stated that the DNA

concentration results of the QFTrio SA were concordant with those of the NQ (NEB) and NQ (BECN1). The alternative hypothesis stated that results were *not* concordant. The results demonstrated (**Figure 11a)** a 63% moderate correlation between QFTrio and NQ (NEB). In addition, a t-test performed also confirmed they were not significantly different with a p-value of .763 (alpha= .05) so the null hypothesis was accepted that the two values were concordant. **Figure 11b** demonstrates a strong correlation of 73% between QFTrio SA and NQ (BECN1). However, a t-test performed confirmed they were significantly different with a p-value of .039 so the null hypothesis was rejected that the two values were concordant. **Figure11c** demonstrates a 93% strong correlation between NQ (NEB) and NQ (BECN). However, a t-test performed confirmed they were significantly different with a p-value of .032 so the null hypothesis was rejected that the two values were concordant. The QFTrio SA CV of 18% indicated moderate variation hence average repeatability, while the NQ (NEB) and NQ (BECN1) CVs of 46.2% and 38%, respectively, indicated extremely high variation and therefore very poor repeatability (**Table 15).** This variation was also demonstrated in **Figure 12** showing varying standard deviations. In addition, when comparing the percentage differences between the assays, all three varied from each other substantially. This high amount of variation could also be due to stochastic effects related to low template DNA having a concentration of 100 pg/µL or less. All bone extracts in this study contained low template DNA [31]. Also, NQ (BECN1) quantified lower than NQ (NEB) and QFTrio SA which could indicate a lack of sensitivity of the assay to the NQ (BECN1) target.

Table 15: *nDNA QFTrio SA vs NQ (NEB) and NQ (BECN1) quantification (nDNA ng/µL)*

nDNA Quantification of Bone Extracts QFTrio (SA), NQ (NEB), and NQ (BECN1)													
	Sample Name	QFTrio SA pg/µL	QFTrio SA Std dev	QFTrio SA CV	NQ (NEB) pg/µL	NQ (NEB) Std dev	NQ (NEB) CV	NQ (BECN1) pg/µL	NQ (BECN1) Std dev	NQ (BECN1) CV	% dif (pg/µL) QFTrio SA/ NEB	% dif (pg/µL) QFTrio SA/ BECN1	% dif (pg/µL) NEB/ BECN1
1	Bone8	67.1	1.450	2.2%	45.4	3.358	7.4%	21.3	9.259	43.4%	32%	68%	53%
2	Bone9	12.1	1.840	15.2%	6.5	0.732	11.3%	4.0	1.384	34.41%	47%	67%	38%
3	Bone10	18.9	1.260	6.7%	54.8	38.248	69.8%	18.5	4.684	25.32%	190%	2%	66%
4	Bone11	22.7	0.090	0.4%	12.0	9.030	75.3%	6.0	4.710	78.37%	47%	74%	50%
5	Bone15	4.6	0.750	16.4%	17.3	2.760	15.9%	9.8	*	*	280%	114%	44%
6	Bone16	16.0	0.640	4.0%	21.0	21.787	103.6%	10.3	1.812	17.57%	31%	36%	51%
7	Bone19	46.0	14.450	31.4%	21.3	6.774	31.8%	11.9	4.896	41.24%	54%	74%	44%
8	Bone20	43.2	1.670	3.9%	27.2	6.830	25.1%	10.0	1.016	10.18%	37%	77%	63%
9	Bone25	13.2	2.890	21.8%	9.9	1.337	13.5%	3.3	2.529	75.49%	25%	75%	66%
10	Bone31	2.3	0.710	30.3%	5.0	1.494	30.1%	1.0	*	*	112%	55%	79%
11	Bone33	6.5	0.130	2.0%	2.9	2.870	98.4%	ND	ND	ND	55%	ND	ND
12	Bone37	2.5	1.440	56.7%	2.7	2.633	99.2%	1.7	0.43578	26.25%	4%	35%	37%
13	Bone41	6.0	0.260	4.3%	15.5	3.017	19.5%	1.7	0.48939	29.31%	158%	72%	89%
14	Bone45	16.0	9.060	56.6%	7.5	*	*	3.1	*	*	53%	81%	59%
			Avg CV	**18%**		**Avg CV**	**46%**		**Avg CV**	**38%**	**80%**	**64%**	**57%**
ND neither replicate quantifed													
* one replicate could not be quantified therefore Std dev and CV could not be calculated													
ND1 mtDNA gene not listed due to qPCR amplification failure													
SA small autosomal													
CV coefficient of variation of the mean													
Std dev standard deviation of the mean													
pg/uL picograms per microliter													
% dif percent difference between quantification (pg/uL) (SA/NEB), (SA/BECN1), (NEB/BECN1)													

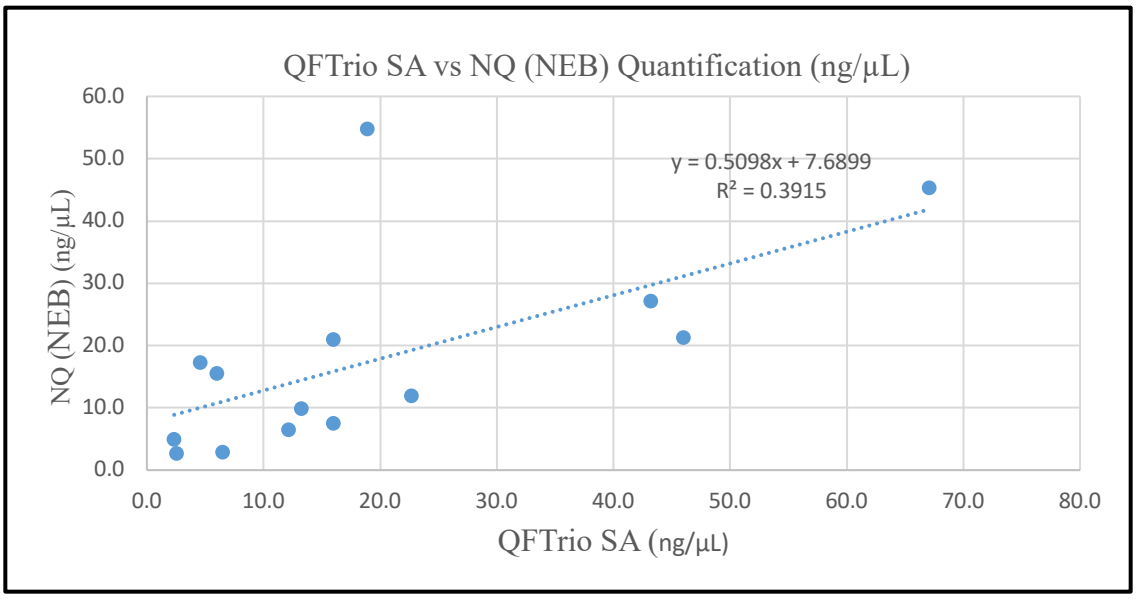


Figure 11a: QFTrio SA vs NQ (NEB) Linear Plot.
A moderate positive correlation was demonstrated between QFTrio SA and NQ (NEB) quantification data. The R^2 value of 0.3915 signifies the correlation of 63%. The QFTrio and NQ (NEB) quantification results were not significantly different (F-test for variances, p= 0.235; t-test assuming equal variances, p=0.763)

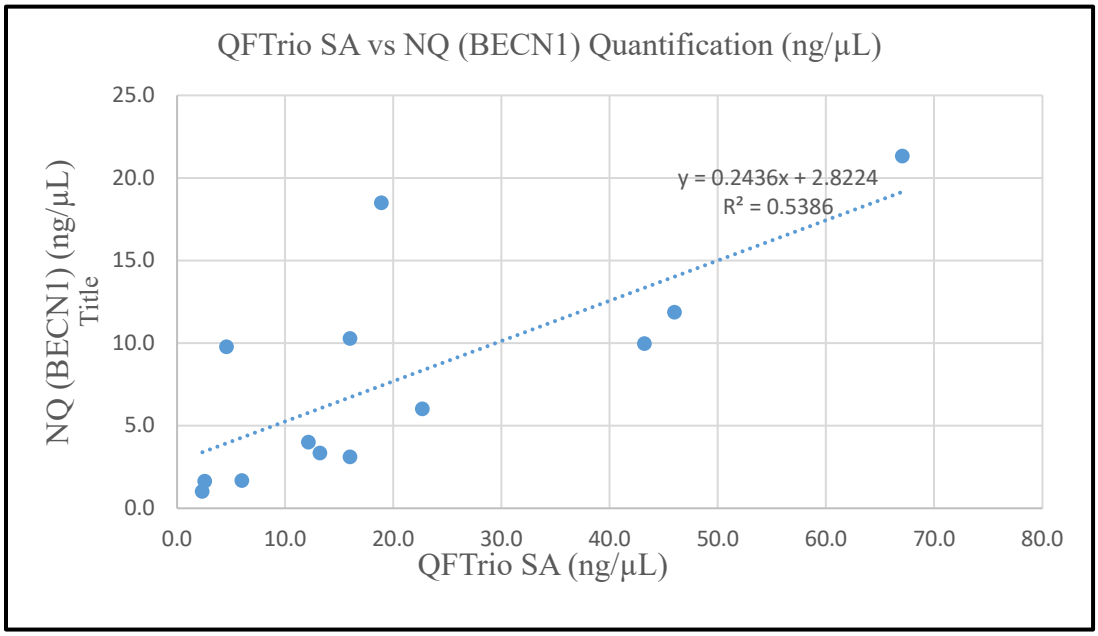


Figure 11b: QFTrio SA vs NQ (BECN1) Linear Plot.
A strong positive correlation was demonstrated between QFTrio SA and NQ (NEB) quantification data. The R^2 value of 0.5386 signifies the correlation of 73%. The QFTrio and NQ (BECN1) quantification results were significantly different (F-test for variances, p= 0.0002; t-test assuming unequal variances, p=0.039)

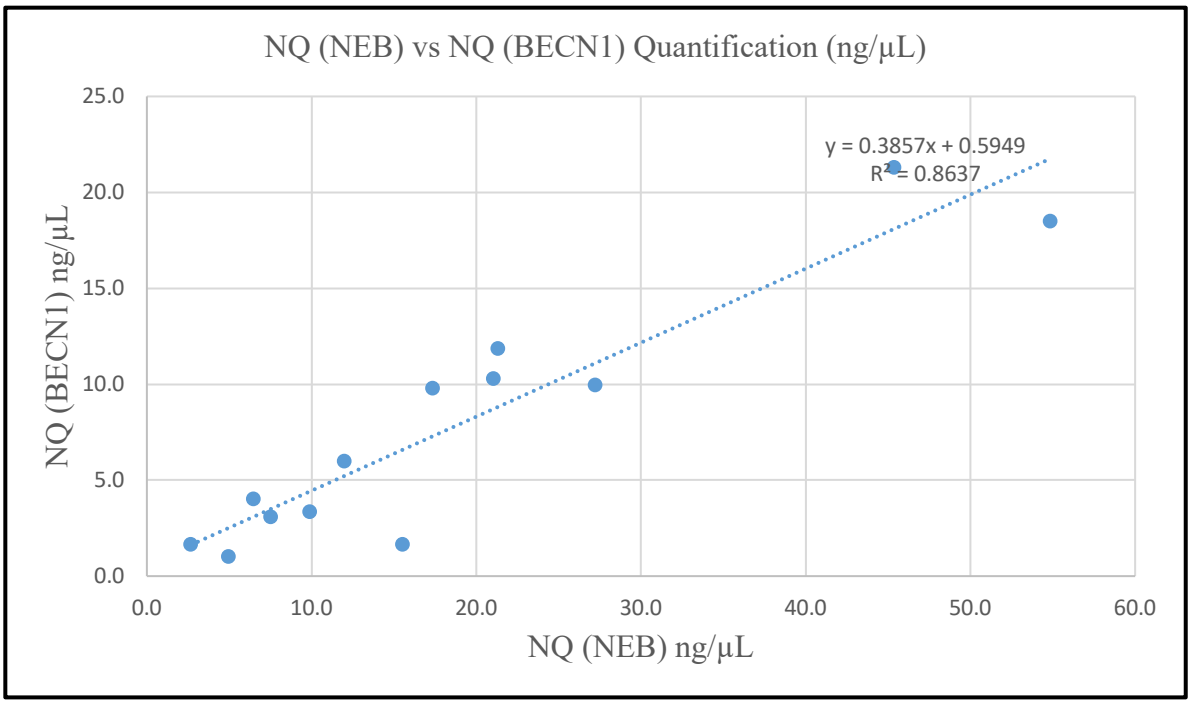


Figure 11c: NQ (NEB) vs NQ (BECN1) Linear Plot.
A strong positive correlation was demonstrated between NQ (NEB) vs NQ (BECN1) quantification data. The R^2 value of 0.8637 signifies the correlation of 93%. The NQ (NEB) and NQ (BECN1) quantification results were significantly different (F-test for variances, p= 0.002; t-test assuming unequal variances, p=0.032)

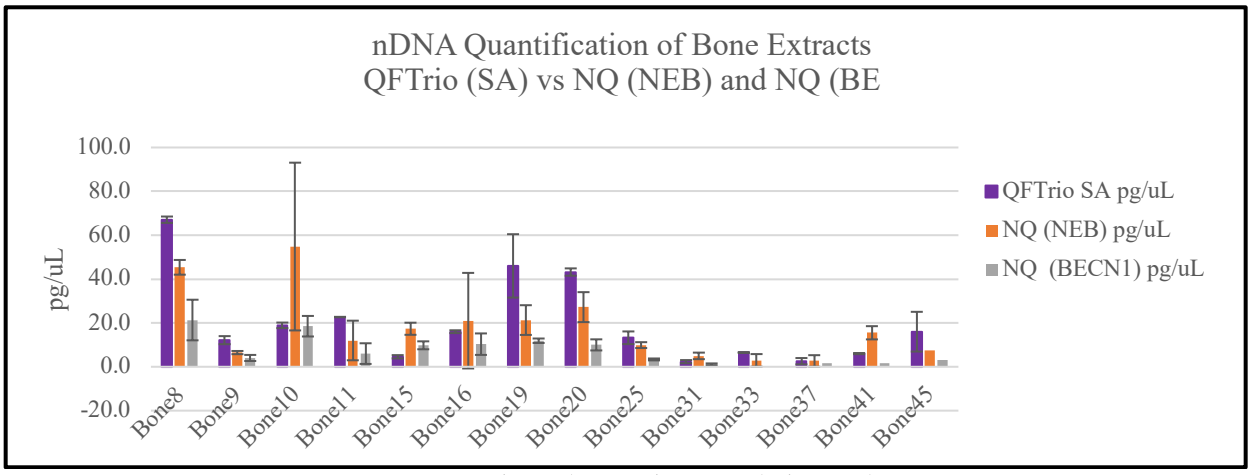


Figure 12: nDNA QFTrio SA vs NQ (NEB) and (BECN1) (pg/µL).
Standard deviation is displayed as error bars.

mtDNA Triplex Previous vs New quantification (mtDNA copies/µL)

This experiment (**Table 16**) examined concordance via correlation testing between previous (MK 2017) quantification [14] and new (EC 2019) quantification of eighteen bone extracts (mtDNA copies/μL) using the Triplex assay. The null hypothesis stated that all results were

concordant. The alternative hypothesis stated that results were *not* concordant. **Figure 13** shows a very strong positive correlation of 96% between previous and new quantification results. In addition, a t-test performed also confirmed they were not significantly different with a p-value of .531 so the null hypothesis was accepted that the two values were concordant. This high positive correlation also indicates very strong reproducibility of the Triplex. This means that quantification results remained consistent between different time periods the Triplex was conducted, between different people conducting the assays, and between different qPCR runs of the assay. The results in **Figure 14** show the low standard deviations indicating low variation between the triplicates and replicates of the previous and new quantification experiments. In addition, (**Table 16**) a percent difference of 22% was calculated between the previous and new.

Table 16: *Triplex Previous vs New Bone Extract Quantification*

	Sample Name	Triplex previous MK (2017) copies/µL	Triplex new EC (2019) copies/µL	% dif between Previous (MK 2017 and new (EC 2019
1	Bone8	4778	4889	2%
2	Bone9	3177	2049	-35%
3	Bone10	9692	8517	-12%
4	Bone11	2165	5907	1.7%
5	Bone15	8890	5962	-32%
6	Bone16	663	1045	58%
7	Bone19	2634	3359	27%
8	Bone20	2824	3211	13%
9	Bone25	1665	1593	-4%
10	Bone31	346	366	5%
11	Bone33	719	961	33%
12	Bone37	8957	5592	-37%
13	Bone41	2410	1905	-21%
14	Bone45	229590	85981	-62%
15	Bone49	203	147	-27%
16	Bone55	2310	1888	-18%
17	Bone57	2234	1369	-38%
18	Bone60	12334	7433	-39%
Correlation	**99.7%**		**Avg % dif**	**22%**

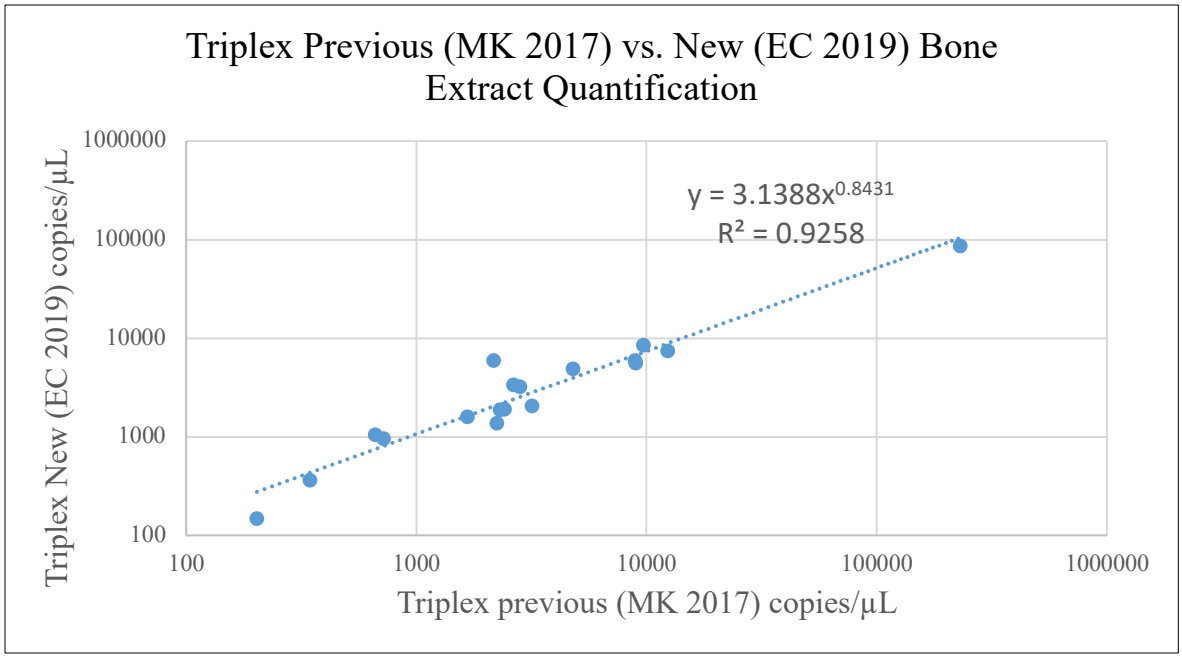


Figure 13: Triplex Previous vs New Bone Extracts Quantification Linear Plot.
A strong positive correlation was demonstrated between Triplex Previous (MK 2017) and New (EC 2019) quantification data. The R^2 value of 0.9258 signifies the correlation of 96%. The new and previous quantification results were not significantly different (F-test for variances, p= 0.00005; t-test assuming unequal variances, p=0.531)

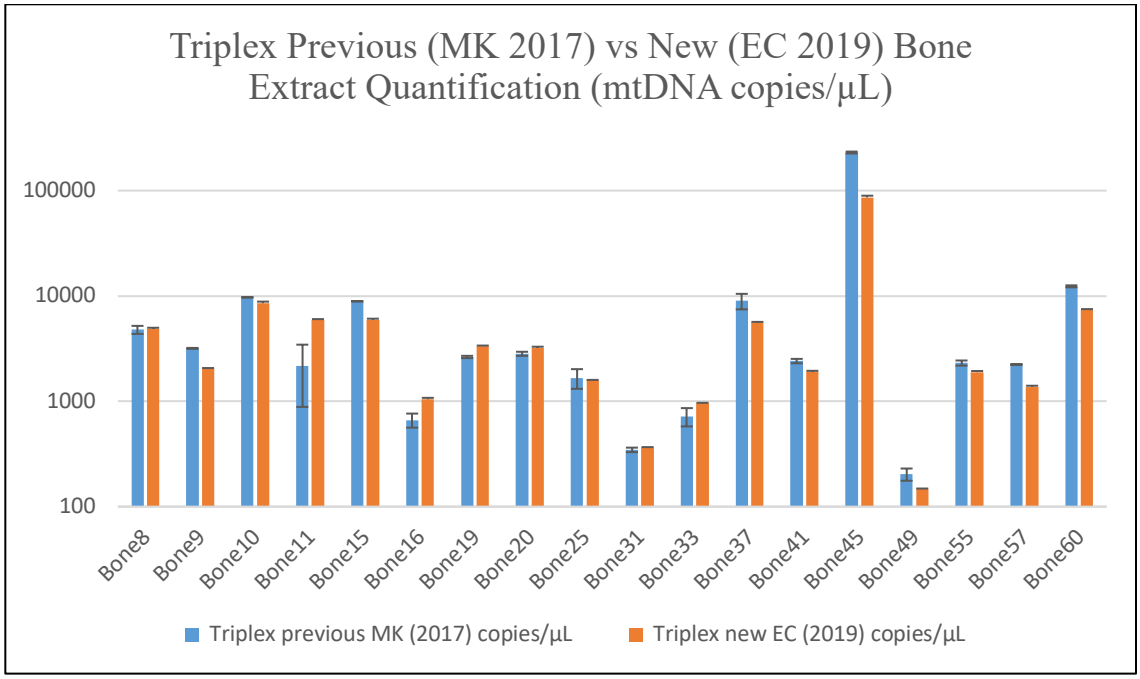


Figure 14: Triplex Previous vs New Bone Extract Quantification (mtDNA copies/µL).
Previous Triplex qPCR performed by Mark Kavlick (MK 2017) in 2017 and new Triplex qPCR performed by Emily Cropper (EC 2019) in 2019. Shown are the averages of triplicate and duplicate testing, respectively. Error bars represent standard deviation of the replicates.

mtDNA Triplex Previous vs New degradation (ΔΔCt)

This experiment (**Table 17)** examined concordance via correlation testing between previous (MK 2017) and new (EC 2019) ΔΔCt values of eighteen bone extracts using the Triplex assay. The null hypothesis stated that all results were concordant. The alternative hypothesis stated that results were *not* concordant. The ΔΔCt values (**Figure 15)** between previous and new results show a strong correlation of 81%. In addition, a t-test performed also confirmed they were not significantly different with a p-value of .246 so the null hypothesis was accepted that the two values were concordant. The results in **Table 17** demonstrate the percent difference between previous and new bone ΔΔCt with the total average percent difference as 9%. This number indicates high accuracy and reproducibility of the Triplex due to the similarity of the low template bone extract previous and new ΔΔCt values. **Figure 16** displays the previous and new ΔΔCt comparisons for each bone. Another observation to note was that the bones sample 9 contained the highest degradation at 8.9.

Table 17: *Triplex Previous vs New Degradation (ΔΔCt)*

	Sample Name	Triplex previous (MK 2017) ΔΔCt	Triplex new (EC 2019) ΔΔCt	% dif previous vs. new
1	Bone8	4.3	4.1	-5%
2	Bone9	8.3	8.9	7%
3	Bone10	6.4	6.0	6%
4	Bone11	7.7	4.1	-47%
5	Bone15	4.1	4.2	3%
6	Bone16	6.5	4.2	-35%
7	Bone19	7.8	5.2	-34%
8	Bone20	4.6	4.4	-4%
9	Bone25	6	6.0	0%
10	Bone31	2.9	3.7	26%
11	Bone33	3.4	3.0	-12%
12	Bone37	2.5	2.4	-5%
13	Bone41	4.7	3.8	-20%
14	Bone45	1.6	1.4	-11%
15	Bone49	7.6	ND	
16	Bone55	4	4.0	0%
17	Bone57	4	3.7	-7%
18	Bone60	3.5	3.2	-10%
	Correlation	**81%**	**Avg % dif**	**9%**

ND, not detected

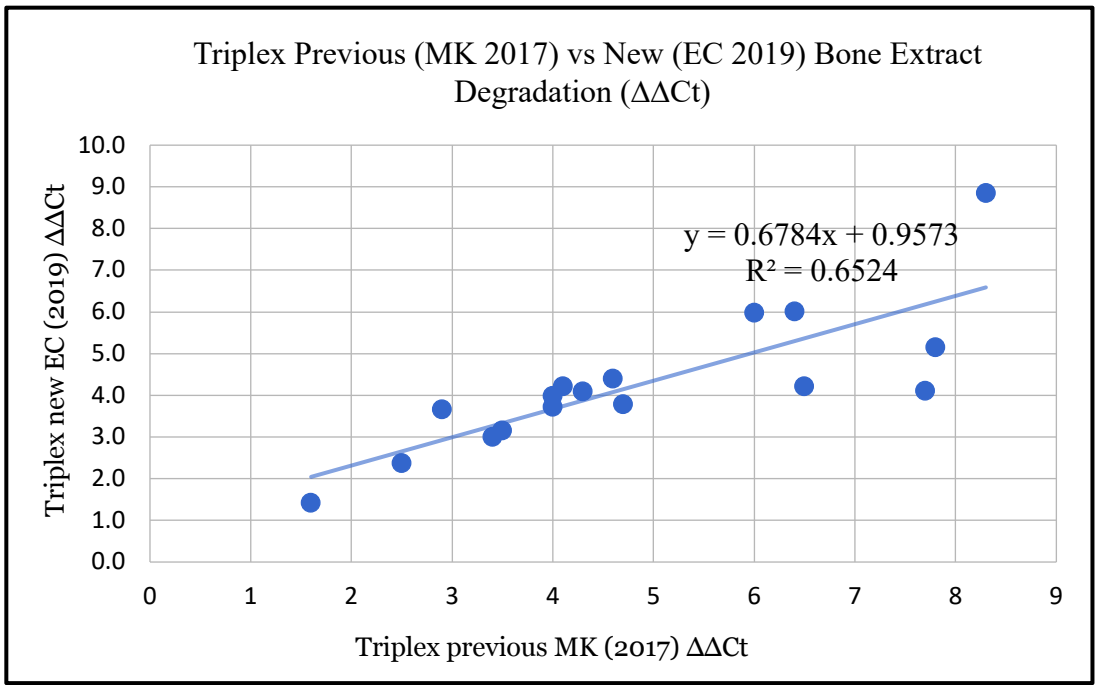


Figure 15: Triplex Bone Extract (ΔΔCt) Previous vs. New Linear Plot

A strong positive correlation was demonstrated between previous and new Triplex ΔΔCt data. The R^2 value of 0.6524 signifies the correlation of 81%. *The new and previous degradation results were not significantly different (F-test for variances, p= 0.246; t-test assuming equal variances, p=0.341)*

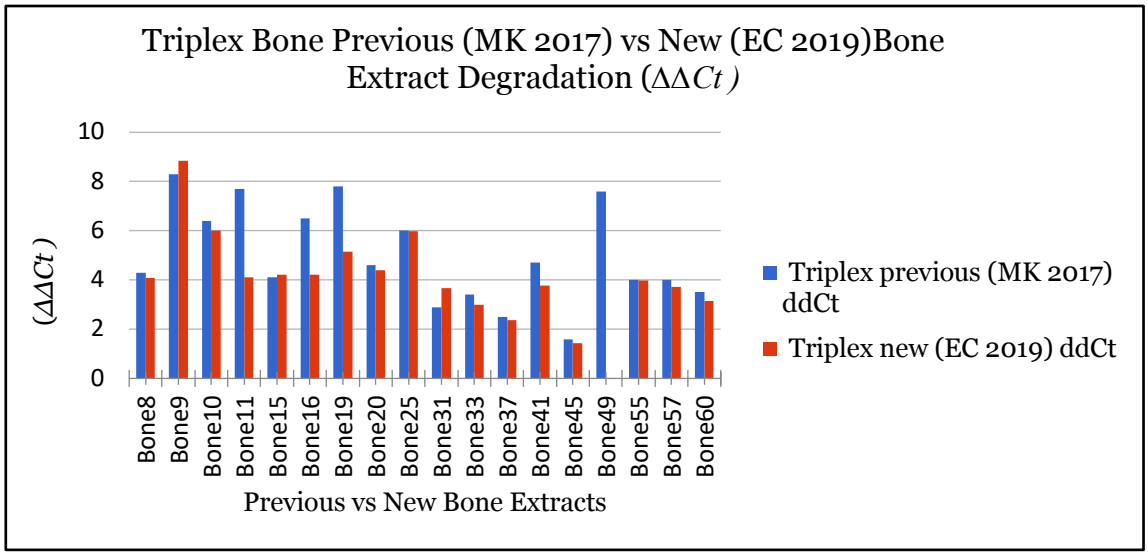


Figure 16: Triplex Bone Extract (ΔΔCt) Previous vs New

Previous Triplex ΔΔCt data (MK 2017) and new Triplex ΔΔCt (EC 2019). Note: the ΔΔCt value for Bone 49 could not be calculated contemporaneously since the large target could not be amplified.

nDNA QFHP Previous vs QFTrio New degradation (DI)

This experiment tested concordance via correlation testing between previous (MK 2017) and new (EC 2019) DI results of eighteen bone extracts using QFHP and QFTrio respectively (**Table 18**). The null hypothesis stated that all results were concordant. The alternative hypothesis stated that results were *not* concordant. Below in **Figure 17** the DI values between previous and new results show a strong correlation of 87.6%. In addition, a t-test performed also confirmed they were not significantly different with a p-value of .256 so the null hypothesis was accepted that the two values were concordant. The results in **Figure 18** show each bone and its corresponding DI between the previous and the new. However, **Table 18** demonstrates the percent difference of 79% between the previous and the new DI values which indicates low reproducibility of measurements. The bones samples that contained the lowest quantity of DNA - Bones 31, 33, and 49 - also had the highest amount of degradation.

Table 18: *QFHP Previous vs. QFTrio New Degradation DI*

	QFHP previous (MK 2017)	QFTrio new (EC 2019)	% dif QFHP previous and QFTrio new
Bone8	5.3	3.8	-29%
Bone9	21.6	20.2	-6%
Bone10	6.9	2.7	-61%
Bone11	4.3	3.0	-30%
Bone15	8.9	8.4	-6%
Bone16	5.1	4.9	-4%
Bone19	4.8	6.5	36%
Bone20	7.2	10.1	41%
Bone25	5.9	6.6	11%
Bone31	33.6	64.7	93%
Bone33	34.2	294.8	762%
Bone37	3.4	3.6	5%
Bone41	1.9	2.6	36%
Bone45	2.1	3.3	59%
Bone49	12.9	66.9	418%
Bone55	4.7	9.2	95%
Bone57	2.4	2.9	21%
Bone60	5.1	4.3	-15%
Correlation	**87.6%**	**Avg % dif**	**79%**

Shown are log_2 of Trio DI values

ND, not detected

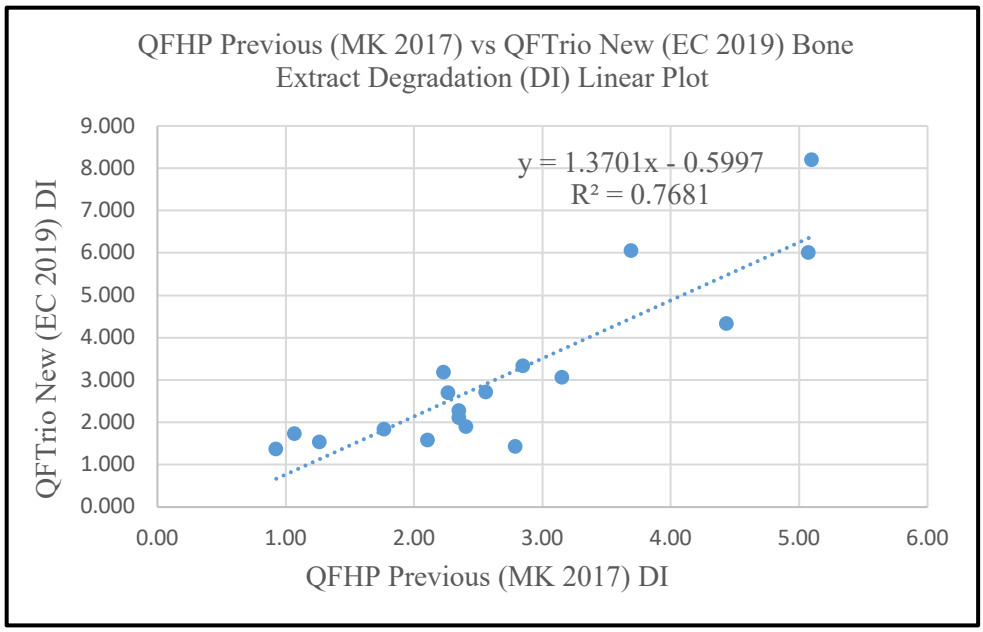


Figure 17: Bone Extract DI QFHP Previous vs. QFTrio New Linear Plot
A strong positive correlation was demonstrated between previous QFHP and new QFTrio DI data. The R^2 value of 0.7681 signifies the correlation of 87.6%. *Shown are log_2 of Trio DI values. The new and previous degradation results were not significantly different (F-test for variances, p= 4.1x10^-11; t-test assuming unequal variances, p=0.256)*

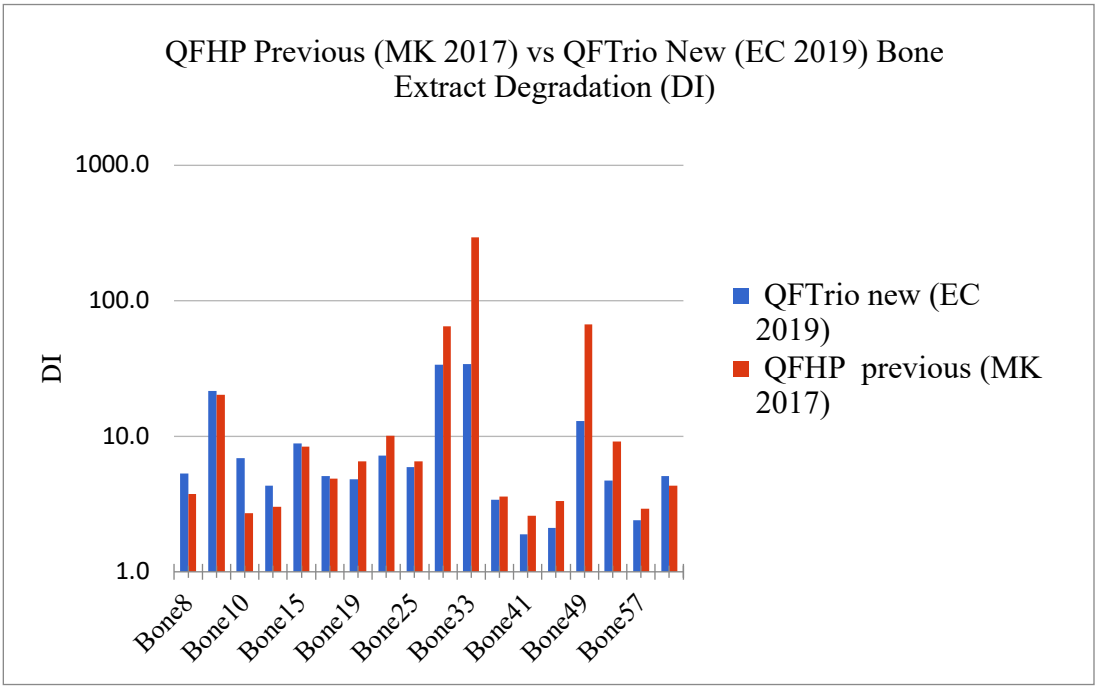


Figure 18: Bone Extract DI QFHP Previous vs. QFTrio New.
Previous QFHP DI data (MK 2017) and new QFTrio DI (EC 2019). Note: Bones 31, 33, and 49 that contained the highest amount of degradation also had the lowest quantity of DNA Shown are log_2 of Trio DI value.

mtDNA Triplex ΔΔCt vs nDNA QFTrio DI converted

This experiment examined concordance via correlation testing between Triplex ΔΔCt and converted QFTrio DI values for eighteen bone extracts. The null hypothesis stated that all results were concordant. The alternative hypothesis stated that results were *not* concordant. **Table 19** shows the Triplex ΔΔCt the QFTrio DI converted degradation values. The original QFTrio DI values were converted by applying a (log2 n) formula [14]so as to be comparable with the Triplex ΔΔCt values. **Figure 19** shows a very low to no correlation of 6%. In addition, a t-test performed showed they were not significantly different with a p-value of .678 so the null hypothesis was accepted that the two values were concordant. The results in **Figure 20** show each bone and its corresponding ΔΔCt or converted DI. However, **Table 19** demonstrates the percent difference of 22% between the assays.

Table 19: *Triplex ΔΔCt vs QFTrio DI Converted*

	Sample Name	Triplex ΔΔCt	QFTrio DI converted	% dif Triplex ΔΔCt vs. QFTrio DI converted
1	Bone8	4.09	3.01	-26%
2	Bone9	8.85	6.83	-23%
3	Bone10	6.00	2.27	-62%
4	Bone11	4.11	2.51	-39%
5	Bone15	4.21	4.82	14%
6	Bone16	4.21	3.60	-15%
7	Bone19	5.16	4.27	-17%
8	Bone20	4.40	5.26	20%
9	Bone25	5.97	4.28	-28%
10	Bone31	3.66	9.47	159%
11	Bone33	3.00	12.92	330%
12	Bone37	2.37	2.89	22%
13	Bone41	3.78	2.16	-43%
14	Bone45	1.42	2.74	93%
15	Bone49	ND	9.55	
16	Bone55	3.98	5.03	26%
17	Bone57	3.73	2.43	-35%
18	Bone60	3.16	3.32	5%
Correlation		**6%**	**Avg % dif**	**22%**

ND, not detected

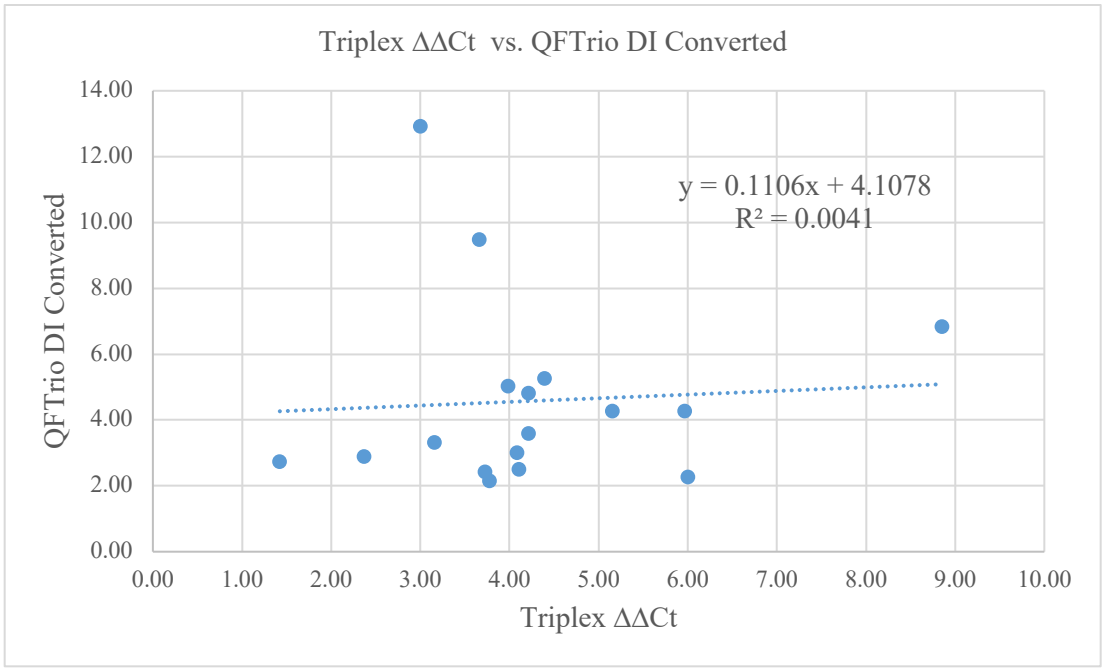


Figure 19: Triplex ΔΔCt vs QFTrio DI Converted Linear Plot

No correlation was demonstrated between Triplex ΔΔCt and QFTrio DI Converted. The R^2 value of 0.0041 signifies the correlation of 6%. *The Triplex* ΔΔCt *and QFTrio DI Converted degradation results were not significantly different (F-test for variances, p= .016; t-test assuming unequal variances, p=0.678)*

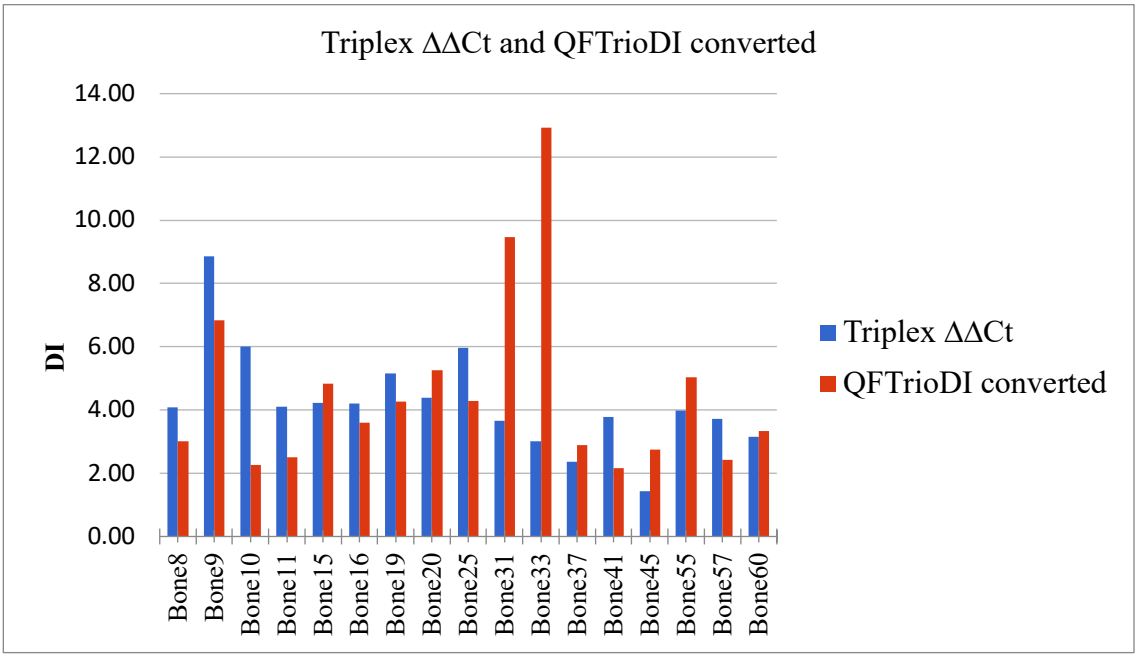


Figure 20: Triplex ΔΔCt vs QFTrio DI Converted

Triplex ΔΔCt vs. QFTrio DI Converted Note: The Triplex ΔΔCt value for Bone 49 could not be calculated contemporaneously since the large target could not be amplified.

Post-HVR Amplification Quantification Bone Extracts (ng/μL)

This experiment examined if eighteen bone extract HVR PCR amplifications would produce adequate DNA amplicon concentrations (≥1 ng/μL) for sequencing and analysis. The Triplex qPCR data was used to determine the optimal HVR amplification of one of four targets in preferred order from largest to smallest region [bp] - WCR [1228], HV2 [401], HV2B [276], or MP4 [108] - depending on the mtDNA virtual copy numbers (VCN) which were calculated using mtDNA copies/μL and state of degradation for each sample (Kavlick 2018).

Examined first was whether eighteen bone extract amplicon lengths were concordant with the expected lengths **Table 20** provides expected amplicon length, actual amplicon length, and the amplicon target that was amplified via capillary electrophoresis for eighteen bone extracts.

Table 20: *Post-Amplification Quantification Bone –Expected [bp] vs. Observed [bp]*

		Expected Amplicon length [bp]	Observed Amplicon length [bp]	Amplicon	% dif Expected vs. Observed
1	Bone9	108	122	MP4	13%
2	Bone49	108	121	MP4	12.5%
3	Bone8	276	283	HV2B	2.5%
4	Bone10	276	281	HV2B	2.0%
5	Bone11	276	282	HV2B	2.4%
6	Bone15	276	284	HV2B	3.1%
7	Bone16	276	286	HV2B	3.8%
8	Bone19	276	283	HV2B	2.7%
9	Bone20	276	282	HV2B	2.2%
10	Bone25	276	282	HV2B	2.2%
11	Bone31	276	283	HV2B	2.7%
12	Bone33	276	283	HV2B	2.7%
13	Bone41	276	286	HV2B	3.8%
14	Bone55	276	283	HV2B	2.5%
15	Bone57	276	284	HV2B	3.1%
16	Bone37	401	415	HV2	3.6%
17	Bone45	401	415	HV2	3.6%
18	Bone60	401	417	HV2	4.1%
	Bone45	1228	1449	WCR	18.0%
		Correlation	**99%**	**Avg % dif**	**4.8%**

Table 20 shows a 99% correlation between expected amplicon length [bp] and the actual eighteen (18) bones amplicon length [bp] of the four amplicon regions of WCR, HV2, HV2B, and MP4.

Next, this experiment examined whether each of the eighteen bone extracts amplified yielded a sufficient DNA concentration (≥ 1 ng/μL) for downstream sequencing and analysis. (**Table 21)** contains replicate DNA concentrations for each bone amplified by capillary electrophoresis. All amplifications met the minimum concentration requirements (≥1ng/µL).

Table 21: *Post-Amplification Quantification Bone – DNA concentration (ng/µL)*

sample name	Rep 1 ng/µL	Rep 2 ng/µL	mean ng/µL	Std dev ng/µL	cv ng/µL	mtDNA Amplicon
Bone8	3.9	4.8	4.4	0.622	14.3%	HV2B
Bone9	3.2	3.5	3.4	0.255	7.6%	MP4
Bone10	5.0	5.4	5.2	0.255	4.9%	HV2B
Bone11	4.1	4.5	4.3	0.290	6.8%	HV2B
Bone15	3.7	3.8	3.7	0.064	1.7%	HV2B
Bone16	3.5	4.3	3.9	0.573	14.8%	HV2B
Bone19	4.7	4.5	4.6	0.163	3.6%	HV2B
Bone20	3.5	2.8	3.1	0.481	15.4%	HV2B
Bone25	6.4	6.7	6.5	0.156	2.4%	HV2B
Bone31	3.2	3.5	3.3	0.205	6.2%	HV2B
Bone33	4.4	4.8	4.6	0.219	4.8%	HV2B
Bone37	15.0	15.0	15.0	0.064	0.4%	HV2
Bone41	3.2	3.2	3.2	0.014	0.4%	HV2B
Bone45	17.2	18.3	17.8	0.764	4.3%	HV2
Bone45	11.9	12.4	12.2	0.368	3.0%	WCR
Bone49	3.7	4.0	3.8	0.191	5.0%	MP4
Bone55	4.9	5.3	5.1	0.304	5.9%	HV2B
Bone57	4.6	4.7	4.7	0.057	1.2%	HV2B
Bone60	19.2	14.5	16.9	3.309	19.6%	HV2
				Avg CV	**6.44%**	

Values obtained using the Agilent 2100 Bioanalyzer and DNA 7500 kit (WCR) or DNA 1000 kit (all others).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

SRM Quantification - Triplex vs QFTrio vs NQ

Differences in performance were observed after comparing the known quantity of DNA (NIST SRM2372a) across the non-commercial Triplex and the commercially available QFTrio, and NQ qPCR assays. Since commercial kits are readily available for the forensic laboratory, one may assume a commercial qPCR product to perform exceedingly well based upon the manufacturer's literature. Interestingly, the only non-commercial assay in this study out-performed the two commercially available qPCR assays. The consistency by which the SRM quantified within the interval (U95 +/-2.5%) of the NIST expected values with the Triplex is most likely due to the robust analytical accuracy, specificity, repeatability, reproducibility, and efficiency of the dsT8sig synthetic standard.

An accurate qPCR assay refers to the closeness of experimentally measured concentrations presented as fold changes. In the case of absolute qPCR, this depends on the accuracy of the standards. The Triplex and the NQ (ND6) mtDNA target quantified the SRM most accurately compared to the NIST expected interval. The success of the Triplex quantification was most likely due to the dsT8sig standard. The accuracy in measurement of the ND6 is unknown since the two NQ nDNA targets quantified higher than expected and the other mtDNA target failed to amplify.

Because QFTrio SA quantified lower than the NIST expected interval in most cases, there may have been an issue with the QFTrio THP standard containing a lower than documented

concentration of (100 ng/µL). The 143B DNA standard provided with NQ was suspected to have a lower quantity than documented since Ct values were higher than expected for all targets. This hypothesis was investigated by testing the 143B standard as an unknown sample with the Triplex and QFTrio. The 143B concentrations remained lower than expected therefore it was replaced by THP DNA for all final NQ experiments. In addition, there is no prior documented research with NQ that utilizes the standard curve method.

The NQ nDNA targets NEB and BECN1 quantified all SRM components higher than expected which may indicate a lack of specificity, an increase of primer dimers, or lack of probes from utilizing a SYBR GREEN master mix. Any non-specific double-stranded DNA product (human and/or non-human) would be amplified in this case with SYBR GREEN. However, when using a human-specific targeted TaqMan master mix (contained in Triplex and QFTrio assays) human DNA will amplify. The failure of the ND1 target and the low performance of NEB could have been potentially due the primers being compromised due to primer dimers or contamination. A poor primer design specific to ND1 may also be a plausible reason for the apparent weak performance given that the other three targets (ND6, BECN1, and NEB) amplified.

With respect to qPCR assay efficiency, 100% efficiency is an exact doubling of amplicon with each cycle. It is directly related to the slope (m) of the standard curve regression line. The targeted slope for an assay that has a 100% efficiency is -3.3219. However, an acceptable efficiency ranges from 90 – 110% ($-3.6 \geq \text{slope} \geq -3.3219$). Efficiencies lower than this range can result from stochastic effects indicating low levels of DNA, poor primer design, poorly constituted serial dilutions, and pipetting errors. Efficiencies above this range could indicate polymerase inhibition, stochastic effects, or perhaps be an internal characteristic of the assay chemistry. The percent efficiency and slope for each of the *pooled SRM dilutions* assay were as

follows: Triplex: 89.8%, ND6 87.5%, QFTrio SA 111.4%, QFTrio LA 101.56, BECN1 86.4%, and NEB 89.7%.

The R^2 value on the standard curve for absolute quantification indicates the closeness of fit between the standard curve regression line and the individual data points. An R^2 of 1.0 is a perfect closeness of fit and indicates an assay which exhibits a good linear dynamic range as well as pipetting accuracy and precision. An R^2 (≥ 0.98) was considered an acceptable closeness of fit for this project. Triplex R^2 was 1.0, ND6 1.0, QFTrio SA 0.996, QFTrio LA 0.998, BECN1,0.996, and NEB 0.987.

Bone Quantification - Triplex vs QFTrio vs NQ

Repeatability is also a major factor in a successful qPCR assay and is measured by the CV (coefficient of variation) (standard deviation divided by the mean) of sample replicates. A low CV indicates low variation and low variation in quantification values show higher repeatability and therefore more reliability. When comparing the three assays, the Triplex had the lowest variation of CVs for the bone extract quantifications. Triplex CV's ranged from 0.2 – 7.9%, ND6 ranged from 0.1 – 57.8%, QFTrio SA from 2.0 – 56.7%, NEB 7.4 – 103.6%, and BECN1 10.2 – 78.4%. Despite the fact that there was a large discrepancy between the CVs, there was a high correlation of bone DNA concentrations Triplex vs ND6 98%, QFTrio vs NQ NEB 63%, QFTrio vs NQ BECN1 73%, and NQ NEB vs NQ BECN1 93%.

Bone quantification - Previous vs New Triplex

CVs also reflect reproducibility, but rather than just considering the sample replicates within the assay, an assay with high reproducibility from an assay occurs between different people, temporally, and different qPCR runs. The re-assayed (via Triplex) eighteen bone extracts were quantified and evaluated for degradation ($\Delta\Delta$Ct) were concordant between the new and previous tests, providing supporting evidence for reproducibility of the Triplex assay. The new

and previous quantification correlation was 96% while the (ΔΔCt) correlation was 81%. The QFTrio new and QFHP previous eighteen bone extract DI (ΔΔCt) had a correlation of 86.7%. No degradation assessment was conducted with the NQ since the targets were so close in size ND1 (153bp), ND6 (96bp), and NEB (116bp). In addition, the target size for BECN1 was never provided by the manufacturer and ND1 failed to amplify.

HVR Post-Amp Quantification

All eighteen bone extracts amplified (using Triplex qPCR data) with their respective amplicons in order of decreasing degradation of mtDNA: WCR, HV2, HV2B, and MP4. The most intact (i.e., least degraded mtDNA) sample, Bone 45, was amplified for the WCR (amplicon size 1228bp); HV2 (amplicon size 401bp) was amplified for mildly degraded mtDNA, Bones 37, 60; HV2B (amplicon size 276bp) was used for the moderately degraded mtDNA. Bones 08,10,11,15,16,19,20,25,31,33,41,55, and 57; and MP4 (amplicon size 108) was used for severely degraded mtDNA, Bones 09 and 49. Despite various degradation indices, every bone extract yielded amplicon concentrations ≥1ng/µL, a sufficient quantity for the analyst to move forward with sequencing and analysis. Determining the quantity and state of degradation of samples via Hypervariable (HV) post-amplification quantification would save time, money, and resources and also would also ensure proper protocols are in place for sequencing and analysis.

In conclusion, when selecting a qPCR assay, there are several key considerations: the quality of the assay, the time available for the project, the experimental goals of the project, and any limitations to the available budget. Commercially available products like QFTrio and NQ are convenient to use, already (presumably) developmentally validated, and relatively easy to obtain results. Unfortunately, for commercially available products, the concentration of the DNA standard is not always in agreement with the stated value provided by the manufacturer.

For example, in this study QFTrio SA consistently quantified lower than expected for the SRM samples. Downstream normalization for PCR amplification would potentially be under-valued (too little DNA would be added to the sequencing reaction) in these instances and could result in poor (or failed) sequencing data and analysis. The 143B DNA standard underperformed from what was expected. The pure, synthetic, well characterized Triplex dsT8sig standard provided the most reliable, reproducible, and accurate results with all three SRM components and bone extracts. Non-commercially available assays such as the Triplex can be more laborious to produce and can take more time to be "bench-ready" for qPCR. However, with convenience quality may sometimes be sacrificed. As observed in this study, non-commercial products can perform equal to, or out-perform, those assays which are commercially available. Once a laboratory is comfortable with the development and quality control of non-commercially available assays, these can provide cost-effective alternative commercial products

REFERENCES

1. Budowle B, Bieber FR, Eisenberg AJ. Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification. *Legal Medicine.* 2005;7(4):230-243.

2. Duewer DL, Kline MC, Romsos EL, Toman BJA, Chemistry B. Evaluating droplet digital PCR for the quantification of human genomic DNA: converting copies per nanoliter to nanograms nuclear DNA per microliter. 2018;410(12):2879-2887.

3. Kline MC, Duewer DL, Travis JC, et al. Production and certification of NIST Standard Reference Material 2372 Human DNA Quantitation Standard. *Analytical & Bioanalytical Chemistry.* 2009;394(4):1183-1192.

4. Andréasson H, Gyllensten U, Allen M. Real-Time DNA Quantification of Nuclear and Mitochondrial DNA in Forensic Analysis. *BioTechniques.* 2002;33(2):402-411.

5. Timken MD, Swango KL, Orrego C, Buoncristiani MR. A duplex real-time qPCR assay for the quantification of human nuclear and mitochondrial DNA in forensic samples: Implications for quantifying DNA in degraded samples. *Journal of Forensic Sciences.* 2005;50(5):1044-1060.

6. Greaves LC, Beadle NE, Taylor GA, et al. Quantification of mitochondrial DNA mutation load. *Aging Cell.* 2009;8(5):566-572.

7. Niederstätter H, Köchl S, Grubwieser P, Pavlic M, Steinlechner M, Parson W. A modular real-time PCR concept for determining the quantity and quality of human nuclear and mitochondrial DNA. *Forensic Science International: Genetics.* 2007;1(1):29-34.

8. Sprouse ML, Phillips NR, Kavlick MF, Roby RK. Internal Validation of Human Mitochondrial DNA Quantification Using Real-Time PCR. *Journal of Forensic Sciences.* 2014;59(4):1049-1056.

9. Kavlick MF, Lawrence HS, Merritt RT, et al. Quantification of Human Mitochondrial DNA Using Synthesized DNA Standards*. *Journal of Forensic Sciences.* 2011;56(6):1457-1463.

10. Romsos E, Kline M, L. Duewer D, Toman B, Farkas N. *Certification of Standard Reference Material 2372a; Human DNA Quantitation Standard.* 2018.

11. Côté HCF, Gerschenson M, Walker UA, et al. Quality assessment of human mitochondrial DNA quantification: MITONAUTS, an international multicentre survey. *Mitochondrion.* 2011;11(3):520-527.

12. Kaur G, Dufour JM. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis.* 2012;2(1):1-5.

13. Hazkani-Covo E, Zeller RM, Martin W. Molecular poltergeists: mitochondrial DNA copies (numts) in sequenced nuclear genomes. *Plos Genetics.* 2010;6(2):e1000834-e1000834.

14. Kavlick MF. Development of a triplex mtDNA qPCR assay to assess quantification, degradation, inhibition, and amplification target copy numbers. *Mitochondrion.* 2018.

15. Butler JM, Levin BC. Forensic applications of mitochondrial DNA. *Trends in Biotechnology.* 1998;16(4):158-162.

16. Anderson S, Bankier AT, Barrell BG, et al. Sequence and organization of the human mitochondrial genome. *Nature.* 1981;290(5806):457-465.

17. Bollongino R, Tresset A, Vigne J-DJCRP. Environment and excavation: Pre-lab impacts on ancient DNA analyses. 2008;7(2-3):91-98.

18. Budimlija ZM. World Trade Center human identification project: experiences with individual body identification cases. *Croatian medical journal.*44(3):259-263.

19. Chaitanya L, Pajnič IZ, Walsh S, Balažic J, Zupanc T, Kayser M. Bringing colour back after 70 years: Predicting eye and hair colour from skeletal remains of World War II victims using the HIrisPlex system. *Forensic Science International: Genetics.* 2017;26:48-57.

20. Ginther C, Issel-Tarver L, King M-CJNg. Identifying individuals by sequencing mitochondrial DNA from teeth. 1992;2(2):135.

21. Pfeiffer H, Hühne J, Ortmann C, Waterkamp K, Brinkmann BJIJoLM. Mitochondrial DNA typing from human axillary, pubic and head hair shafts – success rates and sequence comparisons. 1999;112(5):287-290.

22. Rohland N, Siedel H, Hofreiter M. Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. 2004;36(5):814-821.

23. Coble MD. Mystery solved: the identification of the two missing Romanov children using DNA analysis. *PloS one.* 2009;4(3).

24. Farrugia A, Keyser C, Ludes B. Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue. *Forensic Science International.* 2010;194(1):e25-e28.

25. Holland MM. Development of a quality, high throughput DNA analysis procedure for skeletal samples to assist with the identification of victims from the World Trade Center attacks. *Croatian medical journal.*44(3):264-272.

26. Quantifiler Trio Quantification Manual. 2014; https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-

Assets/LSG/manuals/4485356_Quantifiler_HP_Trio_DNA_QR.pdf. Accessed Feb 03, 2019.

27. NovaQUANT™ Human Mitochondrial to Nuclear DNA Ratio Kit. In: EMD Millipore Corporation; 2011.

28. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry.* 2009;55(4):611-622.

29. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods.* 2001;25(4):402-408.

30. Kavlick MF. Real-Time Quantitative PCR of Human Mitochondrial DNA [mtDNA qPCRv3] 2016.

31. FBI Laboratory Quality System Documents. 2018; Federal Bureau of Investigation, Department of Justice. Available at: http://fbilabqsd.com. Accessed March 06, 2019.