NIST Special Publication 260-189

Certification of Standard Reference Material® 2372a

Human DNA Quantitation Standard

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Abstract

Standard Reference Material (SRM) 2372a is intended for use in the value assignment of human genomic deoxyribonucleic acid (DNA) forensic quantitation materials. A unit of SRM 2372a consists of three well-characterized human genomic DNA materials solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3 propanediol hydrochloride (Tris HCl) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) pH 8.0 buffer (TE^{-4}). The three component genomic DNA materials, labeled A, B, and C, are respectively derived from a single male donor, a single female donor, and 1:3 mixture of a male and a female donor. A unit of the SRM consists of one 0.5 mL tube of each component, each tube containing approximately 55 µL of DNA solution. Each of these tubes is labeled and is sealed with a color-coded screw cap. This publication documents the production, analytical methods, and statistical evaluations involved in production of this SRM.

Keywords

Human mitochondrial DNA (mtDNA);
Human nuclear DNA (nDNA);
Standard Reference Material (SRM)

Technical Information Contact for this SRM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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Purpose and Description

Standard Reference Material (SRM) 2372a is intended for use in the value assignment of human genomic deoxyribonucleic acid (DNA) forensic quantitation materials.

A unit of SRM 2372a consists of three well-characterized human genomic DNA materials solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3 propanediol hydrochloride (Tris HCl) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) pH 8.0 buffer (TE^{-4}). The three component genomic DNA materials, labeled A, B, and C, are respectively derived from a single male donor, a single female donor, and 1:3 mixture of a male and a female donor.

A unit of the SRM consists of one 0.5 mL tube of each component, each tube containing approximately 55 µL of DNA solution. Each of these tubes is labeled and is sealed with a color-coded screw cap.

NIST is guided by and adheres to the ethical principles set forth in the Belmont Report.[1] SRM 2372a was developed after an appropriate human subject research determination by the NIST Human Subjects Protections Office.

Warning: SRM 2372a is a Human Source Material

The three SRM 2372a components are human genomic DNA extracts prepared at NIST from buffy coat white blood cells from single-source anonymous donors. All buffy coats were purchased from Interstate Blood Bank (Memphis, TN). The supplier reported that each donor unit used in the preparation of this product was tested by FDA-licensed tests and found to be negative for human immunodeficiency virus (HIV-1 RNA and Anti-HIV 1/2), hepatitis B (HBV DNA and HBsAg), hepatitis C (HCV RNA and Anti-HCV), West Nile virus (WNV RNA), and syphilis. Additionally, each donor was tested according to FDA guidelines for *Trypanosoma cruzi* (Chagas) and found to be negative/non-reactive. However, no known test method can offer complete assurance that infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 1.[2]

SRM 2372a components and derived solutions should be disposed of in accordance with local, state, and federal regulations.

Storage and Use

Until required for use, SRM 2372a should be stored in the dark between 2 °C and 8 °C.

The SRM 2372a component tubes should be mixed briefly and centrifuged (without opening the tube cap) prior to removing sample aliquots for analysis. For the certified values to be applicable, materials should be withdrawn immediately after opening the tubes and processed without delay. Certified values do not apply to any material remaining in recapped tubes. The certification only applies to the initial use and the same results are not guaranteed if the remaining material is used later.

History and Background

SRM 2372a is the second member of the SRM 2372 Human DNA Quantitation Standard series. The supply of the initial material, SRM 2372, was exhausted in May 2017. SRM 2372a is derived from commercially obtained buffy coat cells from different donors than those used in SRM 2372.

A series of interlaboratory studies coordinated by what was then the Biotechnology Division of the National Institute of Standards and Technology (NIST) during the 1990s demonstrated that the DNA quantitation technologies then in use by the forensic human identification community adequately met the needs of the DNA typing tools then available.[3,4] However, our 2004 study demonstrated that the increasingly sensitive and informative polymerase chain reaction (PCR)-based multiplex typing tools required more reliable determinations of DNA input quantity for best performance.[5]

Quantitative PCR (qPCR) methods for DNA quantitation require calibration to an external standard of defined DNA concentration ([DNA]), expressed in units of nanograms of DNA per microliter of extract solution. The true [DNA] of a commercial secondary standard may vary from its nominal concentration depending on supplier and lot number. To enable providers of secondary standards to more reliably evaluate their materials and forensic testing laboratories to validate their use of commercial quantitation methods and supplies, in 2007 we delivered the human DNA calibration standard SRM 2372: Human DNA Quantitation Standard.[6]

The quantity of DNA in each of the three components of SRM 2372 was originally assigned based on the absorbance of double-stranded DNA (dsDNA) at 260 nm.[7] However, by 2012 the absorbance properties of the materials had increased beyond the certified uncertainty limits due to slow but progressive changes in DNA tertiary structure. Sales of the SRM were restricted until the DNA quantities were reassigned based on measurements of single-stranded DNA (ssDNA) in early 2013.[8-10] Figure 1 displays the sales history of this relatively high-selling SRM.

While SRM 2372 was fit for the practical purpose of harmonizing DNA quantitation measurements to an accessible and reproducible reference material, the conversion of both dsDNA and ssDNA absorbance to mass [DNA] is through conventional proportionality constants that are not metrologically traceable to the modern reference system for measurements: the International System of Units (SI). Responding to requests from the forensic and clinical communities for calibration standards that provide traceability to the SI, we and others have established droplet digital polymerase chain reaction (ddPCR) as a potentially primary measurement process that can provide traceable results.[11-18]

The copy numbers of human nuclear DNA (nDNA) in the reaction mixture per partition, symbolized as λ, were determined by ddPCR using ten PCR assays optimized for use with our ddPCR platform. These assays target loci on eight chromosomes, with three assays on widely separated locations on one chromosome (see Table 1).

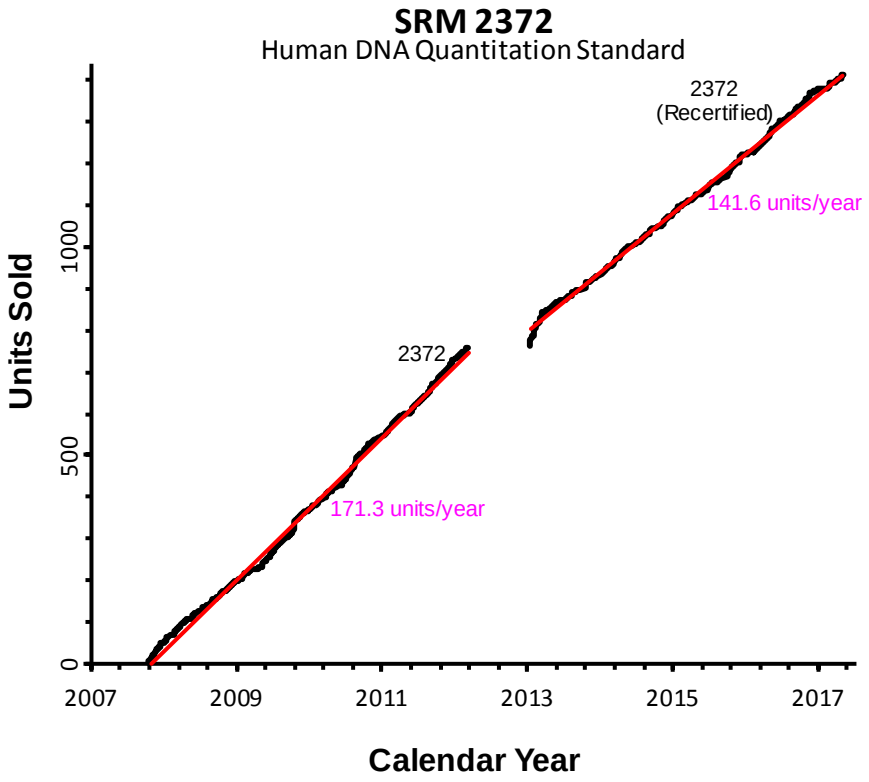

Figure 1: Sales History of SRM 2372

Each small black dot represents the sale of one unit of SRM 2372. The red lines summarize the average rate of sales as originally certified (2007 to 2012) and after re-certification (2013 to 2017). The magenta labels state the average yearly sales rate during these two periods.

The nDNA copy number concentration, human haploid genome equivalents (HHGE) per nanoliter component solution and symbolized here as λ', is calculated as

$$\text{copy number [DNA]} = \lambda' = \lambda/(VF) \text{ HHGE/nL}$$

where V is the average droplet volume and F is the total dilution factor, the volume fraction of the component solution in the ddPCR reaction mixture.

The manufacturer of the ddPCR platform used, a Bio-Rad QX200 Droplet Digital PCR System (Hercules, CA) system, does not provide metrologically traceable droplet volume information. This lack of information prevented metrologically traceable conversion of the number per partition measurements to the desired number per reaction volume units. In consequence, NIST developed a measurement method that provides metrologically traceable droplet volumes.[19]

We show elsewhere that the mass [DNA], nanograms of human DNA per microliter component, can be traceably estimated as

$$\text{mass [DNA]} = 3.031 \times \lambda' \text{ ng/µL}$$

where the constant is the product of the number of nucleotide basepairs (bp) in the human reference genome, the average bp molecular weight, Avogadro's number of bp per mole, and

unit scaling factors.[18] The three components of SRM 2372a are certified for both copy number and mass [DNA].
Mitochondrial DNA (mtDNA) analysis within the forensic community is useful when the nDNA may be degraded or is in low-to-undetectable quantity. The challenges of mtDNA testing include contamination of the standard due to routine laboratory use, degradation of plasmids or synthetic oligomers, or improper commutability of the standard relative to casework samples. Commonly, cell line materials are used as standards for qPCR with human genomic DNA, which may lead to an incorrect mtDNA-to-nDNA ratio (mtDNA/nDNA) for unknown samples. Non-certified values of mtDNA/nDNA for the three components are provided to bridge the current gap between well characterized mitochondrial DNA quantification assays and the availability of commercial standards.

With the understanding that the forensic DNA community primarily uses qPCR as a method for DNA quantification, the performance of the three SRM 2372a materials was evaluated in eleven commercial qPCR chemistries currently used employed within the forensic DNA community.

Experimental Methods

Production and value-assignment of any certified reference material (CRM) is a complex process. In addition to acquiring, processing, and packaging the materials included in the CRM, the materials must be sufficiently homogenous across the units, stable over time within its packaging, and well-characterized to deliver results that are fit-for-purpose within the intended measurement community.[20] The process has been particularly complicated for SRM 2372a because we needed to determine the metrological properties of the required ddPCR measurement systems before we could trust the results they provide.

The following sections describe the methods and processes used to produce and characterize the SRM 2372a materials, including: sample preparation, SRM unit production, PCR assays, ddPCR measurement processes, spectrophotometric absorbance measurements, homogeneity testing, stability testing, ddPCR nDNA and mtDNA/nDNA measurements, and performance in commercial qPCR assessments. Many of these processes were mutually dependent, therefore the sections are presented in a "definitional" order that sequentially introduces terminology.

Polymerase Chain Reaction Assays

Ten PCR assays were developed for characterization of SRM 2372a. Each assay was confirmed to target one locus per HHGE using the National Center for Biotechnology Information's BLAST/blastn system.[21] Primers were purchased from Eurofins Operon (Huntsville, AL). TaqMan probes, labeled with 6-carboxyfluorescein (FAM), were purchased from Thermo Fisher (Waltham, MA). FAM-labeled Blackhole Quencher and Blackhole Quencher+ probes were purchased from LGC Biosearch Technologies (Novato, CA).

Table 1 details the ten nDNA assays used to certify copy number and mass [DNA] of the SRM 2372a components. FAM-labeled TaqMan probes were purchased from Thermo Fisher

and used in monoplex reactions. VIC (a proprietary dye)-labeled probes were purchased from the same source and were used when nDNA and mtDNA were analyzed in the same reaction mixture. Figure 2 displays exemplar droplet patterns for the assays. All assays were developed at NIST and have been optimized for ddPCR.[18]

Table 2 details three mitochondrial DNA (mtDNA) assays used to estimate mtDNA/nDNA in the SRM 2372a components. Figure 3 displays exemplar droplet patterns for the assays. These assays were optimized for ddPCR from real-time quantitative PCR (qPCR) assays from the literature.

All primers probes were diluted with TE^{-4} buffer purchased from Affymetrix-USB Products (Affymetrix, Inc., Cleveland, OH) to a nominal 5 µmol working solution for all single-plex assays (individual nDNA and mtDNA assays). For duplex assays (combined nDNA and mtDNA), primer and probe working solutions were diluted to a nominal 10 µmol.

Table 1: NIST-Developed Human Nuclear DNA Assays

Assay Target	Chromosome, Band Accession #	Primers and Probe [a]	Amplicon Length, bp
NEIF Gene EIF5B	Chr 2, p11.1-q11.1 NC_000002.12	F gccaaacttcagccttctcttc R ctctggcaacatttcacactaca P^{B+} tcatgcagttgtcagaagctg	67
2PR4 Gene RPS27A	Chr 2, p16 NC_000002.12	F cgggtttgggttcaggtctt R tgctacaatgaaaacattcagaagtct P^{B} tttgtctaccacttgcaaagctggccttt	97
POTP STR TPOX	Chr 2, p25.3 NC_000002.12	F ccaccttcctctgcttcacttt R acatgggtttttgcctttgg P^{T} caccaactgaaatatg	60
NR4Q Gene DCK	Chr 4, q13.3-q21.1 NC_000004.12	F tggtgggaatgttcttcagatga R tcgactgagacaggcatatgtt P^{B+} tgtatgagaaacctgaacgatggt	83
D5 STR D5S2500	Chr 5, q11.2 NC_000005.10	F ttcatacaggcaagcaatgcat R cttaaagggtaaatgtttgcagtaatagat P^{T} ataatatcagggtaaacaggg	75
ND6 STR D6S474	Chr 6, q21-22 NC_000006.12	F gcatggctgagtctaaagttcaaag R gcagcctcagggttctcaa P^{B+} cccagaaccaaggaagatggt	82
D9 STR D9S2157	Chr 9, q34.2 NC_000009.12	F ggctttgctgggtactgctt R ggaccacagcacatcagtcact P^{T} cagggcacatgaat	60
HBB1 Gene HBB	Chr 11, p15.5 NC_000011.10	F gctgagggtttgaagtccaactc R ggtctaagtgatgacagccgtacct P^{T} agccagtgccagaagagccaagga	76
ND14 STR D14S1434	Chr 14, q32.13C NC_000014.9	F tccaccactgggttctatagttc R ggctgggaagtcccacaatc P^{B+} tcagactgaatcacaccatcag	109
22C3 Gene PMM1	Chr 22, q13.2 NC_000022.10	F cccctaagaggtctgttgtgttg R aggtctggtggcttctccaat P^{B} caaatcacctgaggtcaaggccagaaca	78

a F: Forward primer, R: Reverse primer, P^{B}: Blackhole quencher probe (FAM labeled), P^{B+}: Blackhole Plus quencher probe (FAM labeled), P^{T}: TaqMan MGB probe (FAM labeled)

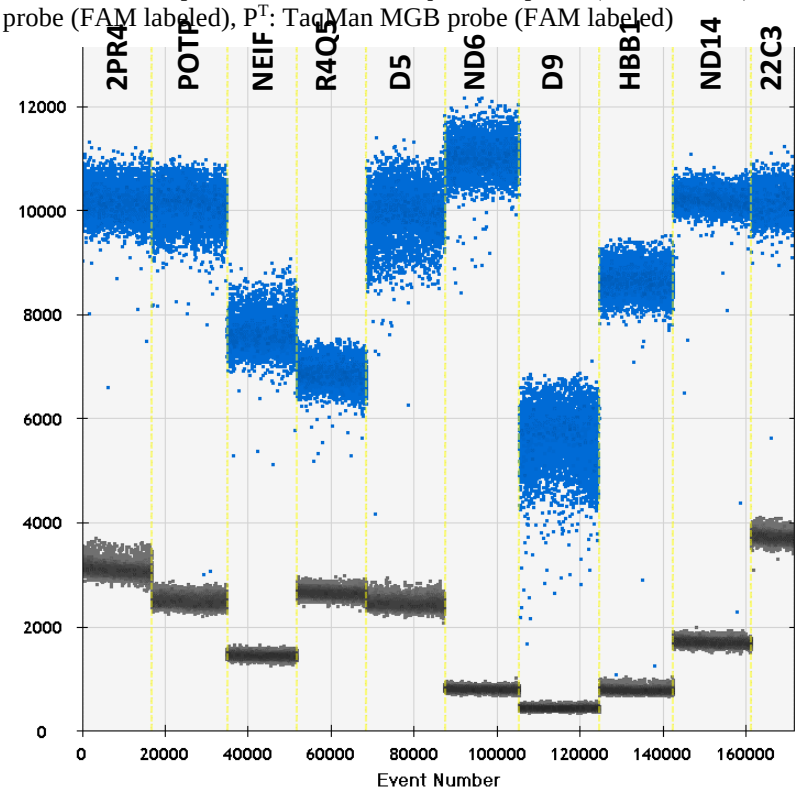

Figure 2: Exemplar Droplet Patterns for the Ten nDNA Assays

Table 2: NIST-Optimized Human Mitochondrial DNA Assays

Assay	Location	Primers and Probe [a]	Amplicon Length, bp	Ref
mtND1	3485:3504 3533:3553 3506:3522	F ccctaaaacccgccacatct R gagcgatggtgagagctaaggt P^{T} ccatcaccctctacatc	69	22
mtBatz	8446:8473 8503:8524 8475:8491	F aatattaaacacaaactaccacctacct R tggttctcagggtttgttataa P^{T} cctcaccaaagcccata	79	23
mtKav	13188:13308 13369:13392 13310:13325	F ggcatcaaccaaccacaccta R attgttaaggttgtggatgatgga P^{T} cattcctgcacatctg	105	24

a F: Forward primer, R: Reverse primer, P^{T}: TaqMan MGB probe (FAM labeled)

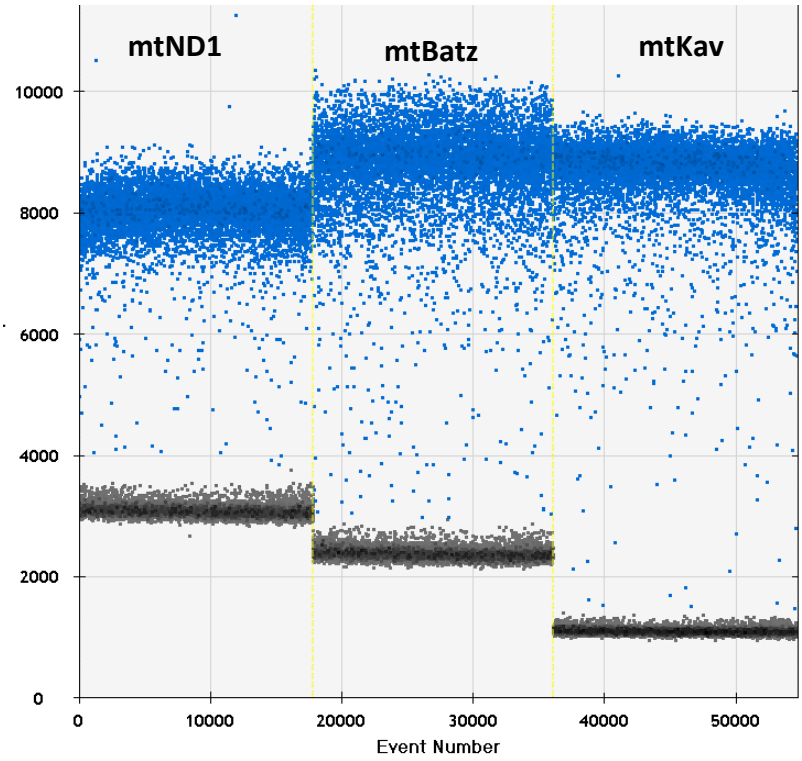

Figure 3: Exemplar Droplet Patterns for the Three mtDNA Assays

Droplet Digital Polymerase Chain Reaction Measurements

Nuclear DNA (nDNA)

All nDNA ddPCR measurements used in this study followed the same measurement protocol:

- Droplets were generated on the Auto Droplet Generator using the Bio-Rad "ddPCR "Supermix for Probes (No dUTP)" (Bio-Rad, cat. # 186-3024, Control 64079080) and "Droplet Generation Oil for Probes" (Bio-Rad, cat # 186-4110, Control 64046051). Note: desoxyuridine 5'-triphosphatase (dUTP) is an enzyme sometimes included in PCR reaction mixtures to minimize PCR carryover contamination.
- Table 3 details the composition of the ddPCR mastermix setup for a given assay and sample, where "mastermix refers to reaction mixture of manufacturer's proprietary PCR reagent "Supermix", forward and reverse PCR primers for a given assay, probe for that assay, the sample DNA, and sometimes additional water to produce the desired value dilution factor, *F*. This setup was used for all assays and samples.
- Non-Template Controls (NTCs) for each assay were included in every analysis.
- Droplets were thermal cycled on a ProFlex thermal cycler (Applied Biosystems) for 95 °C for 10 min followed by 60 cycles of 94 °C for 0.5 min and 61 °C for 1 min, then 98 °C for 10 min before a 4 °C hold until the plate was removed.
- Droplets were read on the QX200 Droplet Reader and analysis was performed using the QuantaSoft Analysis Software version 1.7.4.0917.

- The numbers of positive and negative droplets at the end of 60 cycles were determined and were exported into a spreadsheet for further analysis. Assay-specific intensity thresholds were determined by visual inspection for each assay for each measurement session.

Table 3: Mastermix Setup for Human Nuclear DNA ddPCR Assays

Concentration	nDNA Mastermix	Microliter per Reaction
2X	"Supermix for Probes (no dUTPs)"	12.5
5 µmol/L	Forward Primer	1.875
5 µmol/L	Reverse Primer	1.875
5 µmol/L	Probe (FAM)	1.25
	PCR Grade Water	5.0
	1:4 Diluted Sample	2.5
	Total Volume	25.0

Mitochondrial DNA (mtDNA)

All the mtDNA measurements used in this study followed the nDNA protocol described above, but with different mastermix setups. The duplex mtDNA measurements used the setup described in Table 4. The monoplex mtDNA measurements used the setup described in Table 5.

Table 4: Mastermix Setup for Human Mitochondrial DNA ddPCR Duplex Assays

Concentration	mtDNA Mastermix	Microliter per Reaction
2X	Supermix for Probes (no dUTPs)	12.5
10 µmol/L	Forward Primer	0.9375
10 µmol/L	Reverse Primer	0.9375
10 µmol/L	Probe (VIC)	0.625
	PCR Grade Water	9.5
	nDNA Mastermix from Table 3	0.5
	Total Volume	25.0

Table 5: Mastermix Setup for Human Mitochondrial DNA ddPCR Monoplex Assays

Concentration	mtDNA Mastermix	Microliter per Reaction
2X	Supermix for Probes (no dUTPs)	12.0
10 µmol/L	Forward Primer	0.9
10 µmol/L	Reverse Primer	0.9
10 µmol/L	Probe (FAM)	0.6
	PCR Grade Water	7.2
	Diluted Sample (See Table 7)	2.4

Total Volume	24.0

Sample Preparation

The buffy coat white blood cells used to prepare SRM 2372a materials were purchased from Interstate Blood Bank. These materials were from four single-source anonymous individuals, supplied to us labeled as: $Female_1$, $Female_2$, $Male_1$, and $Male_2$. In our laboratories, each donor buffy coat bag was aliquoted (5 mL per aliquot) into sterile 50 mL conical tubes and stored at 4 °C prior to extraction. The modified salt-out manual extraction protocol described in Appendix A was performed for each aliquot, with rehydration of the DNA in TE^{-4} buffer.

From 150 mL to 250 mL of solubilized DNA for each component was accumulated through the extraction of the 5 mL aliquots. The DNA was stored in perfluoroalkoxy polymer (PFA) containers that had been bleach sterilized, thoroughly rinsed with deionized water, rinsed with ethanol, and air-dried. The minimum volume for production of 2000 units was 120 mL. Preliminary estimates of the mass [DNA] of the re-solubilized components were obtained using a Nanodrop microvolume spectrophotometer. Dilutions of the component materials were made until the absorbance at 260 nm was approximately 1, corresponding to a nominal mass [DNA] of 50 ng/µL of dsDNA.[7] After addition of the TE^{-4} buffer, the containers were placed on an orbital shaker and gently rotated for several hours prior to refrigerated storage at 4 ºC.

The four single-source components were initially screened with the nDNA ddPCR measurement process using four nDNA assays: 2PR4, NR4Q, ND6, and 22C3. Samples of the four single-source materials were gravimetrically prepared as 1→4 dilutions of the bulk materials, followed by a 1→10 volumetric dilution, and run in duplicate. The gravimetric 1→4 dilution was prepared by weighing an empty PFA vial and recording the weight, then pipetting 30 µL TE^{-4} into the vial and recording the weight, then pipetting 10 µL DNA, and recording the final weight. The dilution factor was calculated in a spreadsheet. These screening measurements verified that the individual component materials were fit-for-purpose.

The four single-source components were evaluated with the nDNA ddPCR measurement process using the ten nDNA assays. Samples of the four single-source materials were gravimetrically prepared as 1→4 dilutions of the bulk materials, followed by a 1→10 volumetric dilution, and run in duplicate. Quantitative information was derived from the average of the ten nDNA assays to prepare the component C mixture.

Thirty milliliters of sample $Male_2$ (estimated at 45.2 ng/µL mass [DNA]) were combined with 80 mL of sample $Female_2$ (estimated at 50.7 ng/µL mass [DNA]) and 10 mL of TE^{-4} buffer to create a 1 male nDNA to 3 female nDNA, producing a 1:4 male/human mixture. After addition of the TE^{-4} buffer, the component C container was gently rotated on the orbital shaker for several hours prior to refrigerated storage at 4 ºC.

Absorbance Measurements

In February 2017 absorbance spectra of the recently prepared, fully diluted, nominally dsDNA and ssDNA solutions of the three components were acquired with a BioCary 100 spectrophotometer using the measurement protocol described in Appendix B. In December 2017, additional spectra were acquired for the remaining bulk solution of components A and B and for SRM 2372 components A and B. These solutions had been stored at 4 °C in well-sealed PFA containers. There was no remaining bulk component C solution.

SRM Unit Production

In the morning of the day a component was transferred into the sterile component tubes (Sarstedt Biosphere SC micro tube-0.5 mL, Newton, NC) used to package the SRM, the container holding the diluted component was removed from the refrigerator and placed on a slow orbital shaker for 2 h. The tubes were opened and placed under the laminar flow hood in rows of 5 within 80-hole tube racks for a total of 30 tubes per rack. Tubes were placed in every second row of the racks to facilitate filling with an Eppendorf Repeater Xstream pipette (Eppendorf North America, Inc., Hauppauge NY) fitted with a 1-mL positive displacement tip set to dispense 55 µL per tube. The component container was removed from the shaker, a magnetic stir-bar was added, the container was placed on a magnetic stir plate in the laminar flow hood, and the material was stirred gently. Filling proceeded until 2200 vials were filled, with pipet tip replacement after filling every 200 tubes. The filling process consisted of two individuals manually filling each vial within a biosafety cabinet (one physically pipetting each sample, the other verifying that each tube was filled) and four individuals tightly closing the lids to each tube on a sterilized and covered bench.

When all tubes in a rack were filled, the rack was moved out of the hood, the tubes checked for proper filling, their lids closed, and the tubes transferred to 100-unit pre-labeled storage boxes. As the 100-unit boxes were filled, they were transferred to the labeling room where SRM labels were applied by hand and colored inserts were added to the lids: red for component A, white for component B, and blue for component C.

After all tubes for a given component were labeled, the 22 boxes were placed in a locked refrigerator. Filling of each of the components took 60 min to 90 min. All units were transferred to the refrigerator within 4 hours of beginning the filling process. All units of the three materials were allowed to equilibrate for a minimum of two weeks prior to beginning homogeneity analysis.

Component Homogeneity

The ddPCR measurement protocol described above was used to assess the nDNA homogeneity of the three components. One tube from the approximate center of each of the 22 storage boxes was assayed using the NEIF and ND6 assays (see Table 1). A sample from each tube was gravimetrically diluted 1→4 with TE^{-4}, followed by a volumetric 1→10 dilution into the mastermix. Three technical replicates were obtained for every tube. A total

of six plates were used: two plates per component, one with the NEIF assay and one with the ND6 assay. Figure 4 details the layout of the samples.

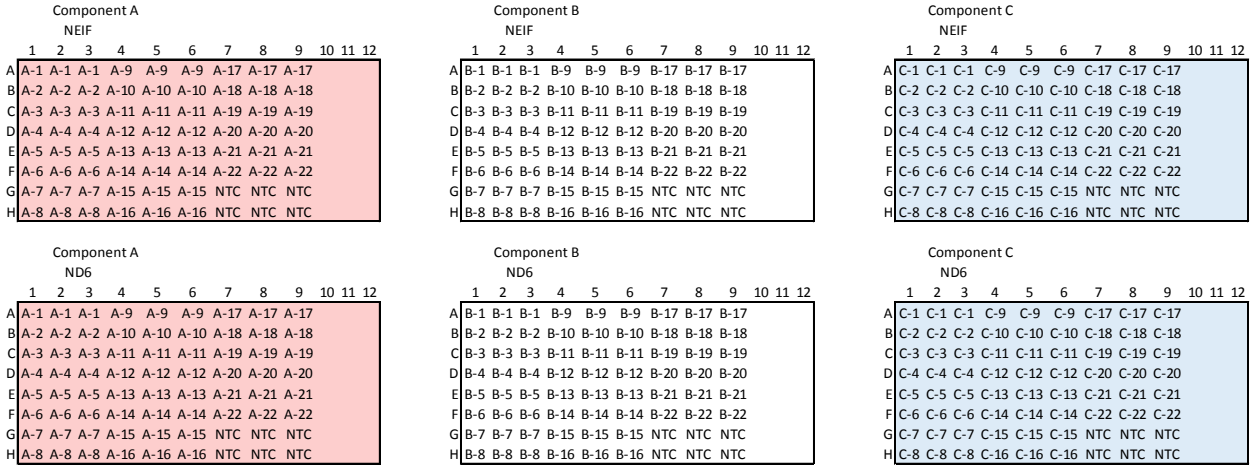

Figure 4: Plate Layout Design for Homogeneity Measurements

Component Stability

Stability of the materials was evaluated at 4 °C, room temperature (RT, ≈ 22 °C), and 37 °C. Tubes from the homogeneity study were divided among the three temperatures for the stability study. Additionally, two never-opened tubes (one from box 8 and one from box 12 of each component) were held at each temperature. Two tubes at each temperature for each component were opened, sampled, and resealed 4 times over the course of 8 weeks. Measurements were made at week 1, week 3, week 5, and week 8. The measurement on week 8 was from an unopened tube (from box 8) for each of the components at all temperatures.

The data from the homogeneity study was used as timepoint 0 in the stability study, anchored to 4 °C by the storage recommendations. A sample from each tube was gravimetrically diluted 1→4 with TE^{-4} followed by a volumetric 1→10 dilution into the mastermix. Two technical replicates were used for both the NEIF and ND6 assays. All temperatures, components, and both assays fit onto one plate for timepoints 1 to 5. Twelve NTCs, placed in rows G and H, were included for each assay. Figure 5 details the sample layout.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	A-7	A-7	B-7	B-7	C-7	C-7	A-7	A-7	B-7	B-7	C-7	C-7	4C
B	A-10	A-10	B-10	B-10	C-10	C-10	A-10	A-10	B-10	B-10	C-10	C-10	
C	A-9	A-9	B-9	B-9	C-9	C-9	A-9	A-9	B-9	B-9	C-9	C-9	RT
D	A-12	A-12	B-12	B-12	C-12	C-12	A-12	A-12	B-12	B-12	C-12	C-12	
E	A-8	A-8	B-8	B-8	C-8	C-8	A-8	A-8	B-8	B-8	C-8	C-8	37C
F	A-11	A-11	B-11	B-11	C-11	C-11	A-11	A-11	B-11	B-11	C-11	C-11	
G	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	
	NEIF						ND6						

Figure 5: Plate Layout Design for Stability Measurements

Component Certification Measurements

After establishing homogeneity and stability, measurements intended to be used to certify nDNA values and inform mtDNA values were started using randomly selected SRM tubes from the boxes listed in Table 7. The ten nDNA assays described in Table 1 and two of the three mtDNA assays described in Table 2 were run simultaneously on the same sample plate. A sample from each tube was gravimetrically diluted 1→4 with TE^{-4} followed by a volumetric 1→10 dilution into the mastermix. Two technical replicates were used in each of the six measurement campaigns.

Table 6: Tubes used for Certification Measurements

	Component		
Plate	A	B	C
dd20170711	Box 1	Box 1	Box 1
dd20170712a	Box 10, tube a	Box 10, tube a	Box 10, tube a
dd20170712b	Box 10, tube b	Box 10, tube b	Box 10, tube b
dd20170713	Box 20	Box 20	Box 20
dd20170714	Box 5	Box 5	Box 5
dd20170801	Box 5	Box 5	Box 5

Every sample was analyzed in duplicate with every assay in all six plates. Component A was always located in rows B and C, component B in rows D and E, component C in rows F and G, and NTCs in rows A and H. Figure 6 displays the basic layout of the ten nDNA and two mtDNA assays. In addition to the plate column assignment scheme shown, the three assay groupings were also ordered as {Group 2, Group 3, Group 1} and {Group 3, Group 1, Group 2} to address potential positional bias.

		Group 1				Group 2				Group 3			
		2PR4	POTP	NEIF	NR4Q	D5	ND6	D9	HBB1	ND14	22C3	mtND1	mtBatz
		1	2	3	4	5	6	7	8	9	10	11	12
	A	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC
Comp A	B												
Comp A	C												
Comp B	D												
Comp B	E												
Comp C	F												
Comp C	G												
	H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

Figure 6: Basic Plate Layout Design for Certification Measurements

Mitochondrial DNA Measurements

A "duplexed reaction" was used to determine mtDNA/nDNA where 1 µL of one of the nDNA mastermix was added to 49 µL of the mtDNA mastermix resulting in a 1→50 volumetric dilution of the nDNA. The nDNA was targeted with a FAM-labeled probe, while the mtDNA was targeted with a VIC labeled probe. The primer and probe concentrations were increased from 5 µmol to 10 µmol in the duplexed reactions.

Additional testing of the mtDNA assays were performed in monoplex with FAM-labeled probes to determine the additional sources of bias between sampling, assay setup, and dilution factors. Table 7 outlines the different methods examined to determine mtDNA/nDNA for the three components.

Table 7: Run Parameters for Mitochondrial DNA

		Droplet	nDNA			mtDNA		
Plate	Component	Generation	Assay	Dilution	Reps	Assay	Dilution	Reps
dd20170824	A	manualDG	HBB1	1→4	3	mtND1	1→200	9
dd20170825	A,B,C	manualDG	HBB1	1→4	4	mtBatz	1→10→100	4
dd20170829	A,B,C	manualDG	HBB1	1→4	4	mtBatz	1→10→100	4
dd20170831	A,B	autoDG	HBB1	1→3	2	mtND1	1→10→100	3
dd20170831	A,B	autoDG	HBB1	1→6	2	mtND1	1→10→100	3
dd20170831	A,B	autoDG	HBB1	1→12	2	mtND1	1→10→100	3
dd20170907	A,B,C	manualDG	HBB1	1→3	2	mtBatz	1→10→100	3
dd20170913	A,B	manualDG	NEIF	1→4	3	mtND1, mtBatz, mtKav	1→10→100	3
dd20170913	C	manualDG	NEIF	1→4	3	mtBatz, mtKav	1→10→100	3

Performance in Commercial qPCR Chemistries

Table 8 lists the 11 qPCR chemistries that were commercially available in late 2017. All three of the SRM 2372a components were evaluated with these chemistries using component A of SRM 2372 as the reference standard.

For each component of SRM 2372a, four tubes were pulled from box 22 and combined into one tube. A 1→5 dilution of all samples was prepared, followed by three serial 1→2 dilutions. A five-point serial dilution of each SRM 2372a component was amplified in triplicate for each chemistry. This consisted of the neat material, the 1→5 dilution, then the three serial 1→2 dilutions. A six-point serial dilution of SRM 2372 Component A was also created for the standard curve for the commercial qPCR chemistries. This consisted of the neat material, followed by a 1→5 dilution, then followed by four 1→2 dilutions serially. The six-point serial dilution series of SRM 2372 Component A was also amplified in triplicate.

These commercial chemistries were amplified in individual plates on an Applied Biosystems 7500 HID Real-Time PCR Instrument (Foster City, CA) following the manufacturers' recommended protocols for reaction mix setup and cycling conditions. Figure 7 details the plate layout for the qPCR plates. Data was analyzed with the HID Real-Time PCR Analysis Software v1.2 and further analyzed in a spreadsheet.

Table 8: Commercial qPCR Chemistries Tested with SRM 2372a

Manufacturer	Commercial Kit	Target	Code[a]
Innogenomics	InnoQuant	Long	IQ:h
		Short	IQ:d
	InnoQuantHY	Long	IQHY:h
		Short	IQHY:d
		Y	IQHY:y
Thermo Fisher	Quantifiler Duo	Human	QFD:h
		Male	QFD:y
	Quantifiler HP	Large	QFHP:h
		Small	QFHP:d
	Quantifiler Human	Human	QFH:h
	Quantifiler Trio	Large	QFT:h
		Small	QFT:d
		Y	QFT:y
Qiagen	Quantiplex	Autosomal	QP:h
	Quantiplex Hyres	Autosomal	QPH:h
		Y	QPH:y
	Quantiplex Pro	Human	QPP:h
		Degradation	QPP:d
		Y	QPP:y
Promega	Plexor	Autosomal	P:h
		Y	P:y
	PowerQuant	Autosomal	PQ:h
		Degradation	PQ:d
		Y	PQ:y

a Assays are coded with a short acronym derived from the kit name followed by the following codes for the type of target: "h": autosomal non-degradation {described as "Autosomal", "Human", or "Long"}
"d": autosomal degradation {described as "Degradation" or "Short"}
"y": Y-chromosome {described as "Male" or "Y"}

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2372-A_57	2372-A_57	2372-A_57	2372a-neat_A	2372a-neat_A	2372a-neat_A	2372a-neat_B	2372a-neat_B	2372a-neat_B	2372a-neat_C	2372a-neat_C	2372a-neat_C
C	2372-A_11.4	2372-A_11.4	2372-A_11.4	2372a-1:5_A	2372a-1:5_A	2372a-1:5_A	2372a-1:5_B	2372a-1:5_B	2372a-1:5_B	2372a-1:5_C	2372a-1:5_C	2372a-1:5_C
D	2372-A_5.7	2372-A_5.7	2372-A_5.7	2372a-1:10_A	2372a-1:10_A	2372a-1:10_A	2372a-1:10_B	2372a-1:10_B	2372a-1:10_B	2372a-1:10_C	2372a-1:10_C	2372a-1:10_C
E	2372-A_2.85	2372-A_2.85	2372-A_2.85	2372a-1:20_A	2372a-1:20_A	2372a-1:20_A	2372a-1:20_B	2372a-1:20_B	2372a-1:20_B	2372a-1:20_C	2372a-1:20_C	2372a-1:20_C
F	2372-A_1.425	2372-A_1.425	2372-A_1.425	2372a-1:40_A	2372a-1:40_A	2372a-1:40_A	2372a-1:40_B	2372a-1:40_B	2372a-1:40_B	2372a-1:40_C	2372a-1:40_C	2372a-1:40_C
G	2372-A_0.7125	2372-A_0.7125	2372-A_0.7125									
H	NTC	NTC	NTC									

Figure 7: Plate Layout Design for Performance Assessments

Measurement Results

After preparing the SRM 2372a bulk solutions, their approximate 50 ng/µL mass [DNA] were confirmed spectrophotometrically. The bulk solutions were then packaged into individual tubes. The materials contained in the tubes were characterized for homogeneity, stability, nDNA copy number [DNA], and mtDNA/nDNA ratio. The following sections detail the results of these measurements.

Absorbance Spectrophotometry

By widely accepted convention, an aqueous DNA solution having an absorbance of 1.0 at 260 nm corresponds to a mass [DNA] of 50 ng/µL for dsDNA and (37 to 40) ng/µL for ssDNA.[7] Absorbance at (230, 270, 280, and 330) nm are traditionally used in the assessment of DNA quality.[7,25] Due to minor baseline shifts during acquisition of some spectra, it was necessary to bias-adjusted all spectra to have absorbance of 0.0 at 330 nm.

Table 9 lists the resulting bias-adjusted values for selected wavelengths and the conventional mass [DNA] conversion of the absorbance at 260 nm.

The close agreement between the mass concentration values for the nominally dsDNA and the converted-to-ssDNA spectra confirm that the bulk solutions were correctly prepared to be ≈ 50 ng/µL dsDNA. The slight increase in absorbance over the 10 months between acquiring the spectra suggests that, like SRM 2372, the tertiary structure of the dsDNA in the SRM 2372a materials may be changing slowly with time. Figure 8 displays the spectra.

Table 9: Absorbance at Selected Wavelengths

			Wavelength, nm				
Form	Date	Component	230	260	270	280	ng/µL
dsDNA	2/14/2017	A	0.394	0.931	0.758	0.488	46.6
		B	0.480	1.112	0.905	0.583	55.6
		C	0.451	1.006	0.815	0.524	50.3
ssDNA	2/14/2017	A	0.390	0.614	0.540	0.316	45.4
		B	0.457	0.723	0.634	0.372	53.5
		C	0.411	0.641	0.563	0.330	47.4
dsDNA	12/14/2017	A	0.402	0.956	0.775	0.503	47.8
		B	0.484	1.128	0.914	0.589	56.4

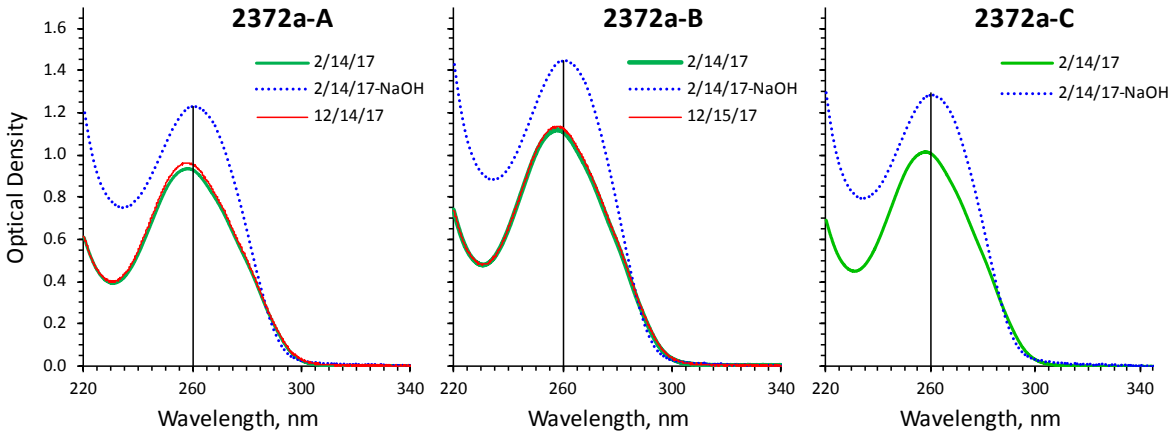

Figure 8: Absorbance Spectra of SRM 2372a Components A, B, and C

dsDNA is denatured to ssDNA by diluting with an equal volume of 0.4 mol/L NaOH; hence, the NaOH-treated spectra displayed are twice the absorbance of the spectra as acquired. The black vertical line marks the 260 nm values used in the conventional conversion of absorbance to DNA mass concentration.

Droplet Volume

Droplet volumes for the two lots of "Supermix" used in the studies described in this report were determined using NIST's Special Test Method 11050S/-D as described in Dagata et al.[19] The lot used for most of the ddPCR analyses produced (0.7349 ± 0.0085) nL droplets, where the "±" value is the combined standard uncertainty. The lot used for analyses performed after October 11, 2017 produced (0.7376 ± 0.0085) nL droplets.

NIST measurements have demonstrated that droplet volumes are not influenced by the presence or absence of DNA in the sample solution. Measurements repeated over time on an earlier lot of the Supermix product used in these studies suggest that droplet volumes remain constant over the shelf-life of the lot. However, droplet volumes for different lots of this product as well as for different Supermix products have been observed to differ by several percent. We intend to document these measurements in a future report.

Other researchers have demonstrated that non-constant droplet volume distributions may contribute to ddPCR measurement imprecision and bias.[26,27] However, these effects become significant only with distributions wider than observed using Special Test Method 11050S/-D[19] and at copy/droplet values larger than the $\lambda \leq 0.5$ copy/droplet typically used in our measurements.

Certification Measurements

Homogeneity Results

Table 10 lists the λ' nDNA copy number values, adjusted for both droplet volume and sample dilution, from the homogeneity measurements. The data was analyzed using the three-level Gaussian hierarchical ANOVA model described in Appendix C. Table 11 lists the values of the within- and between-tube variance components, expressed as percent relative standard deviations. At the 95 % confidence level, all components are homogeneous.

Table 10: Homogeneity Data as λ', Copy Number per Nanoliter of Component

		Component A				Component B				Component C		
Assay	Tube[a]	Rep_1	Rep_2	Rep_3	Tube[a]	Rep_1	Rep_2	Rep_3	Tube[a]	Rep_1	Rep_2	Rep_3
NEIF	A-1-01	16.32	15.29	15.11	B-1-01	17.90	19.09	18.85	C-1-01	14.93	13.85	15.10
ND6		16.16	16.40	15.82		NA[e]	18.70	18.60		15.65	15.62	14.66
NEIF	A-1-02	15.31	15.25	15.49	B-1-02	17.12	17.19	17.04	C-1-02	15.16	15.13	15.77
ND6		16.15	15.89	16.01		17.75	17.18	17.56		14.90	15.47	15.70
NEIF	A-1-03	15.24	15.40	15.33	B-1-03	16.93	17.55	17.49	C-1-03	14.51	15.28	15.06
ND6		15.82	11.52	15.55		18.24	14.83	18.96		14.85	15.05	14.71
NEIF	A-1-04	14.54	14.58	14.27	B-1-04	17.54	17.45	17.39	C-1-04	14.40	14.86	15.20
ND6		16.27	16.05	16.07		16.70	16.32	18.14		15.29	15.08	15.06
NEIF	A-1-05	14.65	15.40	15.04	B-1-05	17.57	16.87	17.35	C-1-05	15.23	14.72	14.83
ND6		16.07	16.33	15.86		16.83	17.59	17.03		15.13	15.20	14.91
NEIF	A-1-06	15.12	14.91	14.91	B-1-06	16.97	17.09	17.14	C-1-06	14.37	14.93	15.22
ND6		15.11	14.83	15.24		16.93	17.23	17.22		14.92	14.81	14.61
NEIF	A-1-07	14.18	14.80	14.55	B-1-07	17.22	16.25	17.03	C-1-07	14.85	14.77	14.63
ND6		14.79	15.57	16.56		16.90	16.84	16.88		14.75	15.47	14.85
NEIF	A-1-08	14.96	15.60	15.01	B-1-08	18.84	18.02	17.72	C-1-08	14.33	14.05	14.78
ND6		15.14	16.15	15.40		18.64	18.87	18.21		14.97	15.01	14.95
NEIF	A-1-09	15.88	15.81	16.37	B-1-09	17.13	17.34	17.27	C-1-09	14.63	14.99	15.05
ND6		14.74	14.38	14.54		17.09	17.56	17.58		15.03	14.91	15.16
NEIF	A-1-10	15.74	15.13	15.70	B-1-10	17.59	18.09	17.38	C-1-10	15.34	14.58	14.54
ND6		15.94	17.13	16.69		17.64	18.16	17.87		14.48	14.65	14.74
NEIF	A-1-11	15.57	15.08	16.33	B-1-11	17.49	17.82	17.40	C-1-11	15.33	15.14	15.55
ND6		15.71	16.11	16.31		17.41	17.92	17.84		14.88	14.70	14.83
NEIF	A-1-12	15.00	14.76	15.15	B-1-12	17.48	17.12	17.18	C-1-12	14.50	15.25	14.97
ND6		16.26	15.86	16.08		17.97	16.14	17.90		14.96	14.83	15.19
NEIF	A-1-13	16.72	16.34	18.04	B-1-13	16.32	16.68	17.01	C-1-13	15.37	15.27	15.15
ND6		14.90	15.16	14.97		16.26	15.66	16.81		14.99	14.79	14.75
NEIF	A-1-14	15.51	15.68	15.38	B-1-14	17.52	15.35	16.73	C-1-14	15.15	14.90	14.94
ND6		16.17	16.14	16.39		17.24	17.41	16.85		14.69	14.80	14.66
NEIF	A-1-15[b]	17.93	17.22	17.33	B-1-15	18.71	18.35	18.87	C-1-15	15.11	15.68	15.94
ND6		18.18	17.53	17.62		17.70	17.52	17.98		15.15	15.43	15.65
NEIF	A-1-16	15.73	15.20	16.17	B-1-16	16.88	17.25	17.43	C-1-16	14.49	14.52	14.71
ND6		16.34	16.27	15.75		17.44	16.97	17.10		15.43	14.48	14.91
NEIF	A-1-17	15.16	15.16	16.28	B-1-17	17.89	17.69	18.25	C-1-17	13.88	14.21	13.94
ND6		16.13	15.67	15.67		17.16	17.62	16.38		14.52	NA[e]	14.35
NEIF	A-1-18	16.12	15.35	16.72	B-1-18	18.26	17.80	18.49	C-1-18	14.75	14.55	14.45
ND6		15.29	14.95	15.28		18.05	18.01	19.31		14.58	15.31	14.59
NEIF	A-1-19	15.98	15.14	15.96	B-1-19	17.51	17.59	18.80	C-1-19	14.68	15.25	14.46
ND6		15.71	15.35	15.11		18.27	17.66	17.86		14.35	14.78	14.21
NEIF	A-1-20	15.75	15.68	15.86	B-1-20	17.38	16.78	17.68	C-1-20	15.49	15.31	14.99
ND6		16.52	15.45	16.27		17.52	17.47	17.68		14.99	14.65	14.68
NEIF	A-1-21	16.58	16.11	15.78	B-1-21	17.42	18.36	17.13	C-1-21	15.30	14.40	14.84
ND6		16.47	16.07	16.12		17.46	17.44	17.64		14.99	14.54	NA[e]
NEIF	A-1-22	15.47	15.28	15.32	B-1-22	17.61	17.43	16.60	C-1-22	14.42	14.87	14.36
ND6		15.37	15.13	15.36		16.66	16.92	18.19		15.89	15.91	15.56
NEIF	A-1-15[c]	14.56	14.48	15.23								
ND6		15.25	14.02	14.36								
NEIF	A-2-15[d]	16.79	15.99	16.74								
ND6		15.81	15.50	15.84								

a Tube coded as Component-Tube-Box

b These results were identified as a probable error in the preparation or volumetric delivery of the sample solution into the microfluidic device and were not used in the homogeneity evaluations.

c Repeat assessment of the solution in sample A-1-15.

d Results for a second tube from box 15, analyzed at the same time as the repeat assessment of A-1-15.

e Value not available due to a technical failure by either the droplet generator or droplet reader

Table 11: Within- and Between-Tube Relative Standard Deviations

Component	Within-Tube NEIF	Within-Tube ND6	Between-Tube
A	2.8 %	4.0 %	3.3 %
B	2.7 %	3.8 %	2.5 %
C	2.3 %	1.8 %	1.9 %

Stability Results

Table 12 lists the λ' volume- and dilution-adjusted nDNA copy number values from the stability measurements; Figure 9 summarizes these results. The results for the tubes held at 4 °C, room temperature (RT, ≈ 22 °C), and 37 °C are very similar for all three components. The absence of between-temperature differences identifies the between-week differences as attributable to variation in the measurement processes rather than sample instability. These results indicate that the SRM 2372a genomic copy number is thermally stable from 4 °C to 37 °C over an extended period.

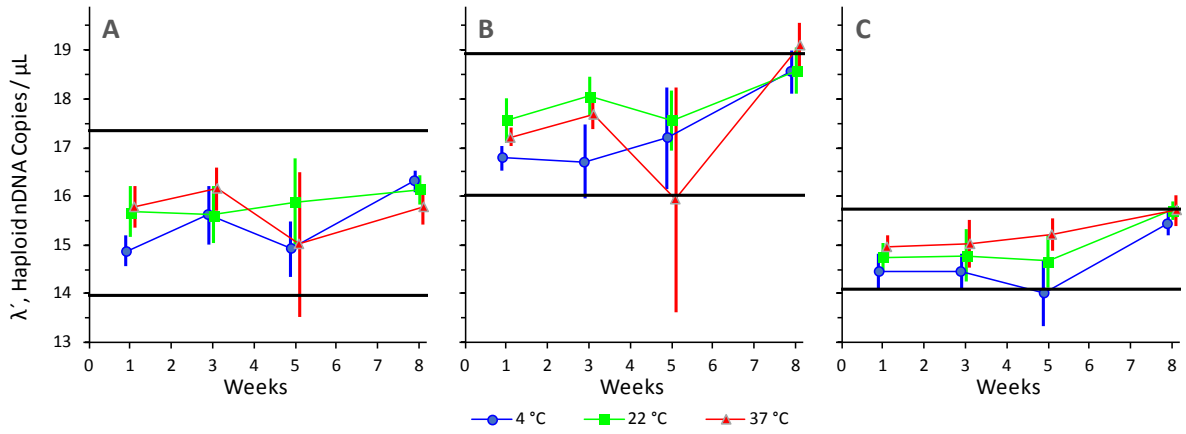

Figure 9: ddPCR Stability Measurements as Function of Time

Each dot-and-bar symbol represents the mean λ' and the approximate 95 % expanded uncertainty for all results for a given component at a given storage temperature. The thick black horizontal lines represent an approximate 95 % confidence interval on the λ' values estimated from the homogeneity data.

Table 12: Stability Data as λ', Copy Number per Nanoliter of Component

			Component A				Component B				Component C			
Week	Temp	Assay	Tube[a]	Rep1	Rep2	Rep3	Tube[a]	Rep1	Rep2	Rep3	Tube[a]	Rep1	Rep2	Rep3
1	4 °C	NEIF	A-10	15.68	14.41		B-10	16.66	16.53		C-10	14.24	13.94	
		ND6	A-10	14.74	14.94		B-10	16.55	17.05		C-10	14.61	13.94	
		NEIF	A-07	15.00	14.63		B-07	17.18	16.40		C-07	14.46	14.52	
		ND6	A-07	14.79	14.79		B-07	17.08	16.86		C-07	15.22	14.82	
	RT (22 °C)	NEIF	A-12	16.74	16.08		B-12	18.05	17.77		C-12	15.06	14.95	
		ND6	A-12	15.22	15.07		B-12	17.59	17.88		C-12	14.78	14.61	
		NEIF	A-09	16.35	15.39		B-09	17.91	17.93		C-09	14.11	NA[b]	
		ND6	A-09	15.43	15.19		B-09	16.61	16.82		C-09	14.82	14.96	
	37 °C	NEIF	A-11	16.14	16.37		B-11	17.48	17.44		C-11	15.15	14.64	
		ND6	A-11	14.94	15.02		B-11	17.40	16.97		C-11	14.95	15.35	
		NEIF	A-08	15.92	16.14		B-08	16.91	17.25		C-08	15.28	15.00	
		ND6	A-08	15.81	15.95		B-08	17.27	16.99		C-08	14.58	14.83	
3	4 °C	NEIF	A-10	15.20	17.06		B-10	NA[b]	17.33		C-10	14.27	14.73	
		ND6	A-10	15.43	16.24		B-10	16.70	17.55		C-10	14.43	13.64	
		NEIF	A-07	15.37	15.70		B-07	15.49	15.65		C-07	14.24	15.12	
		ND6	A-07	15.17	14.79		B-07	17.05	17.23		C-07	14.83	14.39	
	RT (22 °C)	NEIF	A-12	15.21	16.07		B-12	18.17	18.42		C-12	15.66	14.71	
		ND6	A-12	16.44	15.97		B-12	18.45	17.86		C-12	15.04	15.11	
		NEIF	A-09	14.32	16.26		B-09	18.25	18.56		C-09	13.92	13.87	
		ND6	A-09	15.39	15.28		B-09	17.34	17.37		C-09	15.35	14.65	
	37 °C	NEIF	A-11	16.51	16.85		B-11	17.59	17.79		C-11	14.37	14.40	
		ND6	A-11	16.80	15.94		B-11	18.06	18.06		C-11	14.71	15.09	
		NEIF	A-08	15.80	16.13		B-08	17.40	17.91		C-08	15.82	15.90	
		ND6	A-08	15.92	15.24		B-08	17.69	16.94		C-08	14.68	15.20	
5	4 °C	NEIF	A-10	14.86	14.40		B-10	16.26	15.51		C-10	13.81	13.30	
		ND6	A-10	15.55	15.77		B-10	16.73	16.25		C-10	15.47	13.95	
		NEIF	A-07	13.87	14.29		B-07	17.23	17.87		C-07	13.30	13.14	
		ND6	A-07	15.38	15.22		B-07	18.62	19.09		C-07	14.52	14.58	
	RT (22 °C)	NEIF	A-12	16.01	16.31		B-12	17.18	17.52		C-12	15.46	15.41	
		ND6	A-12	16.99	16.77		B-12	17.72	17.21		C-12	14.90	14.71	
		NEIF	A-09	13.81	14.84		B-09	16.75	16.94		C-09	13.71	13.76	
		ND6	A-09	16.25	16.15		B-09	19.04	18.15		C-09	14.68	14.72	
	37 °C	NEIF	A-11	14.56	15.17		B-11	17.20	17.04		C-11	15.05	14.93	
		ND6	A-11	16.50	16.58		B-11	11.48	11.52		C-11	15.27	16.00	
		NEIF	A-08	15.90	16.66		B-08	17.45	17.23		C-08	14.69	15.40	
		ND6	A-08	12.35	12.44		B-08	18.09	17.49		C-08	15.08	15.29	
8	4 °C	NEIF	A-08a	16.67	16.32	15.89	B-08a	18.80	18.84	19.40	C-08a	15.25	14.75	15.42
		ND6	A-08a	15.99	15.93	15.90	B-08a	18.59	18.97	19.72	C-08a	15.50	15.02	15.20
	RT (22 °C)	NEIF	A-08b	16.93	16.66	16.34	B-08b	17.69	18.84	17.71	C-08b	15.95	15.57	15.45
		ND6	A-08b	16.44	16.20	16.47	B-08b	18.62	17.38	18.11	C-08b	16.11	15.85	15.35
	37 °C	NEIF	A-08c	16.20	15.76	16.08	B-08c	19.34	19.52	19.56	C-08c	15.95	16.00	15.69
		ND6	A-08c	15.76	15.53	15.32	B-08c	18.89	18.44	18.89	C-08c	15.69	15.14	15.81

a Tube coded as Component-Tube-Box
b Value not available due to a technical failure by either the droplet generator or droplet reader

Ten-Assay Certification Results

The original intention was to certify the copy number [DNA] of the three components with results from five independently prepared sets of samples analyzed over four days. Each sample of the three components was from a new tube from different boxes and was evaluated with 10 nDNA assays. The assay positions were alternated across these five "10-assay certification" plates. The λ' results for these five plates are systematically somewhat larger than those for the homogeneity and stability data.

On review, we realized that a different workspace and its suite of pipettes had been used to prepare the 1→10 dilution of the 1→4 diluted components into the reaction mastermix. The "high" workstation was used for stability and homogeneity measurements, while the "low" workstation was used for certification measurements. Gravimetric testing of the pipettes established that the volumes delivered by these alternate pipettes was sufficiently biased to account for the observed λ' differences. A sixth "certification" plate was prepared using the original (and now re-verified) pipettes. The λ' results or this plate agreed well with the homogeneity and stability data.

Table 13 lists the λ' volume- and dilution-adjusted nDNA copy number values for all six of the ten-assay datasets; Figure 10 displays the extent of agreement among the ten assays. While there are small-but-consistent differences between the assays, there is only a 4 % difference between the assays providing smallest and largest λ'.

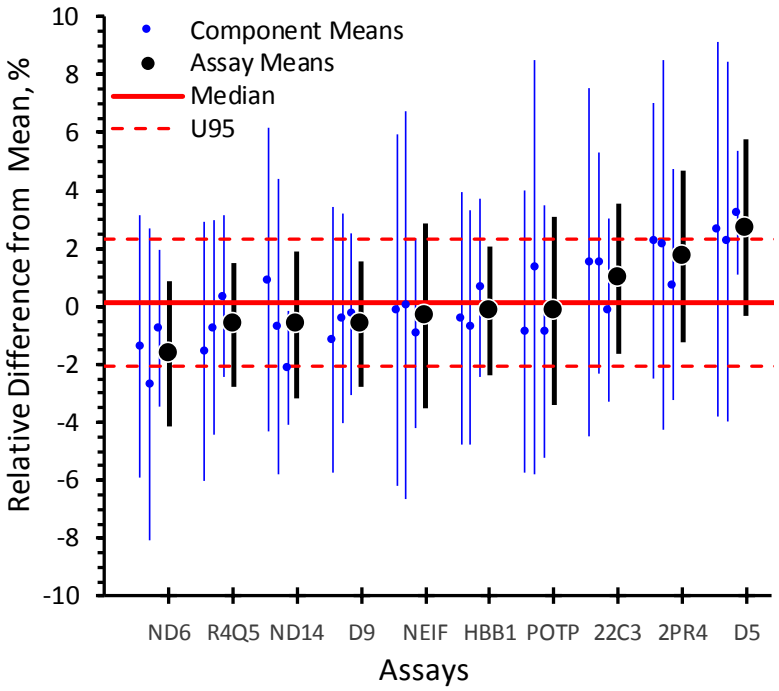

Figure 10: Comparison of Assay Performance

The blue dot-and-bars represent the mean and its combined standard uncertainty for the A, B, and C components for each assay, relative to the over-all grand mean for each component. The black dot-and-bars represent combination of three component results for each assay. The solid horizontal line represents the median difference of the assay means from the grand mean; the dotted horizontal lines bound an approximate 95 % confidence region about the median.

Table 13: Certification Data as λ', Copy Number per Nanoliter of Component

		Component A		Component B		Component C	
Date	Assay	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
7/11/2017	2PR4	16.67	16.98	18.69	19.60	16.30	17.06
	POTP	16.55	16.85	18.50	17.81	15.96	15.76
	NEIF	16.47	16.65	17.09	17.07	15.74	15.38
	R4Q5	15.94	16.58	18.56	17.87	15.90	15.55
	D5	16.55	16.83	18.48	18.29	16.10	16.07
	ND6	16.29	16.47	18.28	18.26	15.89	15.48
	D9	16.55	16.52	18.61	18.58	15.69	15.72
	HBB1	16.44	16.11	18.25	18.69	15.65	16.81
	ND14	16.45	17.07	18.71	17.73	15.64	14.92
	22C3	15.45	16.03	17.76	19.02	15.48	16.55
7/12/2017	2PR4	16.68	17.39	21.74	22.31	14.99	14.97
	POTP	15.87	17.02	22.02	22.92	15.76	16.11
	NEIF	16.78	16.14	21.72	22.16	15.45	15.60
	R4Q5	16.28	17.62	NA	20.78	15.84	15.46
	D5	17.38	16.49	22.30	21.92	15.77	15.29
	ND6	16.67	16.78	20.94	20.34	15.99	14.78
	D9	16.95	16.53	19.49	21.10	15.87	15.04
	HBB1	17.57	17.50	19.71	21.23	15.20	15.51
	ND14	17.71	17.64	21.23	20.66	14.79	14.38
	22C3	17.62	17.59	21.07	20.46	15.12	14.94
7/12/2017	2PR4	16.38	16.67	18.10	18.06	14.52	14.24
	POTP	16.76	16.38	18.65	18.01	13.47	13.97
	NEIF	16.16	16.24	18.06	17.86	13.95	13.50
	R4Q5	15.59	15.87	18.78	17.68	14.42	14.59
	D5	16.08	16.52	18.24	17.90	14.48	16.02
	ND6	16.11	15.89	17.68	16.70	14.63	14.35
	D9	15.17	16.07	18.11	17.49	14.02	14.33
	HBB1	15.57	15.82	17.63	17.41	14.74	14.81
	ND14	16.04	16.25	18.65	17.81	14.66	14.82
	22C3	16.75	16.16	18.15	18.66	14.39	15.68
7/13/2017	2PR4	18.88	19.39	17.84	18.21	14.70	14.44
	POTP	18.68	18.15	17.35	18.13	14.11	14.27
	NEIF	19.53	19.02	17.78	18.13	15.12	14.39
	R4Q5	17.79	18.71	18.35	17.49	14.52	14.68
	D5	19.24	21.24	17.94	18.48	15.67	15.24
	ND6	18.61	17.87	17.00	17.11	14.80	14.24
	D9	18.24	18.44	17.99	17.79	14.42	14.59
	HBB1	18.09	17.99	17.65	17.69	14.30	14.67
	ND14	18.64	18.57	17.54	17.25	14.13	14.11
	22C3	19.30	19.42	17.92	18.14	14.86	14.80
7/14/2017	2PR4	15.97	15.90	17.18	17.68	14.85	14.58
	POTP	15.33	14.04	17.53	16.98	14.22	14.59
	NEIF	14.62	15.33	17.73	17.92	14.38	14.65
	R4Q5	14.93	15.19	17.77	17.40	14.39	14.68
	D5	15.38	15.86	18.03	17.95	15.15	14.57
	ND6	14.92	15.16	17.18	16.93	14.09	14.12
	D9	15.28	15.38	18.02	17.43	14.69	14.74
	HBB1	15.48	15.81	18.34	17.45	14.53	14.28
	ND14	15.13	15.14	17.31	17.14	14.65	14.25
	22C3	15.62	NA	18.42	NA	13.63	13.93

		Component A		Component B		Component C	
Date	Assay	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
10/12/17	2PR4	14.31	14.11	17.91	17.70	13.57	13.64
	POTP	14.07	13.95	17.46	18.20	14.47	13.61
	NEIF	14.87	13.98	18.03	18.03	14.48	14.04
	R4Q5	14.50	14.39	17.67	17.39	14.09	14.11
	D5	13.94	14.22	17.45	17.73	13.89	14.29
	ND6	13.96	13.93	18.24	17.29	14.15	14.17
	D9	14.14	13.90	17.52	17.44	14.31	13.80
	HBB1	14.01	13.82	18.13	17.54	13.81	13.36
	ND14	14.12	14.60	16.48	16.71	14.03	13.87
	22C3	14.35	14.10	18.18	17.41	14.35	13.72

Estimation of Copy Number Concentration

It was decided that all three sets of results (homogeneity, stability, and certification) would be used to derive the certified values, with the λ' of the first five "ten-assay" plates adjusted by a data-driven estimate of the volumetric bias. This bias was incorporated into the statistical model, providing better estimates of the λ' means while capturing all of the variance components (homogeneity, stability, between-assay, and between data sets) as random effects using Bayesian MCMC programmed in OpenBUGS.[28] Table 14 lists the estimated values and their uncertainties. See Appendix C for details.

Table 14: Measured Copy Number Concentrations

Genomic Copies per Nanoliter of Solution

Component	Mean [a]	u(Mean) [b]	U_{95}(Mean) [c]	CV_{95} [d]
A	15.1	0.7	1.4	9.3 %
B	17.5	0.3	0.6	3.4 %
C	14.5	0.5	1.0	6.9 %

a Mean: the experimentally determined most probable estimate of the true value.
b *u*(Mean): the standard uncertainty of the Mean.
c Mean $\pm U_{95}$(Mean): the interval which is believed, with about 95 % confidence, to contain the true value.
d CV_{95}: $100*U_{95}$(Mean)/Mean, the 95 % coefficient of variation (percent relative uncertainty).

These results are metrologically traceable to the International System of Units through 1) the counting unit one,[29] 2) the validity of the Poisson endpoint transformation for digital PCR endpoint assays when applied to samples providing ≤ 0.5 copies per droplet, 3) the determination that the copies are dispersed as dsDNA, and 4) calibrated mean droplet volume measurements made at NIST during sample dilution and mastermix preparation.

Conversion of Copy Number to Mass Concentration

As described in Reference 18, metrologically valid conversion from copies per nanoliter to nanograms per microliter requires quantitative estimates for the number of nucleotide bases per reference human genome and the average nucleotide mass. Using the values estimated in that report and asserting that each target copy corresponds to one HHGE, the required transformation is:

$$\frac{[\text{DNA}]\ \text{ng}}{\mu\text{L}} = \frac{[\text{DNA}]\ \text{HHGE}}{\text{nL}} \times \frac{3.301\ \text{ng}}{\text{HHGE}}.$$

The combined standard uncertainty is:

$$u\left(\frac{[\text{DNA}]\ \text{ng}}{\mu\text{L}}\right) = \sqrt{u^2\left(\frac{[\text{DNA}]\ \text{HHGE}}{\text{nL}}\right) + \left(\frac{0.531}{100} \times \frac{[\text{DNA}]\ \text{HHGE}}{\text{nL}}\right)^2}\ .$$

Both the measured and the conversion factor uncertainties are associated with "large" degrees of freedom. Therefor the approximate 95 % confidence uncertainty is estimated:

$$U_{95}\left(\frac{[\text{DNA}]\ \text{ng}}{\mu\text{L}}\right) = 2 \times u\left(\frac{[\text{DNA}]\ \text{ng}}{\mu\text{L}}\right).$$

Table 15 lists the transformation of the Table 14 copy number values. These values agree well with the conventional 260 nm absorbance values: averaging (4 ± 5) % larger.

Table 15: Estimated Mass Concentrations, Nanograms DNA per Microliters Solution

ng/µL

Component	Value [a]	u(Measurement)[b]	u(Conversion)[c]	U_{95}(Value)[d]	CV_{95}[e]
A	49.8	2.3	0.3	4.7	9.3 %
B	57.8	1.0	0.3	2.1	3.6 %
C	47.9	1.7	0.3	3.3	7.0 %

a Estimated true value.
b Standard uncertainty component from the ddPCR measurements.
c Standard uncertainty estimated from the relative uncertainty of the conversion factor.
d Value ±U_{95}(Value) is believed, with about 95 % confidence, to contain the true value.
e 100*U_{95}(Value)/Value, the 95 % coefficient of variation (percent relative uncertainty).

These results are metrologically traceable to the International System of Units through 1) the measured estimates of copies per nanoliter and 2) determinations made by us from internationally recognized literature resources of the average number of nucleotide bases per HHGE and the mean molecular weights of the bases.[18]

Mitochondrial to Nuclear DNA Ratio (mtDNA/nDNA)

While not intended to provide certified values, mtDNA/nDNA was measured as part of 13 experiments over 5 months. Measurements were made using dilution-adjusted λ for the mtND1, mtBatz, and/or mtKav mitochondrial assays referenced against the dilution-adjusted λ of either the NEIF or HBB1 nDNA assays. By chance, both DNAs in the component C mixture have a binding site mutation that silences the mtND1 probe. Figure 11 graphically summarizes the results by assay and component;

Table 16 lists the data; Table 17 summarizes results from a random effects model that accounts for between-assay variability. See Appendix C for details.

Due to the limited number of mtDNA assays used in these measurements, these results are metrologically traceable only to the assays used.

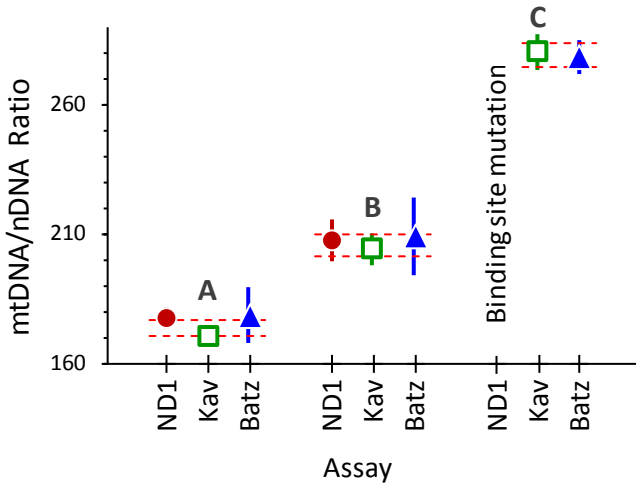

Figure 11: mtDNA/nDNA for Components A, B, and C

Each dot-and-bar symbol represents a mean mtDNA/nDNA and its approximate 95 % expanded uncertainty. The dotted horizontal lines represent approximate 95 % confidence intervals on the ratios.

Table 16: mtDNA/nDNA Ratios

			Component A				Component B				Component C			
Dataset	Aliquot	mtDNA Assay	Rep_1	Rep_2	Rep_3	Rep_4	Rep_1	Rep_2	Rep_3	Rep_4	Rep_1	Rep_2	Rep_3	Rep_4
20170711a	1	mtND1	193.1	187.0			246.1	240.1						
	1	mtBatz	184.5	176.4			255.5	260.8			320.1	329.9		
20170711b	1	mtND1	181.0	190.6			222.9	220.0						
	1	mtBatz	178.5	183.7			210.8	219.2			334.7	328.7		
20170712	1	mtND1	174.3	176.1			212.7	219.8						
	1	mtBatz	182.9	184.7			245.5	250.6			321.7	338.4		
20170713	1	mtND1	171.6	180.0			221.1	219.9						
	1	mtBatz	174.0	181.3			238.0	224.9			320.0	336.3		
20170714	1	mtND1	192.3	183.5			210.5	194.3						
	1	mtBatz	193.5	185.1			213.6	211.9			329.4	301.3		
20170814	1	mtND1	178.0	177.6	170.6		204.6	205.7	202.4					
	1	mtBatz	180.4	175.1	173.8		199.8	204.3	195.2		292.7	277.6	276.2	
	1	mtKav	176.9	178.6	176.1		207.7	211.7	204.1		304.4	283.0	282.5	
20170824	1	mtND1	194.7	192.8	192.2									
	2	mtND1	182.9	189.5	185.1									
	3	mtND1		167.4	161.7									
20170825	1	mtBatz	166.7	181.3	170.8	159.8	192.8	191.6	191.5		283.2	284.0	278.8	272.5
	2	mtBatz	156.6	168.7	166.3	158.9	194.7	194.6	196.1	190.9	283.5	279.0	273.6	267.9
	3	mtBatz	164.3	166.8	160.4	157.3	195.3	195.2	194.1	188.1	280.3	277.3	275.7	275.2
20170829	1	mtBatz	171.9	171.6	167.0	166.6	186.5	186.3	182.8	182.8	259.9	250.8	255.1	260.9
	2	mtBatz	158.1	157.3	160.1	161.2	194.4	196.4	194.7	189.5	261.7	248.3	262.6	260.2
	3	mtBatz	154.6	154.2	153.8	158.7	190.8	190.9	194.0	183.9	266.5	253.5	257.0	265.0
20170831	1	mtND1	161.7	159.9			168.8	178.3						

Dataset	Aliquot	mtDNA Assay	Component A				Component B				Component C			
			Rep1	Rep2	Rep3	Rep4	Rep1	Rep2	Rep3	Rep4	Rep1	Rep2	Rep3	Rep4
	2	mtND1	162.2	157.9			182.3	163.1						
	3	mtND1	157.5	152.8			192.0	188.7						
20170907	1	mtBatz	158.4	154.4			189.0	188.6			250.1	252.2		
20170913	1	mtND1	175.7	172.4	176.8		199.0	192.1	198.2					
	1	mtBatz	174.3	167.1	176.6		199.0	192.4	195.8		280.5	282.5	282.9	
	1	mtKav	169.2	166.2	176.7		190.3	183.1	191.9		270.9	273.8	272.7	
20171016	1	mtND1	192.4	194.4	189.3		230.4	234.1	233.2					
	1	mtBatz	178.6	175.2	174.3		204.8	207.8	206.7		279.7	264.2	279.2	
	1	mtKav	153.0	154.2	152.3		239.9	247.9	249.3		280.4	278.9	284.2	
	2	mtBatz	158.0	155.7	159.2						271.5	260.6	268.7	
	2	mtKav	210.3	210.0	204.9						279.9	262.8	255.9	
	3	mtND1	174.9	176.9	177.7		206.5	216.6	216.4					
	3	mtBatz	196.5	188.9	194.7		231.4	228.0	225.8		263.9	253.5	265.7	
	3	mtKav	181.3	186.4	186.4		186.7	198.7	197.1		295.3	275.7	280.2	

Table 17: mtDNA/nDNA Summary Results

	mtDNA/nDNA			
Component	Value [a]	u(Value)[b]	U_{95}(Value)[c]	CV_{95}[d]
A	173.7	1.6	3.8	1.7 %
B	205.8	2.2	4.4	2.1 %
C	279.1	2.3	4.7	1.7 %

a Estimated true value.
b Standard uncertainty.
c Value $\pm U_{95}$(Value) is believed, with about 95 % confidence, to contain the true value.
d 100*U_{95}(Mean)/Mean, the 95 % coefficient of variation (percent relative uncertainty).

Performance in Commercial qPCR Chemistries

Traditional qPCR assays only quantify relative to a reference standard. Thus, qPCR assays do not provide absolute [DNA] for the individual materials but they can provide [DNA] of one component relative to another. Figure 12 summarizes the B/A and C/A ratios for the commercial autosomal ("Human") targets from the eleven assays evaluated (see Table 8). The Y-chromosome ("Male") assays cannot be summarized in this manner as the (all female) component B does not include a Y chromosome.

Figure 13 summaries the mass [DNA] results for the (all male) component A and (male-female mixture) component C. Since this material was prepared as a nominal 1:3 male:female mixture, the expected male:human (total) ratio is 1:4.
sjc
As displayed in these figures, there is intrinsic variability between the SRM 2372a components and between the commercial qPCR chemistries examined. This reflects the difference in the targets and amplification chemistries for each of the commercial kits. Many of the commercial chemistries on the market to date contain multi-copy targets for both autosomal and Y-chromosomal quantification to increase the sensitivity of the assays.

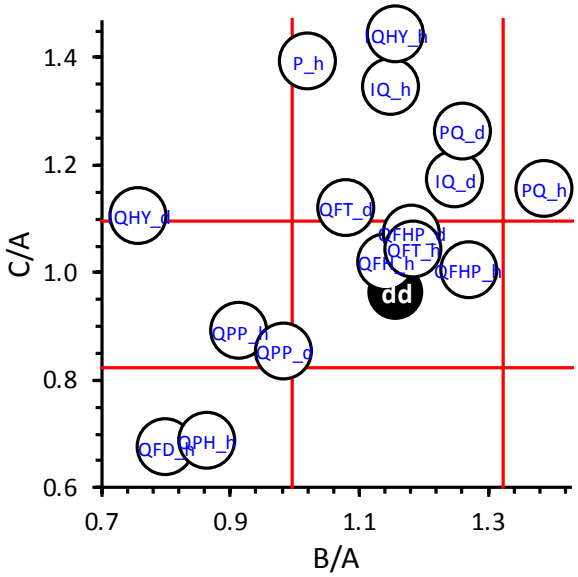

Figure 12: qPCR Autosomal Quantitation Performance Summary

Open circles denote the {B/A, C/A} ratios the 17 commercial qPCR assays evaluated, each labeled with the code listed in Table 8. The solid black circle represents ratios as estimated by ddPCR. The red lines bound the approximate 95 % confidence intervals on the ddPCR-estimated ratios.

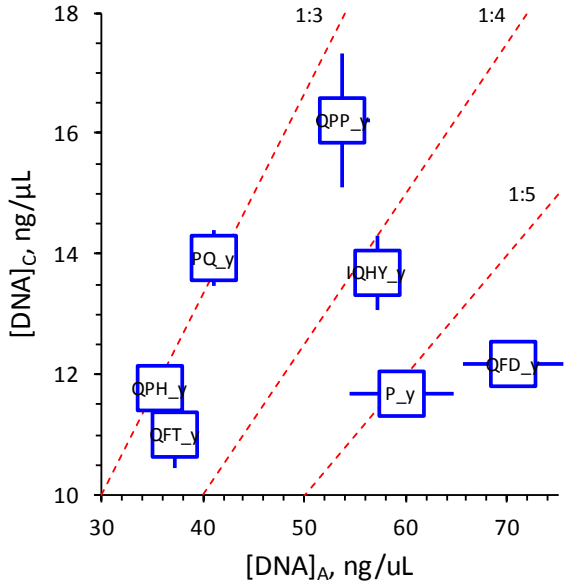

Figure 13: qPCR Y-Chromosome/Autosomal Ratio Performance Summary

Open squares denote the ng/µL values for SRM 2372a Components A (male) and C (mixture of male and female) for the seven commercial Y-chromosome qPCR assays evaluated, each labeled with the code listed in Table 8. The dotted red lines represent male:human ratios of 1:3, 1:4, and 1:5.

Appendix D provides a graphical analysis of all the data generated in the performance assessments for each of the targets amplified in the eleven commercial qPCR chemistries and the [DNA] values for all of the assays.

Qualification of ddPCR as Fit-For-Purpose

We have documented the requirements for establishing metrological traceability of ddPCR human genomic assay results, expressed as copies per nanoliter of sample, in a series of three peer-reviewed papers.[15,16,17] Through the experimental evidence they are based upon, these papers demonstrate that ddPCR can accurately enumerate the number of independently migrating genomic copies in an aqueous extract. Our most recent paper establishes the metrological traceability of these ddPCR results when transformed into the nanogram per microliter units used by the forensic and clinical communities.[18]

However, our previous studies have not addressed a fundamental requirement for correctly interpreting digital PCR measurements. In broad simplification, end-point assay systems count the number of independently partitioning entities regardless of the number of copies the entities contain. With assays that target just one site per HHGE, entities that migrate as ssDNA will yield counts that are twice the value had they migrated as dsDNA.[30] Should some originally dsDNA be converted over time to ssDNA, ddPCR measurements of the number of independently migrating entities would increase although the number of HHGE would be unchanged.

Measurements of mass [DNA] provided by absorbance spectrophotometry and ddPCR reflect different properties: absorbance at 260 nm is sensitive to the spatial configuration of the nucleotides as well as their number, while ddPCR counts independently migrating entities. The SRM 2372a materials have absorbance spectra shown in Figure 8 consistent with being predominantly dsDNA. The SRM 2372 materials now have the absorbance spectra shown in Figure 14 which are consistent with them being entirely ssDNA.

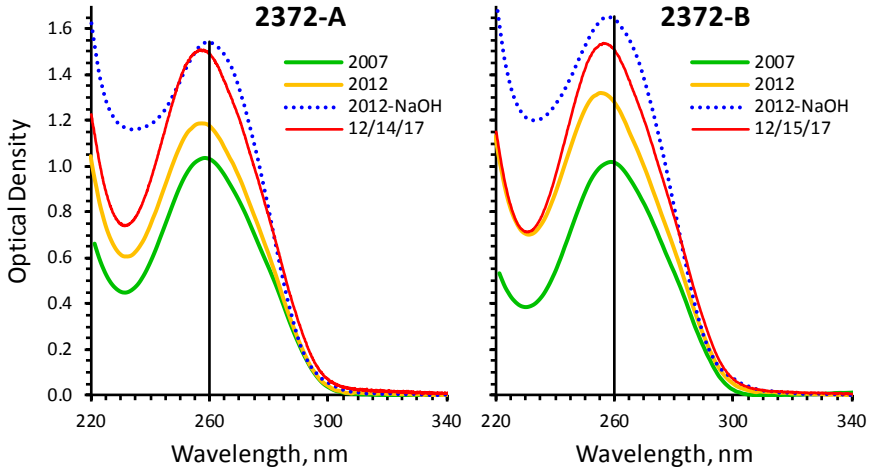

Figure 14: Absorbance Spectra of SRM 2372 Components A and B

dsDNA is denatured to ssDNA by diluting the source material with an equal volume of 0.4 mol/L NaOH; hence, the NaOH-treated spectra displayed are twice the absorbance of the spectra as acquired. The black vertical line marks the 260 nm values used in the conventional conversion of absorbance to mass [DNA].

To ensure the validity of ddPCR-based value assignments it is thus necessary to evaluate the nature of the partitioning entities. Should a solution of what is thought to be dsDNA contain an appreciable fraction of ssDNA then the ddPCR results would require adjustment.[31]

To avoid further delay in making SRM 2372a available to the forensic community, the following sections detail a series of experiments that have enabled us to conclude, with high confidence, that the SRM 2372a components have a dsDNA conformation and that for this material "entities" can be considered dsDNA "copies". The remaining stock of the SRM 2372 components also have a dsDNA conformation; however, the strands in the old materials are much less robustly connected than they are in the new materials.

Digital Polymerase Chain Reaction Assays

Three nDNA assays were used: HBB1, NEIF, and ND6. The HBB1 and NEIF assays reproducibly yield very similar results for DNA extracts from many different donors. The HBB1 target is at the tip of the short (p) arm of chromosome 11. The NEIF target is in the centromere region of chromosome 2. The ND6 assay was selected for use because it typically yields the lowest results of the ten nDNA assays used to certify the SRM 2372a components (see Figure 10, below). The ND6 target is about halfway down the long (q) arm of chromosome 6.

Heat Treatments (Melting of the DNA into Single Strands):

In all experiments the non-heated control and the heat-treated diluted solutions were stored in the same type of PCR reaction tube.

The starting point for the heat treatment was to determine if there was a difference between snap-cooling on ice and slower cooling at 25 °C. Two tubes of the diluted material were placed in a GeneAmp 9700 thermal cycler (Applied Biosystems) and cycled up to 98 °C and held for 15 min. After 15 min one tube was removed and placed on ice while the remaining tube was cooled at 1 °C/s in the thermal cycler to 25 °C then placed on ice until prepared for ddPCR.

Additional heat treatments included cycling samples on a GeneAmp 9700 thermal cycler:

a) from 98 °C for 5 min then cooling to 25 °C for 2 min. During the 25 °C hold a randomly selected tube was removed and placed on ice. The last tube was removed after 9 cycles of treatment resulting in a total of 45 min at 98 °C.
b) from 94 °C for 1 min then cooling to 25 °C for 1 min. During the 1 min hold at 25 °C a random sample was removed and placed on ice. The last tube was removed after 9 cycles of treatment resulting in a total of 9 min at 94 °C.
c) from 90 °C for 1 min then cooling to 25 °C for 1 min for a total of 20 cycles. Samples were pulled at 25 °C after 1 min, 3 min, 5 min, 10 min, and 20 min and placed on ice.

After the above experiments, heat treatments were performed using a ProFlex thermal cycler. This thermal cycler has six temperature zones that can be ≤ 5 °C apart. This enables heating

tubes at the same time but at different temperatures. After treatment, tubes were cooled at 5 °C/s to 4 °C in the thermal cycler. While designed for use with PCR plates, a MicroAmp 96-well tray for the VeriFlex block enabled its use with single PCR reaction tubes.

Comparison of Mass Concentration Estimates for SRM 2372 and SRM 2372a

As a first step in evaluating whether the DNA in the SRM materials disperse as dsDNA or ssDNA, a direct comparison of the five materials was made using the HBB1 and NEIF assays. Table 18 reports summary statistics for the ddPCR measurements, reported as λ, copies/droplet, and the estimated mass [DNA] values from the ddPCR results,[18] from the conventional dsDNA relationship,[7] and from the conventional ssDNA relationship after denaturing with 0.4 mol/L NaOH.[8,9]

While the mass [DNA] values differ somewhat between the three methods, there is no evidence for the SRM materials segregating exclusively or even mostly as ssDNA. The extent of the hypochromic interactions among the nucleotides does not accurately reflect the extent to which the two strands of dsDNA have separated.

Table 18: Comparison of Mass Concentration Estimates for SRM 2372 and 2372a

		λ, copies per droplet			λ, copies per droplet			ng/µL		
Sample	Assay	N_{rep}[a]	Mean	CV[b]	n_{cmb}[c]	Mean	CV[b]	ddPCR[d]	dsDNA[e]	ssDNA[f]
2372-A	HBB1	4	0.3645	1.9 %	8	0.3644	2.0 %	56.6[g]	52.4	57
2372-A	NEIF	4	0.3644	2.3 %						
2372-B	HBB1	4	0.3513	2.3 %	8	0.3487	3.5 %	58.0[h]	53.6	61
2372-B	NEIF	4	0.3461	4.7 %						
2372a-A	HBB1	4	0.2603	2.7 %	8	0.2649	3.0 %	47.4	46.5	45.4
2372a-A	NEIF	4	0.2694	2.4 %						
2372a-B	HBB1	4	0.2908	3.4 %	8	0.2891	3.7 %	51.7	55.6	53.4
2372a-B	NEIF	4	0.2873	4.5 %						
2372a-C	HBB1	4	0.2757	2.8 %	8	0.2711	2.8 %	48.5	50.2	47.4
2372a-C	NEIF	4	0.2665	1.6 %						

a Number of technical replicates for the given assay
b CV = 100(standard deviation)/mean.
c Combined number of technical replicates, ignoring potential differences between the assays.
d Estimated from the ddPCR λ measurements listed in this Table.[18]
e Estimated from the conventional relationship between the mass [DNA] of dsDNA and absorbance at 260 nm,[7] using absorbance spectra acquired shortly after solutions were produced.
f Estimated from the relationship between the mass [DNA] of ssDNA and absorbance at 260 nm after 1:1 dilution with 0.4 mol/L NaOH.[8,9]
g Values adjusted to measured (13.2 ±1.9) % volume loss of archived units due to evaporation.
h Values adjusted to measured (7.1 ±2.5) % volume loss of archived due to evaporation.

Quantitative Evaluation of ssDNA Content

Absorbance spectrophotometry is routinely used to quantitatively assess DNA tertiary structure.[32,33] On storage "at physiological pH, the intrastrand repulsion between the negatively charged phosphate groups forces the double helix into more rigid rodlike conformation."[34] suggesting a mechanism for the observed slow changes in the SRM 2372 materials. As demonstrated in Table 18, absorbance at 260 nm does not necessarily correlate with ddPCR behavior. Yet "furthermore, the repulsion between phosphate groups on opposite strands tends to separate the complementary strands."[34] Therefore, a method that is independent of hypochromism is needed to adequately evaluate the proportion (if any) of "true" (completely disjoint) ssDNA in the SRM materials.

Circular dichroism spectroscopy is sensitive to the conformational structures of DNA in solution,[35] and different DNA configurations migrate differentially in agarose gel electrophoresis.[7] Both techniques have been used to qualitatively discriminate ssDNA from dsDNA in "pure" single-sequence materials, but neither technique appears to be able to quantify small proportions of ssDNA in a complex, multi-chromosome genomic material.

Wilson and Ellison have proposed a real-time chamber digital PCR (cdPCR) technique that can estimate excess proportions of ssDNA copies from the crossing-threshold distribution.[31] However, their method is insufficiently sensitive for low ssDNA proportions.

It is well known that heating DNA beyond the melting temperature separates mammalian dsDNA into ssDNA,[36] albeit we have shown using cdPCR that boiling dsDNA for some minutes also renders some target sites non-amplifiable.[15] In the following section we document a ddPCR-based approach for estimating the proportion, if any, of ssDNA in a sample that involves less destructive heating. While the method is not yet optimized, the results from these preliminary studies demonstrate that the SRM 2372 and 2372a components disperse into the ddPCR droplets as dsDNA.

ddPCR Measurement of ssDNA Proportion

Given two otherwise identical aliquots of a given DNA material, one subjected to some treatment and the other untreated, the ratio between the average number of copies per droplet of treated material and that of its untreated sibling, λ_t/λ_u, quantitatively estimates the change in the number of independently dispersing entities. Should the treatment reduce the number of accessible amplifiable targets (AAT), λ_t/λ_u will be less than 1. Reduction in AAT may result from damage to the target itself and/or conformational changes to the fragment containing the target that render the target inaccessible or non-amplifiable.[16]

Assuming no denaturation of dsDNA to ssDNA, either because of an ineffective treatment or the material having started as "pure" ssDNA, if the treatment does not itself damage targets then λ_t/λ_u will equal 1. Should treatment convert at least some dsDNA to ssDNA without damaging targets, the number of AAT will increase and λ_t/λ_u will be greater than 1. Assuming complete conversion of "pure" dsDNA to "pure" ssDNA without other damage then λ_t/λ_u will equal 2.

Values greater than 1 but less than 2 indicate some conversion of dsDNA to ssDNA but also some combination of: (a) incomplete denaturation of dsDNA to ssDNA, (b) partial renaturation of ssDNA to dsDNA after treatment but before ddPCR analysis, (c) damage to targets during the process, or – of greatest interest – (d) the original sample material was an impure mixture of dsDNA and ssDNA.

The following discussions graphically summarize sets of λ_t/λ_u, followed by tabular listings of the relevant experimental conditions, ddPCR measurements, and the numerical λ_t/λ_u values. The information in these tables includes:

Plate Index	Experiment identifier, indicates date performed in YYYYMMDD format
Material	Sample material: SRM 2372-A, 2372-B, 2372a-A, 2372a-B, or 2372a-C
Assay	ddPCR assay: HBB1, NEIF, or ND6
°C	Denaturation temperature, in °C
Min	Length of time the treated material was held at denaturation temperature, in minutes
Cool	Method used to cool treated material from denaturation temperature: Snap, Slow, R1, or R5 where "Snap" indicates cooling on ice, "Slow" cooling at room temperature to 25 °C, "R1" cooling in a thermal cycler to 25 °C at 1 °C/s, and "R5" cooling in a thermal cycler to 4 °C at 5 °C/s
F	Dilution factor, expressed as (volume Material) per (final volume of diluted solution): 1:2, 1:4, or 1:8
n	Number of technical replicates
Mean	Mean of technical replicates
CV	Coefficient of Variation expressed as a percentage: $100\,\frac{\text{Standard Deviation}}{\text{Mean}}$
λ_t/λ_u	the copies per droplet result of the treated solution, λ_t, divided by the copies per droplet result of its untreated sibling, λ_u
$u(\lambda_t/\lambda_u)$	Standard uncertainty of λ_t/λ_u: $u(\lambda_t/\lambda_u) = \lambda_t/\lambda_u \sqrt{\left(\frac{\mathrm{CV}(\lambda_t)}{100}\right)^2 + \left(\frac{\mathrm{CV}(\lambda_u)}{100}\right)^2}$

Proof of Principle

Figure 15 summarizes two replicate sets of measurements performed to assess whether the SRM 2372 and 2372a materials respond similarly, whether the denatured materials renature if allowed to cool slowly, and whether results are (reasonably) repeatable. Table 19 details the experimental conditions.

The λ_t/λ_u values for the four materials are similar, all much greater than 1 but not approaching 2. Most of the ratios are on or close to the "slow cooling is the same as snap cooling" line, suggesting that renaturation is not a significant concern. While the replicate results for the SRM 2372-A and 2372a-A materials do not overlap as closely as those for SRM 2372-B and 2372a-B, similar treatment conditions produce similar results.

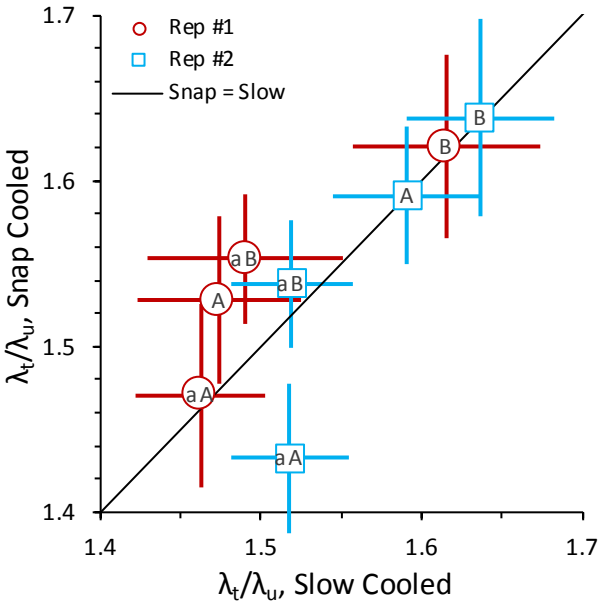

Figure 15: Comparison of Snap-Cooling to Slow-Cooling

Symbols mark the means of replicate measurements made using the same dilutions on successive days, with bars representing ± one standard combined uncertainty. Results are labeled by material: "A" and "B" for SRM 2372 components and "aA", "aB", and "aC" for SRM 2372a components. The diagonal black line represents equality between the snap- and slow-cooling treatments.

Table 19: Comparison of Snap-Cooling to Slow-Cooling

			Treatment				λ			Ratio,	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20171218	2372-A	HBB1				1:4	4	0.3830	2.9		
dd20171218	2372-A	HBB1	98	15	Slow	1:4	2	0.5644	1.8	1.474	0.051
dd20171218	2372-A	HBB1	98	15	Snap	1:4	4	0.5852	1.5	1.528	0.051
dd20171218	2372a-A	HBB1				1:4	4	0.3029	1.8		
dd20171218	2372a-A	HBB1	98	15	Slow	1:4	4	0.4430	2.1	1.463	0.040
dd20171218	2372a-A	HBB1	98	15	Snap	1:4	4	0.4454	3.3	1.471	0.055
dd20171218	2372-B	HBB1				1:4	4	0.3594	3.1		
dd20171218	2372-B	HBB1	98	15	Slow	1:4	4	0.5806	1.8	1.615	0.058
dd20171218	2372-B	HBB1	98	15	Snap	1:4	4	0.5825	1.3	1.620	0.055
dd20171218	2372a-B	HBB1				1:4	4	0.3383	1.5		
dd20171218	2372a-B	HBB1	98	15	Slow	1:4	4	0.5043	3.8	1.491	0.061
dd20171218	2372a-B	HBB1	98	15	Snap	1:4	4	0.5254	2.0	1.553	0.039
dd20171219	2372-A	HBB1				1:4	4	0.3357	2.3		
dd20171219	2372-A	HBB1	98	15	Slow	1:4	4	0.5340	1.6	1.591	0.046
dd20171219	2372-A	HBB1	98	15	Snap	1:4	4	0.5340	1.2	1.591	0.042

Plate Index	Material	Assay	Treatment °C	Min	Cool	F	λ n	Mean	CV	Ratio, λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20171219	2372a-A	HBB1				1:4	4	0.2735	2.4		
dd20171219	2372a-A	HBB1	98	15	Slow	1:4	4	0.4151	0.3	1.518	0.037
dd20171219	2372a-A	HBB1	98	15	Snap	1:4	4	0.3918	2.1	1.433	0.045
dd20171219	2372-B	HBB1				1:4	4	0.3346	2.2		
dd20171219	2372-B	HBB1	98	15	Slow	1:4	4	0.5476	1.7	1.637	0.045
dd20171219	2372-B	HBB1	98	15	Snap	1:4	4	0.5480	2.9	1.638	0.059
dd20171219	2372a-B	HBB1				1:4	4	0.3158	1.9		
dd20171219	2372a-B	HBB1	98	15	Slow	1:4	4	0.4798	1.6	1.519	0.038
dd20171219	2372a-B	HBB1	98	15	Snap	1:4	4	0.4856	1.7	1.538	0.039

Time at Denaturation Temperature

Figure 16 summarizes λ_t/λ_u for the SRM 2372-B material as a function of denaturation temperature and the cumulative time the solution is held at that temperature. Table 20 details the experimental conditions.

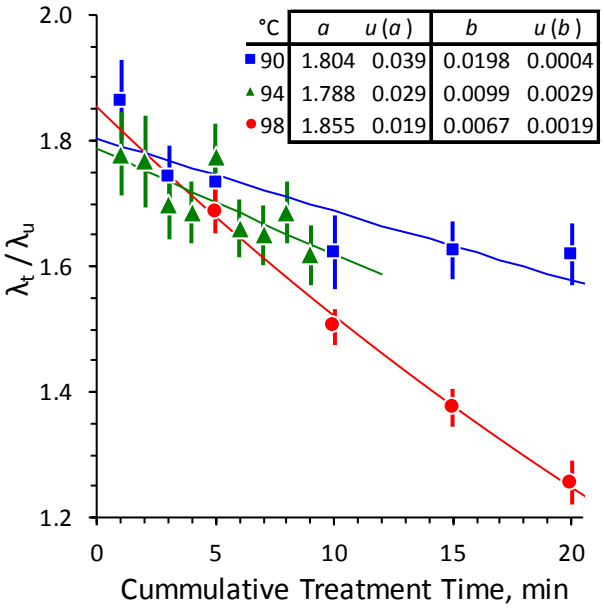

Figure 16: Change in λ_t/λ_u with Increasing Time at Several Denaturation Temperatures

The symbols represent the ratio of the mean result of three technical replicates of the untreated and each of the treated solutions; the bars represent combined standard uncertainties. The lines denote regression fits to the function $\lambda_t/\lambda_u = a \times e^{-bt}$ where t is the cumulative time. Values and the standard uncertainties of the coefficients are listed in the legend.

For all three of the denaturation temperatures studied, the relationship between λ_t/λ_u and cumulative time, *t*, can be modeled as:

$$\lambda_t/\lambda_u = ae^{-bt}$$

where *a* is the extrapolated value of λ_t/λ_u at $t = 0$ and *b* is the rate of change in AAT per minute. As expected, the rate of loss due to the treatment increases with increasing treatment temperature and cumulative time at that temperature.[37]

Given the much longer treatment time for the 98 °C series, the agreement among the three $t = 0$ parameter (*a*) values is remarkable. This suggests that the first 1 min of treatment efficiently separates dsDNA into ssDNA.

The pooled relative standard uncertainty of these regression parameter is 1.7 %, somewhat smaller than the 2.5 % pooled relative standard uncertainties of the individual λ_t/λ_u. However, the ±standard uncertainty interval of the 1-min λ_t/λ_u results for the 94 °C and 90 °C both overlap the extrapolated $t = 0$ value, suggesting that reliable results can be obtained using a single treatment of short duration.

Table 20: Change in λ_t/λ_u with Increasing Time at Several Denaturation Temperatures

			Treatment				λ			Ratio	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20171228	2372-B	HBB1				1:4	3	0.3650	1.7		
dd20171228	2372-B	HBB1	98	5	R1	1:4	3	0.6157	1.2	1.687	0.035
dd20171228	2372-B	HBB1	98	10	R1	1:4	3	0.5493	0.8	1.505	0.028
dd20171228	2372-B	HBB1	98	15	R1	1:4	3	0.5019	1.3	1.375	0.029
dd20171228	2372-B	HBB1	98	20	R1	1:4	3	0.4583	2.1	1.256	0.034
dd20171228	2372-B	HBB1	98	25	R1	1:4	3	0.4154	3.2	1.138	0.041
dd20171228	2372-B	HBB1	98	30	R1	1:4	3	0.3784	1.4	1.037	0.023
dd20171228	2372-B	HBB1	98	35	R1	1:4	3	0.3408	1.0	0.934	0.019
dd20171228	2372-B	HBB1	98	40	R1	1:4	3	0.2980	0.1	0.816	0.014
dd20171228	2372-B	HBB1	98	40	R1	1:4	3	0.2815	0.8	0.771	0.015
dd20171229	2372-B	HBB1				1:4	3	0.3708	2.7		
dd20171229	2372-B	HBB1	94	1	R1	1:4	3	0.6598	2.6	1.779	0.056
dd20171229	2372-B	HBB1	94	2	R1	1:4	3	0.6553	3.2	1.767	0.063
dd20171229	2372-B	HBB1	94	3	R1	1:4	3	0.6301	2.0	1.699	0.045
dd20171229	2372-B	HBB1	94	4	R1	1:4	3	0.6252	1.4	1.686	0.037
dd20171229	2372-B	HBB1	94	5	R1	1:4	3	0.6577	1.4	1.773	0.039
dd20171229	2372-B	HBB1	94	6	R1	1:4	3	0.6156	0.7	1.660	0.030
dd20171229	2372-B	HBB1	94	7	R1	1:4	3	0.6118	1.0	1.650	0.033
dd20171229	2372-B	HBB1	94	8	R1	1:4	3	0.6256	1.2	1.687	0.035
dd20171229	2372-B	HBB1	94	9	R1	1:4	3	0.5999	1.0	1.618	0.032

			Treatment				λ			Ratio	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20180104	2372-B	HBB1				1:4	4	0.3713	2.5		
dd20180104	2372-B	HBB1	90	1	R1	1:4	3	0.6916	2.2	1.863	0.052
dd20180104	2372-B	HBB1	90	3	R1	1:4	3	0.6465	0.6	1.741	0.032
dd20180104	2372-B	HBB1	90	5	R1	1:4	3	0.6431	2.0	1.732	0.045
dd20180104	2372-B	HBB1	90	10	R1	1:4	3	0.6014	2.5	1.620	0.049
dd20180104	2372-B	HBB1	90	15	R1	1:4	3	0.6024	1.0	1.623	0.032
dd20180104	2372-B	HBB1	90	20	R1	1:4	3	0.6003	1.4	1.617	0.035

Temperature

Figure 17 summarizes the effect of denaturation temperature, using a single treatment of 0.5 min, on the 2372-B and 2372a-B materials. Table 21 details the experimental conditions.

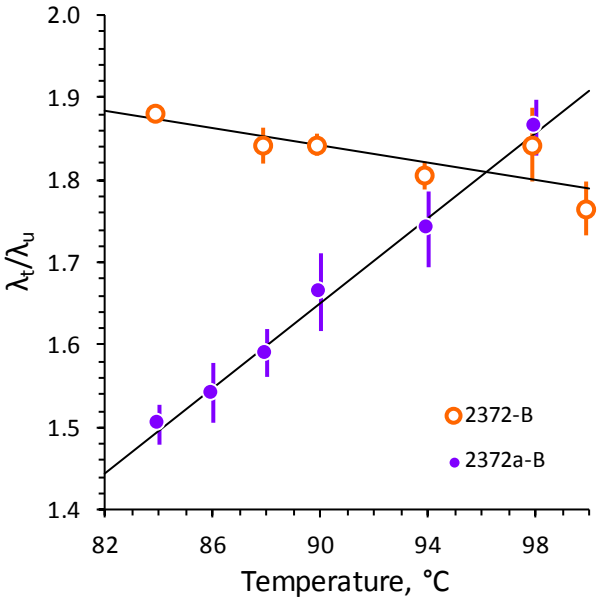

Figure 17: Change in λ_t/λ_u with Denaturation Temperature for SRM 2372 and 2372a

The symbols represent the ratio of the mean result of four technical replicates of the untreated and three replicates of the treated solutions; the bars represent combined standard uncertainties. In all cases, the duration of the treatment was 0.5 min. The lines denote regression fits to the linear function $\lambda_t/\lambda_u = a + bT$ where T is the denaturation temperature.

The SRM 2372 and 2372a materials respond very differently to changes in denaturation temperature. The older, spectrophotometrically ssDNA, material suffers somewhat greater loss in λ_t/λ_u as temperature *increases*. The new, spectrophotometrically dsDNA, material suffers much greater loss in λ_t/λ_u as temperature *decreases*.

Table 21: Change in λ_t/λ_u with Denaturation Temperature for SRM 2372 and 2372a

			Treatment				λ			Ratio,	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20180109	2372-B	HBB1				1:4	4	0.3577	2.5		
dd20180109	2372-B	HBB1	100	0.5	R5	1:4	3	0.6314	1.6	1.765	0.051
dd20180104a	2372-B	HBB1				1:4	4	0.3577	0.5		
dd20180104a	2372-B	HBB1	98	0.5	R5	1:4	3	0.6592	1.7	1.843	0.032
dd20180104a	2372-B	HBB1	94	0.5	R5	1:4	3	0.6459	2.5	1.806	0.046
dd20180104a	2372-B	HBB1	90	0.5	R5	1:4	2	0.6591	0.8	1.842	0.017
dd20180104a	2372-B	HBB1	88	0.5	R5	1:4	3	0.6589	0.5	1.842	0.013
dd20180104a	2372-B	HBB1	84	0.5	R5	1:4	3	0.6729	1.0	1.881	0.022
dd20180105	2372a-B	HBB1				1:4	4	0.3165	1.6		
dd20180105	2372a-B	HBB1	98	0.5	R5	1:4	3	0.5901	0.9	1.864	0.034
dd20180105	2372a-B	HBB1	94	0.5	R5	1:4	3	0.5510	2.2	1.741	0.047
dd20180105	2372a-B	HBB1	90	0.5	R5	1:4	3	0.5270	2.3	1.665	0.047
dd20180105	2372a-B	HBB1	88	0.5	R5	1:4	3	0.5032	0.9	1.590	0.029
dd20180105	2372a-B	HBB1	86	0.5	R5	1:4	3	0.4880	1.8	1.542	0.036
dd20180105	2372a-B	HBB1	84	0.5	R5	1:4	3	0.4760	0.4	1.504	0.025

We hypothesize that in the older SRM 2372 material the between-strand bond is relatively fragile and can be completely severed at relatively low temperature while avoiding much temperature-dependent AAT loss. In contrast, the between-strand bond in the SRM 2372a material requires higher temperature to completely disassociate the strands.

This suggests that there is not a sample-independent "optimum" combination of denaturation temperature and treatment duration. However, 98 °C for 0.5 min appears to provide comparable results for these two materials.

DNA Concentration in the ddPCR Solution

Figure 18 summarizes the effect of [DNA] in the ddPCR solution with SRM 2372-B at a denaturation temperature of 98 °C and treatment duration of 0.5 min. Table 22 details the experimental conditions.

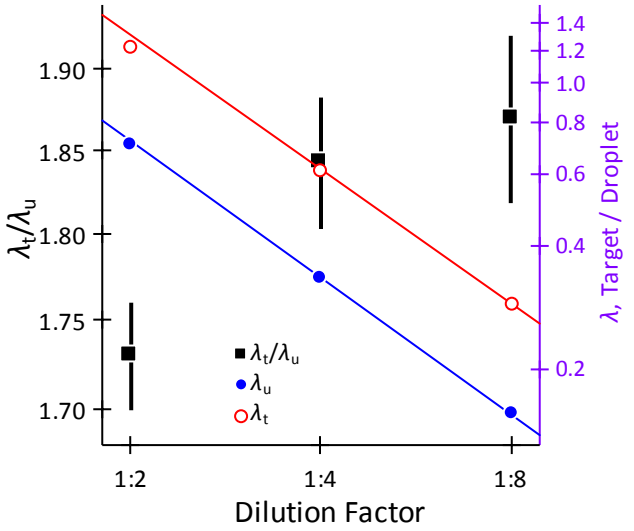

Figure 18: Change in λ_t/λ_u with DNA Concentration in ddPCR Solution

The solid black symbols represent the ratio of the mean result of four technical replicates of the untreated and treated solutions with the λ_t/λ_u values shown on the left-hand vertical axis; the bars represent combined standard uncertainties. The solid blue circles represent λ_u and the open red circles λ_t with values shown on the right-hand vertical axis; at this graphical scale, the standard deviations of the technical replicates are covered by the symbol. The diagonal blue and red lines represent the log-linear relationships between just the 1:8 and 1:4 λ values.

The primary effect of [DNA] appears to be the loss of linearity in λ_t with copies/droplet values greater than about 1 copies/droplet. There may be some advantage to use somewhat greater dilutions than the routine 1:4, however the small increase in λ_t/λ_u may be offset by the increased variability with small λ_u. This suggests the use of dilutions that give λ_u values of (0.1 to 0.4) copies/droplet.

Table 22: Change in λ_t/λ_u with DNA Mass Concentration in Treated Solution

			Treatment				λ			Ratio	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20180117	2372-B	HBB1				1:2	4	0.7127	1.1		
dd20180117	2372-B	HBB1	98	0.5	R5	1:2	4	1.2331	1.4	1.730	0.031
dd20180117	2372-B	HBB1				1:4	4	0.3338	1.5		
dd20180117	2372-B	HBB1	98	0.5	R5	1:4	3	0.6151	1.5	1.843	0.040
dd20180117	2372-B	HBB1				1:8	4	0.1549	1.8		
dd20180117	2372-B	HBB1	98	0.5	R5	1:8	4	0.2897	2.0	1.870	0.051

Equivalence of Assays

Figure 19 summarizes the agreement between the HBB1, NEIF, and ND6 assays at a denaturation temperature of 98 °C and treatment duration of 0.5 min. Table 23 details the experimental conditions.

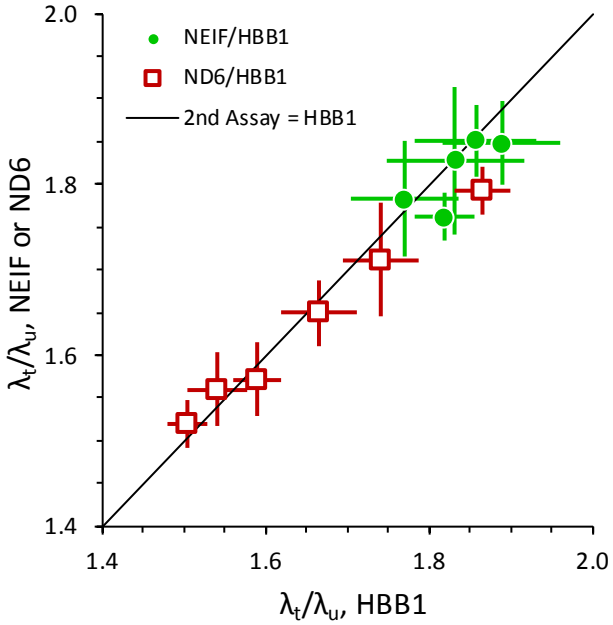

Figure 19: Comparison of Assays

The symbols compared the λ_t/λ_u results from the HBB1 and either the NEIF or ND6 assays. Each λ_t/λ_u value is the ratio of the mean result of four technical replicates of an untreated and three or four replicates of the treated solutions. The bars represent combined standard uncertainties. The diagonal line represents equality between the assays.

There is little or no difference between the λ_t/λ_u results from the HBB1, NEIF, and ND6 assays, suggesting that the chromosomal location of the assay target has little influence. Since the NEIF/HBB1 comparison includes results for both the SRM 2372 and 2372a materials, this equivalence is not material-specific.

Table 23: Comparison of Assays

			Treatment				λ			Ratio	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20180105	2372a-B	HBB1				1:4	4	0.3165	1.6		
dd20180105	2372a-B	HBB1	98	0.5	R5	1:4	3	0.5901	0.9	1.864	0.034
dd20180105	2372a-B	HBB1	94	0.5	R5	1:4	3	0.5510	2.2	1.741	0.047
dd20180105	2372a-B	HBB1	90	0.5	R5	1:4	3	0.5270	2.3	1.665	0.047
dd20180105	2372a-B	HBB1	88	0.5	R5	1:4	3	0.5032	0.9	1.590	0.029
dd20180105	2372a-B	HBB1	86	0.5	R5	1:4	3	0.4880	1.8	1.542	0.036
dd20180105	2372a-B	HBB1	84	0.5	R5	1:4	3	0.4760	0.4	1.504	0.025
dd20180105	2372a-B	ND6				1:4	4	0.3229	2.5		
dd20180105	2372a-B	ND6	98	0.5	R5	1:4	3	0.5790	0.1	1.793	0.045
dd20180105	2372a-B	ND6	94	0.5	R5	1:4	3	0.5526	3.6	1.711	0.074
dd20180105	2372a-B	ND6	90	0.5	R5	1:4	3	0.5326	1.8	1.649	0.051
dd20180105	2372a-B	ND6	88	0.5	R5	1:4	3	0.5077	2.2	1.572	0.052
dd20180105	2372a-B	ND6	86	0.5	R5	1:4	3	0.5038	2.3	1.560	0.053
dd20180105	2372a-B	ND6	84	0.5	R5	1:4	3	0.4906	1.0	1.519	0.041
dd20180110	2372-A	HBB1				1:4	3	0.3949	3.3		
dd20180110	2372-A	HBB1	98	0.5	R5	1:4	4	0.6989	1.6	1.770	0.065
dd20180110	2372-B	HBB1				1:4	4	0.2269	4.4		
dd20180110	2372-B	HBB1	98	0.5	R5	1:4	4	0.4156	1.2	1.832	0.084
dd20180110	2372a-A	HBB1				1:4	4	0.2596	1.3		
dd20180110	2372a-A	HBB1	98	0.5	R5	1:4	4	0.4721	1.5	1.819	0.036
dd20180110	2372a-B	HBB1				1:4	4	0.2845	2.7		
dd20180110	2372a-B	HBB1	98	0.5	R5	1:4	3	0.5283	2.9	1.857	0.074
dd20180110	2372a-C	HBB1				1:4	4	0.2443	2.7		
dd20180110	2372a-C	HBB1	98	0.5	R5	1:4	4	0.4614	2.7	1.889	0.072
dd20180111	2372-A	NEIF				1:4	4	0.3971	1.1		
dd20180111	2372-A	NEIF	98	0.5	R5	1:4	4	0.7080	3.7	1.783	0.069
dd20180111	2372-B	NEIF				1:4	4	0.2324	1.1		
dd20180111	2372-B	NEIF	98	0.5	R5	1:4	4	0.4249	4.6	1.828	0.087
dd20180111	2372a-A	NEIF				1:4	4	0.2698	1.4		
dd20180111	2372a-A	NEIF	98	0.5	R5	1:4	4	0.4755	1.5	1.762	0.035
dd20180111	2372a-B	NEIF				1:4	4	0.2943	2.5		
dd20180111	2372a-B	NEIF	98	0.5	R5	1:4	3	0.5448	1.9	1.851	0.058
dd20180111	2372a-C	NEIF				1:4	4	0.2568	2.3		
dd20180111	2372a-C	NEIF	98	0.5	R5	1:4	4	0.4747	2.4	1.849	0.062

ssDNA Proportion Assessment of the SRM 2372 and 2372a Materials

Figure 20 summarizes the currently available λ_t/λ_u results obtained by denaturing at 98 °C for 0.5 min, rapidly cooling in the thermal cycler, with dilutions that provide ≈ 0.3 copies/droplet. The figure also displays the consensus result and its 95 % confidence region estimated using the Hierarchical Bayes method provided in the NIST Consensus Builder (NICOB) system.[38,39] Table 24 details the experimental conditions.

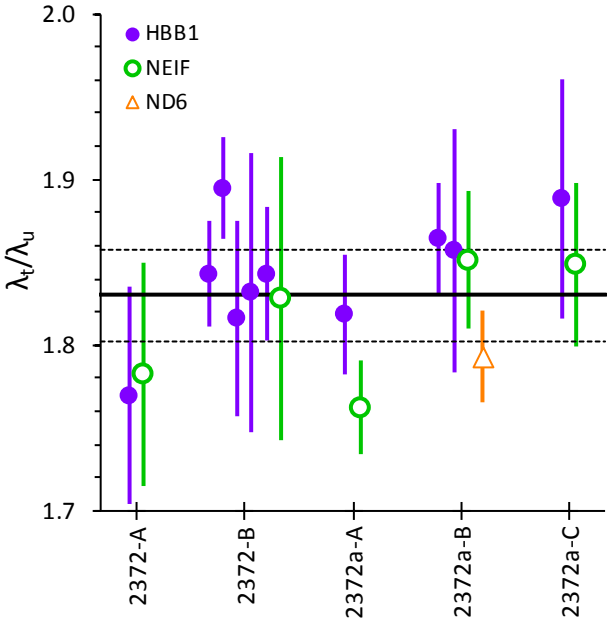

Figure 20: Evaluation of Limiting Ratio

The symbols represent the ratio of the mean result of three or four technical replicates of the untreated and treated solutions; the bars represent combined standard uncertainties. The thick horizontal line represents a consensus value covering all displayed results; the dotted horizontal lines bracket the 95 % confidence interval about the consensus value.

The Hierarchical Bayes consensus result for these values is 1.83 with a 95 % confidence interval of ±0.03. All but three of the 16 λ_t/λ_u values are within one standard uncertainty of this consensus value with most of the λ_t/λ_u means lying within the 95 % confidence interval. This suggests that there are no statistically significant differences between the SRM 2372 and 2372a materials with regard to ssDNA proportion.

Table 24: Evaluation of Limiting Ratio

			Treatment				λ			Ratio	
Plate Index	Material	Assay	°C	Min	Cool	*F*	*n*	Mean	CV	λ_t/λ_u	$u(\lambda_t/\lambda_u)$
dd20180110	2372-A	HBB1				1:4	3	0.3949	3.3		
dd20180110	2372-A	HBB1	98	0.5	R5	1:4	4	0.6989	1.6	1.770	0.065
dd20180111	2372-A	NEIF				1:4	4	0.3971	1.1		
dd20180111	2372-A	NEIF	98	0.5	R5	1:4	4	0.7080	3.7	1.783	0.069
dd20180104a	2372-B	HBB1				1:4	4	0.3577	0.5		
dd20180104a	2372-B	HBB1	98	0.5	R5	1:4	3	0.6592	1.7	1.843	0.032
dd20180108	2372-B	HBB1				1:4	3	0.3463	0.9		
dd20180108	2372-B	HBB1	98	0.5	R5	1:4	3	0.6562	1.4	1.895	0.031
dd20180109	2372-B	HBB1				1:4	3	0.3577	2.5		
dd20180109	2372-B	HBB1	98	0.5	R5	1:4	3	0.6495	2.1	1.816	0.059
dd20180110	2372-B	HBB1				1:4	4	0.2269	4.4		
dd20180110	2372-B	HBB1	98	0.5	R5	1:4	4	0.4156	1.2	1.832	0.084
dd20180111	2372-B	NEIF				1:4	4	0.2324	1.1		
dd20180111	2372-B	NEIF	98	0.5	R5	1:4	4	0.4249	4.6	1.828	0.087
dd20180117	2372-B	HBB1				1:4	4	0.3338	1.5		
dd20180117	2372-B	HBB1	98	0.5	R5	1:4	4	0.6151	1.5	1.843	0.040
dd20180110	2372a-A	HBB1				1:4	4	0.2596	1.3		
dd20180110	2372a-A	HBB1	98	0.5	R5	1:4	4	0.4721	1.5	1.819	0.036
dd20180111	2372a-A	NEIF				1:4	4	0.2698	1.4		
dd20180111	2372a-A	NEIF	98	0.5	R5	1:4	4	0.4755	1.5	1.762	0.035
dd20180105	2372a-B	HBB1				1:4	4	0.3165	1.6		
dd20180105	2372a-B	HBB1	98	0.5	R5	1:4	3	0.5901	0.9	1.864	0.034
dd20180110	2372a-B	HBB1				1:4	4	0.2845	2.7		
dd20180110	2372a-B	HBB1	98	0.5	R5	1:4	3	0.5283	2.9	1.857	0.074
dd20180111	2372a-B	NEIF				1:4	4	0.2943	2.5		
dd20180111	2372a-B	NEIF	98	0.5	R5	1:4	3	0.5448	1.9	1.851	0.058
dd20180105	2372a-B	ND6				1:4	4	0.3229	2.5		
dd20180105	2372a-B	ND6	98	0.5	R5	1:4	3	0.5790	0.1	1.793	0.045
dd20180110	2372a-C	HBB1				1:4	4	0.2443	2.7		
dd20180110	2372a-C	HBB1	98	0.5	R5	1:4	4	0.4614	2.7	1.889	0.072
dd20180111	2372a-C	NEIF				1:4	4	0.2568	2.3		
dd20180111	2372a-C	NEIF	98	0.5	R5	1:4	4	0.4747	2.4	1.849	0.062

The agreement among the measured λ_t/λ_u is more directly visualized using estimated unilateral "Degrees of Equivalence," d. Each d is the absolute difference between the measured and consensus values

$$d = |\lambda_t/\lambda_u - \text{consensus}|$$

and is associated with a 95 % confidence interval, $U_{95}(d)$, that combines the uncertainty estimates of the measured and consensus values. The $U_{95}(d)$ calculation details are specific to the consensus estimation method.[39] Values of $d \pm U_{95}(d)$ that include 0 indicate that the λ_t/λ_u result does not differ from the majority at about a 95 % level of confidence. Figure 21 displays the d for the λ_t/λ_u results for the Hierarchical Bayes consensus value.[39] By this figure of merit, all the λ_t/λ_u can be considered as members of a single population.

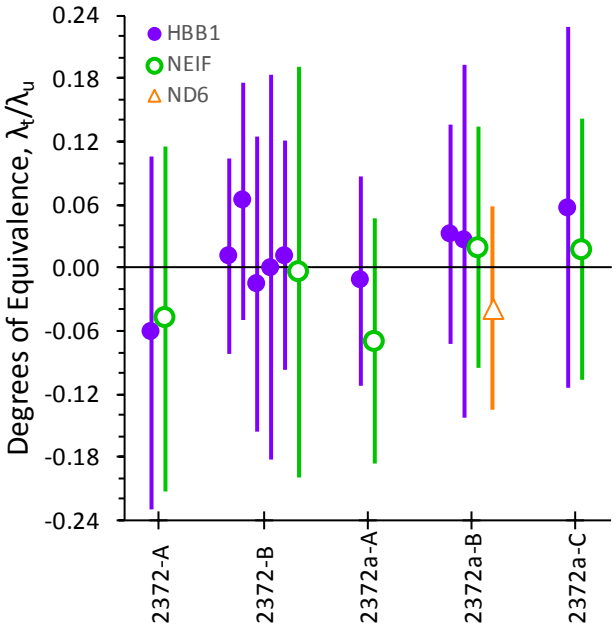

Figure 21: Degrees of Equivalence

The symbols represent the Degree of Equivalence, d, for the λ_t/λ_u results relative to the Hierarchical Bayes consensus value. The bars represent 95 % level of confidence uncertainty intervals, $d \pm U_{95}(d)$. The horizontal line marks the $d = 0$ "no difference" value. Results with $d \pm U_{95}(d)$ that include this line are not significantly different from the majority.

Conclusions and Recommendations

With high confidence, all tubes of the SRM 2372a solution have the same nDNA copy number content within measurement repeatability. Additionally, within measurement repeatability all tubes of the SRM 2372a have the same mtDNA/nDNA.

With high confidence, the nDNA copy number content of the SRM 2372a solution is thermally stable from 4 °C to 37 °C over an extended period. The solution should not be shipped or stored below 4 °C.

With high confidence, the ddPCR measurements used to establish the entity and mass concentrations and the mtDNA/nDNA ratios are adequately precise and unbiased.

With high confidence, the DNA in the SRM 2372a solutions is now dsDNA and can be expected to remain dsDNA for at least ten years when stored at 4 °C.

Recommended Certification Values

Since the component materials are believed to be homogeneous, are of very similar composition, and were prepared and processed similarly, the observed differences in the measured entity concentration uncertainties are likely artifacts of the measurement process. To limit over-interpretation of these differences, we recommend that the copy per nanoliter values of the three components be assigned same 95 % relative uncertainty interval (CV_{95}), based on the largest of the measured CV_{95} values (9.3 %) rounded up to 10 %. Since the ng/µL transformation does not appreciably inflate this relative uncertainty, we recommend that the mass concentration values also be assigned CV_{95} of 10 %.

Similarly, we recommend that the mtDNA/nDNA ratios be rounded to integer values, all three components be assigned the same CV_{95}, and that this uncertainty be based on the largest (2.1 %) of the measured CV_{95} rounded up to 2.5 %.

Table 25 lists the values that we recommend be used in the Certificate of Analysis.

Table 25: Recommended Values and 95 % Uncertainties for Certification

Component	Units	Value	U_{95}(Value) [a]
A	Copies per nanoliter	15.1	1.5
B	Copies per nanoliter	17.5	1.8
C	Copies per nanoliter	14.5	1.5
A	ng/µL	49.8	5.0
B	ng/µL	57.8	5.8
C	ng/µL	47.9	4.8
A	mtDNA/nDNA	174	4
B	mtDNA/nDNA	206	5
C	mtDNA/nDNA	279	7

a The interval Value ± U_{95}(Value) is believed, with about 95 % confidence, to contain the true value of the measurand.

Use of SRM 2372a for Value-Assigning In-House Reference Materials

The within-component and between-assay variability observed for the commercial qPCR assays is not unexpected since different qPCR methods exploit qualitatively different molecular targets. Given the intrinsic variability of the human genome, the prospect of developing a human DNA source material that responds equally to all current and future qPCR assays is improbable. All three components should be used to elucidate potential bias when using these materials to value assign in-house DNA reference and control materials for qPCR assays.

Certificate of Analysis

In accordance with ISO Guide 31: 2000, a NIST SRM certificate is a document containing the name, description, and intended purpose of the material, the logo of the U.S. Department of Commerce, the name of NIST as a certifying body, instructions for proper use and storage of the material, certified property value(s) with associated uncertainty(ies), method(s) used to obtain property values, the period of validity, if appropriate, and any other technical information deemed necessary for its proper use. A Certificate is issued for an SRM certified for one or more specific physical or engineering performance properties and may contain NIST reference, information, or both values in addition to certified values. A Certificate of Analysis is issued for an SRM certified for one or more specific chemical properties. Note: ISO Guide 31 is updated periodically; check with ISO for the latest version. [https://www.nist.gov/srm/srm-definitions]

For the most current version of the Certificate of Analysis for NIST SRM 2372a Human DNA Quantification Standard, please visit: https://www-s.nist.gov/srmors/view_detail.cfm?srm=2372a.

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Appendix A: DNA Extraction Method

The following method has been adapted from Miller et al. (1988)[A1]

Procedure

1. Transfer approximately 5 mL of buffy coat into a 50 mL sterile polypropylene centrifuge tube, and add 30 mL Red Blood Cell Lysis Buffer.
2. Mix by gentle inversion and let stand at room temperature for 15 min.
3. Centrifuge at approximately 209 rad/s (2000 rpm) for 20 min.
4. Discard supernatant.
5. Add 9 mL of Nucleic Lysis Buffer and re-suspend the pelleted cells by agitating with a plastic transfer pipette.
6. Add 300 µL of 20 % sodium dodecyl sulfate (SDS) and 1.8 mL of Protease K Solution (freshly prepared 2 mg to 5 mg Protease K per 1 mL Protease K Buffer).
7. Mix by inversion, followed by incubation overnight or longer at 37 ºC to 40 ºC, in a shaking water bath. The length of incubation is determined by the time required to solubilize the cells present in the tube.
8. Add 4 mL saturated ammonium acetate solution (in deionized water, stored at 2 ºC to 8 ºC). Shake vigorously for 15 s.
9. Centrifuge at approximately 471 rad/s (4500 rpm) for 10 min.
10. Pipette supernatant into a 50 mL sterile polypropylene centrifuge tube and add two volumes of room temperature absolute ethanol. Mix by gentle inversion until the DNA aggregates and floats to the top of the tube.
11. Spool the aggregated DNA from the current tube into a fresh tube of 80 % ethanol using a sterile inoculation loop. At this step, multiple tubes of precipitated DNA may be pooled to make a large lot of the purified material. Mix by gentle inversion.
12. Transfer the aggregated DNA to a fresh dry 50 mL polypropylene centrifuge tube. Remove as much ethanol as possible by squeezing the DNA against the wall of the tube with a sterile inoculation loop. Air-dry the material in a laminar flow hood.
13. Resolubilize DNA in TE^{-4} buffer and store material at 4 ºC.

Reagents

Red Blood Cell Lysis Buffer:
144 mmol/L ammonium chloride,
1 mmol/L sodium bicarbonate
in deionized (DI) water.
Store at 2 ºC to 8 ºC.

Nucleic Lysis Buffer:
10 mmol/L Tris-HCl,
400 mmol/L sodium chloride,
2.0 mmol/L disodium ethylenediaminetetraacetic acid
in DI Water, adjust pH to 8.2.
Store at 2 ºC to 8 ºC.

Protease K Buffer:
2.0 mmol/L disodium ethylenediaminetetraacetic acid,
1.0 % (mass per volume) Sodium Dodecyl Sulfate (SDS)
in DI water.
Store at 2 ºC to 8 ºC.

Protease K:
Sigma-Aldrich cat# P6556, *Tritirachium album*, lyophilized powder.
Store at –20 ºC.

Ammonium Acetate:
19.2 mol/L ammonium acetate (saturated) in DI water,
Store at 2 ºC to 8 ºC.

Absolute Ethanol (200 proof)
Store at room temperature.

Reference

A1 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215. https://doi.org/10.1093/nar/16.3.1215

Appendix B: Spectrophotometric Measurement Protocol

The following is for use with a scanning recording spectrophotometer. The procedures include the spectrophotometer calibration, measurement of the "native" dsDNA and measurement of the NaOH denatured ssDNA. The procedure for evaluating solutions after treatment with NaOH is adapted from references.[8,9]

Spectrophotometer Calibration

Establish Baseline

Set the spectrophotometer to acquire data from 220 nm to 345 nm in steps of 0.1 nm. Zero the instrument with nothing (air) in the sample and reference cells. Acquire an air-air baseline of the instrument with nothing (air) in the sample and reference cells. If the trace has significant structure or excessive noise, take corrective action. If the trace is acceptable, proceed with data collection.

Enable Verification of Wavelength Calibration

SRM 2034 is a solution of holmium oxide in 10 % perchloric acid contained in a sealed quartz cuvette, delivering an intrinsic UV/Vis wavelength standard for absorption spectrophotometry.[B1] Evaluation of the location of peak maxima in the spectra of this solution enables verification of the spectrophotometer's wavelength calibration.

Place SRM 2034 in the sample cell. Create and name a file to hold the verification spectra. Perform three replicate SRM 2034-to-air scans, saving each scan to the file. Remove SRM 2034 from the spectrophotometer and store appropriately.

Enable Verification of Absorbance Calibration

SRM 2031 consists of three metal-on-fused silica filters held in cuvette-sized filter holders plus an empty filter holder. The three filters are certified for transmittance and transmittance density ($-\log_{10}$(transmittance), effectively absorbance) at several wavelengths, with nominal wavelength-independent transmittances of 10 %, 30 %, and 90 %. Evaluation of the absorbance of each of these three filters at the certified 250 nm, 280 nm, and 340 nm wavelengths enables verification of the spectrophotometer's absorbance calibration.

Place the empty SRM 2031 filter holder in the reference cell. Remove the protective covers from the 10 % transmittance filter and place in the sample cell. Perform three replicate scans, saving each scan to file. Remove the filter from the spectrophotometer, replace the protective covers, and store appropriately. Repeat for the 30 % and 90 % filters. Close the file containing the verification spectra.

For "native" dsDNA Samples

Verify Optical Balance of Cuvettes

The measurements of the DNA-containing samples are made using a 100 µL quartz cuvette in the sample cell compared to an optically matched cuvette in the reference cell. The optical equivalence of these two cuvettes can be verified by filling both cuvettes with appropriate volumes of a sample solution matrix (TE^{-4} buffer) that does not contain DNA. Fill two dry,

clean quartz cuvettes with this solution. Visually inspect the cell windows to ensure that no bubbles or stray material are present. Zero the instrument with buffer in both the sample and reference cells. Acquire a blank-to-blank baseline scan. If the trace has significant structure or excessive noise, take corrective action. Once the trace is acceptable, create and suitably name a file to hold the spectra for this series. Perform three replicate scans, saving each scan to file.

Leave the filled reference cuvette in place. Remove the sample cuvette clean and dry with a series of DI water washes followed by a methanol rinse in a cuvette washer; dry the cuvette with a vacuum assist.

To the dry cuvette add 100 µL of the DNA solution to be measured. Place the loaded cuvette in the spectrophotometer sample cell. Perform three replicate scans, saving each scan to file. Remove the sample cuvette and repeat the cleaning and re-filling procedure for each sample measured. After the last measurement of the "native" dsDNA, remove and clean both the reference cuvette and the sample cuvette.

For NaOH denatured ssDNA Samples

Prepare a 0.4 mol/L NaOH Stock Solution.

To a 15 mL disposable centrifuge tube add 0.16 g NaOH pellets, add deionized (DI) water to the 10 mL line and mix until all the NaOH pellets have dissolved. Centrifuge at 524 rad/s (5000 rpm) for 5 min. For every 9 samples to be analyzed, aliquot 1 mL of this 0.4 mol/L NaOH stock solution into a 1.5 mL screw top tube. Use these tubes to prepare the NaOH blanks and DNA samples.

In a 1.5 mL screw cap vial mix 200 µL TE^{-4} buffer with 200 µL 0.4 mol/L sodium hydroxide solution to achieve a final NaOH concentration of 0.2 mol/L. Vortex 5 s then centrifuge at 1257 rad/s (12 000 rpm) for 1 min. Fill two dry, clean quartz cuvettes with this solution. Visually inspect the cell windows to ensure that no bubbles or stray material are present. Zero the instrument with buffer in both the sample and reference cells. Acquire a blank-to-blank baseline scan. If the trace has significant structure or excessive noise, take corrective action. Once the trace is acceptable, create and suitably name a file to hold the spectra for this series. Perform three replicate scans, saving each scan to file.

Prepare the Sample Solutions

For each unit of material to be measured (Tube or vial):

a) Vortex the unit for 5 s
b) Centrifuge at 1257 rad/s (12 000 rpm) for 1 min
c) Remove a 100 µL aliquot and place in a new labeled vial
d) Add 100 µL of the freshly prepared 0.4 mol/L NaOH solution (with the same pipet)
e) Vortex 5 s
f) Centrifuge at 1257 rad/s (12 000 rpm) for 1 min

Measure Sample Solutions

Leave the filled reference cuvette in place. Remove the sample cuvette, clean and dry with a series of DI water washes, rinse with methanol in a cuvette washer, dry the cuvette with a vacuum assist.

To the dry cuvette, add 100 µL of the NaOH denatured DNA solution to be measured. Place the loaded cuvette in the spectrophotometer sample cell. Perform three replicate scans, saving each scan to file. Remove the sample cuvette and repeat the cleaning and re-filling procedure for each sample measured. After the last measurement of the "denatured" ssDNA, close the file holding the spectra for this series. Remove and clean both the reference cuvette and the sample cuvette.

After All Sample Measurements are Complete

Enable Verification of Spectrophotometer Stability

The stability of the spectrophotometer over the course of the data collection can be evaluated by comparing spectra for SRM 2034 and 2031 taken at the start of data collection with spectra taken at the end of data collection. Repeat the wavelength and absorbance verification measurements described above, collecting the spectra in a suitably named file.

Clean Up

Transfer all relevant data files from the spectrophotometer's storage to a transfer device. Clean the spectrophotometer work area as necessary and restore the SRMs and cuvettes to their appropriate storage locations.

Evaluation

Export the files to a spreadsheet. Average the replicate spectra. Subtract the average absorbance at A_{320} from the absorbance at A_{260} resulting in the corrected absorbance at A_{260}. The conventional spectrophotometric estimate of the mass concentration of the dsDNA in the sample is achieved by multiplying the corrected absorbance by 50. The conventional estimate for the NaOH-treated ssDNA is achieved by multiplying the corrected absorbance by 37×2, where the "×2" adjusts for the 1→2 volumetric dilution of the sample.

Note: reliable spectrophotometric measurements require corrected A_{260} to be greater than 0.05.

Reference

B1 Travis JC, Acosta JC, Andor G, Bastie J, Blattner P, Chunnilall CJ, Crosson SC, Duewer DL, Early EA, Hengstberger F, Kim C-S, Liedquist L, Manoocheri F, Mercader F, Metzdorf J, Mito A, Monardh LAG, Nevask S, Nilsson M, Noël M, Rodriguez AC, Ruíz A, Schirmacher A, Smith MV, Valencia G, van Tonder N, Zwinkels J. Intrinsic Wavelength Standard Absorption Bands in Holmium Oxide Solution for UV/visible Molecular Absorption Spectrophotometry. J Phys Chem Ref Data 2005;34(1):41-56.

Appendix C: SRM 2372a Statistician's report

Extracted from: Blaza Toman
November 30, 2017

1. Introduction

Candidate material for SRM 2372a is composed of three components labeled A, B, and C. All components are human genomic DNA and prepared at NIST from buffy coat white blood cells from single-source anonymous donors. Component A and B are single source (male/female, respectively), and Component C is a 1 male to 3 female mixture of two single-source donors. Droplet digital PCR (ddPCR) assays were used to obtain measurements of the concentration of copies per microliter of sample. Ten NIST-developed human nuclear DNA and three literature-derived human mitochondrial DNA PCR assays were used in this study.

1.1. OpenBUGS Analysis

All parameters in the following sections were estimated using Markov Chain Monte Carlo (MCMC) implemented in the OpenBUGS freeware.[C1] OpenBUGS is a software application for the Bayesian analysis of complex statistical models.

1.2. OpenBUGS Exemplar Output

All following sections list the OpenBUGs model ("code"), initialization values ("inits") if they are needed, and the data used to evaluate the various data sets discussed in the body of this report: homogeneity, stability, nDNA certification, and mtDNA/nDNA value assignment. The provided information in each of the following sections is sufficient to produce estimates, but the estimates may not exactly reproduce the exemplar results provided. The MCMC paradigm does not produce "analytical" values, but approaches them asymptotically as the number o model updates increases.

All exemplar results presented here are based upon the following:

- 10-fold "thinning" to minimize autocorrelation, there were 10 MCMC estimates for every model update used in parameter estimation;
- a "burn in" of 5 000 updates before beginning parameter estimation; and
- 10 000 model updates.

The following statistics are provided in the tables listing the exemplar results:

mean.....arithmetic mean of the parameter's model update values
sd.....standard deviation of the parameter's model update values
MC_error ....Monte Carlo standard deviation of the mean, mean/√(number of model updates)
val2.5pc.....2.5th percentile of the model update values
median.....50th percentile of the model update values
val97.5pc.....97.5th percentile of the model update values
start.....number of "burn in" updates
sample .....number of model update values

2. Homogeneity assessment

2.1. Data

Samples of the material of all three components were drawn from 22 different boxes. Two assays (NEIF, ND6) were used to obtain 3 replicates of the measurement in copies per nanoliter for each sample and component. *For the complete homogeneity data, see:*

Table 10: Homogeneity Data as λ′, Copy Number per Nanoliter of Component.

2.2. Model

The data were analyzed using a three-level Gaussian hierarchical ANOVA model:

$$y_{ijk} \sim N\left(\alpha_{ijk}, \sigma^2_{within\ box\ ij}\right)$$

where ~N(,) denotes "distributed as a normal distribution of some mean μ and variance"*;*
i denotes component (1 for A, 2 for B, 3 for C),
j denotes assay (1 for NEIF, 2 for ND6),
k denotes box (1,…,22), and
$\alpha_{ijk} \sim N\left(\mu_{ij}, \sigma^2_{between\ box_i}\right)$.

All variance components were given a Half Cauchy prior as described in Reference C2.

Note: The Half Cauchy prior is specified by "~dnorm(0, 0.0016)I(0.001,)". The "I" notation is for censoring. It cuts off any random draws from the Normal distribution that are lower than 0.001. An informative prior is needed when there are less than four data per distribution and the half Cauchy is a good general-purpose prior.

2.3. OpenBUGS code and data

```
##Homogeneity code
ModelBegin {
for(i in 1:3){xiNsb[i] ~ dnorm(0, 0.0016)I(0.001,)
  chSqNsb[i] ~ dgamma(0.5,0.5); sigb[i] <- xiNsb[i]/sqrt(chSqNsb[i])}
for(i in 1:3){for(j in 1:2){mu[i,j]~dnorm(0,1.0E-5)}}
for(i in 1:3){ muA[i]<-mean(mu[i,]);sigbP[i]<-100/(muA[i]*sqrt(sigb[i]))}
#
for(i in 1:3){for(j in 1:2){xiNs[i,j] ~ dnorm(0, 0.0016)I(0.001,)
  chSqNs[i,j]~dgamma(0.5,0.5); sigt[i,j]<-xiNs[i,j]/sqrt(chSqNs[i,j])
  sigtP[i,j]<-100/(mu[i,j]*sqrt(sigt[i,j]))}}
#
for(i in 1:3){for(j in 1:2){for(k in 1:22){alpha[i,j,k]~dnorm(mu[i,j],sigb[i])}}}
#
for(idx in 1:393){lamb[idx]~dnorm(alpha[cmp[idx],asy[idx],box[idx]],sigt[cmp[idx],asy[idx]])}
} ModelEnd

## Inits
None required.
```

##Homogeneity Data

lamb[]	asy[]	box[]	cmp[]	rep[]
16.32	1	1	1	1
15.29	1	1	1	2
15.11	1	1	1	3
16.16	2	1	1	1
16.40	2	1	1	2
15.82	2	1	1	3
15.31	1	2	1	1
15.25	1	2	1	2
15.49	1	2	1	3
16.15	2	2	1	1
15.89	2	2	1	2
16.01	2	2	1	3
15.24	1	3	1	1
15.40	1	3	1	2
15.33	1	3	1	3
15.82	2	3	1	1
11.52	2	3	1	2
15.55	2	3	1	3
14.54	1	4	1	1
14.58	1	4	1	2
14.27	1	4	1	3
16.27	2	4	1	1
16.05	2	4	1	2
16.07	2	4	1	3
14.65	1	5	1	1
15.40	1	5	1	2
15.04	1	5	1	3
16.07	2	5	1	1
16.33	2	5	1	2
15.86	2	5	1	3
15.12	1	6	1	1
14.91	1	6	1	2
14.91	1	6	1	3
15.11	2	6	1	1
14.83	2	6	1	2
15.24	2	6	1	3
14.18	1	7	1	1
14.80	1	7	1	2
14.55	1	7	1	3
14.79	2	7	1	1
15.57	2	7	1	2
16.56	2	7	1	3
14.96	1	8	1	1
15.60	1	8	1	2
15.01	1	8	1	3
15.14	2	8	1	1
16.15	2	8	1	2
15.40	2	8	1	3
15.88	1	9	1	1
15.81	1	9	1	2
16.37	1	9	1	3
14.74	2	9	1	1
14.38	2	9	1	2
14.54	2	9	1	3
15.74	1	10	1	1
15.13	1	10	1	2
15.70	1	10	1	3
15.94	2	10	1	1
17.13	2	10	1	2
16.69	2	10	1	3
15.57	1	11	1	1
15.08	1	11	1	2
16.33	1	11	1	3
15.71	2	11	1	1
16.11	2	11	1	2
16.31	2	11	1	3
15.00	1	12	1	1
14.76	1	12	1	2
15.15	1	12	1	3
16.26	2	12	1	1
15.86	2	12	1	2
16.08	2	12	1	3
16.72	1	13	1	1
16.34	1	13	1	2
18.04	1	13	1	3
14.90	2	13	1	1
15.16	2	13	1	2
14.97	2	13	1	3
15.51	1	14	1	1
15.68	1	14	1	2
15.38	1	14	1	3
16.17	2	14	1	1
16.14	2	14	1	2
16.39	2	14	1	3
14.56	1	15	1	1
14.48	1	15	1	2
15.23	1	15	1	3
15.25	2	15	1	1
14.02	2	15	1	2
14.36	2	15	1	3
15.73	1	16	1	1
15.20	1	16	1	2
16.17	1	16	1	3
16.34	2	16	1	1
16.27	2	16	1	2
15.75	2	16	1	3
15.16	1	17	1	1
15.16	1	17	1	2
16.28	1	17	1	3
16.13	2	17	1	1
15.67	2	17	1	2
15.67	2	17	1	3
16.12	1	18	1	1
15.35	1	18	1	2
16.72	1	18	1	3
15.29	2	18	1	1
14.95	2	18	1	2
15.28	2	18	1	3
15.98	1	19	1	1
15.14	1	19	1	2
15.96	1	19	1	3
15.71	2	19	1	1
15.35	2	19	1	2
15.11	2	19	1	3
15.75	1	20	1	1
15.68	1	20	1	2
15.86	1	20	1	3
16.52	2	20	1	1
15.45	2	20	1	2
16.27	2	20	1	3
16.58	1	21	1	1
16.11	1	21	1	2
15.78	1	21	1	3
16.47	2	21	1	1
16.07	2	21	1	2
16.12	2	21	1	3
15.47	1	22	1	1
15.28	1	22	1	2
15.32	1	22	1	3
15.37	2	22	1	1
15.13	2	22	1	2
15.36	2	22	1	3
17.90	1	1	2	1
19.09	1	1	2	2
18.85	1	1	2	3
18.70	2	1	2	2
18.60	2	1	2	3
17.12	1	2	2	1
17.19	1	2	2	2
17.04	1	2	2	3
17.75	2	2	2	1
17.18	2	2	2	2
17.56	2	2	2	3
16.93	1	3	2	1
17.55	1	3	2	2
17.49	1	3	2	3
18.24	2	3	2	1
14.83	2	3	2	2
18.96	2	3	2	3
17.54	1	4	2	1
17.45	1	4	2	2
17.39	1	4	2	3
16.70	2	4	2	1
16.32	2	4	2	2
18.14	2	4	2	3
17.57	1	5	2	1
16.87	1	5	2	2
17.35	1	5	2	3
16.83	2	5	2	1
17.59	2	5	2	2
17.03	2	5	2	3
16.97	1	6	2	1
17.09	1	6	2	2
17.14	1	6	2	3
16.93	2	6	2	1
17.23	2	6	2	2
17.22	2	6	2	3
17.22	1	7	2	1
16.25	1	7	2	2
17.03	1	7	2	3
16.90	2	7	2	1
16.84	2	7	2	2
16.88	2	7	2	3
18.84	1	8	2	1
18.02	1	8	2	2
17.72	1	8	2	3
18.64	2	8	2	1
18.87	2	8	2	2
18.21	2	8	2	3
17.13	1	9	2	1
17.34	1	9	2	2
17.27	1	9	2	3

17.09	2	9	2	1
17.56	2	9	2	2
17.58	2	9	2	3
17.59	1	10	2	1
18.09	1	10	2	2
17.38	1	10	2	3
17.64	2	10	2	1
18.16	2	10	2	2
17.87	2	10	2	3
17.49	1	11	2	1
17.82	1	11	2	2
17.40	1	11	2	3
17.41	2	11	2	1
17.92	2	11	2	2
17.84	2	11	2	3
17.48	1	12	2	1
17.12	1	12	2	2
17.18	1	12	2	3
17.97	2	12	2	1
16.14	2	12	2	2
17.90	2	12	2	3
16.32	1	13	2	1
16.68	1	13	2	2
17.01	1	13	2	3
16.26	2	13	2	1
15.66	2	13	2	2
16.81	2	13	2	3
17.52	1	14	2	1
15.35	1	14	2	2
16.73	1	14	2	3
17.24	2	14	2	1
17.41	2	14	2	2
16.85	2	14	2	3
18.71	1	15	2	1
18.35	1	15	2	2
18.87	1	15	2	3
17.70	2	15	2	1
17.52	2	15	2	2
17.98	2	15	2	3
16.88	1	16	2	1
17.25	1	16	2	2
17.43	1	16	2	3
17.44	2	16	2	1
16.97	2	16	2	2
17.10	2	16	2	3
17.89	1	17	2	1
17.69	1	17	2	2
18.25	1	17	2	3
17.16	2	17	2	1
17.62	2	17	2	2
16.38	2	17	2	3
18.26	1	18	2	1
17.80	1	18	2	2
18.49	1	18	2	3
18.05	2	18	2	1
18.01	2	18	2	2
19.31	2	18	2	3
17.51	1	19	2	1
17.59	1	19	2	2
18.80	1	19	2	3
18.27	2	19	2	1
17.66	2	19	2	2
17.86	2	19	2	3
17.38	1	20	2	1
16.78	1	20	2	2
17.68	1	20	2	3
17.52	2	20	2	1
17.47	2	20	2	2
17.68	2	20	2	3
17.42	1	21	2	1
18.36	1	21	2	2
17.13	1	21	2	3
17.46	2	21	2	1
17.44	2	21	2	2
17.64	2	21	2	3
17.61	1	22	2	1
17.43	1	22	2	2
16.60	1	22	2	3
16.66	2	22	2	1
16.92	2	22	2	2
18.19	2	22	2	3
14.93	1	1	3	1
13.85	1	1	3	2
15.10	1	1	3	3
15.65	2	1	3	1
15.62	2	1	3	2
14.66	2	1	3	3
15.16	1	2	3	1
15.13	1	2	3	2
15.77	1	2	3	3
14.90	2	2	3	1
15.47	2	2	3	2
15.70	2	2	3	3
14.51	1	3	3	1
15.28	1	3	3	2
15.06	1	3	3	3
14.85	2	3	3	1
15.05	2	3	3	2
14.71	2	3	3	3
14.40	1	4	3	1
14.86	1	4	3	2
15.20	1	4	3	3
15.29	2	4	3	1
15.08	2	4	3	2
15.06	2	4	3	3
15.23	1	5	3	1
14.72	1	5	3	2
14.83	1	5	3	3
15.13	2	5	3	1
15.20	2	5	3	2
14.91	2	5	3	3
14.37	1	6	3	1
14.93	1	6	3	2
15.22	1	6	3	3
14.92	2	6	3	1
14.81	2	6	3	2
14.61	2	6	3	3
14.85	1	7	3	1
14.77	1	7	3	2
14.63	1	7	3	3
14.75	2	7	3	1
15.47	2	7	3	2
14.85	2	7	3	3
14.33	1	8	3	1
14.05	1	8	3	2
14.78	1	8	3	3
14.97	2	8	3	1
15.01	2	8	3	2
14.95	2	8	3	3
14.63	1	9	3	1
14.99	1	9	3	2
15.05	1	9	3	3
15.03	2	9	3	1
14.91	2	9	3	2
15.16	2	9	3	3
15.34	1	10	3	1
14.58	1	10	3	2
14.54	1	10	3	3
14.48	2	10	3	1
14.65	2	10	3	2
14.74	2	10	3	3
15.33	1	11	3	1
15.14	1	11	3	2
15.55	1	11	3	3
14.88	2	11	3	1
14.70	2	11	3	2
14.83	2	11	3	3
14.50	1	12	3	1
15.25	1	12	3	2
14.97	1	12	3	3
14.96	2	12	3	1
14.83	2	12	3	2
15.19	2	12	3	3
15.37	1	13	3	1
15.27	1	13	3	2
15.15	1	13	3	3
14.99	2	13	3	1
14.79	2	13	3	2
14.75	2	13	3	3
15.15	1	14	3	1
14.90	1	14	3	2
14.94	1	14	3	3
14.69	2	14	3	1
14.80	2	14	3	2
14.66	2	14	3	3
15.11	1	15	3	1
15.68	1	15	3	2
15.94	1	15	3	3
15.15	2	15	3	1
15.43	2	15	3	2
15.65	2	15	3	3
14.49	1	16	3	1
14.52	1	16	3	2
14.71	1	16	3	3
15.43	2	16	3	1
14.48	2	16	3	2
14.91	2	16	3	3
13.88	1	17	3	1
14.21	1	17	3	2
13.94	1	17	3	3
14.52	2	17	3	1
14.35	2	17	3	3
14.75	1	18	3	1
14.55	1	18	3	2
14.45	1	18	3	3
14.58	2	18	3	1

15.31	2	18	3	2	15.31	1	20	3	2	14.54	2	21	3	2
14.59	2	18	3	3	14.99	1	20	3	3	14.42	1	22	3	1
14.68	1	19	3	1	14.99	2	20	3	1	14.87	1	22	3	2
15.25	1	19	3	2	14.65	2	20	3	2	14.36	1	22	3	3
14.46	1	19	3	3	14.68	2	20	3	3	15.89	2	22	3	1
14.35	2	19	3	1	15.30	1	21	3	1	15.91	2	22	3	2
14.78	2	19	3	2	14.40	1	21	3	2	15.56	2	22	3	3
14.21	2	19	3	3	14.84	1	21	3	3	END				
15.49	1	20	3	1	14.99	2	21	3	1					

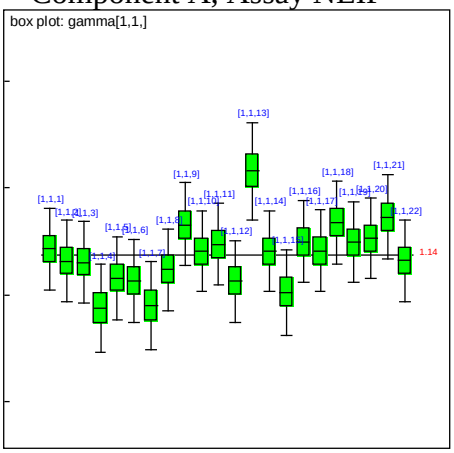
Table C-1: Example OpenBUGS Homogeneity Summary

	mean	**sd**	**MC_error**	**val2.5pc**	**median**	**val97.5pc**	**start**	**sample**
mu[1,1]	15.460	0.122	0.00123	15.22	15.46	15.70	5001	10000
mu[1,2]	15.640	0.137	0.00141	15.37	15.64	15.91	5001	10000
mu[2,1]	17.490	0.113	0.00118	17.27	17.49	17.72	5001	10000
mu[2,2]	17.480	0.129	0.00125	17.22	17.48	17.74	5001	10000
mu[3,1]	14.870	0.073	0.00073	14.73	14.87	15.01	5001	10000
mu[3,2]	14.960	0.069	0.00063	14.82	14.96	15.09	5001	10000
muA[1]	15.550	0.092	0.00092	15.36	15.55	15.73	5001	10000
muA[2]	17.490	0.085	0.00086	17.32	17.49	17.65	5001	10000
muA[3]	14.910	0.050	0.00046	14.81	14.91	15.01	5001	10000
sigbP[1]	3.269	0.4985	0.00514	2.376	3.244	4.324	5001	10000
sigbP[2]	2.548	0.4385	0.00461	1.733	2.533	3.472	5001	10000
sigbP[3]	1.850	0.292	0.00260	1.329	1.832	2.477	5001	10000
sigtP[1,1]	2.774	0.298	0.00260	2.268	2.750	3.442	5001	10000
sigtP[1,2]	4.036	0.429	0.00438	3.290	4.005	4.986	5001	10000
sigtP[2,1]	2.664	0.301	0.00321	2.155	2.640	3.313	5001	10000
sigtP[2,2]	3.807	0.395	0.00377	3.116	3.772	4.662	5001	10000
sigtP[3,1]	2.313	0.250	0.00209	1.880	2.291	2.873	5001	10000
sigtP[3,2]	1.812	0.195	0.00170	1.473	1.797	2.243	5001	10000

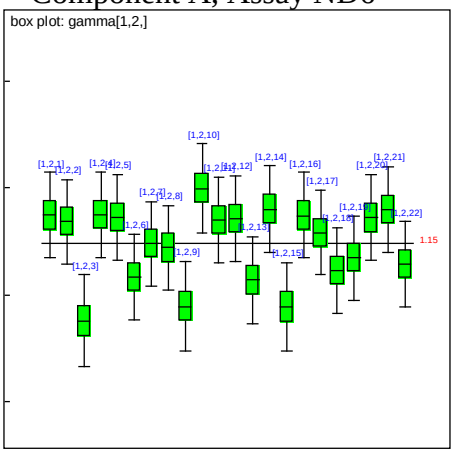
2.4. Results

The material appears to be homogeneous as can be seen in Figure C-1. Based on these results, the between-tube variability is of the same order as within-tube variability.

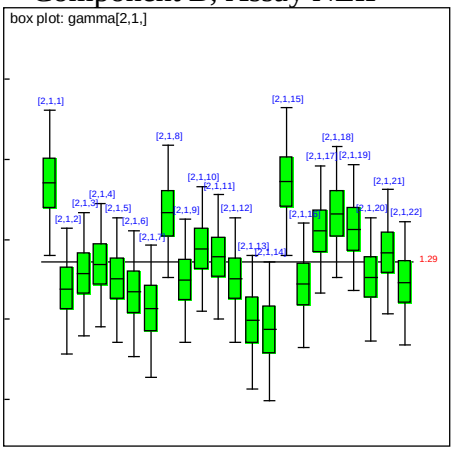
Component A, Assay NEIF



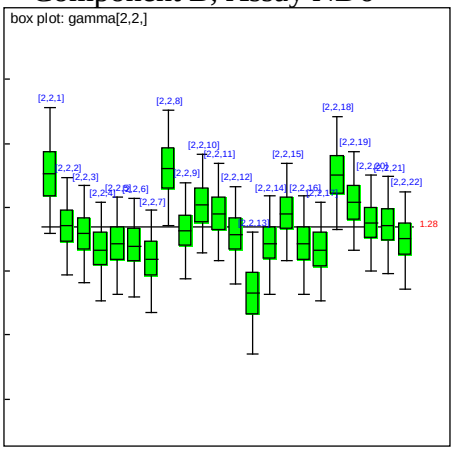
Component A, Assay ND6



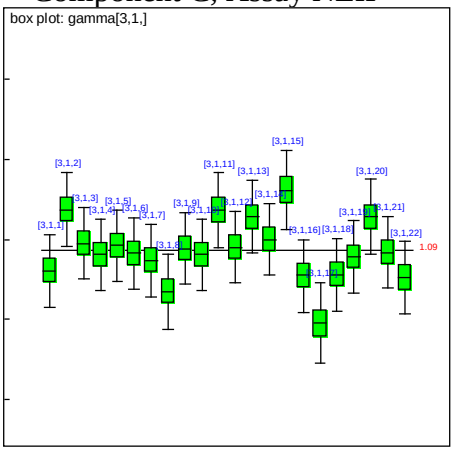
Component B, Assay NEIF



Component B, Assay ND6



Component C, Assay NEIF



Component C, Assay ND6

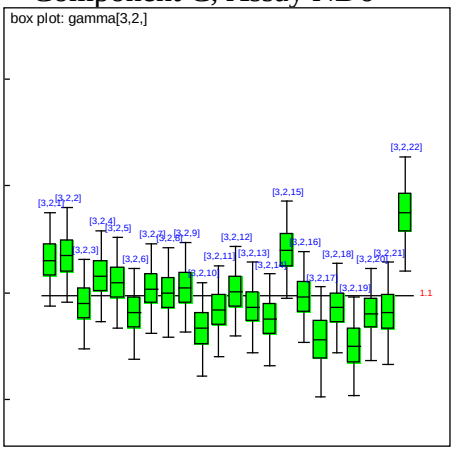


Figure C-1: Homogeneity Assessment

Note: These results were obtained using the 1-4 gravimetric dilution-adjusted λ but uncorrected for droplet volume or the 1-10 volumetric dilution. That is, the vertical axis in each panel should be multiplied by 0.7349×10 = 7.349. This constant difference in scale does not impact the homogeneity analysis. Error bars span approximate 95 % confidence intervals.

3. Certification measurements

3.1. Data

Originally, the experiment consisted of 5 plates containing the 3 components (A, B, C) and 12 assays. Ten of the assays were for genomic DNA identification (for the certified value) and two of the assays were for mitochondrial detection which is used for informational values (and are not included in this data set). Each of the plates contained samples from a new vial from different boxes (noted in the "vial used" portion, the number references the box). The assay positions were alternated across the five plates as well. *For the plate design and which tubes were used, see:*

Figure 6: Basic Plate Layout Design for Certification Measurements

It was later determined that the homogeneity and stability data sets were obtained using a different set of pipettes than the certification data set, and since all data was to be combined for certification, another plate was obtained using the original pipette. *For the complete certification data, see:*

Table 13: Certification Data as λ′, Copy Number per Nanoliter of Component

Stability data was measured at different temperatures over several weeks using assays NEIF and ND6. Only the 4°C data was also used for certification. *For the complete stability data, see:*

Table 12: Stability Data as λ′, Copy Number per Nanoliter of Component

3.2. Model

It was decided that all three sets of data should be used to derive the certified values. In this way, the uncertainty of the certified values would better reflect any potential heterogeneity in the samples as well as any additional unknown factors that may have effected the results. Because two different pipettes were used to obtain different sets of data, there was a significant bias in some of the measurements. Specifically, the "certification" data from the first five plates was obtained using pipette set "1", and the remaining measurements were obtained using pipette set "2". This bias was introduced into the statistical model as part of the mean. Possible effects of different assays, and type of data (heterogeneity, certification, and stability) were accounted for in the statistical model in terms of a random effect. Specifically, the response for each component (A, B, C) was represented as:

$$y_{ijk} \sim N\left(\gamma_{ijk}, \sigma_i^2\right)$$

where ~N(,) denotes "distributed as a normal distribution of some mean and variance"*;*
i = {1,2,3} denotes type of measurement {heterogeneity, certification, and stability};
j = {1,…,10} denotes assay {2PR4, POTP, NEIF, R4Q5, D5, ND6, D9, HBB1, ND14, 22C3};
k = {1, 2} denotes pipette set;
$\gamma_{ijk} = \alpha_{ij} + \beta_k$, $\alpha_{ij} \sim N(\mu_i, \sigma_\alpha^2)$, $\beta_k = \beta$ for data type 1, and $\beta_k = 0$ for data types 2 and 3.
For a given 2372a component the variance σ_α^2 measures the between assay variability and σ_i^2 measures the repeatability variability for each data type.

The data was analyzed using Bayesian MCMC programmed in OpenBUGS,[C2] with non-informative Gaussian priors for the means and Gamma priors for the variance components.

3.3. OpenBUGS code, inits, and data

```
## Certification code
ModelBegin{
for(i in 1:3){mu[i]~dnorm(0,1.0E-5); sig[i]~dgamma(1.0E-5,1.0E-5)}
sigalph~dgamma(1.0E-5,1.0E-5);
for(i in 1:10){alpha[1,i]~dnorm(mu[1], sigalph)}
for(i in 1:2){alpha[2,i]~dnorm(mu[2], sigalph); alpha[3,i]~dnorm(mu[3],sigalph)}
b~dnorm(0,1.0E-5); beta[1]<-b; beta[2]<-0.0
for(i in 1:Ntot){mean[i]<-alpha[typ[i],asy[i]]+beta[pip[i]]}
#
for(i in 1:Ntot){lamb[i]~dnorm(mean[i],sig[typ[i]])}
}ModelEnd

## Certification Inits
list(sig=c(1,1,1), sigalph=1)

## Certification Data for Component A
list(Ntot=275)
```

lamb[]	asy[]	cmp[]	typ[]	pip[]
16.67	1	1	1	1
16.55	2	1	1	1
16.47	3	1	1	1
15.94	4	1	1	1
16.55	5	1	1	1
16.29	6	1	1	1
16.55	7	1	1	1
16.44	8	1	1	1
16.45	9	1	1	1
15.45	10	1	1	1
16.98	1	1	1	1
16.85	2	1	1	1
16.65	3	1	1	1
16.58	4	1	1	1
16.83	5	1	1	1
16.47	6	1	1	1
16.52	7	1	1	1
16.11	8	1	1	1
17.07	9	1	1	1
16.03	10	1	1	1
16.68	1	1	1	1
15.87	2	1	1	1
16.78	3	1	1	1
16.28	4	1	1	1
17.38	5	1	1	1
16.67	6	1	1	1
16.95	7	1	1	1
17.57	8	1	1	1
17.71	9	1	1	1
17.62	10	1	1	1
17.39	1	1	1	1
17.02	2	1	1	1
16.14	3	1	1	1
17.62	4	1	1	1
16.49	5	1	1	1
16.78	6	1	1	1
16.53	7	1	1	1
17.5	8	1	1	1
17.64	9	1	1	1
17.59	10	1	1	1
16.38	1	1	1	1
16.76	2	1	1	1
16.16	3	1	1	1
15.59	4	1	1	1
16.08	5	1	1	1
16.11	6	1	1	1
15.17	7	1	1	1
15.57	8	1	1	1
16.04	9	1	1	1
16.75	10	1	1	1
16.67	1	1	1	1
16.38	2	1	1	1
16.24	3	1	1	1
15.87	4	1	1	1
16.52	5	1	1	1
15.89	6	1	1	1
16.07	7	1	1	1
15.82	8	1	1	1
16.25	9	1	1	1
16.16	10	1	1	1
18.88	1	1	1	1
18.68	2	1	1	1
19.53	3	1	1	1
17.79	4	1	1	1
19.24	5	1	1	1
18.61	6	1	1	1
18.24	7	1	1	1
18.09	8	1	1	1
18.64	9	1	1	1
19.3	10	1	1	1
19.39	1	1	1	1
18.15	2	1	1	1
19.02	3	1	1	1
18.71	4	1	1	1
21.24	5	1	1	1
17.87	6	1	1	1
18.44	7	1	1	1
17.99	8	1	1	1
18.57	9	1	1	1
19.42	10	1	1	1
15.97	1	1	1	1
15.33	2	1	1	1
14.62	3	1	1	1
14.93	4	1	1	1
15.38	5	1	1	1
14.92	6	1	1	1
15.28	7	1	1	1
15.48	8	1	1	1
15.13	9	1	1	1
15.62	10	1	1	1
15.9	1	1	1	1
14.04	2	1	1	1
15.33	3	1	1	1
15.19	4	1	1	1
15.86	5	1	1	1
15.16	6	1	1	1
15.38	7	1	1	1
15.81	8	1	1	1
15.14	9	1	1	1
14.31	1	1	1	2
14.07	2	1	1	2
14.87	3	1	1	2
14.5	4	1	1	2
13.94	5	1	1	2
13.96	6	1	1	2
14.14	7	1	1	2
14.01	8	1	1	2
14.12	9	1	1	2
14.35	10	1	1	2
14.11	1	1	1	2
13.95	2	1	1	2
13.98	3	1	1	2
14.39	4	1	1	2

14.22	5	1	1	2
13.93	6	1	1	2
13.9	7	1	1	2
13.82	8	1	1	2
14.6	9	1	1	2
14.1	10	1	1	2
16.32	1	1	2	2
15.31	1	1	2	2
15.24	1	1	2	2
14.54	1	1	2	2
14.65	1	1	2	2
15.12	1	1	2	2
14.18	1	1	2	2
14.96	1	1	2	2
15.88	1	1	2	2
15.74	1	1	2	2
15.57	1	1	2	2
15	1	1	2	2
16.72	1	1	2	2
15.51	1	1	2	2
15.73	1	1	2	2
15.16	1	1	2	2
16.12	1	1	2	2
15.98	1	1	2	2
15.75	1	1	2	2
16.58	1	1	2	2
15.47	1	1	2	2
15.29	1	1	2	2
15.25	1	1	2	2
15.4	1	1	2	2
14.58	1	1	2	2
15.4	1	1	2	2
14.91	1	1	2	2
14.8	1	1	2	2
15.6	1	1	2	2
15.81	1	1	2	2
15.13	1	1	2	2
15.08	1	1	2	2
14.76	1	1	2	2
16.34	1	1	2	2
15.68	1	1	2	2
15.2	1	1	2	2
15.16	1	1	2	2
15.35	1	1	2	2
15.14	1	1	2	2
15.68	1	1	2	2
16.11	1	1	2	2
15.28	1	1	2	2
15.11	1	1	2	2
15.49	1	1	2	2
15.33	1	1	2	2
14.27	1	1	2	2
15.04	1	1	2	2
14.91	1	1	2	2
14.55	1	1	2	2
15.01	1	1	2	2
16.37	1	1	2	2
15.7	1	1	2	2
16.33	1	1	2	2
15.15	1	1	2	2
18.04	1	1	2	2
15.38	1	1	2	2
16.17	1	1	2	2
16.28	1	1	2	2
16.72	1	1	2	2
15.96	1	1	2	2
15.86	1	1	2	2
15.78	1	1	2	2
15.32	1	1	2	2
16.16	2	1	2	2
16.15	2	1	2	2
15.82	2	1	2	2
16.27	2	1	2	2
16.07	2	1	2	2
15.11	2	1	2	2
14.79	2	1	2	2
15.14	2	1	2	2
14.74	2	1	2	2
15.94	2	1	2	2
15.71	2	1	2	2
16.26	2	1	2	2
14.9	2	1	2	2
16.17	2	1	2	2
16.34	2	1	2	2
16.13	2	1	2	2
15.29	2	1	2	2
15.71	2	1	2	2
16.52	2	1	2	2
16.47	2	1	2	2
15.37	2	1	2	2
16.4	2	1	2	2
15.89	2	1	2	2
11.52	2	1	2	2
16.05	2	1	2	2
16.33	2	1	2	2
14.83	2	1	2	2
15.57	2	1	2	2
16.15	2	1	2	2
14.38	2	1	2	2
17.13	2	1	2	2
16.11	2	1	2	2
15.86	2	1	2	2
15.16	2	1	2	2
16.14	2	1	2	2
16.27	2	1	2	2
15.67	2	1	2	2
14.95	2	1	2	2
15.35	2	1	2	2
15.45	2	1	2	2
16.07	2	1	2	2
15.13	2	1	2	2
15.82	2	1	2	2
16.01	2	1	2	2
15.55	2	1	2	2
16.07	2	1	2	2
15.86	2	1	2	2
15.24	2	1	2	2
16.56	2	1	2	2
15.4	2	1	2	2
14.54	2	1	2	2
16.69	2	1	2	2
16.31	2	1	2	2
16.08	2	1	2	2
14.97	2	1	2	2
16.39	2	1	2	2
15.75	2	1	2	2
15.67	2	1	2	2
15.28	2	1	2	2
15.11	2	1	2	2
16.27	2	1	2	2
16.12	2	1	2	2
15.36	2	1	2	2
15.68	1	1	3	2
15.2	1	1	3	2
14.86	1	1	3	2
14.41	1	1	3	2
17.06	1	1	3	2
14.4	1	1	3	2
15	1	1	3	2
15.37	1	1	3	2
13.87	1	1	3	2
14.63	1	1	3	2
15.7	1	1	3	2
14.29	1	1	3	2
16.67	1	1	3	2
16.32	1	1	3	2
15.89	1	1	3	2
14.74	2	1	3	2
15.43	2	1	3	2
15.55	2	1	3	2
15.99	2	1	3	2
14.94	2	1	3	2
16.24	2	1	3	2
15.77	2	1	3	2
15.93	2	1	3	2
14.79	2	1	3	2
15.17	2	1	3	2
15.38	2	1	3	2
15.9	2	1	3	2
14.79	2	1	3	2
14.79	2	1	3	2
15.22	2	1	3	2

END

Table C-2: Example OpenBUGS Certification Summary for Component A

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
b	2.584	0.2948	0.005376	2.001	2.588	3.158	100001	100000
mu[1]	14.17	0.2706	0.005366	13.64	14.16	14.7	100001	100000
mu[2]	15.59	0.08368	4.776E-4	15.43	15.59	15.76	100001	100000
mu[3]	15.33	0.1504	0.001419	15.04	15.33	15.63	100001	100000

Certification Data for Component B

list(Ntot=278)

lamb[]	asy[]	cmp[]	typ[]	pip[]
18.69	1	2	1	1
18.5	2	2	1	1
17.09	3	2	1	1
18.56	4	2	1	1
18.48	5	2	1	1
18.28	6	2	1	1
18.61	7	2	1	1
18.25	8	2	1	1
18.71	9	2	1	1
17.76	10	2	1	1
19.6	1	2	1	1
17.81	2	2	1	1
17.07	3	2	1	1
17.87	4	2	1	1
18.29	5	2	1	1
18.26	6	2	1	1
18.58	7	2	1	1
18.69	8	2	1	1
17.73	9	2	1	1
19.02	10	2	1	1
21.74	1	2	1	1
22.02	2	2	1	1
21.72	3	2	1	1
22.3	5	2	1	1
20.94	6	2	1	1
19.49	7	2	1	1
19.71	8	2	1	1
21.23	9	2	1	1
21.07	10	2	1	1
22.31	1	2	1	1
22.92	2	2	1	1
22.16	3	2	1	1
20.78	4	2	1	1
21.92	5	2	1	1
20.34	6	2	1	1
21.1	7	2	1	1
21.23	8	2	1	1
20.66	9	2	1	1
20.46	10	2	1	1
18.1	1	2	1	1
18.65	2	2	1	1
18.06	3	2	1	1
18.78	4	2	1	1
18.24	5	2	1	1
17.68	6	2	1	1
18.11	7	2	1	1
17.63	8	2	1	1
18.65	9	2	1	1
18.15	10	2	1	1
18.06	1	2	1	1
18.01	2	2	1	1
17.86	3	2	1	1
17.68	4	2	1	1
17.9	5	2	1	1
16.7	6	2	1	1
17.49	7	2	1	1
17.41	8	2	1	1
17.81	9	2	1	1
18.66	10	2	1	1
17.84	1	2	1	1
17.35	2	2	1	1
17.78	3	2	1	1
18.35	4	2	1	1
17.94	5	2	1	1
17	6	2	1	1
17.99	7	2	1	1
17.65	8	2	1	1
17.54	9	2	1	1
17.92	10	2	1	1
18.21	1	2	1	1
18.13	2	2	1	1
18.13	3	2	1	1
17.49	4	2	1	1
18.48	5	2	1	1
17.11	6	2	1	1
17.79	7	2	1	1
17.69	8	2	1	1
17.25	9	2	1	1
18.14	10	2	1	1
17.18	1	2	1	1
17.53	2	2	1	1
17.73	3	2	1	1
17.77	4	2	1	1
18.03	5	2	1	1
17.18	6	2	1	1
18.02	7	2	1	1
18.34	8	2	1	1
17.31	9	2	1	1
18.42	10	2	1	1
17.68	1	2	1	1
16.98	2	2	1	1
17.92	3	2	1	1
17.4	4	2	1	1
17.95	5	2	1	1
16.93	6	2	1	1
17.43	7	2	1	1
17.45	8	2	1	1
17.14	9	2	1	1
17.91	1	2	1	2
17.46	2	2	1	2
18.03	3	2	1	2
17.67	4	2	1	2
17.45	5	2	1	2
18.24	6	2	1	2
17.52	7	2	1	2
18.13	8	2	1	2
16.48	9	2	1	2
18.18	10	2	1	2
17.7	1	2	1	2
18.2	2	2	1	2
18.03	3	2	1	2
17.39	4	2	1	2
17.73	5	2	1	2
17.29	6	2	1	2
17.44	7	2	1	2
17.54	8	2	1	2
16.71	9	2	1	2
17.41	10	2	1	2
17.9	1	2	2	2
17.12	1	2	2	2
16.93	1	2	2	2
17.54	1	2	2	2
17.57	1	2	2	2
16.97	1	2	2	2
17.22	1	2	2	2
18.84	1	2	2	2
17.13	1	2	2	2
17.59	1	2	2	2
17.49	1	2	2	2
17.48	1	2	2	2
16.32	1	2	2	2
17.52	1	2	2	2
18.71	1	2	2	2
16.88	1	2	2	2
17.89	1	2	2	2
18.26	1	2	2	2
17.51	1	2	2	2
17.38	1	2	2	2
17.42	1	2	2	2
17.61	1	2	2	2
19.09	1	2	2	2
17.19	1	2	2	2
17.55	1	2	2	2
17.45	1	2	2	2
16.87	1	2	2	2
17.09	1	2	2	2
16.25	1	2	2	2
18.02	1	2	2	2
17.34	1	2	2	2

18.09	1	2	2	2	17.41	2	2	2	2	17.84	2	2	2	2
17.82	1	2	2	2	17.97	2	2	2	2	17.9	2	2	2	2
17.12	1	2	2	2	16.26	2	2	2	2	16.81	2	2	2	2
16.68	1	2	2	2	17.24	2	2	2	2	16.85	2	2	2	2
15.35	1	2	2	2	17.7	2	2	2	2	17.98	2	2	2	2
18.35	1	2	2	2	17.44	2	2	2	2	17.1	2	2	2	2
17.25	1	2	2	2	17.16	2	2	2	2	16.38	2	2	2	2
17.69	1	2	2	2	18.05	2	2	2	2	19.31	2	2	2	2
17.8	1	2	2	2	18.27	2	2	2	2	17.86	2	2	2	2
17.59	1	2	2	2	17.52	2	2	2	2	17.68	2	2	2	2
16.78	1	2	2	2	17.46	2	2	2	2	17.64	2	2	2	2
18.36	1	2	2	2	16.66	2	2	2	2	18.19	2	2	2	2
17.43	1	2	2	2	18.7	2	2	2	2	16.66	1	2	3	2
18.85	1	2	2	2	17.18	2	2	2	2	16.26	1	2	3	2
17.04	1	2	2	2	14.83	2	2	2	2	18.8	1	2	3	2
17.49	1	2	2	2	16.32	2	2	2	2	16.53	1	2	3	2
17.39	1	2	2	2	17.59	2	2	2	2	17.33	1	2	3	2
17.35	1	2	2	2	17.23	2	2	2	2	15.51	1	2	3	2
17.14	1	2	2	2	16.84	2	2	2	2	18.84	1	2	3	2
17.03	1	2	2	2	18.87	2	2	2	2	17.18	1	2	3	2
17.72	1	2	2	2	17.56	2	2	2	2	15.49	1	2	3	2
17.27	1	2	2	2	18.16	2	2	2	2	17.23	1	2	3	2
17.38	1	2	2	2	17.92	2	2	2	2	19.4	1	2	3	2
17.4	1	2	2	2	16.14	2	2	2	2	16.4	1	2	3	2
17.18	1	2	2	2	15.66	2	2	2	2	15.65	1	2	3	2
17.01	1	2	2	2	17.41	2	2	2	2	17.87	1	2	3	2
16.73	1	2	2	2	17.52	2	2	2	2	16.55	2	2	3	2
18.87	1	2	2	2	16.97	2	2	2	2	16.7	2	2	3	2
17.43	1	2	2	2	17.62	2	2	2	2	16.73	2	2	3	2
18.25	1	2	2	2	18.01	2	2	2	2	17.05	2	2	3	2
18.49	1	2	2	2	17.66	2	2	2	2	17.55	2	2	3	2
18.8	1	2	2	2	17.47	2	2	2	2	16.25	2	2	3	2
17.68	1	2	2	2	17.44	2	2	2	2	17.08	2	2	3	2
17.13	1	2	2	2	16.92	2	2	2	2	17.05	2	2	3	2
16.6	1	2	2	2	18.6	2	2	2	2	18.62	2	2	3	2
17.75	2	2	2	2	17.56	2	2	2	2	16.86	2	2	3	2
18.24	2	2	2	2	18.96	2	2	2	2	17.23	2	2	3	2
16.7	2	2	2	2	18.14	2	2	2	2	19.09	2	2	3	2
16.83	2	2	2	2	17.03	2	2	2	2	18.59	2	2	3	2
16.93	2	2	2	2	17.22	2	2	2	2	18.97	2	2	3	2
16.9	2	2	2	2	16.88	2	2	2	2	19.72	2	2	3	2
18.64	2	2	2	2	18.21	2	2	2	2	END				
17.09	2	2	2	2	17.58	2	2	2	2					
17.64	2	2	2	2	17.87	2	2	2	2					

Table C-3: Example OpenBUGS Certification Summary for Component B

		mean	**sd**	**MC_error**	**val2.5pc**	**median**	**val97.5pc**	**start**	**sample**
b		0.9337	0.3387	0.007504	0.2695	0.9371	1.592	100001	100000
mu[1]		17.64	0.3101	0.007483	17.04	17.63	18.25	100001	100000
mu[2]		17.48	0.08014	4.719E-4	17.33	17.48	17.64	100001	100000
mu[3]		17.35	0.2338	0.003093	16.88	17.36	17.81	100001	100000

Certification data for Component C

list(Ntot=280)

lamb[]	asy[]	cmp[]	typ[]	pip[]
16.3	1	3	1	1
15.96	2	3	1	1
15.74	3	3	1	1
15.9	4	3	1	1
16.1	5	3	1	1
15.89	6	3	1	1
15.69	7	3	1	1
15.65	8	3	1	1
15.64	9	3	1	1
15.48	10	3	1	1
17.06	1	3	1	1
15.76	2	3	1	1
15.38	3	3	1	1
15.55	4	3	1	1
16.07	5	3	1	1
15.48	6	3	1	1
15.72	7	3	1	1
16.81	8	3	1	1
14.92	9	3	1	1
16.55	10	3	1	1
14.99	1	3	1	1
15.76	2	3	1	1
15.45	3	3	1	1
15.84	4	3	1	1
15.77	5	3	1	1
15.99	6	3	1	1
15.87	7	3	1	1
15.2	8	3	1	1
14.79	9	3	1	1
15.12	10	3	1	1
14.97	1	3	1	1
16.11	2	3	1	1
15.6	3	3	1	1
15.46	4	3	1	1
15.29	5	3	1	1
14.78	6	3	1	1
15.04	7	3	1	1
15.51	8	3	1	1
14.38	9	3	1	1
14.94	10	3	1	1
14.52	1	3	1	1
13.47	2	3	1	1
13.95	3	3	1	1
14.42	4	3	1	1
14.48	5	3	1	1
14.63	6	3	1	1
14.02	7	3	1	1
14.74	8	3	1	1
14.66	9	3	1	1
14.39	10	3	1	1
14.24	1	3	1	1
13.97	2	3	1	1
13.5	3	3	1	1
14.59	4	3	1	1
16.02	5	3	1	1
14.35	6	3	1	1
14.33	7	3	1	1
14.81	8	3	1	1
14.82	9	3	1	1
15.68	10	3	1	1
14.7	1	3	1	1
14.11	2	3	1	1
15.12	3	3	1	1
14.52	4	3	1	1
15.67	5	3	1	1
14.8	6	3	1	1
14.42	7	3	1	1
14.3	8	3	1	1
14.13	9	3	1	1
14.86	10	3	1	1
14.44	1	3	1	1
14.27	2	3	1	1
14.39	3	3	1	1
14.68	4	3	1	1
15.24	5	3	1	1
14.24	6	3	1	1
14.59	7	3	1	1
14.67	8	3	1	1
14.11	9	3	1	1
14.8	10	3	1	1
14.85	1	3	1	1
14.22	2	3	1	1
14.38	3	3	1	1
14.39	4	3	1	1
15.15	5	3	1	1
14.09	6	3	1	1
14.69	7	3	1	1
14.53	8	3	1	1
14.65	9	3	1	1
13.63	10	3	1	1
14.58	1	3	1	1
14.59	2	3	1	1
14.65	3	3	1	1
14.68	4	3	1	1
14.57	5	3	1	1
14.12	6	3	1	1
14.74	7	3	1	1
14.28	8	3	1	1
14.25	9	3	1	1
13.93	10	3	1	1
13.57	1	3	1	2
14.47	2	3	1	2
14.48	3	3	1	2
14.09	4	3	1	2
13.89	5	3	1	2
14.15	6	3	1	2
14.31	7	3	1	2
13.81	8	3	1	2
14.03	9	3	1	2
14.35	10	3	1	2
13.64	1	3	1	2
13.61	2	3	1	2
14.04	3	3	1	2
14.11	4	3	1	2
14.29	5	3	1	2
14.17	6	3	1	2
13.8	7	3	1	2
13.36	8	3	1	2
13.87	9	3	1	2
13.72	10	3	1	2
14.93	1	3	2	2
15.16	1	3	2	2
14.51	1	3	2	2
14.4	1	3	2	2
15.23	1	3	2	2
14.37	1	3	2	2
14.85	1	3	2	2
14.33	1	3	2	2
14.63	1	3	2	2
15.34	1	3	2	2
15.33	1	3	2	2
14.5	1	3	2	2
15.37	1	3	2	2
15.15	1	3	2	2
15.11	1	3	2	2
14.49	1	3	2	2
13.88	1	3	2	2
14.75	1	3	2	2
14.68	1	3	2	2
15.49	1	3	2	2
15.3	1	3	2	2
14.42	1	3	2	2
13.85	1	3	2	2
15.13	1	3	2	2
15.28	1	3	2	2
14.86	1	3	2	2
14.72	1	3	2	2
14.93	1	3	2	2
14.77	1	3	2	2
14.05	1	3	2	2
14.99	1	3	2	2
14.58	1	3	2	2
15.14	1	3	2	2
15.25	1	3	2	2
15.27	1	3	2	2
14.9	1	3	2	2
15.68	1	3	2	2
14.52	1	3	2	2
14.21	1	3	2	2
14.55	1	3	2	2
15.25	1	3	2	2
15.31	1	3	2	2
14.4	1	3	2	2
14.87	1	3	2	2
15.1	1	3	2	2
15.77	1	3	2	2
15.06	1	3	2	2
15.2	1	3	2	2
14.83	1	3	2	2
15.22	1	3	2	2
14.63	1	3	2	2
14.78	1	3	2	2
15.05	1	3	2	2
14.54	1	3	2	2
15.55	1	3	2	2
14.97	1	3	2	2
15.15	1	3	2	2
14.94	1	3	2	2
15.94	1	3	2	2

14.71	1	3	2	2	14.81	2	3	2	2	14.21	2	3	2	2
13.94	1	3	2	2	15.47	2	3	2	2	14.68	2	3	2	2
14.45	1	3	2	2	15.01	2	3	2	2	15.56	2	3	2	2
14.46	1	3	2	2	14.91	2	3	2	2	14.24	1	3	3	2
14.99	1	3	2	2	14.65	2	3	2	2	14.27	1	3	3	2
14.84	1	3	2	2	14.7	2	3	2	2	13.81	1	3	3	2
14.36	1	3	2	2	14.83	2	3	2	2	13.94	1	3	3	2
15.65	2	3	2	2	14.79	2	3	2	2	14.73	1	3	3	2
14.9	2	3	2	2	14.8	2	3	2	2	13.3	1	3	3	2
14.85	2	3	2	2	15.43	2	3	2	2	14.46	1	3	3	2
15.29	2	3	2	2	14.48	2	3	2	2	14.24	1	3	3	2
15.13	2	3	2	2	15.31	2	3	2	2	13.3	1	3	3	2
14.92	2	3	2	2	14.78	2	3	2	2	14.52	1	3	3	2
14.75	2	3	2	2	14.65	2	3	2	2	15.12	1	3	3	2
14.97	2	3	2	2	14.54	2	3	2	2	13.14	1	3	3	2
15.03	2	3	2	2	15.91	2	3	2	2	15.25	1	3	3	2
14.48	2	3	2	2	14.66	2	3	2	2	14.75	1	3	3	2
14.88	2	3	2	2	15.7	2	3	2	2	15.42	1	3	3	2
14.96	2	3	2	2	14.71	2	3	2	2	14.61	2	3	3	2
14.99	2	3	2	2	15.06	2	3	2	2	14.43	2	3	3	2
14.69	2	3	2	2	14.91	2	3	2	2	15.47	2	3	3	2
15.15	2	3	2	2	14.61	2	3	2	2	13.94	2	3	3	2
15.43	2	3	2	2	14.85	2	3	2	2	13.64	2	3	3	2
14.52	2	3	2	2	14.95	2	3	2	2	13.95	2	3	3	2
14.58	2	3	2	2	15.16	2	3	2	2	15.22	2	3	3	2
14.35	2	3	2	2	14.74	2	3	2	2	14.83	2	3	3	2
14.99	2	3	2	2	14.83	2	3	2	2	14.52	2	3	3	2
14.99	2	3	2	2	15.19	2	3	2	2	14.82	2	3	3	2
15.89	2	3	2	2	14.75	2	3	2	2	14.39	2	3	3	2
15.62	2	3	2	2	14.66	2	3	2	2	14.58	2	3	3	2
15.47	2	3	2	2	15.65	2	3	2	2	15.5	2	3	3	2
15.05	2	3	2	2	14.91	2	3	2	2	15.02	2	3	3	2
15.08	2	3	2	2	14.35	2	3	2	2	15.2	2	3	3	2
15.2	2	3	2	2	14.59	2	3	2	2	END				

Table C-4: Example OpenBUGS Certification Summary for Component C

	mean	**sd**	**MC_error**	**val2.5pc**	**median**	**val97.5pc**	**start**	**sample**
b	0.9626	0.1701	0.002428	0.6285	0.9633	1.293	100001	100000
mu[1]	13.99	0.1566	0.002413	13.69	13.99	14.3	100001	100000
mu[2]	14.91	0.05701	2.386E-4	14.8	14.91	15.03	100001	100000
mu[3]	14.49	0.132	0.001181	14.23	14.49	14.75	100001	100000

3.4. Results

Table C-5 lists the results, in Copies/nL, for the three components.

Table C-5: Parameter Values for Components A, B, and C

		Posterior Distribution Summary Statistics				
Component	Parameter	Mean	SD	2.5 %	50 %	97.5 %
A	β	2.60	0.29	2.03	2.60	3.18
	μ_1	14.15	0.27	13.62	14.15	14.67
	μ_2	15.59	0.10	15.39	15.59	15.8
	μ_3	15.33	0.16	15.01	15.33	15.65
B	β	0.95	0.34	0.29	0.95	1.62
	μ_1	17.62	0.31	17.01	17.62	18.22
	μ_2	17.48	0.10	17.28	17.48	17.69
	μ_3	17.35	0.24	16.87	17.35	17.83
C	β	0.97	0.17	0.64	0.97	1.30
	μ_1	13.99	0.16	13.68	13.99	14.29
	μ_2	14.91	0.08	14.76	14.91	15.07
	μ_3	14.49	0.14	14.21	14.49	14.77

Since in each case, the three data set types represent independent measurements, the three estimates can be combined to obtain a consensus value. In this way a between data set type variability is included in the final standard uncertainty. Table C-6 lists the results in Copies per Nanoliter obtained using the NIST Consensus Builder (NICOB)[C3]

Table C-6: Certified Values for Components A, B, and C

Component	Mean	u(Mean)	$U_{k=2}$(Mean)
A	15.1	0.7	1.4
B	17.5	0.3	0.6
C	14.5	0.5	1.0

4. Mito-to-nuclear DNA ratios (mtDNA/nDNA)

4.1. Data

Measurements were also obtained on the same samples using three mitochondrial detection assays mtND1, mtBatz, and mtKav, and a single nDNA assay (genomic) to compute the ratios mtDNA/nDNA. *For the complete mtDNA/nDNA data, see:*

Table 16: mtDNA/nDNA Ratios

4.2. Model

For each component, the data was analyzed using the following random effects model to account for between assay variability.

$$r_i \sim N(\alpha_i, \sigma^2_{assay})$$

where i denotes assay (for A and B: 1 = mtND1, 2 = mtBatz, 3 = mtKav, for C: 1 = mtBatz, 2 = mtKav), and $\alpha_i \sim N(\mu, \sigma^2_{between\ assay})$.

4.3. OpenBUGS code, inits, and data

Two versions of the same model are required, one for the results from the three mtDNA assays available for components A and B {mtND1, mtBatz, and mtKav} and the other for the results from the two mtDNA assays available for component C {mtBatz and mtKav}.

```
## mtDNA/nDNA code for components A and B
ModelBegin{
mu ~ dnorm(0, 0.0016)I(0.001,); chSqNs ~ dgamma(0.5,0.5); sigr <- mu/sqrt(chSqNs)

for(i in 1:3){alpha[i]~dnorm(mu,sigr); sigt[i]~dgamma(1.0E-5,1.0E-5)}

for(i in 1:Ndata){rat[i]~dnorm(alpha[asy[i]],sigt[asy[i]])}
}ModelEnd

## mtDNA/nDNA inits for components A and B
list(sigt=c(1,1,1))
```

##Data for Component A

list(Ndata=102)

set[]	rep[]	asy[]	rat[]
1	1	1	193.1
1	2	1	187
1	1	2	184.5
1	2	2	176.4
2	1	1	181
2	2	1	190.6
2	1	2	178.5
2	2	2	183.7
3	1	1	174.3
3	2	1	176.1
3	1	2	182.9
3	2	2	184.7
4	1	1	171.6
4	2	1	180
4	1	2	174
4	2	2	181.3
5	1	1	192.3
5	2	1	183.5
5	1	2	193.5
5	2	2	185.1
6	1	1	175.7
6	2	1	172.4
6	3	1	176.8
6	1	2	174.3
6	2	2	167.1
6	3	2	176.6
6	1	3	169.2
6	2	3	166.2
6	3	3	176.7
7	1	1	192.4
7	2	1	194.4
7	3	1	189.3
7	1	2	178.6
7	2	2	175.2
7	3	2	174.3
7	1	3	153
7	2	3	154.2
7	3	3	152.3
7	1	2	158
7	2	2	155.7
7	3	2	159.2
7	1	3	210.3
7	2	3	210
7	3	3	204.9
7	1	1	174.9
7	2	1	176.9
7	3	1	177.7
7	1	2	196.5
7	2	2	188.9
7	3	2	194.7
7	1	3	181.3
7	2	3	186.4
7	3	3	186.4
8	1	1	178
8	2	1	177.6
8	3	1	170.6
8	1	2	180.4
8	2	2	175.1
8	3	2	173.8
8	1	3	176.9
8	2	3	178.6
8	3	3	176.1
9	1	2	158.4
9	2	2	154.4
10	1	1	194.7
10	2	1	192.8
10	3	1	192.2
10	1	1	182.9
10	2	1	189.5
10	3	1	185.1
10	1	1	167.4
10	2	1	161.7
11	1	2	166.7
11	2	2	181.3
11	3	2	170.8
11	4	2	159.8
11	1	2	156.6
11	2	2	168.7
11	3	2	166.3
11	4	2	158.9
11	1	2	164.3
11	2	2	166.8
11	3	2	160.4
11	4	2	157.3
12	1	2	171.9
12	2	2	171.6
12	3	2	167
12	4	2	166.6
12	1	2	158.1
12	2	2	157.3
12	3	2	160.1
12	4	2	161.2
12	1	2	154.6
12	2	2	154.2
12	3	2	153.8
12	4	2	158.7
13	1	1	161.7
13	2	1	159.9
13	1	1	162.2
13	2	1	157.9
13	1	1	157.5
13	2	1	152.8

END

Table C-7: Example OpenBUGS mtDNA/nDNA Summary for Component A

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
mu	173.0	1.443	0.04419	170.3	173.0	175.9	100001	100000

##data for Component B

list(Ndata=87)

set[]	rep[]	asy[]	rat[]
1	1	1	246.1
1	2	1	240.1
1	1	2	255.5
1	2	2	260.8
2	1	1	222.9
2	2	1	220.0
2	1	2	210.8
2	2	2	219.2
3	1	1	212.7
3	2	1	219.8
3	1	2	245.5
3	2	2	250.6
4	1	1	221.1
4	2	1	219.9
4	1	2	238.0
4	2	2	224.9
5	1	1	210.5
5	2	1	194.3
5	1	2	213.6
5	2	2	211.9
6	1	1	199.0
6	2	1	192.1
6	3	1	198.2
6	1	2	199.0
6	2	2	192.4
6	3	2	195.8
6	1	3	190.3
6	2	3	183.1
6	3	3	191.9
7	1	1	230.4
7	2	1	234.1
7	3	1	233.2
7	1	2	204.8
7	2	2	207.8
7	3	2	206.7
7	1	3	239.9
7	2	3	247.9
7	3	3	249.3
7	1	1	206.5
7	2	1	216.6
7	3	1	216.4
7	1	2	231.4
7	2	2	228.0
7	3	2	225.8
7	1	3	186.7
7	2	3	198.7
7	3	3	197.1
8	1	1	204.6
8	2	1	205.7
8	3	1	202.4
8	1	2	199.8
8	2	2	204.3
8	3	2	195.2
8	1	3	207.7
8	2	3	211.7
8	3	3	204.1
9	1	2	189.0
9	2	2	188.6
10	1	2	192.8
10	2	2	191.6
10	3	2	191.5
10	1	2	194.7
10	2	2	194.6
10	3	2	196.1
10	4	2	190.9
10	1	2	195.3
10	2	2	195.2
10	3	2	194.1
10	4	2	188.1
11	1	2	186.5
11	2	2	186.3
11	3	2	182.8
11	4	2	182.8
11	1	2	194.4
11	2	2	196.4
11	3	2	194.7
11	4	2	189.5
11	1	2	190.8
11	2	2	190.9
11	3	2	194.0
11	4	2	183.9
12	1	1	168.8
12	2	1	178.3
12	1	1	182.3
12	2	1	163.1
12	1	1	192.0
12	2	1	188.7

END

Table C-8: Example OpenBUGS mtDNA/nDNA Summary for Component B

	mean	sd	MC_error	val2.5pc	median	val97.5pc	start	sample
mu	204.5	2.246	0.111	199.9	204.5	208.8	100001	100000

```
## mtDNA/nDNA code for component C
ModelBegin{
mu ~ dnorm(0, 0.0016)I(0.001,)
chSqNs ~ dgamma(0.5,0.5)
sigr <- mu/sqrt(chSqNs)

for(i in 1:2){alpha[i]~dnorm(mu,sigr)
sigt[i]~dgamma(1.0E-5,1.0E-5)}

for(i in 1:Ndata){rat[i]~dnorm(alpha[asy[i]],sigt[asy[i]])}
}ModelEnd

## mtDNA/nDNA inits for component C
list(sigt=c(1,1))

## mtDNA/nDNA data for component C
list(Ndata=66)
set[]  rep[]  asy[]  rat[]
1  1  1  320.1
1  2  1  329.9
2  1  1  334.7
2  2  1  328.7
3  1  1  321.7
3  2  1  338.4
4  1  1  320.0
4  2  1  336.3
5  1  1  329.4
5  2  1  301.3
6  1  1  280.5
6  2  1  282.5
6  3  1  282.9
6  1  2  270.9
6  2  2  273.8
6  3  2  272.7
7  1  1  279.7
7  2  1  264.2
7  3  1  279.2
7  1  2  280.4
7  2  2  278.9
7  3  2  284.2
7  1  1  271.5
7  2  1  260.6
7  3  1  268.7
7  1  2  279.9
7  2  2  262.8
7  3  2  255.9
7  1  1  263.9
7  2  1  253.5
7  3  1  265.7
7  1  2  295.3
7  2  2  275.7
7  3  2  280.2
8  1  1  292.7
8  2  1  277.6
8  3  1  276.2
8  1  2  304.4
8  2  2  283.0
8  3  2  282.5
9  1  1  250.1
9  2  1  252.2
10  1  1  283.2
10  2  1  284.0
10  3  1  278.8
10  4  1  272.5
10  1  1  283.5
10  2  1  279.0
10  3  1  273.6
10  4  1  267.9
10  1  1  280.3
10  2  1  277.3
10  3  1  275.7
10  4  1  275.2
11  1  1  259.9
11  2  1  250.8
11  3  1  255.1
11  4  1  260.9
11  1  1  261.7
11  2  1  248.3
11  3  1  262.6
11  4  1  260.2
11  1  1  266.5
11  2  1  253.5
11  3  1  257.0
11  4  1  265.0
END
```

Table C-9: Example OpenBUGS mtDNA/nDNA Summary for Component C

	mean	**sd**	**MC_error**	**val2.5pc**	**median**	**val97.5pc**	**start**	**sample**
mu	277.4	2.367	0.1176	272.9	277.4	282.1	100001	100000

Table C-10: Results for mtDNA/nDNA

Posterior Distribution Summary Statistics

Component	Mean	Std Dev	2.5 %	50 %	97.5 %
A	173.0	1.4	170.3	173.0	175.8
B	204.5	2.3	199.4	204.7	208.7
C	277.4	2.3	272.9	277.4	282.1

5. References

C1 Lunn DJ, Spiegelhalter D, Thomas A, Best N. *Statistics in Medicine* 2009;28:3049–3082

C2 Polson NG, Scott JG. *Bayesian Analysis* 2012;7:887–902.

C3 NIST Consensus Builder. Software and user's guide freely available at: https://consensus.nist.gov/

Appendix D: Performance in Commercial qPCR Chemistries

The performance of the SRM 2372a materials in commercial qPCR chemistries was assessed using manufacturer's recommended protocols. Each panel in Figures D-1 to D-4 displays the average cycle threshold (Ct) as a function of DNA concentration for the 5-point dilutions of SRM 2372a components. In all cases, the external calibrant was SRM 2372 component A. Error bars represent approximate 95 % confidence intervals.

Table D-1 lists the estimated [DNA] for the three SRM 2372a components for all commercial assays evaluated. This Table also lists the Component B/Component A (B/A) and Component C/Component A (C/A) ratios calculated from the [DNA] estimates.

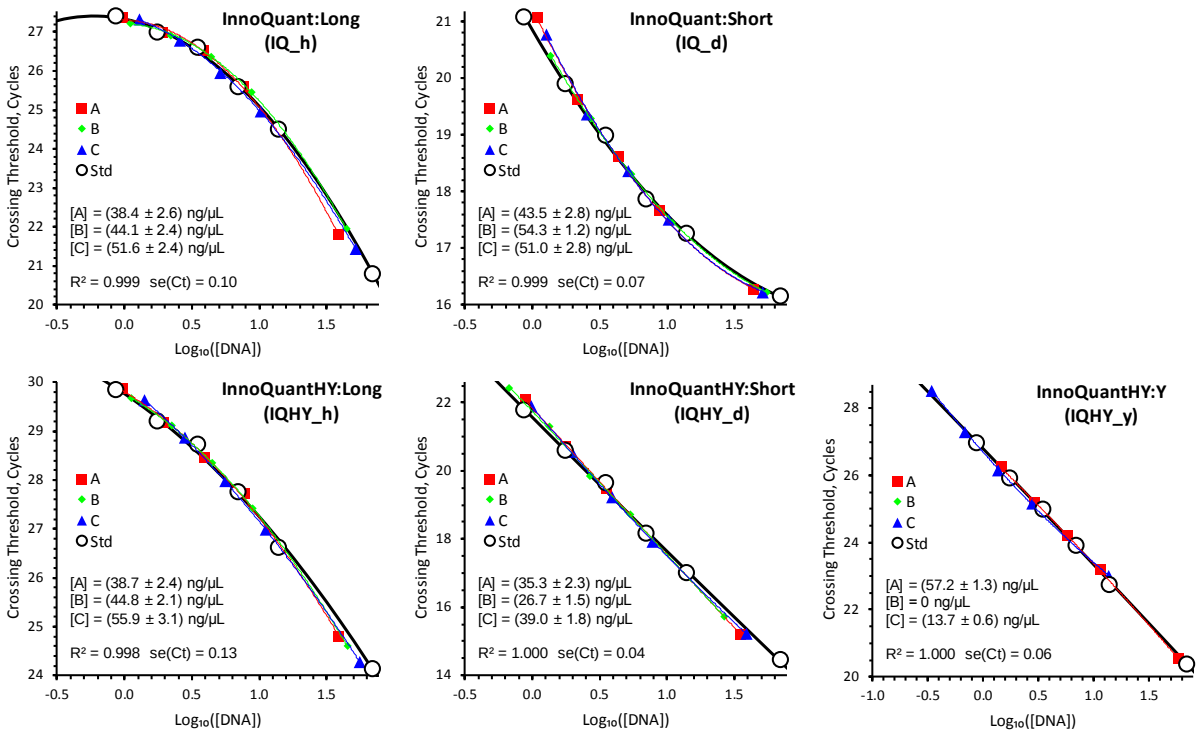


Figure D-1: Results for the Innogenomics Commercial qPCR assays

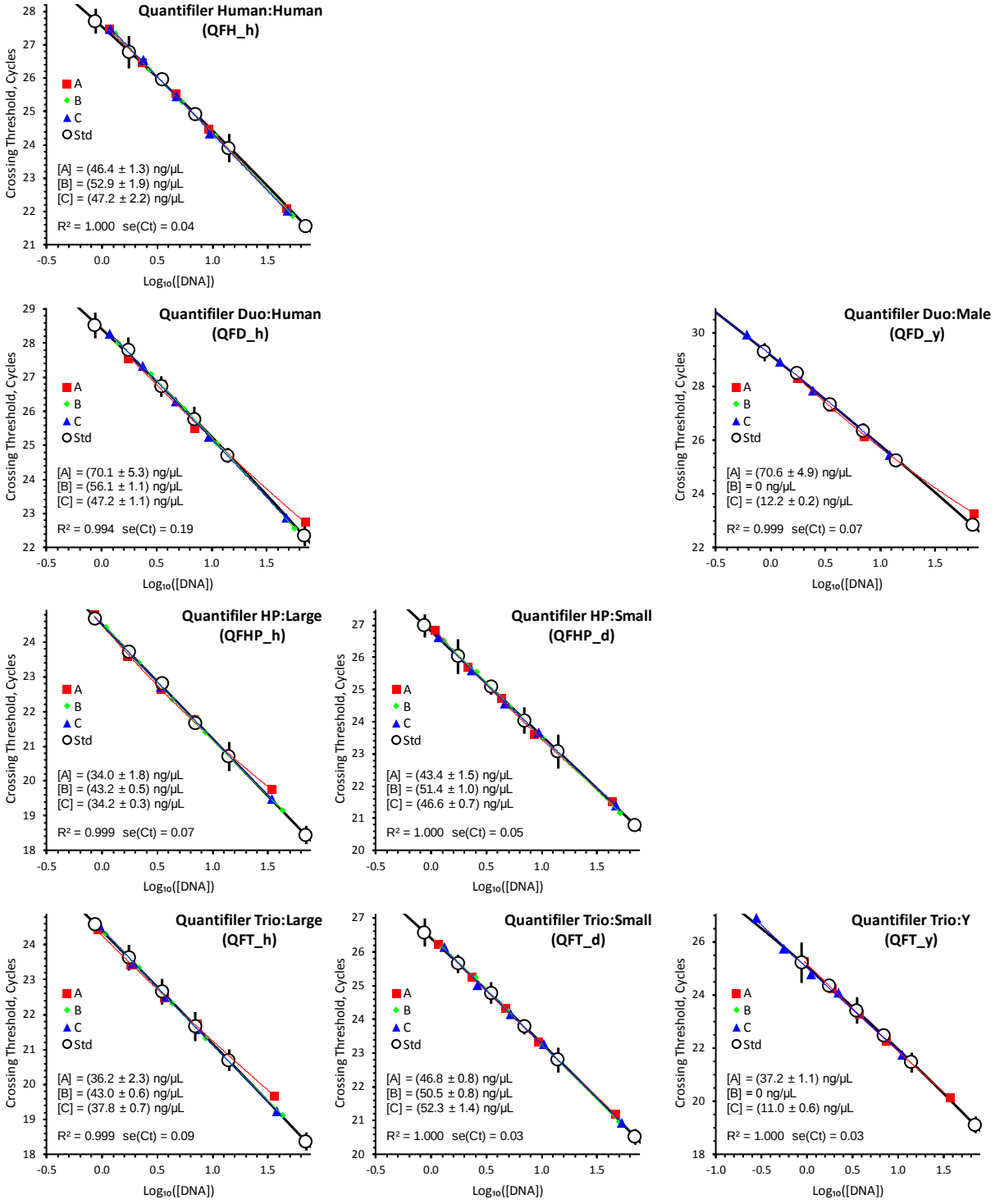


Figure D-2: Results for the Thermo Fisher Commercial qPCR assays

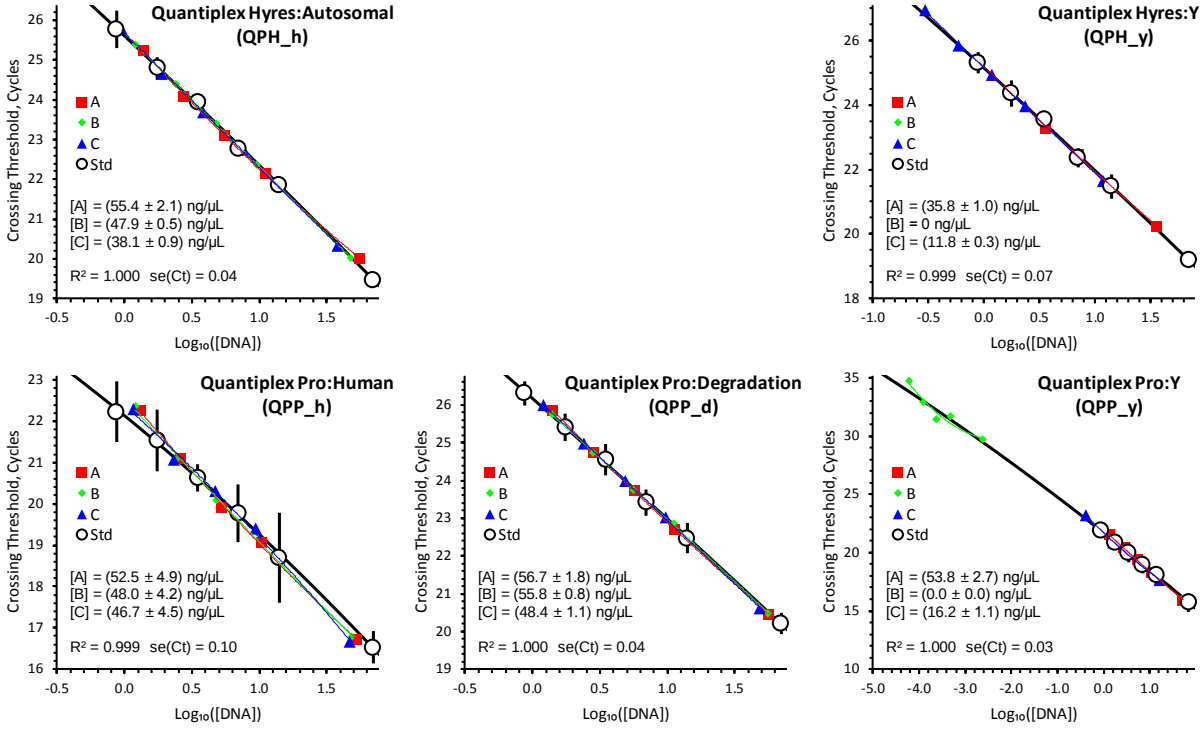


Figure D-3: Results for the Qiagen Commercial qPCR assays

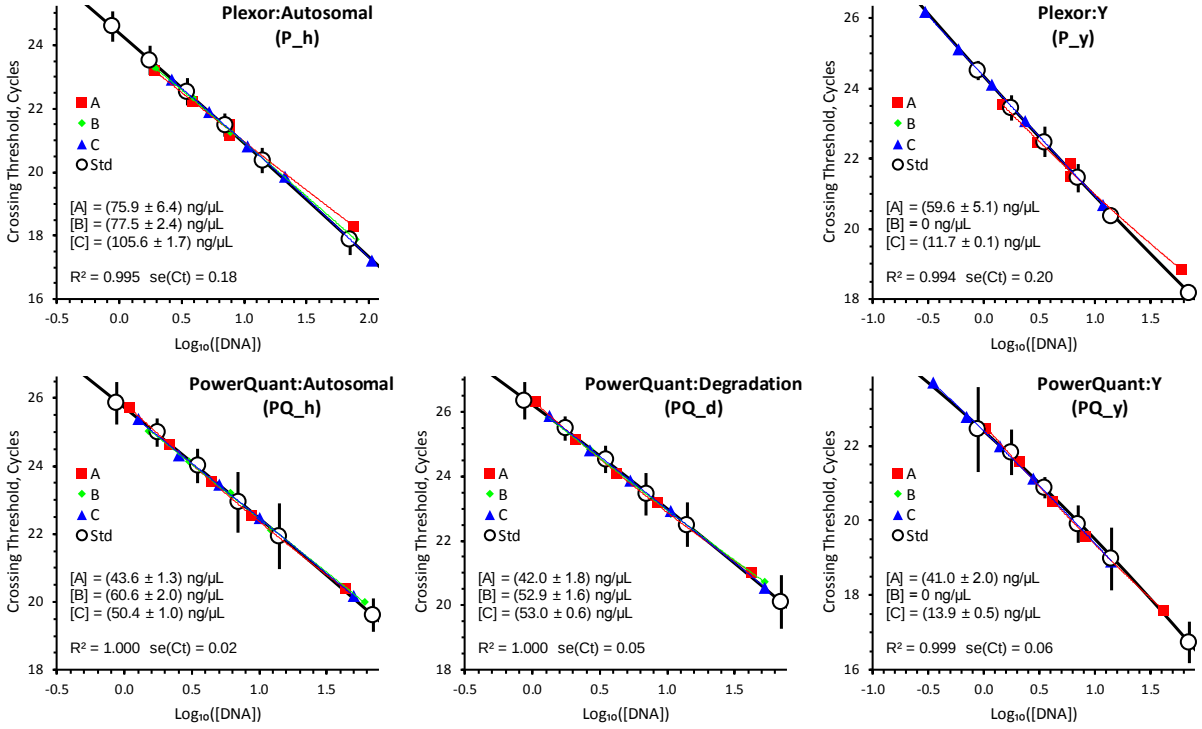


Figure D-4: Results for the Promega Commercial qPCR assays

Table D-1: Summary [DNA] Results for the Commercial qPCR Assays

	Estimated [DNA], ng/µL						Ratios			
	A		B		C		B/A		C/A	
Assay[a]	x^b	$u(x)^c$	x^b	$u(x)^c$	x^b	$u(x)^c$	y^d	$u(y)^e$	y^d	$u(y)^e$
IQ:d	43.5	2.8	54.3	1.2	51.0	2.8	1.25	0.08	1.17	0.10
IQ:h	38.4	2.6	44.1	2.4	51.6	2.4	1.15	0.10	1.34	0.11
IQHY:d	35.3	2.3	26.7	1.5	39.0	1.8	0.76	0.06	1.11	0.09
IQHY:h	38.7	2.4	44.8	2.1	55.9	3.1	1.16	0.09	1.44	0.12
P:h	75.9	6.4	77.5	2.4	105.6	1.6	1.02	0.09	1.39	0.12
PQ:d	42.0	1.8	52.9	1.6	53.0	0.6	1.26	0.07	1.26	0.06
PQ:h	43.6	1.3	60.6	2.0	50.4	1.0	1.39	0.06	1.16	0.04
QFD:h	70.1	5.3	56.1	1.1	47.2	1.1	0.80	0.06	0.67	0.05
QFH:h	46.4	1.3	52.9	1.9	47.2	2.2	1.14	0.05	1.02	0.06
QFHP:d	43.4	1.5	51.4	1.0	46.6	0.7	1.18	0.05	1.07	0.04
QFHP:h	34.0	1.8	43.2	0.5	34.2	0.3	1.27	0.07	1.01	0.05
QFT:d	46.8	0.8	50.5	0.8	52.3	1.4	1.08	0.03	1.12	0.04
QFT:h	36.2	2.3	43.0	0.6	37.8	0.7	1.18	0.08	1.04	0.07
QPH:h	55.4	2.1	47.9	0.5	38.0	0.9	0.86	0.03	0.69	0.03
QPP:d	56.7	1.8	55.8	0.8	48.4	1.1	0.98	0.03	0.85	0.03
QPP:h	52.5	4.9	48.0	4.2	46.7	4.5	0.92	0.12	0.89	0.12
IQHY:y	57.2	1.3	ND[f]		13.7	0.6			0.24	0.01
P:y	59.6	5.1	ND[f]		11.7	0.1			0.20	0.02
PQ:y	41.0	2.0	ND[f]		13.9	0.5			0.34	0.02
QFD:y	70.6	4.9	ND[f]		12.2	0.2	NR[g]		0.17	0.01
QFT:y	37.2	1.1	ND[f]		11.0	0.6			0.30	0.02
QPH:y	35.8	1.0	ND[f]		11.8	0.3			0.33	0.01
QPP:y	53.8	2.7	0.002	0.001	16.2	1.1			0.30	0.03

a Assay codes are listed in Table 8
b Mean of dilution-adjusted results from five dilutions of the SRM 2372a component
c Standard uncertainty of the mean, estimated from the stdanrd deviation of the five dilutions
d Ratio of the estimated [DNA] of component B or C relative to that of component A
e Standard uncertainty of the ratio, estimated from the standard uncertainties of the individual components.
f Not detected. Component B was prepared using tissue from only female donors; it is not expected to respond to male-specific assays.
g Not relevant