Development of the Next Generation of Forensic DNA Standard Reference Material: SRM 2372a

Erica Romsos

Potomac Regional Symposium

April 27, 2018

NIST APPLIED GENETICS

Disclaimer

I will mention commercial platforms, but am in no way attempting to endorse any specific product.

NIST Disclaimer: Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Development of NIST SRM 2372a

- Review of SRM 2372a and why it benefits forensic laboratories
- Examination of the next generation of certification measurements
  - From UV absorbance to Digital PCR
- Overview of the development process of SRM 2372a

What is SRM 2372 Human DNA Quantitation Standard?

SRM 2372 was originally released in 2007

Component A: Single-source male
Component B: Multi-source female
Component C: Multi-source male/female mixture

Certified for spectroscopic traceability in units of decadic attenuance, $D_{10.}$

National Institute of Standards & Technology

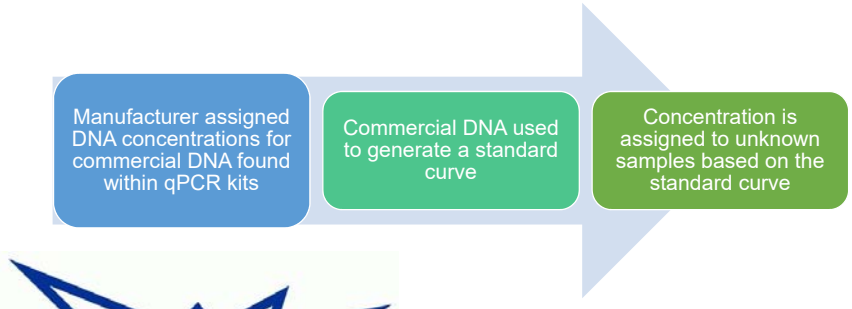
Certificate of Analysis

Standard Reference Material® 2372

Human DNA Quantitation Standard

Parameter	A	B	C
2012 DNA Mass Concentration	57 ng/µL	61 ng/µL	59 ng/µL

Forensic Need for SRM 2372a



Is the manufacturer assigned concentration accurate?

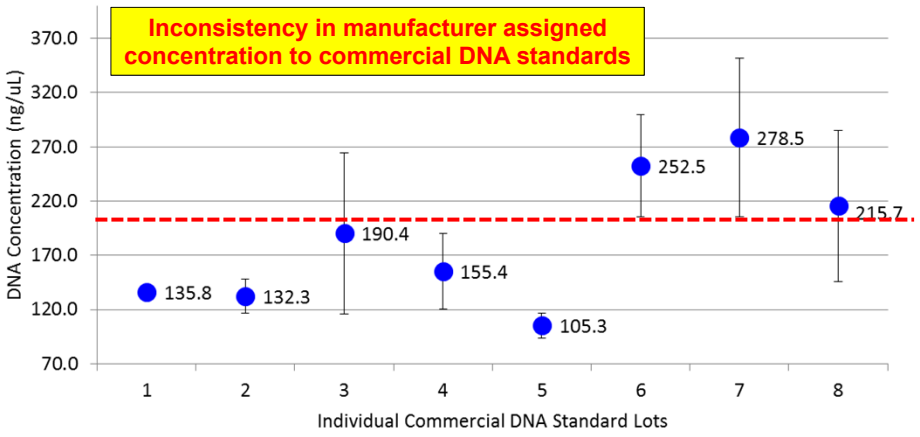
Standard DNA within Quant Kits

- Examined 8 different lots of standard DNA within one commercial quantitation kit
  - 8 individual lots
  - Never opened/used
  - 1:5, 1:10, and 1:20 dilutions were made
    - To allow for the samples to fall within the standard curve
- SRM 2372 component A used to generate standard curve
- All **commercial quantitation kit dilutions** were run in triplicate and per manufacturer's recommendations

8 Commercial DNA Standards

DNA Standard derived from a human cell line found in commercial qPCR kits

Nominal value assigned from manufacturer: 200 ng/µL



qPCR vs. Digital PCR

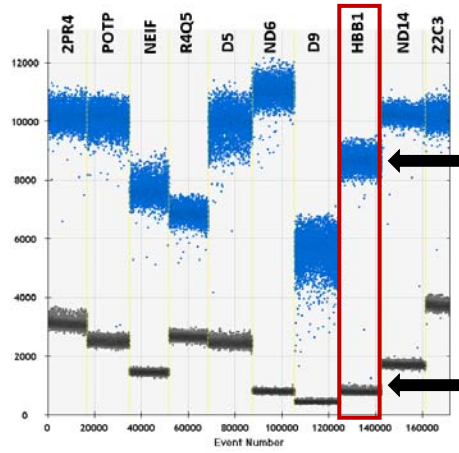
- Quantification of the same 8 DNA standards
- No calibration curve – absolute quantification

- Alternate target from hTERT
  - HBB1 – housekeep gene on chromosome 11

Assay Target	Chromosome, Band Accession #	Primers and Probe [a]	Amplicon Length, bp
HBB1 Gene HBB	Chr 11, p15.5 NC_000011.10	F gctgagggtttgaagtccaactc R ggtctaagtgatgacagccgtacct P^T agccagtgccagaagagccaagga	76

What is Digital PCR?

Partitioning of samples into individual chambers or droplets



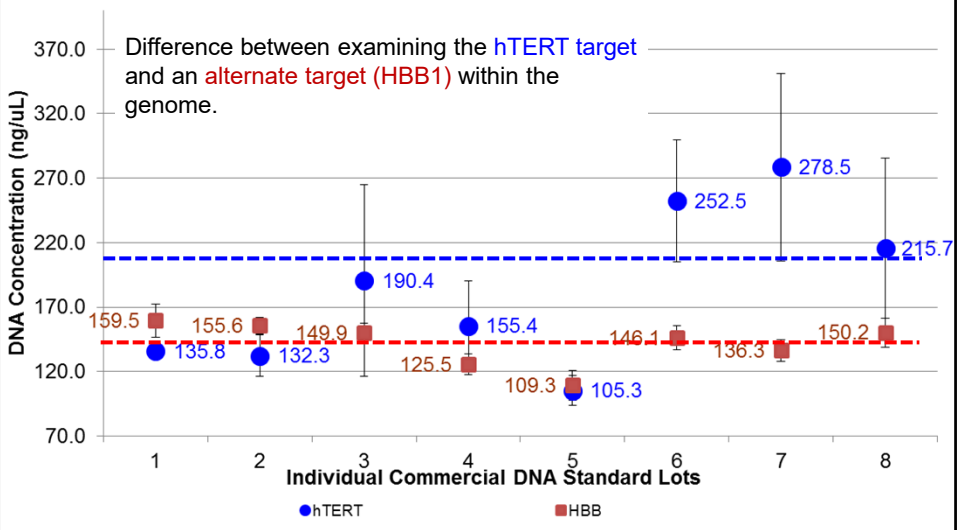
No need for a standard curve

Positives
(1 copy of the target)

Negatives
(0 copies of the target)

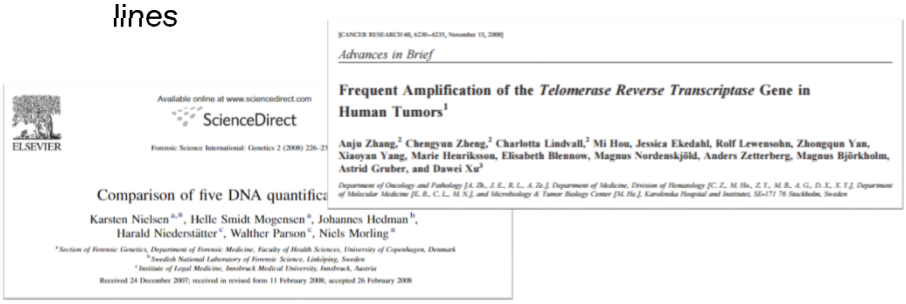
dPCR is counting *accessible* amplifiable targets

hTERT (qPCR) vs. HBB1 (ddPCR)



Example of DNA Standard Bias

- Use of cell lines for production of commercial DNA standards—deviation from wild type DNA due to characteristics of cell lines
- Example: Raji cell line used for a commercial DNA standard
  - More copies in ~85% of all tested immortalized cell lines



Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER

Comparison of five DNA quantifica

Karsten Nielsen[a,*], Helle Smidt Mogensen[a], Johannes Hedman[b], Harald Niederstätter[c], Walther Parson[c], Niels Morling[a]

Received 24 December 2007; received in revised form 11 February 2008; accepted 26 February 2008

[CANCER RESEARCH 60, 6230–6235, November 15, 2000]

Advances in Brief

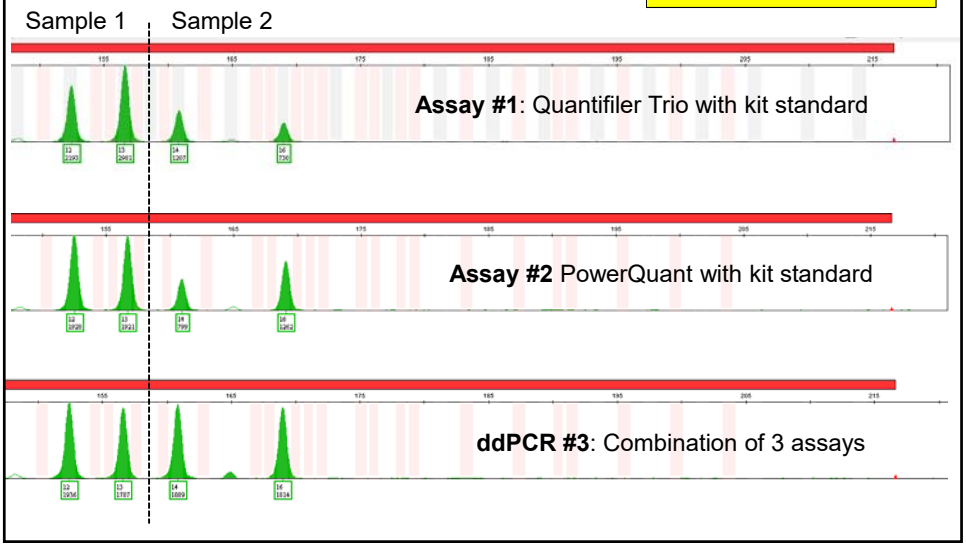
Frequent Amplification of the *Telomerase Reverse Transcriptase* Gene in Human Tumors[1]

Anju Zhang,[2] Chengyun Zheng,[2] Charlotta Lindvall,[2] Mi Hou, Jessica Ekedahl, Rolf Lewensohn, Zhongqun Yan, Xiaoyan Yang, Marie Henriksson, Elisabeth Blennow, Magnus Nordenskjöld, Anders Zetterberg, Magnus Björkholm, Astrid Gruber, and Dawei Xu[3]

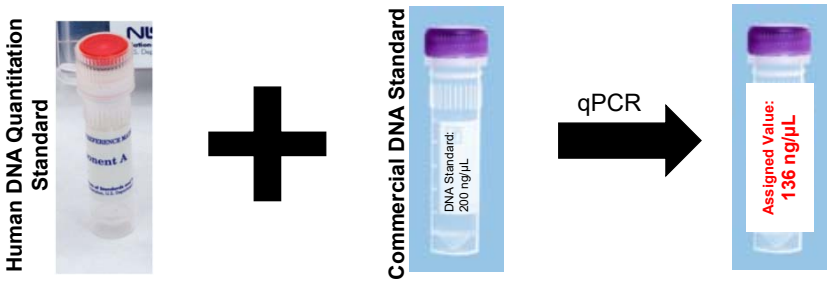
Practical Effect

Goal of 1:1 mixture

2.5x difference



What can laboratories do to ensure more accurate quantitation results?



Standard Curve | **"Unknown"** | **Reassign Commercial DNA Standard**

Proper Use of SRM 2372a

STANDARD CURVE

Dilution	µL TE^{-4}	µL DNA	A [DNA] ng/µL
neat	0	10	57
1→5	16	4	11.4
1→5	16	4	2.28
1→2	10	10	1.14
1→2	10	10	0.57
1→2	10	10	0.28
1→2	10	10	0.14

qPCR Assay

Calculate and assign the NEW concentration to the commercial DNA Standard

This is not necessarily what is written on the tube by the manufacturer

DILUTION OF "UNKNOWN"

Diluted sample	Serial Dilution
Unk 1	1→10
Unk 2	1→5
Unk 3	1→2
Unk 4	1→2

qPCR Assay

Assigning a value to your material

Diluted sample	Serial Dilution	qPCR Result ng/µL	Std dev ng/µL	Dilution Factor	[DNA] ng/µL	Std dev ng/µL
Unk 1	1→10	12.6	0.58	x10	126	5.8
Unk 2	1→5	2.9	0.02	x50	145	0.8
Unk 3	1→2	1.4	0.01	x100	138	0.5
Unk 4	1→2	0.7	0.02	x200	137	3.9

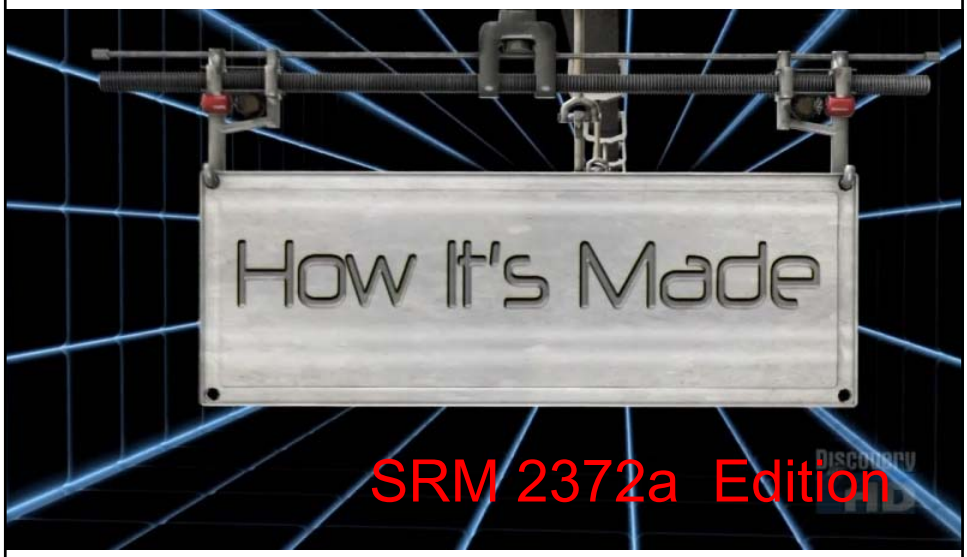
Newly assigned value to your daily use calibrant is the mean of the [DNA] column
= 136 ng/µL

When using this calibrant in the future, the starting concentration will be 136 ng/µL

Concentration of commercial DNA standards needs to be **performed with each lot** of material

Conclusions

- Multiple sources of bias exist in qPCR, some of which cannot be remediated
  - Bias from commercial DNA standards can be remediated with calibration to SRM 2372a
  - Additionally, SRM 2372a may aid in identifying forms of bias within qPCR technologies during internal validation within laboratories
- SRM 2372a should be used to make an outside material NIST Traceable for everyday use within a laboratory to limit the bias between commercial DNA standards
- It is important to keep in mind that using **DNA quantitation as a gate keeper** is impacted by new qPCR targets and STR kit PCR buffer formulations
  - Insensitive qPCR assays or inaccurate DNA standards may not accurately reflect the ability of new, more sensitive STR kits to obtain results



SRM 2372a: Human DNA Quantitation Standard

NIST

Date of Issue:
13 March 2018

Standard Reference Material® 2372a
Human DNA Quantitation Standard
CERTIFICATE OF ANALYSIS

To be used as a qPCR calibrant
OR to assign a value to a 'pot' of DNA – in house or commercial

Table 1. Certified Values of Number and Mass Concentration for SRM 2372a[a]
The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume. The DNA mass concentration values are metrologically traceable to the natural units count and ratio 1 and SI derived units of mass and volume.

Component	Copy Number[b] (per nL)	DNA[c] (ng/µL)
A (red cap)	15.1 ± 1.5	49.8 ± 5.0
B (white cap)	17.5 ± 1.8	57.8 ± 5.8
C (blue cap)	14.5 ± 1.5	47.9 ± 4.8

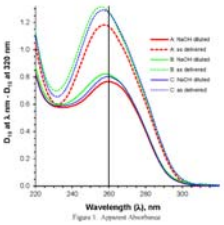
Genomic DNAs from blood

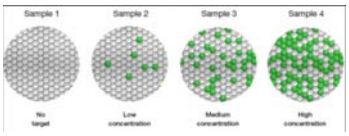
SRM 2372a became available for purchase March 26, 2018

What has changed with SRM 2372a?

UV Absorbance

Digital PCR (dPCR)





How did we get here?

Steps to Develop SRM 2372a

Development and validation of digital PCR assays and platforms → File paperwork with Human Subjects (MTA, SRI, Determination) → With approval, buffy coat samples are ordered and extracted manually

↓

Preliminary screening of bulk material, UV measurements, dilution to target concentration → Bottling and labeling → Homogeneity Testing, Certification, and Stability Studies performed

↓

Report of Analysis, NIST Special Publication, Certificate of Analysis complete and approved

Why use dPCR for certification?

- No need for an external calibrant

- Multiple dPCR assays can be used for characterization
  - Establish reasonable estimates of uncertainty

- More accurate form of concentration measurement for end user

dPCR platforms at NIST

Droplet Digital - ddPCR

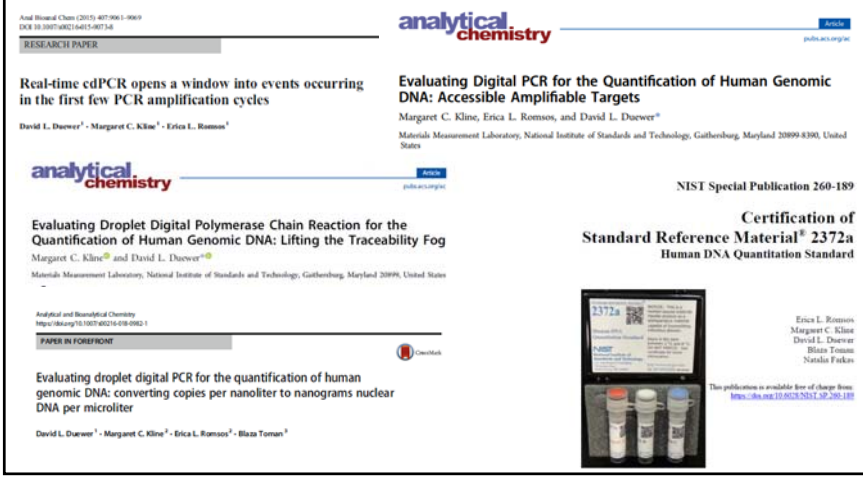
BIO-RAD QX200

Chamber Digital - cdPCR

Fluidigm BioMark

“Absolute” Quantitation at NIST

NIST has developed and optimized >10 dPCR assays for absolute quantitation



RESEARCH PAPER

Real-time cdPCR opens a window into events occurring in the first few PCR amplification cycles

David L. Duewer[1] · Margaret C. Kline[1] · Erica L. Romsos[1]

analytical chemistry

Evaluating Digital PCR for the Quantification of Human Genomic DNA: Accessible Amplifiable Targets

Margaret C. Kline, Erica L. Romsos, and David L. Duewer*

Materials Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899-8300, United States

analytical chemistry

Evaluating Droplet Digital Polymerase Chain Reaction for the Quantification of Human Genomic DNA: Lifting the Traceability Fog

Margaret C. Kline and David L. Duewer*

Materials Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, United States

PAPER IN FOREFRONT

Evaluating droplet digital PCR for the quantification of human genomic DNA: converting copies per nanoliter to nanograms nuclear DNA per microliter

David L. Duewer[1] · Margaret C. Kline[2] · Erica L. Romsos[2] · Blaza Toman[3]

NIST Special Publication 260-189

Certification of Standard Reference Material® 2372a

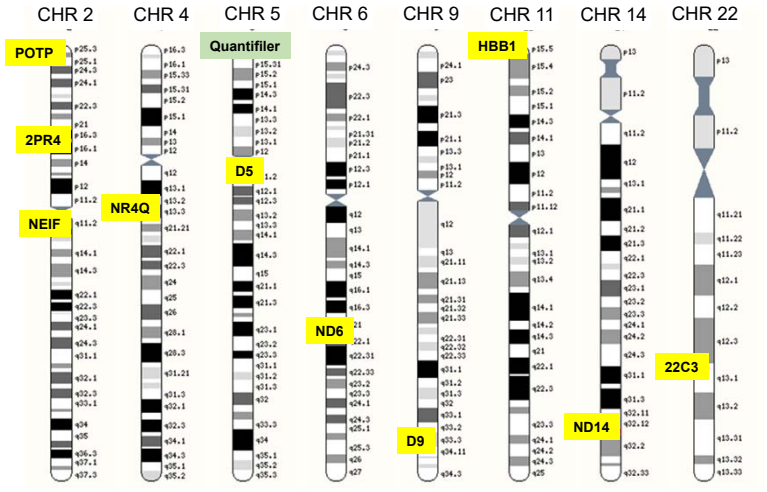
Human DNA Quantitation Standard

Erica L. Romsos
Margaret C. Kline
David L. Duewer
Blaza Toman
Natalia Farkas

Optimized Assays for SRM 2372a

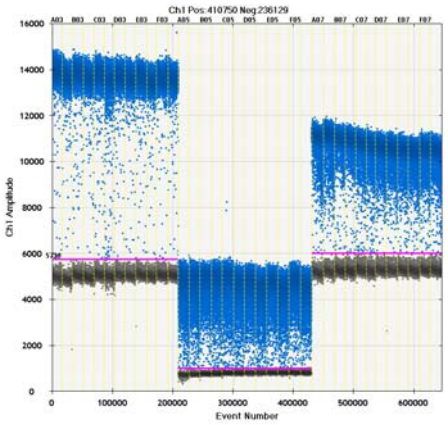
10 assays across 8 different chromosomes

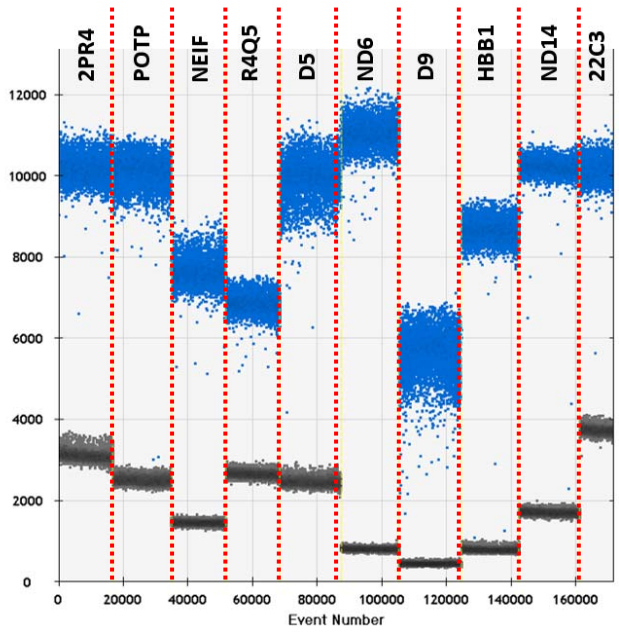
All assays are single copy, and Human, or Primate specific



Importance Assay Design

- Single copy target assays only (for Abs quant)
  - NCBI BLAST search to assess genomic targets
- Not expecting all assays designed to give the same target number (genome accessibility)





Converting copies per nanoliter to nanograms nuclear DNA per microliter

This allows for SRM 2372a to be certified for ng/µL

$$[\text{nDNA}]\frac{\text{ng}}{\mu\text{L}} = \left(\frac{\lambda \text{ copies of target}}{\text{droplet}}\right)\left(\frac{\mu\text{L mixture}}{F\ \mu\text{L sample}}\right)\left(\frac{\text{droplet}}{V\ \text{mixture}}\right)\left(\frac{\text{HHGE}}{r\ \text{target}}\right)\left(\frac{n \text{ base pairs}}{\text{HHGE}}\right)\left(\frac{\overline{w}\,\text{g}}{\text{mol base pairs}}\right)\left(\frac{\text{mol base pairs}}{6.022\ 10^{23}\ \text{base pairs}}\right)\left(\frac{10^3\ \text{nL}}{\mu\text{L}}\right)\left(\frac{10^9\ \text{ng}}{\text{g}}\right) \quad (1)$$

where r is the number of assay targets per human haploid genome equivalents (HHGE), n is the number of nucleotide base pairs (bp) per double-stranded HHGE, and $\bar{w}$ is the average molar mass of a bp in the DNA polymer.

For independent multiplicative factors such as these, the combined relative uncertainty of their product can be estimated from the square root of the sum-of-squares of the individual relative uncertainties [14, Section 5.1.6]:

$$\frac{u([\text{nDNA}])}{[\text{nDNA}]} = \sqrt{\left(\frac{u(\lambda)}{\lambda}\right)^2 + \left(\frac{u(F)}{F}\right)^2 + \left(\frac{u(V)}{V}\right)^2 + \left(\frac{u(r)}{r}\right)^2 + \left(\frac{u(n)}{n}\right)^2 + \left(\frac{u(\overline{w})}{\overline{w}}\right)^2} \quad (2)$$

Duewer *DL, Kline MC, Romsos EL, Toman B. Anal Bioanal Chem. 2018 May;410(12):2879-2887*

Converting copies per nanoliter to nanograms nuclear DNA per microliter

This allows for SRM 2372a to be certified for ng/µL

Copies per nanoliter

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

Table 1. Certified Values of Number and Mass Concentration for SRM 2372a[a]

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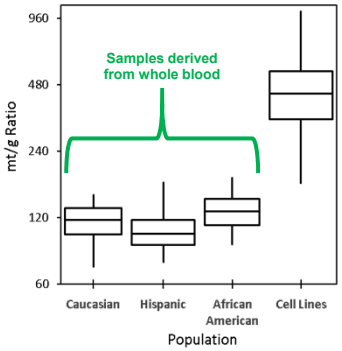
Duewer *DL, Kline MC, Romsos EL, Toman B. Anal Bioanal Chem. 2018 May;410(12):2879-2887*

Mitochondrial DNA Quantification

- Challenging to create a commutable standard
  - Degradation of plasmids
  - Contamination
  - Inefficient amplification
  - Cell line vs. genomic DNA

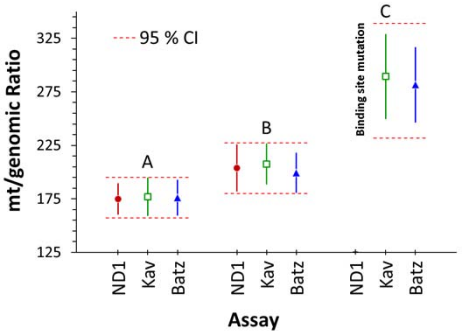


Population (US)	n	Mean (mtDNA/gDNA)	SD (mtDNA/gDNA)
Caucasian	27	115	22
Hispanic	30	106	22
African American	26	130	22
Cell Lines	30	457	176

Mitochondrial DNA Quantification

Mitochondrial to genomic DNA ratio information included in SRM 2372a

Optimized 3 qPCR assays into digital PCR assays



Component	mtDNA/nDNA
A (red cap)	174 ± 4
B (white cap)	206 ± 5
C (blue cap)	279 ± 7

SRM 2372a provides the ratio of mtDNA to gDNA

Steps to Develop SRM 2372a

Development and validation of digital PCR assays and platforms → File paperwork with Human Subjects (MTA, SRI, Determination) → With approval, buffy coat samples are ordered and extracted manually

↓

Preliminary screening of bulk material, UV measurements, dilution to target concentration → Bottling and labeling → Homogeneity Testing, Certification, and Stability Studies performed

↓

Report of Analysis, NIST Special Publication, Certificate of Analysis complete and approved

Before Beginning SRM 2372a

There was Human Subject Protections paperwork

Excluded Human Data/Specimens Form

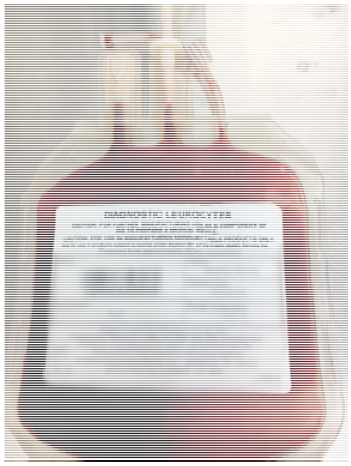
Complete this form when your research study fits into one of the categories below **and** this is the primary use of the specimens and/or data. The form should be routed through your OU for approval and submitted to the HSPO for acknowledgement and tracking before beginning the work on this study.

MML-16-0045-EXCL

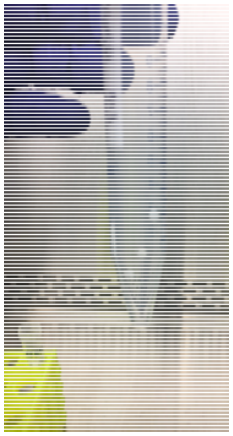
And approval

The HSPO has received your proposed project using only excluded specimens and/or data that meet the criteria for *not human subjects research* as defined in Department of Commerce Regulations, 15 CFR 27, also known as the Common Rule (45 CFR 46, Subpart A), for the Protection of Human Subjects. As indicated in your documentation, these specimens and/or data are from 1) deceased individual(s), 2) established cell lines, 3) human embryonic stem cells from the NIH hESC registry, and/or 4) derivatives of material originally obtained from humans and do not contain information identifying the subjects providing the specimens associated with the data. This determination is valid only for this project. You are responsible for conducting this project as outlined in the above documents. This project may proceed with no further requirement for review by the HSPO, but may require other agreements (MOU, MTA, DUA etc.), grant, contract, RACO (IAA) and/or OU approvals before your project may begin. In the event that there is a change to the above-described project that may affect this determination status, send a description of the change to the HSPO. The HSPO will re-evaluate the project, if necessary.

Acquiring Materials and Extraction

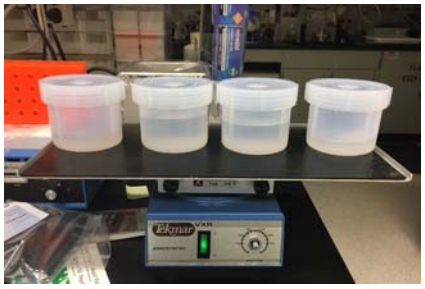


Buffy coat samples were purchased from a blood bank



DNA was extracted using a manual method for high quality and high yield

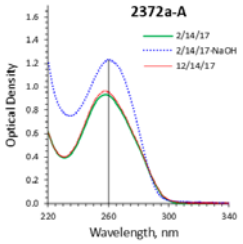
The beginning stages



DNA was allowed to solubilize and reconstitute in Teflon pots in TE^{-4}

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

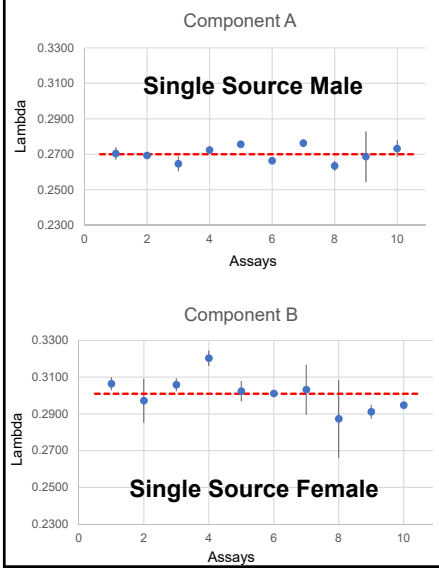


Preliminary screening and UV measurements

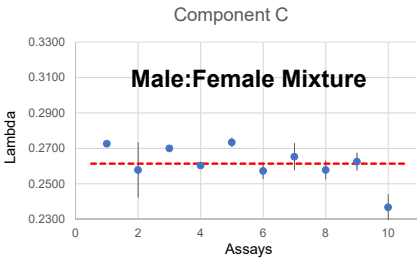
The beginning stages cont.

- UV measurements were made on the bulk pots of the four DNAs
  - DNA were diluted to ~50 ng/µL
- Component C mixture was gravimetrically prepared as a 1→3 mixture (Male:Female)
  - Preliminary testing was repeated
- Once material is proven to behave as expected with ddPCR the bottling process begins

ddPCR Preliminary Screening



Screening 10 human genomic assays with candidate materials for SRM 2372a



All samples behave as expected with human dPCR assays

Bottling and Labeling Process

Ready for bottling

Labelers check the caps, add a SRM label, and verify volume of product.

Completed units for testing

Homogeneity, stability and certification

2100 units of each component produced

Steps to Develop SRM 2372a

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Report of Analysis, NIST Special Publication, Certificate of Analysis complete and approved

SRM 2372a became available for purchase March 26, 2018

Homogeneity and Stability

- Homogeneity: 22 vials in duplicate with 2 assays

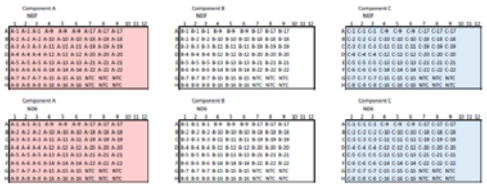


Figure 4: Plate Layout Design for Homogeneity Measurements

	Within-Tube		
Component	NEIF	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:
  - 3 temperatures (4 °C, 22 °C, 37 °C)
  - Two vials in duplicate with 2 assays

Certification Measurements

- Once materials were determined to be homogeneous and stable certification measurements were made

		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

10 genomic DNA assays and 3 mitochondrial DNA assays
6 independent measurements

Value Assignment

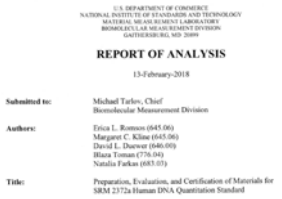
- Data from the homogeneity, stability, and certification measurements were compiled for value assignment
  - Uncertainty assigned to all measurements

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

Paperwork Stage

Internal Report of Analysis



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NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY
MATERIAL MEASUREMENT LABORATORY
BIOMOLECULAR MEASUREMENT DIVISION
GAITHERSBURG, MD 20899

REPORT OF ANALYSIS

13-February-2018

Submitted to: Michael Tarlov, Chief
Biomolecular Measurement Division

Authors: Erica L. Romsos (645.06)
Margaret C. Kline (645.06)
David L. Duewer (646.00)
Blaza Toman (776.04)
Natalia Farkas (683.03)

Title: Preparation, Evaluation, and Certification of Materials for SRM 2372a Human DNA Quantitation Standard

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REPORT OF ANALYSIS

14-February-2018

Submitted to: Michael Tarlov, Chief
Biomolecular Measurement Division

Authors: Margaret C. Kline (645.06)
Erica L. Romsos (645.06)
David L. Duewer (646.00)

Title: Qualification of SRM 2372a materials for certification with droplet digital Polymerase Chain Reaction (ddPCR).

NIST Special Publication 260-189

This details the production, evaluation, and certification measurements for SRM 2372a

NIST Special Publication 260-189

Certification of Standard Reference Material® 2372a
Human DNA Quantitation Standard

Erica L. Romsos
Margaret C. Kline
David L. Duewer
Blaza Toman
Natalia Farkas

This publication is available free of charge from:
https://doi.org/10.6028/NIST.SP.260-189

NIST

Date of Issue:
13 March 2018

Standard Reference Material® 2372a
Human DNA Quantitation Standard
CERTIFICATE OF ANALYSIS

Purpose: This Standard Reference Material (SRM) is intended for use in the value assignment of human genomic deoxyribonucleic acid (DNA) quantitation materials, primarily those used for quantitative polymerase chain reaction (qPCR).

Description: A unit of SRM 2372a consists of three well-characterized human genomic DNA materials in pH 8.0 aqueous buffer. The components are derived from human buffy coat samples and labeled A, B, and C. Com
A consists of genomic DNA from a single male donor. Component B consists of genomic DN
donor. Component C consists of a gravimetric mixture of genomic DNA (1
A unit of the SRM consists of one sterile 0.5 mL vial of
of DNA solution. Each of these vials is lab
cap.

SRM 2372a became available for purchase March 26, 2018

Certified V
able 1. These certified values were determined based on droplet
CR) assay counts of ten unique targets on eight chromosomes, dilution factors,
measurements. A NIST certified value is a value for which NIST has the highest confidence in
all known or suspected sources of bias have been accounted for.

Table 1. Certified Values of Number and Mass Concentration for SRM 2372a[a]
The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume. The DNA mass concentration values are metrologically traceable to the natural units count and ratio 1 and SI derived units of mass and volume.

Component	Copy Number[b] (per nL)	DNA[c] (ng/µL)
A (red cap)	15.1 ± 1.5	49.8 ± 5.0
B (white cap)	17.5 ± 1.8	57.8 ± 5.8
C (blue cap)	14.5 ± 1.5	47.9 ± 4.8

NIST

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Standard Reference Material® 2372a
Human DNA Quantitation Standard
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Component	mtDNA/nDNA
A (red cap)	174 ± 4
B (white cap)	206 ± 5
C (blue cap)	279 ± 7

SRM 2372a provides the ratio of mtDNA to gDNA

NIST Special Publication 260-189

Certification of
Standard Reference Material® 2372a
Human DNA Quantitation Standard

Digital PCR at NIST

- Digital PCR has become our 'go to' method for the quantification of nucleic acid-based materials
- Replacing UV spectroscopy (indirect method)
- The typical downstream application of our ***reference materials*** is PCR or sequencing-based

  We care about ***intact (and accessible) genomic targets***
- SRM 2372a provides a **certified value for DNA concentration in ng/µL**
  - **mtDNA/nDNA ratio** now provided

Acknowledgments

NIST Team for This Work


Margaret Kline


Dave Duewer


Blaza Toman


Pete Vallone

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