SRM 2372: How the Human DNA Quantitation Standard was Characterized at NIST and How it Can be Used to Calibrate qPCR Measurements in Your Laboratory

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2007 MAAFS Meeting May 23, 2007, Washington, DC

Disclaimers

Funding: Interagency Agreement 2003-IJ-R-029 between the National Institute of Justice and NIST Office of Law Enforcement Standards

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Our publications and presentations are made available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

SRM 2372
Human DNA Quantitation Standard

Components

A: Male/single donor/RNased/NIST
B: Female/multiple donors/NIST
C: Mixture/male & female/commercial

Quantities supplied:
110 µL of Human Genomic DNA ≈ 50ng/µL

Certification

Decadic Attenuance (**Absorbance**) by a US National Reference Spectrophotometer
Homogeneity by a Cary 100 Bio Spectrophotometer
Validation of conventional [DNA] by Interlaboratory Study and NIST qPCR studies.


National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2372

Human DNA Quantitation Standard

The paperwork is progressing!

The Statistician is reviewing the data.

We are going down the NIST checklist for a Certified Reference Material (aka SRM)

Requirements for NIST SRM 2372 Human DNA Quantitation Standard

Material must be fit for purpose:

- **Homogeneity** — Tested Random Samples
  - All tubes are the same
- **Stability** — Sarstedt Tubes (2.0 mL)
  - Will withstand shipping and normal storage
- **Recoverability** — Interlaboratory Study & Tube Study
  - What went in the tubes comes out
- **Traceability** — Analysis by Reference Spectrophotometer
  - Values assigned are traceable to the designated certification method.

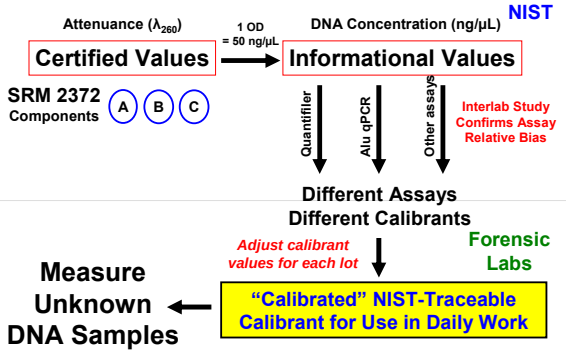
Selection of DNA Sources

- Based on previous work, more consistent DNA calibration solutions are obtained from human Buffy coats than from cell lines.

Presented at 58th Annual AAFS meeting:
http://www.cstl.nist.gov/biotech/strbase/pub_pres/Vallone_AAFS2006_qPCR.pdf

Examining Candidate DNA Quantitation Standards with Real-Time Quantitative PCR Assays
Peter M. Vallone, Margaret C. Kline, Amy E. Decker, David L. Duewer, and John M. Butler
February 23,2006 58th Annual AAFS Meeting Seattle,WA

Overview of SRM 2372 Values and Use



Component Preparation

- Components A and B were prepared from Buffy coats at NIST using a modified "Salt out" procedure of Miller et al. (1988).
- After the initial extraction and EtOH ppt, the material was re-extracted to assure purity.
- Additionally Component A was treated with RNase prior to the second extraction.

Miller, S.A., Dykes, D.D., and Polesky, H.F. (1988) Nucleic Acids Res. 16 (3) p. 1215.

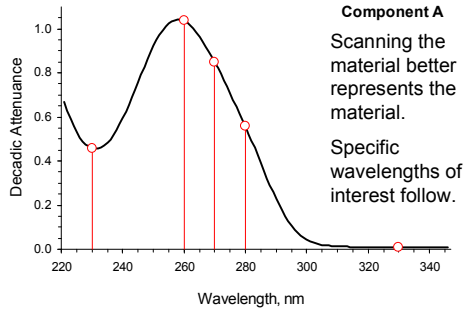
Isolated DNA of Component B in Ethanol

DNA extracted from Buffy coats from anonymous female donors. The image is the re-extracted DNA in the final ethanol wash. The extracted DNA is floating toward the top of the 50 mL polypropylene centrifuge tube.

Component Preparation

- All components were solubilized from an air dried state in TE^{-4} buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) that had been autoclaved.
- Volume prepared was from 210 mL to 250 mL of each component in Teflon containers.
- Materials were allowed to equilibrate several days prior to initial [DNA] determination by scanning from A_{345} to A_{220} and determining A_{260}.

Preliminary Scan of Candidate SRM Material



Scanning the material better represents the material.

Specific wavelengths of interest follow.

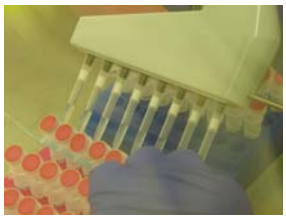
Spectrophotometric Determination

- 260 nm & 280 nm readings
- 260 nm allows calculation of the conventional [DNA]
- OD = 1 ≈ 50 µg/mL dsDNA
  ≈ 40 µg/mL ssDNA
  ≈ 33 µg/mL oligos
- 260 / 280 ratio ≈ 1.8 to 2.0
  (Provides an estimate of contaminating protein)

Additional Wavelengths:

- 230 nm significant absorbance indicates:
  - Phenolate ion
  - Thiocyanates
  - And other organic compounds
- 270 nm
  - Water saturated with phenols absorbs
  - 260:270 ~ 1.2 indicates preparation free of phenol
    Stulnig and Amberger 1994 BioTechniques;16:403-404
- 330 nm and higher absorbance
  - Caused by light scattering indicating presence of particulate matter

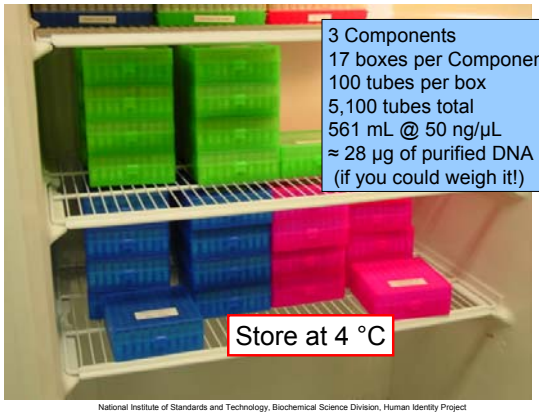
Bottling SRM 2372 Materials



Teflon container holding ≈ 250 mL of Candidate SRM 2372. *It's not an SRM until it passes all testing.*

With a multi-channel pipettor 8 tubes can be filled at a time. That's ≈ 214 reps to fill 1700 tubes per component.

The assembly line closing the recently filled tubes



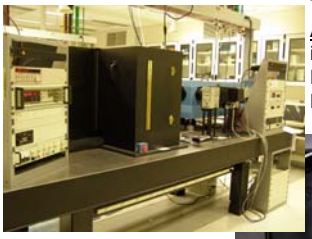
The Blister Brigade

Closing the component tubes caused some blisters even while wearing gloves. Band-aids applied prior to closing SRM tubes the next session helped reduce the number of blisters formed!

Calibration of Spectrophotometers

- Spectrophotometers were calibrated prior to any absorbance readings.
  - SRM 2031 Metal-on-Quartz Filters
    - Verification of transmittance and absorbance scales
  - SRM 2034 Holmium Oxide Solution Wavelength from 240 nm to 650 nm
    - Verification and calibration of wavelength scales

US National Spectrophotometer HAS II



This 2nd generation High Accuracy Spectrophotometer is one of two National Level Reference Instruments at NIST

HAS II Certified Values of Decadic Attenuance for SRM 2372

5 mL were required to fill 2 cuvettes per component, each run in duplicate (4 replicate measurements). Two vials from each component box were pooled for these measurements.

Component	λ_{260}	λ_{260} uncertainty
A	1.049	± 0.0xx
B	1.073	± 0.0xx
C	1.086	± 0.0xx

Nominal DNA Concentrations

Using 1 OD = 50 ng/µL double stranded DNA.
(We do not know the uncertainty in this conversion.)

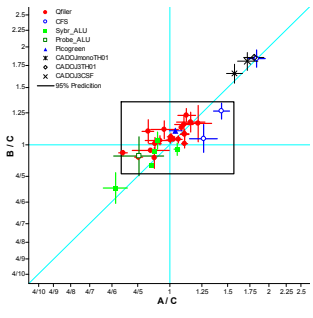
Informational Values

Component	Nominal [DNA], ng/µL
A	52.5
B	53.6
C	54.3

Interlaboratory Study

- 32 laboratories participated
- This limited study was advertised at the NIJ Grantees meeting, June of 2006
- All laboratories provided data (Thank You!)
- Net result of the study: the SRM materials are appropriate for use with different qPCR methods

Interlaboratory Data



While the Interlaboratory data for Component B looks good, it failed homogeneity testing resulting in all 1700 vials being **disposed** of and a **new lot** of Component B was produced.

All labs participating in this Interlaboratory study should discard component B if they have any remaining.

Original Component B Failed Homogeneity Testing

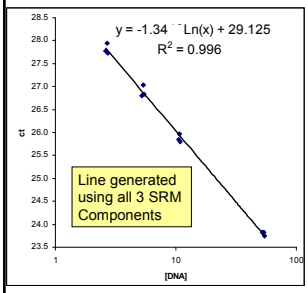
- Higher variability of the Cary UV measurements were seen for the original Component B material.
- Close inspection of the ≈ 1700 tubes reveal particulate matter in too many of the units.
- Original Component B units were discarded and a new material was produced.

Variability of the Homogeneity UV measurements

nm	A	B_old	C	B_new
230	1.0 %	11 %	0.5 %	1.2 %
260	0.4 %	10 %	0.3 %	0.8 %
270	0.5 %	11 %	0.3 %	0.9 %
280	0.6 %	11 %	0.3 %	0.9 %

* **%** Variability at the different wavelengths during homogeneity testing, this is why the 1st lot of B failed.

qPCR testing of SRM 2372



Quantifiler Human Kit was used in conjunction with a 7900 instrument.

Results from two runs were combined to produce the following results for 17 boxes of each component.

n = 34	Ct	sd	%
A_boxes	26.95	**0.09**	0.3
B_boxes	27.02	**0.10**	0.4
C_boxes	26.90	**0.10**	0.4

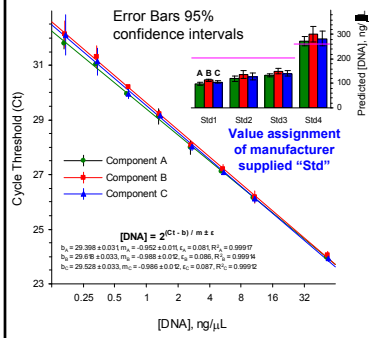
So how will you use this SRM?

- You are going to calibrate your materials and make them NIST Traceable by using SRM 2372.
- How?
  - By analyzing **your materials** with **your DNA Quantification Methods** and assigning a [DNA] based on the values obtained using SRM 2372 materials to generate your standard curve.

Examples of Value Assignment

- We had 4 different commercial materials that can be used for qPCR calibration.
- Serial dilutions of these materials were made: 1:10, 1:5, 1:2, and 1:2
- The SRM components were used as the calibration standards.
- All samples and standards were analyzed in duplicate.

Qfiler Assay



With qPCR, 100% reaction efficiency results in a doubling of the DNA target each cycle. Using $\log_2$ the "perfect" fit regression slope would be -1.0 Ct per $\log_2$.

	Slope	R^2
A	-0.952	0.999
B	-0.988	0.999
C	-0.986	0.999

Quantifiler Human results: value assignment

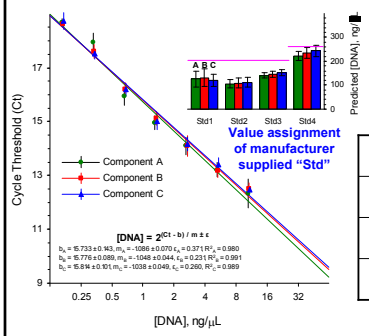
Dilution	1 [DNA]	SD	2 [DNA]	SD	3 [DNA]	SD	4 [DNA]	SD
1:10	**105**	3.2	**122**	1.0	**126**	5.8	**256**	10.1
1:5	**105**	3.3	**122**	7.3	**145**	0.8	**272**	7.8
1:2	**99**	6.2	**113**	11.6	**138**	0.5	**270**	10.5
1:2	**100**	1.7	**137**	18.5	**137**	3.9	**311**	3.7

n=8

Assigned value	102	123	136	277
% deviation from stated	-49%	-38%	-32%	6%

These are the assigned [DNA] of the unknowns for this assay in our hands.
YOU need to perform this analysis for YOUR assays.

Alu SybrGreen assay



Error Bars 95% confidence intervals

	Slope	R^2
A	-1.086	0.980
B	-1.048	0.991
C	-1.038	0.989

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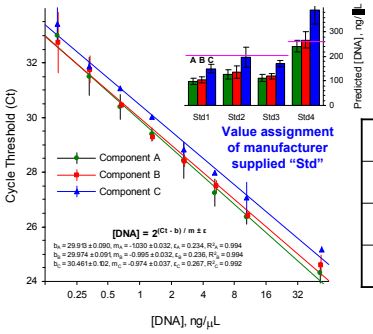
CFS (Th01 Probe) Assay



Error Bars 95% confidence intervals

	Slope	R^2
A	-1.030	0.994
B	-0.995	0.994
C	-0.974	0.992

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Use of SRM 2372

- DO NOT freeze
  - Store at 4 °C. We know it is stable at this temperature
  - We will continue tracking stability
- DO NOT aliquot into new containers
  - We know about our containers; we do not know about yours
- Use to calibrate commercial and in-house materials
  - **Not intended for daily use!**
  - Intended for **you** to evaluate each new calibrant lot

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Thank you for your Attention!!

Acknowledgments

Funding:

Interagency Agreement between National Institute of Justice and NIST Office of Law Enforcement Standards

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