SRM 2372: Past, Present, and Future

Erica Butts, Margaret Kline, and David Duewer

National Institute of Standards and Technology

Mid-Atlantic Association of Forensic Scientists
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NIST

Outline

- What is SRM 2372 and why is it used?
- What happened in 2012?
- Making the next generation of 2372
  - Digital PCR

What is SRM 2372 Human DNA Quantitation Standard?

Genomic DNA prepared to be double-stranded DNA (dsDNA)

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

All solubilized in TE^{-4} buffer (10mM Tris, 0.1 mM EDTA, pH 8.0)

Certified for spectroscopic traceability in units of decadic attenuance, D_{10}. The D_{10} scale is a measure of absorbance and is traceable to the unit 1.

The conventional conversion factor for aqueous DNA:
dsDNA 1.0 D_{10} at 260 nm = 50 ng/µL DNA
ssDNA 1.0 D_{10} at 260 nm = 37 ng/µL DNA

SRM 2372 Certificate of Analysis

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2372

Human DNA Quantitation Standard

First released October 2007

DNA was certified in the double-stranded form for UV absorbance

Temporarily taken off of the market March 2012

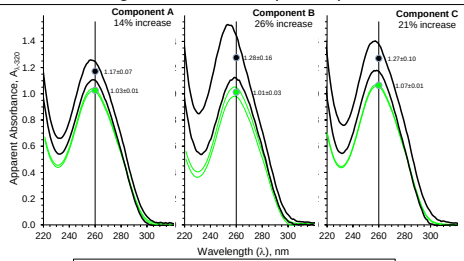
Why was SRM 2372 Taken off the Market in 2012?

- During measurement of the DNA samples to verify stability of certified values the UV absorbance values for the sample increased
  - Not due to degradation of the DNA
  - Due to unraveling or opening up of the DNA strands in the TE^{-4} Buffer
    - Single-stranded DNA absorbs more UV light than double-stranded DNA
  - SRM 2372 is certified for UV absorbance

The changes over time which impacted the UV absorbance, did not appear to affect the qPCR performance

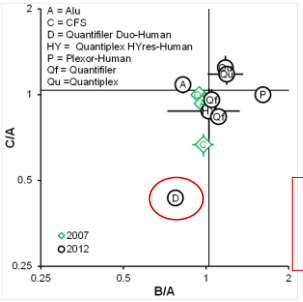
Why did SRM 2372 need to be re-certified?

Six years after production the D_{10} absorbance of these dsDNA solutions had *increased* significantly, suggesting partial conversion to single-stranded DNA (ssDNA)



Green Traces 2006 low/high absorbance spectra
Black Traces 2012 low/high absorbance spectra

Does ssDNA vs dsDNA affect qPCR?



No

The **green diamonds represent assays performed in 2007** while the black circles represent the current results.

Quantifiler Duo was released *AFTER* 2007 release of SRM 2372

We've previously noted the difference seen with component C and Quant Duo
http://www.cstl.nist.gov/strbase/pub_pres/Promega2009poster.pdf

How did we re-certify SRM 2372?

- Material was forced to all ssDNA conformation
- Measurements were made using a modification of ISO 21571 Annex B "Methods for the quantitation of the extracted DNA"
  - Combine equal volumes of the DNA extract and freshly prepared 0.4 mol/L NaOH
  - Measure against equal volumes of TE^{-4} buffer and the 0.4 mol/L NaOH
- Apparent Absorbance is $D_{10\ (260\ nm)} - D_{10\ (320\ nm)}$

Component A	Component B	Component C
0.777 (0.725 – 0.829)	0.821 (0.739 – 0.903)	0.804 (0.753 – 0.855)

Conversion of Apparent Absorbance to ng/µL

- Assertion that a solution of ssDNA with an absorbance of 1.0 at 260 nm and a pathlength of 1.0 cm has a DNA mass concentration of 37 µg/mL (37 ng/µL)

Parameter	A	B	C
2012 DNA Mass Concentration	57	61	59
2007 DNA Mass Concentration	52.4	53.6	54.3
Theoretical difference, %	9 %	14 %	9 %
Theoretical difference, Ct	0.12 cycle	0.19 cycle	0.12 cycle

The difference between the original value and re-certified values is within the noise of the assay.

SRM 2372 went back on sale December 31, 2012

The Plan for the Next Generation

SRM 2372a

Digital PCR (dPCR) Overview

- A sample is partitioned so that individual nucleic acid targets within the sample are localized
  - Microfluidic (Fluidigm BioMark)
  - Emulsion/droplet PCR (Bio-Rad QX100, RainDance)
- Each partition will contain a negative or positive PCR reaction
- Nucleic acid targets may be quantified by counting the regions that contain PCR end-product
  - a standard curve is not required

- Sykes, PJ et al. AA (1992) "Quantitation of targets for PCR by use of limiting dilution". Biotechniques 13 (3): 444–449
- Kalinina, O et al. (1997) "Nanoliter scale PCR with TaqMan detection". Nucleic Acids Research 25 (10): 1999–2004
- Vogelstein and Kinzler (1999) "Digital PCR". Proc Natl Acad Sci U S A. 96 (16): 9236–9241
- Pohl and Shih (2004) "Principle and applications of digital PCR". Expert Rev Mol Diagn 4 (1): 41–47
- Dressman et al. (2003). "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations". Proc Natl Acad Sci USA 100 (15): 8817–8822

Instruments available for dPCR at NIST

Chamber Digital - cdPCR

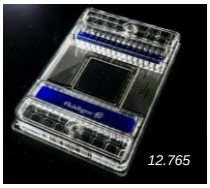
Droplet Digital - ddPCR

Fluidigm BioMark

BIO-RAD QX100

Fluidigm BioMark

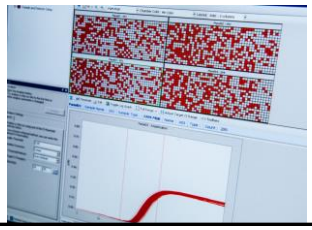
Chamber Digital PCR



- Fluidic module transfers PCR mastermix onto chip
- 'Reader' performs thermal cycling and fluorescence detection (real-time PCR)

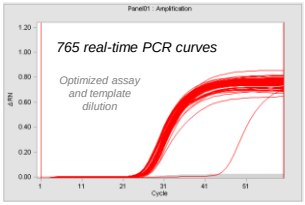
Fluidigm Digital Array

12.765 = 765 chambers × 12 panels (samples)
48.770 = 770 chambers × 48 panels (samples)



- Well volumes
  6 nL (12 sample)
  0.85 nL (48 samples)
- TaqMan compatible chemistry
- FAM-VIC dye detection

Fluorescent signal as a function of amplification cycle in **765 cdPCR reactions**

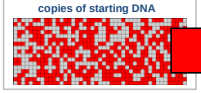


Majority of the wells amplify within a narrow range of C_T values

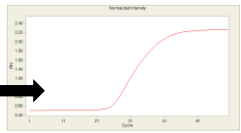
Later amplification may be due to:
Damaged target
Partially blocked target
Secondary binding sites

Grey lines are no amplification

Number of wells with signal relates to the number of copies of starting DNA



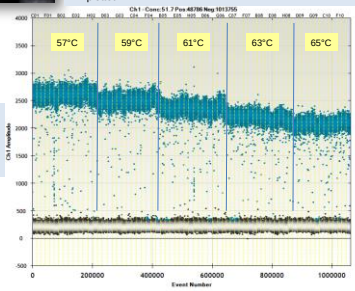
Each red box represents an individual amplification curve



BioRad QX100 (Droplet Digital PCR)

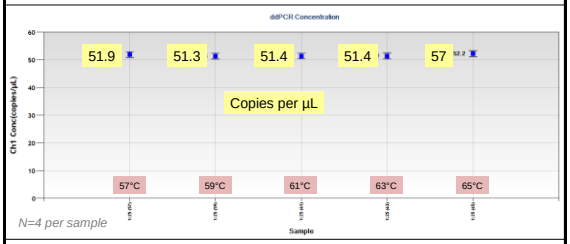
- PCR master mix and DNA template are partitioned into droplets
- 8 strip tubes - up to 96 samples/run
- Thermal cycling is performed on a standard cycler (9700, Veriti)
- Fluorescence from up to 20,000 droplets are detected in the reader (3.5 h)
- Fluorescence intensity for positive and negative droplets are plotted

Validating annealing temperatures for the validation of a digital PCR assay



BioRad QX100

Effect of annealing temperature on copy number estimate



How do we get from positive counts to ng/µL?

255 positive chambers
765 total chambers

$$\text{Copies per microliter of diluted material} = \frac{-\ln\left(1-\frac{\text{total number positives}}{\text{total number of wells}}\right)}{\text{volume of all PCR reactions (microliters)}}$$

$$\text{Copies per microliter of diluted material} = \frac{-\ln\left(1-\frac{255}{765}\right)}{0.006} = 74 \text{ copies/µL}$$

50.7 ng/µL of original starting material

0 dil into reaction
5 dil of stock DNA

$$\text{ng/µL} = \frac{\text{Copies per microliter of diluted material} * \text{Master Mix Dilution} * \text{Original Sample Dilution}}{333 \text{ dsDNA copies/ng}}$$

$$\text{ng/µL} = \frac{74 * 10 * 25}{333 \text{ dsDNA copies/ng}}$$

How will you use SRM 2372a?

Making your material NIST Traceable

- Analyze **your materials** (eg. a standard DNA provided within a commercial quant kit) with **your DNA Quantification Methods** (eg. Quantifiler, Plexor, Quantiplex, etc)
- Assign a [DNA] of **your material** based on the values obtained using SRM 2372a materials to generate your standard curve
  - **Your material** will be the unknowns on the qPCR plate

Example

- 4 different commercial materials (standard DNAs found within commercial qPCR kits)
- The SRM components were used as the calibration standards to generate the standard curve
- All samples (4 commercial standards) and standards (SRM components) were analyzed in duplicate

qPCR plate setup

	1	2	3	4	5	6
A	A_57.0	A_57.0	S1 unknown	S2 unknown	S3 unknown	S4 unknown
B	A_28.5	A_28.5	S1 unknown	S2 unknown	S3 unknown	S4 unknown
C	A_14.3	A_14.3	S1 unknown	S2 unknown	S3 unknown	S3 unknown
D	A_7.1	A_7.1	S1 unknown	S2 unknown	S3 unknown	S4 unknown
E	A_3.6	A_3.6	S1 unknown	S2 unknown	S3 unknown	S4 unknown
F	A_1.8	A_1.8	S1 unknown	S2 unknown	S3 unknown	S4 unknown
G	A_0.9	A_0.9	S1 unknown	S2 unknown	S3 unknown	S4 unknown
H	A_0.4	A_0.4	S1 unknown	S2 Unknown	S3 unknown	S4 unknown

Dilution of commercial DNAs to make NIST Traceable for qPCR

SRM 2372a

Commercial DNAs to make NIST Traceable

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

Average of [DNA] across all dilutions

Assigned value	102	123	136	277

These values are now the assigned NIST Traceable concentration of the commercial DNA provided and can be used for generation of future standard curves

Digital PCR (dPCR) as the Next Certification Method

- The next generation of SRM 2372 (SRM 2372a) will be certified for "copy/target number"
- It is important to realize that there is no one human genomic material that will have the same "target number" for all assays; **lots of variability is being discovered at the genome level in terms of copy number variants and chromosomal rearrangements**
  - This is the need for multiple assays for assessment of the next generation of SRM 2372a candidate material
- SRM 2372 and SRM 2372a should be used to make an outside material NIST Traceable for everyday use within a laboratory

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Dave Duewer

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Contact Info:
erica.butts@nist.gov
301-975-5107