

SRM 2372: Past, Present, and Future

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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 attenuation, D₁₀. The D₁₀ scale is a measure of absorbance and is traceable to the unit 1.

The conventional conversion factor for aqueous DNA:
dsDNA 1.0 D₁₀ at 260 nm = 50 ng/μL DNA
ssDNA 1.0 D₁₀ 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**

Standard Reference Material (SRM) 2372 is assigned primarily for use in the value assignment of human genomic deoxyribonucleic acid (DNA) forensic quantitation systems. It is not intended for use in forensic clinical diagnostic use. SRM 2372 consists of three multi-characterized human genomic DNA material solubilized in Tris-EDTA buffer (pH 8.0) containing 10 mM Tris, 0.1 mM EDTA, and 1 mM β-mercaptoethanol. Each decadal unit (decade) of DNA mass concentration is assigned to pH 8.0 Tris-EDTA buffer. The three component genomic DNA materials, labeled A, B, and C, are represented by a single male donor, multiple female donors, and multiple male and female donors. A unit of the SRM consists of one male (component A) and one female (component B) containing approximately 10 μL of DNA solution. Each of these vials is labeled and is sealed with a color-coded screw cap.

Certified Values: Table 1 lists the certified decadic attenuation (D₁₀) values for the three materials in the ultraviolet spectral region for a cell pathlength of 1.0 cm, at a spectral bandwidth of 0.5 nm, and a temperature of 22 °C ± 1 °C. These values are calculated from measured component concentrations (C_A, C_B, C_C) and are indicated by the absorbance scale of the spectrophotometer when the materials are measured against TE⁻⁴ pH 8.0 buffer. The D₁₀ scale is traceable to the unit 1. See "Primary Measurement of Decadic Attenuance" before further information.

A NIST certified value is a value for which NIST has the highest confidence in its accuracy so that all known or expected errors in the use are negligible or accounted for by NIST. The expanded uncertainty defines an interval within which the reference value of attenuation can be expected to lie with a level of confidence of approximately 95%. The expanded uncertainty of these reference values are illustrated by the horizontal segments of spectral error detection and sample heterogeneity. See "Determinations of Expanded Uncertainty" before further information.

Indicative Values: Table 2 lists indicative values for the DNA mass concentration of SRM 2372 components. These values are derived from the conventional conversion factor of double-stranded DNA with an absorbance of 1.0 at 260 nm and a pathlength of 1.0 cm for a DNA mass concentration of 50 ng/μL. (C_A)(C_B)(C_C). An indicative value is considered to be a value that will be close to the SRM mass, but sufficient information is available to assess the uncertainty associated with the value.

Expression of Certification: The certification of SRM 2372 is valid, within the measurement uncertainties indicated, until 31 Dec 2012, upon which the SRM is labeled and issued in accordance with the accompanying green in this certificate (the "Green" and "Transition" to the "Black"). However, the certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Why was SRM 2372 Taken off the Market in 2012?

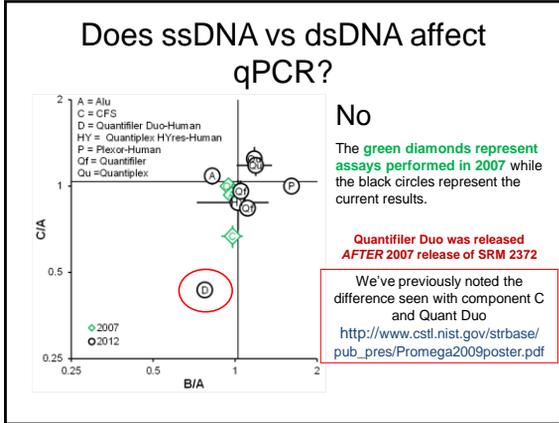
- During measurement of the DNA samples to verify stability of certified values the UV absorbance values for the sample increased
 - Not due to degradation of the DNA
 - Due to unraveling or opening up of the DNA strands in the TE⁻⁴ Buffer
 - Single-stranded DNA absorbs more UV light than double-stranded 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 D₁₀ 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



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 $D_{10}(260\text{ nm}) - D_{10}(320\text{ nm})$

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, P.J et al. AA (1992) "Quantitation of targets for PCR by use of limiting dilution". Biotechniques 13 (3): 444-449
 • Kalinina, O et al. (1997) "Nanometer 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 Shah (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

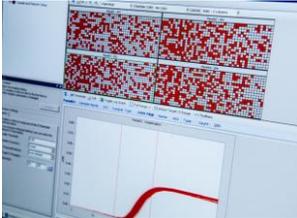


12.765

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

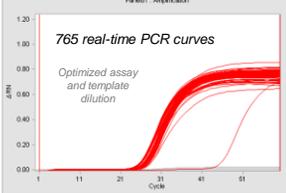
Fluidigm Digital Array

- 12.765 = 765 chambers x 12 panels (samples)
- 48.770 = 770 chambers x 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



765 real-time PCR curves

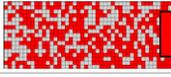
Optimized assay and template dilution

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

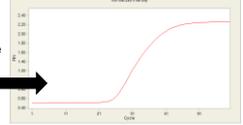
Later amplification may be due to:
Damaged target
Partially blocked target
Secondary binding sites

Grey lines are no amplification

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

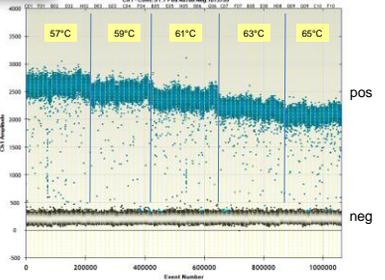


Each red box represents an individual amplification curve



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

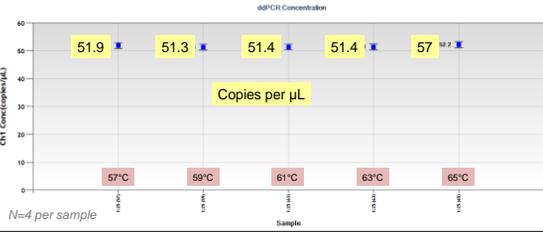
57°C 59°C 61°C 63°C 65°C

pos

neg

BioRad QX100

Effect of annealing temperature on copy number estimate

dPCR Concentration

Ct1 Conc (copies/μL)

51.9 51.3 51.4 51.4 57

Copies per μL

57°C 59°C 61°C 63°C 65°C

N=4 per sample

Sample

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

255 positive chambers
765 total chambers

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

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

50.7 ng/μL of original starting material

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

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

SRM 2372a Commercial DNAs to make NIST Traceable

Dilution of commercial DNAs to make NIST Traceable for qPCR

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
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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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