Recertification of the NIST Standard Reference Material® 2372: Human DNA Quantitation Standard

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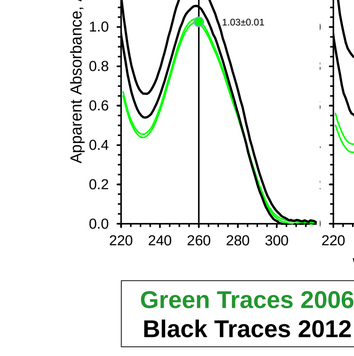
SRM 2372 was designed for use in the value assignment of human genomic DNA forensic quantitation materials. SRM 2372 consists of three human genomic DNA extracts in TE^{-4} buffer, each originally certified to have spectrophotometric absorbance of 1 at 260 nm. However, by five years after production the absorbance in all materials had increased to the point that the certified values were no longer valid. Investigation revealed that the absorbance increases resulted from the slow conversion of double-stranded DNA (dsDNA) to single-stranded (ssDNA). The conventional conversion factor for dsDNA is 50 ng/µL per absorbance unit while that for ssDNA is 37 ng/uL. There was no evidence of any decrease in fragment size or change in behavior of the materials in numerous qPCR assays. Since the materials remain fit for their designed use, the remaining SRM 2372 units have been recertified for the spectroscopic properties of ssDNA. Users interested in the spectroscopic properties of these materials are instructed to force complete conversion to ssDNA with sodium hydroxide (NaOH). Users interested in using the materials to benchmark qPCR assays should use the materials as supplied but note that use of the ssDNA measurement procedure resulted in the "Information values" for conventional mass concentration supplied in the SRM 2372 Certificate changing from ≈50 ng/µL to ≈60 ng/µL.

What is SRM 2372 Human DNA Quantitation Standard?

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



Originally released in September 2007

3 components:

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

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

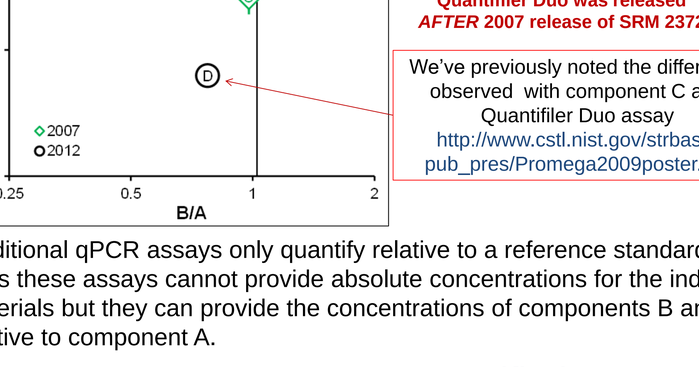
Certified for spectroscopic traceability in units of decadic attenuance, D_{10}.
The D_{10} scale is a measure of absorbance and is traceable to the unit 1.

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

The purpose of SRM 2372 is to allow labs to prepare a NIST traceable calibrant (standard curve) to be used in qPCR assays (Qfiler, Plexor, Quantiplex, etc)
It is not necessarily to be used as a positive control (daily use).
The SRM should be used to assign an accurate value to a lab's internal standard DNA calibrant.

Why did SRM 2372 need to be recertified?

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



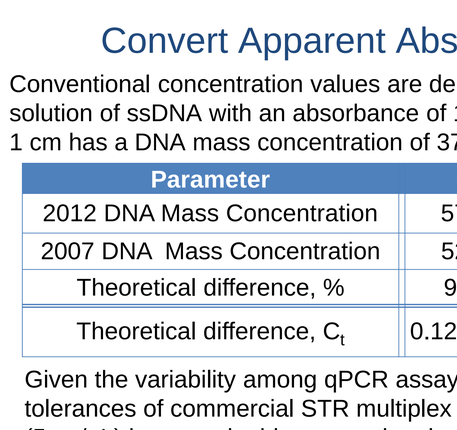
Green Traces 2006 low/high absorbance spectra
Black Traces 2012 low/high absorbance spectra

The increase in absorbance was not due to degradation of the DNA but rather unraveling or opening up of the DNA strands in the TE^{-4} buffer (single-stranded DNA absorbs more UV light than double-stranded DNA)

The sample changes over time that impact UV absorbance do not appear to affect qPCR sample performance (<0.2 C_t)

However, since SRM 2372 is certified for UV absorbance (D_{10}) and one application of this SRM is for calibration of UV spectrophotometers (e.g. nanodrop) the material was restricted from sale until the discrepancy was resolved

The ssDNA vs dsDNA state of the material does not affect qPCR assays



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 observed with component C and Quantifiler Duo assay
http://www.cstl.nist.gov/strbase/pub_pres/Promega2009poster.pdf

Traditional qPCR assays only quantify relative to a reference standard. Thus these assays cannot provide absolute concentrations for the individual materials but they can provide the concentrations of components B and C relative to component A.

SRM 2372 was recertified for single stranded absorbance

The material was converted to an 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 0.4 mol/L NaOH

Measure against a reference of equal volumes of TE^{-4} buffer and the 0.4 mol/L NaOH.

Recertified Apparent Absorbance

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

Convert Apparent Absorbance to ng/µL

Conventional concentration values are derived from the assertion that a solution of ssDNA with an absorbance of 1 at 260 nm and a path length of 1 cm has a DNA mass concentration of 37 µg/mL (37 ng/µL).

Parameter	A	B	C
2012 DNA Mass Concentration	57.5	60.8	59.5
2007 DNA Mass Concentration	52.4	53.6	54.3
Theoretical difference, %	9 %	12 %	9 %
Theoretical difference, C_t	0.12 cycle	0.16 cycle	0.12 cycle

Given the variability among qPCR assays and the rather wide tolerances of commercial STR multiplex assays, the approximately 10% (5 ng/µL) increase in this conventional concentration estimate will have minimal practical impact.

Moving Forward with Digital PCR

The next generation of SRM 2372 will be certified for "copy/target number" not UV absorbance.

Digital PCR will allow for a more direct determination of accessible genomic targets versus an indirect method such as UV absorbance.

It is important to realize that there is no one human genomic material that will have the same "target number" for all assays; **variability is being discovered at the genome level in terms of copy number variants and chromosomal rearrangements.**

What is Digital PCR?

A method to estimate the number of ***accessible amplifiable*** targets ***without*** an external calibrant.

With dPCR, a *sample is partitioned* so that individual nucleic acid molecules within the sample are localized and concentrated within many separate regions. The partitioning of the sample allows one to count the molecules by estimating according to Poisson distribution. As a result, each part will contain "0" or ≥ "1" molecules, or a negative or positive reaction, respectively. After PCR amplification, nucleic acids may be quantified by counting the regions that contain PCR end-product, positive reactions.

In conventional qPCR, starting copy number is proportional to the number of PCR amplification cycles. dPCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and providing absolute quantification.
(*http://digital-pcr.gene-quantification.info/*)

qPCR v dPCR Comparison

Quantitative PCR	Digital PCR
Quant is based on a calibrant; as the calibrant goes so will sample values	Quant is based on Poisson sampling statistics (i.e. calibrant free)
Samples must be bracketed by calibrant dilution curve	Samples must be within a range of concentrations
Older technology Widely accepted	New technology Gaining acceptance
Currently less expensive	Currently more expensive
Larger dynamic range	Smaller dynamic range

dPCR Applications

Absolute Quantification of Nucleic Acid Standards.
Absolute Quantification of Viral Load.
Absolute Quantification of Next-Gen Sequencing Libraries.
Rare Allele Detection.
Low-Fold Copy Number Discrimination.

Advantages of Digital PCR

No calibration curves are needed to interpret the results.
Desired precision can be achieved by increasing total number of PCR replicates.
More tolerant to inhibitors.
Unlike traditional qPCR, digital PCR provides a linear response to the number of copies present to allow for small fold change differences to be detected.

Experimental

Singleplex assays were developed and optimized for dPCR on the BioMark platform.

Assays/targets

Commercial Multiplex Assay Kit: Quantifiler.

Three in house optimized assays: D6S474, D9S2157, D14S1434
1x TaqMan Universal PCR Master Mix No AmpErase® UNG, PCR primers 0.5 µM, 0.2 µM of FAM-MGB probe, total reaction volume 20 µL.

The 3 components of SRM 2372 were characterized with each of the optimized dPCR assays (8 replicates for each).

Results of dPCR Measurements

CN = Copy Number$_{assay}$, dsDNA Templates/µL

	Component A		Component B		Component C	
	CN	*u*(CN)	CN	*u*(CN)	CN	*u*(CN)
[DNA] D6S474	19,100	800	19,600	600	16,000	800
[DNA] D9S2157	21,100	1,700	20,500	800	10,400	800
[DNA] D14S1434	23,200	1,200	22,400	500	24,900	1,800
[DNA] Quantifiler	18,500	1,300	18,600	200	19,200	1,100
[DNA] Average	**20,500**	**1,000**	**20,300**	**700**	**17,600**	**3,000**

Quantifiler performs somewhat lower in the dPCR reactions, this could be due the IPC interfering with overall PCR efficiency.

Copies converted to mass
DNA concentration$_{assay}$, ng/µL

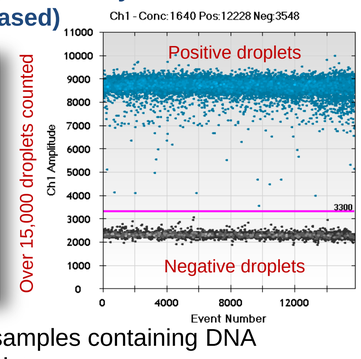
	Component A		Component B		Component C	
	mean	*u*(mean)	mean	*u*(mean)	mean	*u*(mean)
[DNA] D6S474	57.2	2.3	58.9	1.7	48.1	2.4
[DNA] D9S2157	63.2	5.0	61.6	2.5	31.2	2.5
[DNA] D14S1434	69.7	3.7	67.2	1.4	74.7	5.5
[DNA] Quantifiler	55.6	3.9	55.9	0.5	57.7	3.3
[DNA] Average	**61.4**	**3.0**	**60.9**	**2.2**	**52.9**	**9.0**

Values vary based on components and assay/target; additional studies are underway using different assays developed/optimized in-house.

Digital PCR Instrumentation

Digital PCR depends on partitioning PCR reactions which can be accomplished using either a microfluidic or droplet-based approach. Currently we have access to two digital PCR platforms:

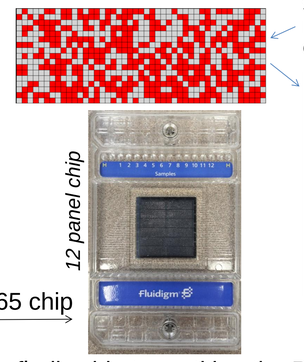
BioRad QX100 Droplet Digital PCR System (Droplet PCR based)



The QX100 droplet generator partitions samples containing DNA template into 20,000 nanoliter-sized droplets.
After PCR in a standard thermal cycler (9700, Veriti, etc), the droplets from each sample are streamed in single file through the QX100 droplet reader.
The PCR-positive and PCR-negative droplets are counted to provide absolute quantification of target DNA in digital form.

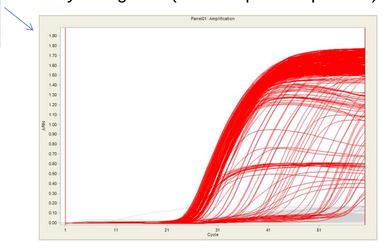
The QX100 performs end point analysis (does not collect real-time PCR curves).

Fluidigm BioMark System (microfluidic based)



Single 765 chamber panel
Red = positive (PCR product present)
Gray = negative (no PCR product present)

BioMark 12.765 chip



View of 765 amplification curves

Utilizes a microfludic chip to partition the PCR mastermix into 765 chambers (volume of each chamber = 6 nL).

Each chip contains 12 unique panels of 765 chambers.

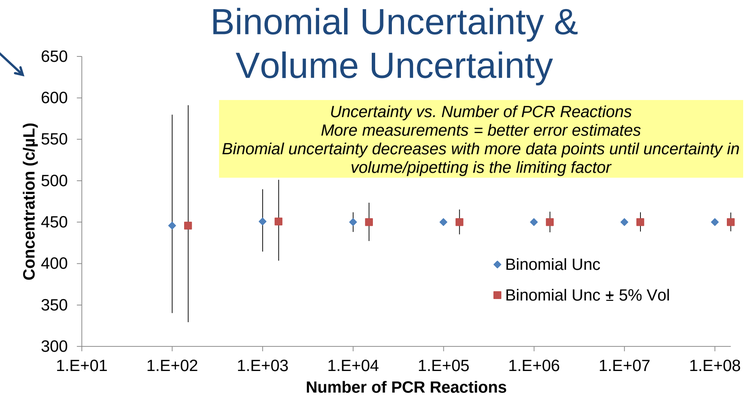
The instrument performs PCR thermal cycling and collects fluorescence signal after each cycle. 765 real-time PCR curves are collected for each panel.

Absolute Quantitation

Using Poisson statistics an estimation of number of copies can be determined. Volume is given by manufacturer (research indicates this estimate is reasonable)

$$\text{Concentration (copies per microliter)} = \frac{\text{total number of wells} \cdot \ln\left(\frac{\text{total number of wells}}{\text{total number of negative wells}}\right)}{\text{volume of all PCR reactions (microliters)}}$$

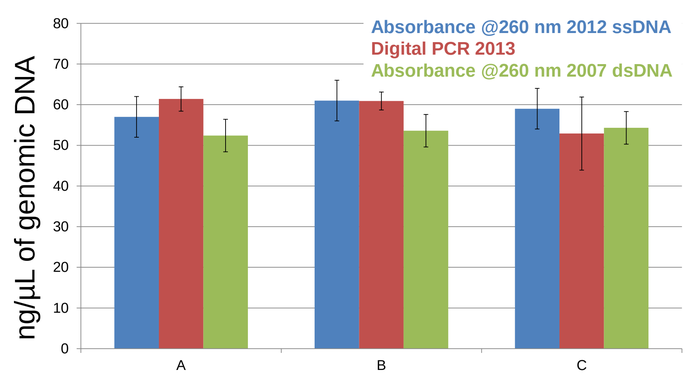
Binomial Uncertainty & Volume Uncertainty



Number of PCR reactions	n=100	n=1000	n=10k	n=100k	n=1m	n=10m	n=100m
Binomial Unc	53.75%	16.67%	5.26%	1.66%	0.53%	0.17%	0.05%
Binomial Unc + 5% Vol Unc	58.75%	21.67%	10.27%	6.67%	5.53%	5.17%	5.06%

Pinheiro et al. Anal Chem. 2012 Jan 17;84(2):1003-11.
Bhat et al. Anal Bioanal Chem. 2009 May;394(2):457-67.

dPCR DNA Concentration Estimates Comparison to Absorbance



Conclusions

The good agreement between the ng/µL values inferred from the digital counts and the conventional ng/µL values inferred from UV absorbance measurements suggests that the dPCR technology can provide unbiased counts. However, further studies will be required before this technology can be used to help certify the "genomic copy number" of a human genomic material.

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