Re-certification of NIST Standard Reference Material® 2372

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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 dsDNA is: 1.0 D_{10} at 260 nm = 50 ng/µL DNA

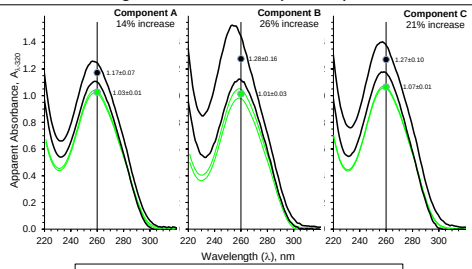
In March 2012, SRM 2372 was taken off the market and work performed to re-certify the materials

Why Was SRM 2372 Taken Off the Market?

- During measurement of the DNA samples to verify stability of certified values we observed **that the UV absorbance values for the samples had changed significantly**
  - 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**)
  - SRM 2372 is certified for UV absorbance (decadic attenuance)
    - One application of this SRM is for calibration of UV spectrophometers
- **The sample changes over time that impact UV absorbance do not appear to affect qPCR sample performance**

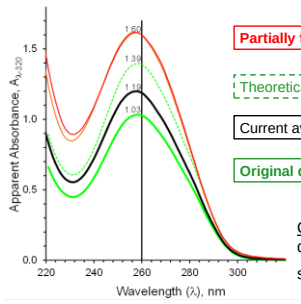
Why did SRM 2372 need to be re-certified?

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

Evidence of dsDNA/ssDNA mixture



Component A

Partially fragmented DNA profile

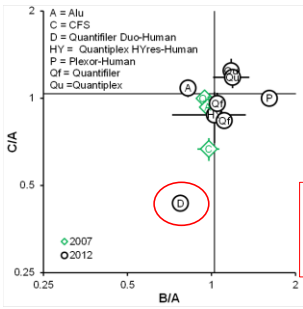
Theoretical ssDNA profile

Current average DNA profile

Original dsDNA profile

Conventional Conversion Factors
dsDNA: $D_{10\ (260\ nm)} = 1$ ➔ 50 ng/µL
ssDNA: $D_{10\ (260\ nm)} = 1$ ➔ 37 ng/µL
(some references say 40 ng/µL)

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?

- **Force the material 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 freshly prepared 0.4 mol/L NaOH
  - Measure against a reference of equal volumes of TE^{-4} buffer and the 0.4 mol/L NaOH
    - Microvolume spectrometers may have issues with NaOH solutions
- 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)

Convert Apparent Absorbance to ng/µL

- Conventional concentration values are derived from the 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

Difference between the original and re-certified values is within the noise of the assay

SRM 2372 back on Sale December 31, 2012

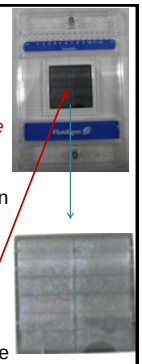
Do we measure ng/µL or amplifiable targets or accessible amplifiable targets?

- qPCR methods have evolved to try to establish the link between "quality/quantity" of the DNA extract and the resulting STR profiles
- The STR profiles generated are based on the *accessible amplifiable* targets
- We propose using digital PCR (dPCR) to **directly** assess the number of *accessible amplifiable* targets
  - This measurement technique has been shown to work well with plasmid DNA
  - Not yet demonstrated to work with human genomic DNA

Digital PCR (dPCR) Overview

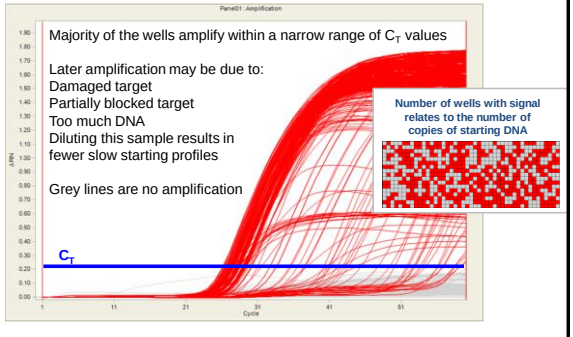
- Combination of:
  - Limiting dilution
  - End point PCR
  - Poisson statistics (no standard curve required)
- Need to dilute and partition templates so molecules can be amplified individually
  - Microfluidics (Fluidigm)
  - Emulsion/droplet PCR (Bio-Rad)

Digital PCR (dPCR) Overview



- Estimates the number of *accessible amplifiable* targets without an external calibrant
- Samples are split into 100s to 1000s of reaction chambers
  - Fluidigm 12.765 Digital Array
  - 765 chambers × 12 panels = **9180 dPCR reactions**
- The count of the number of chambers containing at least 1 target can be used to estimate the total number of targets in a sample

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



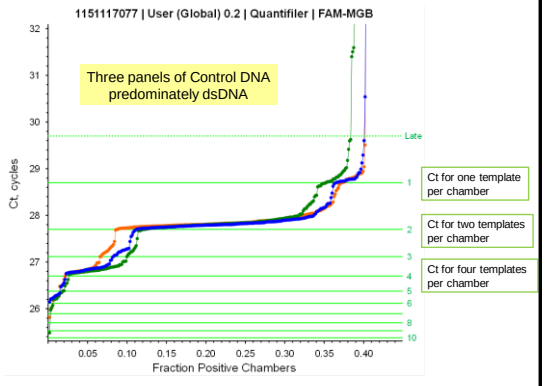
"Detail Table" from an export file

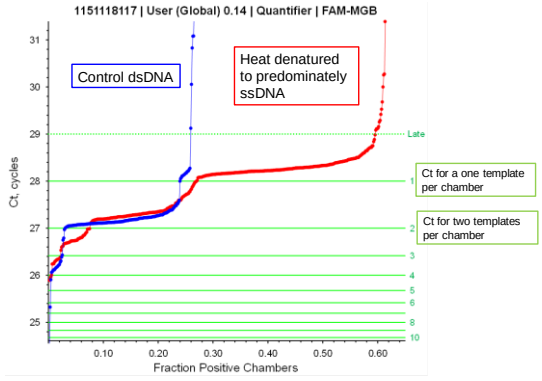
Chip Run Info	1151117077
Date	4/12/2012 18:34
Kit	Quantifiler
Export Type	Detailed Table Result:
Quality Threshold	0.01
Baseline Correction Method	Linear
Ct Threshold Method	User (Global)
FAM Threshold	0.2
VIC Threshold	0.14

Ct for Chamber P01-X01-Y01

Experiment Information		FAM-MGB Ct					VIC-MGB Ct				
Chamber and Panel											
Chamber ID	Panel ID	Value	Quality	Call	Target	Threshold	Value	Quality	Call	Target	Threshold
P01-X01-Y01	1.00	29.04	0.97	Pass	Hit	0.20	999.00	0.00	Fail	No Hit	0.14
P01-X02-Y01	1.00	999.00	0.00	Fail	No Hit	0.20	36.11	0.84	Pass	Hit	0.14
P01-X03-Y01	1.00	49.89	0.92	Pass	Hit	0.20	999.00	0.00	Fail	No Hit	0.14
P01-X04-Y01	1.00	999.00	0.00	Fail	No Hit	0.20	35.58	0.93	Pass	Hit	0.14
P01-X05-Y01	1.00	999.00	0.00	Fail	No Hit	0.20	35.45	0.91	Pass	Hit	0.14
P01-X06-Y01	1.00	28.05	0.96	Pass	Hit	0.20	40.57	0.79	Pass	Hit	0.14
P01-X07-Y01	1.00	27.84	0.96	Pass	Hit	0.20	41.64	0.77	Pass	Hit	0.14
P01-X08-Y01	1.00	27.88	0.97	Pass	Hit	0.20	37.65	0.66	Pass	Hit	0.14

Evaluate 'rank ordered' Ct data





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

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

http://www.nist.gov/mml/bmd/genetics/upload/Digital-PCR-Ross-Haynes.pdf

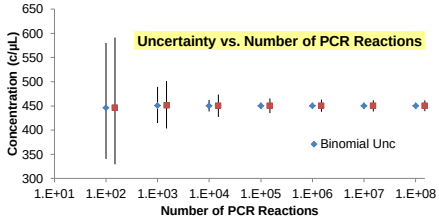
Absolute Quantitation

theoretical example

- PCR amplify → 1000 reactions
- Count positive wells → 594 reactions amplified
- Poisson stats → 900 copies
- Divide by total volume → 20 µL
- Correct for dilutions → 10 fold dilution
- Concentration → 450 c/µL = (900/20)x10
- Uncertainty is based on binomial statistics → 95 % CI: 415 to 489 c/µL

http://www.nist.gov/mml/bmd/genetics/upload/Digital-PCR-Ross-Haynes.pdf

Binomial Uncertainty & Volume Uncertainty



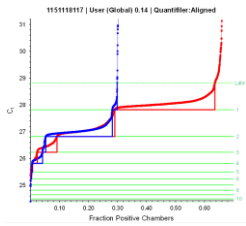
Number of PCR reactions	n=100	n=1000	n=10k	n=100k	n=1m	n=10m	n=100m
Binomial Unc	53.75%	16.67%	5.26%	1.66%	0.53%	0.17%	0.05%
Binomial Unc + 5% Vol Unc	58.75%	21.67%	10.27%	6.67%	5.53%	5.17%	5.06%

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

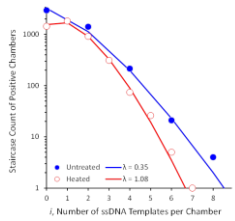
Poisson model fits the data well

Validation of the dPCR analysis

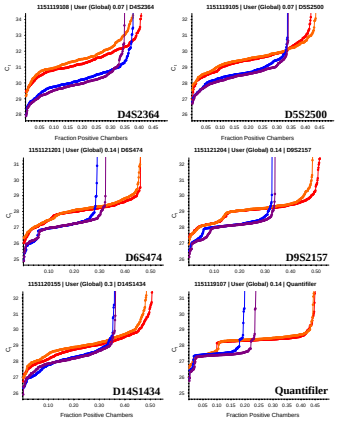
Rank ordered Ct count



Model (lines) fits the data (symbols)



Validation of the dPCR analysis
Performed by Dave Duewer



Six qPCR assay results:

We developed our own monoplex assays

Duplicate panels for control dsDNA (blue-violet) and heat treated ssDNA (red-orange)

Results were obtained over several months for the different assays.

We determined we wanted to dilute the DNA to obtain approximately 0.3 fraction positive chambers in future experiments

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 is Planned as the Next Certification Method

- The next generation of SRM 2372 will be certified for "copy/target number" not UV absorbance
  - dPCR assays require optimization to improve measurement accuracy and reproducibility
- 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**

dPCR Copy Number Estimates

Table 6: CN_{assay} and CN_{genome} Estimates, dsDNA Templates/µL

Unique Target Assay	Component A		Component B		Component C	
Measurand	CN	u(CN)	CN	u(CN)	CN	u(CN)
$[DNA]_{D5S474}$	19100	800	19600	600	16000	800
$[DNA]_{D9S2157}$	21100	1700	20500	800	10400	800
$[DNA]_{D14S1434}$	23200	1200	22400	500	24900	1800
$[DNA]_{Quantifiler}$	18500	1300	18600	200	19200	1100
$[DNA]_{Genome}$	20500	1000	20300	700	17600	3000

Use of multiple target assays for dPCR copy number estimates
Each assay may perform differently

dPCR DNA Concentration Estimates

Table 5: $[DNA]_{assay}$ and $[DNA]_{genome}$ Estimates, ng/µL

Unique Target Assay	Component A			Component B			Component C		
Measurand	$\bar{X}$	$u(\bar{X})$	P	$\bar{X}$	$u(\bar{X})$	P	$\bar{X}$	$u(\bar{X})$	P
$[DNA]_{D5S474}$	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	
$[DNA]_{Genome}$	61.4	3.0	0.13	60.9	2.2	0.02	52.9	9.0	0

	Comp A	Comp B	Comp C
2012 DNA Mass Concentration (ng/µL)	57	61	59

Summary

- NIST SRM 2372 has been re-certified through forcing dsDNA to become ssDNA in order to improve the UV absorbance measurements
- qPCR measurements have not been significantly impacted by the new certified (and DNA concentration) values
- Digital PCR will be used to certify copy number for future DNA quantitation SRMs
- Quantitation is impacted by new qPCR targets and STR kit PCR buffer formulations
  - Insensitive qPCR assays may not accurately reflect ability of new, more sensitive STR kits to obtain results

Thanks for your attention!

Questions?
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