Certification of a DNA Quantitation Standard Using Digital PCR

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The current version of SRM 2372 was designed for use in the value assignment of human genomic DNA forensic quantitation materials and was originally certified for spectrophotometric absorbance of 260 nm [1]. However in 2012, five years after production, the measured absorbance in all materials had increased (by approximately 9 % to 14 %) to the point that the certified values were no longer within the stated error. Investigation revealed that the increase resulted from deshielding due to tertiary structure changes [2,3]. Due to this phenomenon and the projected depletion of SRM 2372, the next iteration of SRM 2372 will be certified for copy/target number using digital PCR (dPCR). Digital PCR depends on partitioning PCR reactions either a microfluidic platform (chamber digital PCR, cdPCR) or in a droplet emulsion (droplet digital PCR, ddPCR). This partitioning allows the estimation of the number of accessible amplifiable targets without an external calibrant. The use of digital PCR enables direct determination of accessible amplifiable genomic targets. Initial experiments suggest that the nine PCR assays agree within 5 % to 10 % across all platforms. This presentation will discuss the importance of assessing the variability at the genome level in terms of copy number variants by utilizing nine single copy assays evaluated and optimized for amplifiable accessibility across three platforms (qPCR, cdPCR, ddPCR).

What is SRM 2372 Human DNA Quantitation Standard?

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

SRM 2372 Human DNA Quantitation Standard

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 SI unit ratio 1.

The conventional conversion factors 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

The NIST SRM 2372 Human DNA Quantitation Standard was produced to support the need for a human-specific DNA quantitation standard in forensic casework as a calibrant for commercially produced DNA standards.

Planning for SRM 2372a

The current supply of SRM 2372 is expected to be exhausted in 2017.

Initial examination of material for SRM2372a:

- Number of components to be decided
- Single source, multiple sources to be decided
- Material must be stable over the lifetime of the SRM (≈5 to 10 years)
- Material must remain homogenous over the lifetime of the SRM (≈5 to 10 years)
- Material must be fit-for-purpose (quantification of human genomic DNA)

qPCR and dPCR assay development needs to take place to include optimization and validation on multiple platforms. Additionally, dPCR technologies need to be evaluated and validated prior to certification.

SRM 2372a will be characterized using Digital PCR

The technique of Digital PCR allows for a direct determination of accessible genomic targets versus an indirect method such as UV absorbance. The estimation of genome copies per microliter is directly proportional to the STR targets during PCR amplification.

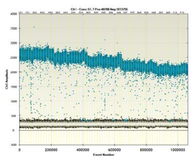
Digital PCR works by partitioning the sample into droplets (droplet digital) or in a microfluidic chamber (chamber digital). Signal is amplified through a qPCR assay and allows 'positive' partitions to be detected and enumerated. dPCR estimates the number of accessible amplifiable targets without an external calibrant.

Droplet Digital - ddPCR

BIO-RAD
QX100/200

In ddPCR

- Sample/mastermix is placed in a droplet generator
- Individual droplets in an oil emulsion are formed
- PCR amplification is performed (end point)
- Droplets are read as being positive or negative

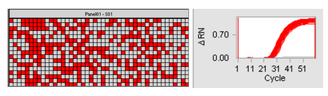


Chamber Digital - cdPCR

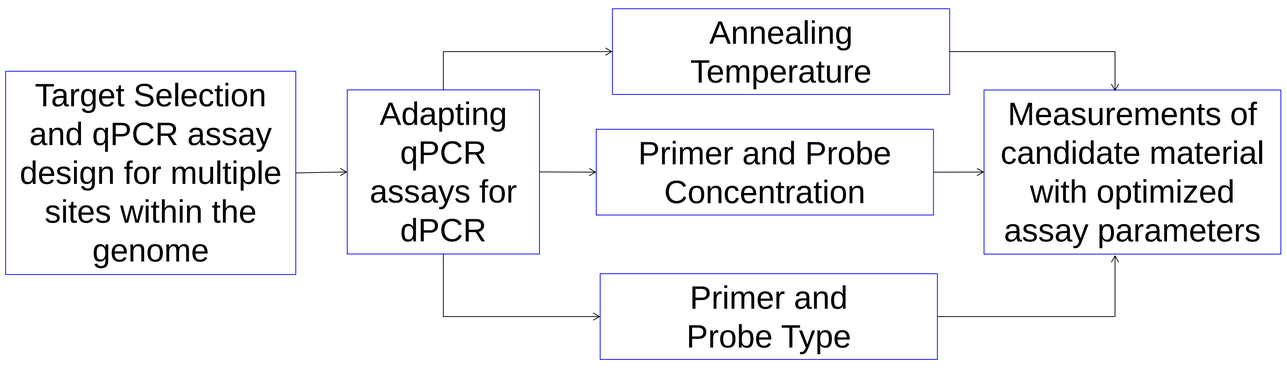
Fluidigm
BioMark

In cdPCR

- Sample/mastermix is placed in a sample inlet
- Sample inlets are pressurized to distribute mixture into 770 chambers
- PCR amplification is performed with real time data collection of the fluorescent signal per cycle
- Chambers are counted as positive or negative

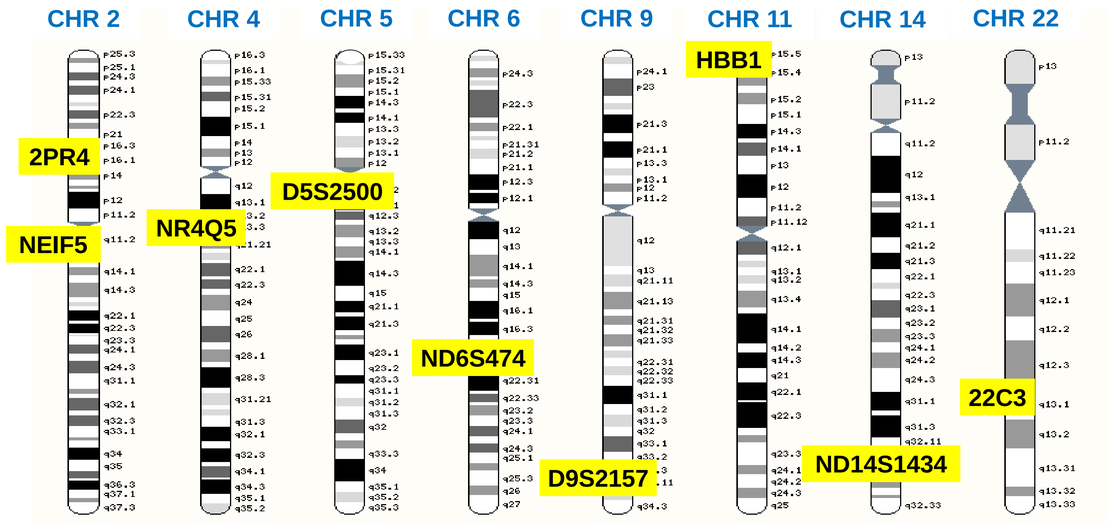


Assay Design and Optimization Approach



Target Selection and Assay Design

9 assays covering 8 different chromosomes

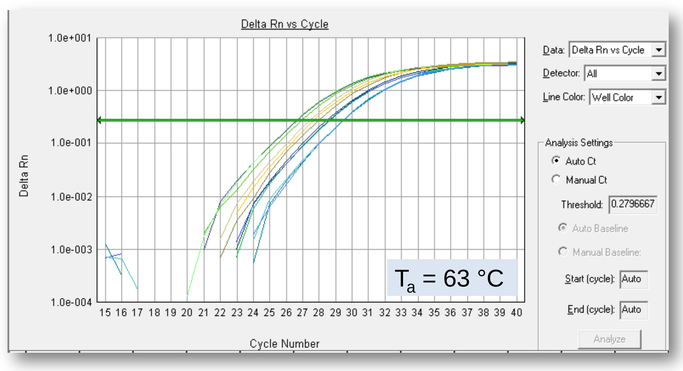


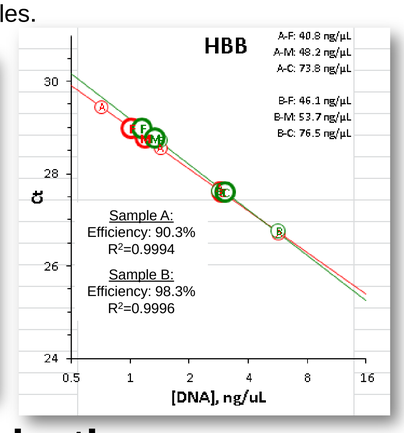
Individual assays count ***number of specific targets***. Value certified will be ***number of genomes***.

There is a **need to use multiple assays** to establish reasonable estimates of uncertainty. We do not expect the different assays to yield the exact same number of targets because of the variability within the genome.

qPCR Assay Design

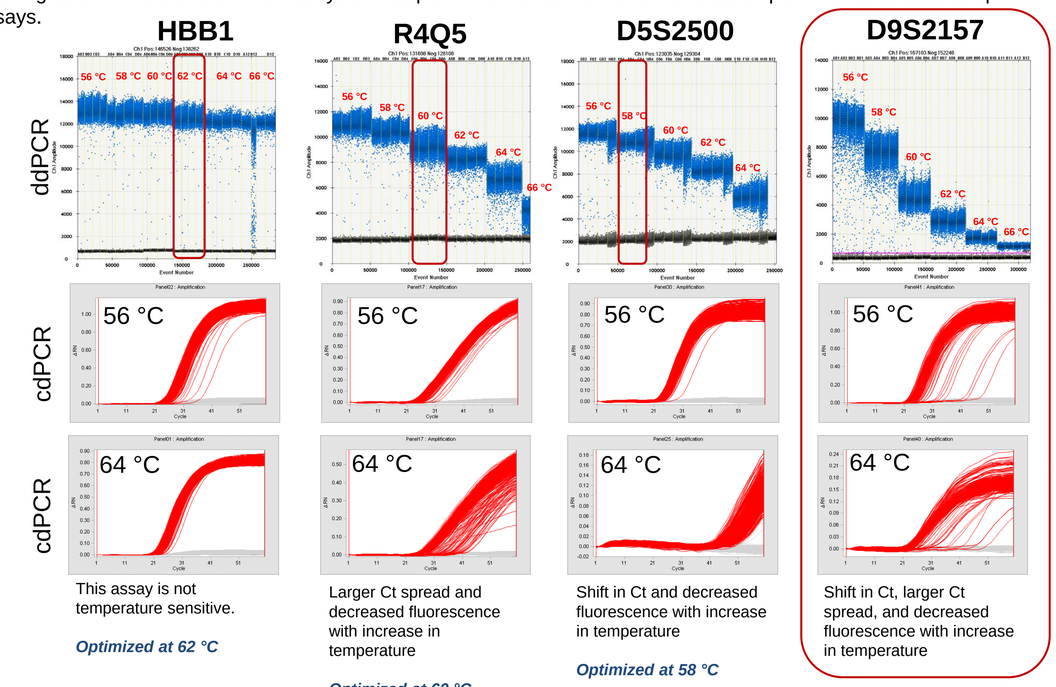
Assays are first tested with qPCR for linearity and efficiency with a dilution series. Below represents one assay optimized with triplicates of a dilution series of two different calibrating materials. These two materials yielded similar concentrations for unknown samples.





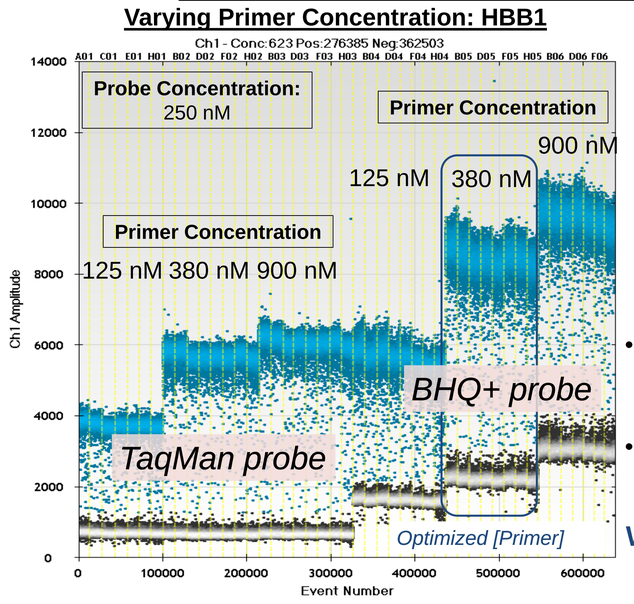
Temperature Optimization

Annealing temperature gradients were performed to determine optimal annealing temperature required for each assay.
A temperature gradient on a standard thermal cycler was performed for ddPCR and individual chips were run at each temperature for all cdPCR assays.

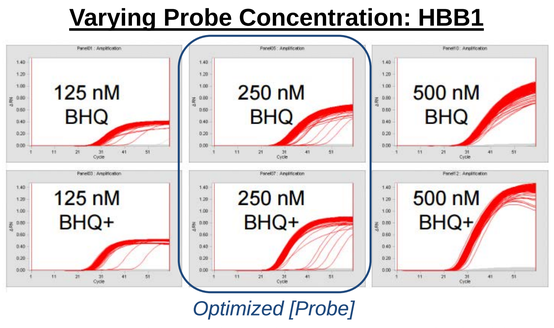


Primer Concentration and Probe Type (HBB1)

Varying Primer Concentration: HBB1



Varying Probe Concentration: HBB1

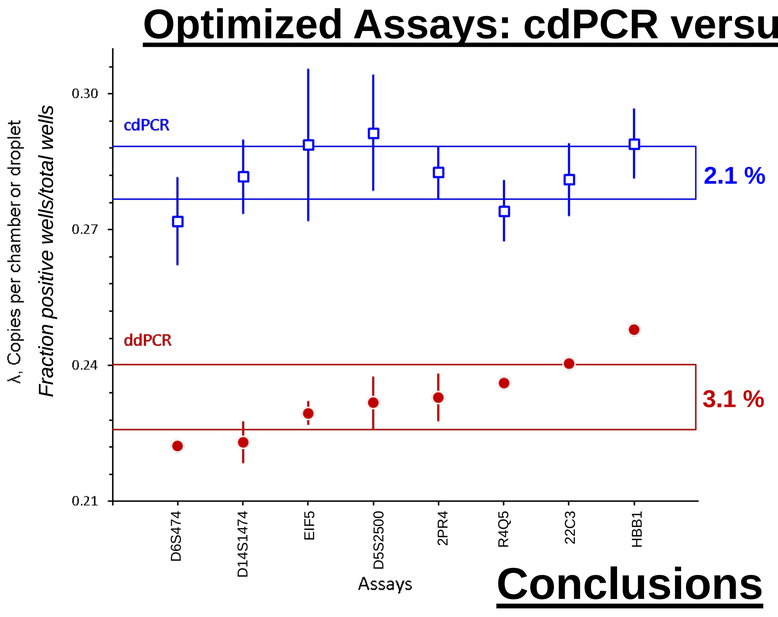


Optimized [Probe]

- Concentration of the primers were varied with two different probe types (TaqMan and BHQ+) (*Left*)
- Probe concentration was tested with a primer concentration of 380 nM (*Above*)

While intensity of the probe varies across the data, the final copies/µL remain similar.

Optimized Assays: cdPCR versus ddPCR platforms



The copies of DNA per chamber or droplet are represented for the optimized assays, along with the standard deviation of each individual assay for both ddPCR and cdPCR.

The lines for both cdPCR and ddPCR represent the 95% confidence level.

The difference (< 10%) between the cdPCR and ddPCR values for each assay may reflect the need to accurately measure the volume of the chamber/droplet.

Conclusions

The next iteration of SRM 2372 (SRM 2372a) will be certified for copy/target number rather than UV absorbance. Using Poisson statistics the estimation of number of copies is determined allowing for absolute quantitation. A thorough optimization and validation of eight PCR assays, two digital PCR platforms, and performance with qPCR is being conducted. Eight assays have been optimized with examination of appropriate annealing temperature, primer and probe concentration, and primer and probe type. **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 genomic level in terms of copy number variants and chromosomal rearrangement.** Further work is being conducted to determine the volume of the chambers and droplets and the appropriate uncertainty around the volume measurement, which is needed for digital PCR to be used to certify the next iteration of the SRM.

References:
1. Kline, M.C., Duewer, D.L., Travis, J.C., Smith, M.V., Redman, J.W., Vallone, P.M., Decker, A.E., Butler, J.M. (2009) Production and certification of NIST Standard Reference Material 2372 Human DNA Quantitation Standard. Anal. Bioanal. Chem. 394: 1183-1192.
2. Vallone, P.M., Butts, E.L.R., Duewer, D.L., Kline, M.C. (2013) Recertification of the NIST Standard Reference Material 2372®, human DNA quantitation standard. Forensic Sci. Int. Genet.: Suppl. Ser. 4(1): e256-e257.
3.Tinoco I Jr. Hypochromism in Polynucleotides. (1960) J Am Chem Soc 82(18):4785–4790.

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http://www.cstl.nist.gov/biotech/strbase/pub_pres/RomsosISFG2015SRM2372.pdf