The Use of Digital PCR (dPCR) for SRM Characterization

Forensics @ NIST
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U.S. Department of Commerce

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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.

We have re-certified the D_{10} as single stranded DNA (01/13)

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

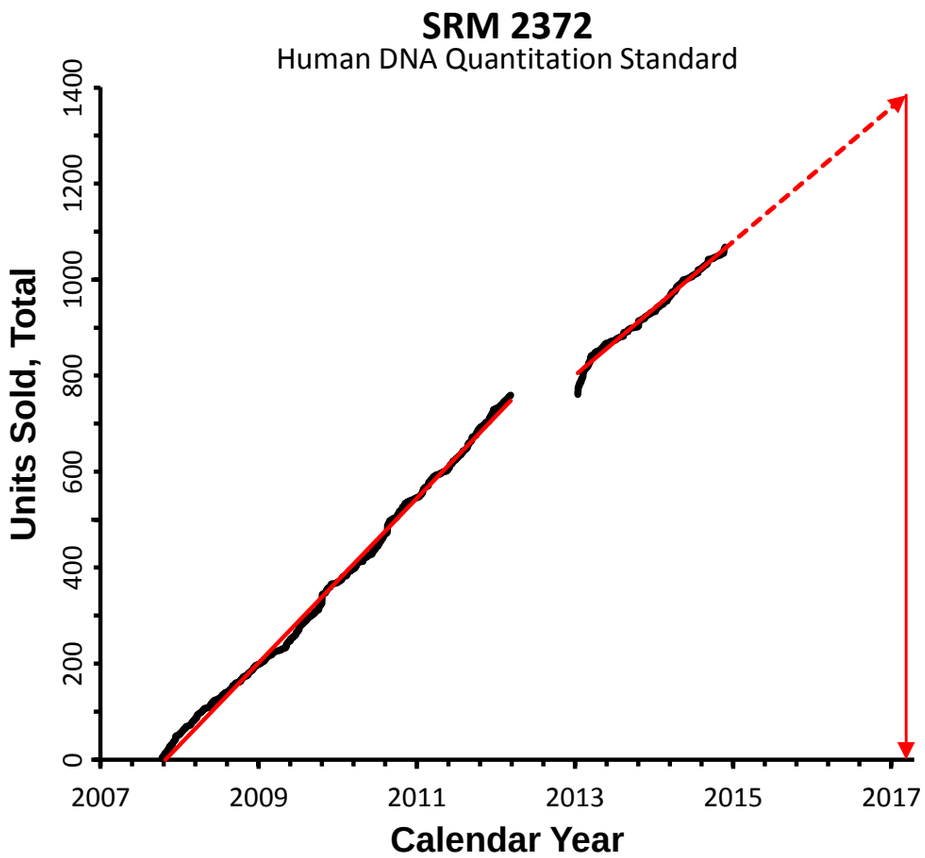
Planning for 2372a: Why?

STR amplification depends on number of accessible, amplifiable targets (AAT), not “amount DNA”

ssDNA in strong NaOH not convenient sample for “nanodrop” spectrometry

Supply of 2372 expected to be exhausted in 2017

Sales History

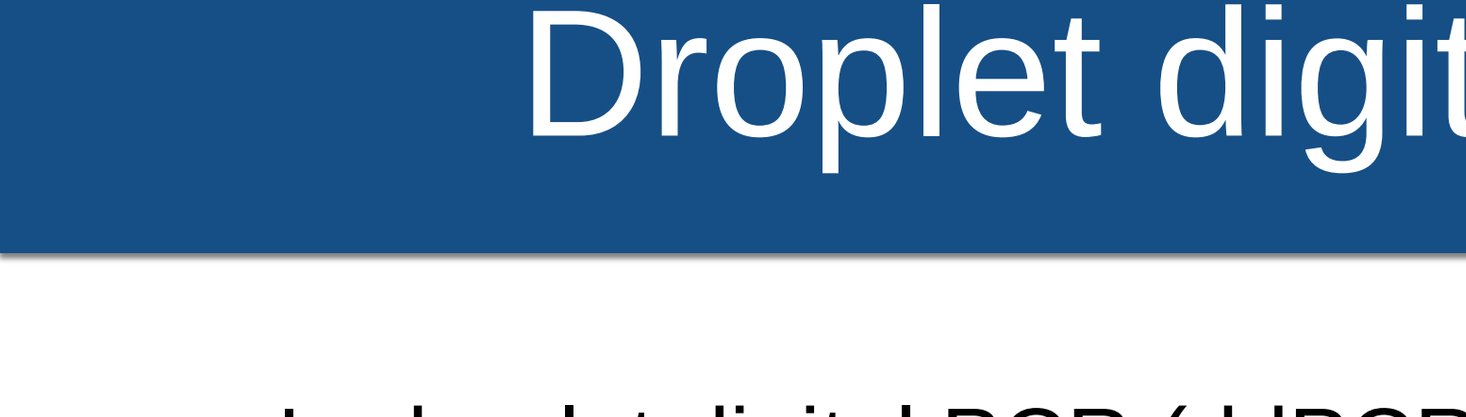


dPCR Technology

- Limiting dilution assays
  - Samples divided into many partitions (droplets or chambers)
  - With suitably diluted samples, each partition contains either zero, one, or a few PCR targets before amplification.
  - Results are categorized as Positive or Negative
  - Poisson statistics transform observed number of Positives to number of PCR targets
- Two basic “flavors”
  - Droplet digital (ddPCR)
  - Chamber digital (cdPCR)

Droplet digital PCR

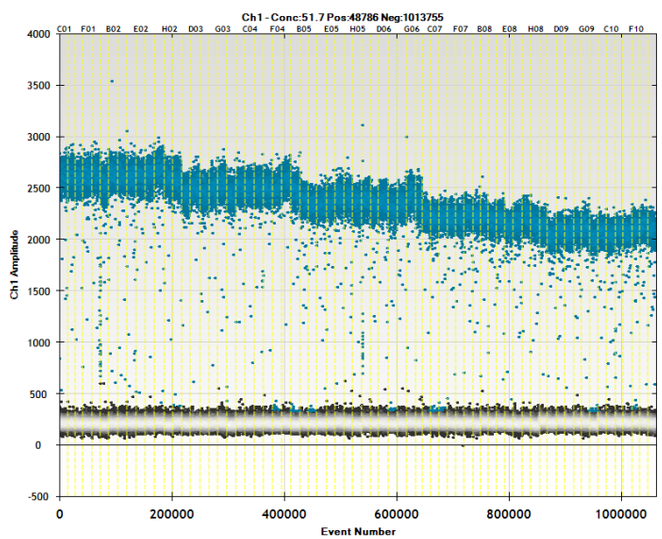
- In droplet digital PCR (ddPCR)
  - Sample/mastermix is placed in a droplet generator
  - Individual droplets in an oil emulsion are formed
  - PCR amplification is performed (end point)
  - Droplets are read as being positive or negative



V. Patel/Bio-Rad

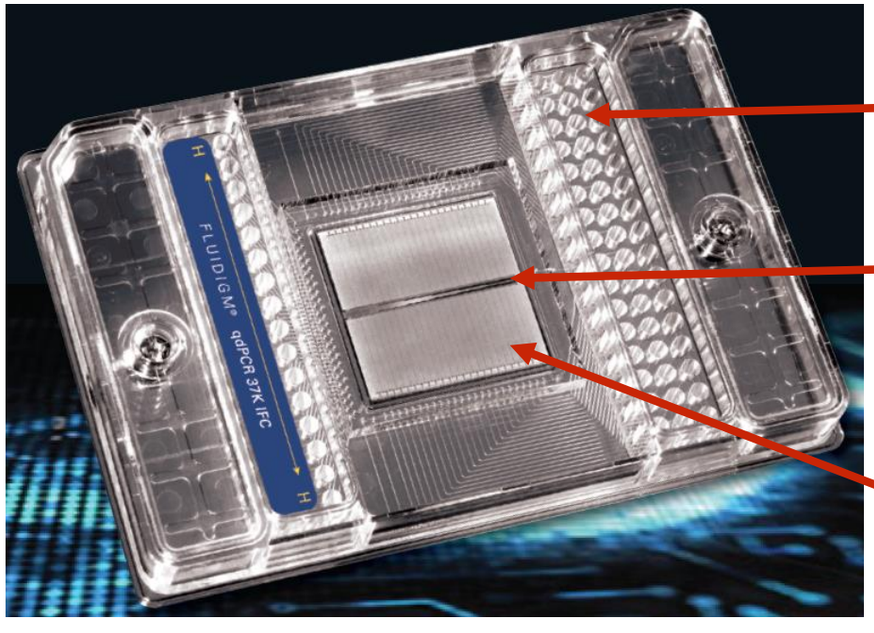
In Bio-Rad's dPCR workflow, reaction mixes are partitioned into droplets (left). The PCR reaction takes place in each droplet (center). If the target sequence is amplified, a reporter dye emits a fluorescent signal, which is read (right).

Up to 20,000 droplets per 20 µL sample/mastermix

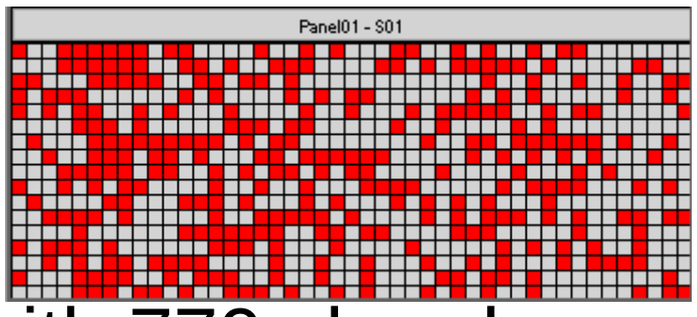


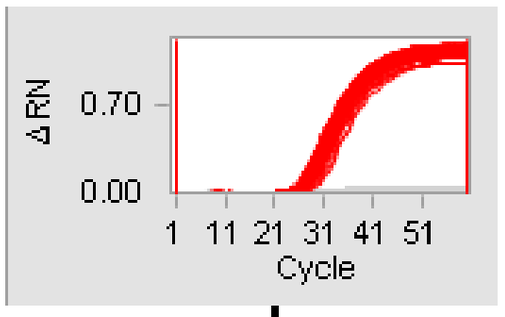
Chamber digital PCR

- In chamber digital PCR (cdPCR)
  - Sample/mastermix is placed in a sample inlet
  - Sample inlets are pressurized to distribute mixture into 765 or 770 chambers depending on chip
  - PCR amplification is performed with <u>real time data collection</u> of the fluorescent signal per cycle
  - Chambers are counted as positive or negative

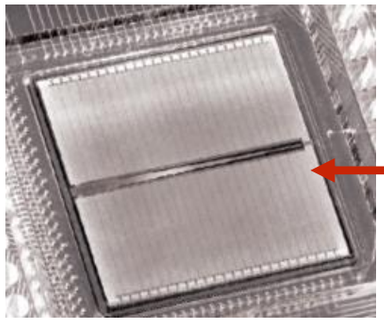


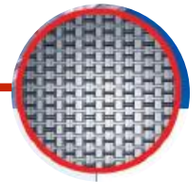
Sample inlet





48 panels with 770 chambers per panel





4 µL sample/master mix per panel

Applied Genetics Group's Instruments

Droplet Digital - ddPCR

BIO-RAD
QX100/200

Chamber Digital - cdPCR

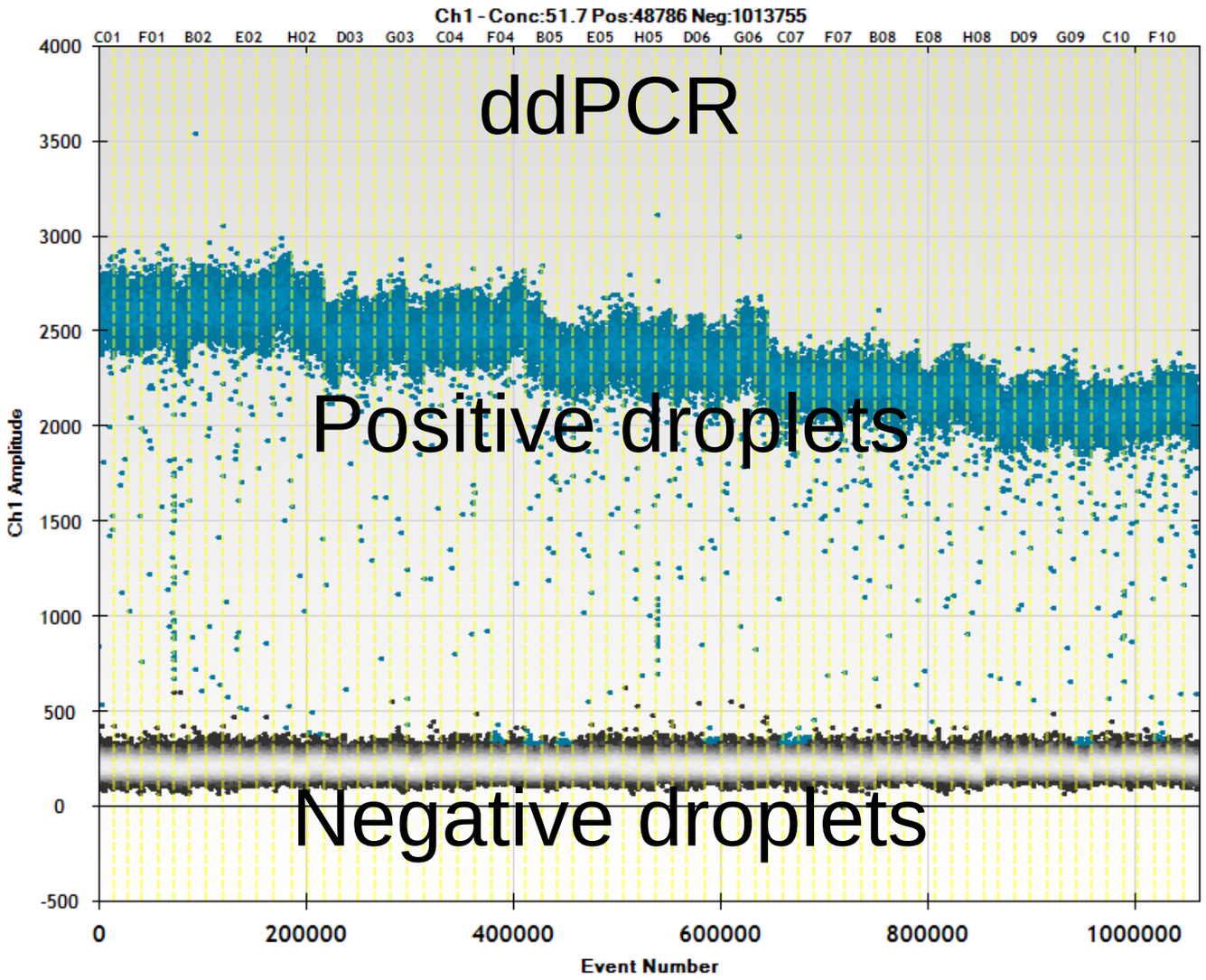
Fluidigm
BioMark

Why use dPCR as a certification approach?

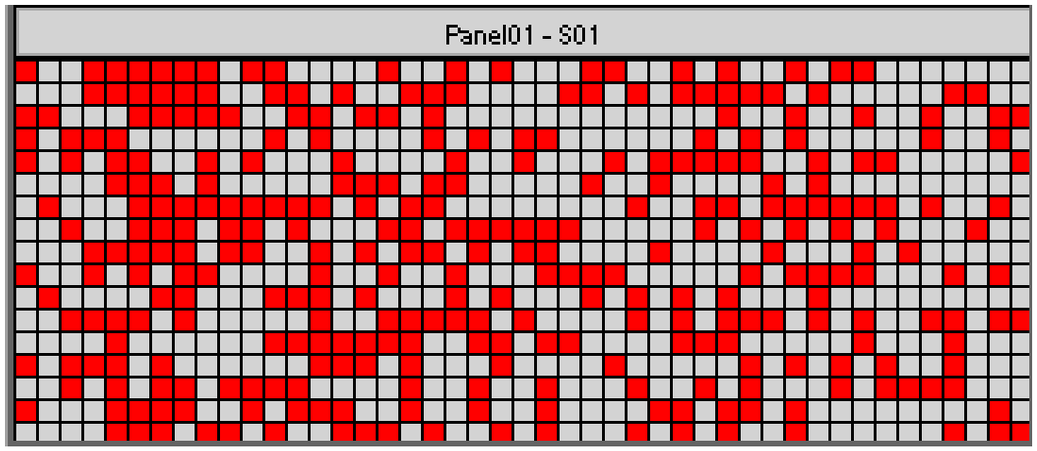
- dPCR estimates the number of *accessible amplifiable* targets *<u>without</u>* an external calibrant
    - Direct Counting of targets
    - STR profiles are generated based on the *accessible amplifiable* targets
- Use multiple dPCR assays
    - Establish reasonable estimates of uncertainty
    - We do not expect different assays to yield the exact same number because of variability of the genome
- Use multiple dPCR platforms
    - Check for bias between platforms

dPCR measures Counts/µL…

- What's a positive partition?



Red chambers: Positives
Gray chambers: Negatives



Positives are reaction partitions where the fluorescence intensity exceeds the threshold after a set number of amplification cycles

dPCR measures Counts/µL…

- What's a count?
- From Poisson statistics (i.e., 18th century voodoo)

$$\text{Counts} = -ln\left(1 - \left(\frac{\text{Number Positives}}{\text{Number Partitions}}\right)\right)$$

Converting Counts/µL to ng/µL

255 positive chambers
765 total chambers

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

$$\text{ng/µL} = \frac{\text{Copies per microliter of diluted material} * \text{Master Mix Dilution} * \text{Original Sample Dilution}}{\text{305 dsDNA copies/ng}}$$

Dolezel et al. Cytometry Part A 51A:127–128 (2003); Human Genome NCBI Build 38: 3,203,286,105 bp

Converting Counts/µL to ng/µL

255 positive chambers

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

$$\text{Copies per microliter of diluted material} = \frac{-\ln\left(1-\frac{255}{765}\right)}{0.006} = 68 \text{ copies/µL}$$

1→10 dil into reaction

1→25 dil of stock DNA

$$\text{ng/µL} = \frac{\text{Copies per microliter of diluted material} * \text{Master Mix Dilution} * \text{Original Sample Dilution}}{\text{305 dsDNA copies/ng}}$$

$$\text{ng/µL} = \frac{68 * 10 * 25}{\text{305 dsDNA copies/ng}}$$

Dolezel et al. Cytometry Part A 51A:127–128 (2003); Human Genome NCBI Build 38: 3,203,286,105 bp

Converting Counts/µL to ng/µL

255 positive chambers

$$\text{Copies per microliter of diluted material} = \frac{-\ln\left(1-\frac{\text{total number positives}}{\text{total number of partitions}}\right)}{\text{PCR r}}$$

$$\text{Copies per microliter of diluted} \quad \frac{-\ln\left(1-\frac{255}{765}\right)}{6} = 68 \text{ copies/µL}$$

55.4 ng/µL of original starting material

dil into reaction

1→25 dil of stock DNA

$$\text{ng/µL} = \frac{\text{Copies per microliter of diluted material} * \text{Master Mix Dilution} * \text{Original Sample Dilution}}{\text{305 dsDNA copies/ng}}$$

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Converting Counts/µL to ng/µL

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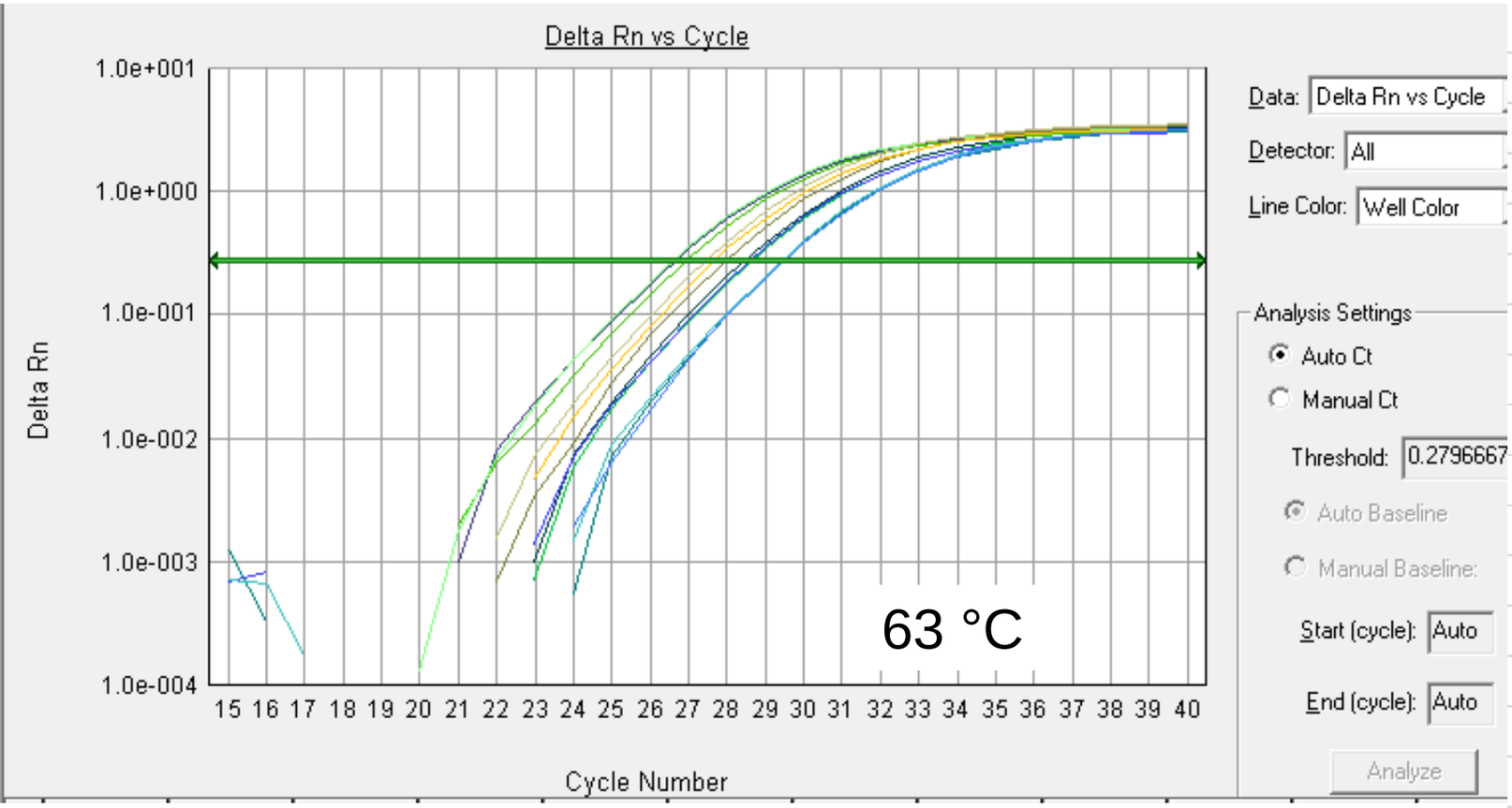
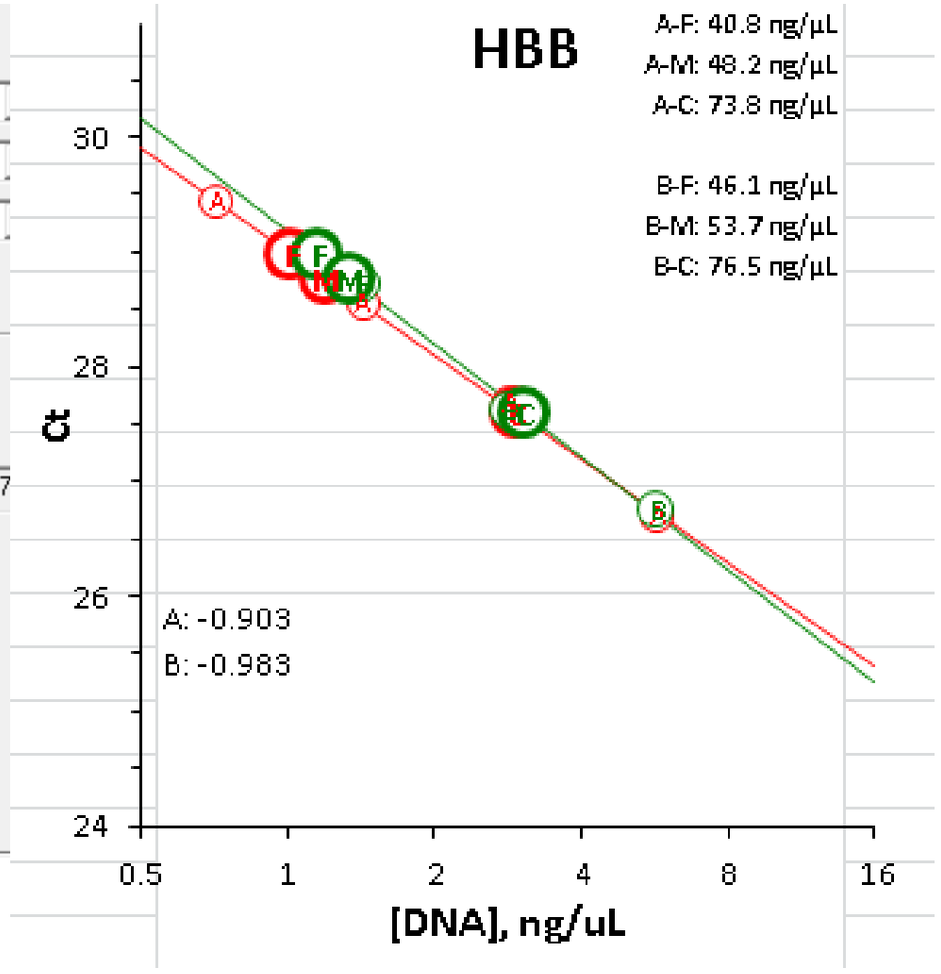
Proposed certification approach: Assays

- Single copy target only
    - Multiple copy assays are not useful
- Primer binding efficiency
    - Effects amplification of template
    - Effects amplification of amplicon
- Probe binding efficiency
    - Effects the “reporting” of amplification
- Concentration of analyte
    - Must be within the linear analytical range

HBB1 qPCR (Hemoglobin, beta)



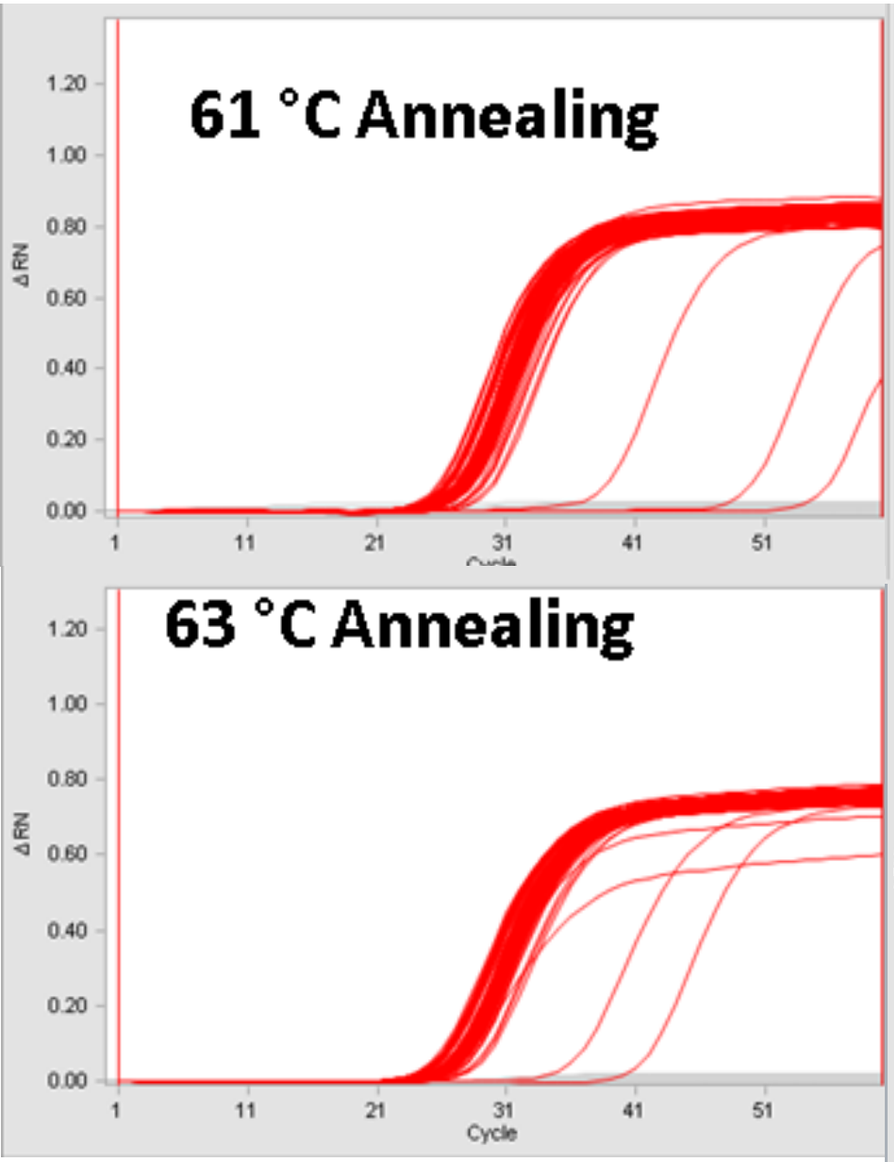


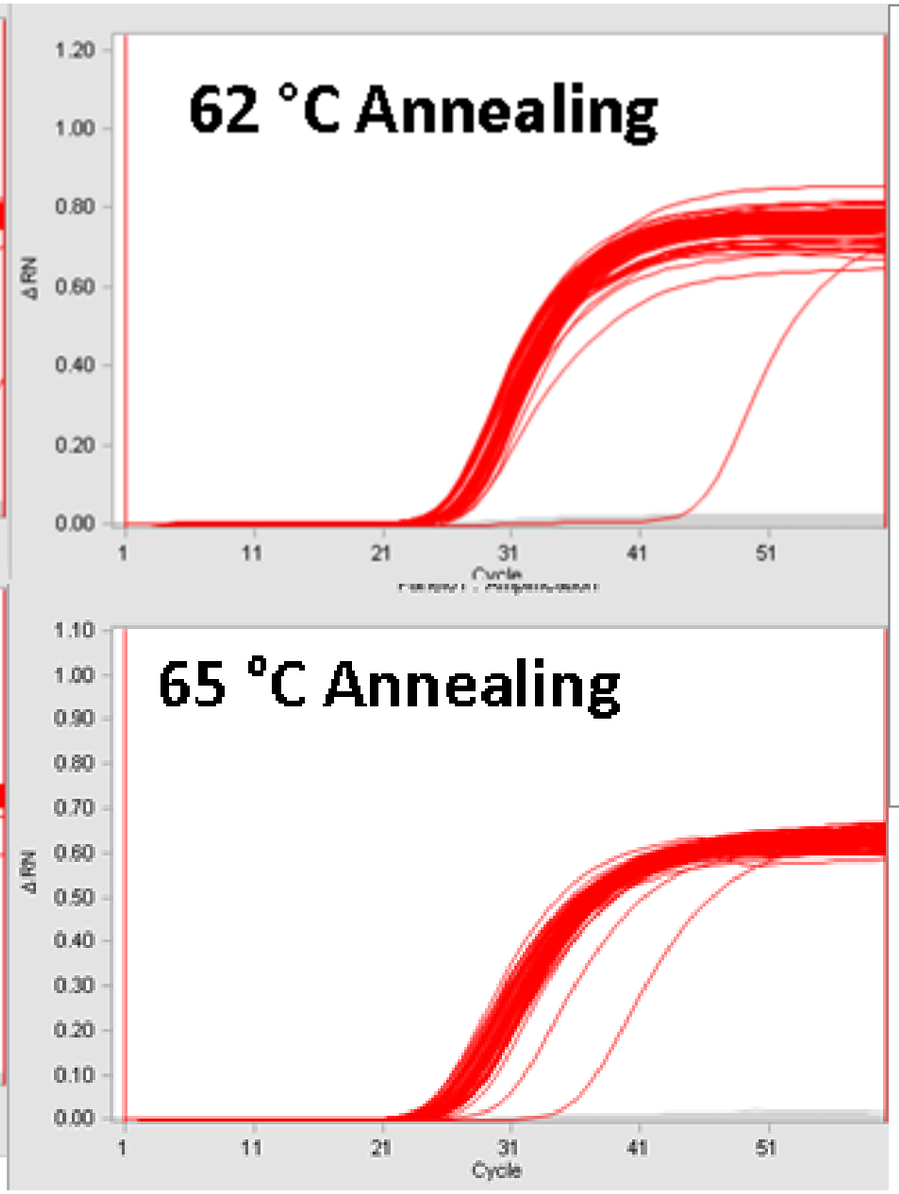
Triplicates of a dilution series of SRM 2372 Component A : 1:10, 1:20, 1:40, and 1:80

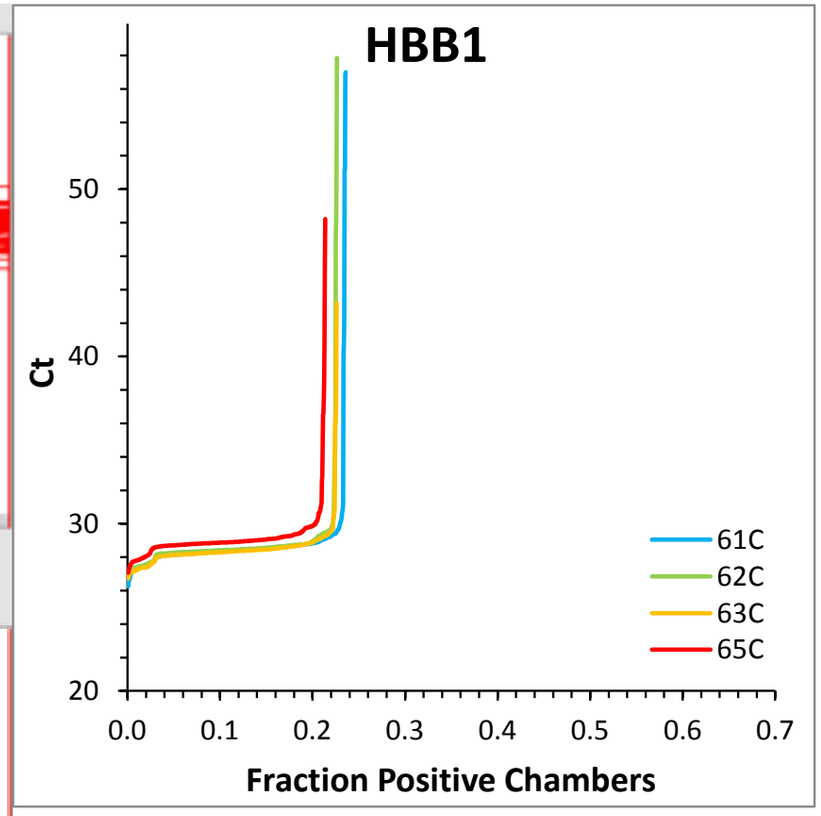
1:10 dilution of Component A 26.6 Cts

Results from 2 different calibrating materials yield similar concentrations for unknown samples

HBB1 cdPCR-Temperature Gradient

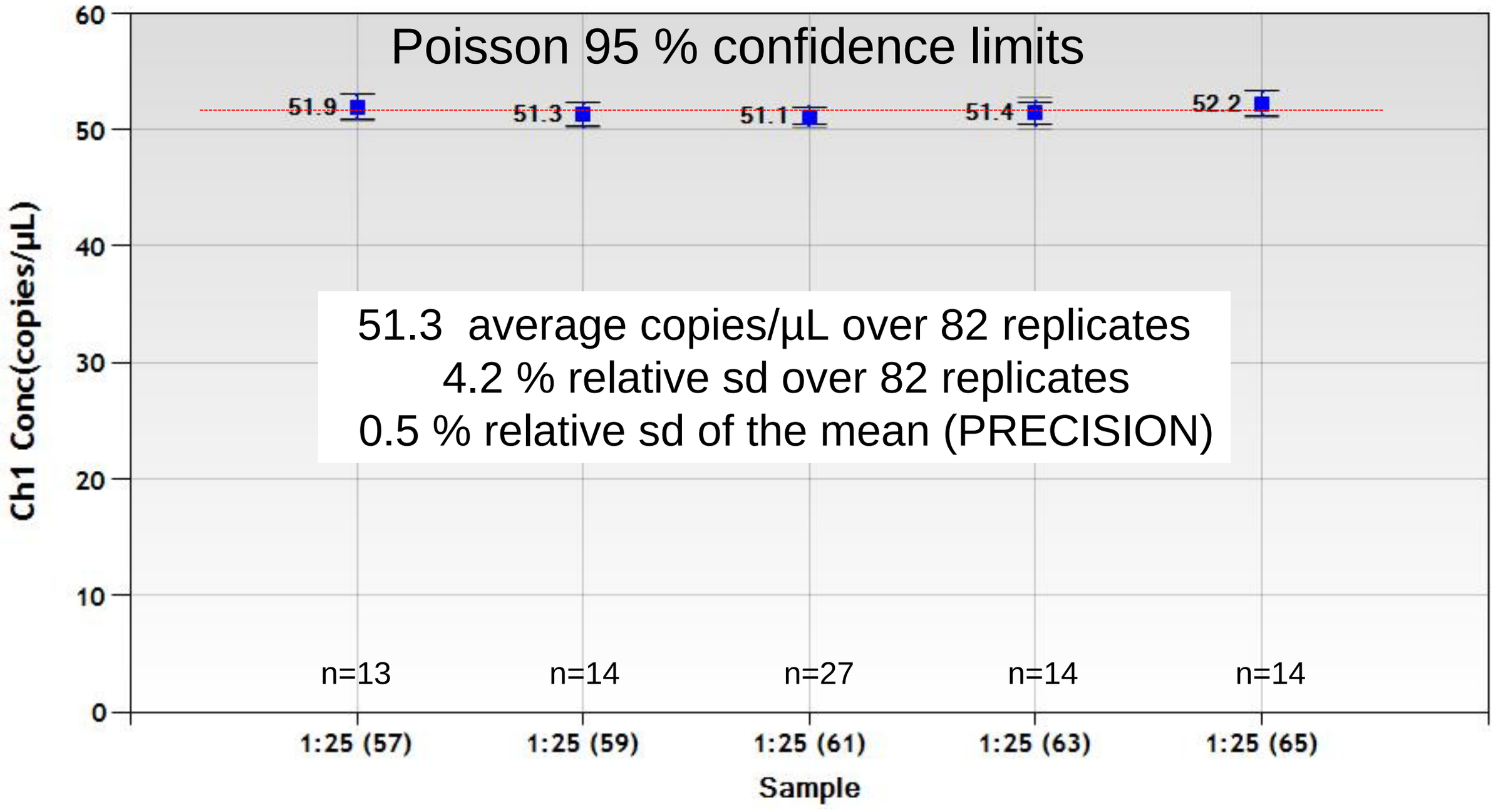






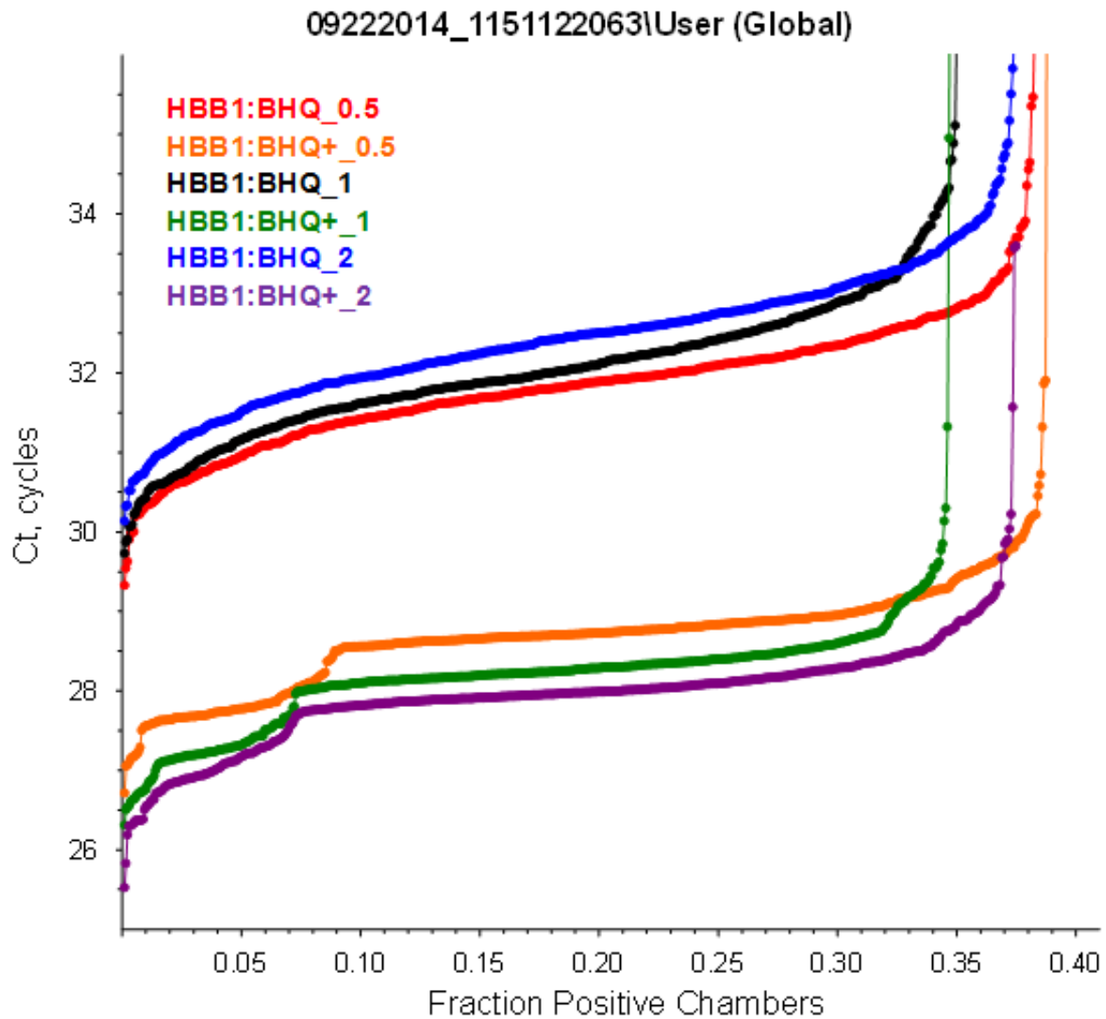
No significant observed difference between temperatures tested for HBB1 cdPCR assay
12.765 chip

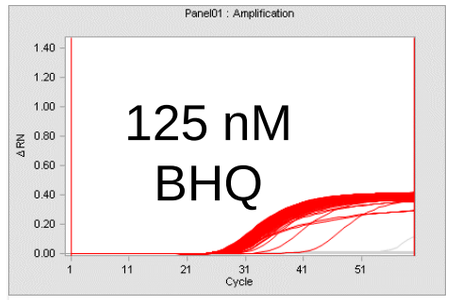
ddPCR HBB1 Temperature Study

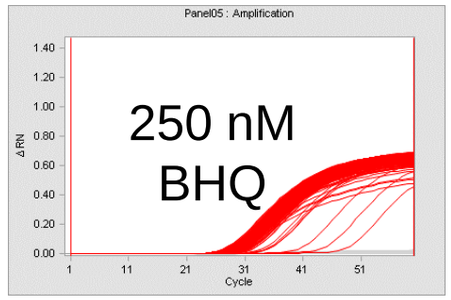


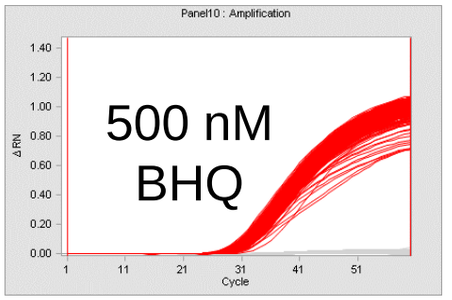
Varying Probe Concentration

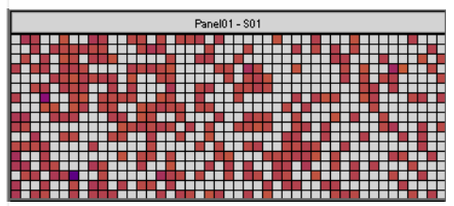
NCBI primers used at 59 °C annealing temperature.

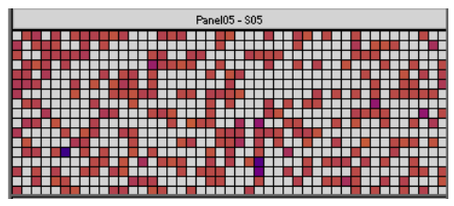


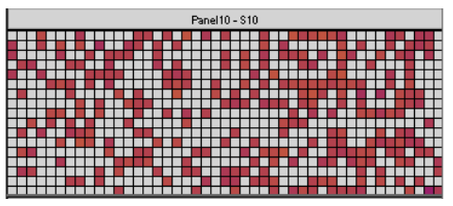


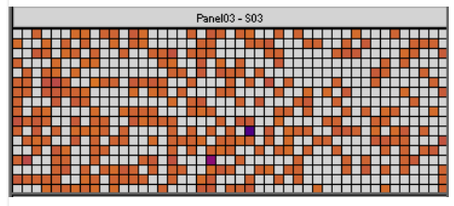


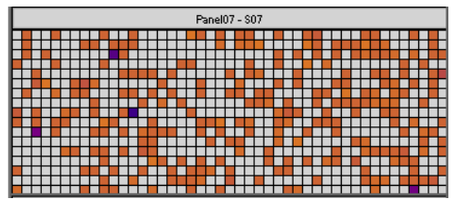


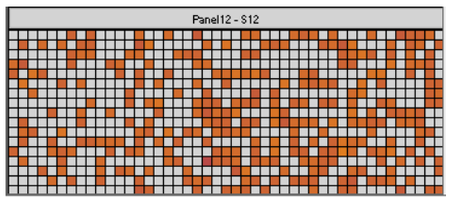




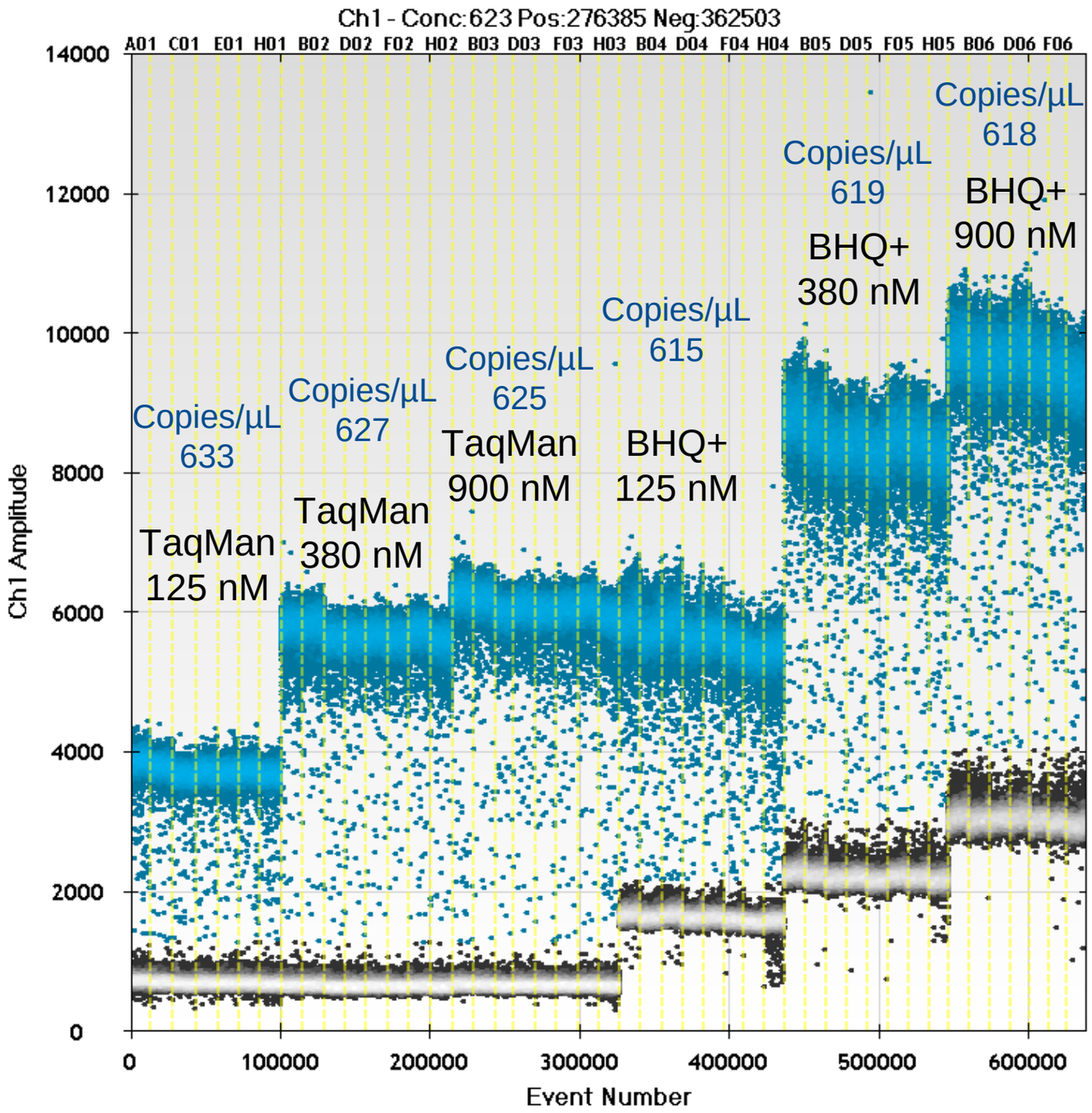








HBB1 ddPCR
Primers (concentration)/Probes (types)



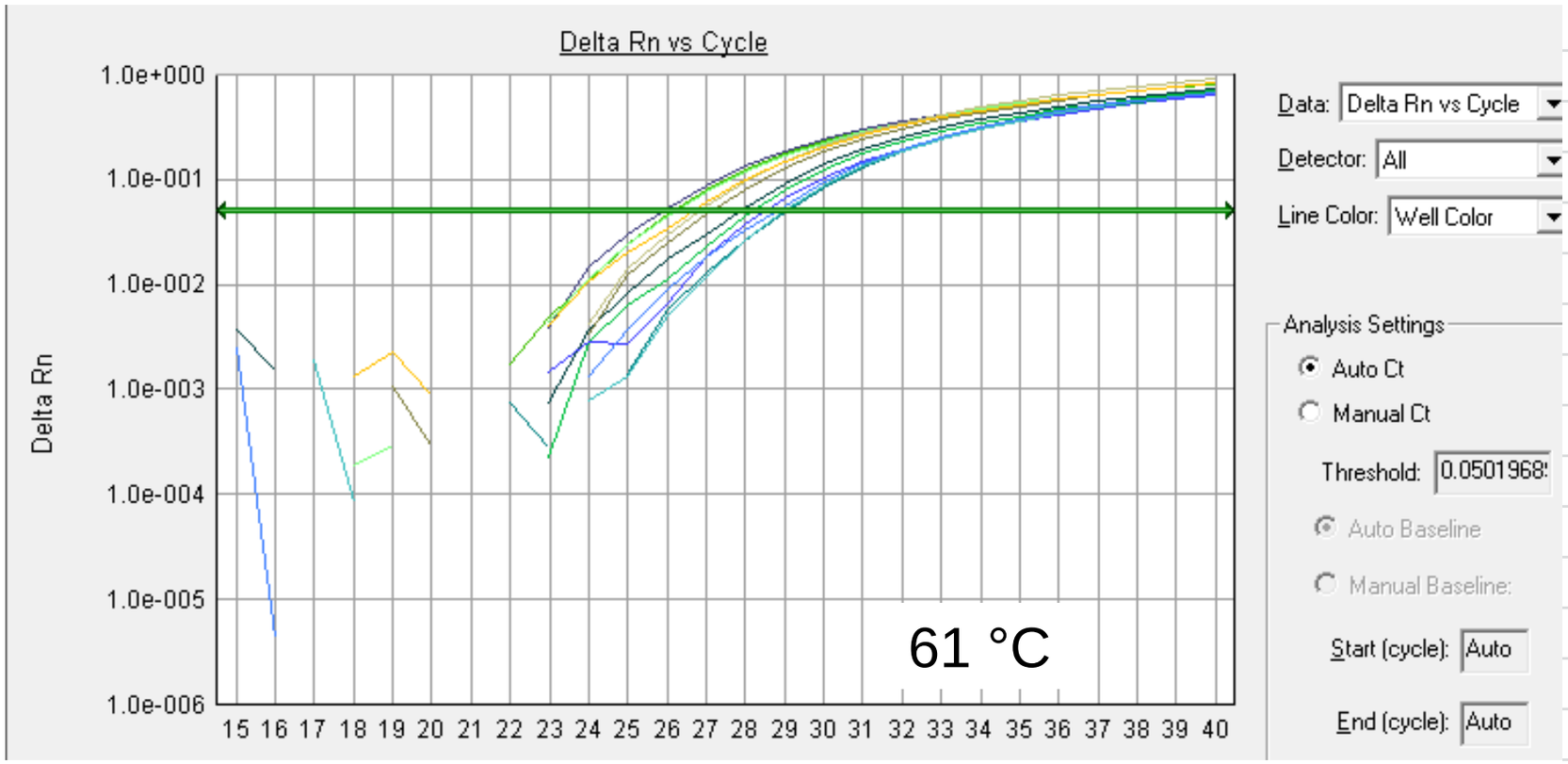
Concentration of the primers varied

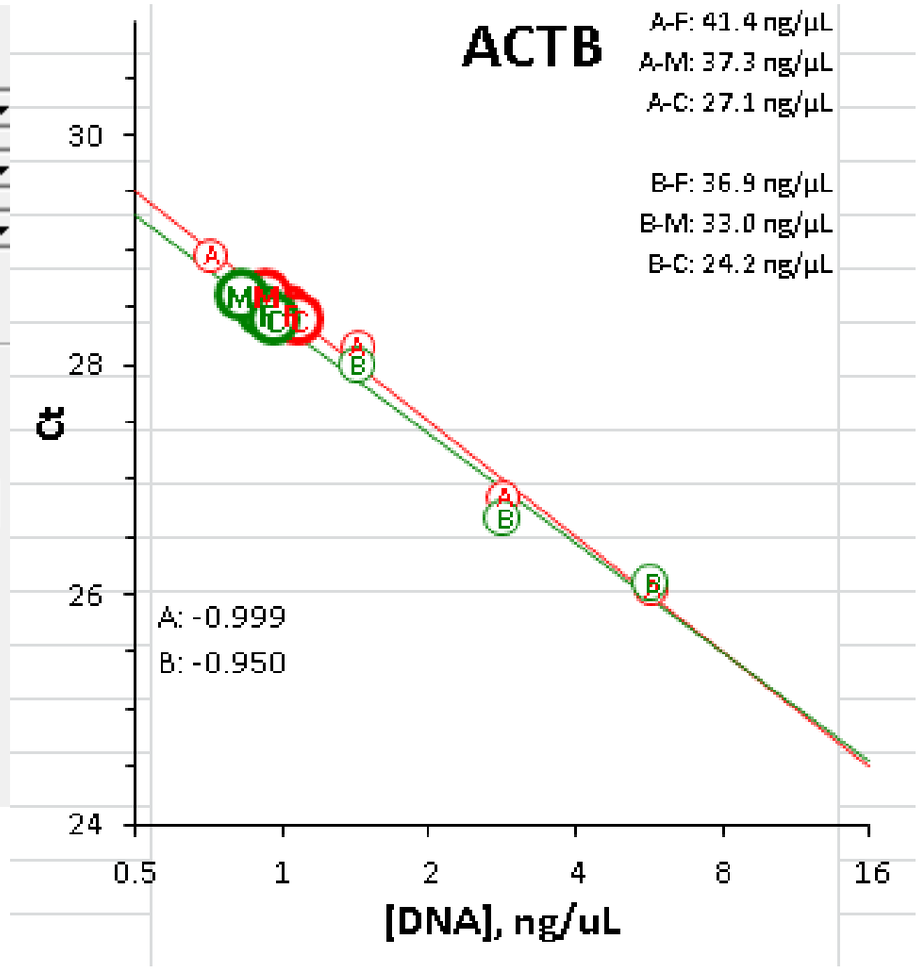
Two different primer pairs

Two different probes used primer specific

While intensity varies final copies/µL do not

ACTB qPCR (actin, beta)



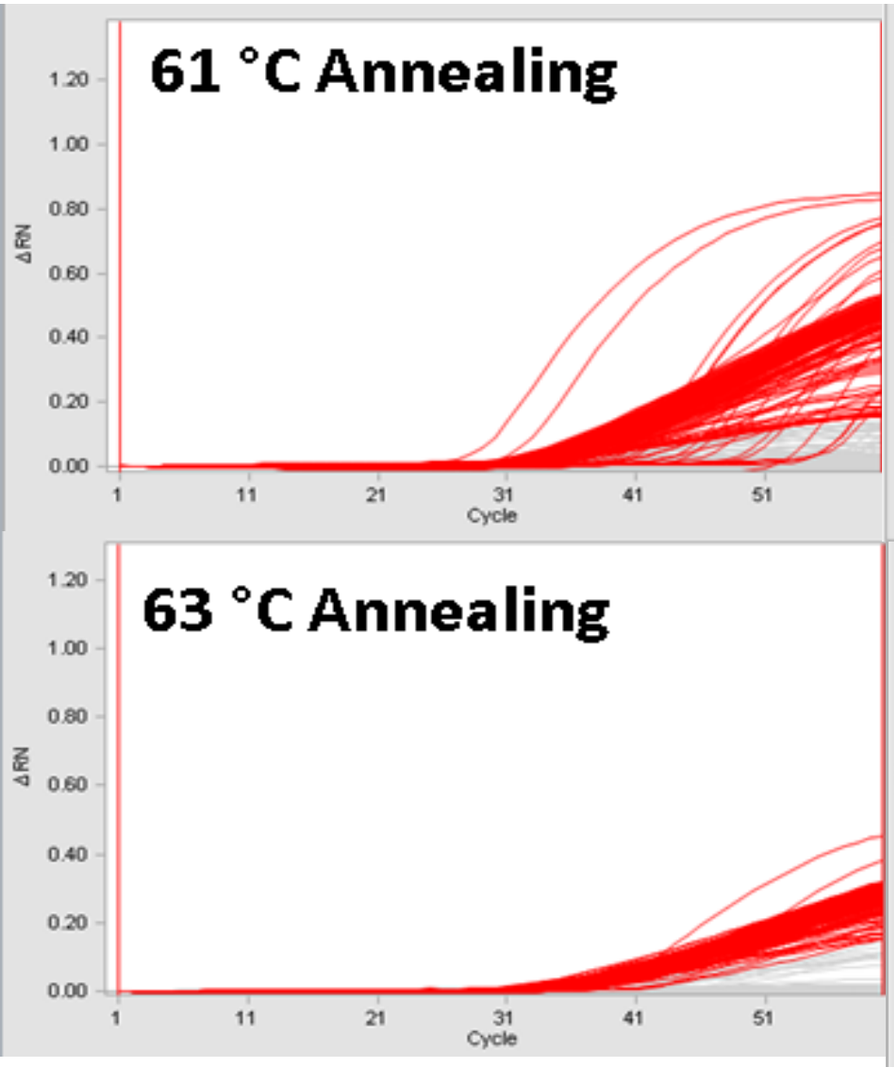


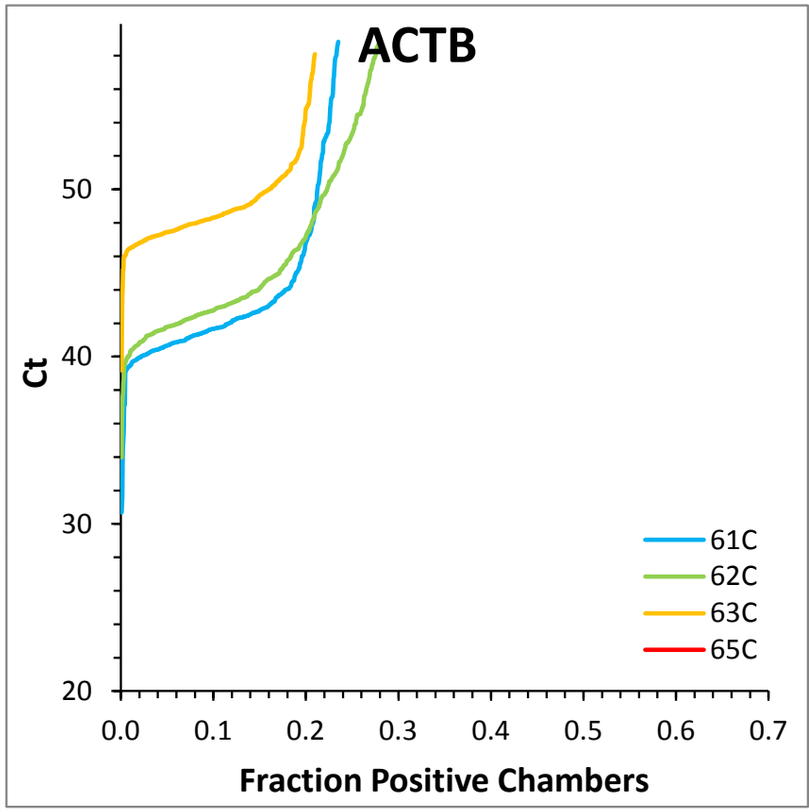
Triplicates of a dilution series of SRM 2372 Component A : 1:10, 1:20, 1:40, and 1:80

1:10 dilution of Component A 26 Cts

Results from 2 different calibrating materials yield similar concentrations for unknown samples

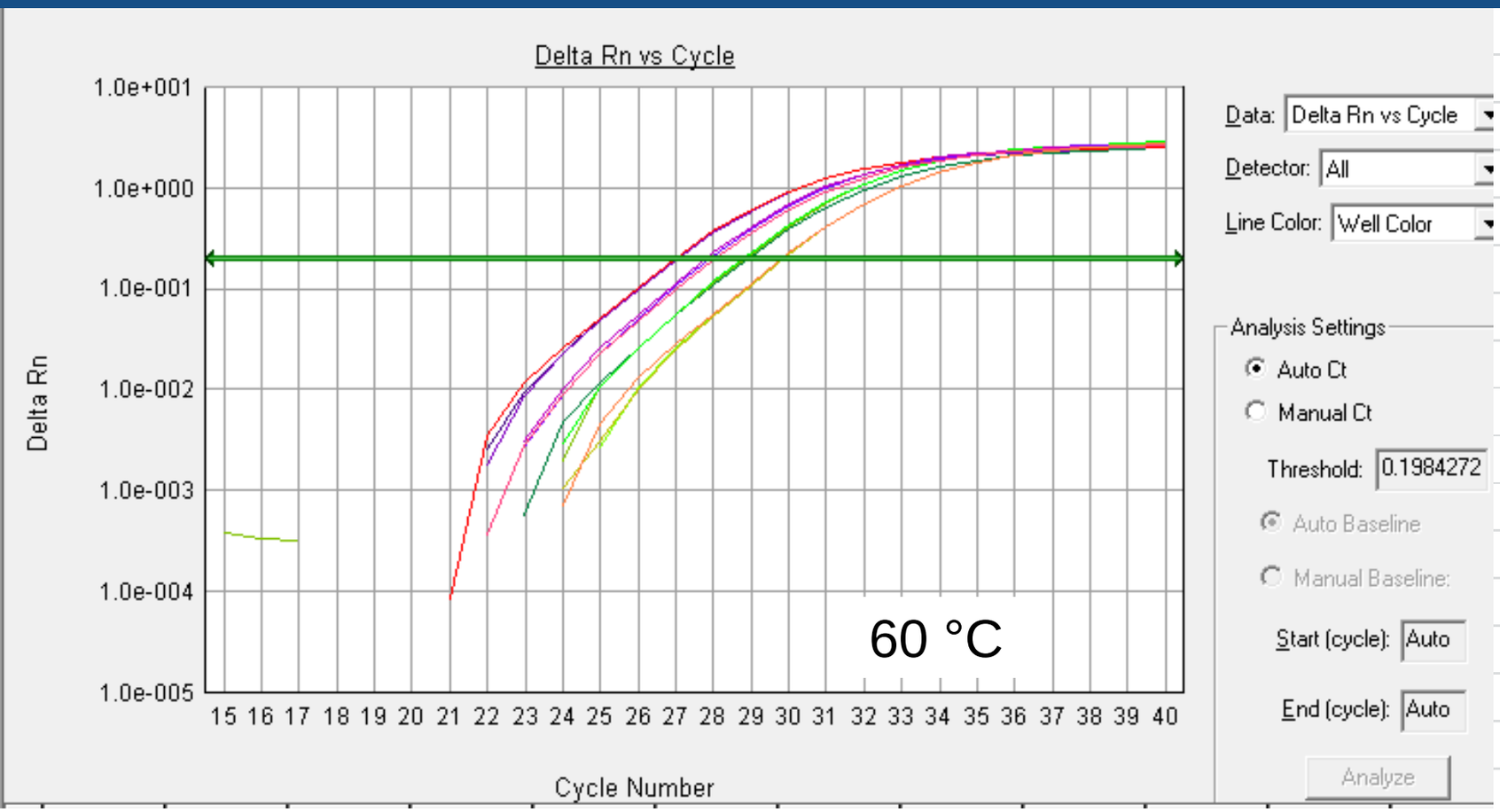
ACTB cdPCR

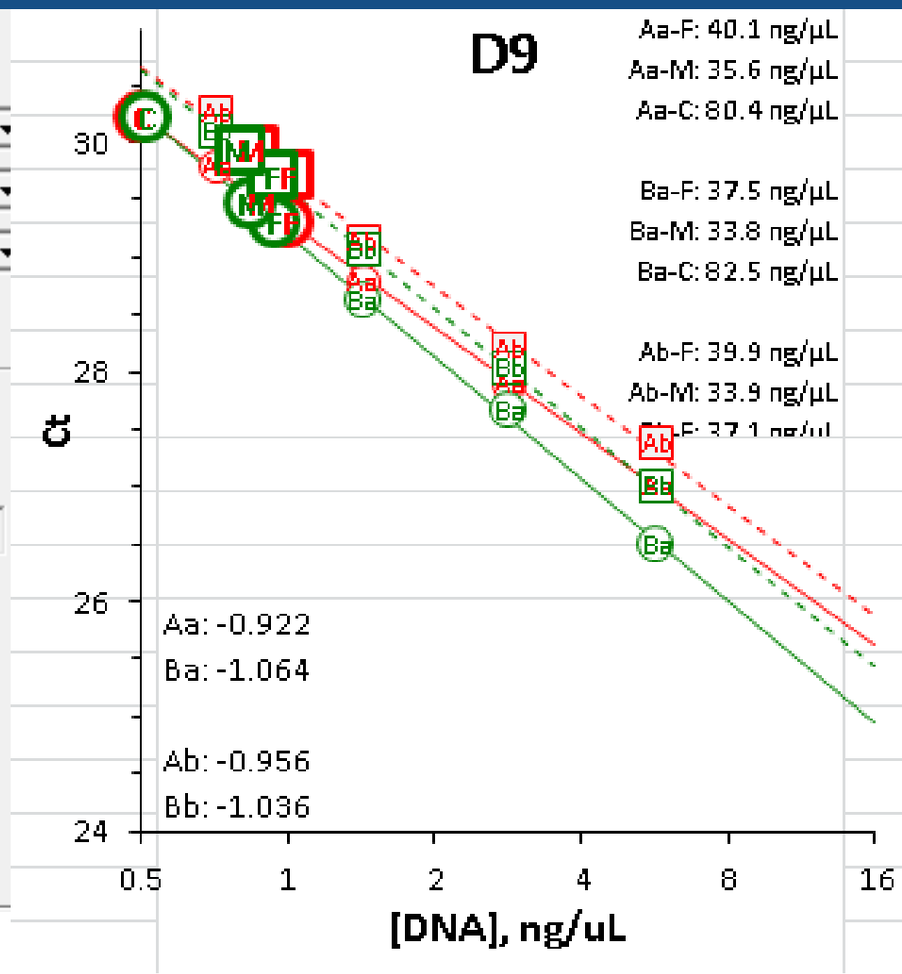




Assay not suitable for cdPCR

D9S2157 qPCR (flanking region)



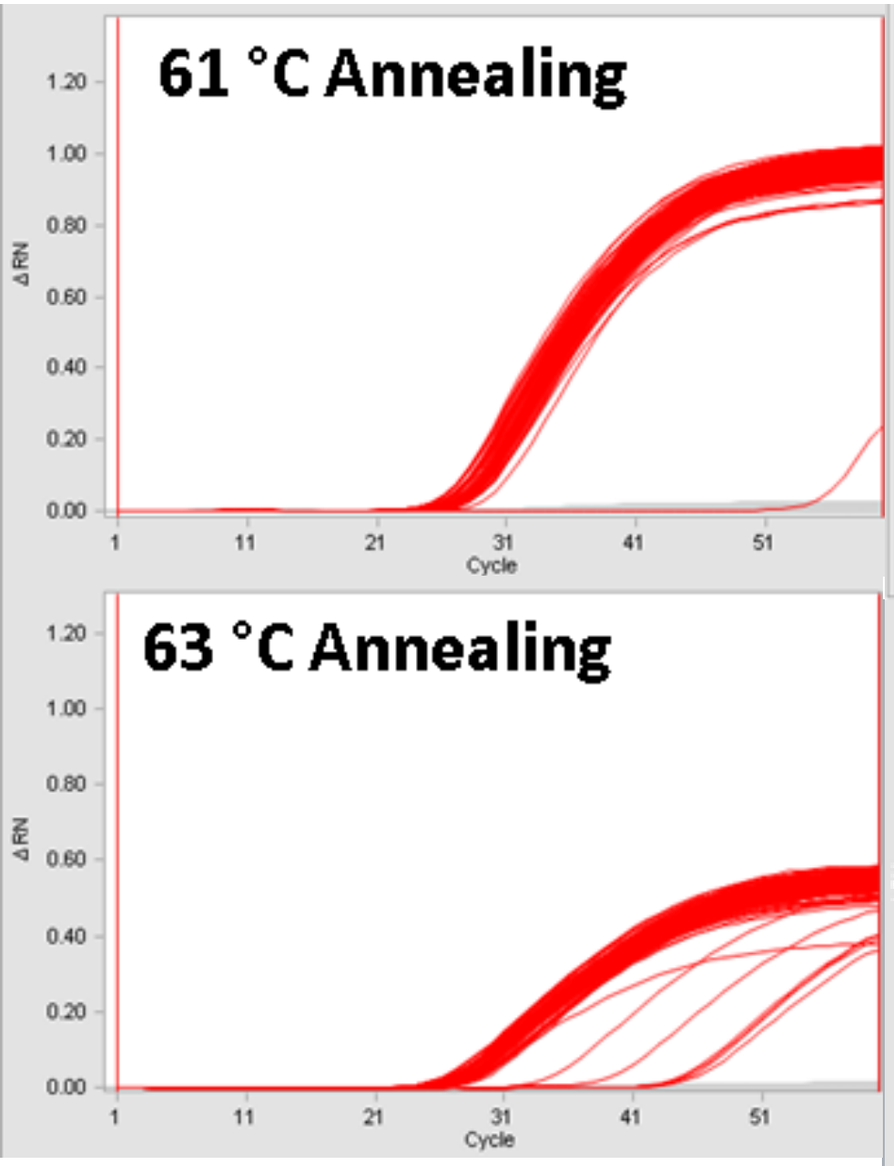


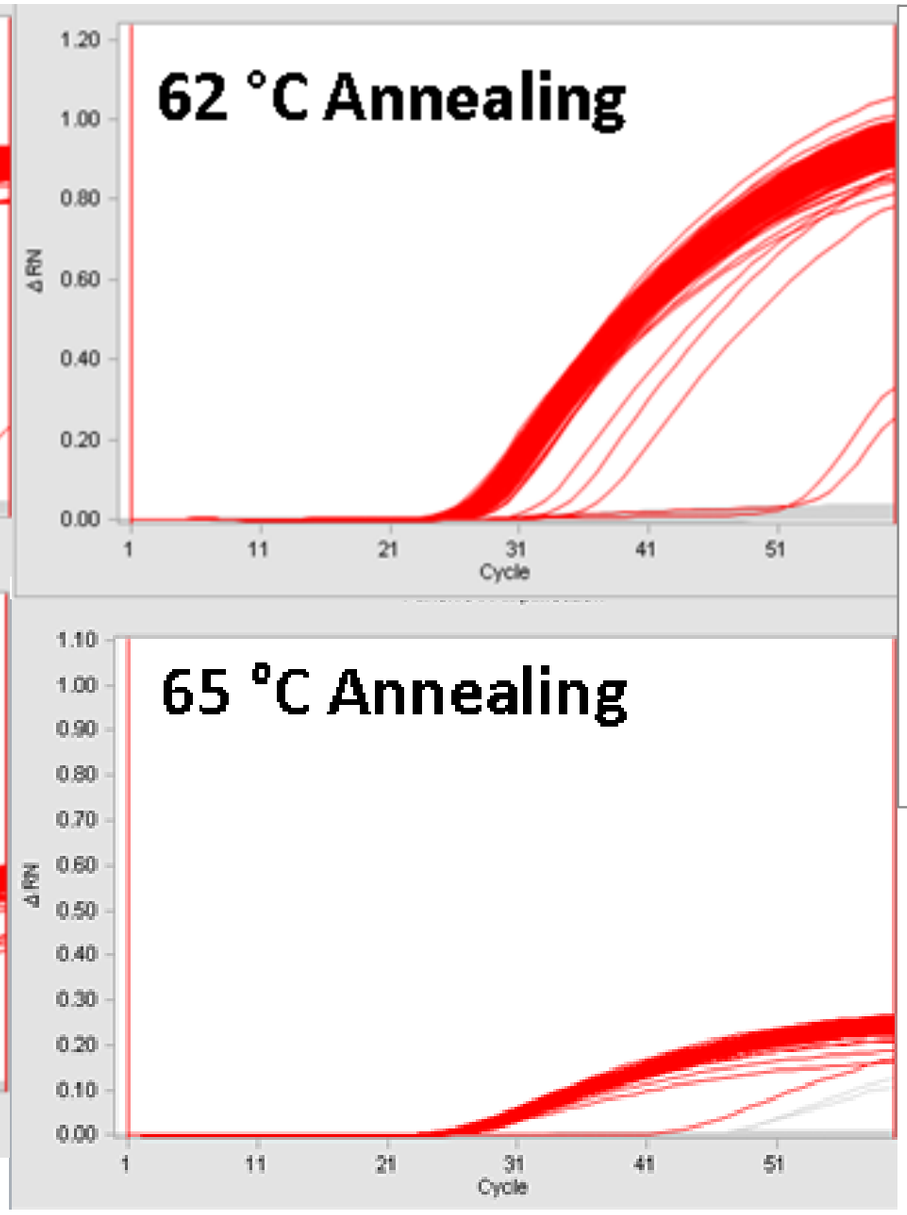
Triplicates of a dilution series of SRM 2372 Component A : 1:10, 1:20, 1:40, and 1:80

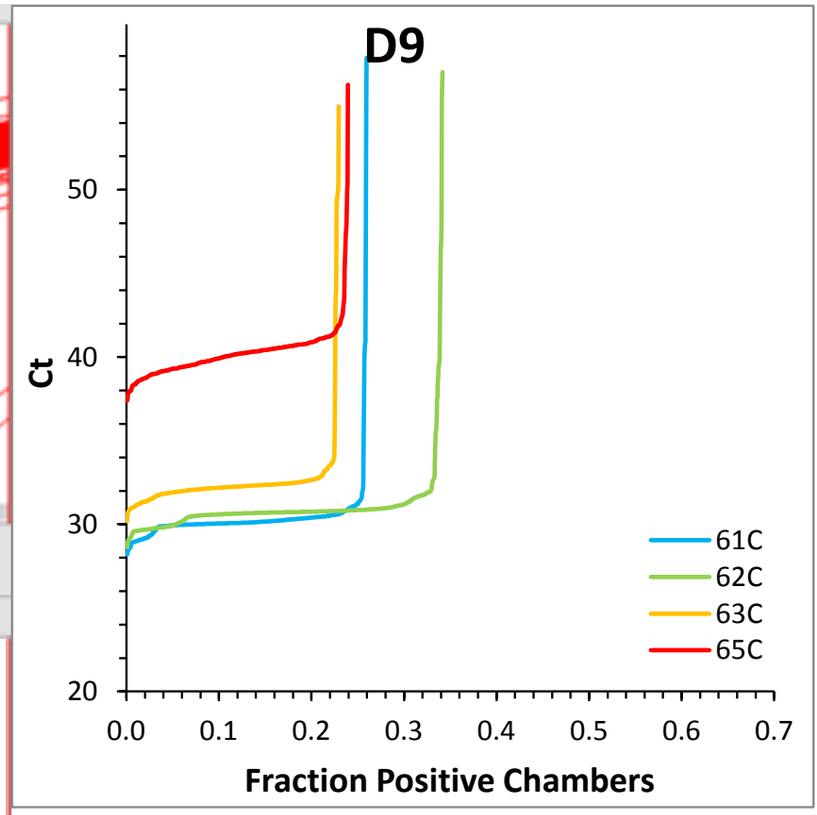
1:10 dilution of Component A 27 Cts

Results from 2 different calibrating materials yield different concentrations for unknown samples

D9S2157 cdPCR





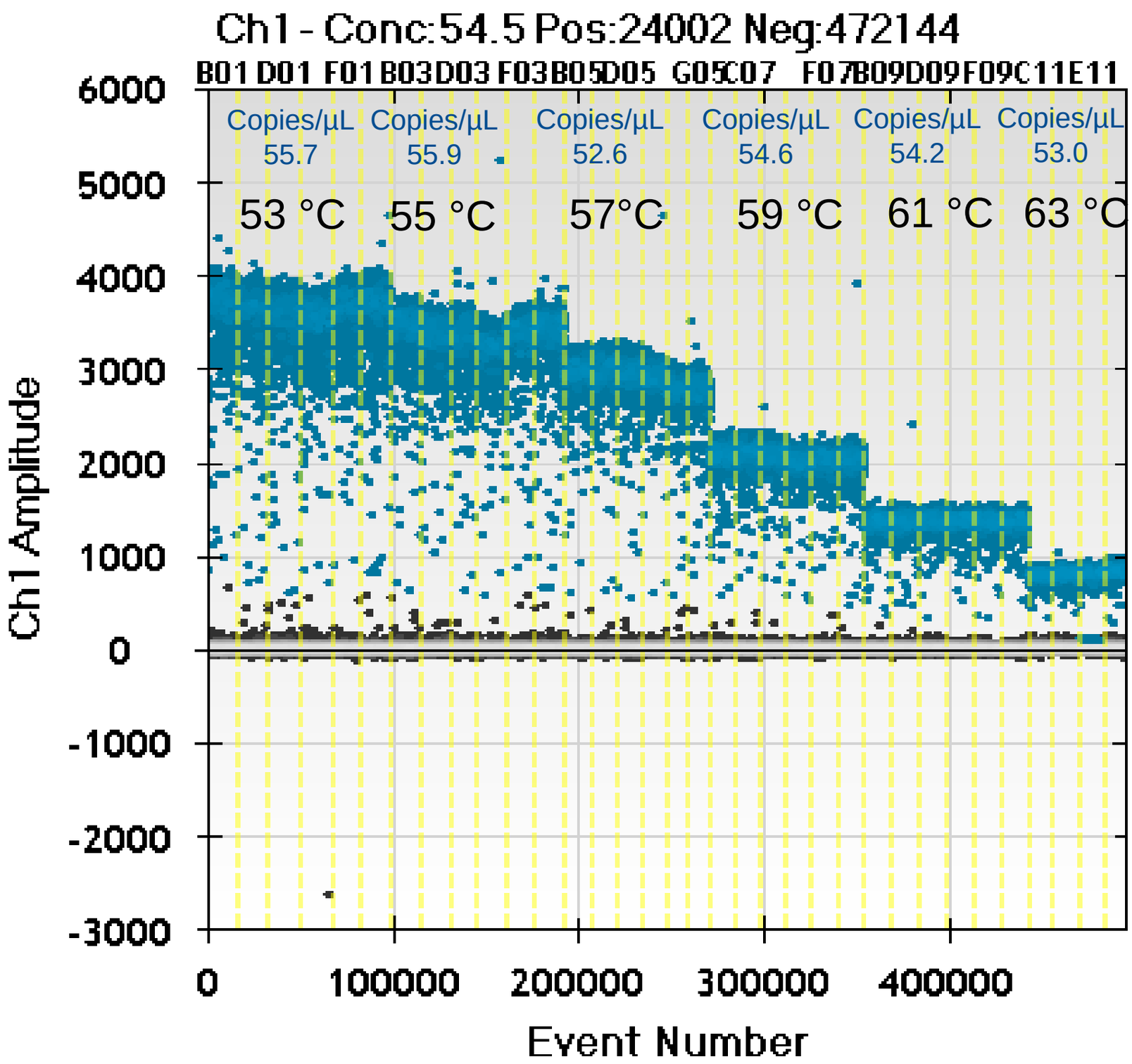


D9 is tracking HBB1 reasonably well with the exception of the 62°C data (to be examined further)
12.765 chip

D9S2157 ddPCR

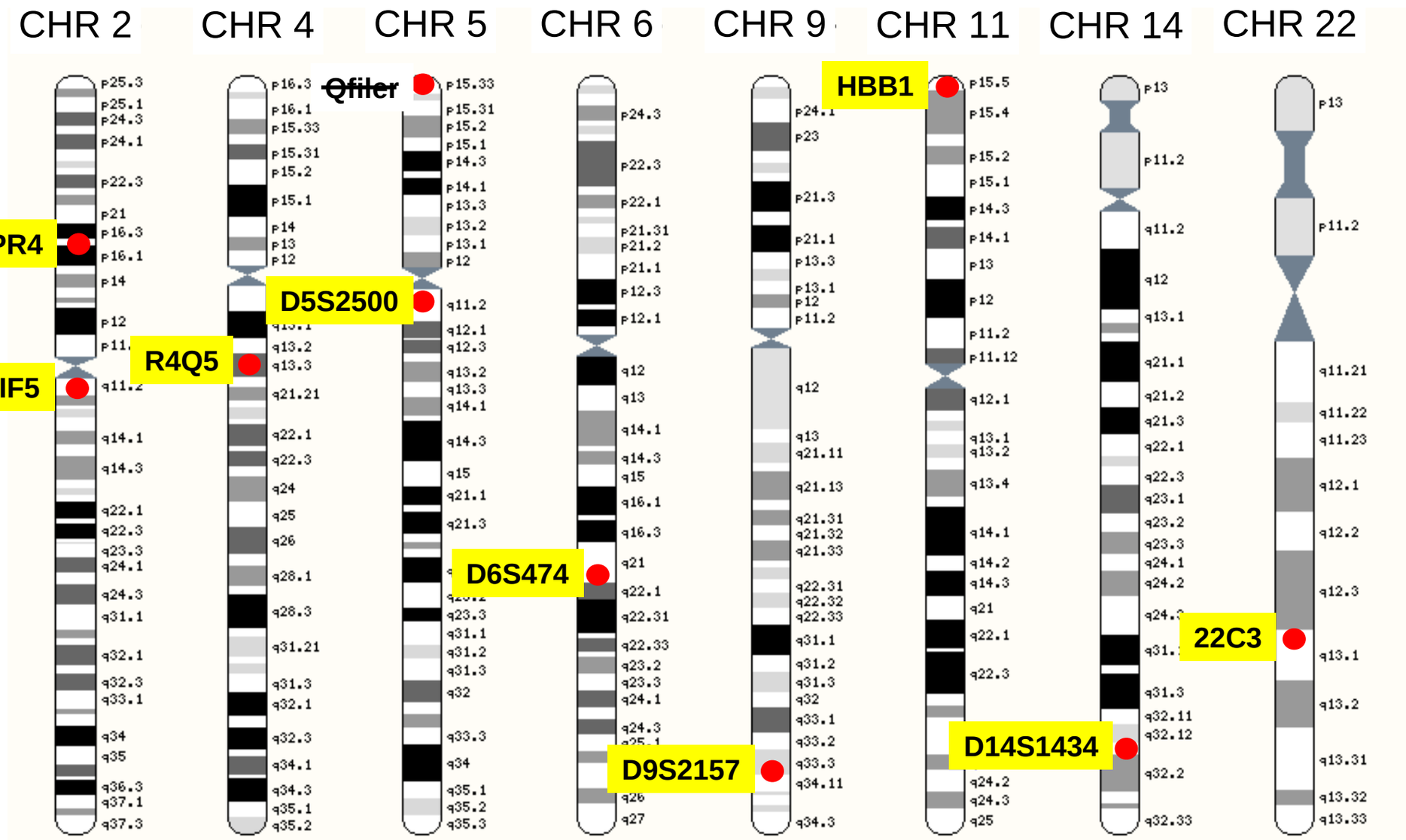
Annealing Temperatures:
53 °C, 55 °C, 57 °C, 59 °C, 61 °C, 63 °C

As the temperature increases there is a decrease in the separation of positives and negatives However these yield the same counts

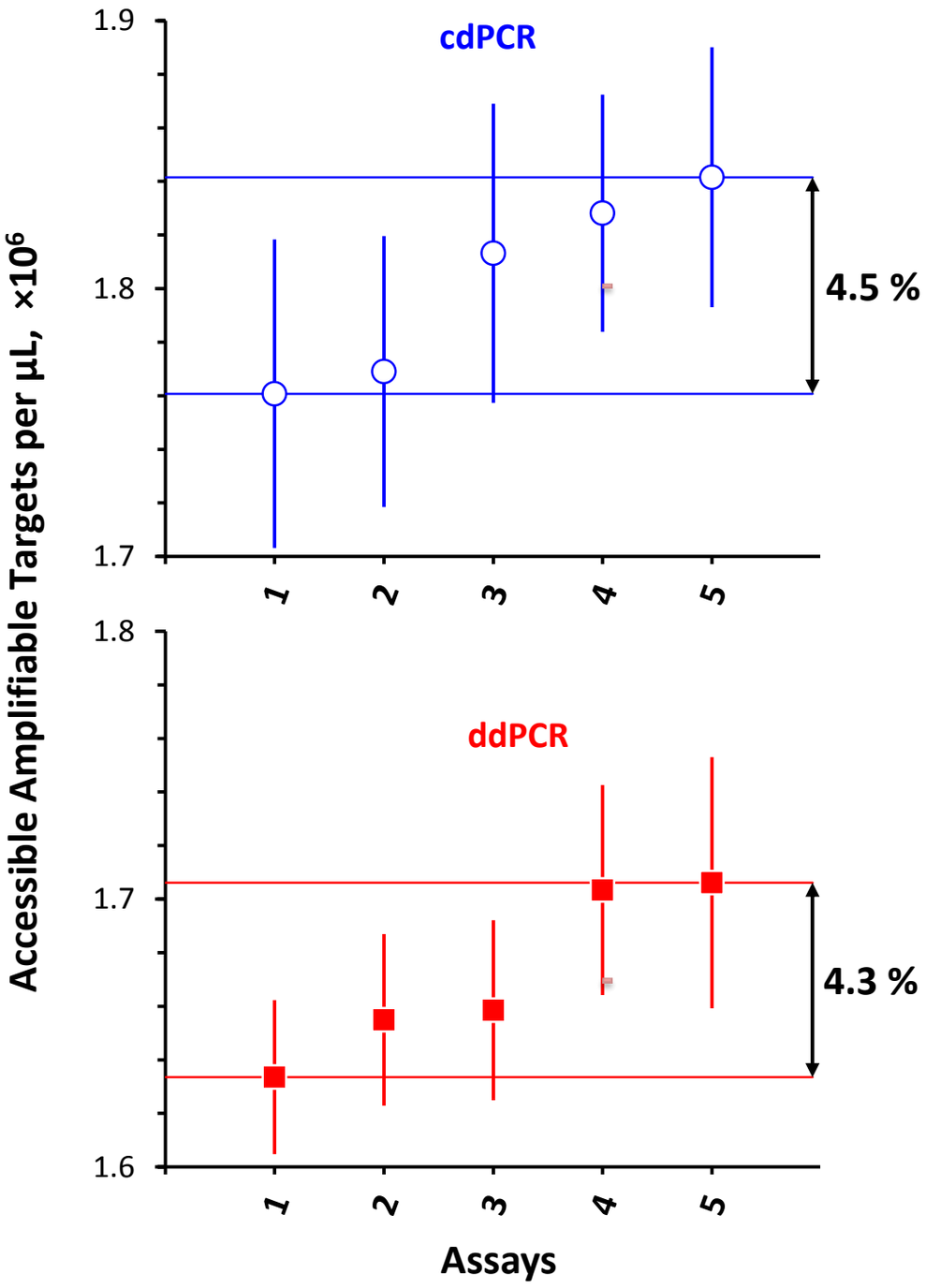


dPCR optimized Assays for SRM 2372a

9 assays spread across 8 different chromosomes
All assays are single copy, and Human, or Primate specific checked in silico at NCBI BLAST website



Bias Between Assays

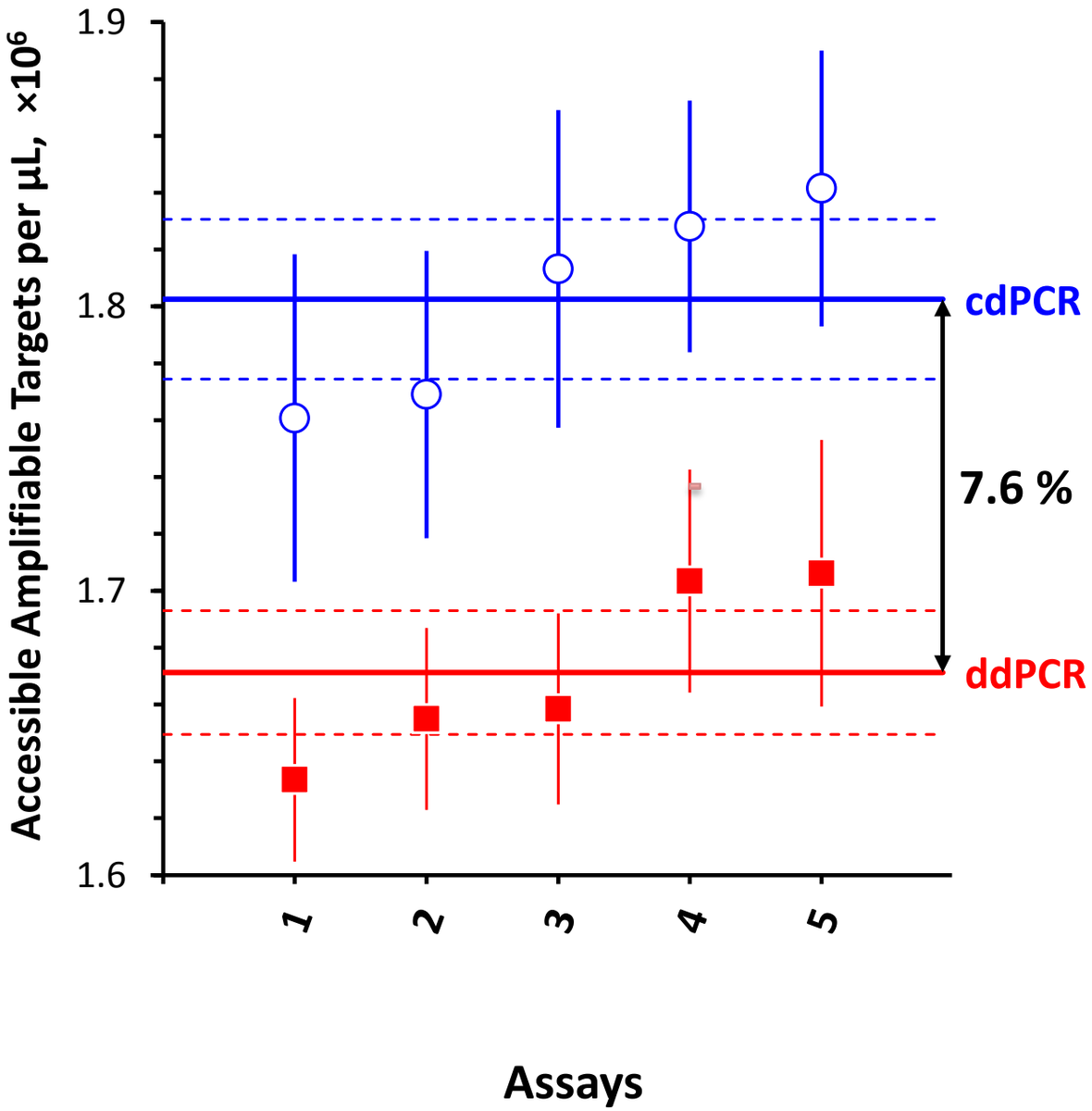


5 assays used for a clinical SRM
≈4.5 % bias between assays
within a platform

Error bars represent ± 1 SD of measurements made over 5 weeks

The thousands of droplets in ddPCR give slightly smaller error bars then cdPCR chambers

Bias Between Platforms



7.6 % Bias between cdPCR and ddPCR averages

One possible source of this bias is the volume estimates of the ddPCR droplets and the cdPCR chip chambers

Volume as a source of Bias

Question:

How well is the volume of chambers or droplets known?

Answer:

Manufacturers give volumes with no estimates of uncertainty

Bio-Rad Droplet: Manufacturer: 0.91 nL

As measured by NMI-A: 0.868 nL ± 2 %

Pinheiro et al. *Anal. Chem.* 2012, 84, 1003-1011

Fluidigm: 6 nL 12 panel Chip

0.85 nL 48 panel Chip

12 panel Chips as measured by NMI-A: (6.0±0.6) nL & (5.6±0.6) nL

Bhat et al. *Anal. Bioanal. Chem.* 2009, 394, 457-467

Our colleagues in the Physics Laboratory here at NIST are working on measurements of the ddPCR droplets and the cdPCR 48 panel chip

Backdoor Volume determination

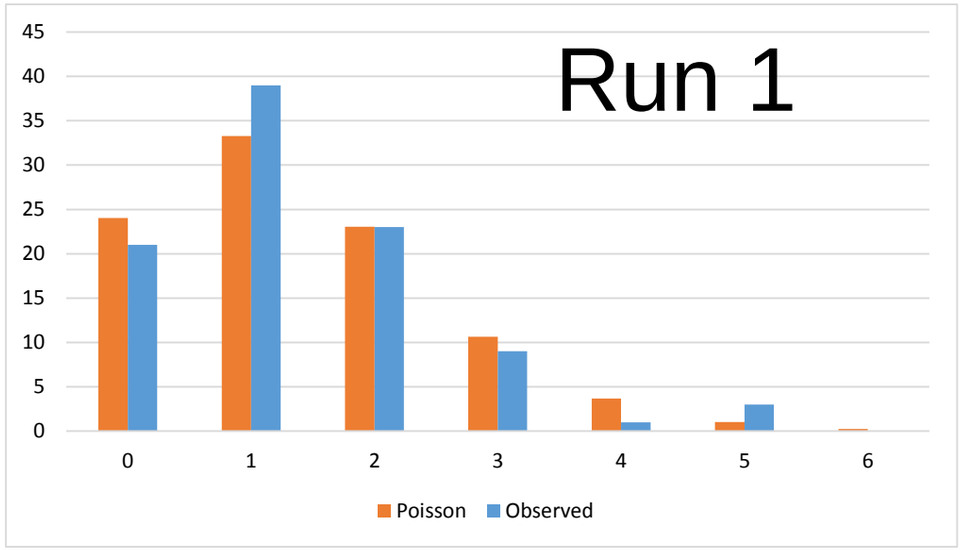
- Take a material where you know the copies/µL
- Analyze that material on both dPCR platforms
- Determine the volumes of the platforms based on the known copies/µL material
- Wait Wait Wait a minute is that not what we are trying to determine with dPCR?
  - …but if we had an assay system that uses 96-well plates
  - …where we can directly measure all the volumes
  - …we can calibrate the ddPCR and cdPCR volumes!

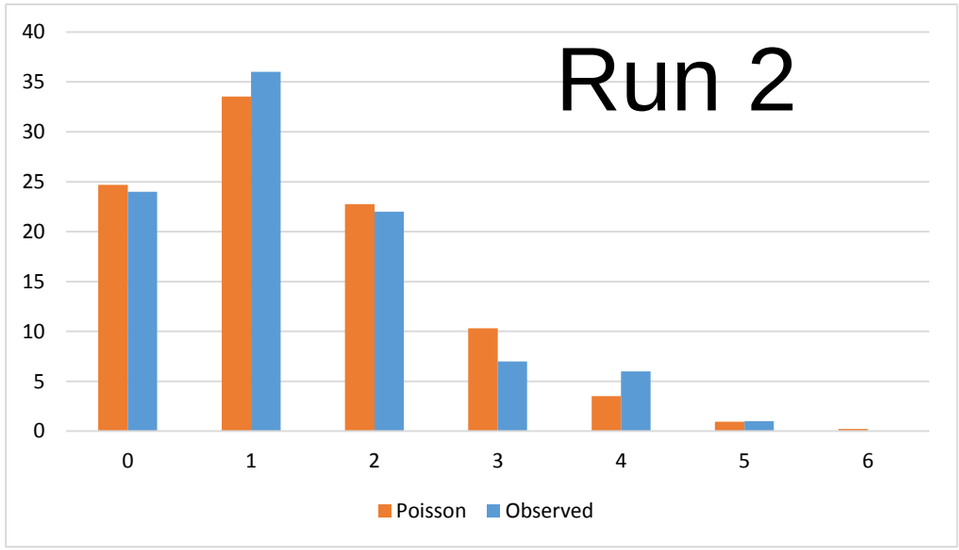
Counting PCR (cPCR)

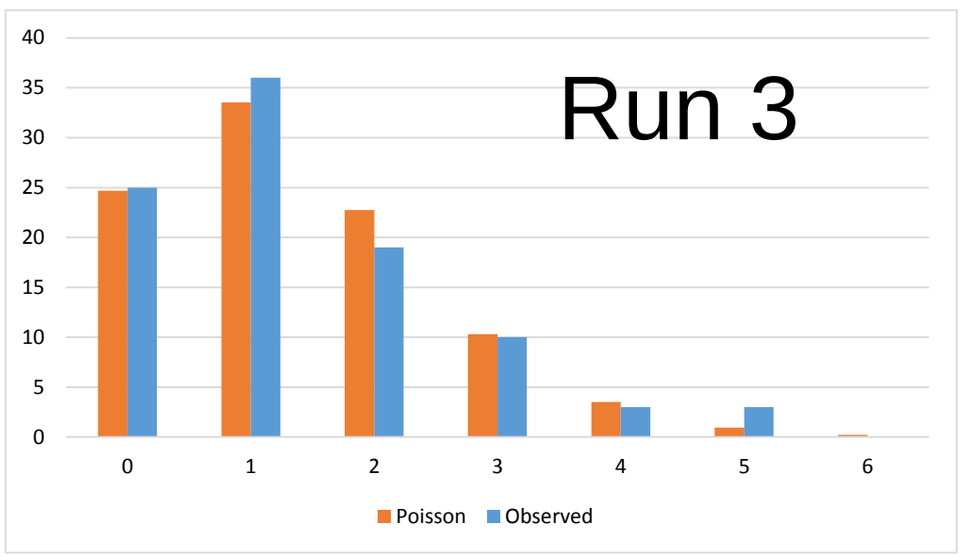
- cPCR developed by dnasoftware (Ann Arbor, MI)
- Uses data from all cycles of a qPCR instrument
- “qPCR CopyCount” software
- Calibration uses a 96-well plate
  - ≈ 1.5 targets/well
  - 60 amplification cycles
- We can determine the uncertainty associated with these volumes! (2 to 20) µL
- How reproducible is it?

CopyCount Calibration Data

	Run 1	Run 2	Run 3
Mean copy #	1.3854	1.3579	1.3579
Calibration error %	8.7	8.8	8.8







Method appears reproducible
We are still working on this

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