The use of microfluidic and droplet-based digital PCR platforms for DNA quantitation

Standardisation of Genome Amplification Technologies Meeting
29 May 2014

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National Institute of Standards and Technology
U.S. Department of Commerce

Background on Digital PCR

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9236–9241, August 1999
Genetics

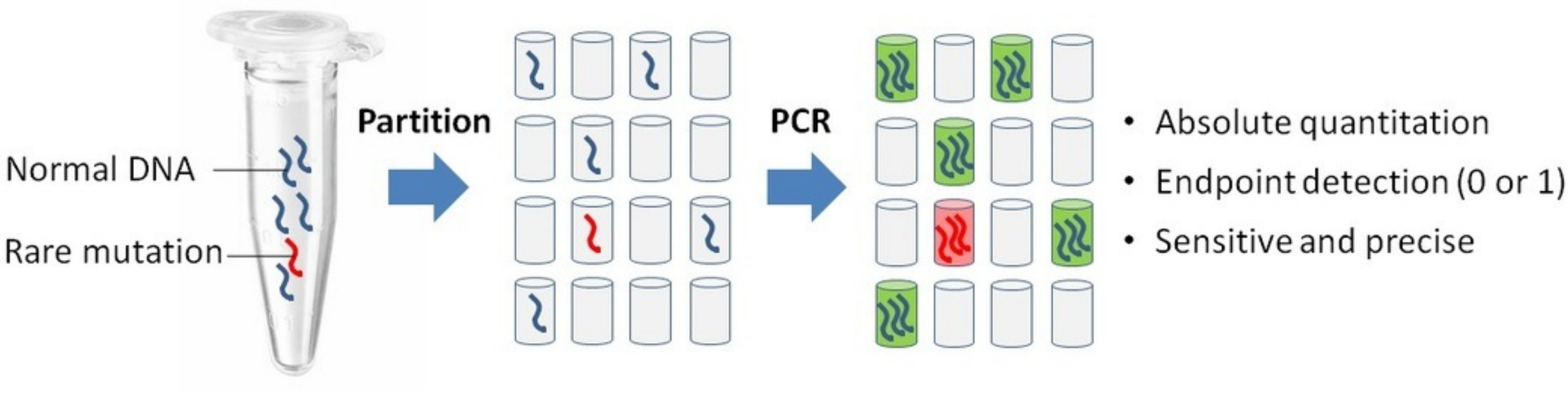
1st paper on digital PCR

Digital PCR

BERT VOGELSTEIN* AND KENNETH W. KINZLER

The Howard Hughes Medical Institute and the Johns Hopkins Oncology Center, Baltimore, MD 21231

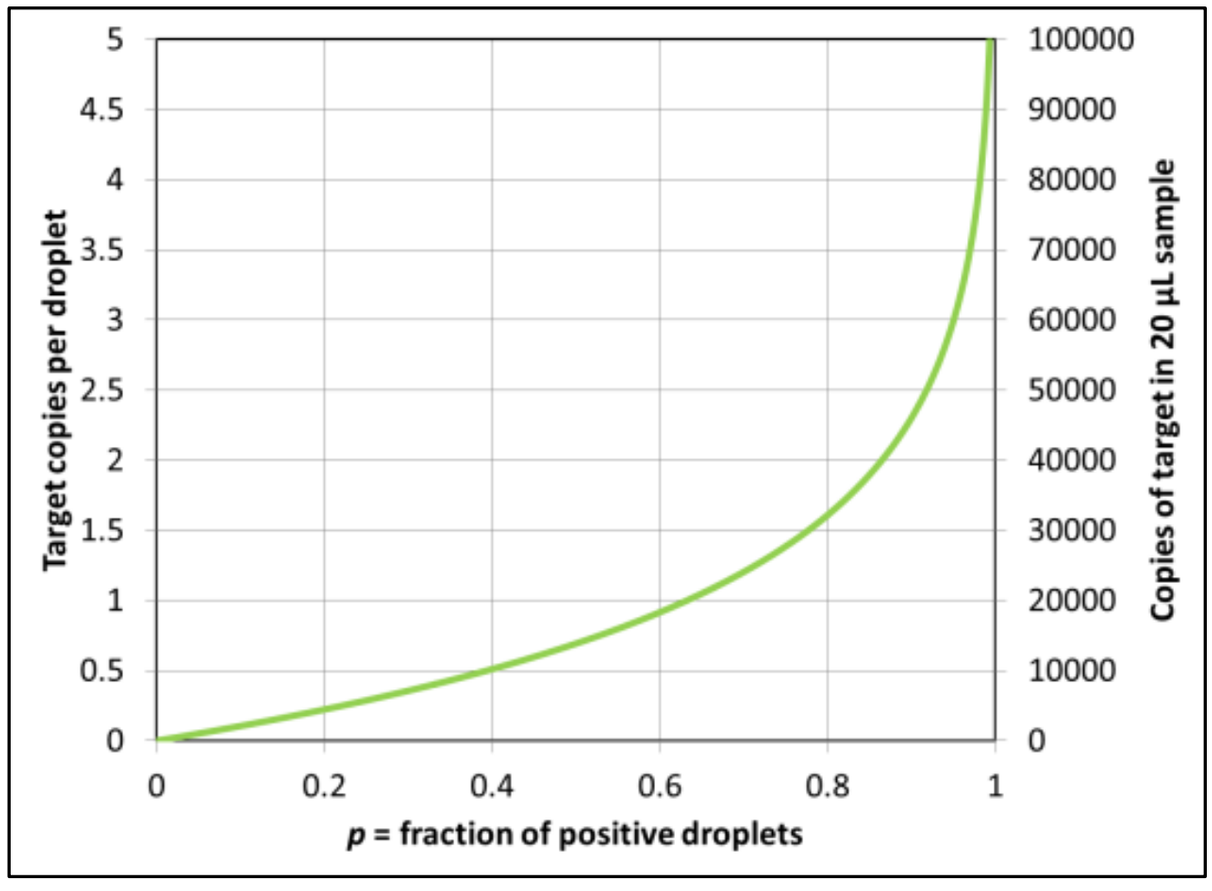
- Diluted DNA is partitioned into many volumes, ideally containing 0 or 1 template, thermalcycled, and ***accessible*** targets are counted



Background on Digital PCR

- Poisson statistics are used to determine an starting DNA copy number without the use of a calibrant

- Derived units, **counts per *µ*L**, are traceable to the SI unit, count <u>one</u> and the <u>meter</u> (1 L = 10^{-3} m^3)



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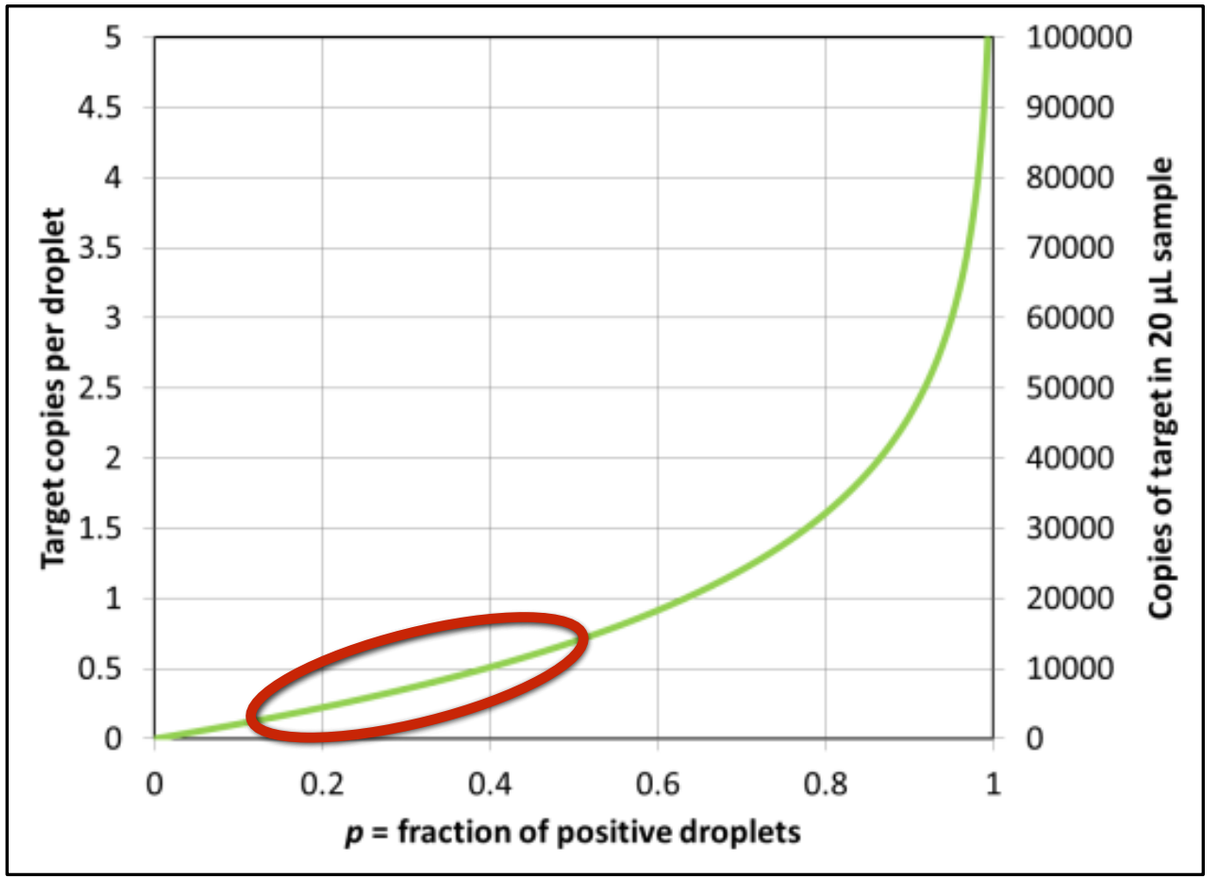


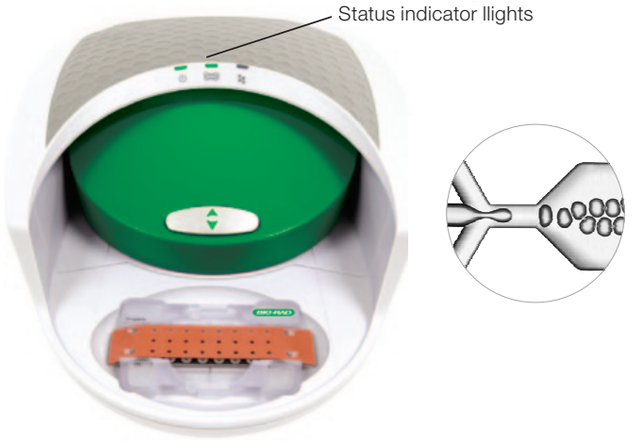
Linearity between ~10-50 % positive droplets/chambers increases accuracy of measurement by decreasing the standard error

Droplet digital PCR (ddPCR)

QX100/200 by Bio-Rad Laboratories

- 96 well PCR plate; 10,000-20,000 droplets per sample
  - Bio-Rad estimates: {0.89, 0.91, 1.0} nL droplets
  - NMI Australia: 0.868 +/- 2 % nL droplets
- end-point DNA quantitation

Fill Cartridge & Generate Droplets



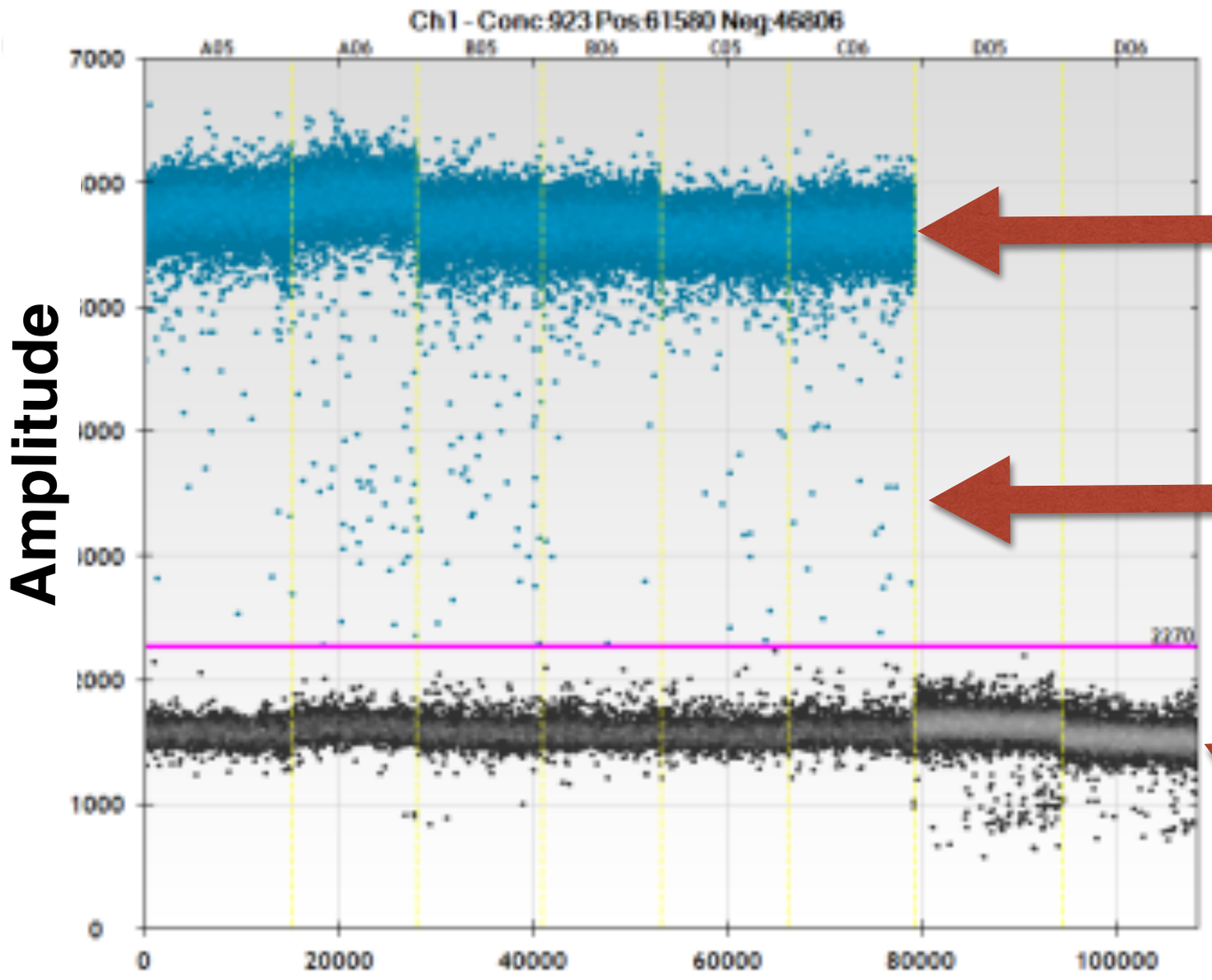
Thermal cycle

Read Droplets

ddPCR Data Output

samples

NTCs



positive droplets

"rain" = positive droplets: "late starters"

manual threshold

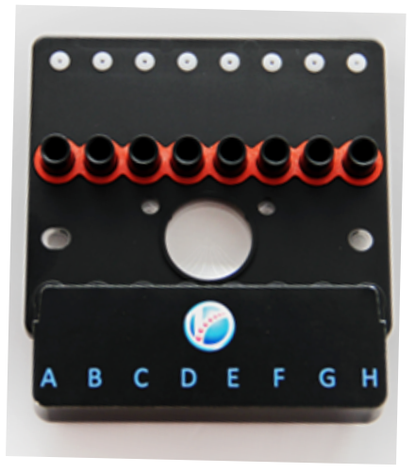
negative droplets

Droplet digital PCR (ddPCR)

RainDrop™ System by RainDance Technologies

- 8 panel chip
- up to 1 million droplets per sample — advantageous for detecting rare mutations
- real-time data collection and droplet size estimation during droplet generation

Fill chip with samples **Generate Droplets** **Thermal cycle & Read Droplets**

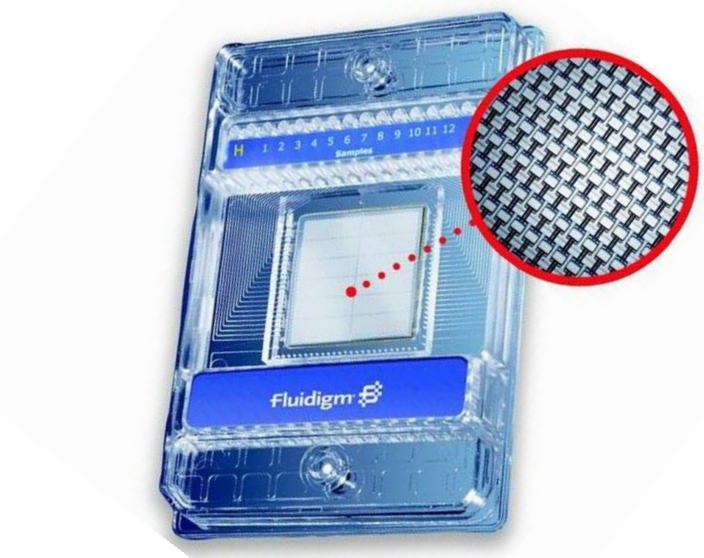


Microfluidics: chamber digital PCR (cdPCR)

Biomark^{TM} HD System by Fluidigm Corporation

- 12x765 (6 nL) or 48x770 (0.85 nL)
  - 9,180 or 36,960 chambers
- Real-time data collection at every PCR cycle

Fill chip with samples

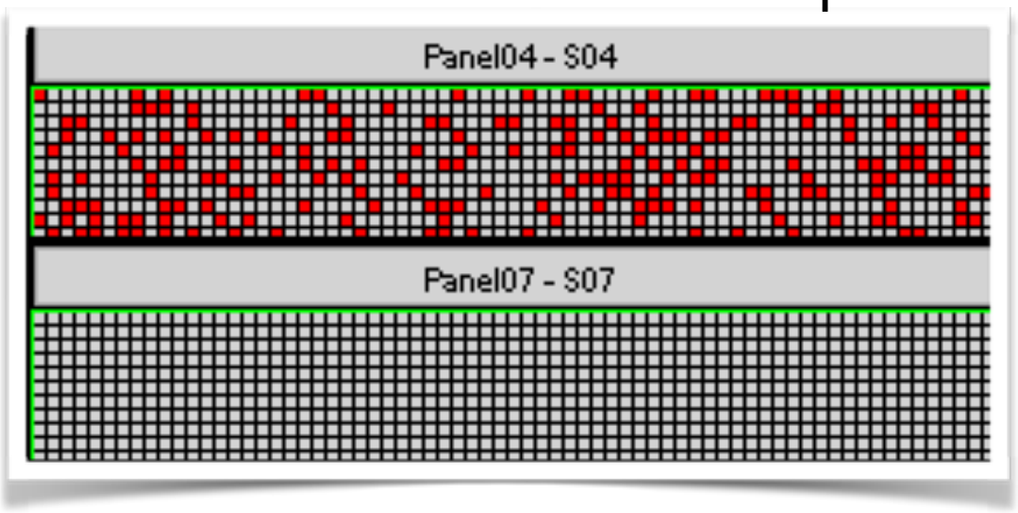


Load samples into chambers

Thermal cycle

cdPCR Data Output & Analysis

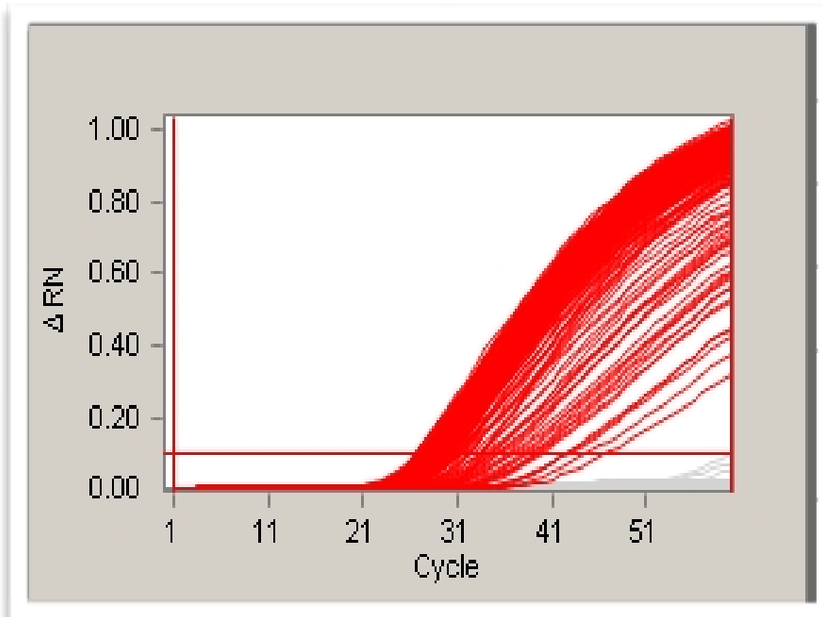
chamber heat maps



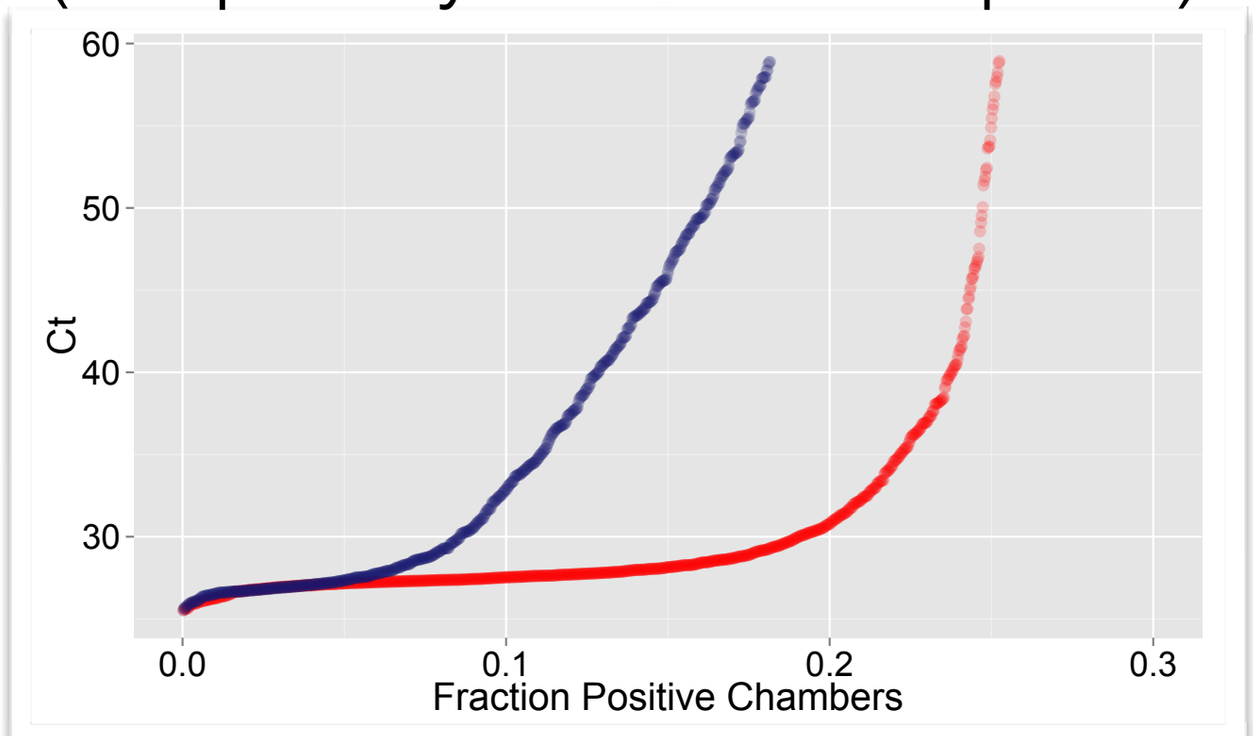
sample

NTC

real-time PCR curves



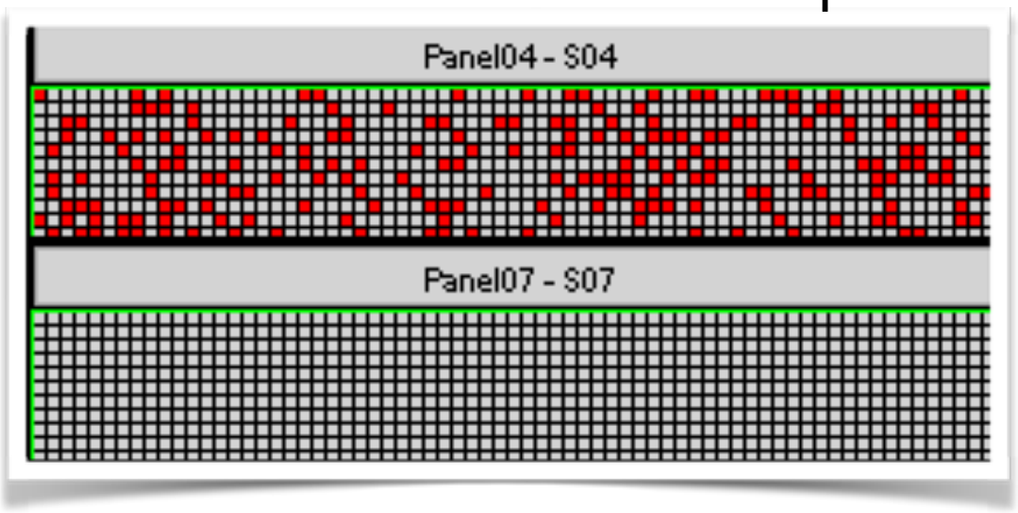
ogives (frequency distribution plots)



(Jo Lynne Harenza, Dave Duewer)

cdPCR Data Output & Analysis

chamber heat maps



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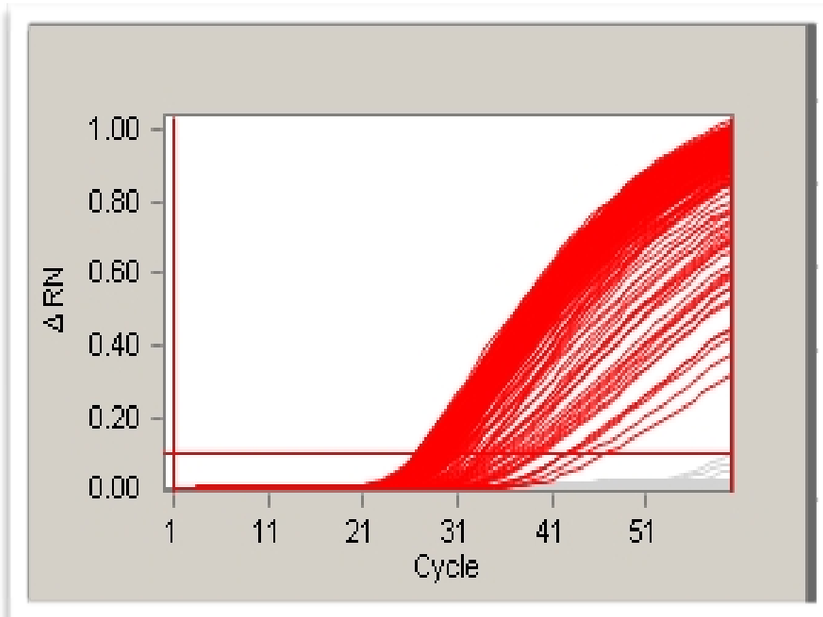

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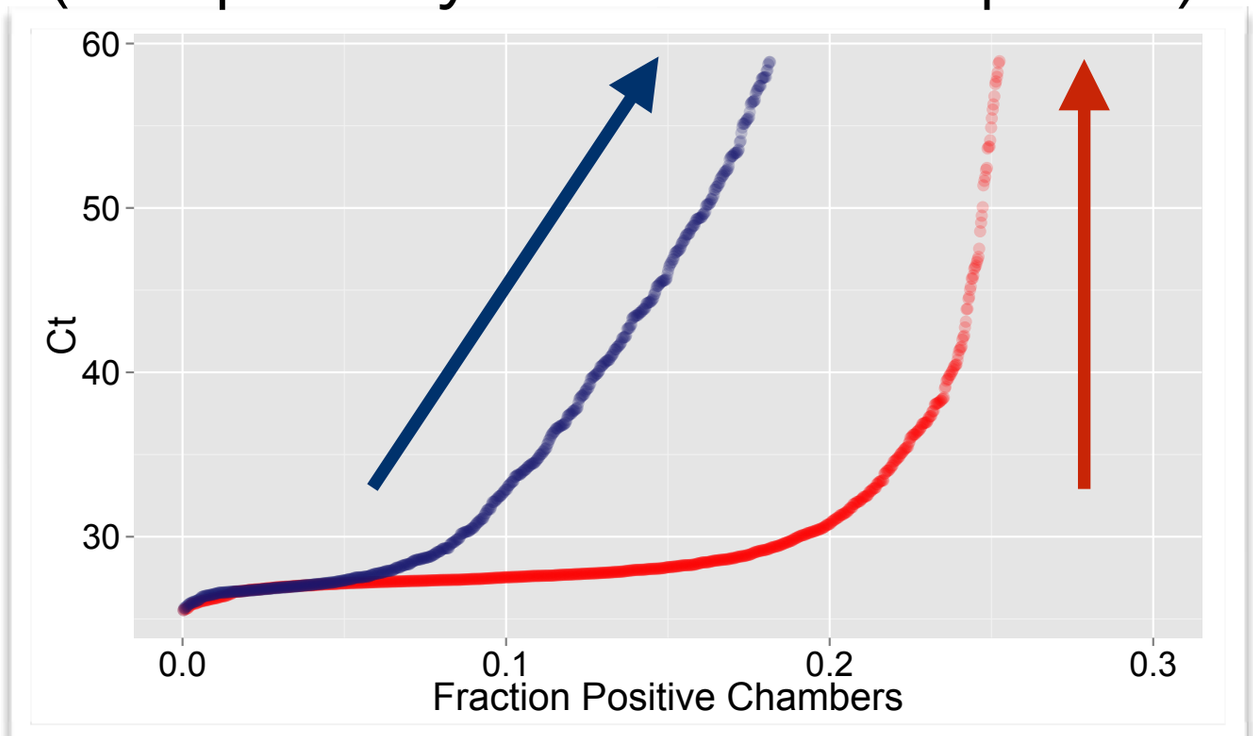


(Jo Lynne Harenza, Dave Duewer)

Interesting Findings

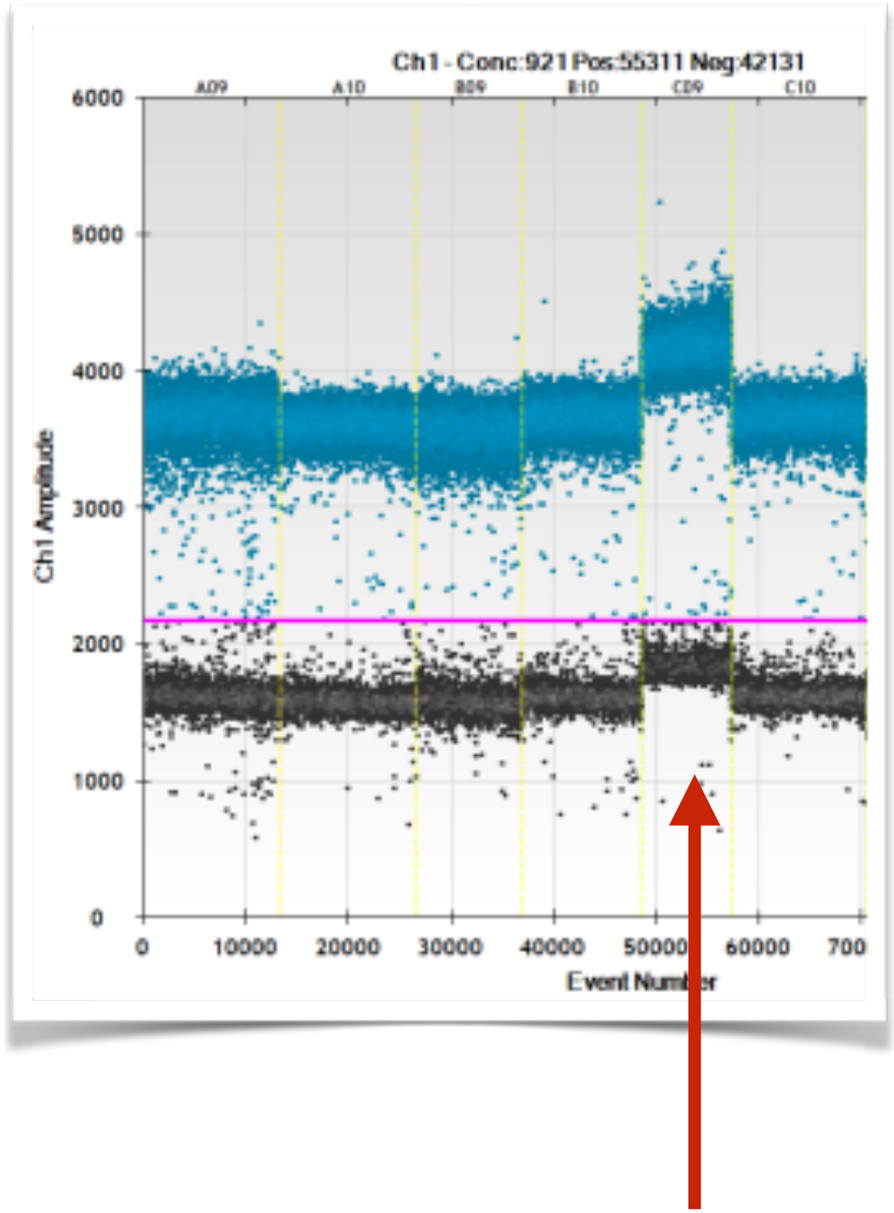
- Artifacts on the Bio-Rad ddPCR system
- Assay Optimization: qPCR v. digital PCR
- Plasmid DNA: digested v. supercoiled plasmid DNA
    - Attempt to relax supercoiled plasmid DNA with DMSO
- Assays with multiple targets are revealed with dPCR
- Master mix contamination

Artifacts of ddPCR

- **Shifting** of positive and negative droplets

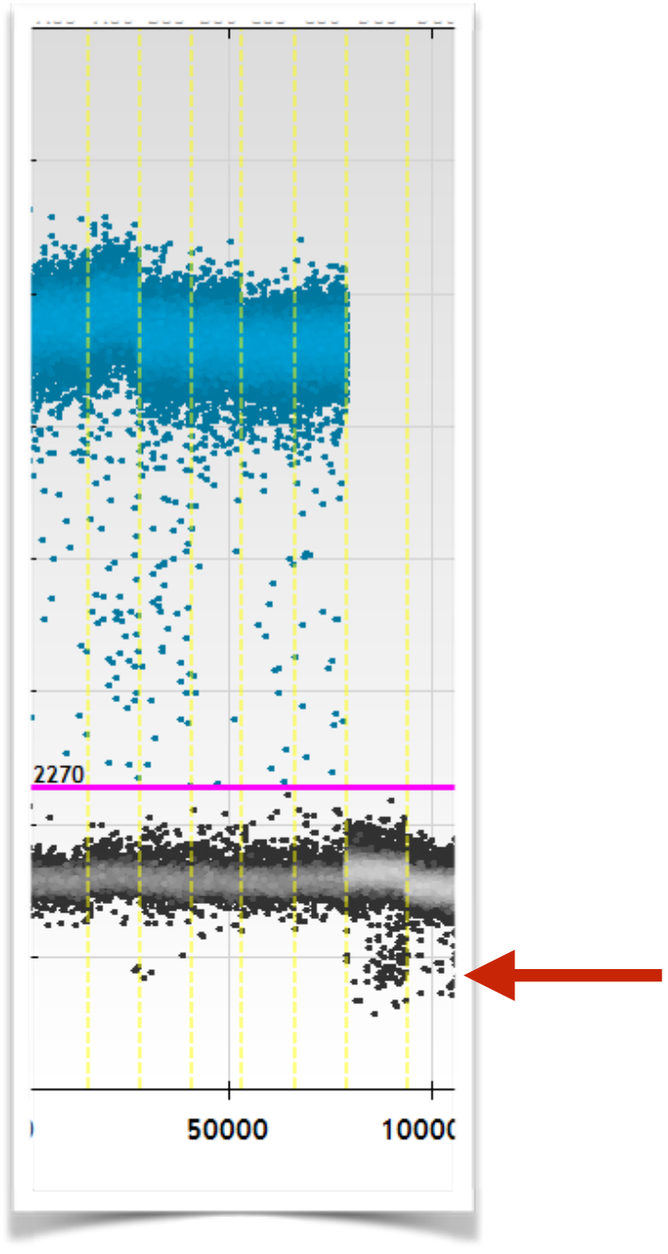
  - Larger than average size droplets generated - caused by a problem with the first generation of cartridges

- Results in concentration inflation — omit samples from analysis



Artifacts of ddPCR

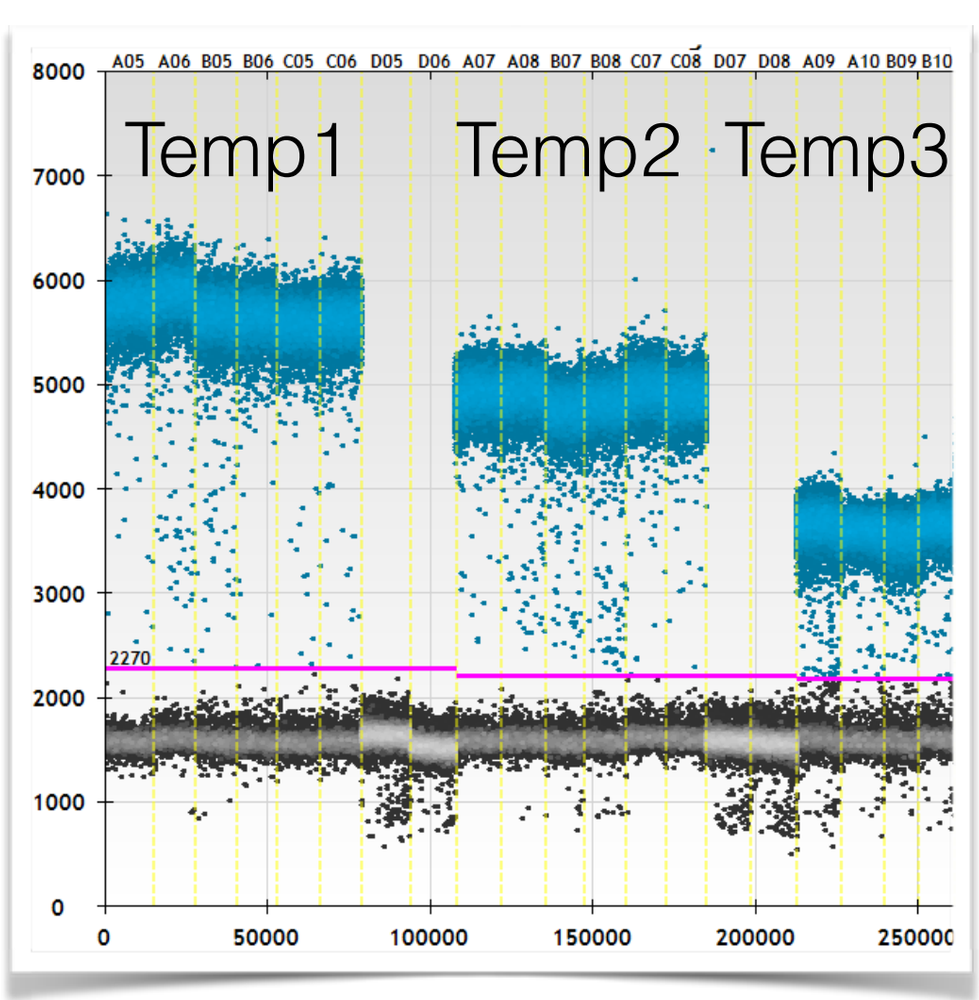
- Droplet **shearing**
  - Happens during pipetting of droplets — either into the cartridge or into the PCR plate
  - Can occur when bubbles in the sample mixture rise within the tip and burst/shear droplets
  - affect?

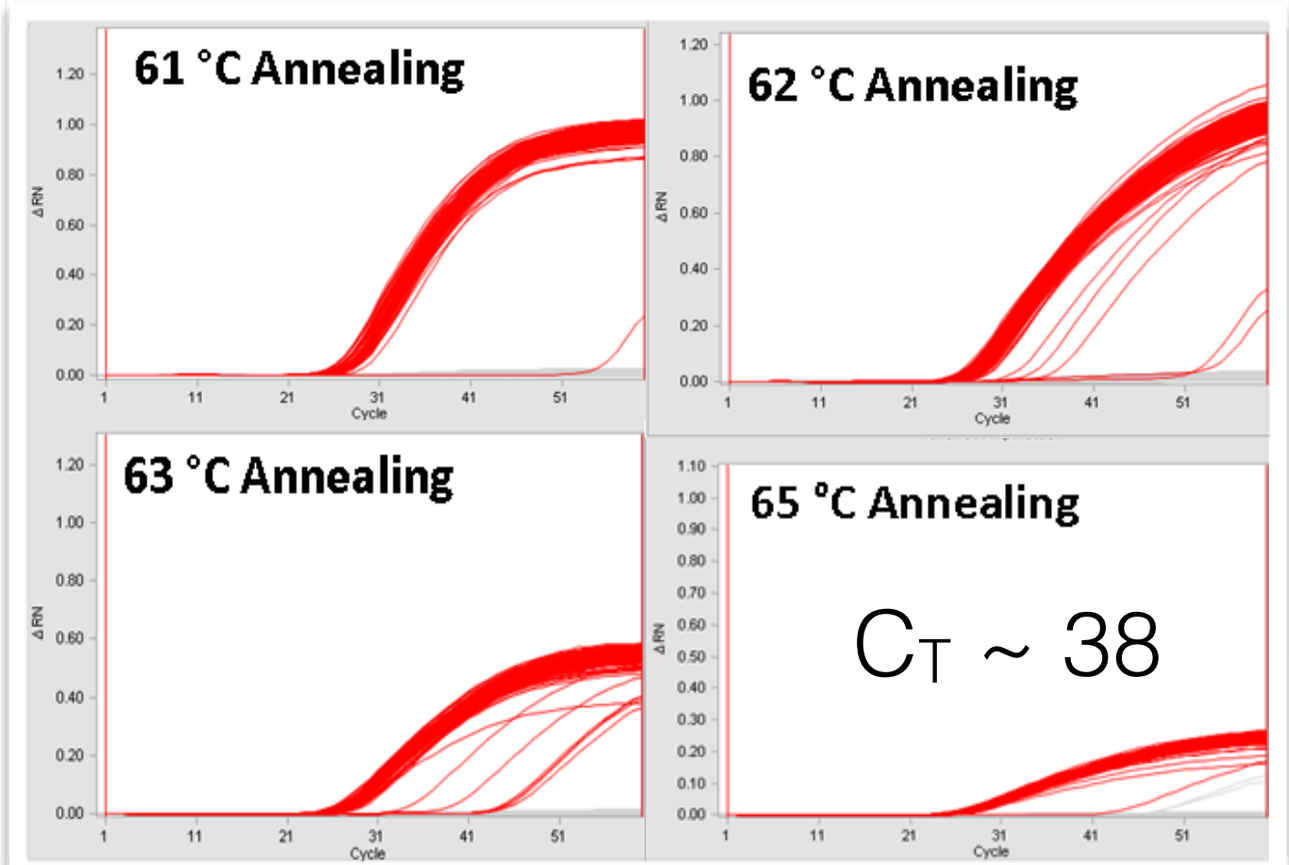


Temperature gradients are still required to optimize dPCR assays

- For ddPCR, want separation of positive and negative droplets

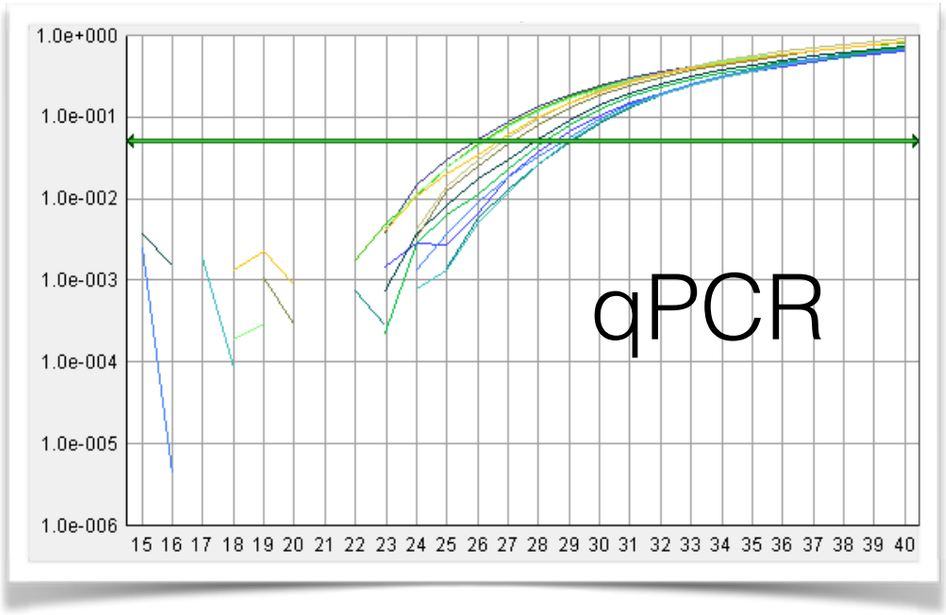
- For cdPCR, want efficient amplification at a reasonable C_T and good curve morphologies



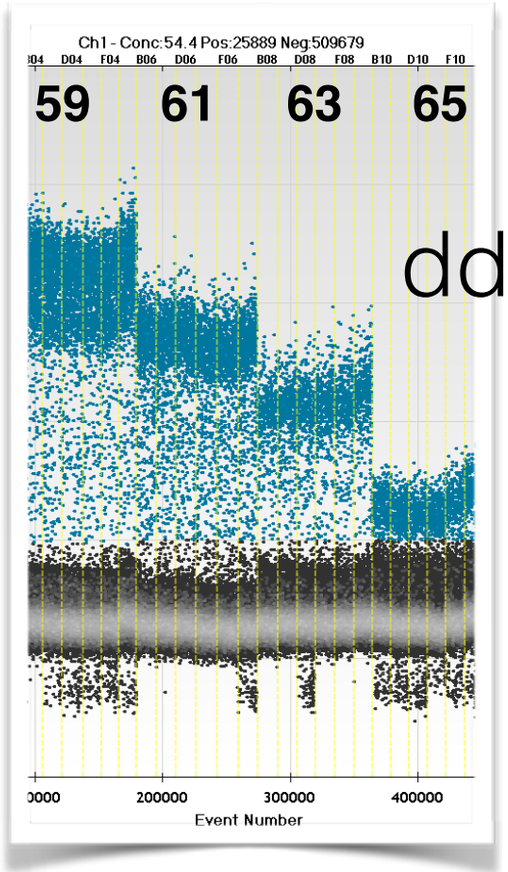


(Erica Butts & Margaret Kline)

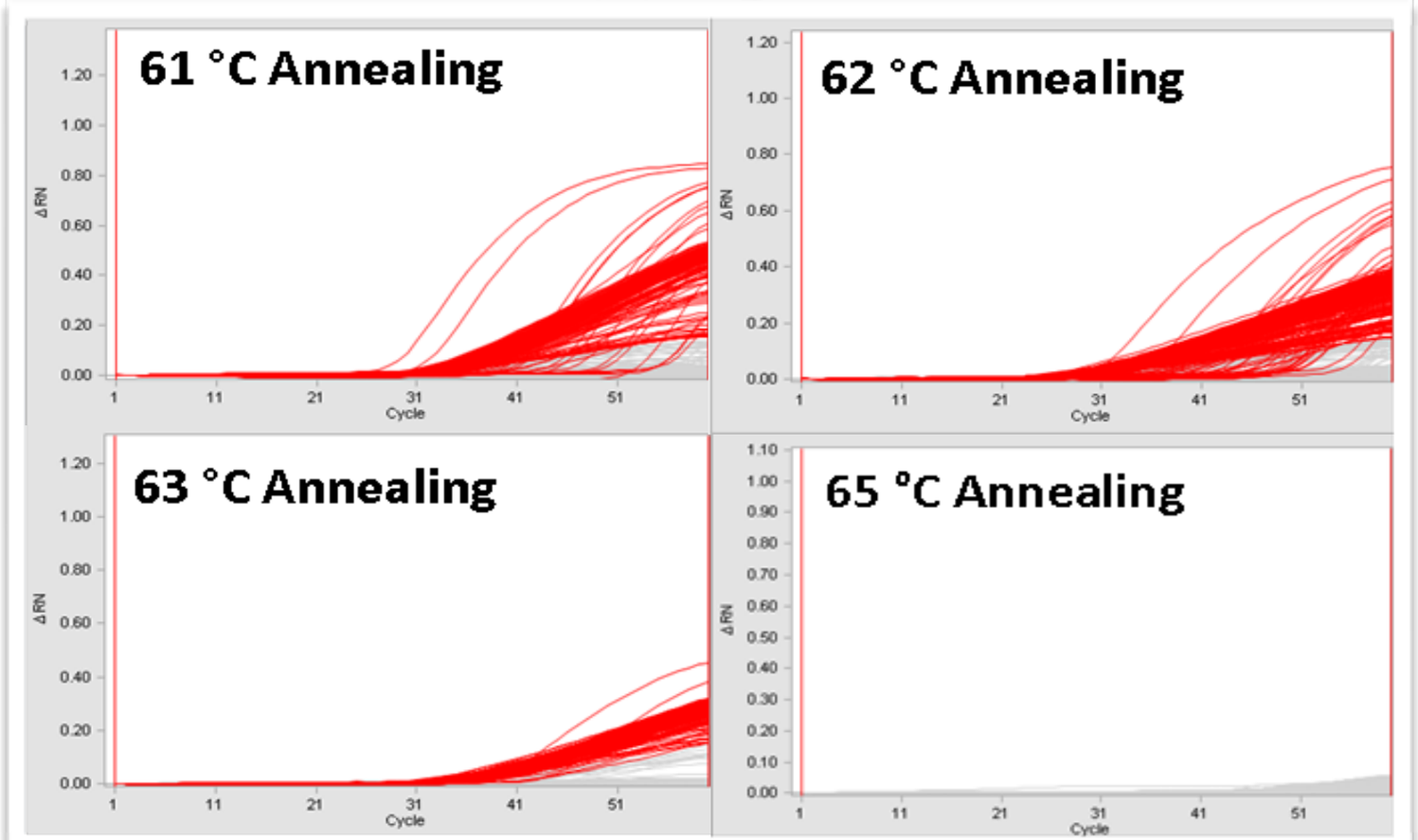
Efficient qPCR assays are not always good assays for dPCR



human *ACTB*



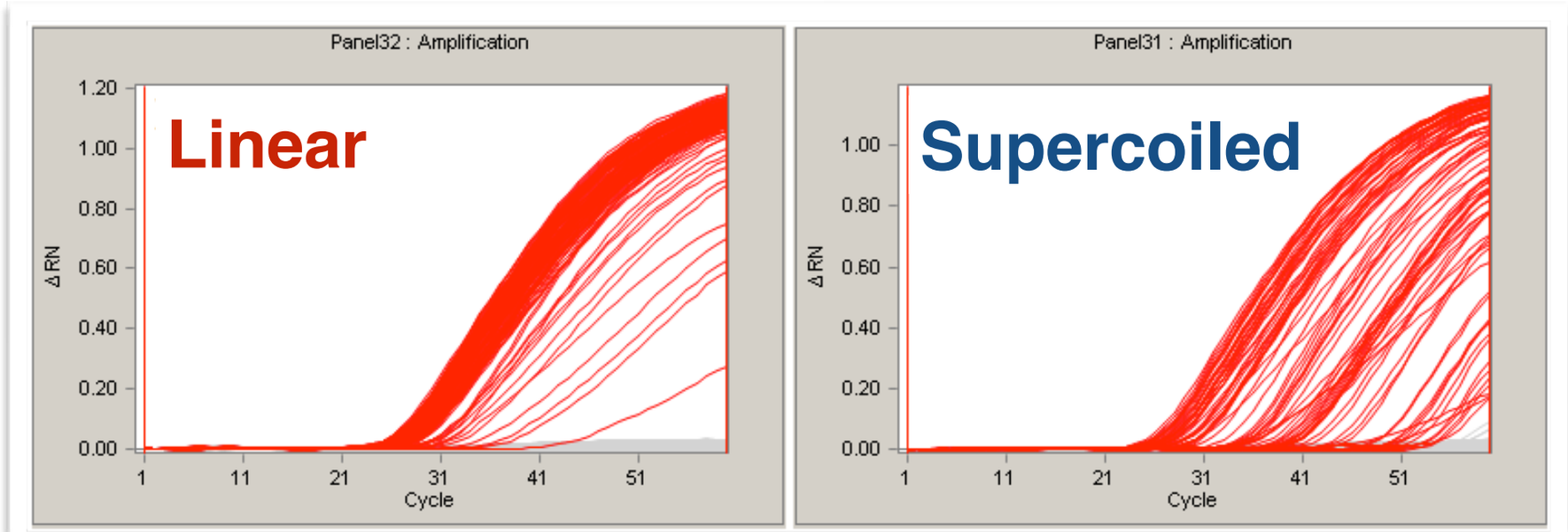
cdPCR

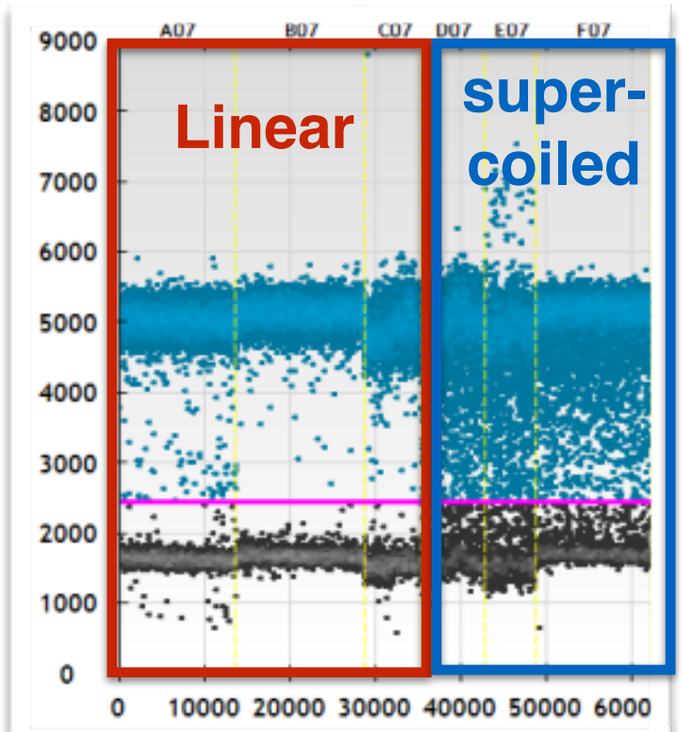


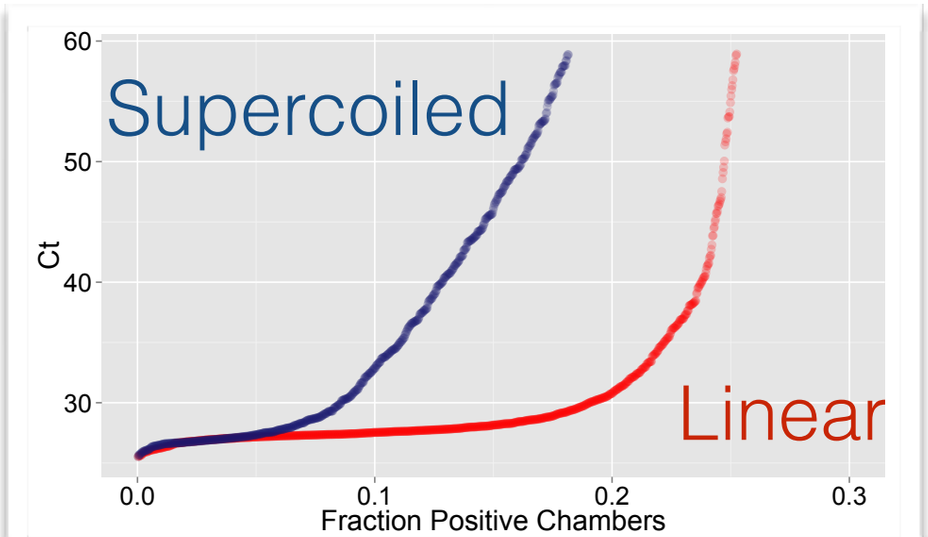
(Erica Butts & Margaret Kline)

Linear v. Supercoiled DNA

- Supercoiled DNA contains many "**late-starters**" and therefore, the target never fully amplifies

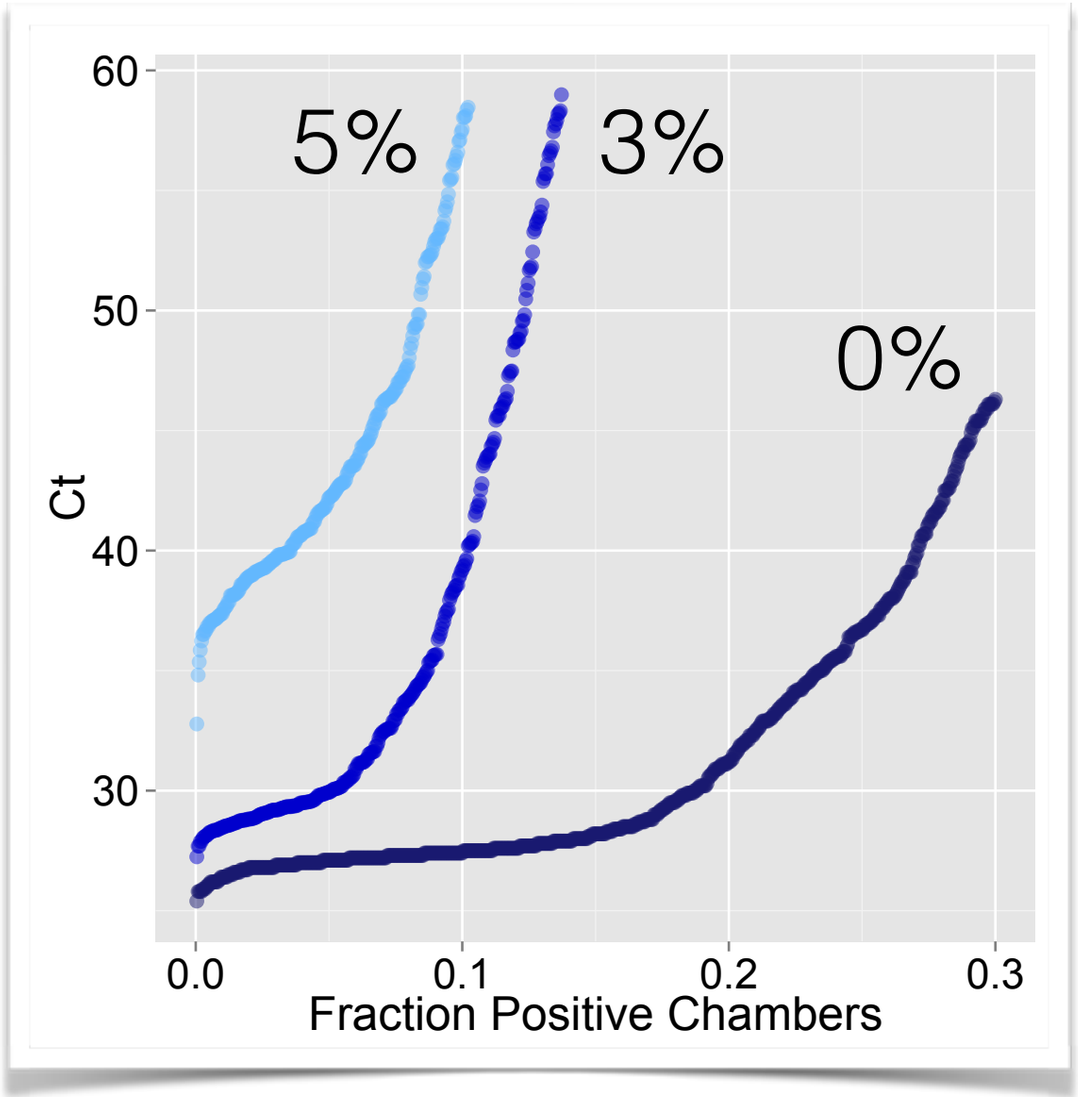






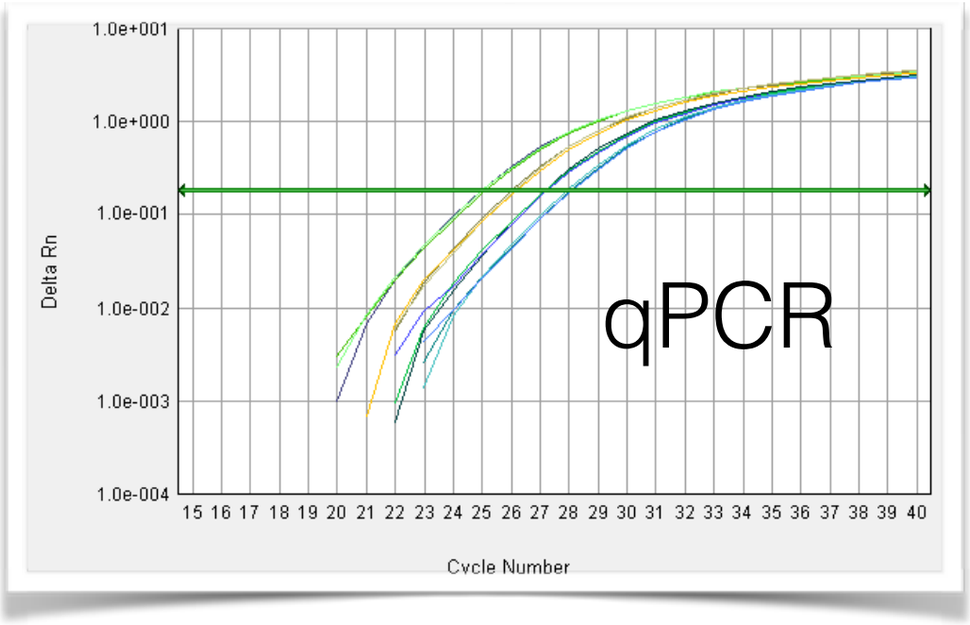
DMSO relaxation of supercoiled pDNA

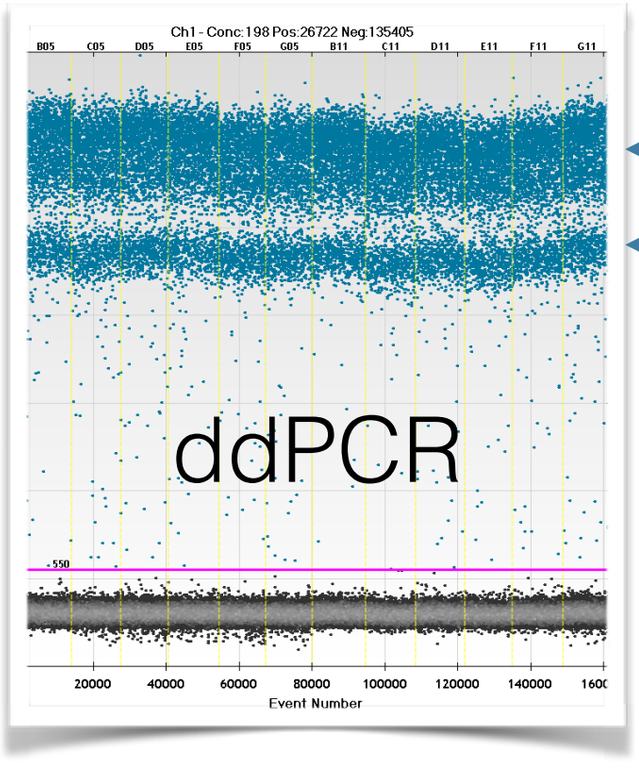
- DMSO is commonly used to relax plasmid DNA in qPCR [3, 5, 7, 9%]
- DMSO reduced the efficiency of cdPCR reactions
  - Concentrations of 7% and 9% completely inhibited amplification



(Jo Lynne Harenza, Dave Duewer)

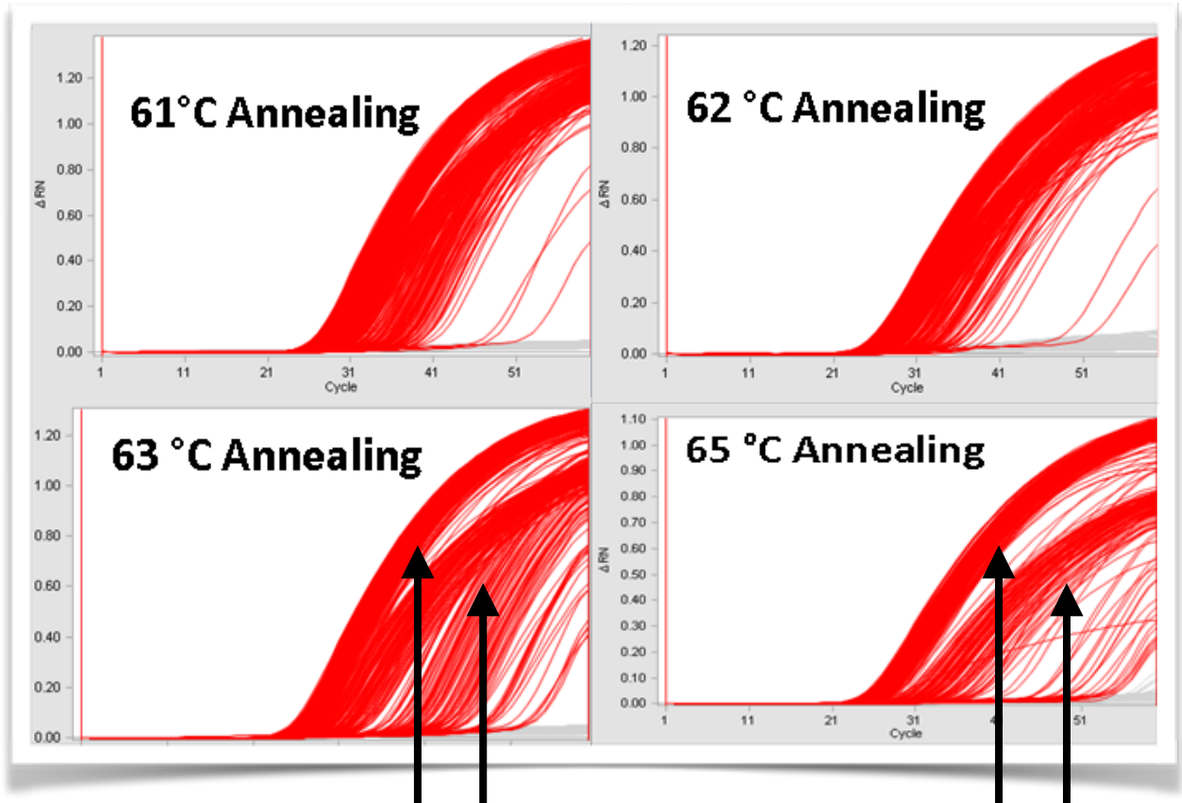
Assays with multiple targets can be detected with digital PCR





two distinct populations being amplified

human *RPLP0*



two distinct populations being amplified

(Erica Butts & Margaret Kline)

Many commercial master mixes contain leftover plasmid DNA from recombinant *Taq* production

JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2005, p. 530–531 Vol. 43, No. 1
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.1.530–531.2005

Presence of β-Lactamase Gene TEM-1 DNA Sequence in Commercial *Taq* DNA Polymerase

Biotechnology Letters (2006) 28: 321–325 © Springer 2006
DOI 10.1007/s10529-005-5931-3

The occurrence of antibiotic resistance genes in *Taq* polymerases and a decontamination method applied to the detection of genetically modified crops

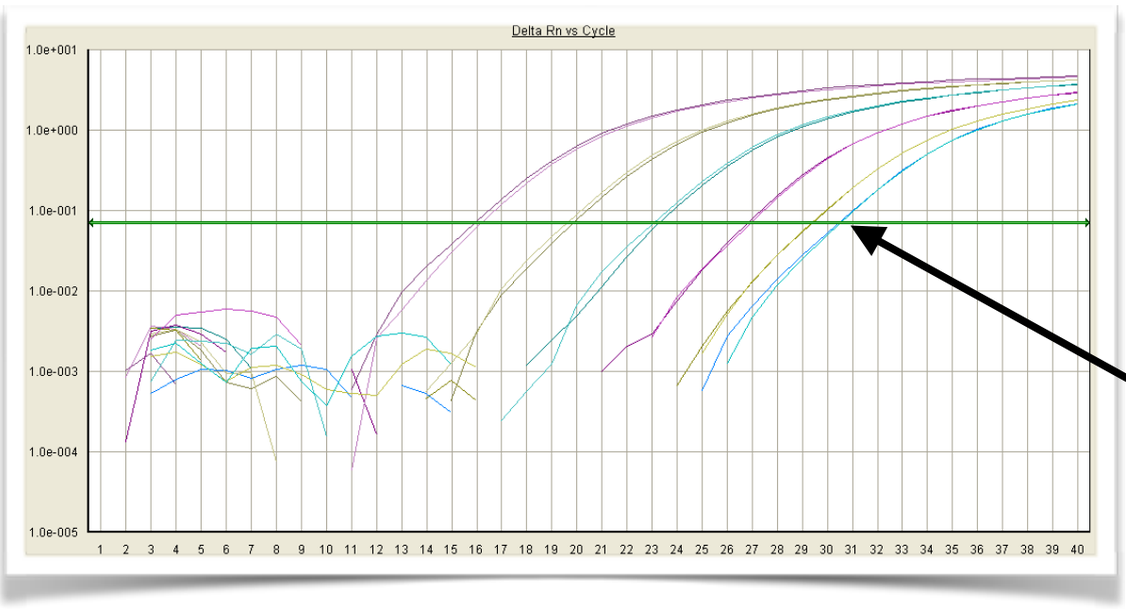
André Perron, Philippe Raymond* & Robin Simard
St-Hyacinthe Laboratory, Canadian Food Inspection Agency, Casavant Blvd West, J2S 8E3 3400, St-Hyacinthe, Quebec, Canada

Negative Control Contamination

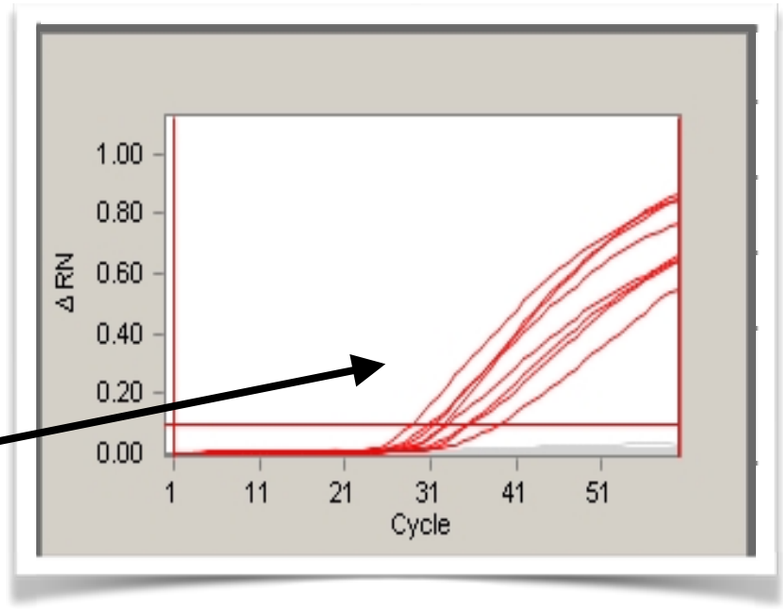
qPCR Taqman

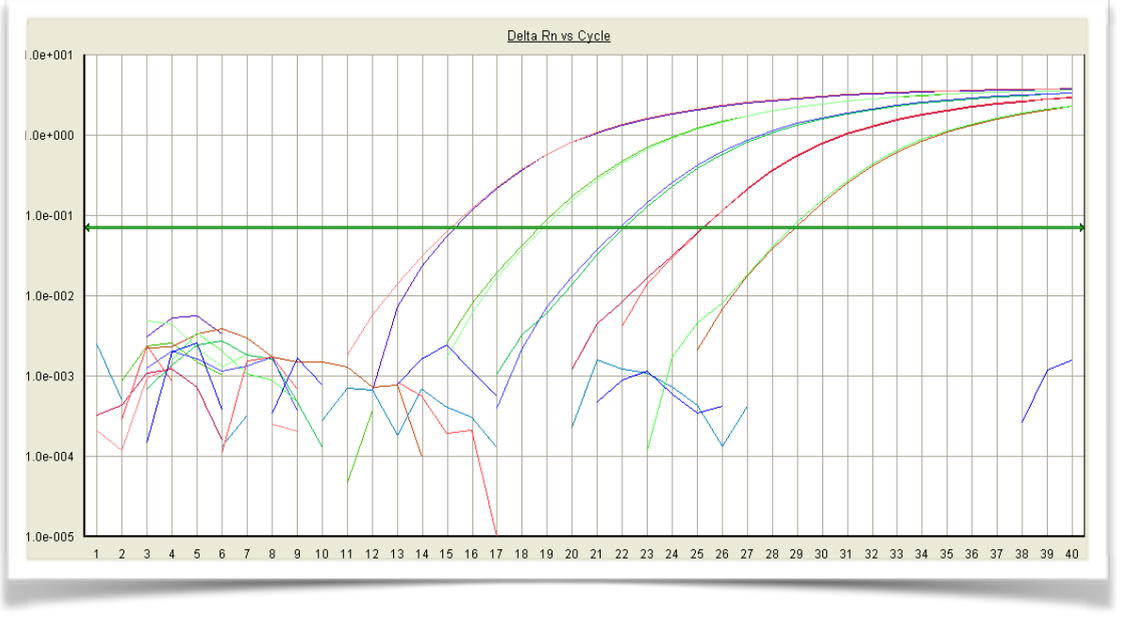
Assay 1

cdPCR



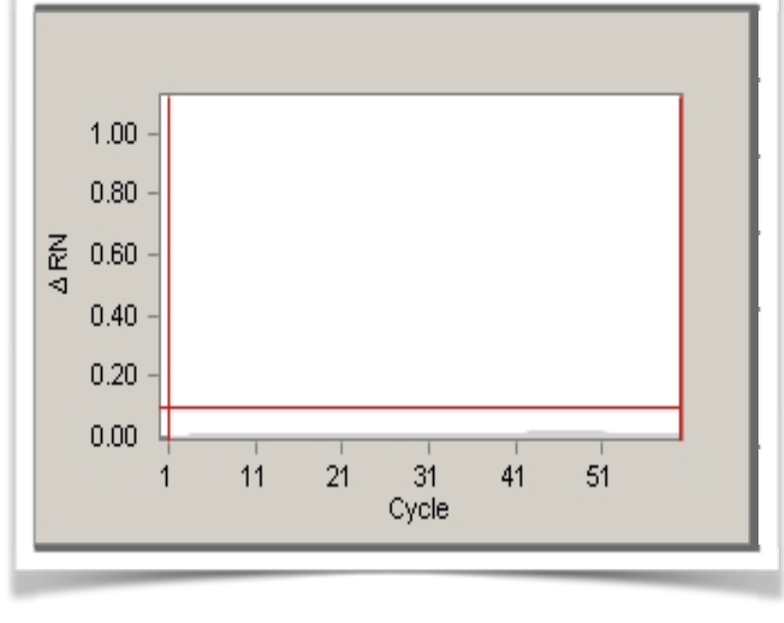
NTC amplification





Assay 2

No NTC amplification



Master mixes can be decontaminated

- ArcticZyme PCR Decontamination Kit — DNase specific to dsDNA, followed by heat inactivation with DTT

- Restriction enzyme digestion of dsDNA such that the target is no longer in tact (requires *a priori* knowledge of contaminant sequences)#

- UV irradiation — sequence and length-dependent*

- γ -irradiation induces dsDNA breaks, but high levels destroy *Taq's* enzymatic activity^

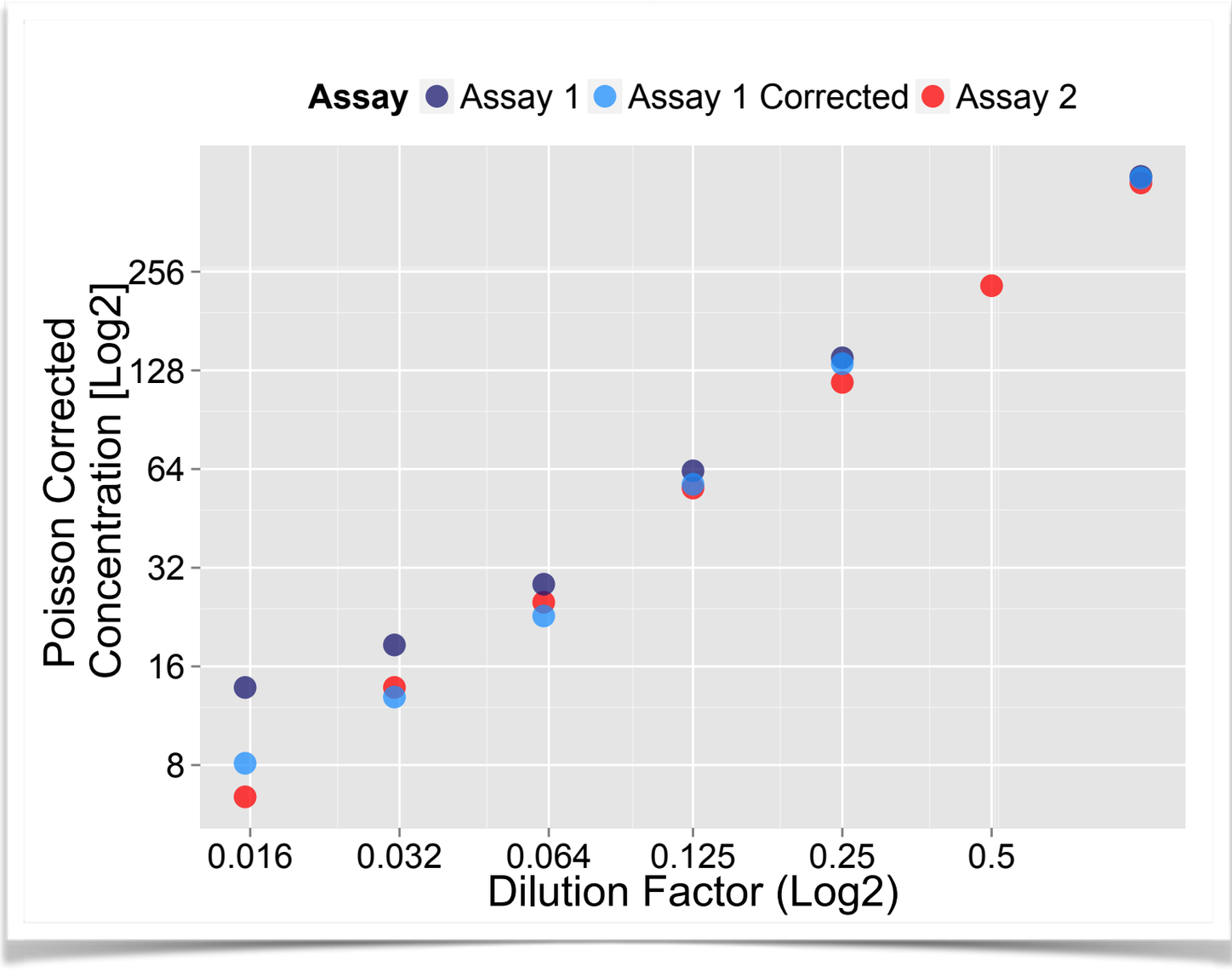
#Sharma, et al, 1992. A simple method for elimination of unspecific amplifications in polymerase chain reaction. *Nucleic Acids Res.* **20**: 6117–6118.
*Corless, et al, 2000. Contamination and sensitivity issues with a real-time universal 16S rRNA PCR. *J. Clin. Microbiol.* **38**: 1747–1752.
^Champlot, et al, 2010. An Efficient Multistrategy DNA Decontamination Procedure of PCR Reagents for Hypersensitive PCR Applications. *PLoS ONE*, **5**(9), e13042.

Negative control correction is also valid for contaminated master mixes

- A linearity study can be used to validate NTC correction

- Before correction, the regression line is appreciably curved

  - Compared an assay free of NTC contamination with one exhibiting contamination



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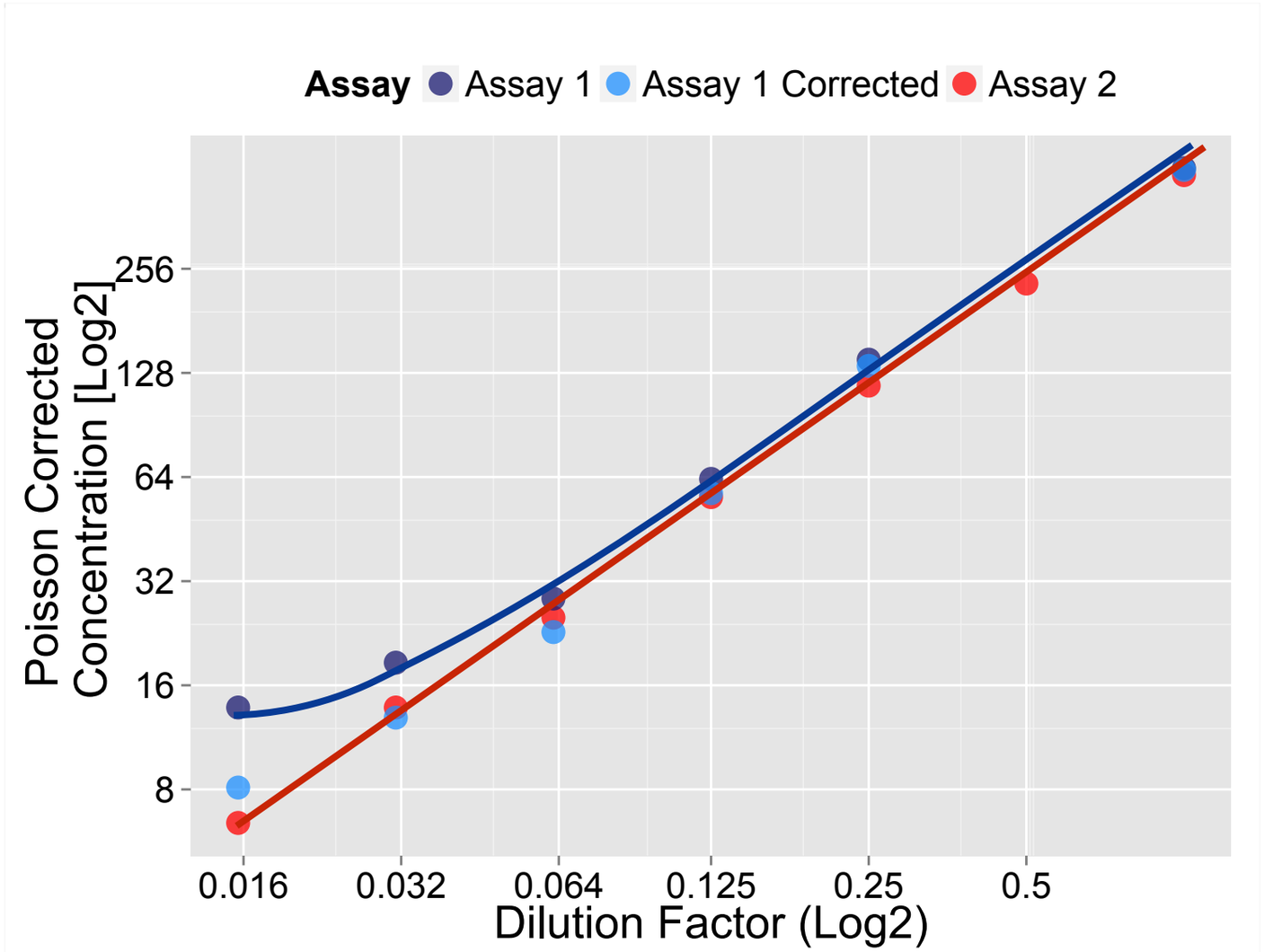

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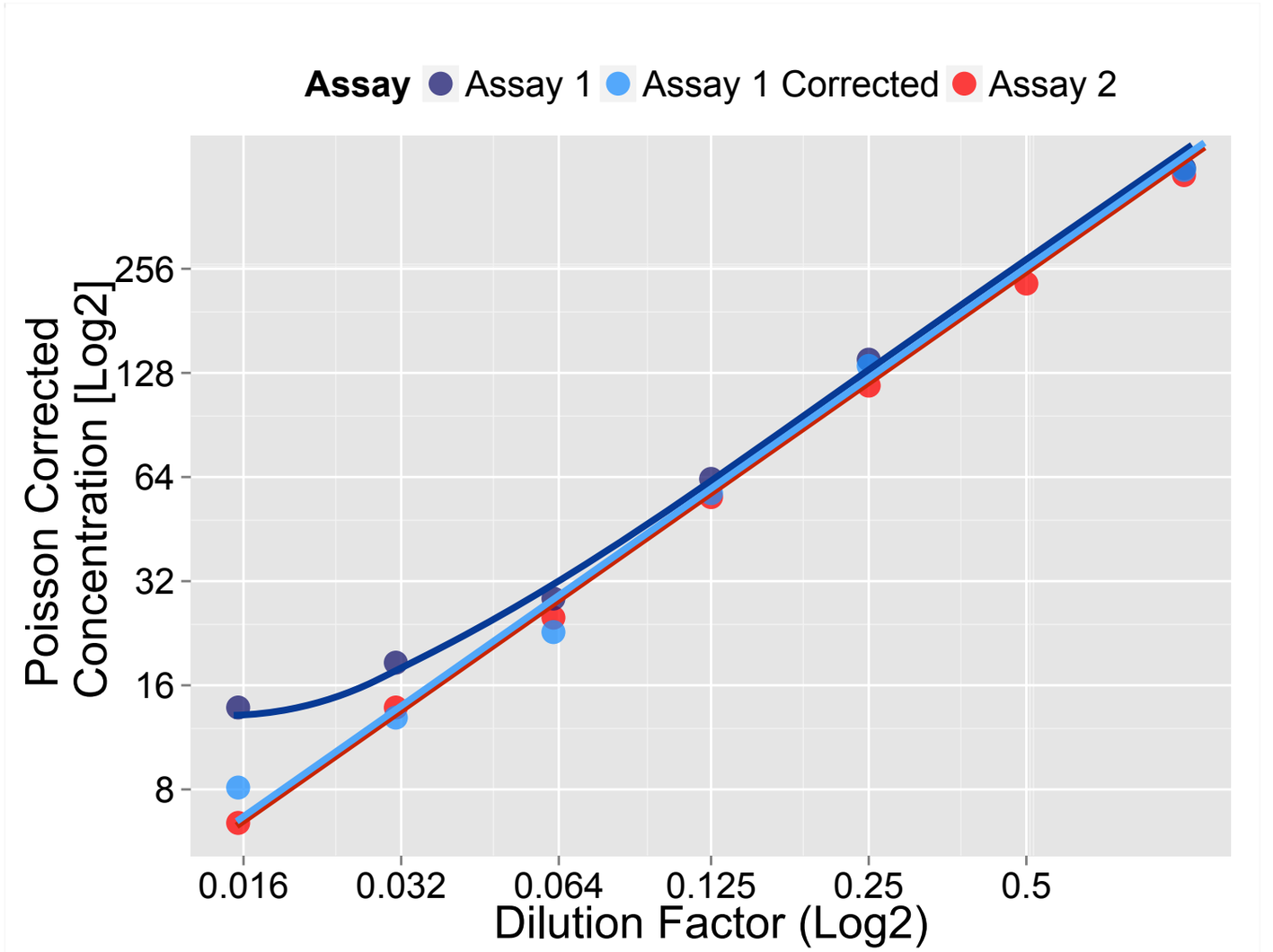
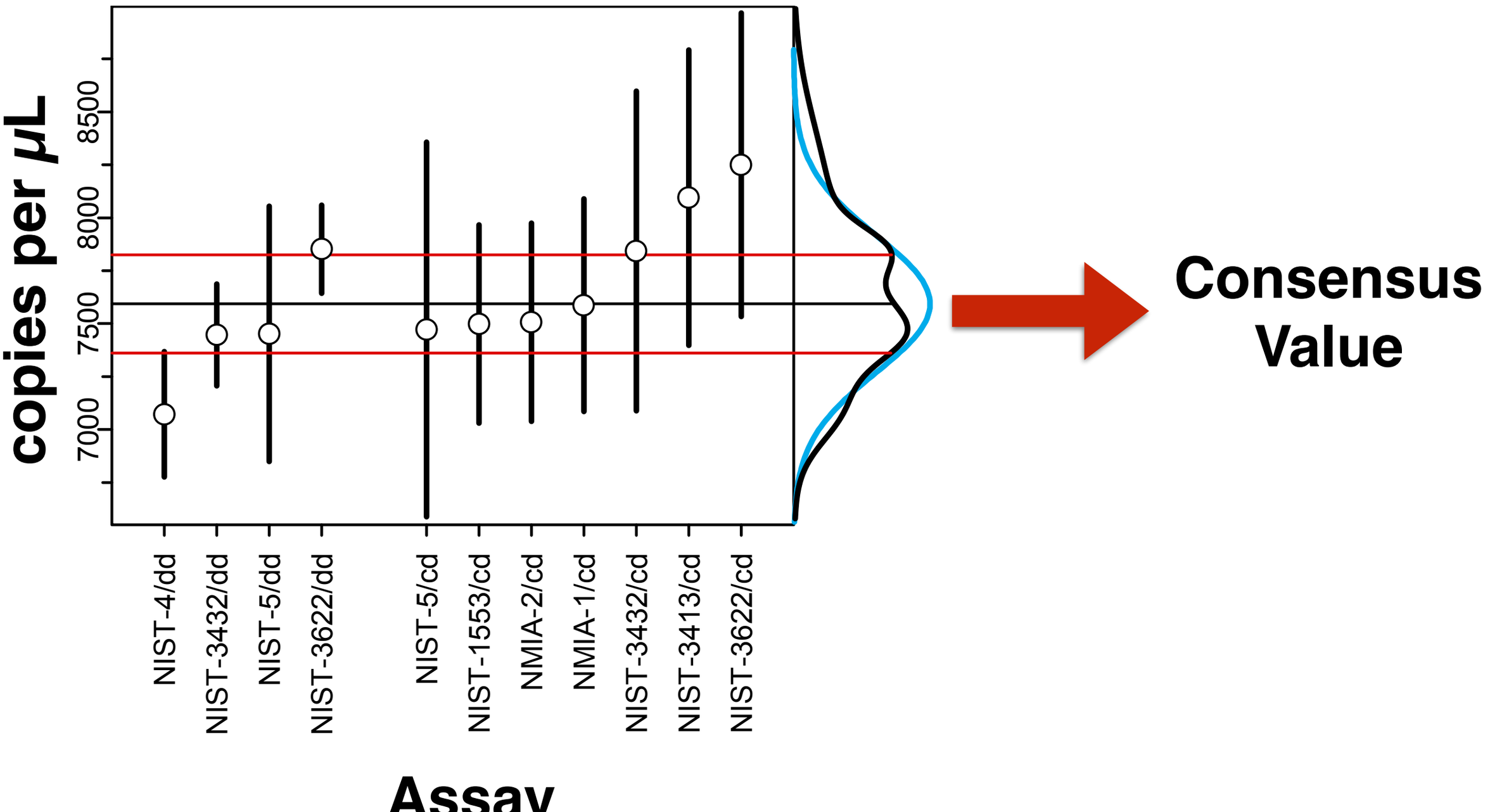


(Jo Lynne Harenza, Dave Duewer)

Use multiple assays and treating results interlaboratory study to determine at consensus value



(Margaret Kline, Ross Haynes, Jo Lynne Harenza, Dave Duewer)

Summary

- Useful information can be gained from combining cdPCR with ddPCR

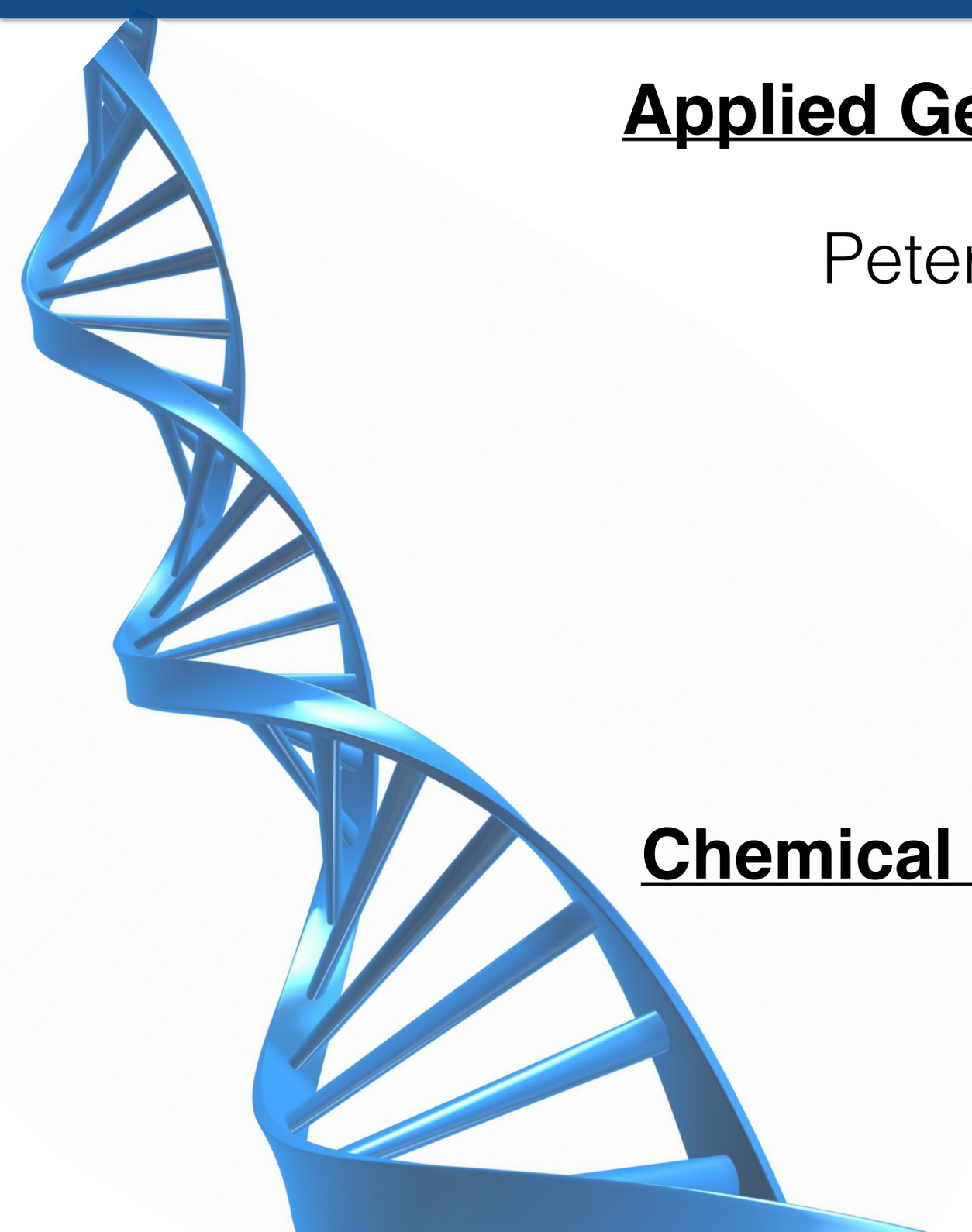
  - It is important to examine droplet and chamber traces to uncover artifacts or amplification biases

- Concordance between digital PCR instruments can be achieved following proper assay optimization

- Digital PCR measures only ***accessible*** targets and therefore, may underestimate the true DNA quantity in certain samples

- Digital PCR quantitation is SI-traceable

Acknowledgements



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Margaret Kline, M.S.

Chemical Sciences Division - Statistics

David Duewer, Ph.D.