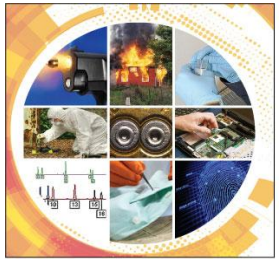

FORENSICS@NIST
November 28-30, 2012 • #NISTForensics

Digital PCR and Quantitation

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Research Biologist, Applied Genetics Group

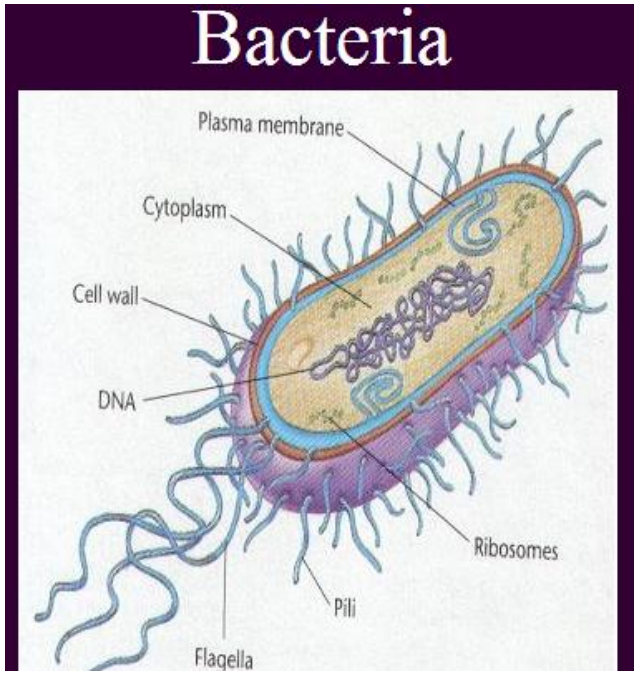
Forensics@NIST 2012 Meeting
Gaithersburg, MD
November 28, 2012

Agenda

- Why quantitate with qPCR?
- How digital PCR Will Help Quantitation
- Quantitative PCR versus Digital PCR
- Digital PCR at NIST
- Instruments at NIST
- Benefits of digital PCR

Why Quantitate with qPCR

- Forensic samples often have non-human DNA
  - Forensic standards require human specific DNA quantitation
  - qPCR measures specific (e.g. human) DNA targets

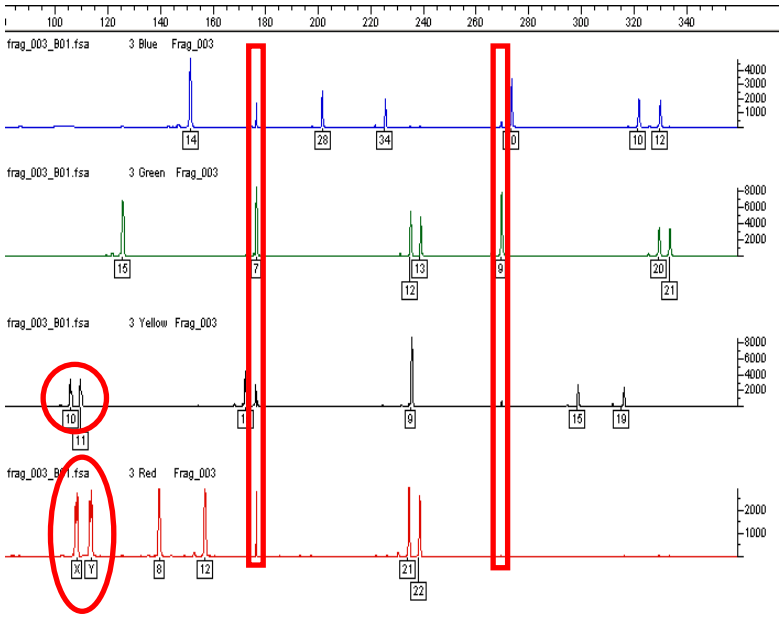


Pet Hair

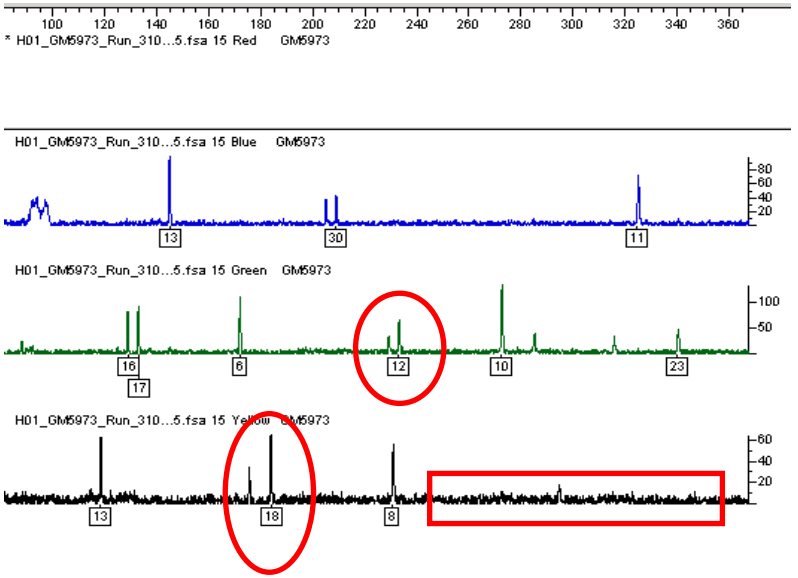
Why Quantitate with qPCR

- STR kits have a narrow working range for amount of DNA (0.5 ng to 2 ng DNA)

Too much DNA
→ artifacts/noise



Too little DNA
→ incomplete profile

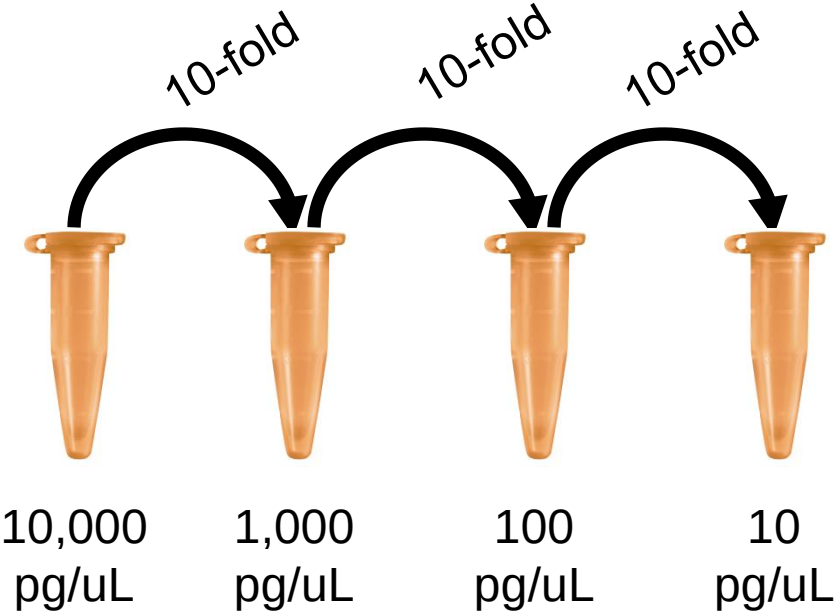


How dPCR Will Help Quantitation

- Predicted Major Uses of Digital PCR:
  - Quantify higher order reference materials
    - Standard Reference Materials
  - Quantify calibrant materials for qPCR
    - Manufacturers of calibrant materials
  - Quantify DNA solutions for critical and sensitive processes
    - E.g. Next Generation Sequencing

qPCR

- Calibrant concentration is independently determined (e.g. UV spectrophotometer)
- Prepare a dilution curve of calibrant

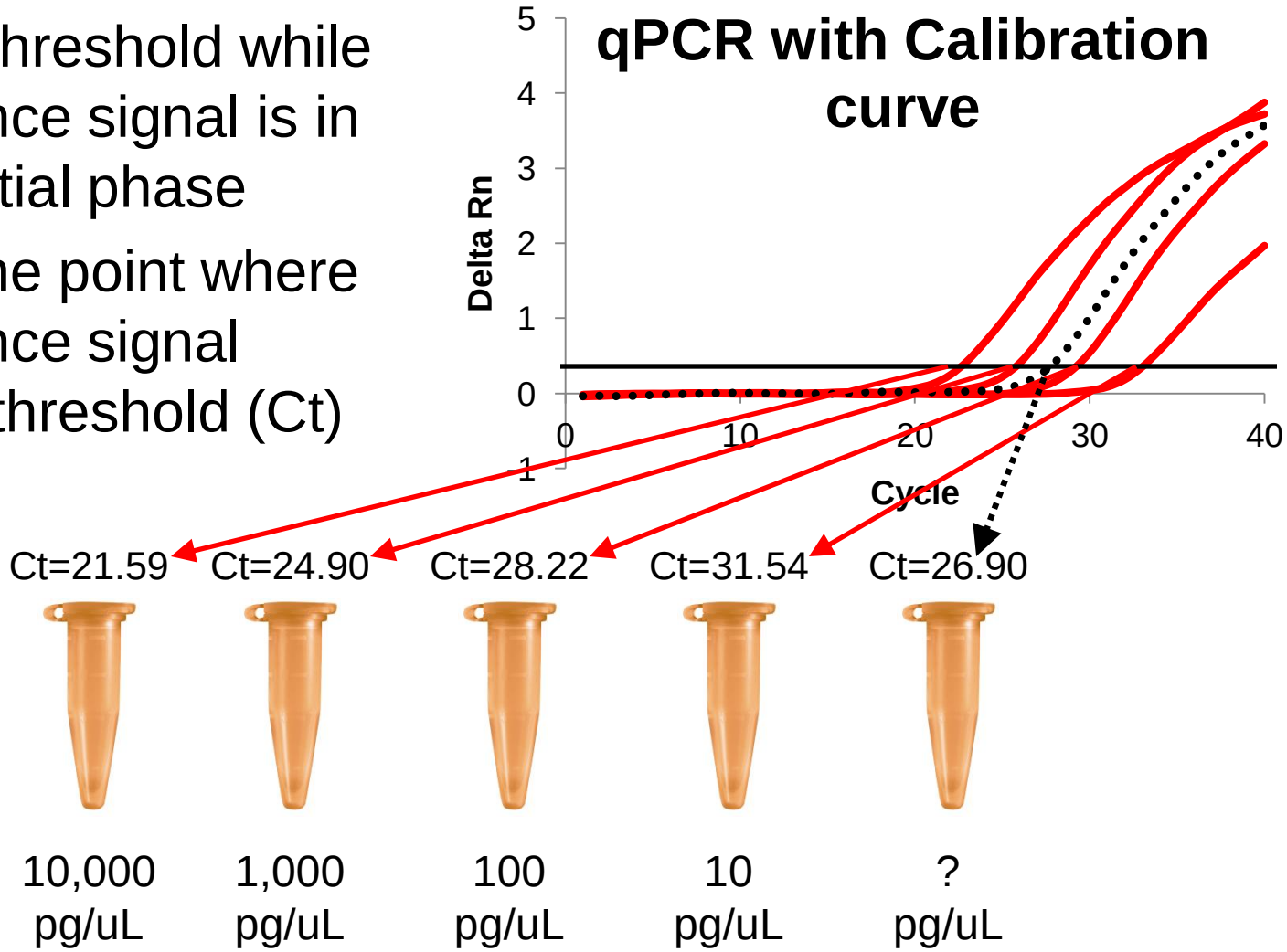


qPCR

- Use calibration dilutions plus samples of unknown concentration as template for qPCR
- Thermal cycle and measure florescence signal after each cycle of PCR

qPCR

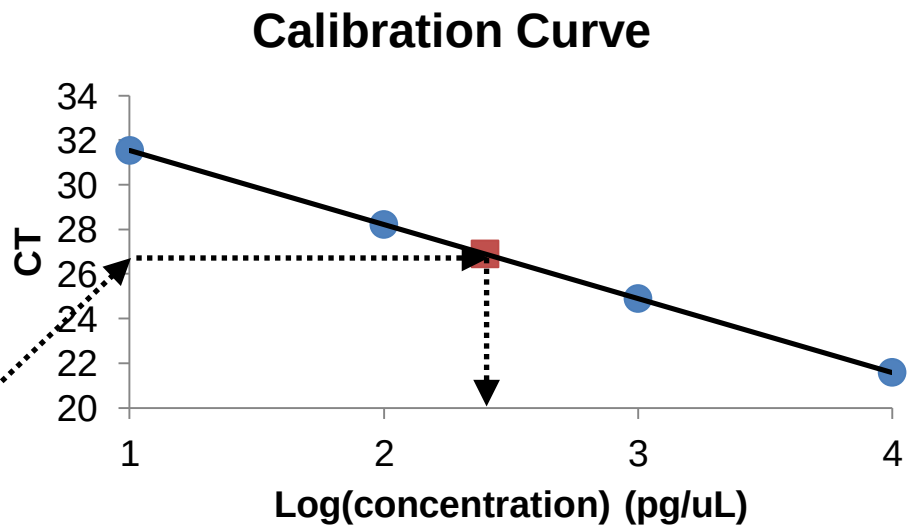
- Apply a threshold while florescence signal is in exponential phase
- Determine point where florescence signal crosses threshold (Ct)



qPCR

- Log transform concentration
- Plot Log(conc.) vs Ct

Conc. (pg/µL)	Log(conc.)	Ct
10,000	4	21.59
1,000	3	24.90
100	2	28.22
10	1	31.54
unknown	unknown	26.90



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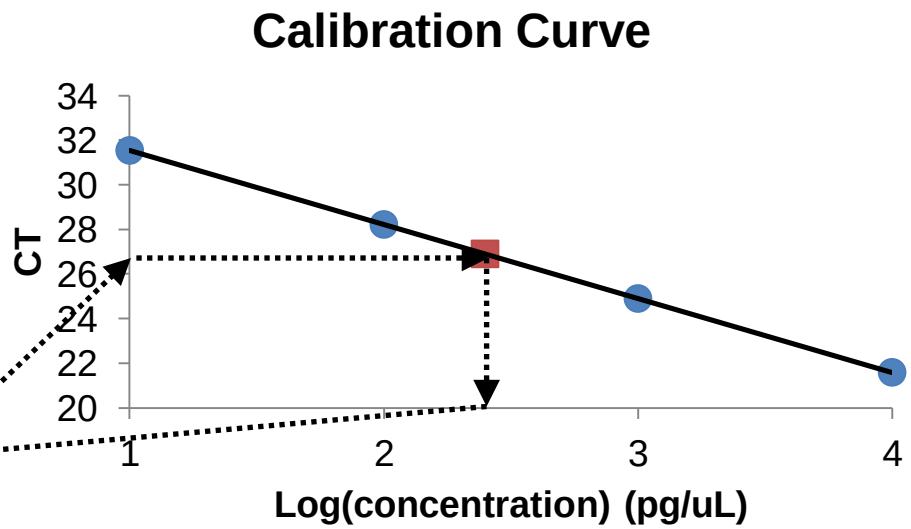

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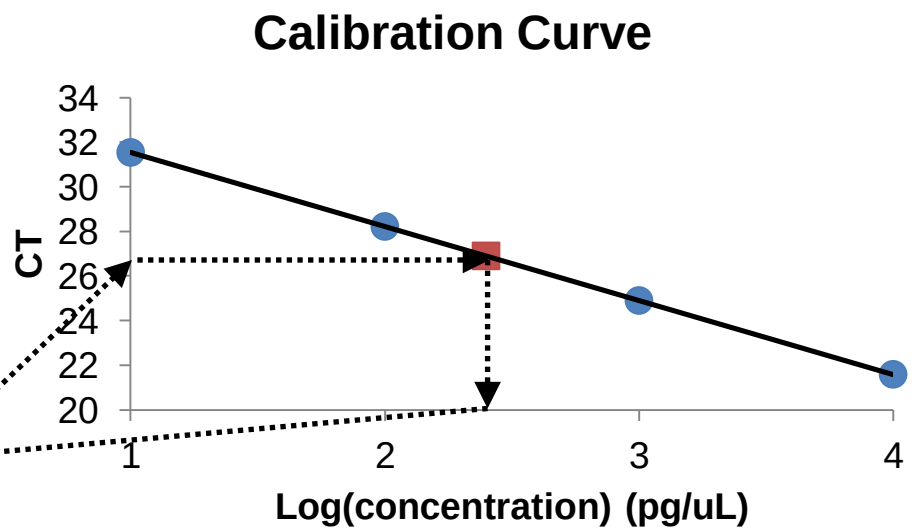
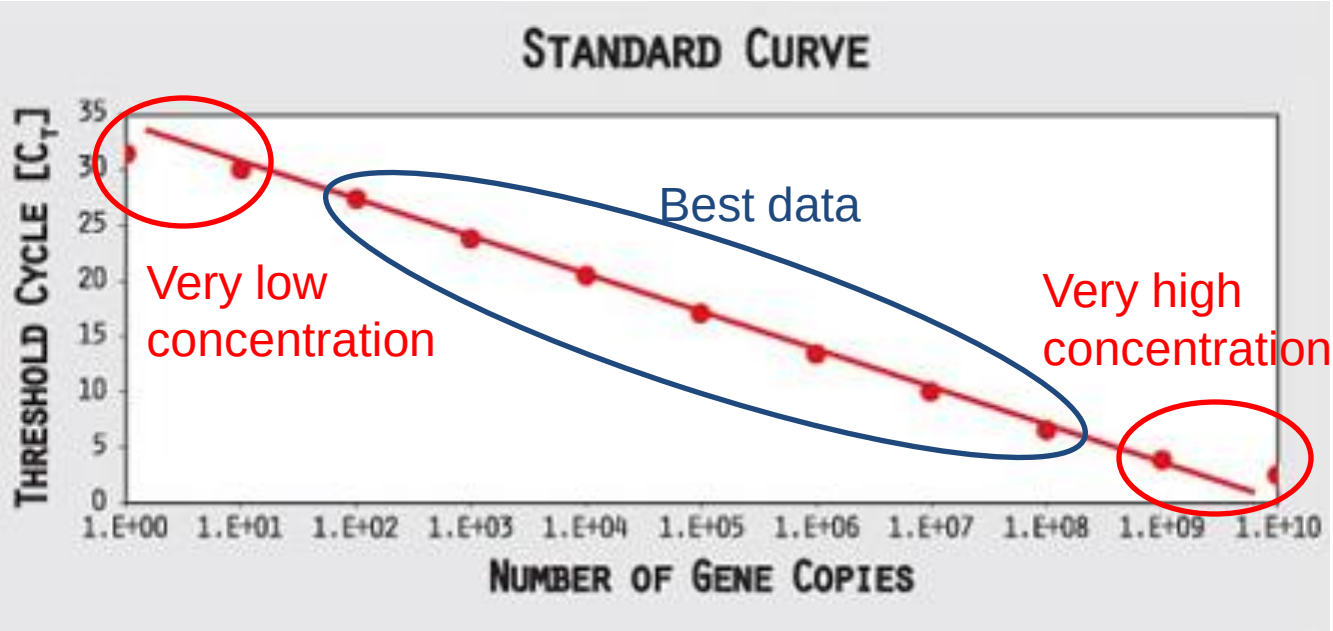


qPCR "Goldilocks Zone"

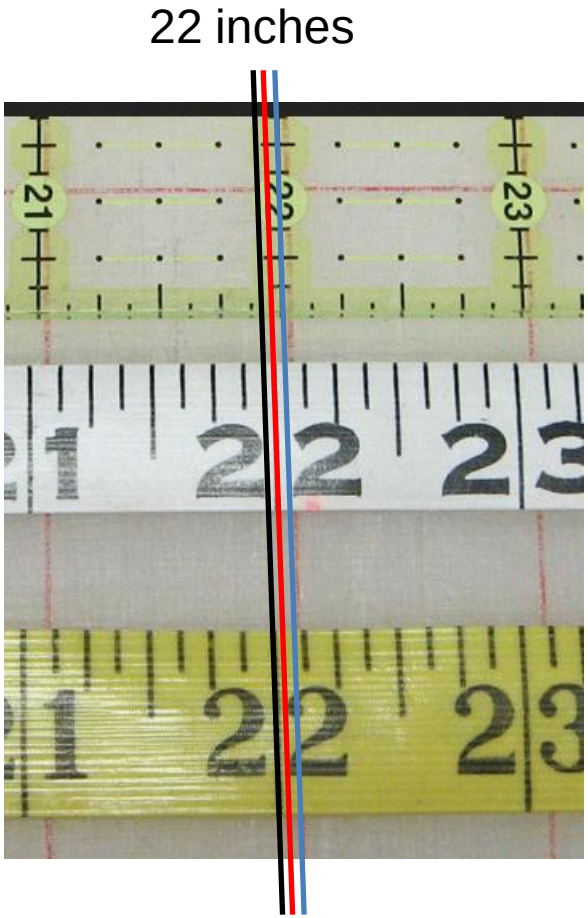
- Very high and very low concentrations do not fit on the line
- Best data obtained from the middle



http://www.sabiosciences.com/pathwaymagazine/pathways7/designing-validating-real-time-pcr-primers.php

qPCR

- Relative quantitation between calibrant of known concentration (aka standard) and samples of unknown concentration
  - Just as using a tape measure is a relative measurement if the calibrant is inaccurate the measurement will be inaccurate
- Spectrophotometer measures everything that absorbs at 260 nm (i.e. DNA, RNA, protein, monomers)



http://vickiwelsh.typepad.com/field_trips_in_fiber/tips/

dPCR

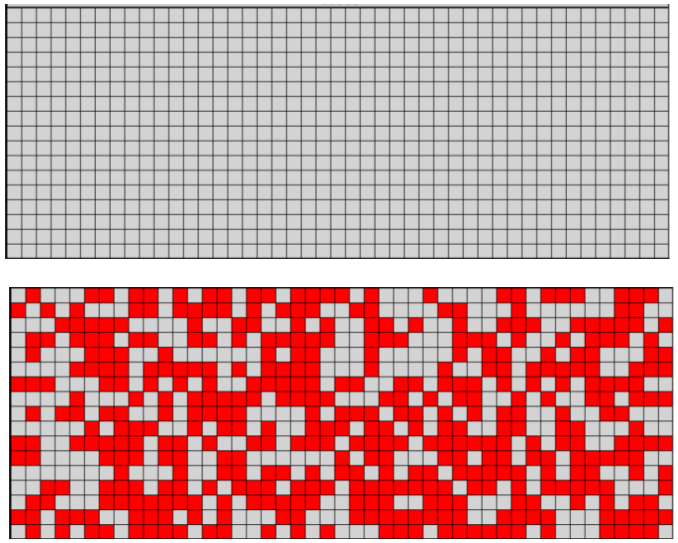
1) Create a PCR mastermix as if for qPCR

2) Aliquot across 100s or 1000s of wells

3) Thermal cycle as if for qPCR & count wells with detectible amplification at any cycle

4) Use Poisson statistics to determine concentration of starting material

?
pg/uL



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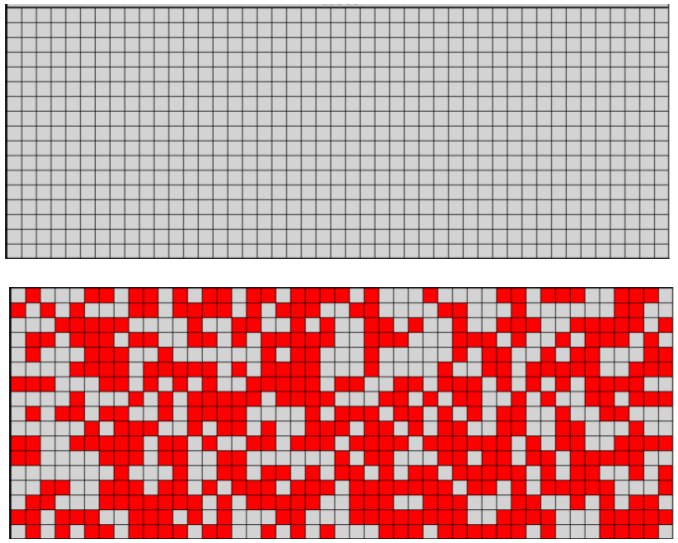

$$\text{Concentration (c/µL)} = \frac{\cancel{\text{Number of copies}} \times \text{Number of wells} \times \ln\left(\frac{\text{Number of wells}}{\text{Number of negative wells}}\right)}{\text{Volume of all PCR reactions}}$$

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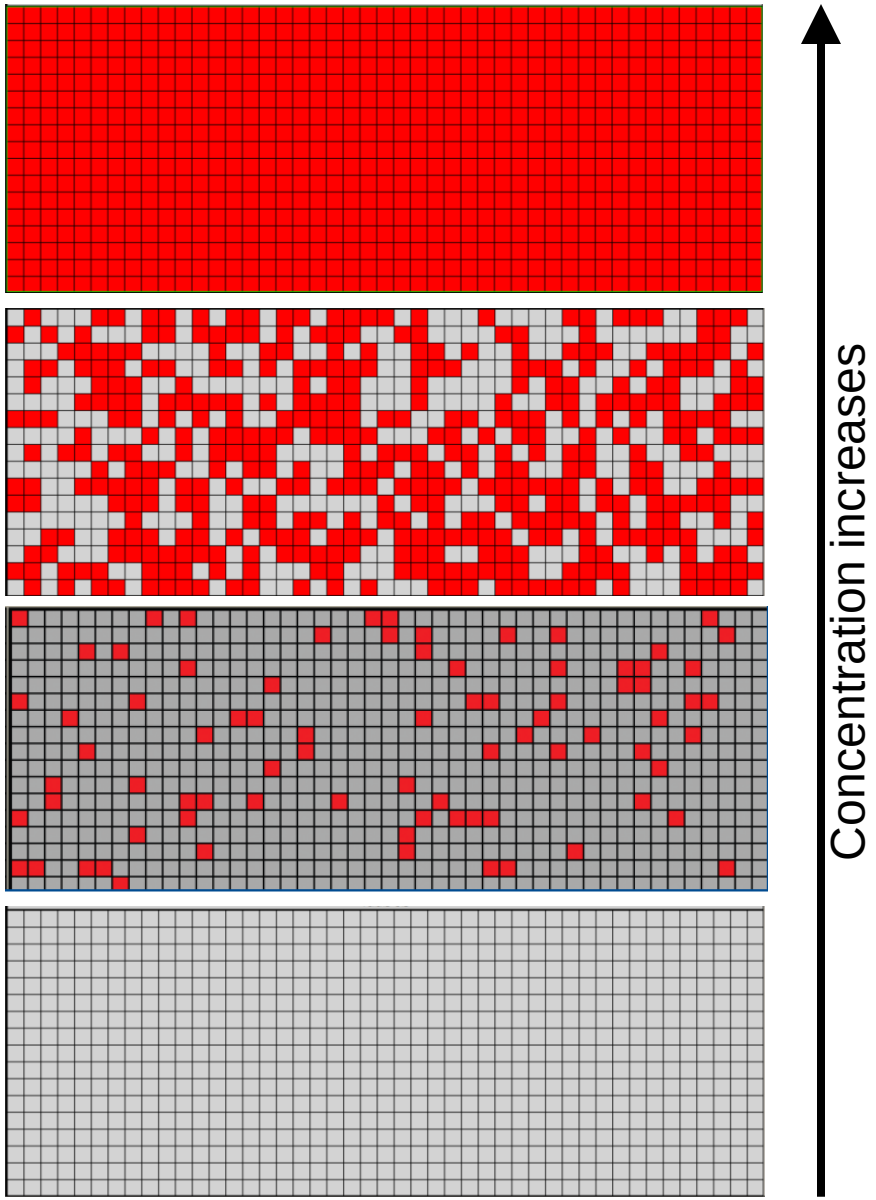
Range of Concentrations

- Saturated
  - Every well has at least one copy
- Binary detection
  - Calculate concentration
- No amplification
  - < 1 copy/total volume



dPCR

- Absolute quantitation of target sequence
- Relies on PCR amplification
  - Only detects specific target DNA or RNA
  - Will not detect proteins or monomers
  - Will not detect fragmented or degraded DNA molecules
  - Affected by PCR inhibitors

Comparison

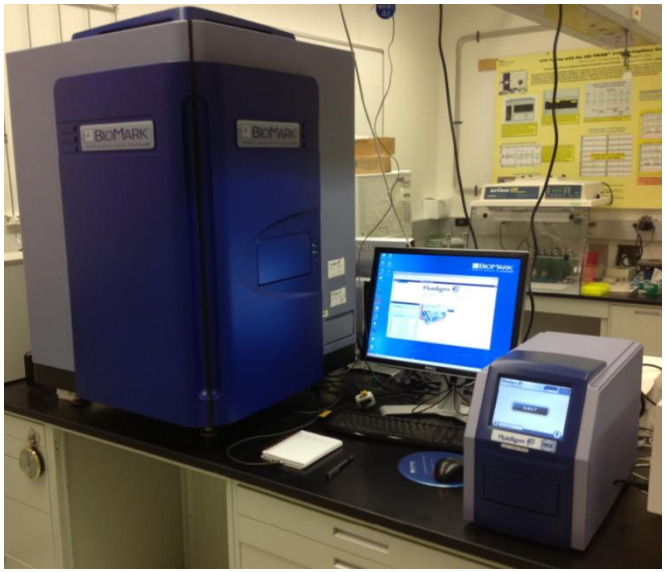
Quantitative PCR	Digital PCR
Quant of unknown is based on amplifiable DNA	
Quant is based on a previously characterized material (a.k.a “calibrant”)	Quant is based on Poisson sampling statistics (i.e. calibrant free)
Samples must be bracketed by calibrant dilution curve	Samples must be within “digital” range
Older technology Widely accepted	New technology Gaining acceptance
Currently less expensive	Currently more expensive

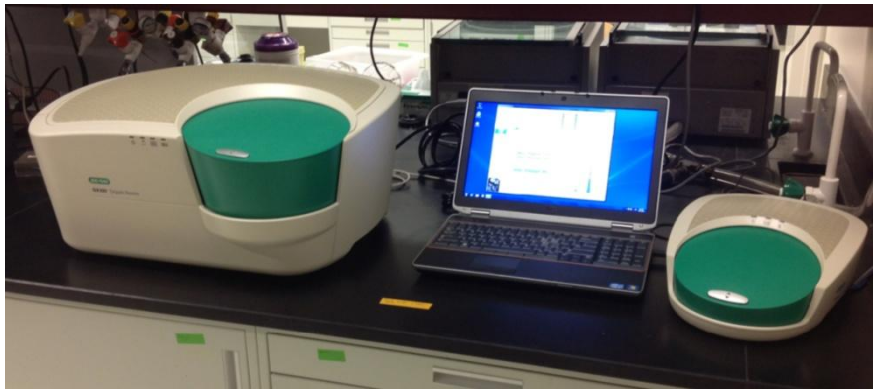
dPCR at NIST

- Digital PCR has been used at NIST:
  - To certify the concentration of a Standard Reference Material for Cytomegalovirus SRM 2366
- Evaluated using dPCR to measure the concentration of the SRM 2372: Human DNA Quantitation Standard
  - Further validation of targets required

Instruments at NIST

- Fluidigm BioMark
  - Array based PCR reactions
  - Gathers real-time data
    - Better for validation of PCR conditions
- Bio-Rad QX100
  - Emulsion based PCR reactions
  - End-point data
    - Less expensive
    - Better statistics





Benefits of dPCR

- Digital PCR:
  - Is a PCR based quantitation method
    - Specificity of DNA target
  - Uses Poisson counting statistics to determine number of molecules of DNA
    - Does not require a calibrant
    - Primarily a counting method with statistics to compensate for PCR reactions that had more than one template molecule
  - Used to characterize higher order reference materials and qPCR calibrants
    - Standard Reference Materials
    - Commercial qPCR calibrants

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Acknowledgments

Applied Genetics Group: Clinical Team


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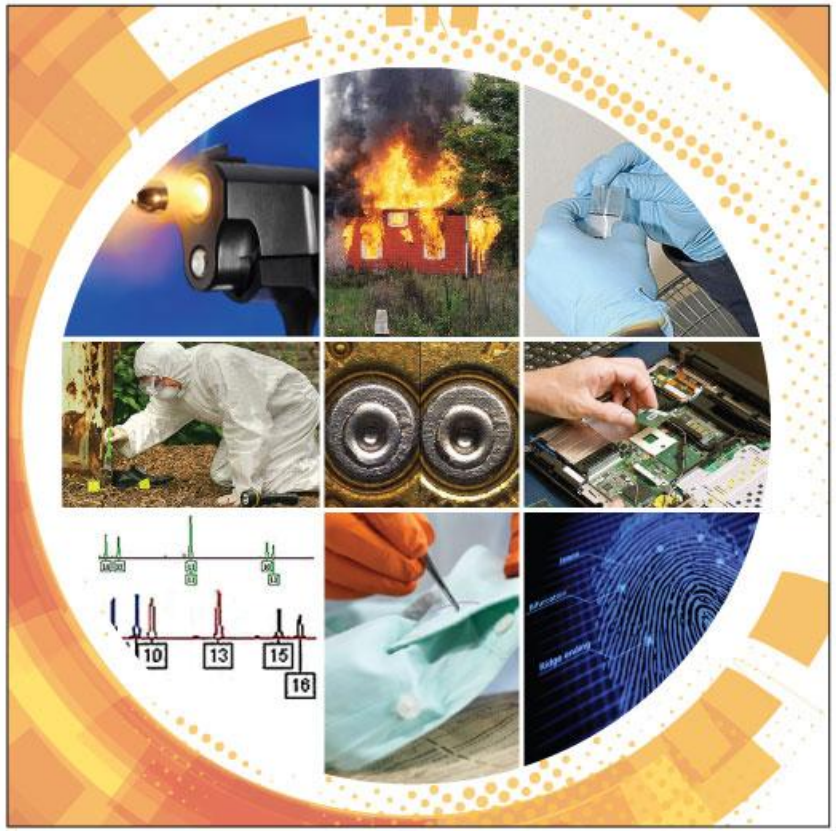
Thank you for your attention!



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