Issues Concerning Extraction Efficiency, Methods, and Direct dPCR

by Ross Haynes
National Institute of Standards and Technology

April 10, 2013
Sample Prep & Target Enrichment
Boston, MA

Disclaimer

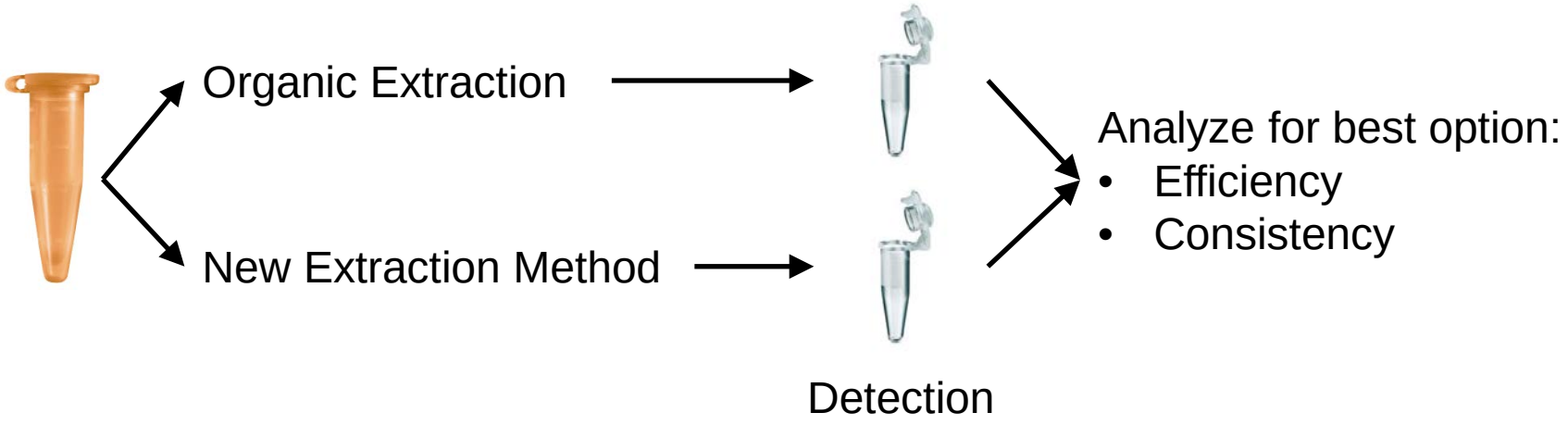
- Views expressed in this presentation are the author’s opinion and do not represent the opinion of NIST or the Department of Commerce.
- Any mention of commercial products within this presentation is for information only; it does not imply recommendation or endorsement by NIST, nor does it imply that the products are the best available.

Agenda

- Extraction
  - Efficiency (relative vs. absolute)
  - Overview of traditional methods
- Alternates to traditional extractions
  - Liquid based
  - Direct PCR methods
- Direct digital PCR
  - NIST experiences
  - Considerations

Extraction Efficiency

- Relative: compared to another technique
  - New technique > Organic extraction



http://www.humpath.com/spip.php?article123

Extraction Efficiency

- Absolute: compared to amount of input material
- Mumy et al found ~ 15 % efficiency using 3 commercial kits (range 0 % to 45 %)
  - Lambda DNA in plasmid

Sample + known amount of control DNA

New Extraction Method

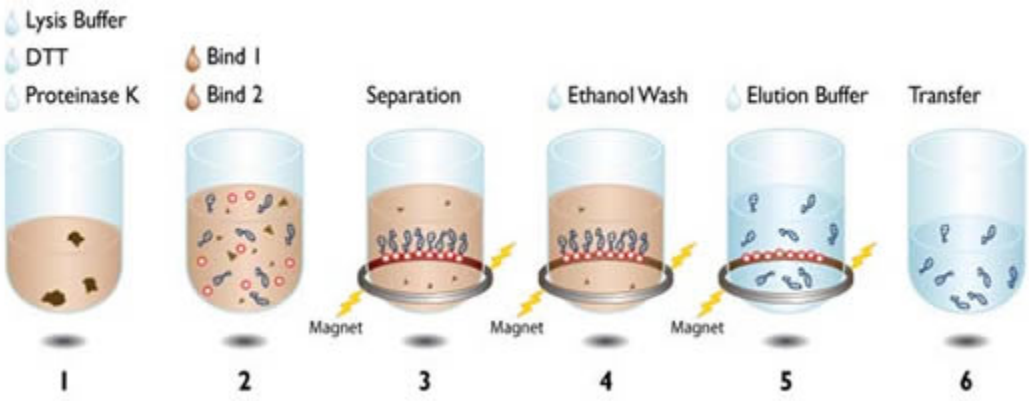
Detection

Analyze method:
- Efficiency
- Consistency

K.L. Mumy, R.H. Findlay / Journal of Microbiological Methods 57 (2004) 259–268

DNA Extractions

- Steps:
  - Lysis
  - Separation
  - Purification/wash
  - Recovery
- Benefits
  - Clean DNA
- Limitations
  - No method is 100 % efficient



https://www.beckmancoulter.com/

DNA Extractions

- Steps:
  - Lysis
  - Separation
  - Purification/wash
  - Recovery
- Benefits
  - Clean DNA
- Limitations
  - No method is 100 % efficient

90 % efficient
90 % efficient
90 % efficient
90 % efficient

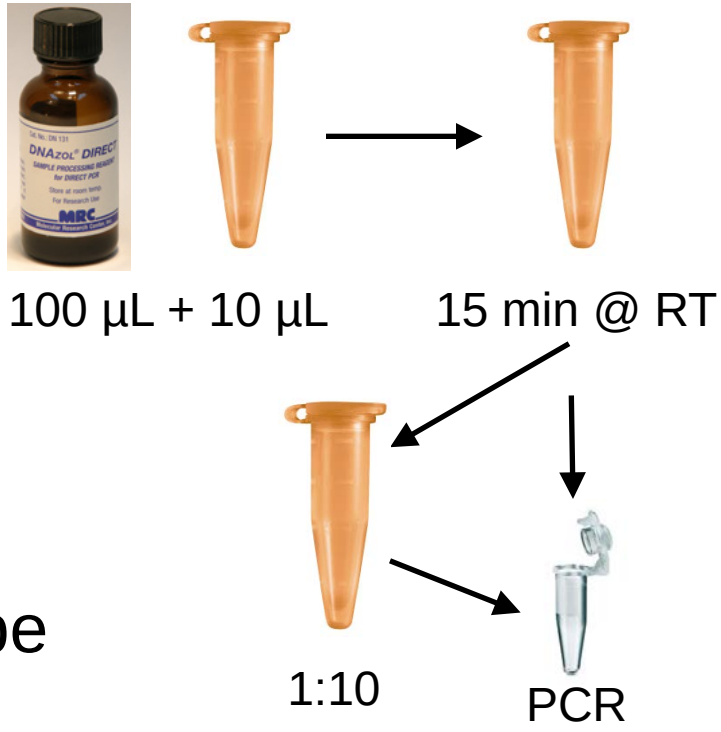
Overall 66 % efficient

Efficiency probably not uniform
But we have to consider that lysis may not be 100 % efficient.

https://www.beckmancoulter.com/

Alternate Methods

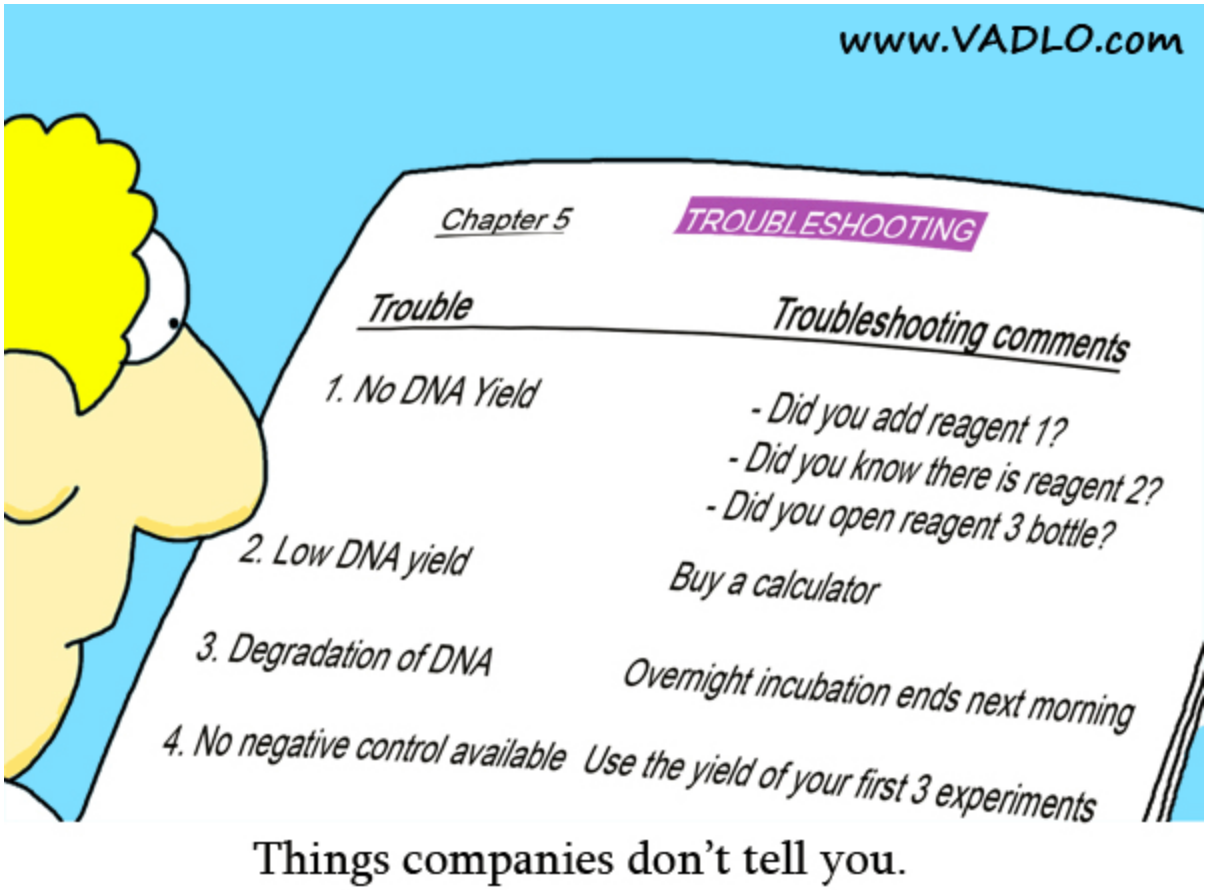
- Liquid based methods
  - E.g. DNAzol Direct
    - Add reagent to sample
    - Incubate
    - Add directly to PCR
- Benefits
  - All DNA contained in one tube
- Limitations
  - Reagents may not lyse all cell or virus particles
  - Regents may contain PCR inhibitors
    - 1/10 dilution required



http://www.mrcgene.com/dnazoldirect.htm

Extraction Efficiency

- People
  - Training
  - Education
  - Motivation
  - Sleep
- Robots
  - Set-up
  - Maintenance



http://vadlo.com/cartoons.php?id=108

Direct PCR

- Sample added directly to PCR mix
- Hot start used as lysis method
- Polymerases resistant to inhibition

- Thermo Scientific – Phusion polymerase
  - “Tolerant of many PCR inhibitors”
  - End point PCR protocols
  - Research Use Only
  - No 5’ to 3’ nuclease activity (not suitable for TaqMan probes)

dPCR

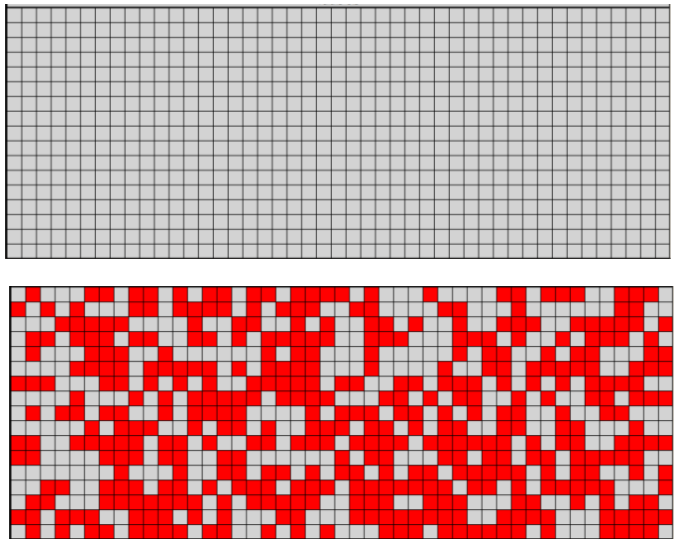
1) Create a PCR mastermix as if for qPCR

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



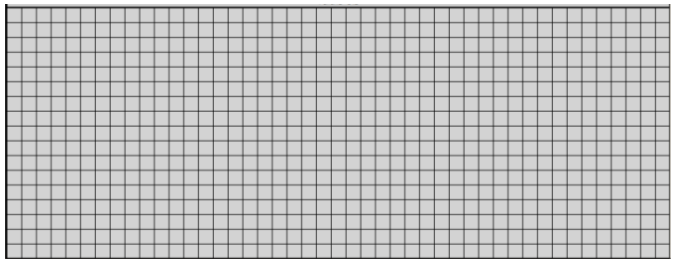
Direct dPCR

1) Create a PCR mastermix as if for qPCR

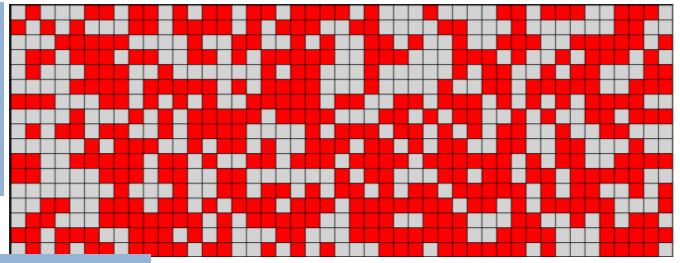
Virus particles instead of template DNA

? pg/uL

2) Aliquot across 100s or 1000s of wells



Hot start to lyse virus particles



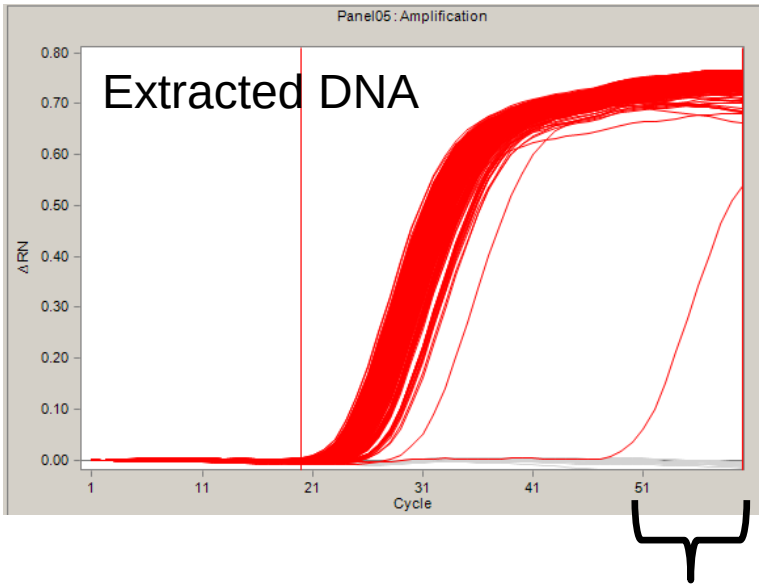
3) Thermal cycle as if for qPCR & count wells with detectible amplification at any cycle

Exhaustive cycling to ensure lysis & amplification of all target molecules

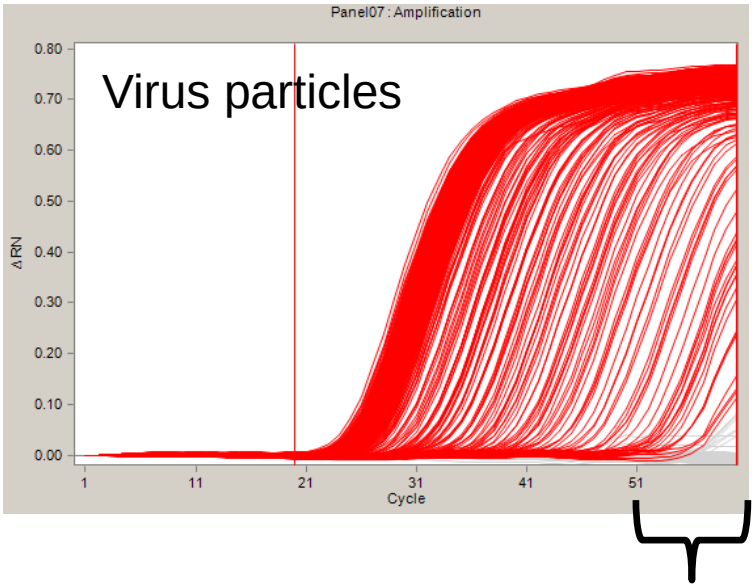
4) Use Poisson statistics to determine concentration of starting material

1st experiment direct dPCR

- NIST standard protocol Fluidigm 12.765
  - 10 minute hot start and 60 cycles
- Many late amplifications – inefficient lysis



0.03 % of amplifications
were in the last 10 cycles
1 out of 3763



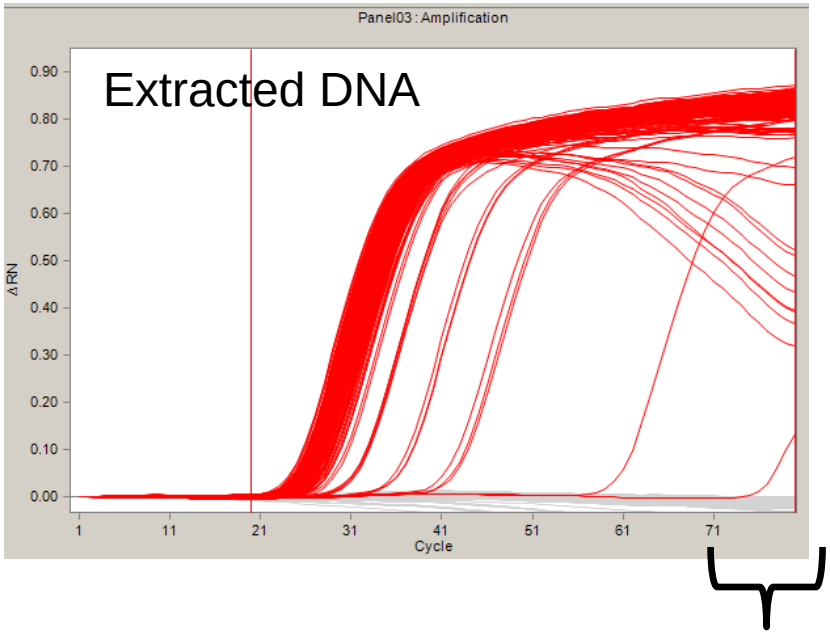
3.6 % of amplifications
were in the last 10 cycles
126 out of 3476

1st experiment direct dPCR

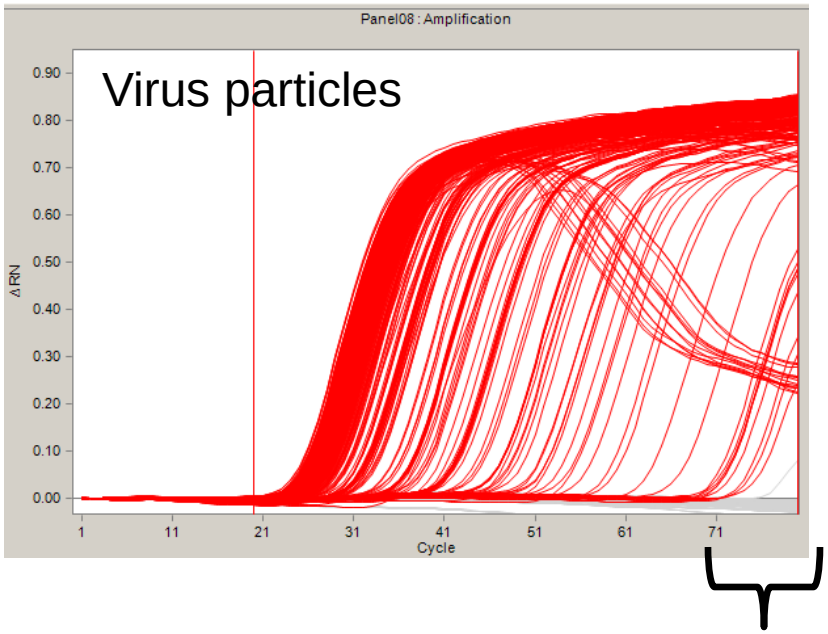
- Are we detecting all virus particles?
  - No, late amplifications indicate the hot start is inefficient at lysing viruses
- Solution add more cycles & lysing steps
  - 10 min hot start
  - Every 5 cycles 2 min at 95 °C (first 25 cycles)
  - 85 cycles total

2nd series direct dPCR

- Extra incubations at 95 °C
- 85 cycles total



0.04 % of amplifications
were in the last 10 cycles
1 out of 2443



0.75 % of amplifications
were in the last 10 cycles
28 out of 3739

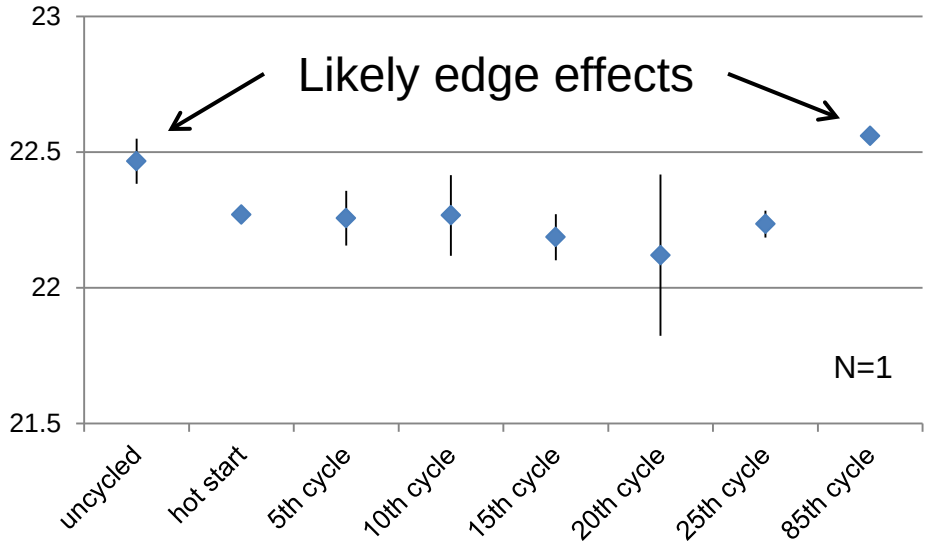
2nd series direct dPCR

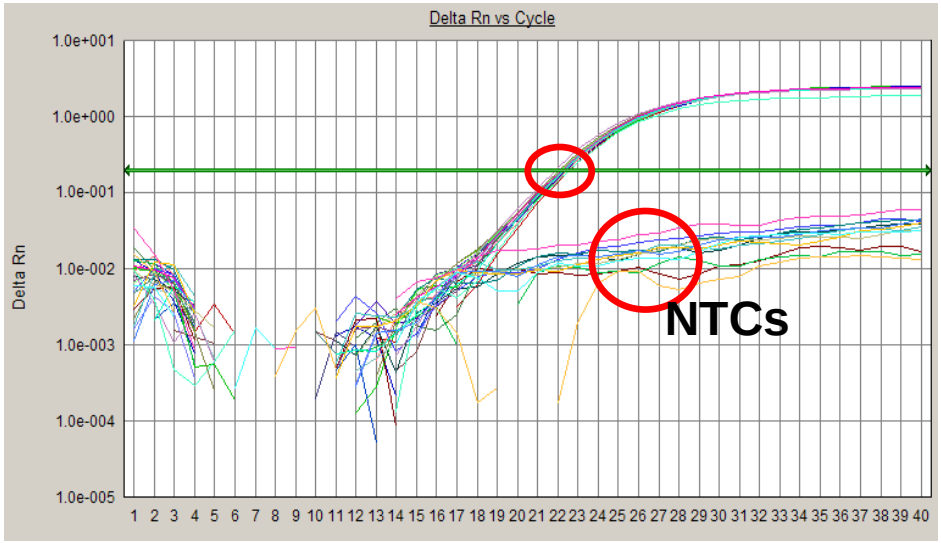
- Still some evidence that all virus particles have not been lysed
  - Additional cycles may not be enough
- Solution: run excessive number of cycles
  - 100 cycles with extra “hot starts”
- Question: will enzyme (Taq Gold – ABI Gene Expression MM) be active at 100 cycles?

Stress Test Polymerase

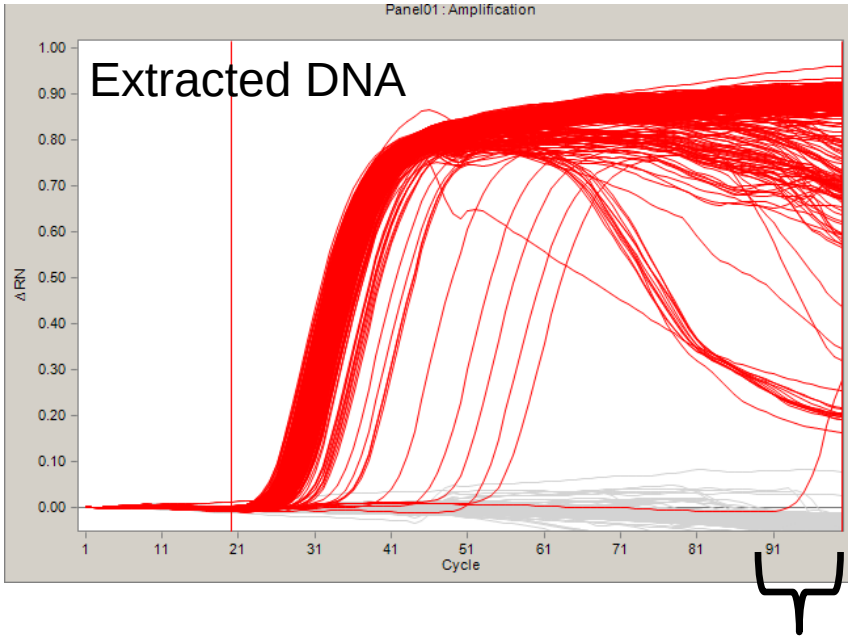
- Master mix (sans DNA) cycled on standard thermal cycler
- Template DNA added
- Run qPCR on 7500

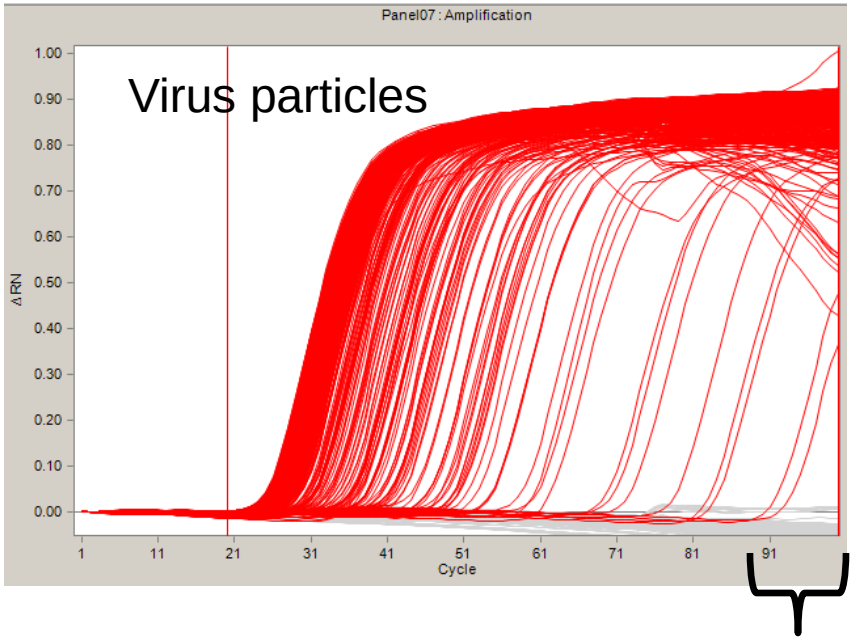
Conclusion: cycling has little to no effect on polymerase activity
Plateau likely due to consuming dNTPs





100 cycle direct dPCR



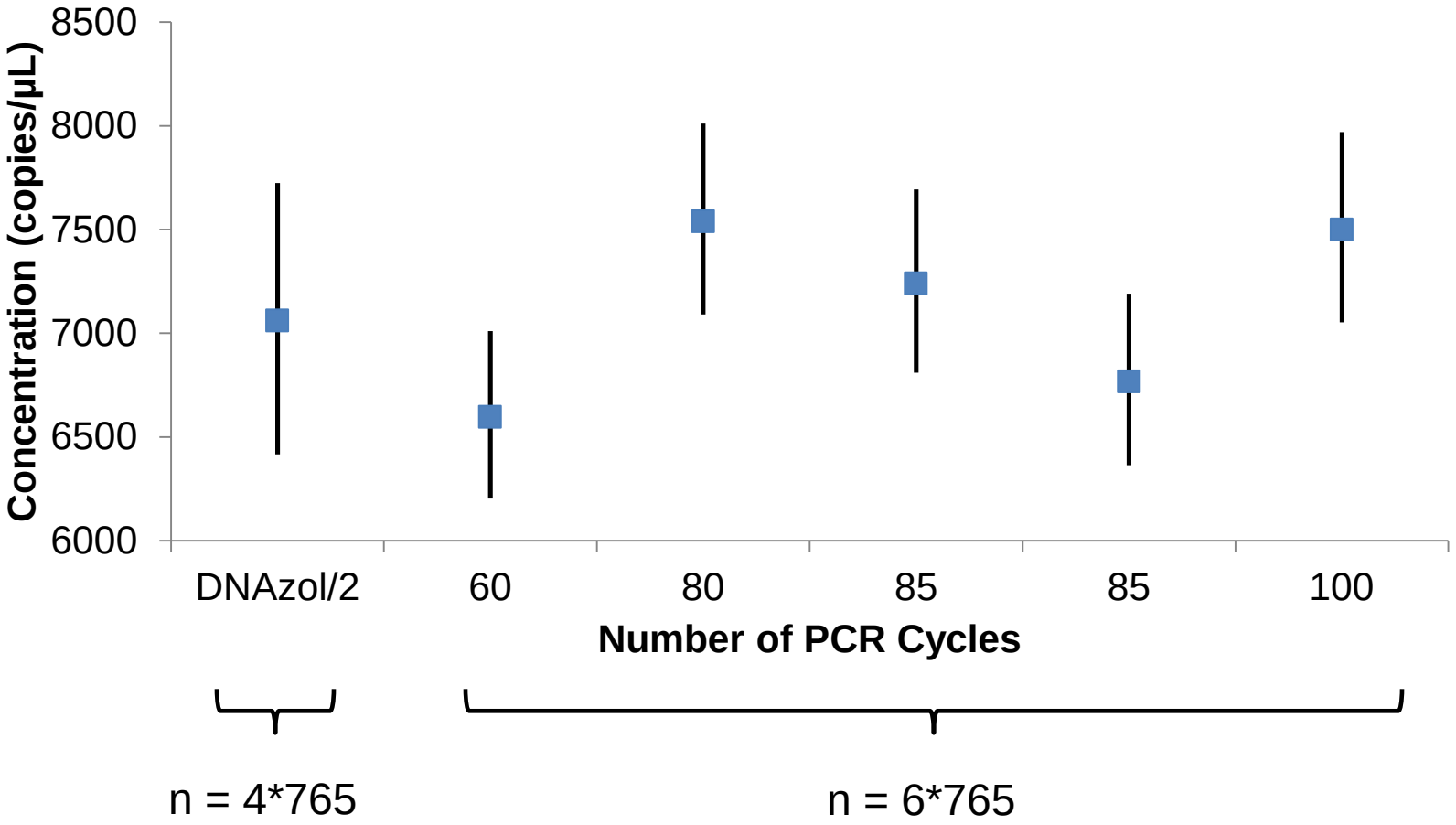


0.04 % of amplifications
were in the last 10 cycles
1 out of 2446

0.20 % of amplifications
were in the last 10 cycles
7 out of 3501

Concentration

- Do additional cycles change result?



Considerations

- Adding cycles adds time & reduces throughput
- Exhaustive cycles gives confidence that all DNA molecules present were amplified
- Principle of diminishing returns
  - Rare very late amplifications may not be significant
- End point systems: Are additional cycles significantly changing the measured concentration?

Considerations (cont.)

- What are you trying to do?
  - Quantifying standard – correct answer
  - Patient sample – would change/variation affect medical decisions?
    - Is ±0.5 log close enough? ±5%? ±1%?
- Dead volume – portion of the sample is not analyzed

	Fluidigm 12.765	Bio-Rad QX100	Life Technologies Quant Studio 3D
Input volume	8 µL	20 µL	variable
Volume analyzed	4.59 µL	10 to 18 µL	up to 20 µL
% Analyzed	57%	50 to 90 %	up to 100 %

Future Directions

- Correlate particle (or cell) count with direct dPCR measurement
- Estimate of absolute extraction efficiency comparing direct dPCR with extraction followed by dPCR.

Conclusions

- Direct dPCR may be acceptable with heat lysis
- Modifications may be necessary
  - Additional cycles
  - Additional heating (lysing) steps
  - Polymerases resistant to inhibition with 5’ to 3’ nuclease activity
- Purpose and required accuracy may affect optimization scheme

Questions

Ross Haynes
Ross.Haynes@nist.gov

This presentation will be available online at
http://www.nist.gov/mml/bmd/genetics/clinical_dna.cfm
Or Google “CDIR NIST”