Clinical Uses of Digital PCR and Measurement Issues

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Integrating Digital PCR
Boston, MA

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Clinical Uses of dPCR

- Absolute quant – absolute concentration
  - Viral load – how much is there?
- Copy Number Variation (CNV)
  - Caner prognosis and treatment options
  - How many HER2 copies are present?
- Minority target detection
  - Fetal DNA in material blood
  - Circulating tumor cells
  - Tumor surrounded by normal cells

Clinical Adoption of dPCR

1. Use a reference material (RM) that is certified by digital PCR
   - You don't have to own or operate a dPCR system
   - Use the RM to quant your patient samples
   - Use the RM to quant your in-house calibrant
2. Use dPCR on your in-house calibrant
   - qPCR is cheaper than dPCR as many components are required for both
3. Use dPCR on your patient samples
   - Sensitivity/Specificity issues
   - Dead volume issue

Clinical Uses of dPCR

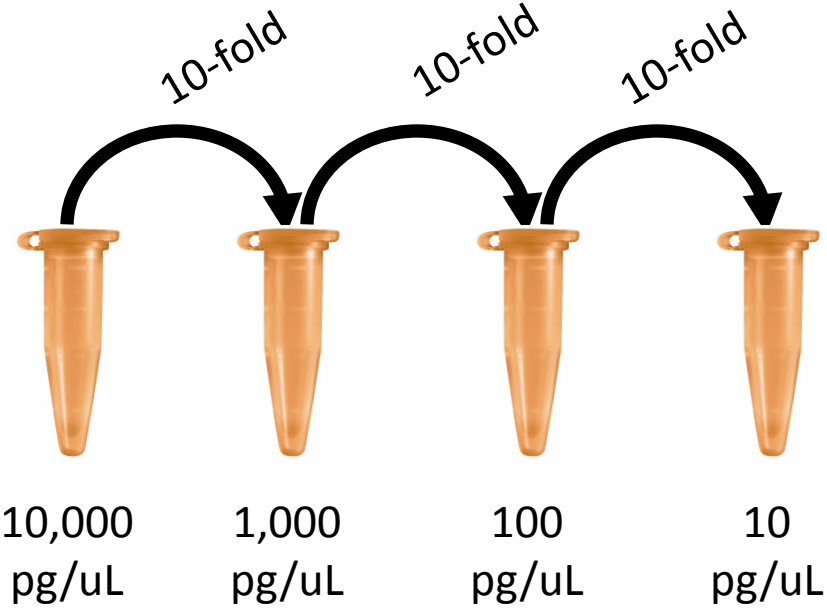
- Detailed knowledge of dPCR including measurement issues
  - Help you design intelligent experiments
  - Utilize dPCR for its strengths
  - Avoid weaknesses of dPCR
  - All of which
    - Save time
    - Save money

Agenda

- Quantitative PCR versus Digital PCR
- Digital PCR Applications
- Poisson Statistics
- Measurement Issues
- Technology Types

qPCR

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

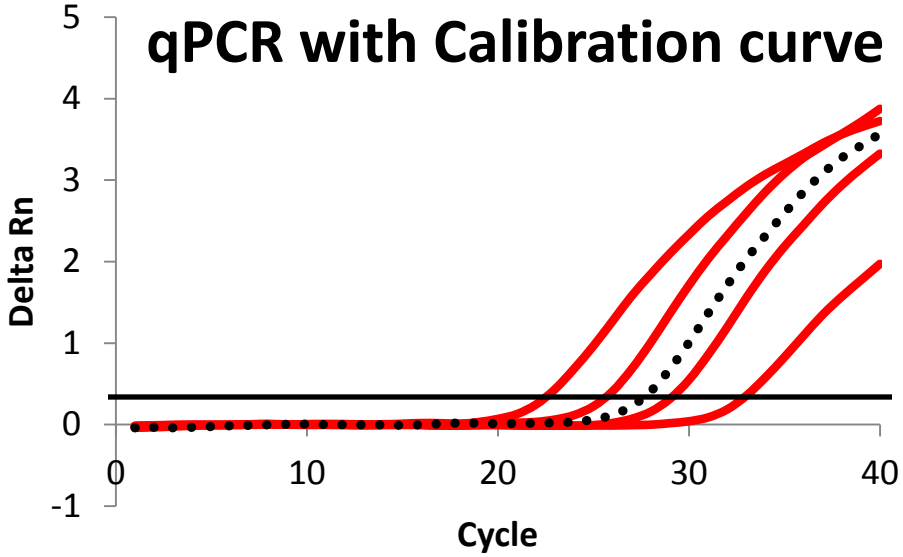


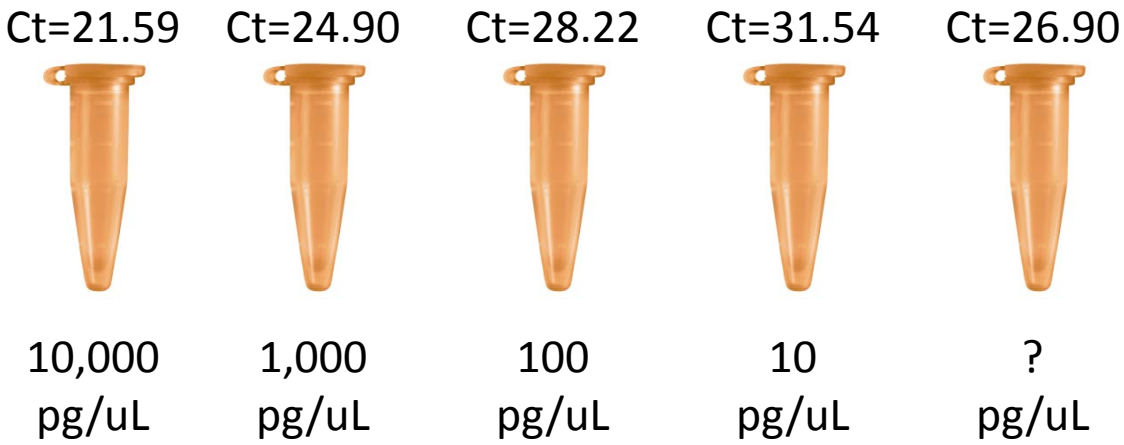
qPCR

- 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)





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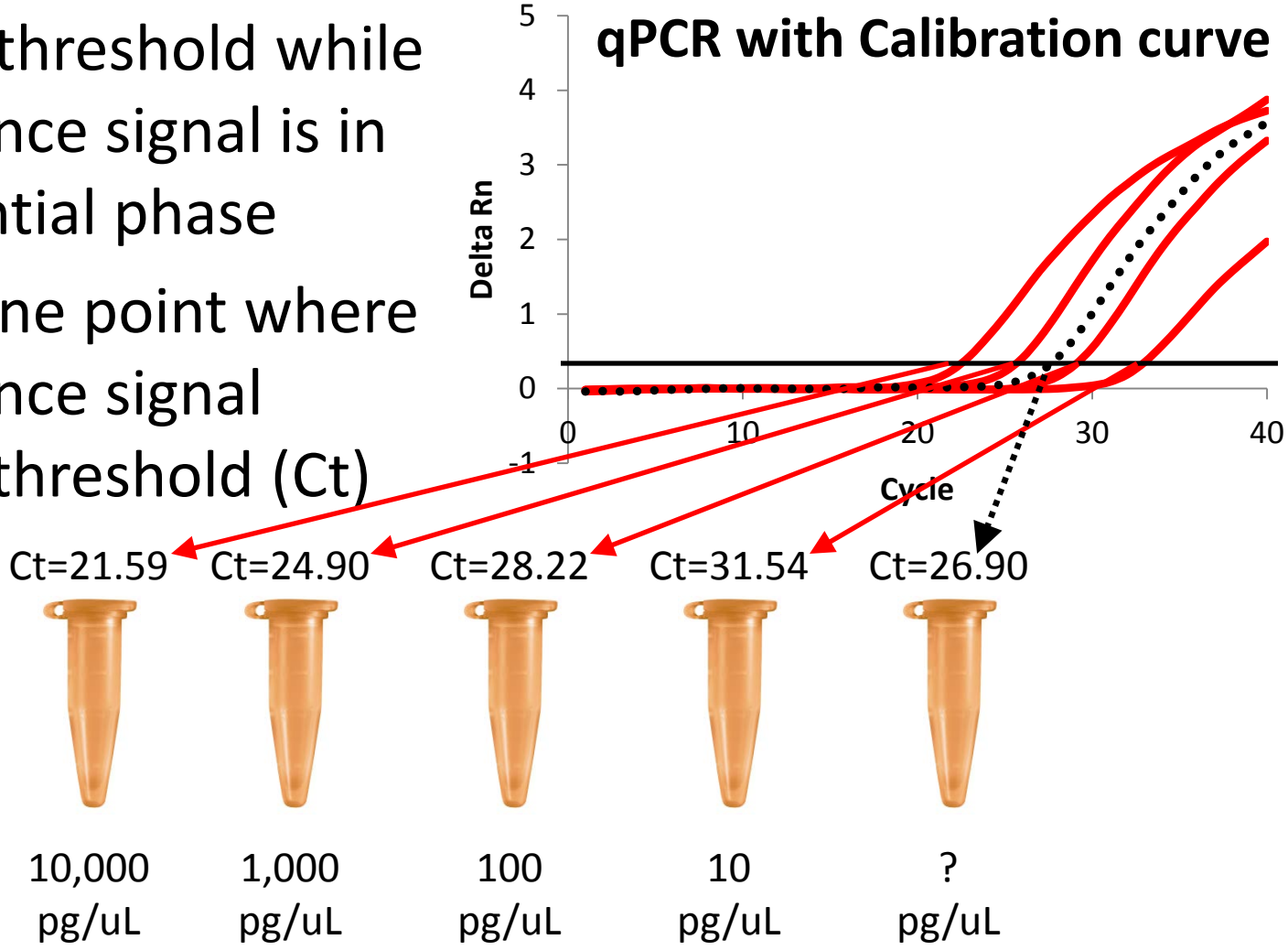
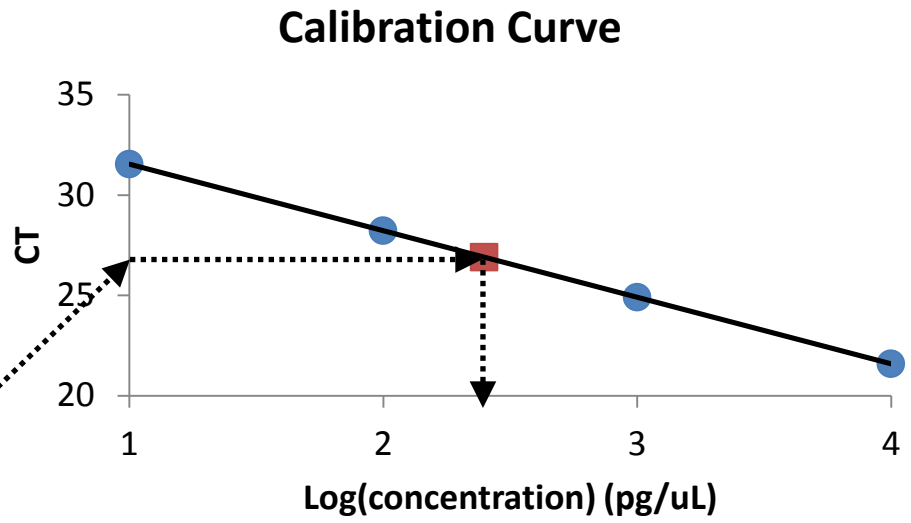


qPCR

- 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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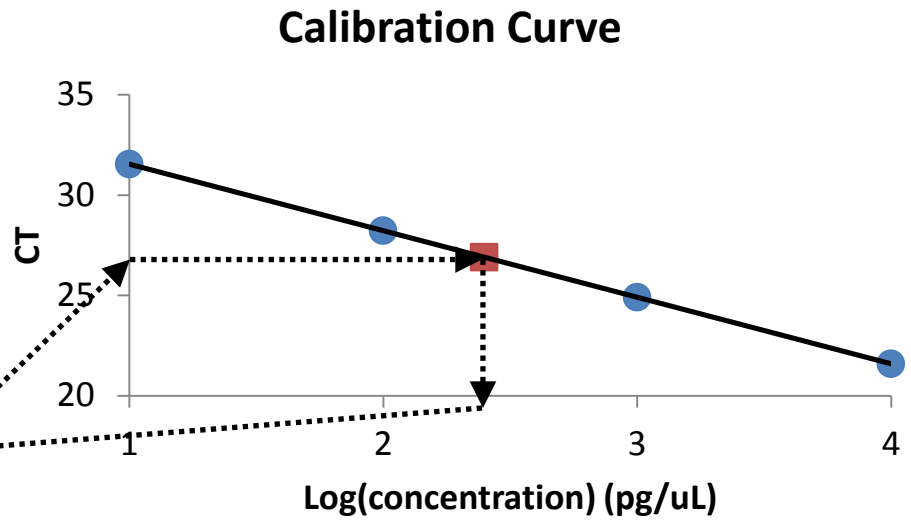

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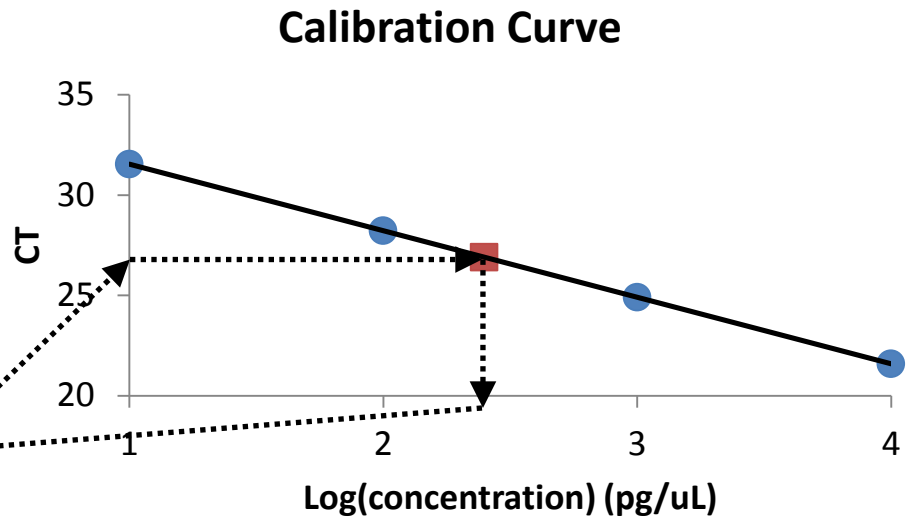
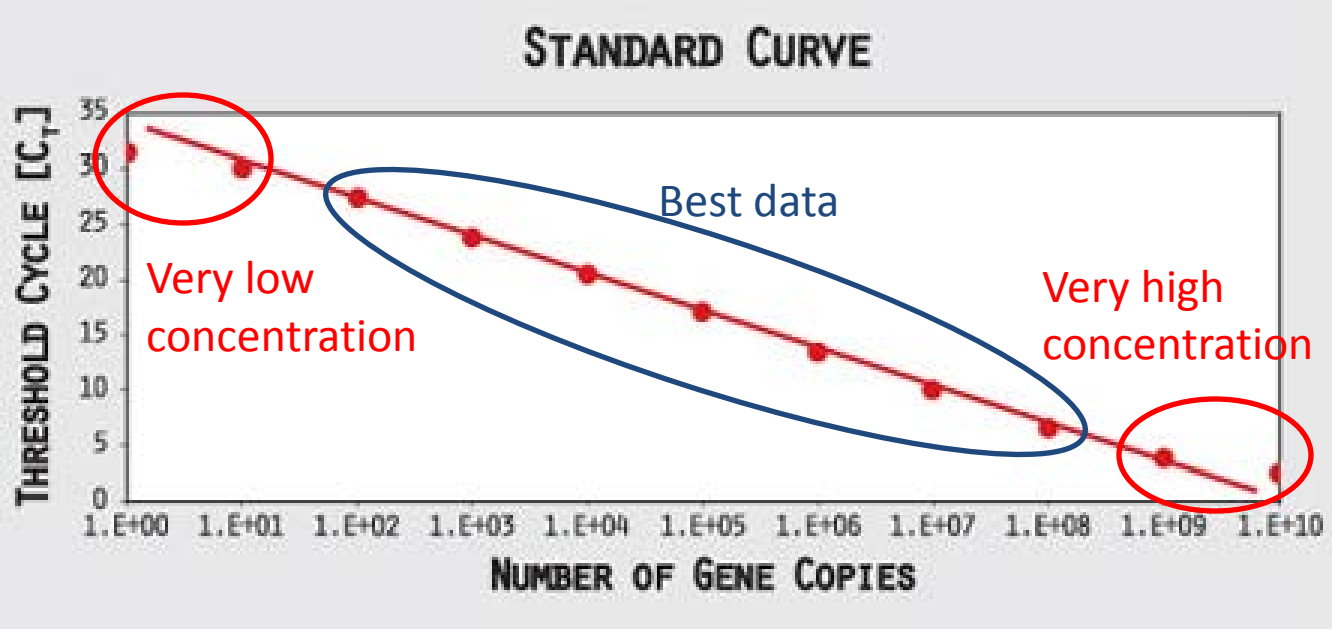


qPCR “Goldilocks Zone”

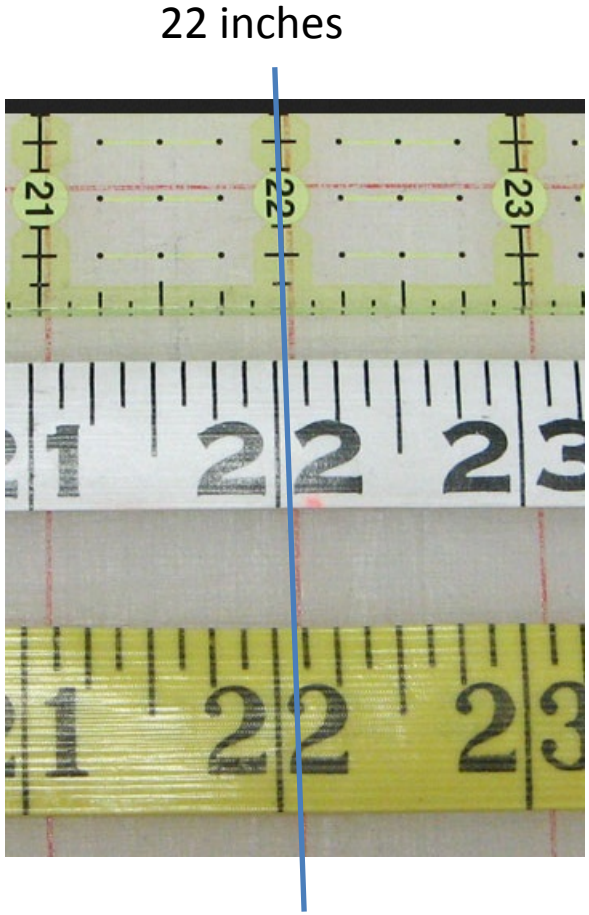
- Very high and very low concentrations do not fit on the line
- Best data obtained from the middle



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/

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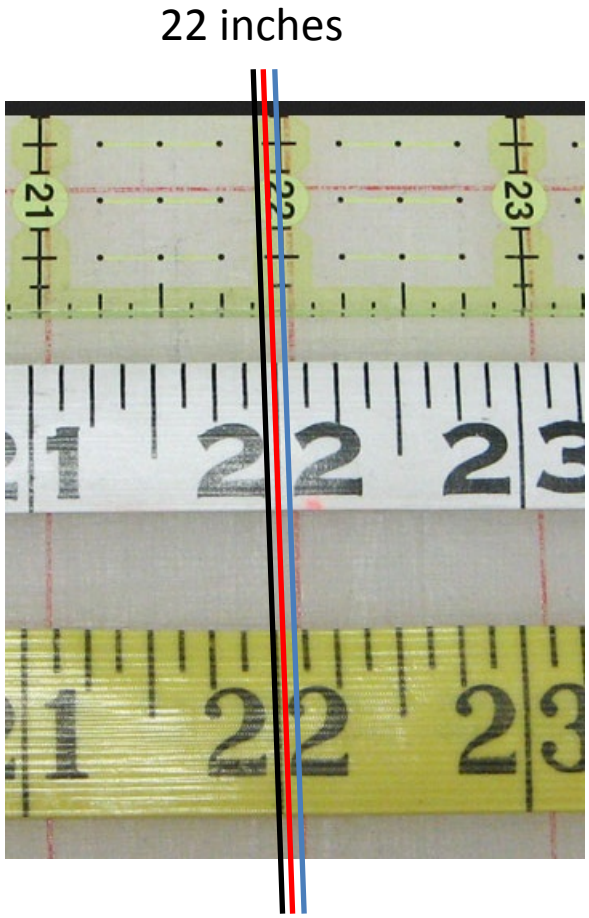

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dPCR

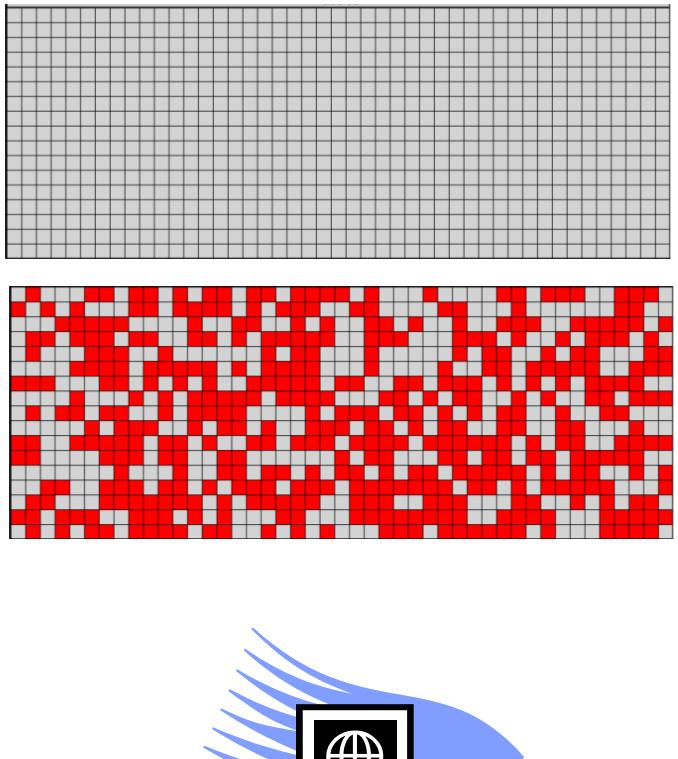
1) Create a PCR mastermix as if for qPCR

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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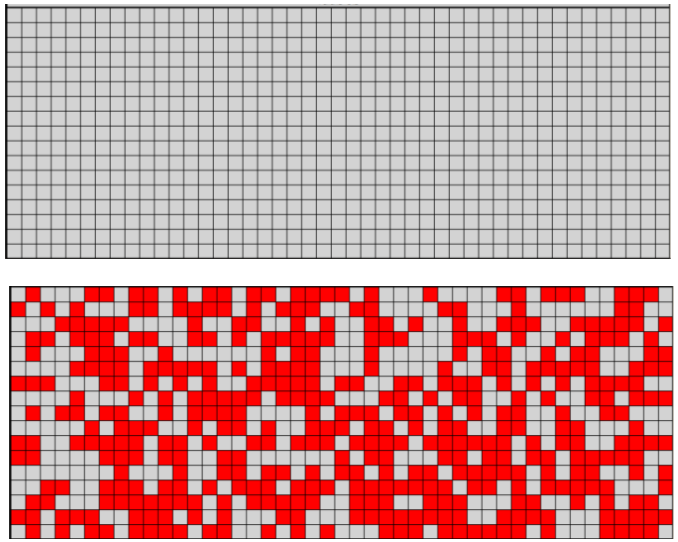
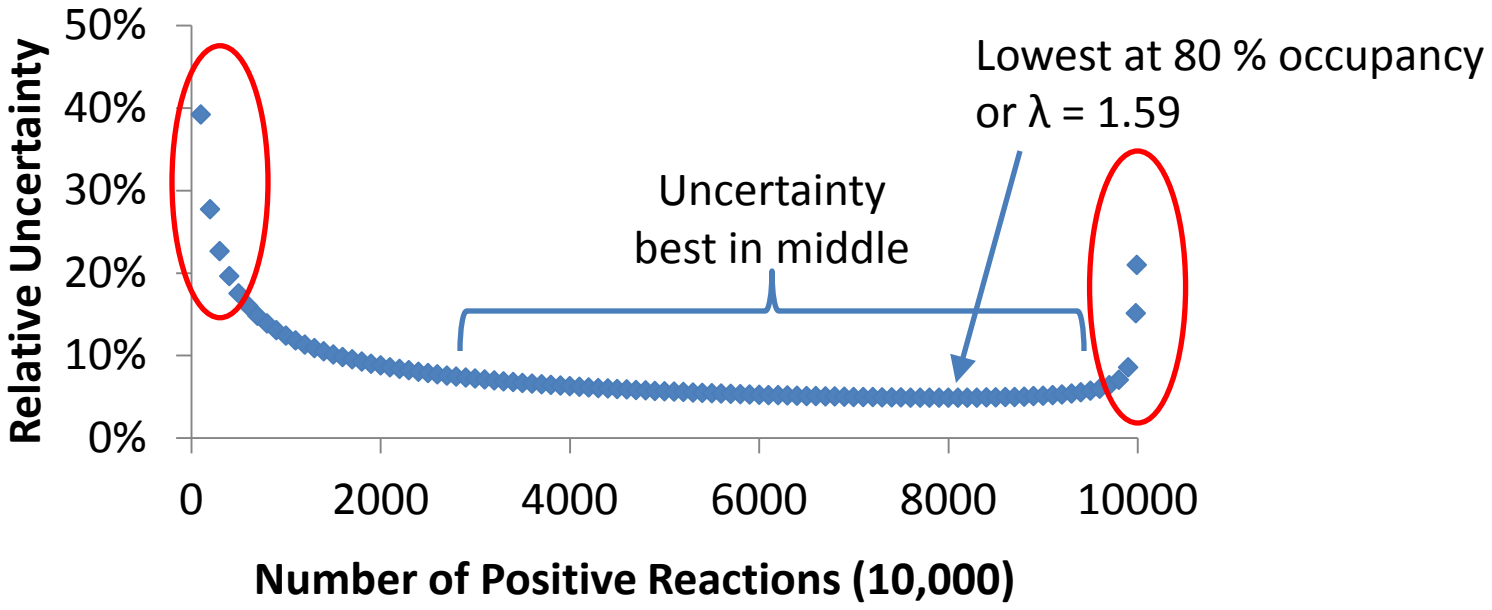


$$\text{Concentration } (c/\mu L) = \frac{\cancel{\text{Number of copies}}}{\text{Volume of all PCR reactions}} = \frac{\text{Number of wells}}{\text{Volume of all PCR reactions}} \ln\left(\frac{\text{Number of wells}}{\text{Number of negative wells}}\right)$$

dPCR "Goldilocks Zone"

- Very high and very low proportion of positive PCR reactions give increased uncertainty
- Uncertainty is lowest in the middle



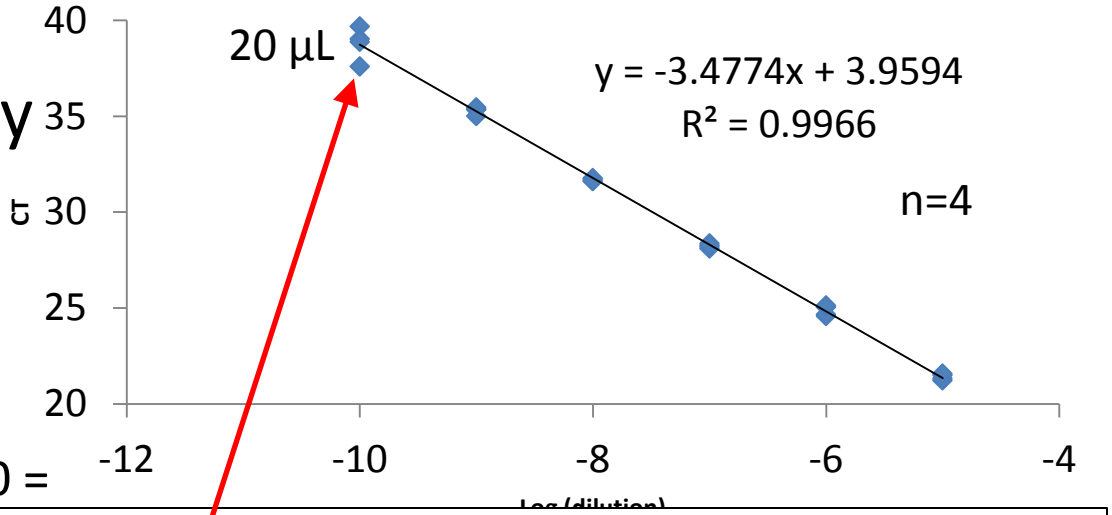
Calculations by Ross Haynes (NIST)

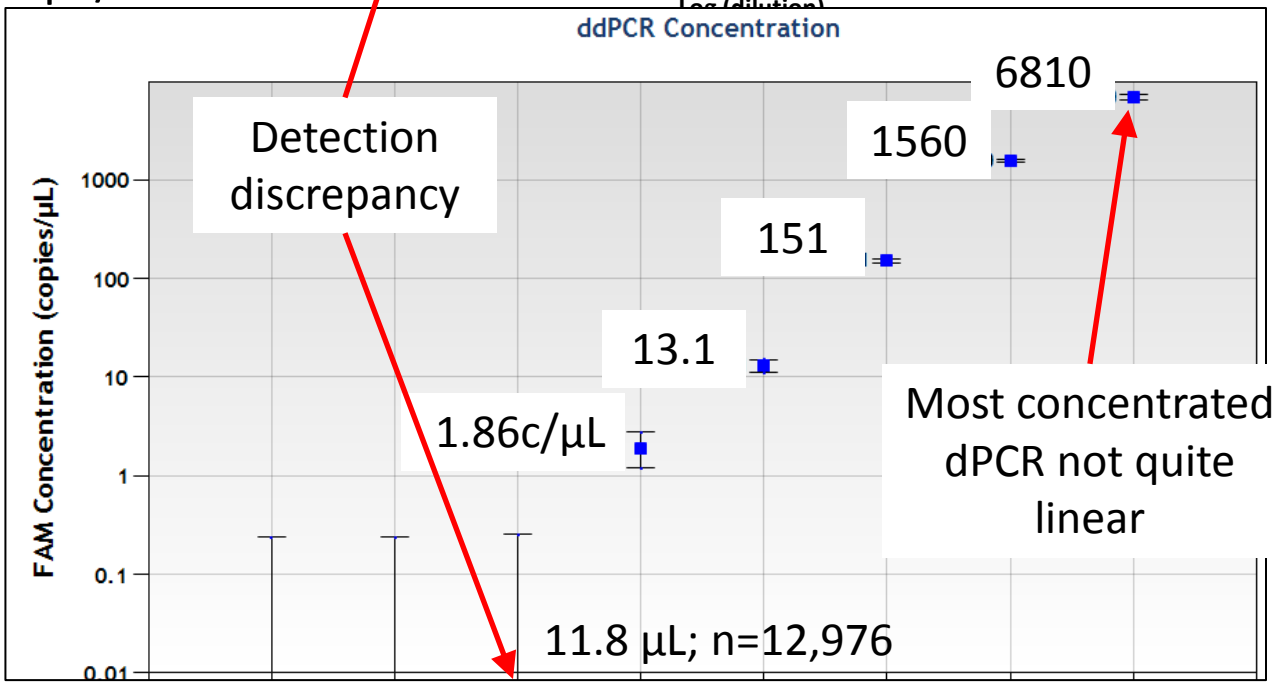
dPCR

- 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

Dynamic Range

- Detection discrepancy
  - Analyzed:
    - qPCR 20 µL
    - dPCR 11.8 µL (60 %)
      - 1.86 c/µL x 11.8 µL/10 = 1.77 copies
      - 22 pos/10 = 2
- Linearity
  - qPCR 10-fold
  - dPCR 4.4-fold
    - 22 neg





Comparison

Quantitative PCR	Digital PCR
Quant is based on amplifiable DNA	Quant is based on amplifiable DNA
Quant is based on a calibrant; as the calibrant goes so will sample values	Quant is based on Poisson sampling statistics (i.e. calibrant free)
Samples must be bracketed by calibrant dilution curve	Samples must be within a range of concentrations
Older technology Widely accepted	New technology Gaining acceptance
Currently less expensive	Currently more expensive
Larger dynamic range	Smaller dynamic range

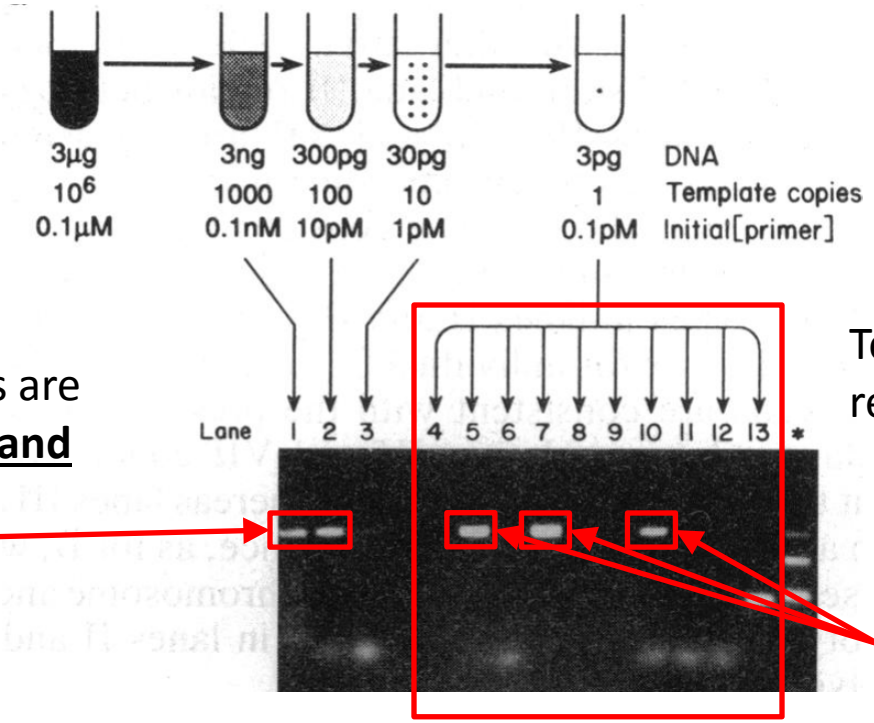
Applications of dPCR

- Absolute quant – absolute concentration
  - Viral load – how much is there?
- Copy Number Variation (CNV)
  - Caner prognosis and treatment options
  - How many HER2 copies are present?
- Minority target detection
  - Fetal DNA in material blood
  - Circulating tumor cells
  - Tumor surrounded by normal cells

Original Application

- Single Molecule Dilution (SMD) PCR
  - Resolve maternal/paternal sequence

Figure 3a



These PCR fragments are mixture of maternal **and** paternal sequences

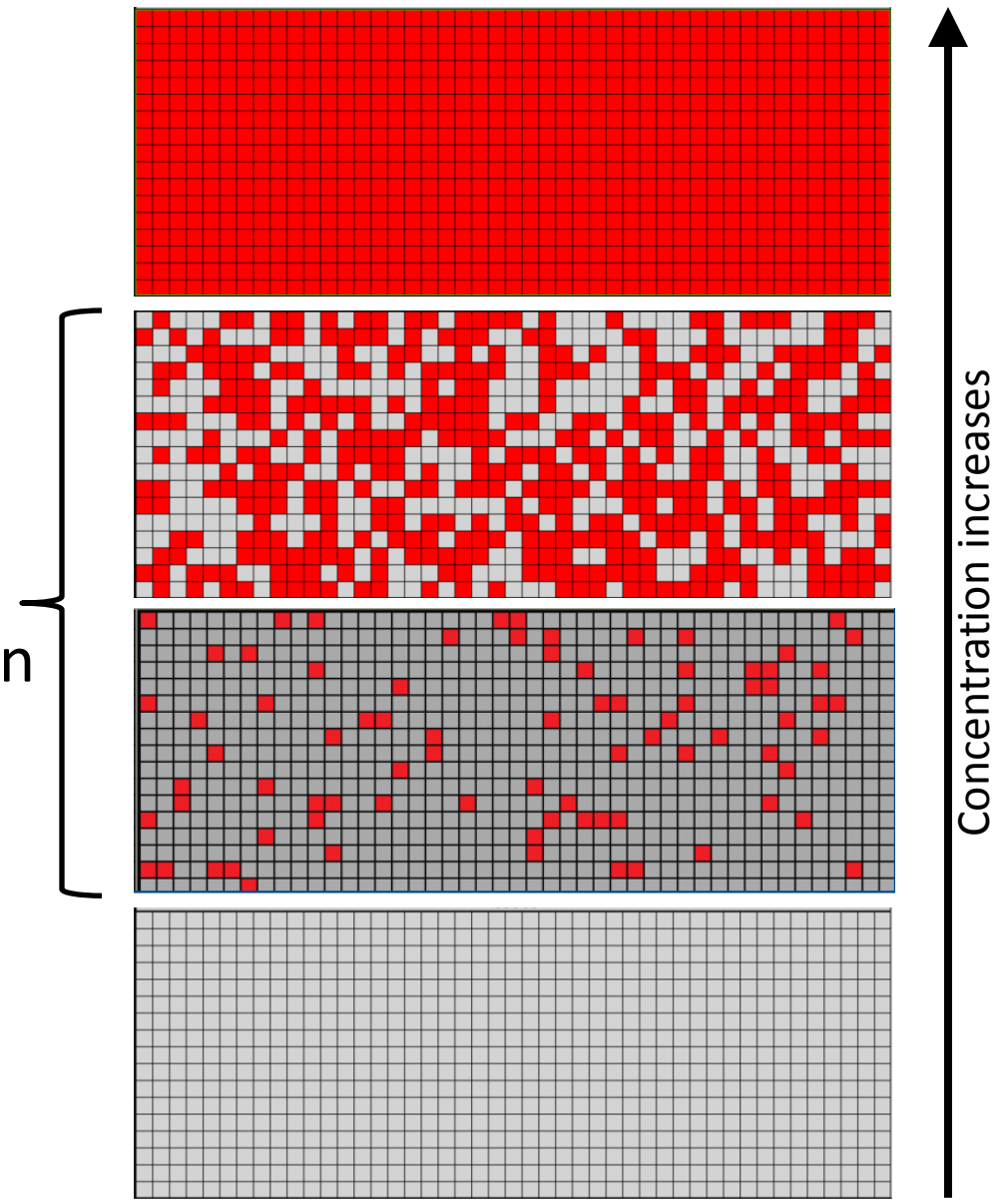
Ten replicate PCR reactions

These PCR fragments are likely either maternal **or** paternal sequence.

Ruano et al. Proc Natl Acad Sci U S A. 1990 Aug;87(16):6296-300.

Range of Concentrations

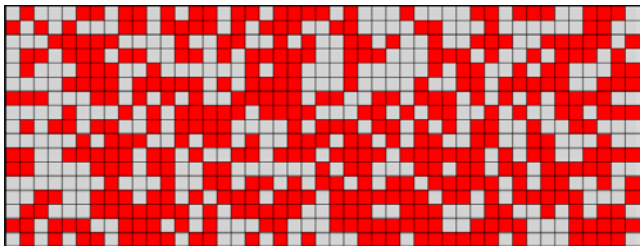
- Saturated
  - Every well has at least one copy
- Binary detection
  - Calculate concentration
- No amplification
  - < 1 copy/total volume

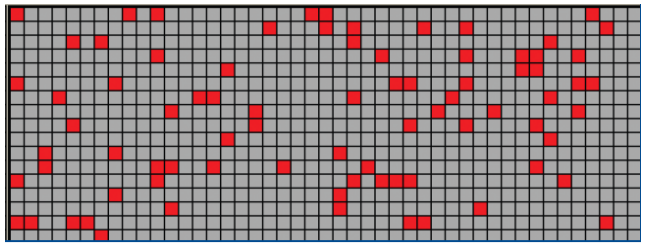


Absolute Quantitation

- Using Poisson statistics an estimation of number of copies can be determined
- Volume is given by manufacturer
  - Research indicates this estimate is reasonable

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





Absolute Quantitation

- PCR amplify → 1000 reactions
- Count positive wells → 594 reactions amplified
- Poisson stats → 900 copies
- Divide by total volume → 20 µL
- Correct for dilutions → 10 fold dilution
- Concentration → 450 c/µL = (900/20)x10
- Uncertainty is based on binomial statistics → 95 % CI: 415 to 489 c/µL

Relative Quantitation

- Copy Number Variations (CNVs)

$$\text{CNV of gene 1} = \frac{\text{Absolute quant gene 1}}{\text{Absolute quant gene 2}}$$

  - E.g. number of EGFR copies has implications in some cancers*

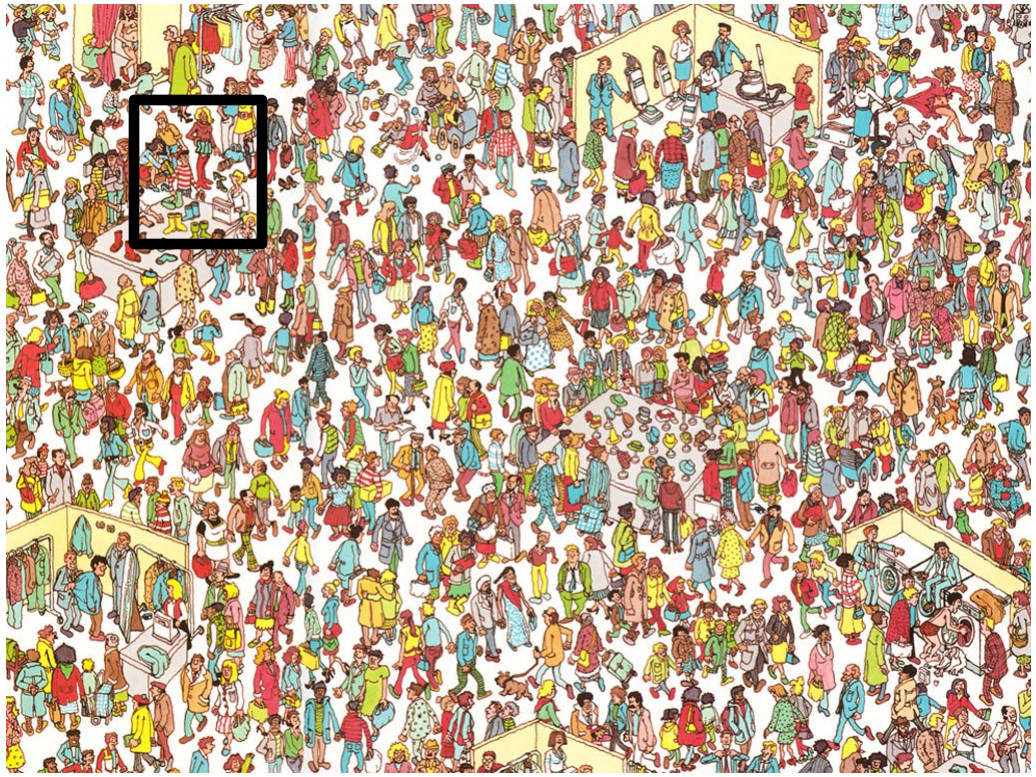
- Relative Gene Expression Levels

$$\text{Expression of gene 1} = \frac{\text{Absolute quant RNA 1}}{\text{Absolute quant RNA 2}}$$

*Cappuzzo et al. J Natl Cancer Inst. 2005 May 4;97(9):643-55.

Minority Target Measurement

- Reduce background by partitioning
- More productive than an undergrad



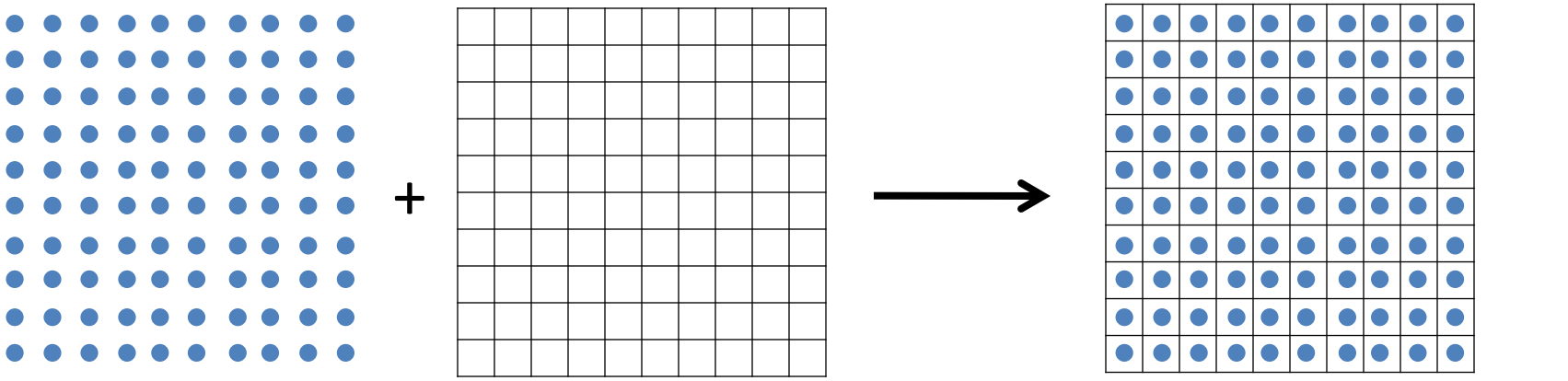
http://www.findwaldo.com/fankit/graphics/IntlManOfLiterature/Scenes/DepartmentStore.jpg

Statistics

- Why do we need them?
  - “Aren’t we just counting?”
- Why use Poisson statistics
  - Random distribution
- Assumptions of dPCR and potential issues

Even vs. Random Distributions

- Even distribution



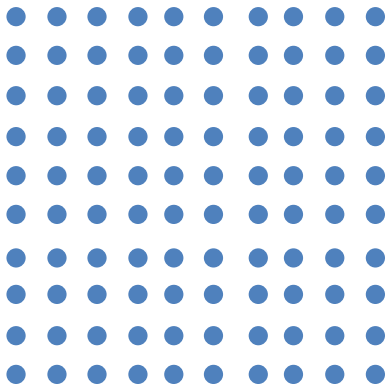
100 molecules

100 wells

All 100 well have exactly one molecule

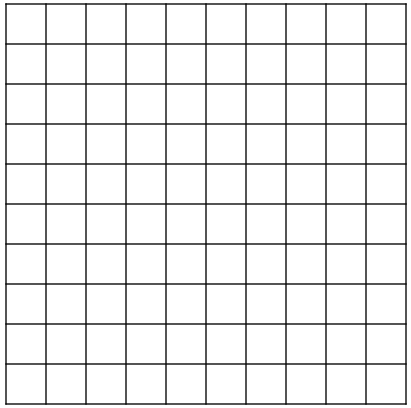
Even vs. Random Distributions

- Random distribution

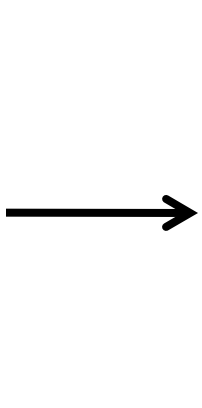


100 molecules

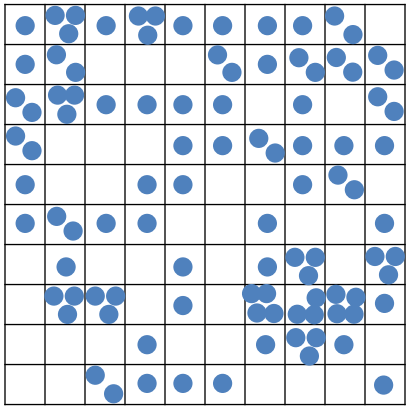
+



100 wells



One possibility

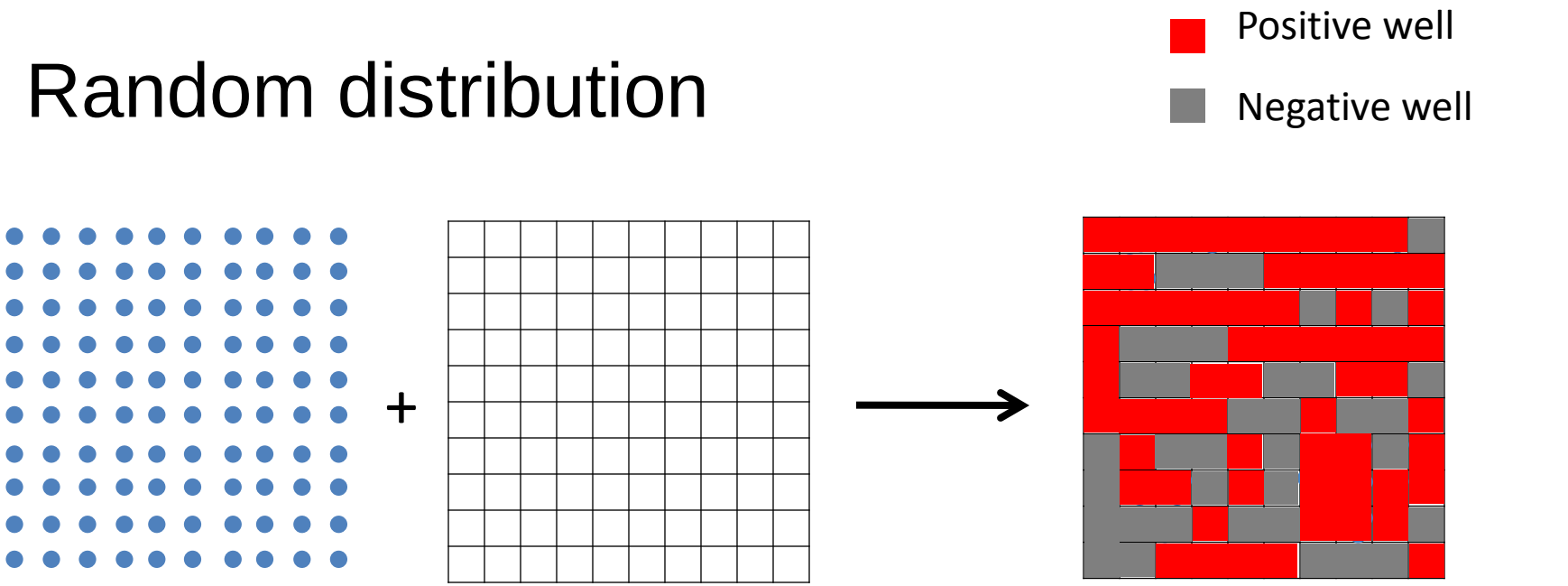


37 wells with 0 molecules
39 wells with 1 molecule
13 wells with 2 molecules
9 wells with 3 molecules
2 wells with 4 molecules
Total = 100 molecules

If we could count the individual molecules we would not need to use Poisson statistics or dPCR

Even vs. Random Distributions

- Random distribution



100 molecules

100 wells

37 wells with 0 molecules
63 wells with ≥1 molecules
Poisson calculated 99 molecules
95%CI 77 to 129 molecules

Poisson Statistics

- Look it up from a table of values

Concentration = μ/volume of one PCR reaction

Estimate the average number of copies per PCR reaction (μ)

Proportion of negative wells

TABLE A.2
Poisson probabilities

	μ									
k	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
0	0.6065	0.3679	0.2231	0.1353	0.0821	0.0498	0.0302	0.0183	0.0111	0.0067

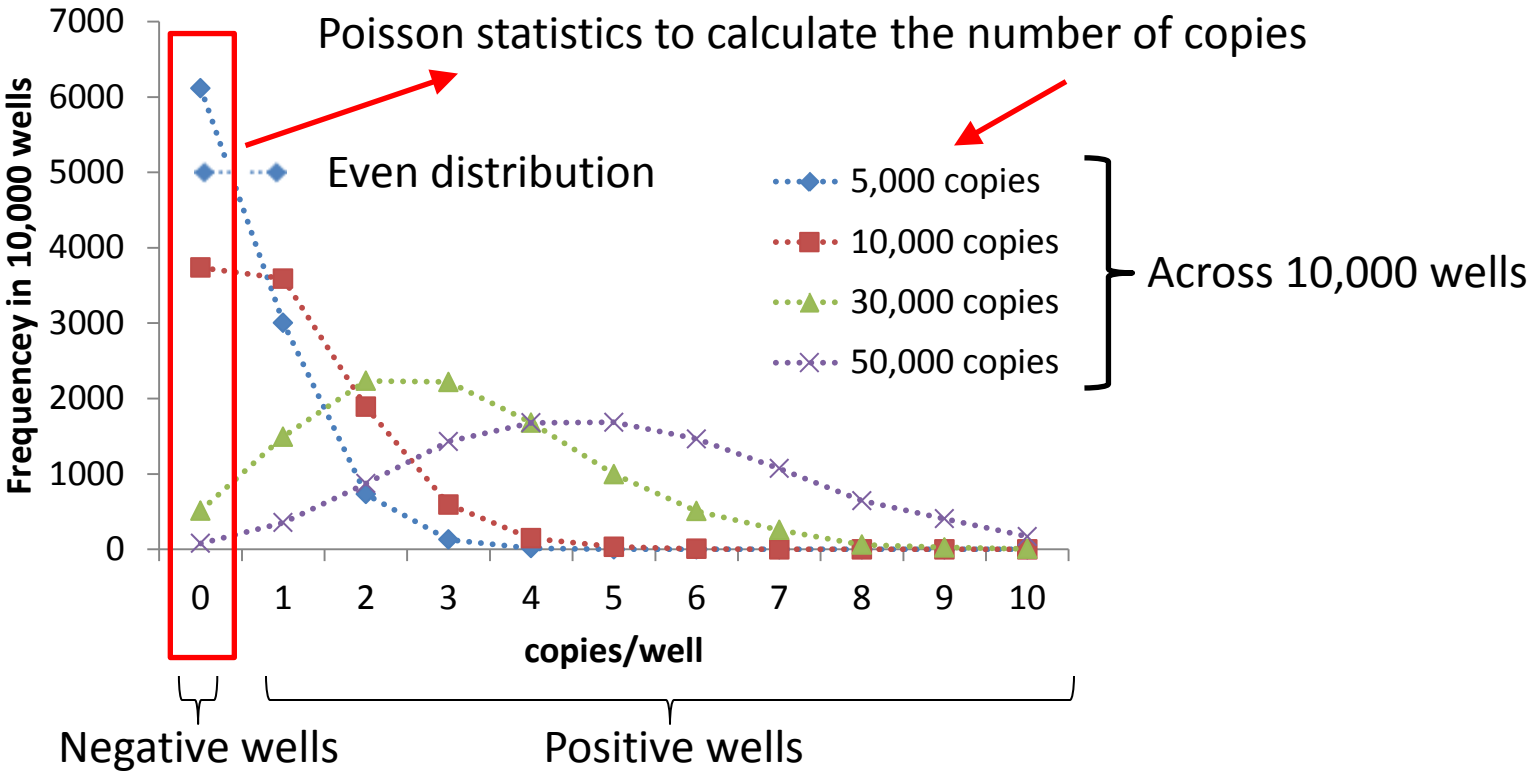
	μ									
k	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
0	0.0041	0.0025	0.0015	0.0009	0.0006	0.0003	0.0002	0.0001	0.0001	0.0000
1	0.0225	0.0149	0.0098	0.0064	0.0041	0.0027	0.0017	0.0011	0.0007	0.0005
2	0.0618	0.0446	0.0318	0.0223	0.0156	0.0107	0.0074	0.0050	0.0034	0.0023
3	0.1133	0.0892	0.0688	0.0521	0.0389	0.0286	0.0208	0.0150	0.0107	0.0076
4	0.1558	0.1339	0.1118	0.0912	0.0729	0.0573	0.0443	0.0337	0.0254	0.0189
5	0.1714	0.1606	0.1454	0.1277	0.1094	0.0916	0.0752	0.0607	0.0483	0.0378
6	0.1571	0.1606	0.1575	0.1490	0.1367	0.1221	0.1066	0.0911	0.0764	0.0631
7	0.1234	0.1377	0.1462	0.1490	0.1465	0.1396	0.1294	0.1171	0.1037	0.0901
8	0.0849	0.1033	0.1188	0.1304	0.1373	0.1396	0.1375	0.1318	0.1232	0.1126
9	0.0519	0.0688	0.0858	0.1014	0.1144	0.1241	0.1299	0.1318	0.1300	0.1251
10	0.0285	0.0413	0.0558	0.0710	0.0858	0.0993	0.1104	0.1186	0.1235	0.1251
11	0.0143	0.0225	0.0330	0.0452	0.0585	0.0722	0.0853	0.0970	0.1067	0.1137
12	0.0065	0.0113	0.0179	0.0263	0.0366	0.0481	0.0604	0.0728	0.0844	0.0948
13	0.0028	0.0052	0.0089	0.0142	0.0211	0.0296	0.0395	0.0504	0.0617	0.0729

1 negative in 10,000

Pagano & Gauvreau "Principles of Biostatistics" 2nd ed. Appendix A pA-6

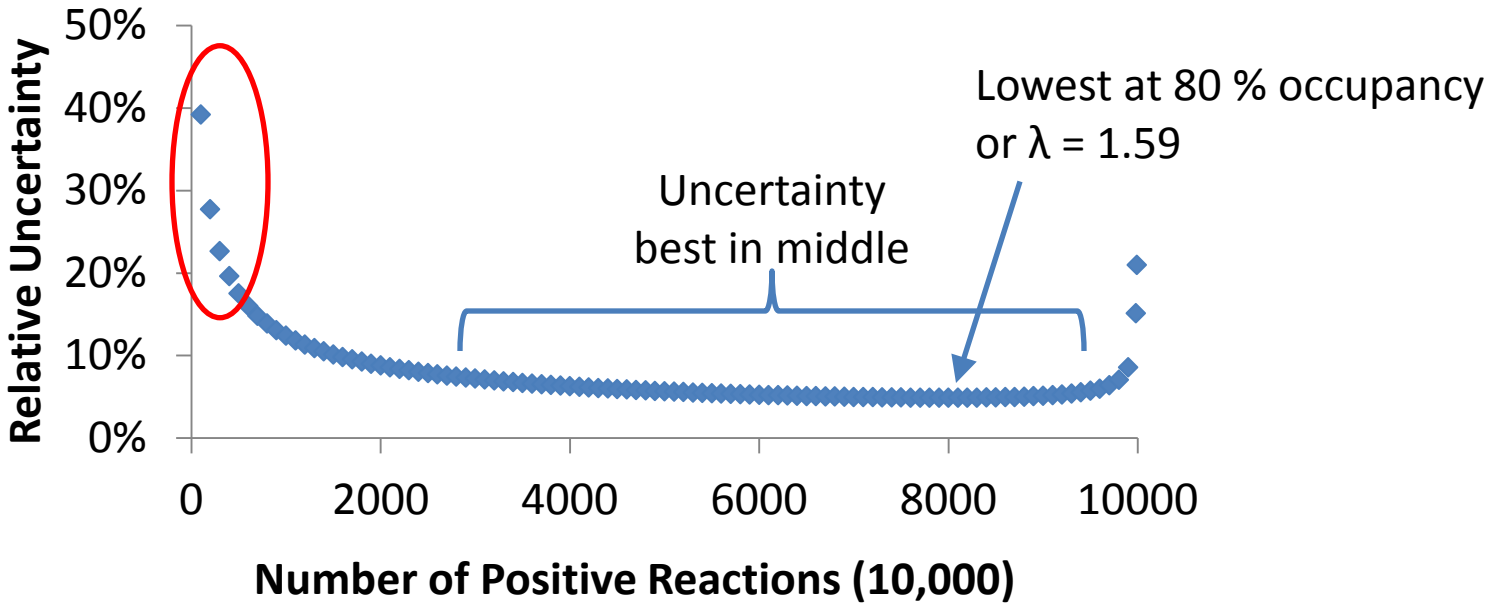
Poisson Statistics

- Poisson Distributions look like a normal distribution crashing into zero
  - When negative values are impossible



Why do we need Poisson Stats?

- Molecules distribute randomly
- Diluting to either 0 to 1 copy would be at the extremely dilute & have high uncertainty



Calculations by Ross Haynes (NIST)

Poisson Statistics

- $N\lambda = N \ln(N/N-x)$
  - $N\lambda$ = number of copies across all wells
  - N = number of wells
  - x = number of positive wells
  - λ = average number of copies in a well
- Concentration (copies/µL) =

  Number of copies / total volume x dilution factor

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

Heyries et al. Nat Methods. 2011 Jul 3;8(8):649-51.

Uncertainty Calculations

- If number of PCR reactions (N) is large enough, calculate the uncertainty for a binomial

$$\hat{p} \pm z_c \sqrt{\frac{\hat{p}(1-\hat{p})}{N}}$$

$N\lambda = N \ln(N/N-x)$

- Z_c is 1.96 for 95 % confidence interval
- $\hat{p}$ is the proportion of PCR reactions that amplified target; equal to x/N
- Solve for x_{hi} and x_{lo} & plug into Poisson equation

Dube et al. PLoS One. 2008 Aug 6;3(8):e2876.

Absolute Quantitation

- PCR amplify → 1000 reactions
- Count positive wells → 594 reactions amplified
- Poisson stats → 900 copies
- Divide by total volume → 20 µL
- Correct for dilutions → 10 fold dilution
- Concentration → 450 c/µL = (900/20)x10
- Uncertainty is based on binomial statistics → 95 % CI: 415 to 489 c/µL

Uncertainty Calculations

Define parameters:

- N = 1000 reactions
- X = 594 reactions amplified
- Z_c is 1.96 (for 95% CI)
- 10-fold dilution; 20 µL rxn

$$\hat{p} \pm z_c \sqrt{\frac{\hat{p}(1-\hat{p})}{N}}$$

$$0.594 \pm 1.96 \sqrt{\frac{0.594 \text{ X } 0.406}{1000}}$$

0.594±0.0304 = (0.564, 0.624)

X_{lo} = 564 X_{hi} = 624

Copies = Nλ = N ln(N/N-x)

Nλ = 1000 ln(1000/406) = 900

900 c/20 µL x 10 = 450 c/µL

$N\lambda_{hi}$ = 1000 ln(1000/376) = 978

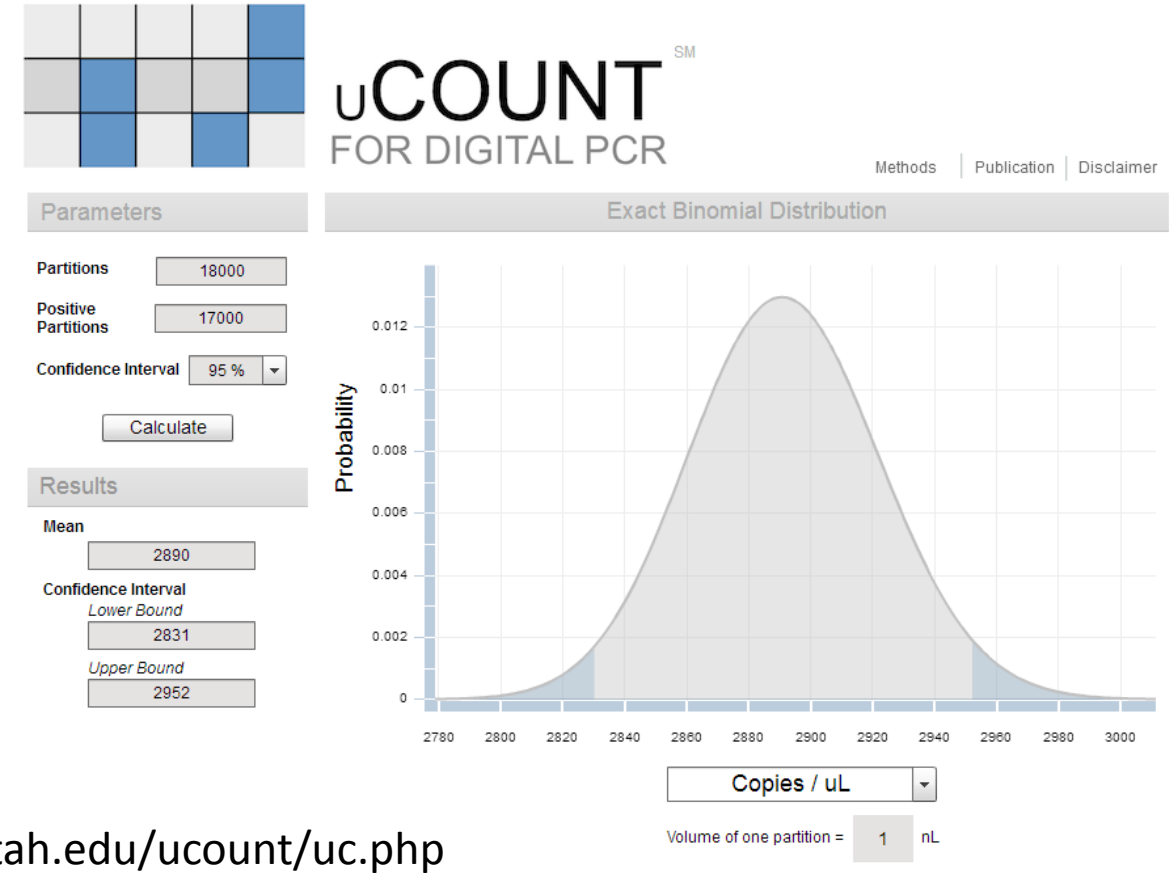
978 c/20 µL x 10 = 489 c/µL

$N\lambda_{lo}$ = 1000 ln(1000/436) = 830

830 c/20 µL x 10 = 415 c/µL

Exact Binomial Statistics

- uCOUNT(SM) for Digital PCR: U. of Utah
- Exact Binomial Distribution



https://dna.utah.edu/ucount/uc.php

Poisson Statistics

dPCR has several assumptions some of which relate to Poisson statistics

Assumptions:

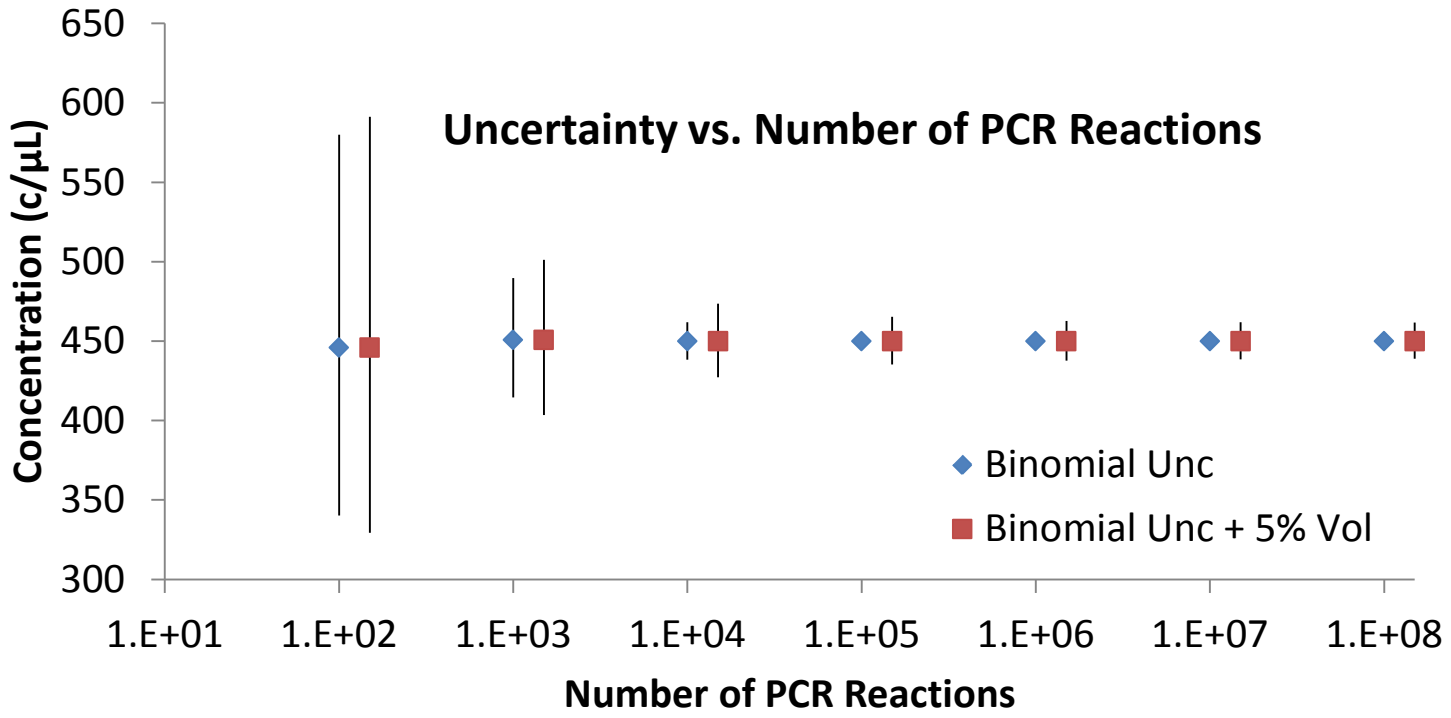
1. Large number of PCR reactions
   - As with most statistics a larger n means more power to discriminate small differences
2. Random distribution
   - It is easier to think in arithmetic division rather than random distribution
3. Independent segregation of molecules
4. Every copy gives a signal
5. Every molecule is dsDNA

Assumption 1
Large number of PCR reactions

- Student's t table n > 120 is considered large*
- dPCR 100s to 1000s to 1,000,000s of PCR reactions
- Accuracy and precision requirements will dictate number of PCR reactions

*Pagano & Gauvreau "Principles of Biostatistics" 2nd ed. Appendix A pA-10

Binomial Uncertainty & Volume Uncertainty



Number of PCR reactions	n=100	n=1000	n=10k	n=100k	n=1m	n=10m	n=100m
Binomial Unc	53.75%	16.67%	5.26%	1.66%	0.53%	0.17%	0.05%
Binomial Unc + 5% Vol Unc	58.75%	21.67%	10.27%	6.67%	5.53%	5.17%	5.06%

Pinheiro et al. Anal Chem. 2012 Jan 17;84(2):1003-11.
Bhat et al. Anal Bioanal Chem. 2009 May;394(2):457-67.

Assumption 2
Random distribution

- Use Poisson statistics because molecules distribute randomly
- For validating dPCR principles:
  - Random distribution is testable with Ripley's K function
  - Detects clustering or ordering of positive wells
  - See paper below for details

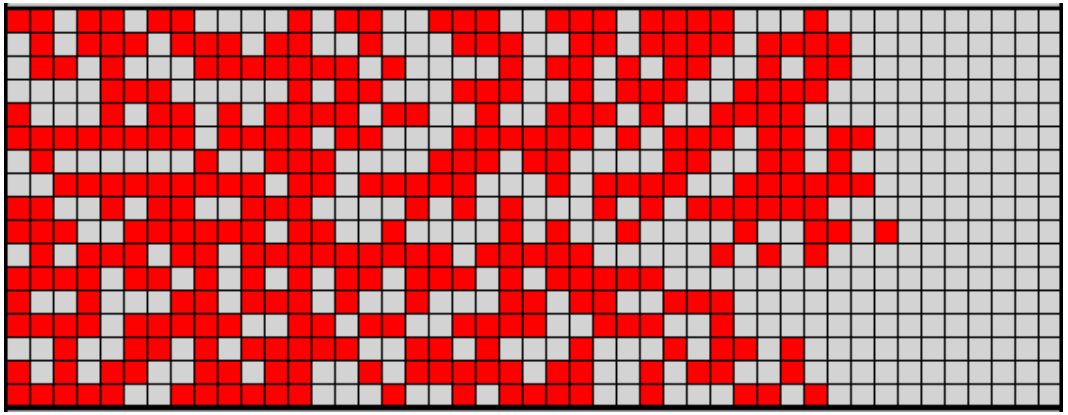
Bhat et al. Anal Bioanal Chem. 2009 May;394(2):457-67.

Assumption 2
Random distribution

- Everyday testing, use eyeball method

This area empty

Clustered



Fluidigm 12.765 digital array 1151-120-151 panel 5

Loaded this direction

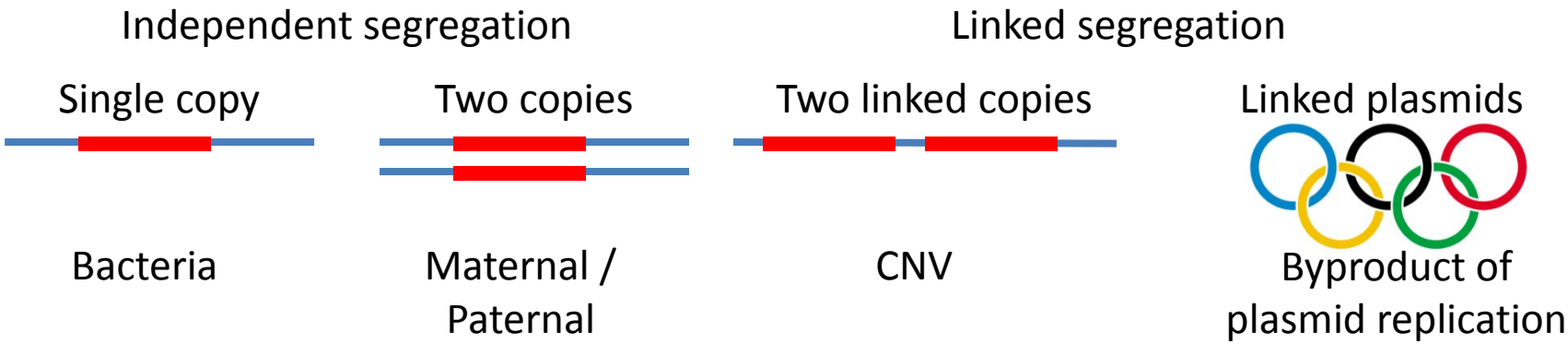
Looks like a loading problem

Ordered

http://www.newretrodining.com/retro_laminates.htm

Assumption 3
Independent Segregation

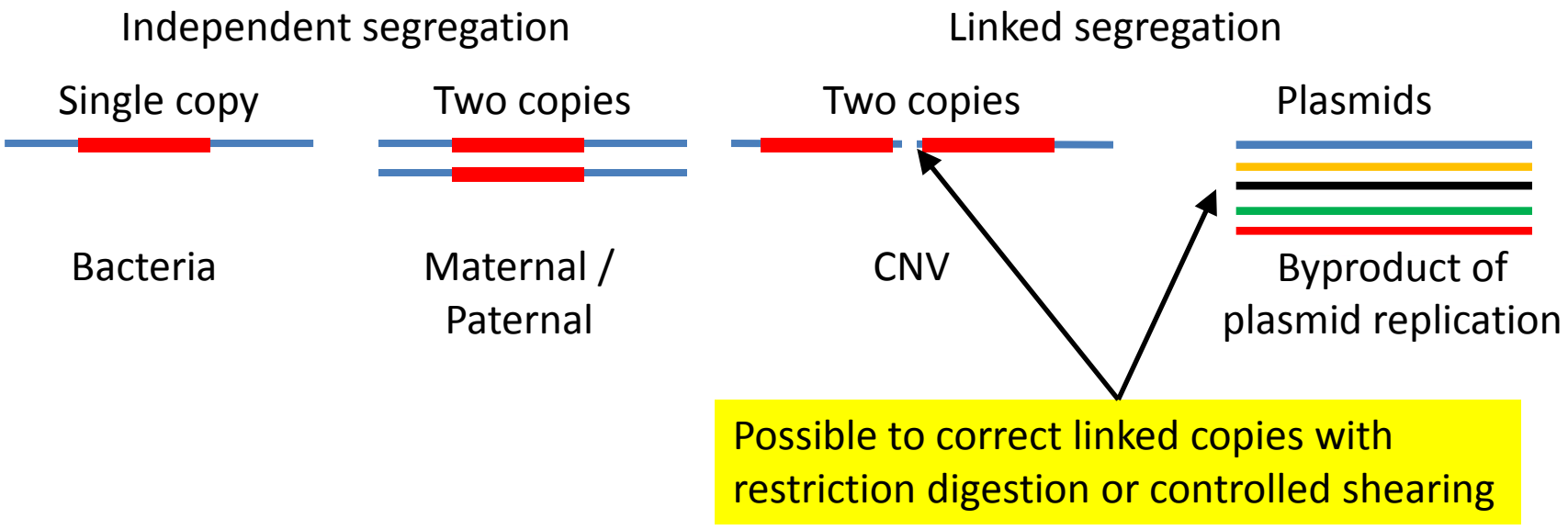
- If molecules are concatemers or physically bound to one another one “amplification-forming-unit” will consist of multiple copies
  - i.e. two linked copies will be counted as one copy



Possible to correct linked copies with restriction digestion or controlled shearing

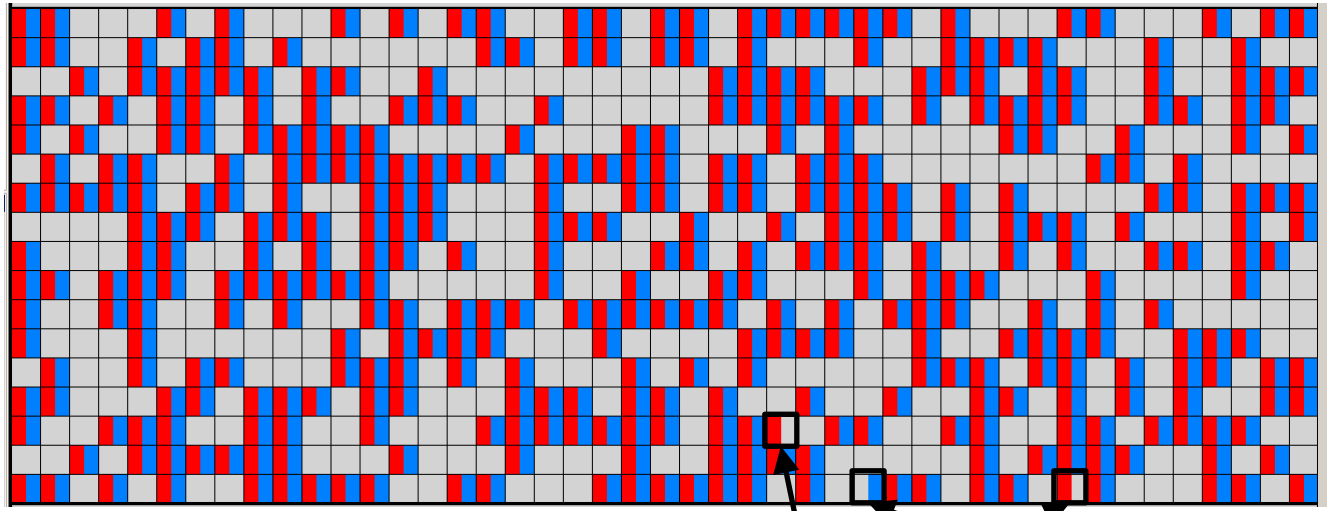
Assumption 3
Independent Segregation

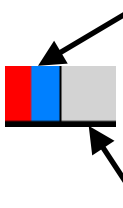
- If molecules are concatemers or physically bound to one another one “amplification-forming-unit” will consist of multiple copies
  - i.e. two linked copies will be counted as one copy



Assumption 4
Every Copy Gives a Signal

- Two linked genes 
- Duplex dPCR: each PCR reaction should have both or neither





Linear plasmid ~0.7 % only one target was detected
Supercoiled plasmid ~3 %

Red Gene only

Blue Gene only

Data generated by Ross Haynes (NIST)
IRMM CRM-AD413 certified to have 1:1 ratio of HMG and MON810 Maize genes

Assumption 4
Every Copy Gives a Signal

Plasmid type	Only one target detected	Possible cause(s)
Linear	~0.7 %	dPCR bias Fragmentation
Supercoiled	~3 %	dPCR bias Inaccessibility of target due to supercoiling

Fragmentation

If blue gene is cut

Then only the red gene will be detected

dPCR bias

Both genes present

But only the red gene is detected

Assumption 4
Every Copy Gives a Signal

Plasmid type	Only one target detected	Possible cause(s)
Linear	~0.7 %	dPCR bias Fragmentation
Supercoiled	~3 %	dPCR bias Inaccessibility of target due to supercoiling

Supercoiling

Reagents can not “see” blue gene

Then only the red gene will be detected

dPCR bias

Both genes present

But only the red gene is detected

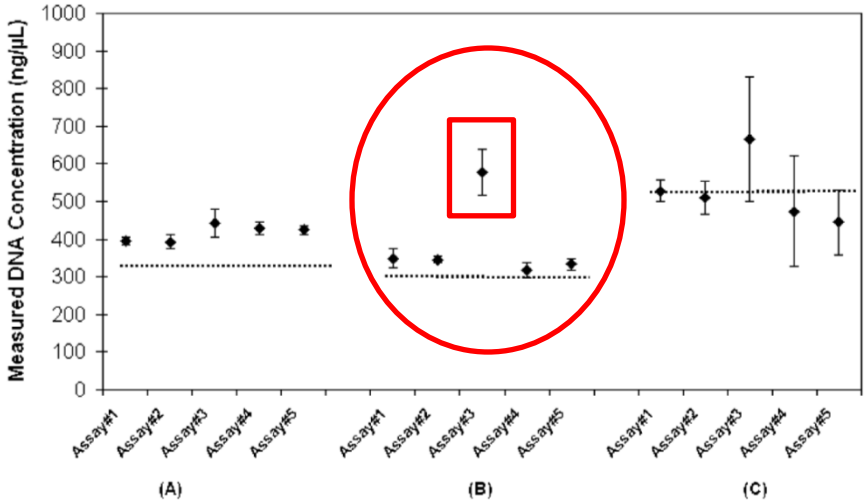
http://bitesizebio.com/articles/is-supercoiled-dna-derailing-your-cloning/

Assumption 4
Every Copy Gives a Signal

- Extraction method may leave PCR inhibitors
  - Also a problem for qPCR
  - Some direct extraction methods use alkaline solution to free DNA from cell
    - DNAzol® Direct: 10-fold dilution of sample into master mix required to avoid PCR inhibition

Assumption 5
Every Molecule is dsDNA

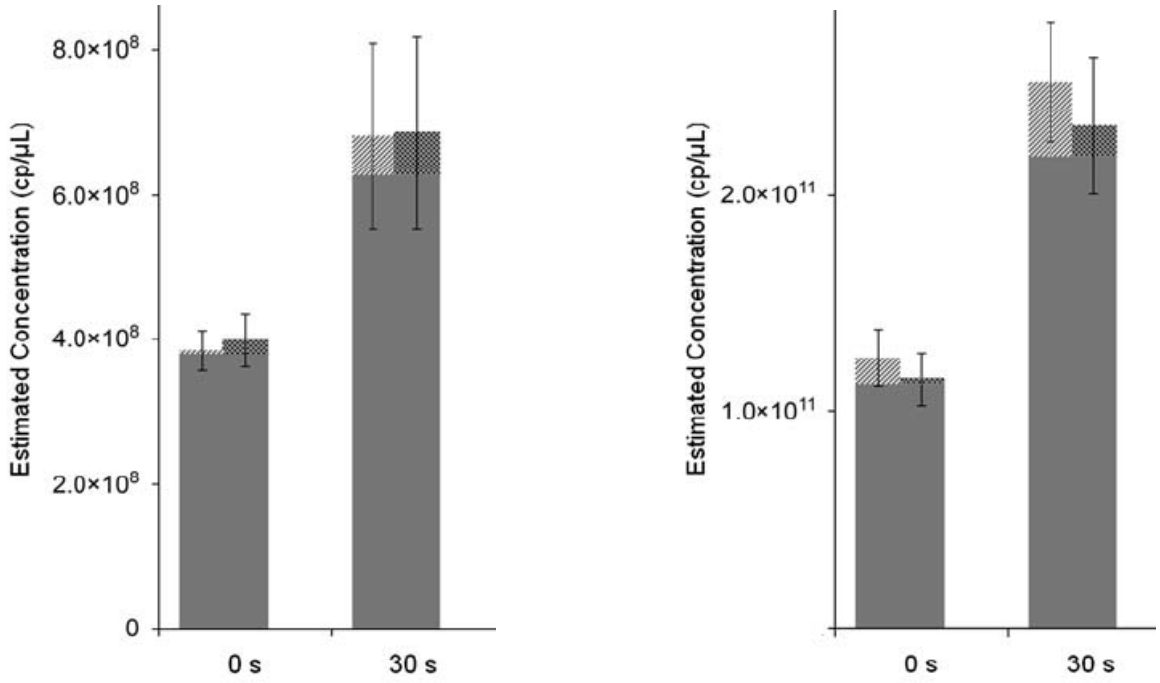
- NMI Australia:
  - Five (5) targets across genome used to quantify amount of DNA; one assay gave a 2-fold increase in concentration
  - Traced to low local GC-content; ssDNA



Bhat et al. Anal. Chem. 2010, 82, 7185-7192

Assumption 5
Every Molecule is dsDNA

- NMI Australia:
  - dsDNA vs. ssDNA gave 2-fold difference in concentration (95 °C for 30 sec and snap cooled)



Bhat et al. Analyst. 2011 Feb 21;136(4):724-32.

Assumption 5
Every Molecule is dsDNA

- Extraction method may leave ssDNA
  - Some direct extraction methods use alkaline solution or heat inactivating enzymes
    - DNAzol® Direct – alkaline solution
    - ZyGEM forensicGEM™ – heat inactivation of protease
- Options:
  - Validate method that produces 100% ssDNA then apply a 2-fold factor to concentration calculations
  - Validate method that produces 100% dsDNA

Holden et al. J Agric Food Chem. 2009 Aug 26;57(16):7221-6.

Technology Types

- Types of dPCR systems
- Advantages of each
- Examples of advantages
- Comparison of specifications

Technology Types

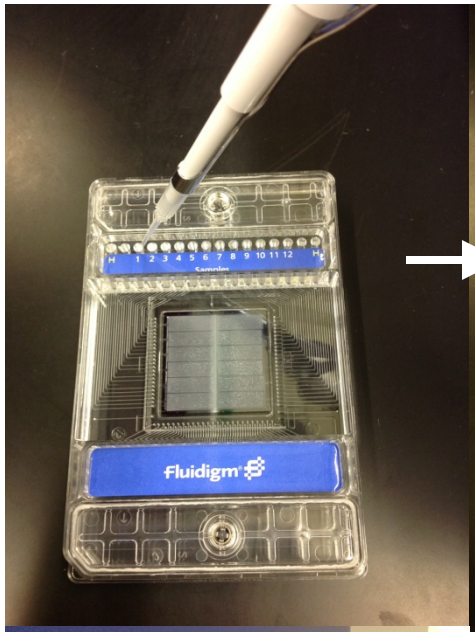
- Pre-manufactured microfluidic chambers
  - wells are pre-machined and static in space
    - Fluidigm – BioMark™ & EP1™
    - Life Technologies – QuantStudio® 12K Flex
    - Life Technologies – QuantStudio® 3D (end point only)
- Emulsion based chambers
  - oil in water emulsion with reactions of same size
    - BioRad – QX100™
    - RainDance – RainDrop™ System

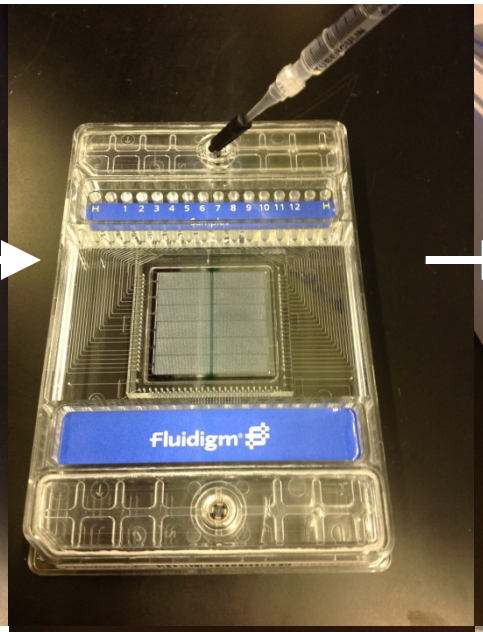
*This is not an exclusive list of manufacturers. This is only a list of known manufacturers by the author at the time this presentation was created.

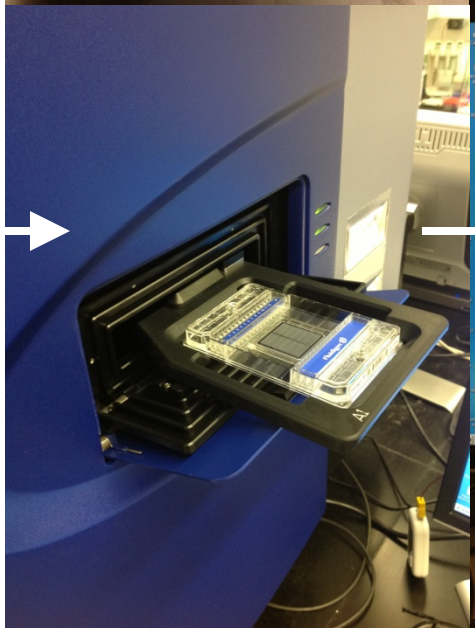
Pre-manufactured microfluidic chambers

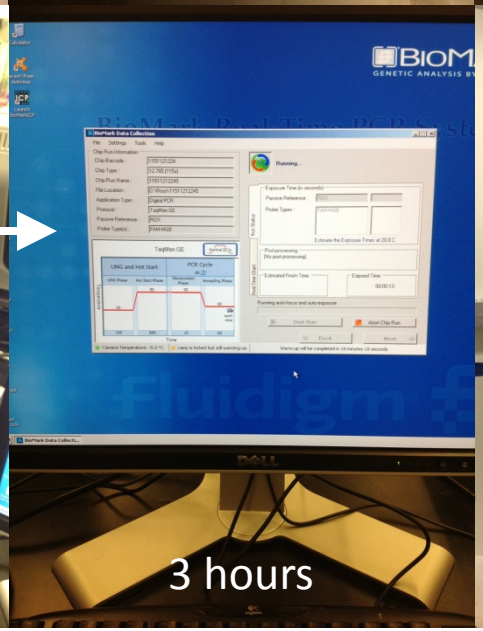
- Microfluidic technologies used to aliquot sample into massive number of PCR reactions
- Geometry of the well dictates volume of chamber – can not fit 20 nL in 10 nL space
- Chambers are fixed in space – therefore images can be taken after each cycle, just like real-time qPCR
  - Troubleshooting
  - Assay optimization
  - Multi-purpose instruments (not just dPCR)

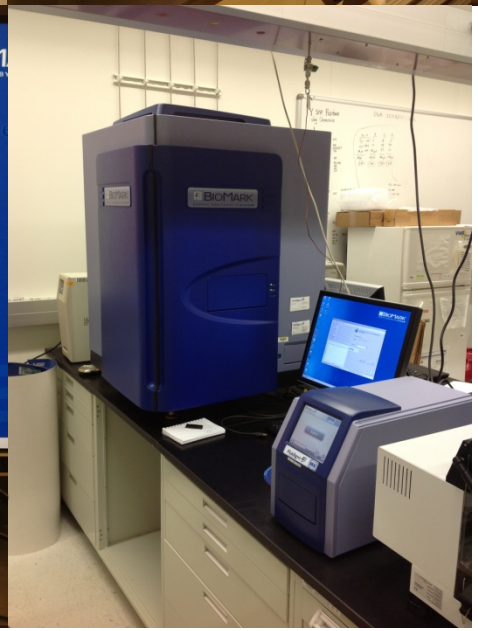
Fluidigm Workflow



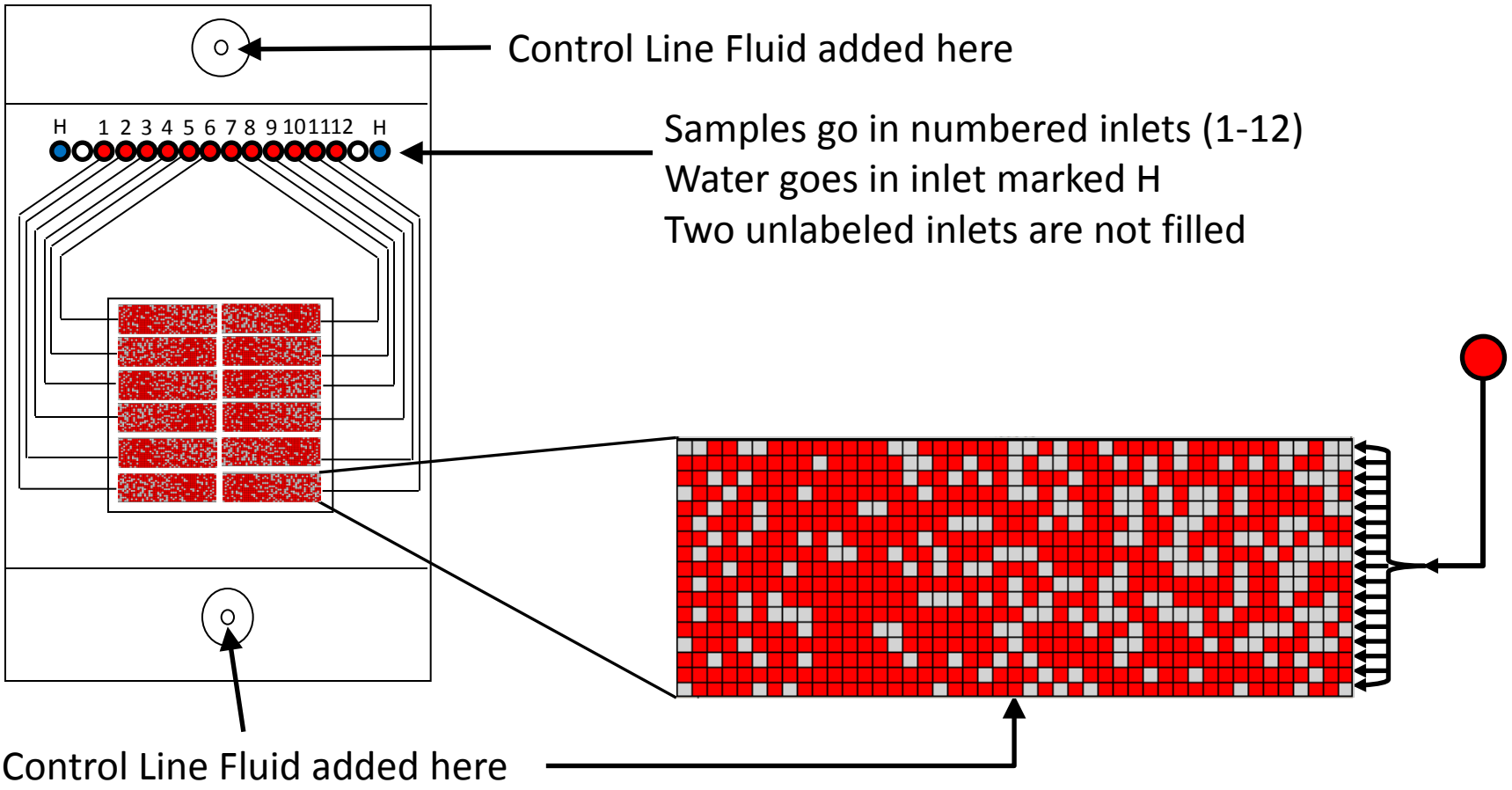








Fluidigm 12.765 Digital Array

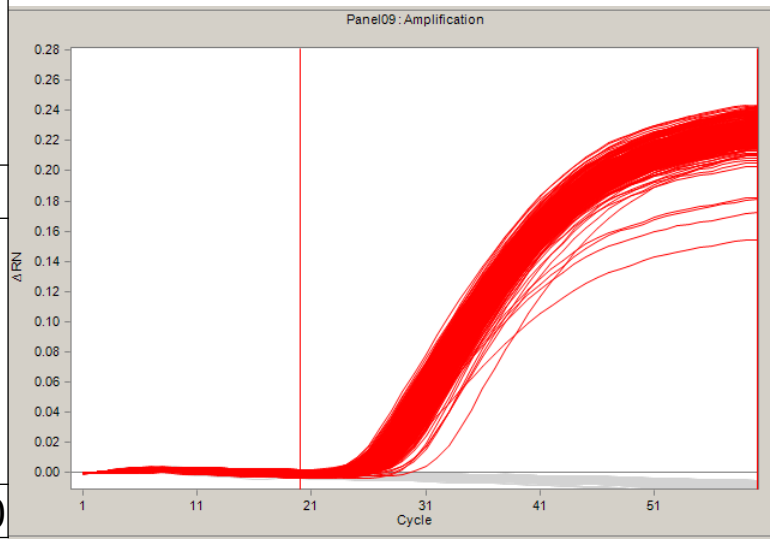


See a video here: http://www.youtube.com/watch?v=UwzDc6wcGZg&feature=relmfu

Fluidigm Analysis

- Same algorithms for qPCR are used
- Factor for dilutions & divide by 4.59 µL to get concentration

	Count	Est. Targets	DNA	Dilution	C/µL	Avg C/µL
1	551	976			11,072	
2	577	1075			12,195	
3	585	1109	RS5	1:10	12,581	11,962
4	581	1092			12,388	
5	563	1020			11,571	
6	0	0	ntc	n/a	-	-
7	556	994			112,646	
8	564	1024			116,045	
9	589	1126	RS6	1:100	127,605	116,589
10	551	976			110,606	
11	564	1024			116,045	
12	0	0	ntc	n/a	0	0



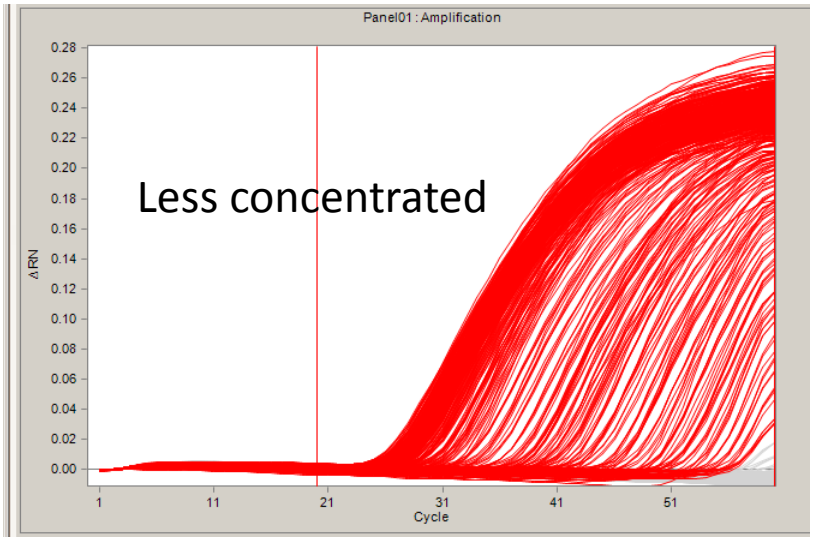
Troubleshooting

- Intact and linear plasmid diluted the same amount are run on dPCR

Intact plasmid DNA

Positive chambers = 429

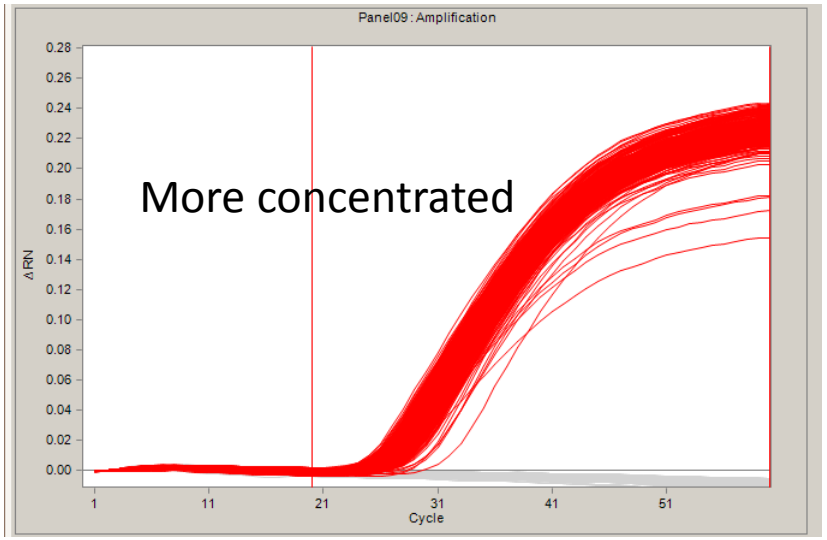
Conc. 1.2 x 10^6 copies/µL



Linear plasmid DNA

Positive chambers = 534

Conc. 2.0 x 10^6 copies/µL



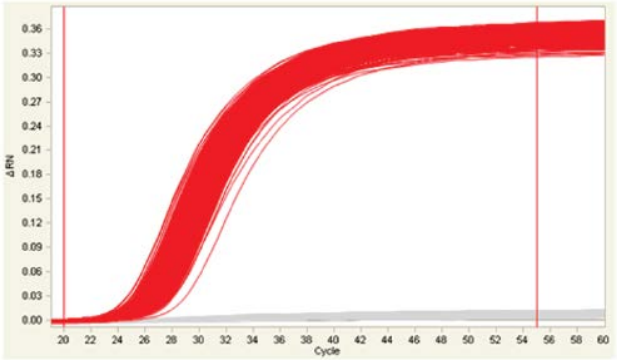
Would more chambers be positive if more cycles were run?
Is this a true difference in concentration?

Multi-Use Instrument

48 samples

48 qPCR assays

2304 qPCR curves

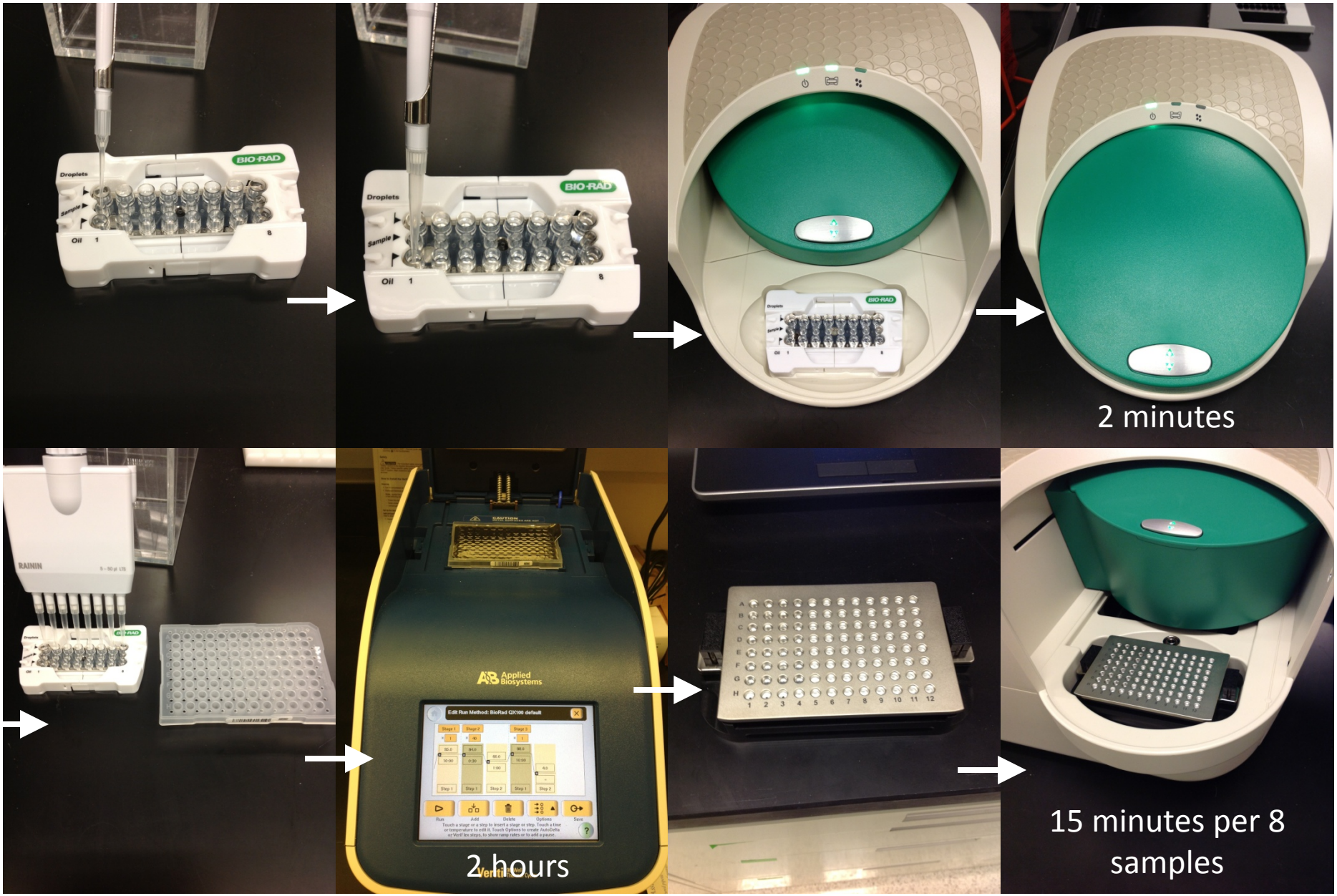


http://www.youtube.com/watch?v=s9HUhuCbbhU

Emulsion based chambers

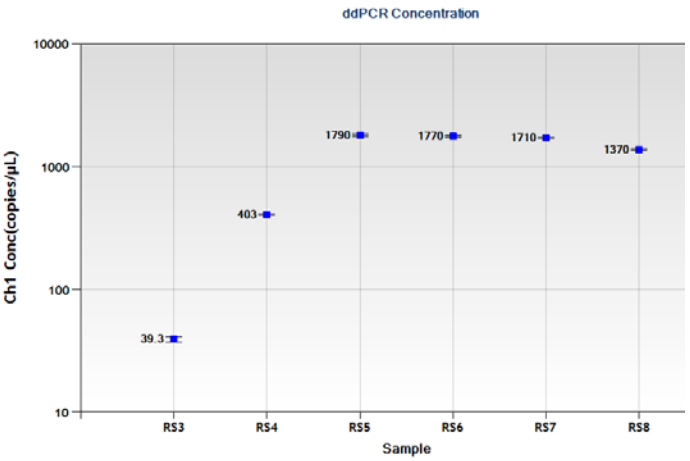
- Emulsion generator produces water-in-oil emulsion chambers of equal size
- Geometry of the generator and stability of emulsion determines chamber size
- Chambers are not fixed in space – end-point detection only
  - Cheaper to create emulsion chambers
  - More reactions – better statistics
  - All dPCR technologies use end-point data for Poisson calculations

Bio-Rad Workflow



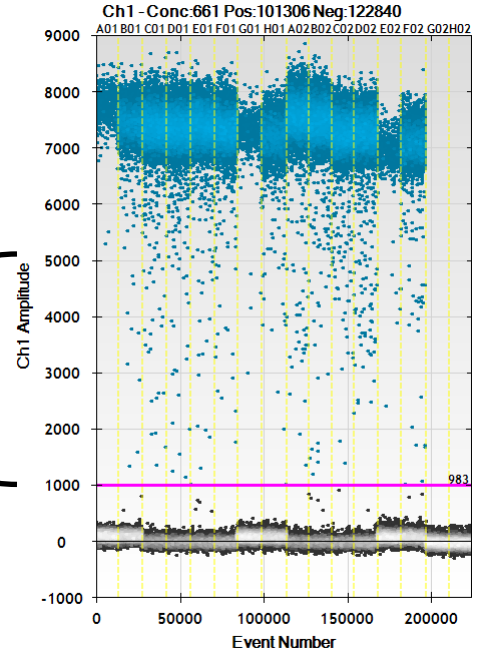
Bio-Rad Analysis

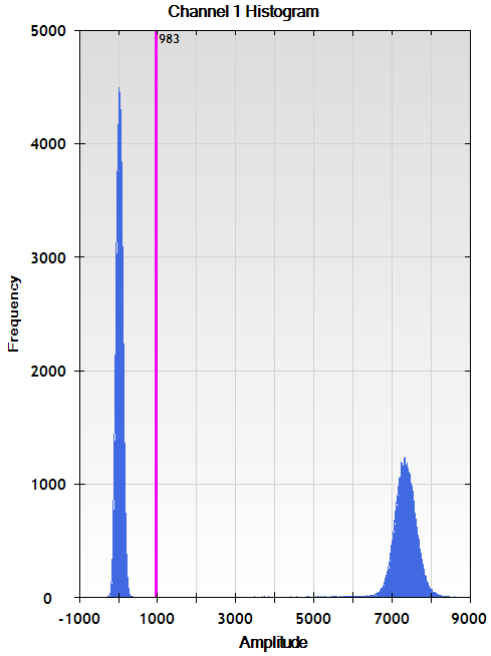
	Dilution	c/µL	Positives	Negatives	Total
RS3	2.2	86	1,458	39,993	41,451
RS4	2.2	884	13,719	30,923	44,642
RS5	5.0	8,914	22,352	5,438	27,790
RS6	50.9	90,079	22,058	5,534	27,592
RS7	534.6	914,199	21,748	5,787	27,535
RS8	6513.7	8,923,788	19,971	8,064	28,035
ntc		-	-	27,101	27,101



259 droplets (0.13 %)

197,045 total droplets

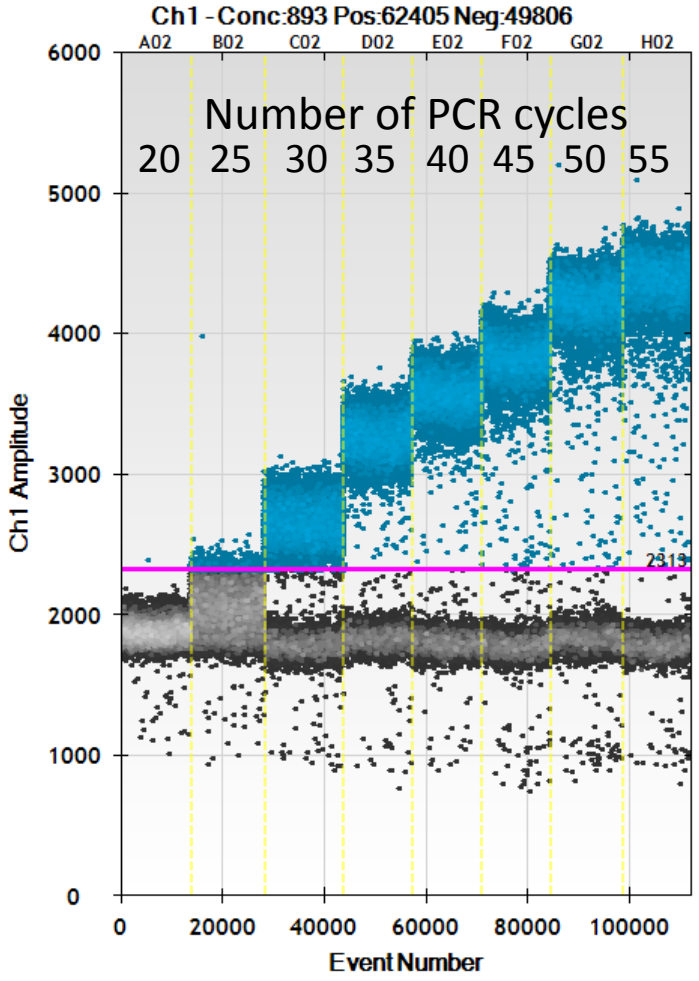




Emulsion based chambers

Troubleshooting must rely on statistics rather than curve shape

- Validate different sample types (plasmid, genomic, viral, etc) for number of cycles required
- Restriction digestion has been shown to improve qPCR and dPCR efficiency*
- Additional confidence can be gained by using multiple target genes across the genome



*Bhat et al. Anal Bioanal Chem. 2009 May;394(2):457-67.

Comparison of Specifications

System	Fluidigm	Life Technologies 12K Flex	Life Technologies 3D	BioRad	RainDance Technologies
Type	Pre-MFR'ed	Pre-MFR'ed	Pre-MFR'ed	Emulsion	Emulsion
Reactions per sample	765 or 770	64 to 9,216	20,000	20,000	10 million
Number of samples	12 or 48	1 to 144	1 to 24	8 to 96	8 to 96
Total number of reactions	9,180 or 36,960	9216	20,000 to 480,000	160,000 to 1.9 million	80 million to 960 million
Detection	Real-Time or End Point	Real-Time	End Point	End Point	End Point
Reaction volume	6 nL or 0.85 nL	33 nL	1 nL	1 nL	1 pL
Total volume	4.6 µL or 4µL	304 µL	20 µL	20 µL	25 µL
Cost per run	$320[%] or $100	$150[&#]	$5[^3]	$40*[1]	$240*[2]
Cost per 10,000 reactions	$348 or $27	$488	$2.50	$2.50	$0.03

[%]based on quote for 12.765 digital arrays 2012
[&]list price Sept 2012
[#]based on one OpenArray (n=3072 reactions)

*cost for 8 samples
[1]based on quote 2012
[2]based on pre-commercial market research
[^3]based on 1 sample; quote 2013

Technology Types: Conclusions

Lab requirements may influence system requirements:

- Uncertainty
- Throughput
- Cost
- Space
- Sample types

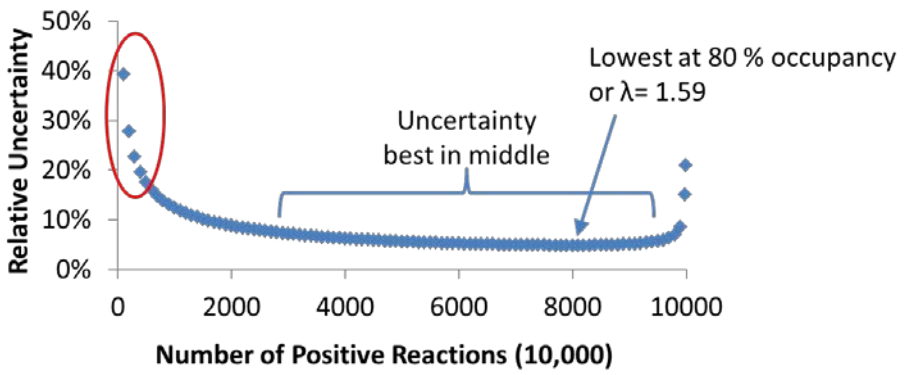
End Point Only Detection	Real-time Detection
Cheaper → More reactions → More Power to Discriminate Small Differences	More expensive per reaction → Fewer Reactions → Less Power to Discriminate Small Differences
Poisson calculations only use end-point data	Real-time data collection gives more data for troubleshooting and optimizing
Single-purpose instrument	Multi-purpose instrument (space saver)

Conclusions

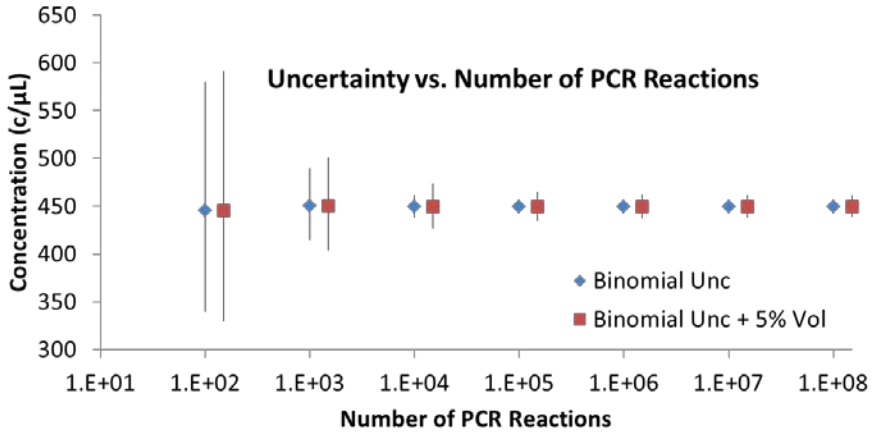
- Levels of dPCR use:
  - Purchasing dPCR certified Reference Materials
  - Certifying your Reference Materials with dPCR
  - Measuring patient samples with dPCR
- Applications:
  - Absolute quantitation
  - Relative quantitation (DNA or RNA)
  - Minority target detection
  - Investigation of individual alleles; haploid typing

Conclusions

- Poisson Statistics:
  - "Large" depends on uncertainty required
  - More PCR reactions give power to differentiate smaller differences in concentration
  - Uncertainty in volume – absolute quantitation
  - Used because molecules distribute randomly



Calculations by Ross Haynes (NIST)



Conclusions

- Measurement Issues:
  - Extraction issues:
    - ssDNA left by heat or alkaline solution
    - PCR inhibitors
  - Sample type:
    - Difference in PCR efficiency → bias in dPCR measurement
    - E.g. supercoiled versus linear plasmid
  - Linked copies:
    - Measured per amplification-forming-unit
    - Fixed by restriction digestion or controlled shearing
  - Number of copies:
    - ssDNA gives two amplification-forming-units per dsDNA

Conclusions

- Technology Types:
  - Laboratory needs will dictate “best” system
  - End-point: more PCR reactions → more power to discriminate small differences in concentration
  - Real-time: data familiar for optimization and troubleshooting
    - Multipurpose instrument (e.g. SNP detection, 96-well qPCR)

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”

Select References (1)

- Ruano et al. Proc Natl Acad Sci U S A. 1990 Aug;87(16):6296-300.
  - Single molecule PCR for separating maternal and paternal chromosomes before sequencing – heredity
- Monckton et al. Genomics. 1991 Oct;11(2):465-7.
  - Single molecule PCR for separating maternal and paternal chromosomes before sequencing – human ID
- Vogelstein et al. Proc Natl Acad Sci U S A. 1999 Aug;96(16):9236-41.
  - Investigation of rare mutant KRAS alleles
  - First paper to suggest dPCR could be used for quantitating DNA
- Dube et al. PLoS One. 2008 Aug 6;3(8):e2876.
  - Uncertainty calculations for digital PCR

Select References (2)

- Bhat et al. Anal Bioanal Chem. 2009 May;394(2):457-67.
  - Ripley's K to measure random distribution of molecules
  - Restriction digestion to increase dPCR efficiency
- Holden et al. J Agric Food Chem. 2009 Aug 26;57(16):7221-6.
  - Discusses how ssDNA can affect DNA quantitation
- Bhat et al. Anal. Chem. 2010, 82, 7185-7192
  - ssDNA can over estimate concentration of dPCR
- Bhat et al. Analyst. 2011 Feb 21;136(4):724-32.
  - Heating of DNA can effect dPCR, extended heating can damage DNA

Select References (3)

- Heyries et al. Nat Methods. 2011 Jul 3;8(8):649-51.
  - Statistics of digital PCR in supplementary section
- Pagno & Gauvreau “Principles of Biostatistics” 2nd ed. ISBN-10: 0534229026
  - Statistics textbook
- https://www-s.nist.gov/srmors/view_detail.cfm?srm=2366
  - SRM 2366 webpage
- uCOUNT(SM) University of Utah https://dna.utah.edu/ucount/uc.php
  - Exact binomial distribution for dPCR

Select References (4)

- http://www.youtube.com/watch?v=s9HUhuCbbhU
  - Loading of Fluidigm 48.48 array
- http://www.youtube.com/watch?v=UwzDc6wcGZg&feature=relmfu
  - Loading of Fluidigm 12.765 digital array
- http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6237.pdf
  - Product sheet for Bio-Rad QX100