Droplet Digital PCR

Precise Counting of Targeted Nucleic Acids has Never Been Easier

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Digital Biology Center

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- ATCC® collaborates with and supports the scientific community with industry-standard products and innovative solutions
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  - Establishing sensitivity, linearity, and specificity during assay validation or implementation
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Preparations are authenticated and characterized to ensure identity, integrity, purity, functional activity, and concentration

www.atcc.org/GenuineNucleics

Droplets enable thousands of digital measurements

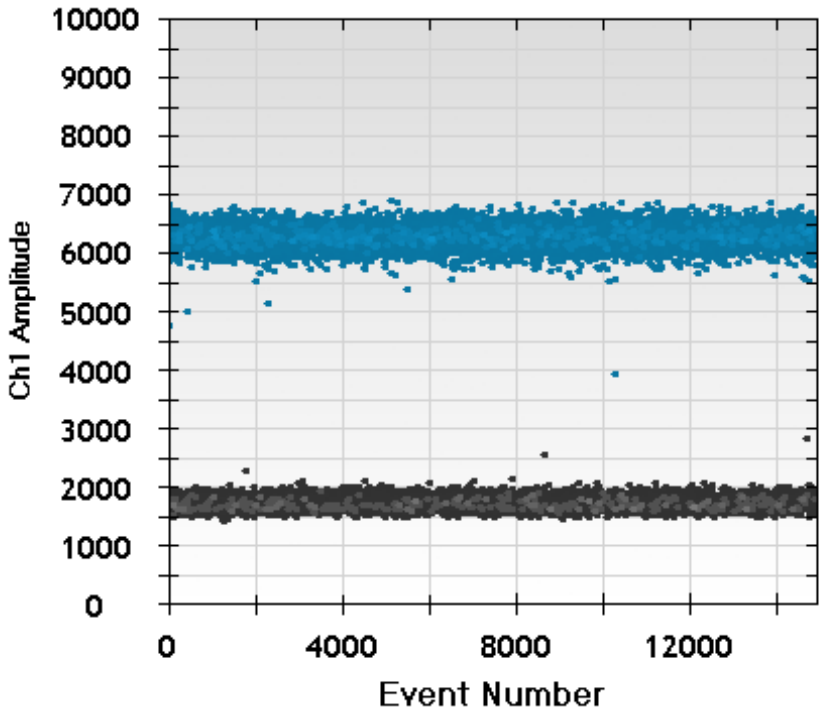
One measurement

Nanodroplet PCR reactions are independent, single amplification events

Many thousands of discrete measurements

Droplet readings converted to a digital signal

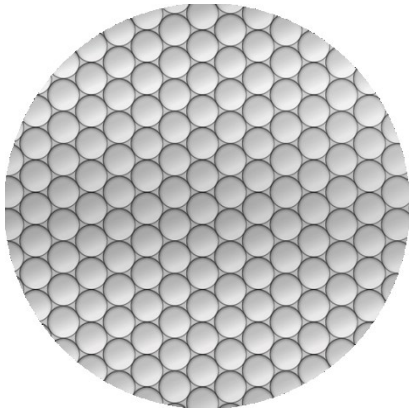
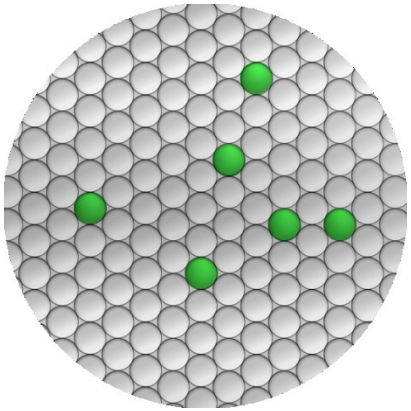
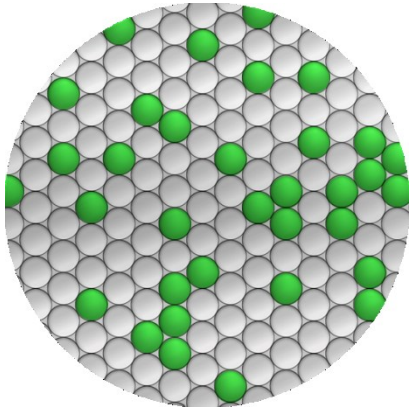
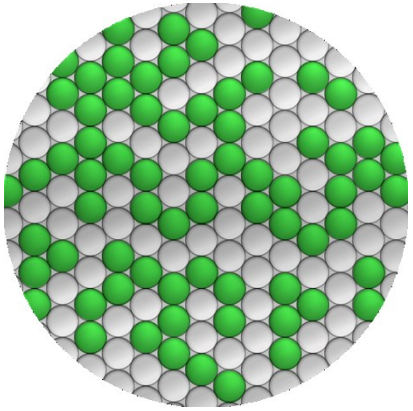
- Positive droplets contain at least one copy of target DNA (cDNA)
- Positive droplets have increased fluorescence vs. negatives
- Quantasoft software measures the number of positive and negative droplets per fluorophore per sample



Each positive counted as 1

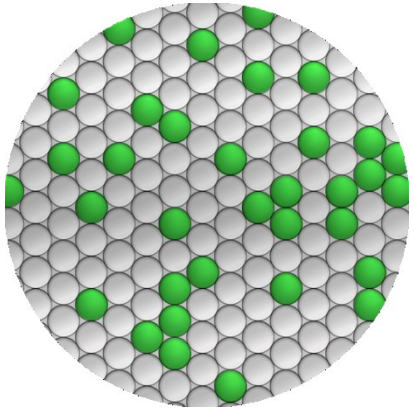
Each negative counted as 0

Counting positives to estimate target concentration

Sample 1	Sample 2	Sample 3	Sample 4
			
NO targets	Low concentration	Medium concentration	High concentration
$p=0$ *positive*/143 *total*	$p=6/143$	$p=34/143$	$p=70/143$
	Poisson corrected **6.2**/143	Poisson corrected **38**/143	Poisson corrected **96**/143

Increased number of partitions

Sample 3



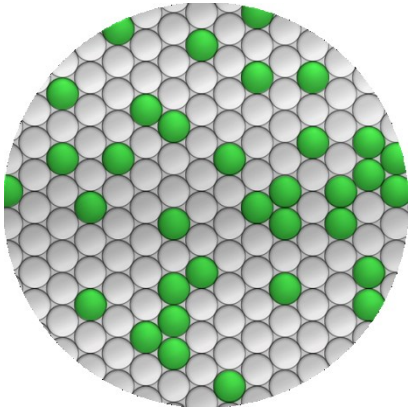
Medium concentration

$p=34/143$

Poisson corrected
38

Increased number of partitions

Sample 3

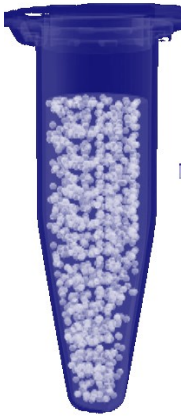


Medium concentration

$p=34/143$

Poisson corrected
38

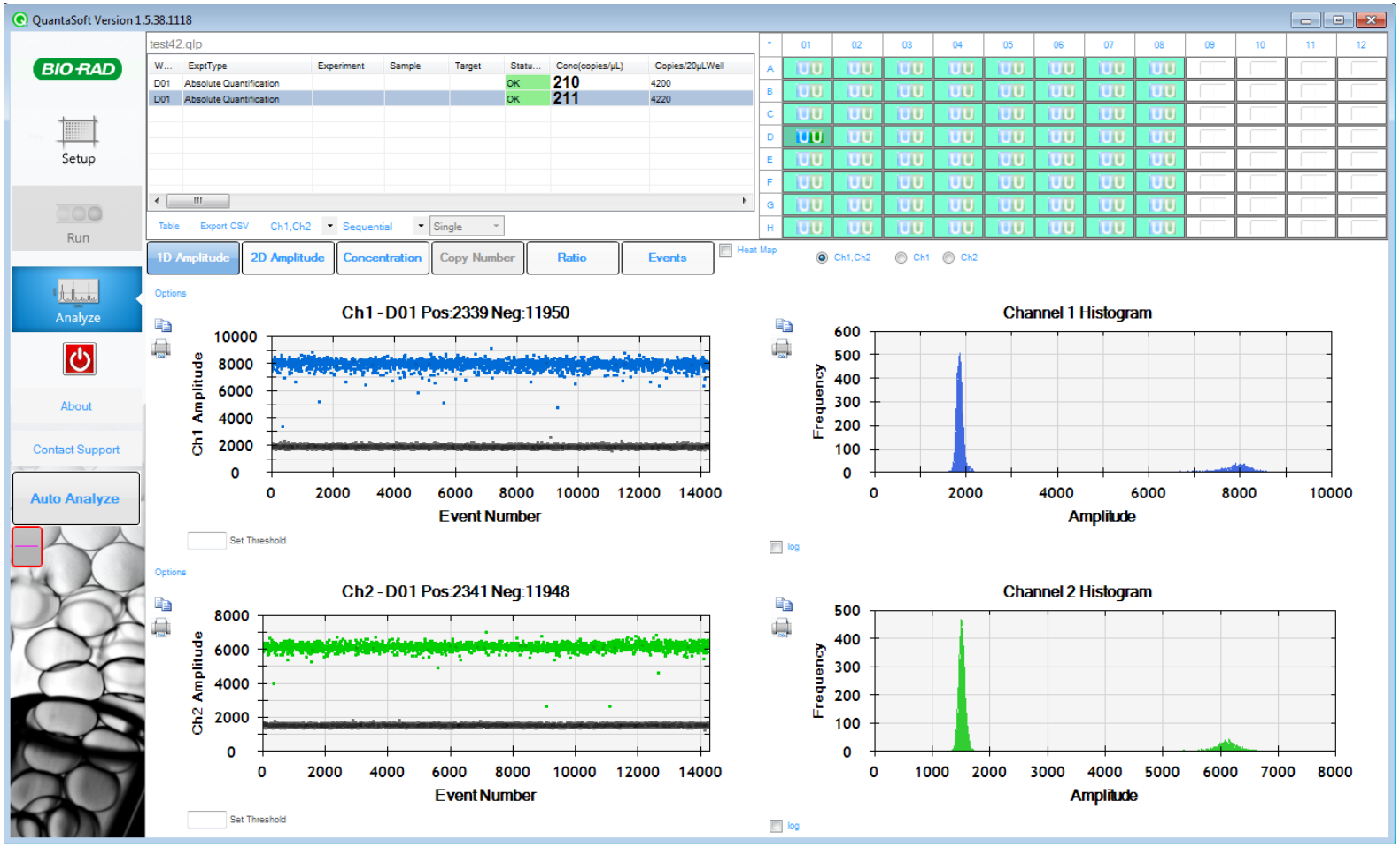
Well A3



P=16 076 /17 451

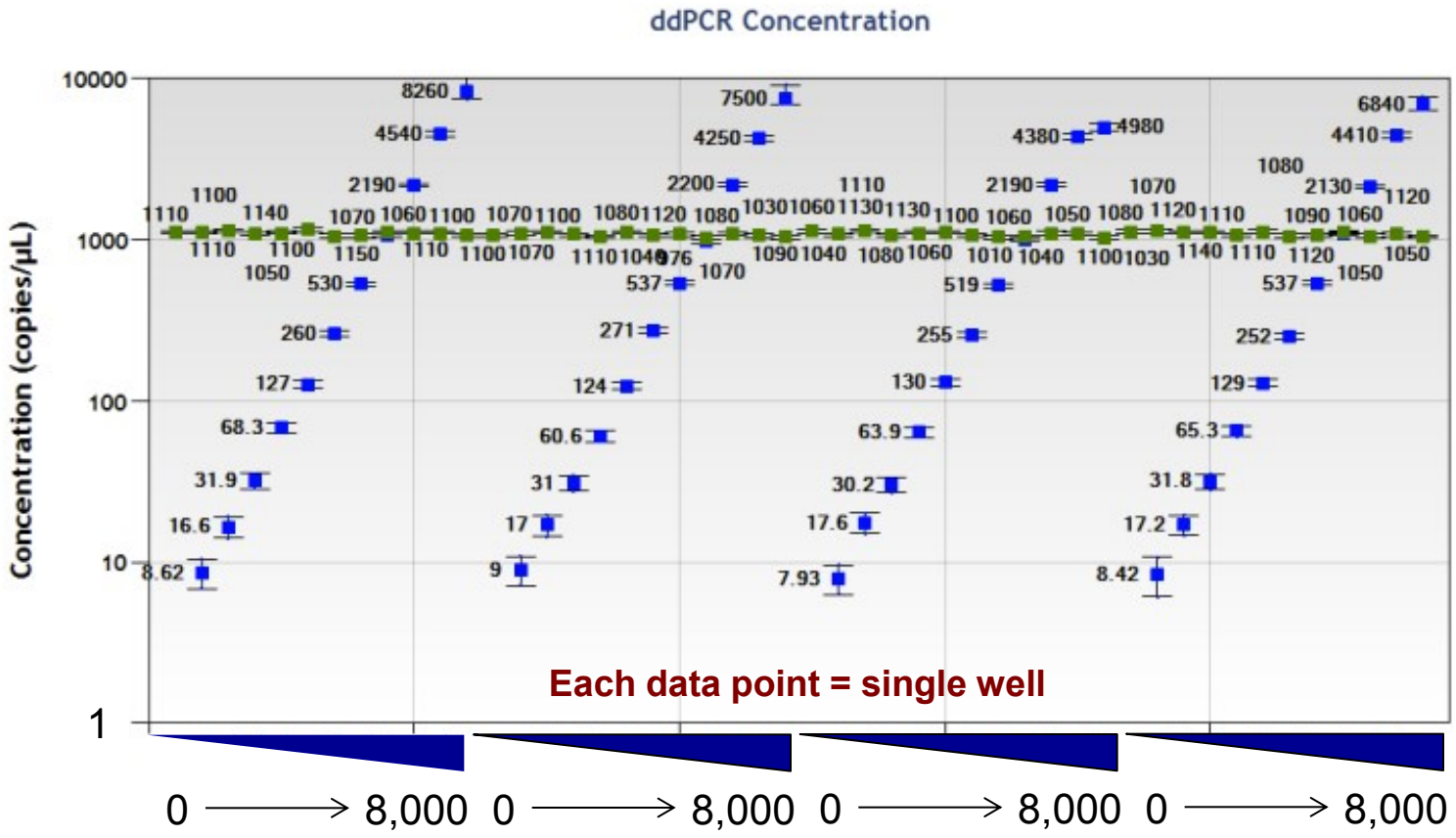
Poisson corrected
55 800

Software calculates number of target molecules



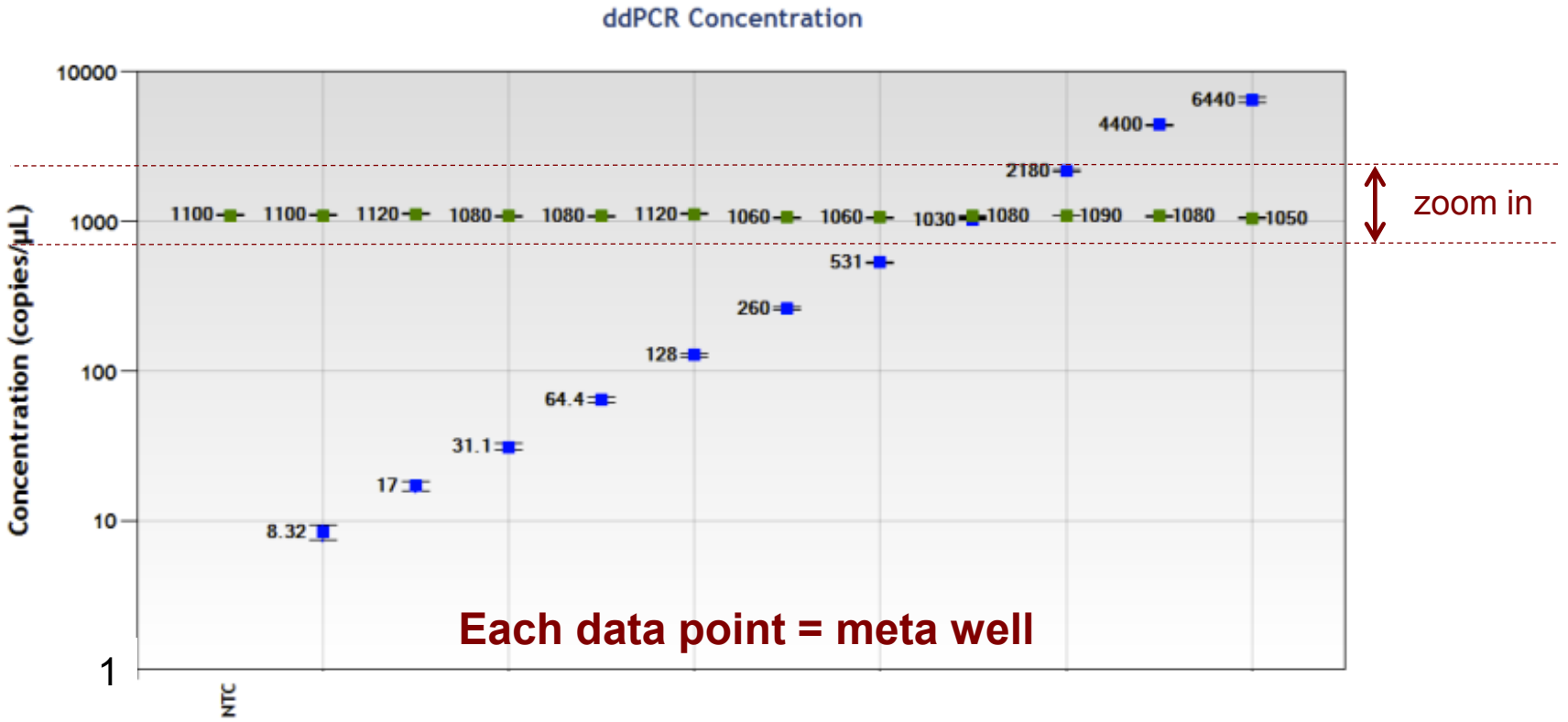
What kind of results can we expect from Digital PCR?

2-fold serial dilutions of *S. aureus*



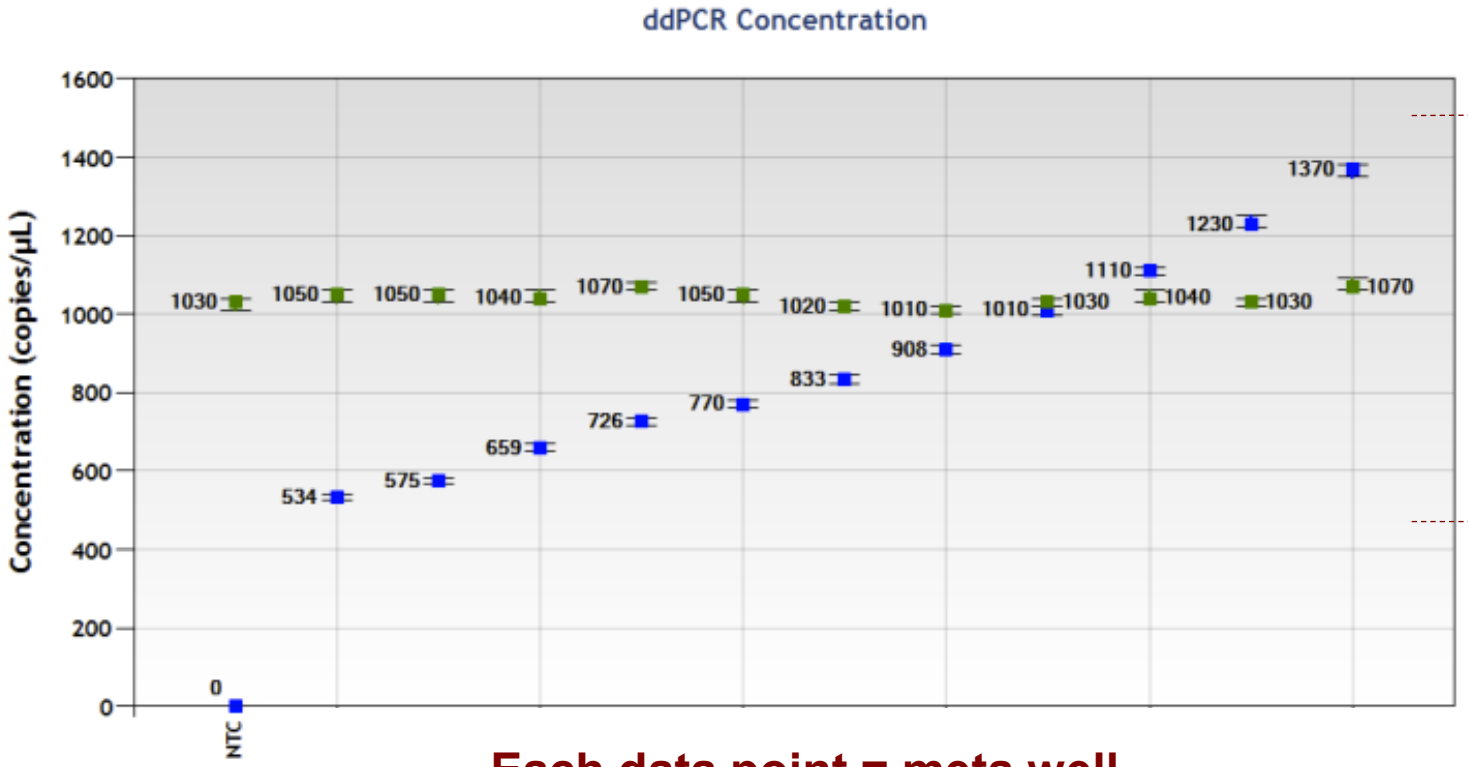
S. aureus dilutions (copies/µl)
Constant human gDNA (RPP30)

4 merged wells of each 2-fold serial dilution



S. aureus dilutions (copies/µl)
Constant human gDNA (RPP30)

Excellent precision from 10% dilutions



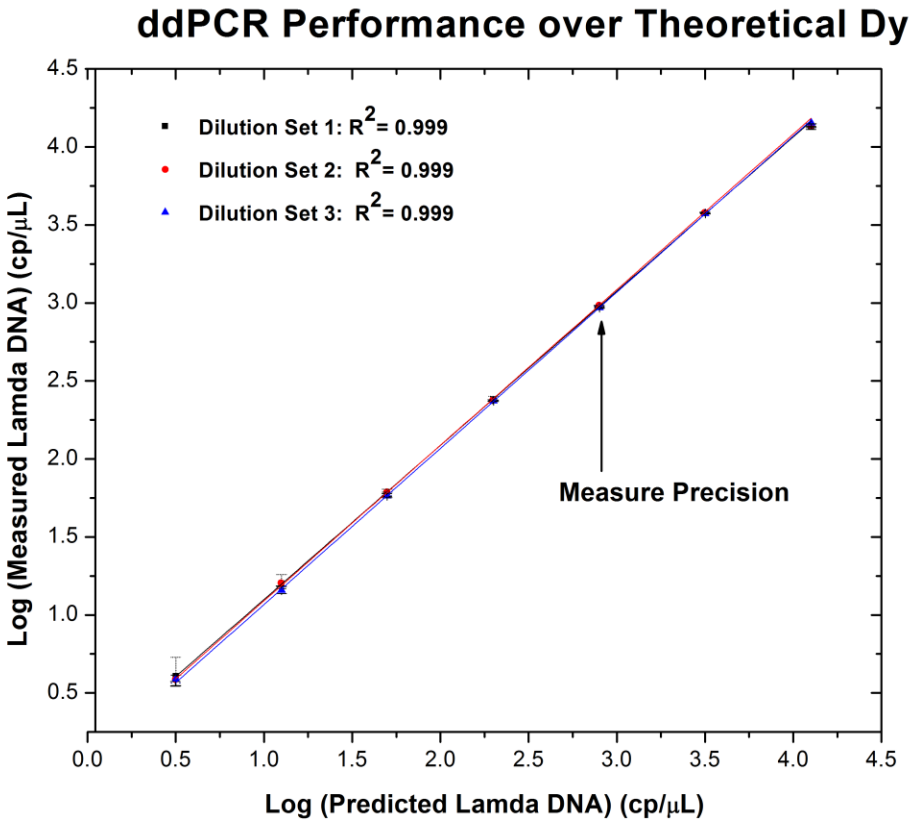
zoomed in

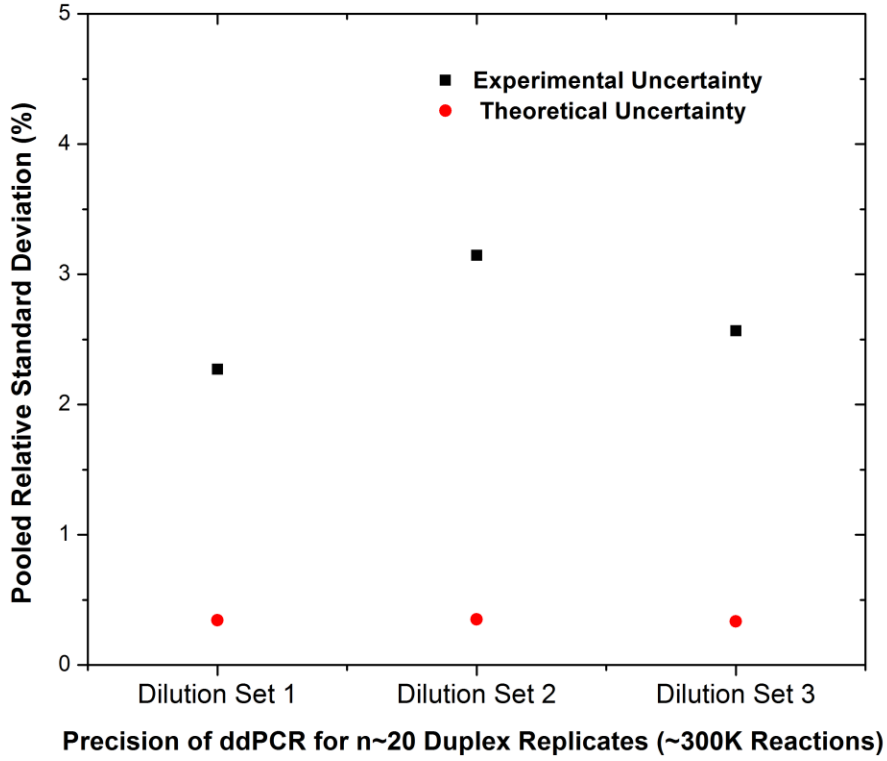
Each data point = meta well

S. aureus dilutions (copies/µl)
Constant human gDNA (RPP30)

BIO-RAD

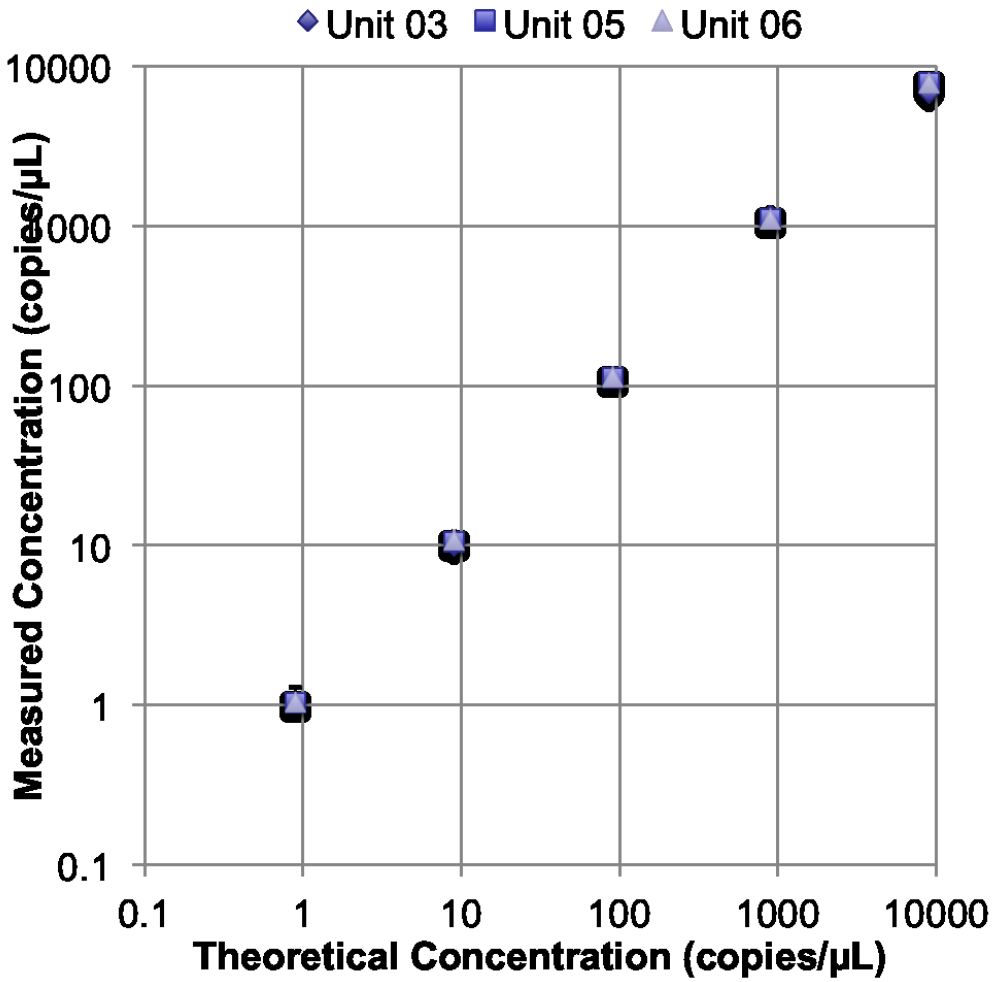
Precision independently verified and observed, +/- 1.5% uncertainty over theoretical value





Gravimetric Experiments Conducted at National Measurement Institute, NSW (Australia)

Excellent reproducibility and linearity across concentrations and instruments



Quantitative analysis of food and feed samples with droplet digital PCR, Morisset D *et al*, PLoS One 2013 May 2;8(5)

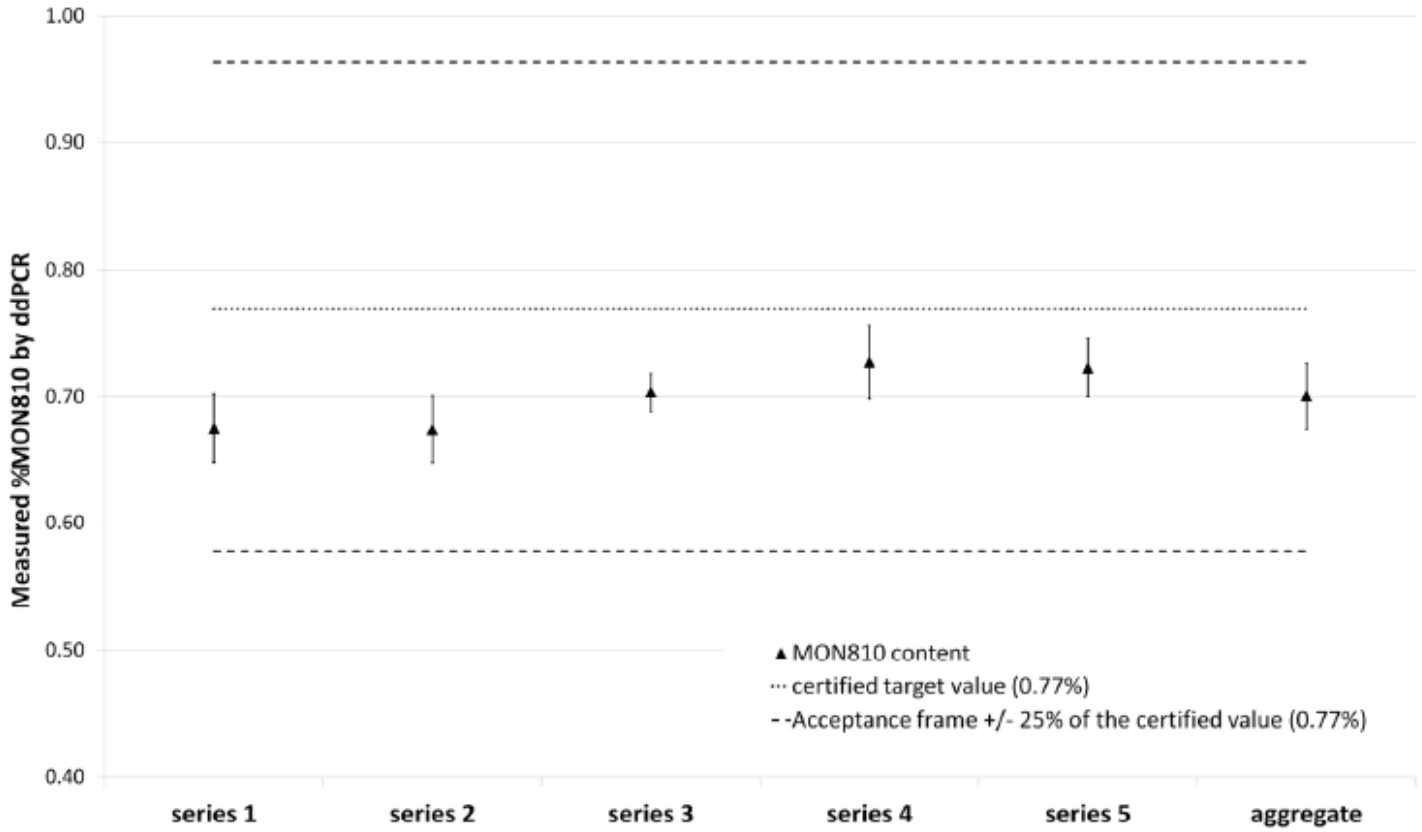
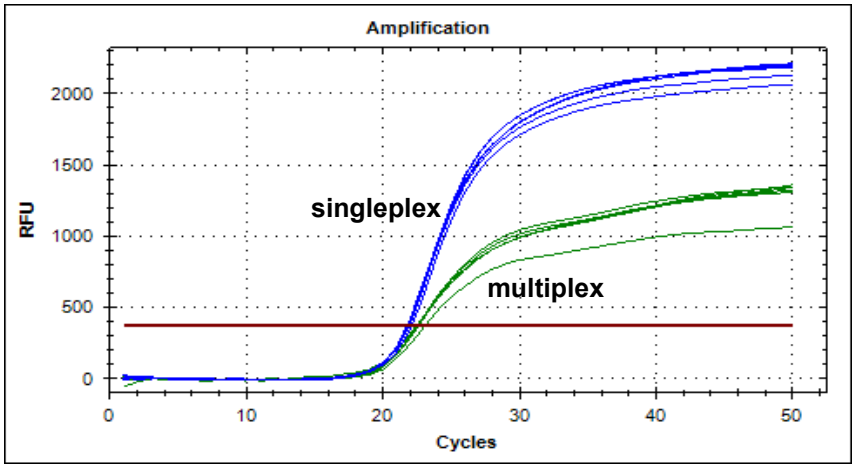
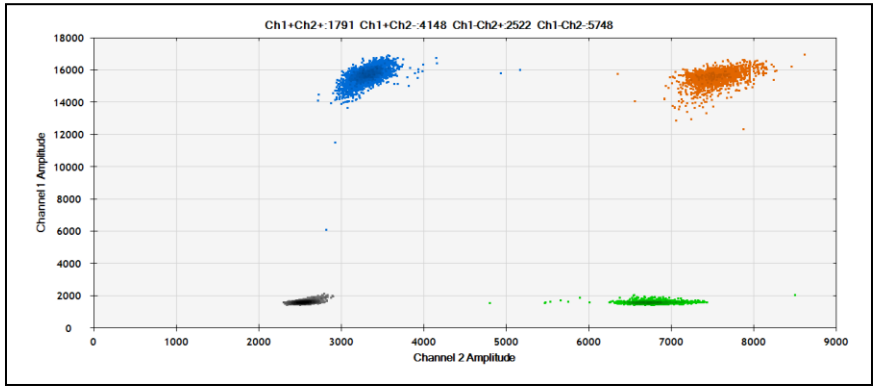
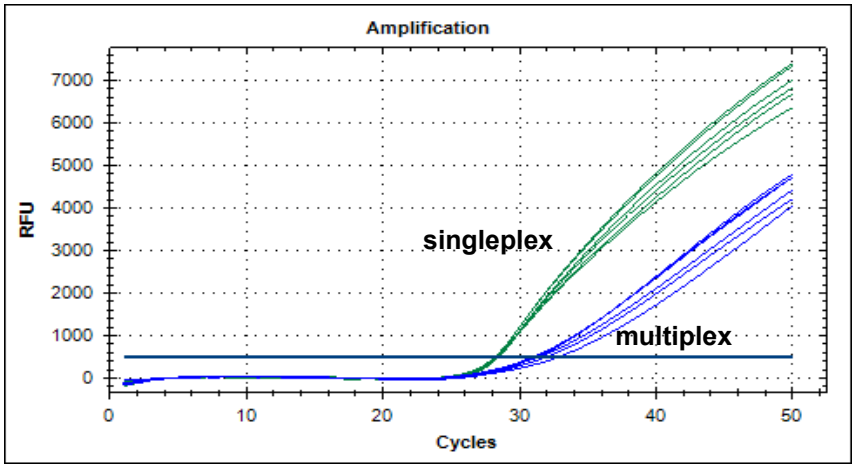


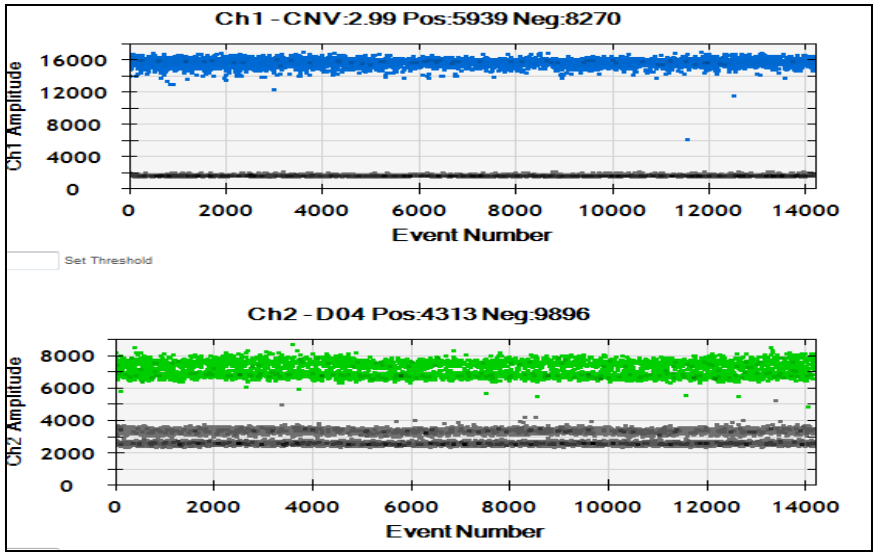
Figure 2. Repeatability results of the ddPCR duplex assay. MON810 content measured by ddPCR in five series of seven replicates. The aggregate represents the sum of the five series. The target certified MON810 content (0.77%) is indicated by a dotted line. Acceptance criterion for repeatability is ±25% of the target content (from 0.58% to 0.96%) represented by the dashed lines. Error bars represent the standard deviation between the replicates for each series or in the aggregate.
doi:10.1371/journal.pone.0062583.g002

Multiplexing



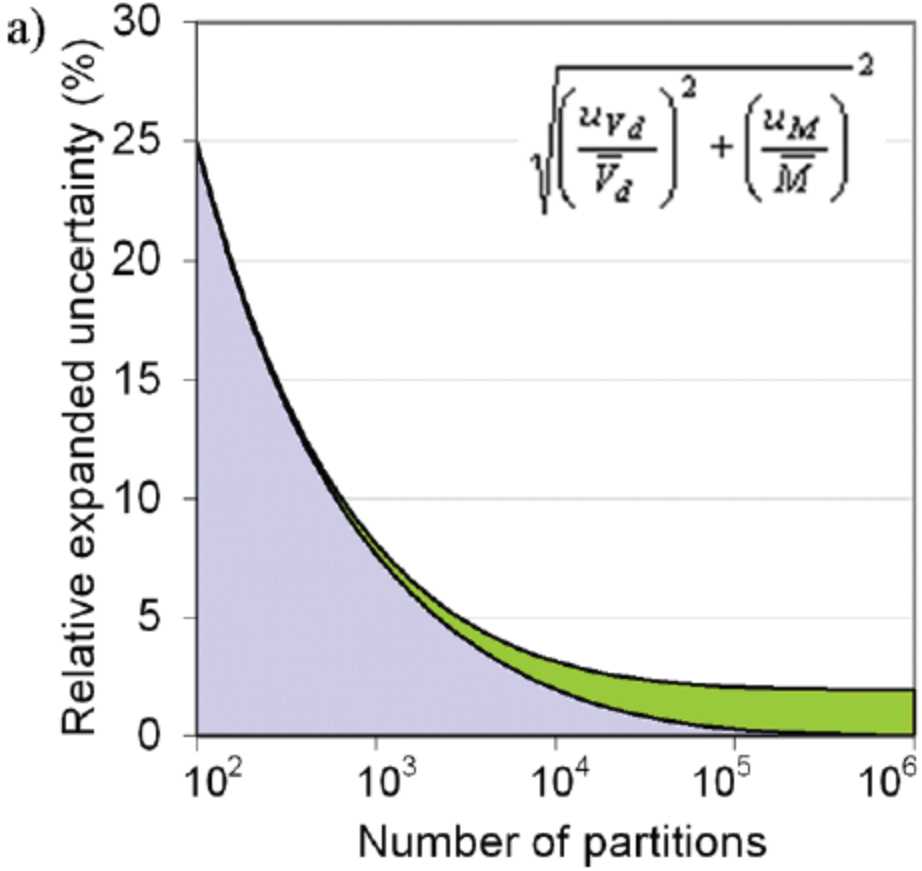






BIO-RAD

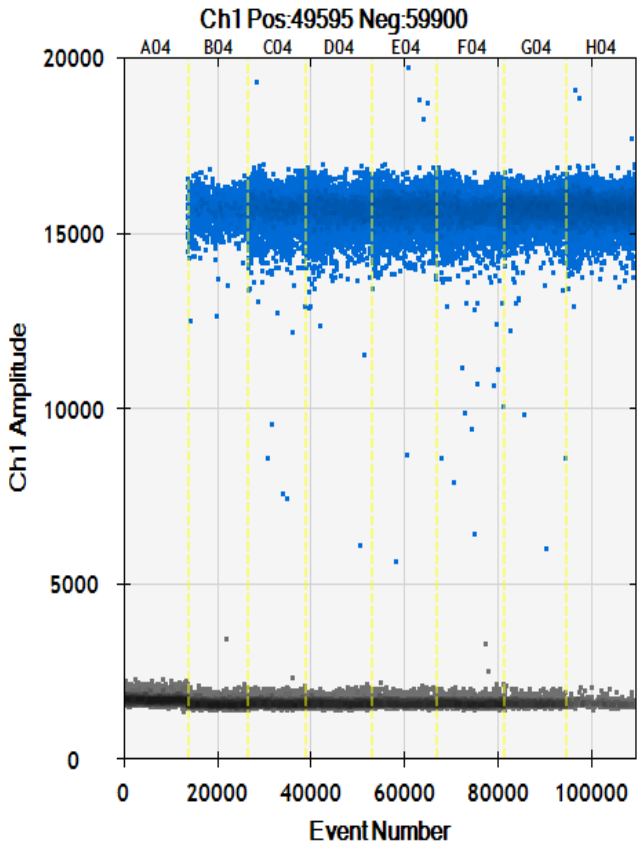
Why use 20 000 partitions?



Pinheiro et al, Anal.Chem(2012) 84,1003

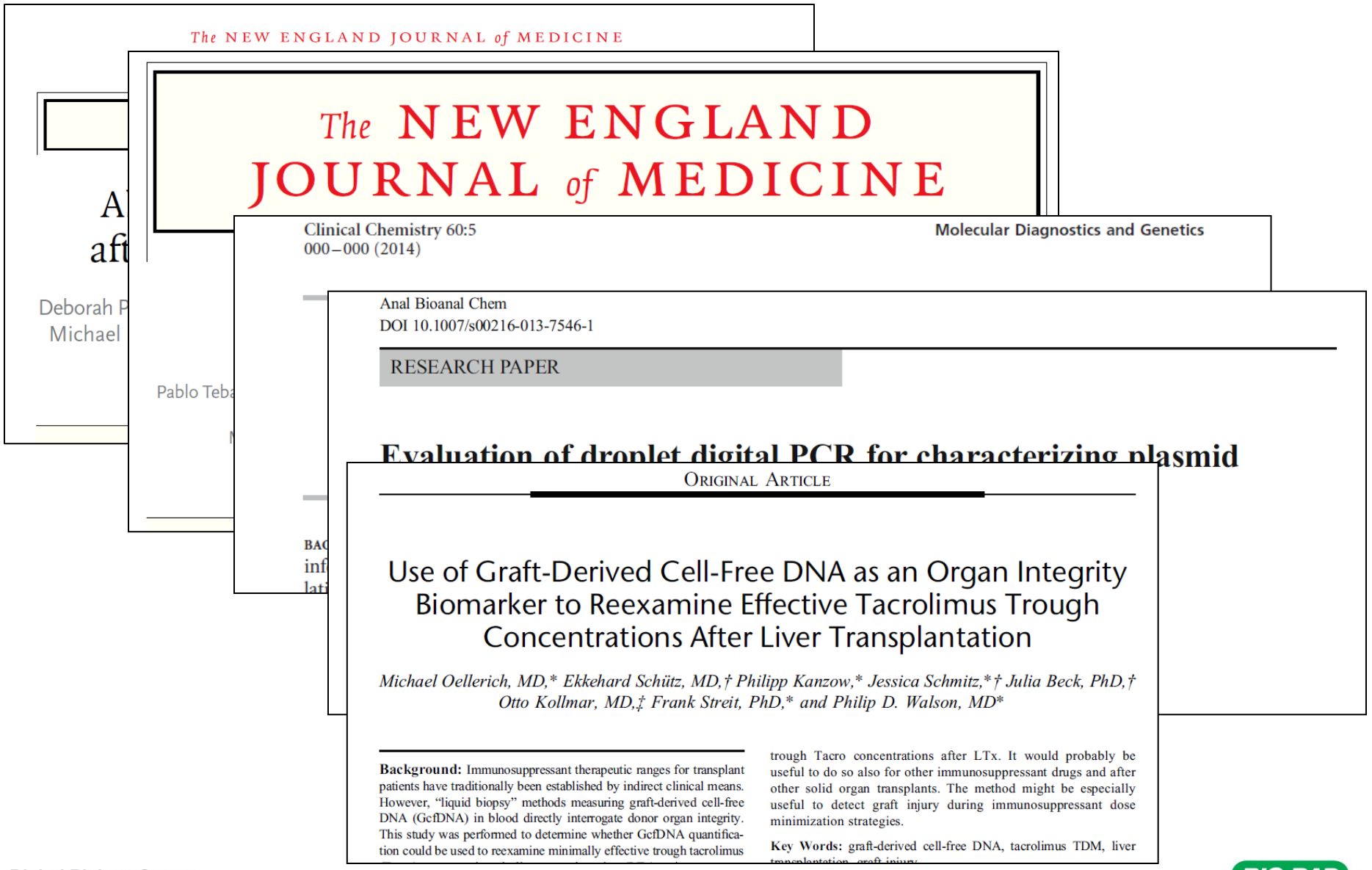
- 10 000 partitions is a “sweet” spot where Poisson distribution uncertainty is low (blue area) and uncertainty due to droplet variability is also low (green area).
- Increasing droplet number (as long as they are of uniform size) does can decrease Poisson error somewhat.
- If partitions are not of uniform size, partition variability and limiting dilution error (not shown) contribute to total uncertainty.

Advantages of ddPCR over other quantitative methods



- Quantitative resolution
- Absolute quantitation not dependent on standards and other comparative templates that may or may not properly represent the matrix the experimental sample is in
- Tolerance to minor inhibitors that affect amplification
- Unforeseen point mutations on primer annealing sites have less impact on quantitative accuracy
- Multiplex reactions are less prone to assay reagent depletion causing false negatives in low abundance targets

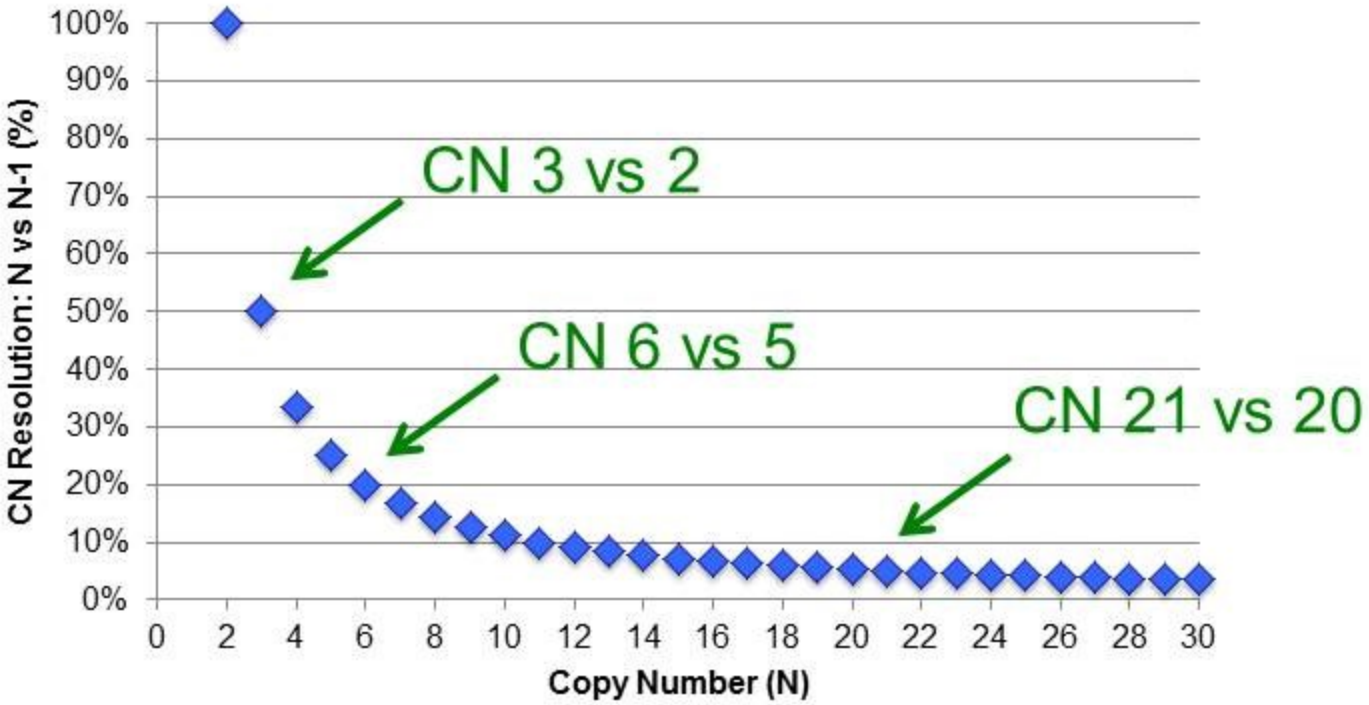
Examples in the literature



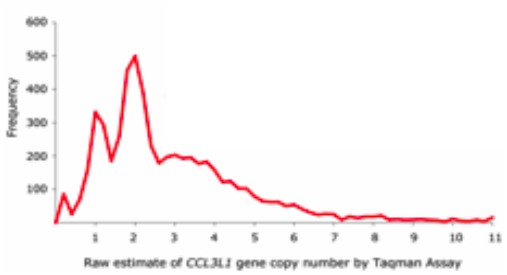
Copy Number Variation

Copy Number Variation: What is the challenge?

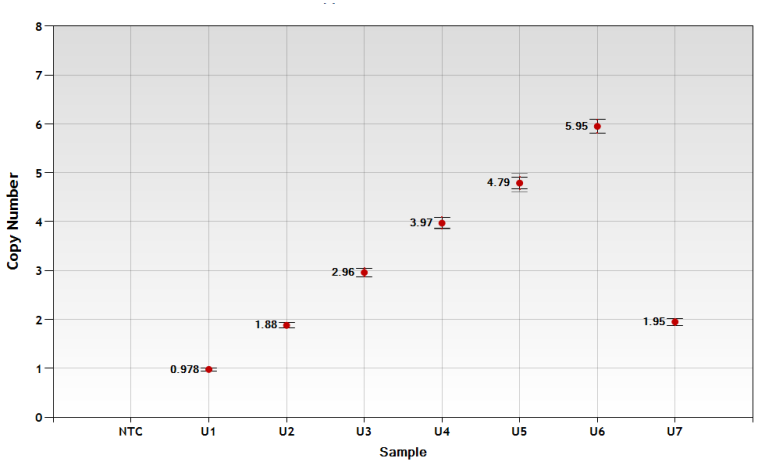
Homogeneous Samples: Discrimination between consecutive copy number states is more difficult at higher order copy number.



Challenge



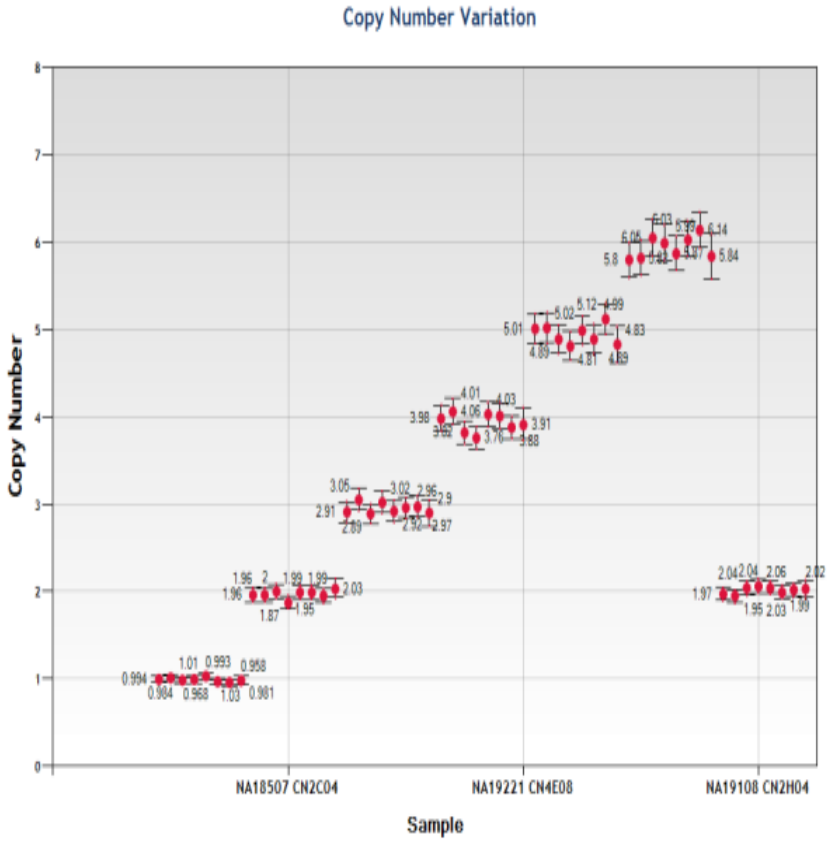
Gonzales et al., 2005.



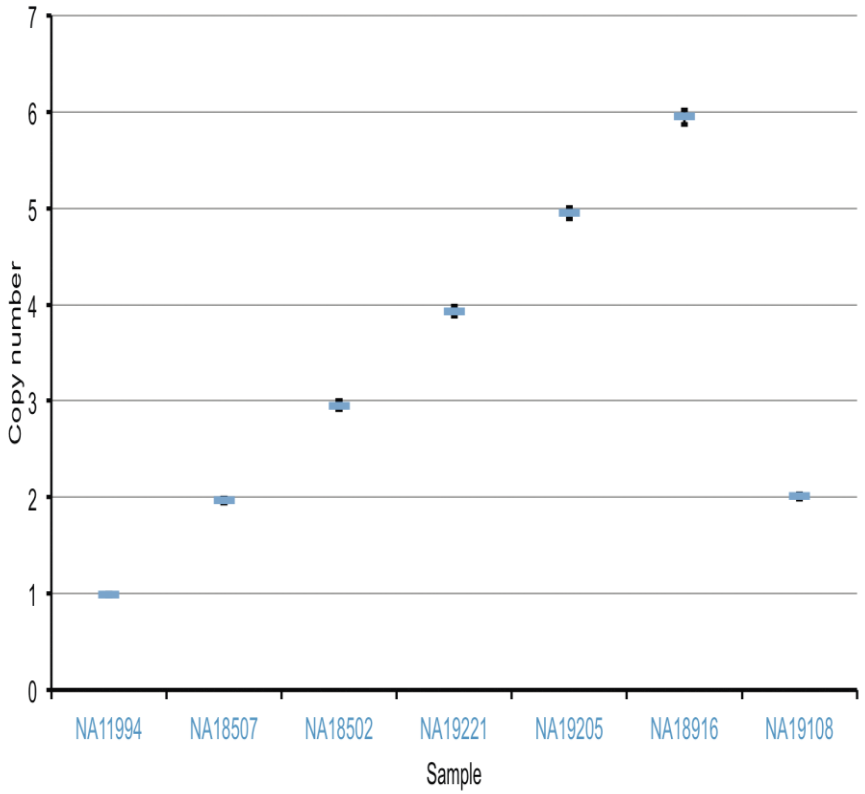
- Techniques such as qPCR can differentiate 1 from 2 from 3 copies with relative ease when using robust assays and reasonable amounts of template.
- Accurate quantification at higher levels (ex 5 from 6 copies) can prove difficult. The difference in C(q) is small and these values need to be normalized to reference genes. Propagation error form standards and efficiencies add to the complexity.

Measuring copy number for MRGPRX1

ddPCR individual wells

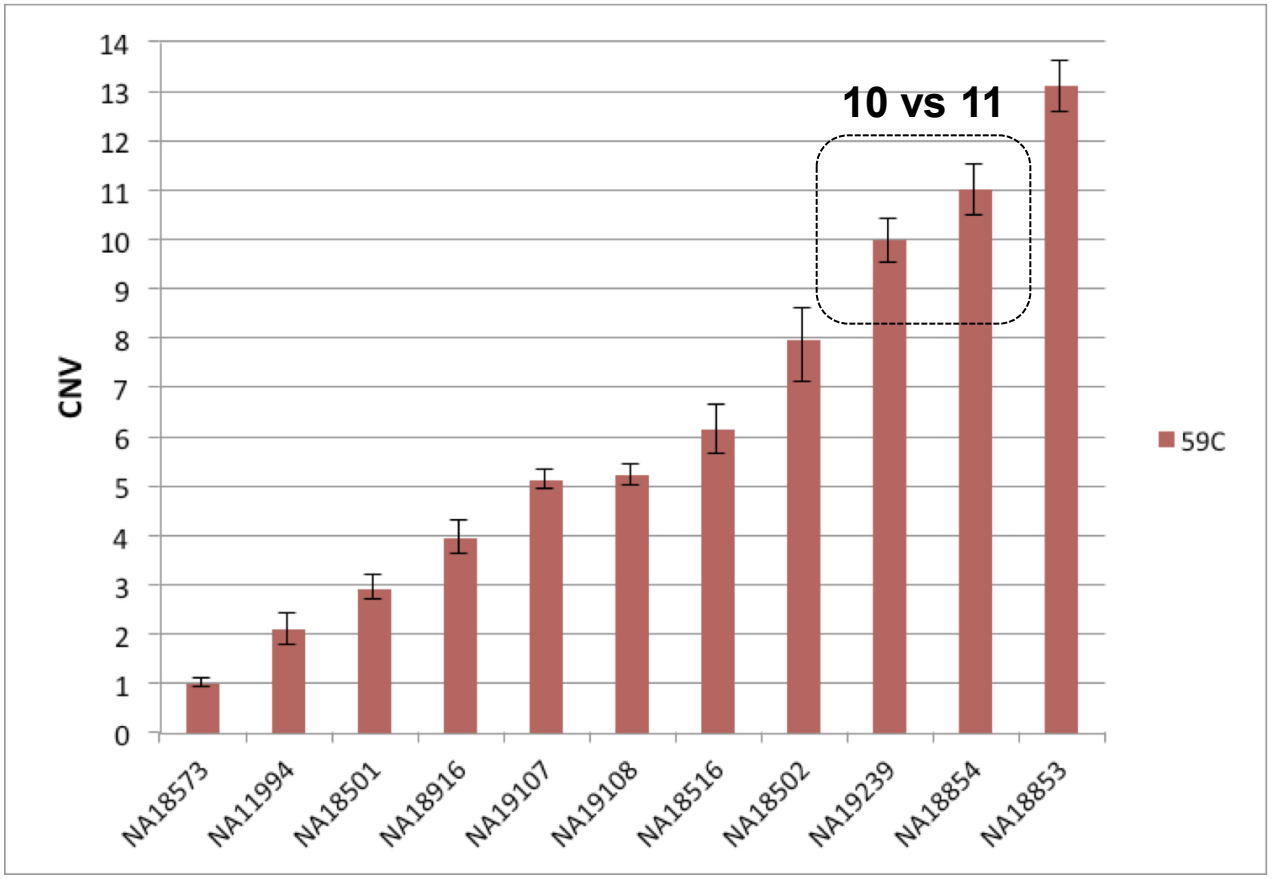


ddPCR merged wells



Higher CNV level discrimination

CCL3L1 Copy Number Analysis of 11 HapMap samples

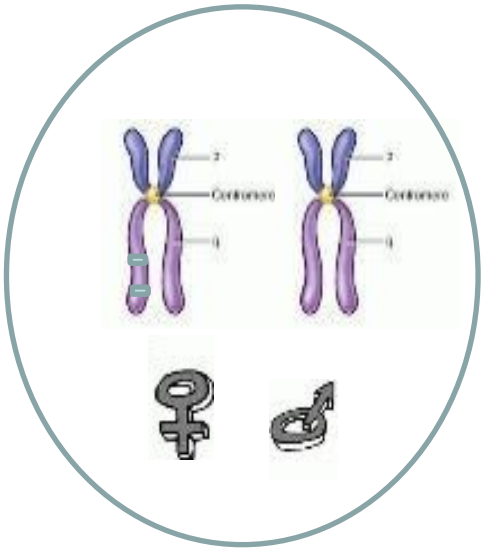


- Merged duplicate wells w/ 16.5ng DNA each – 95% CI's

Can we tell if gene copies are on different chromosomes?

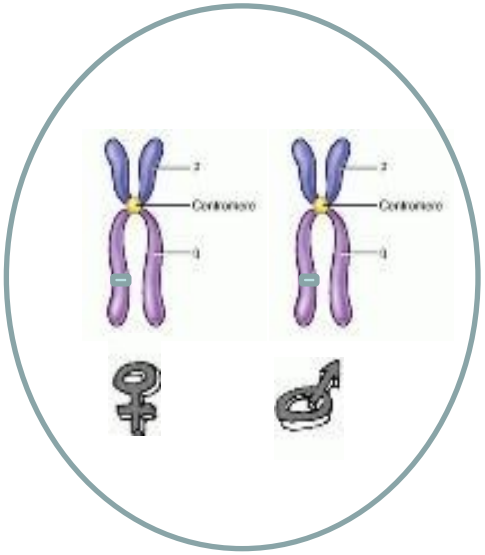
Problem: determine if a normal-seeming CNV=2 is a deletion carrier:

Deletion Carrier



VS

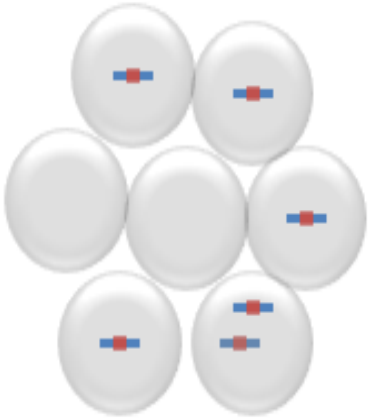
Normal



Approach: compare CNV estimates with and without restriction digestion.

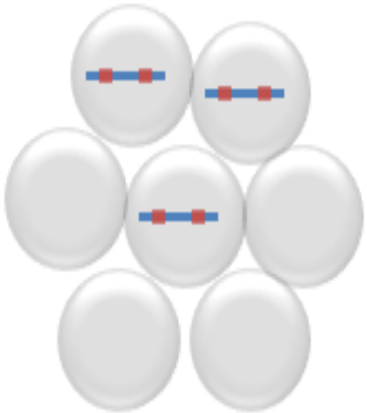
Can we tell if gene copies are on different chromosomes?

With restriction digestion



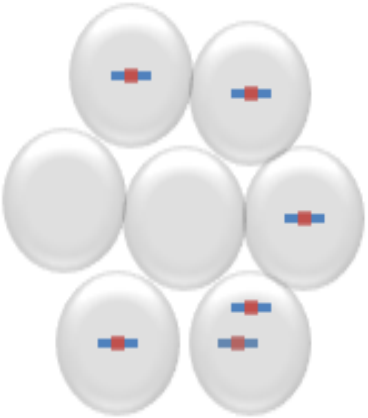
No restriction digestion

Two tandem copies:



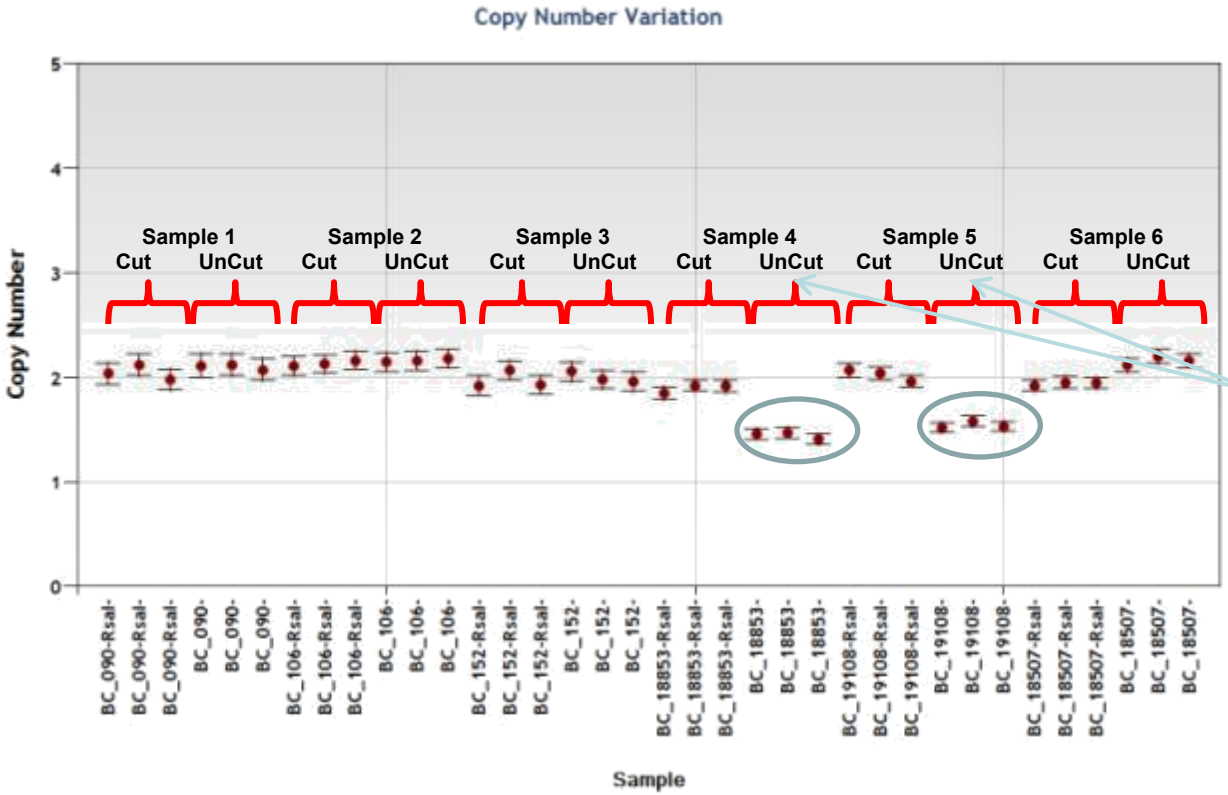
Expect lower CNV estimate

Two unlinked copies:



Expect higher CNV estimate

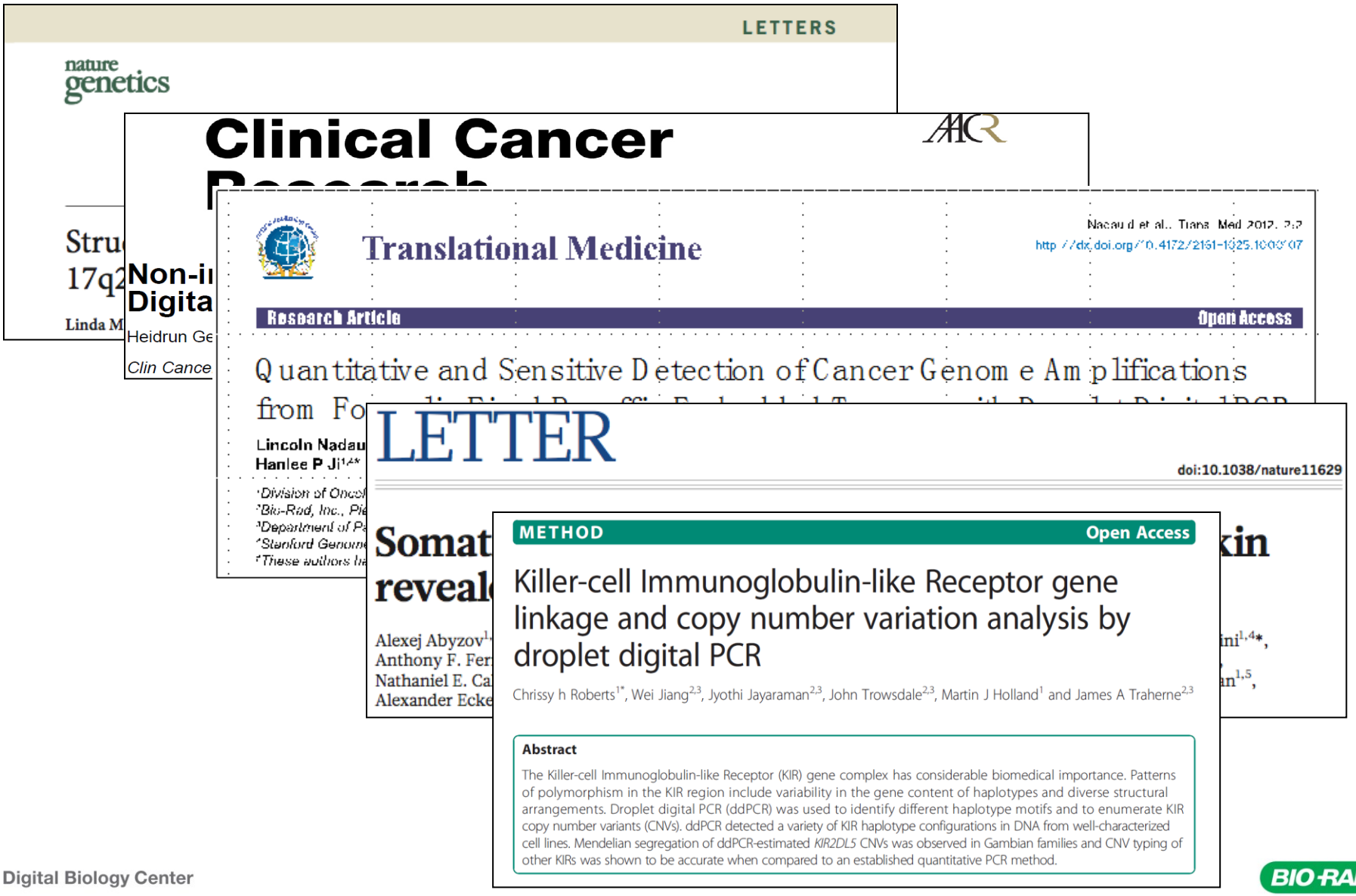
The precision of ddPCR uniquely allows haplotyping of CNV copies



Lower CNV values when sample is not digested suggests that both copies are proximal or on the same chromosome.

* Data for MRGPRX1

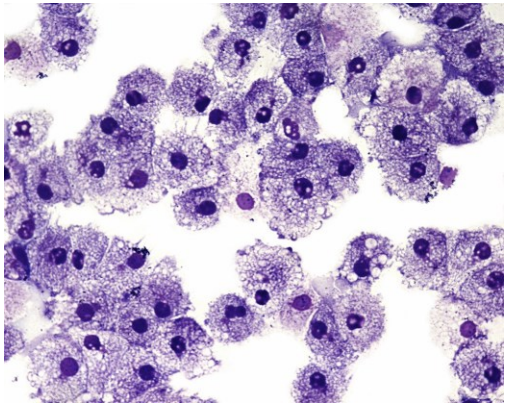
Examples in the literature

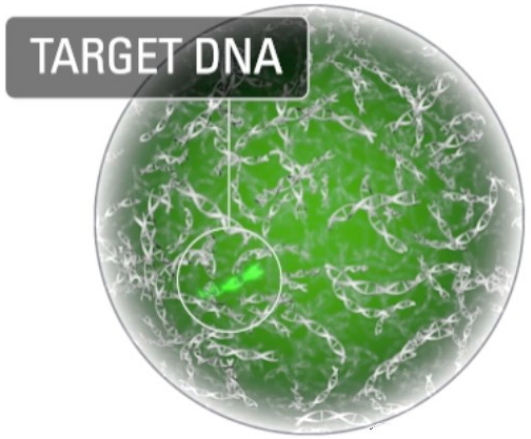


BIO-RAD

Rare Mutation Detection

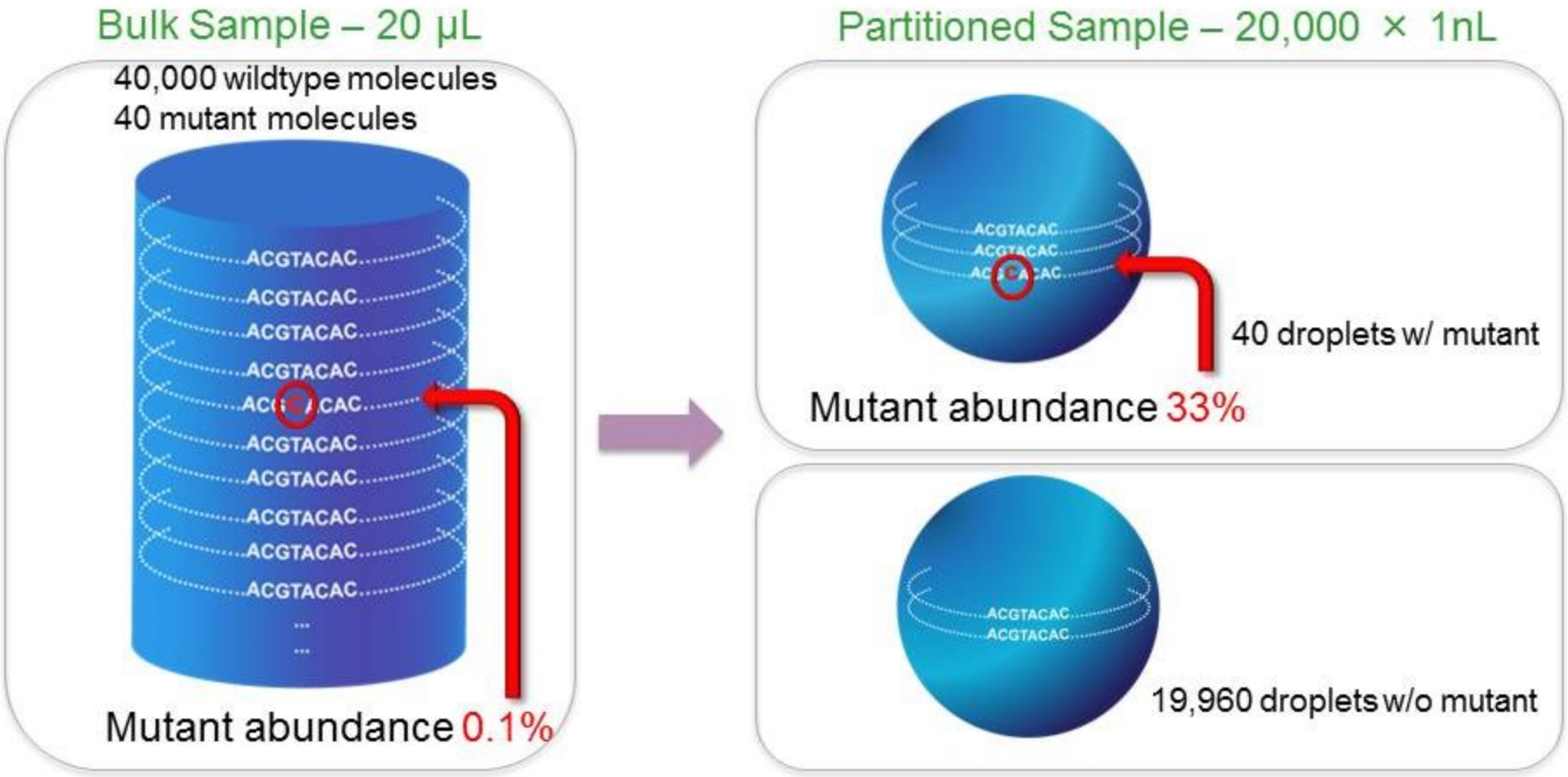
Rare mutation detection challenge





- Growing set of somatic mutations are of key importance for diagnostics, prognostics, and therapeutics
  - Kinases (serine, tyrosine)
  - Phosphatases
- Biggest application is in clinical diagnostics
  - Body fluids
    - Whole blood, serum, plasma, urine
  - Peripheral Blood Mononuclear Cells (PBMCs)
  - Biopsies and FFPEs
- The detection of mutations in heterogeneous samples increases in difficulty as the abundance of mutant genes decreases
  - Needle biopsies where most of the sample is normal tissue
  - Blood samples where aberrant cells are highly diluted

Rare mutation detection challenge



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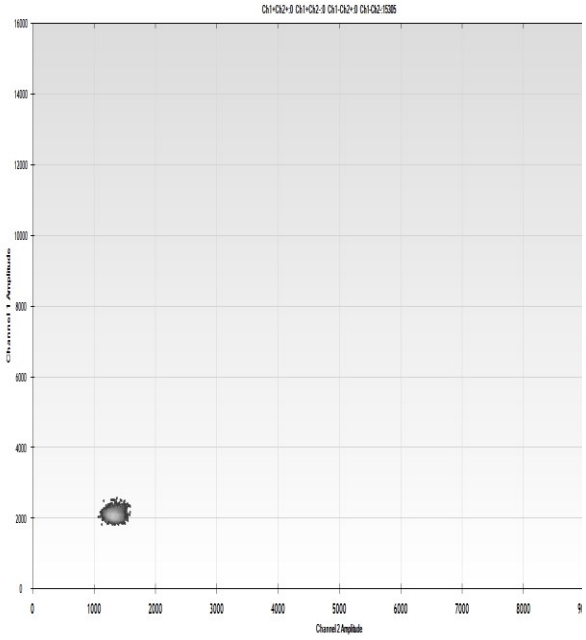
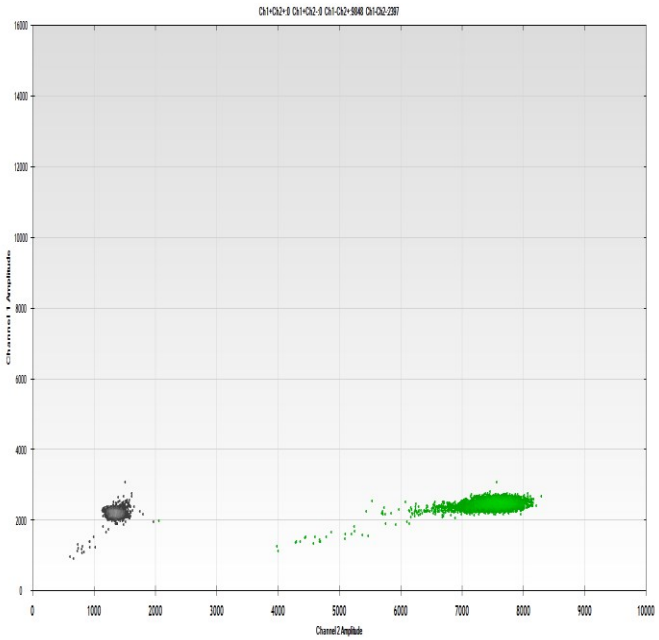
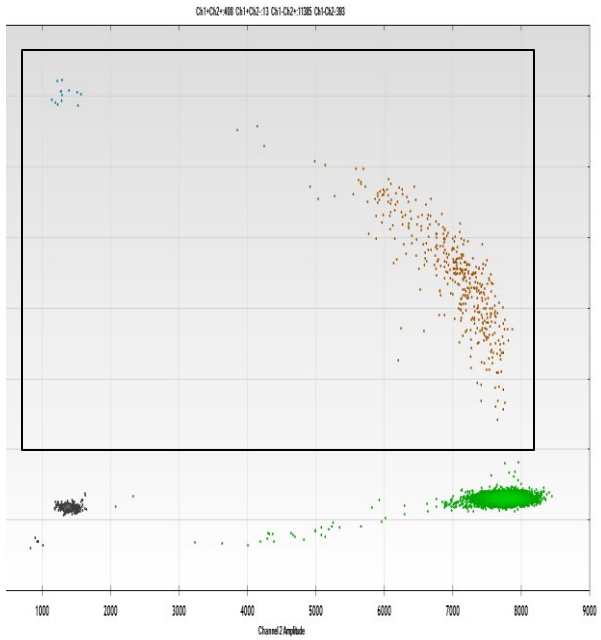
Cancer/rare mutation detection -RMD

KRASG12A assay







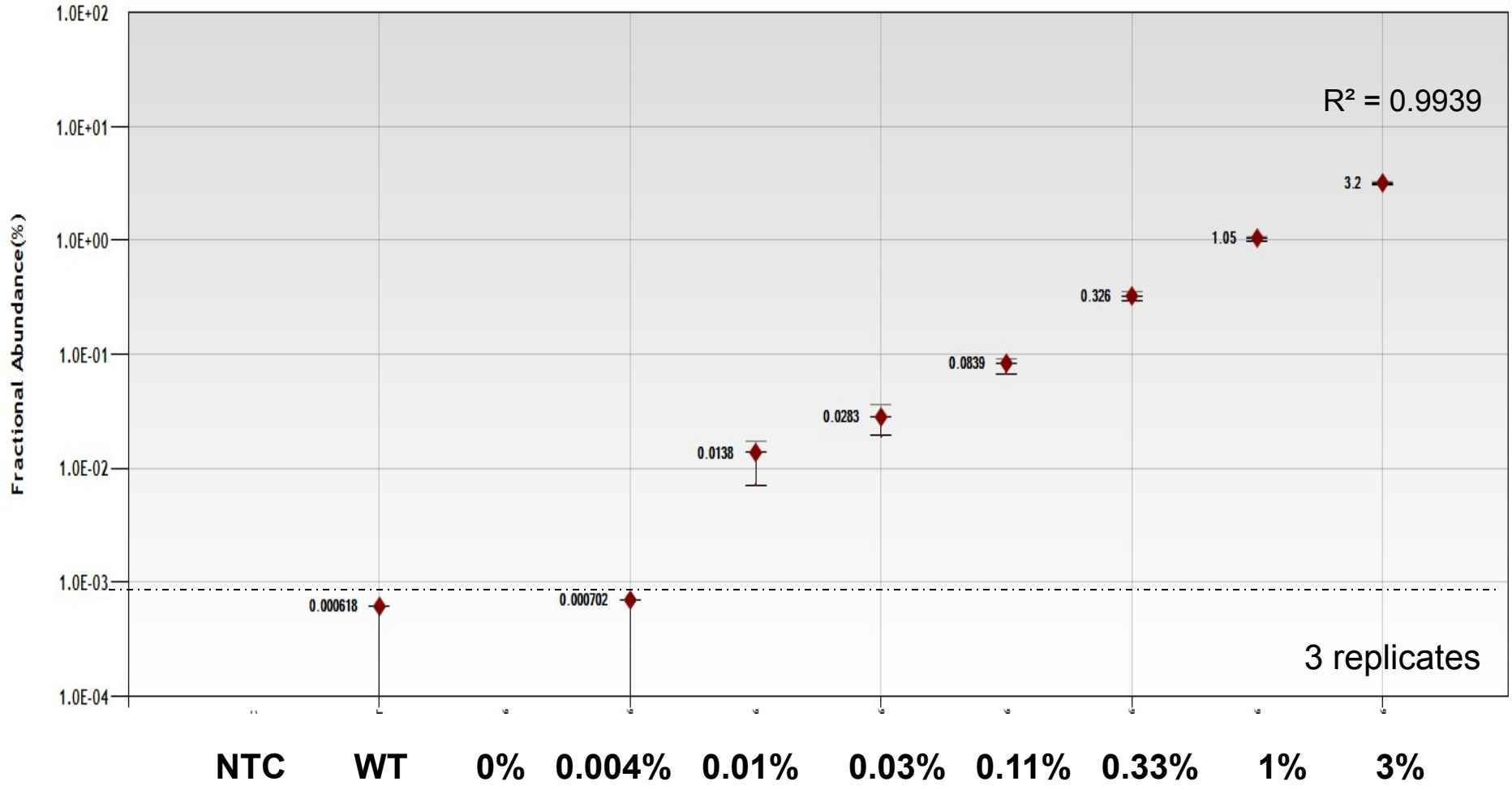
(~270ng)

NTC

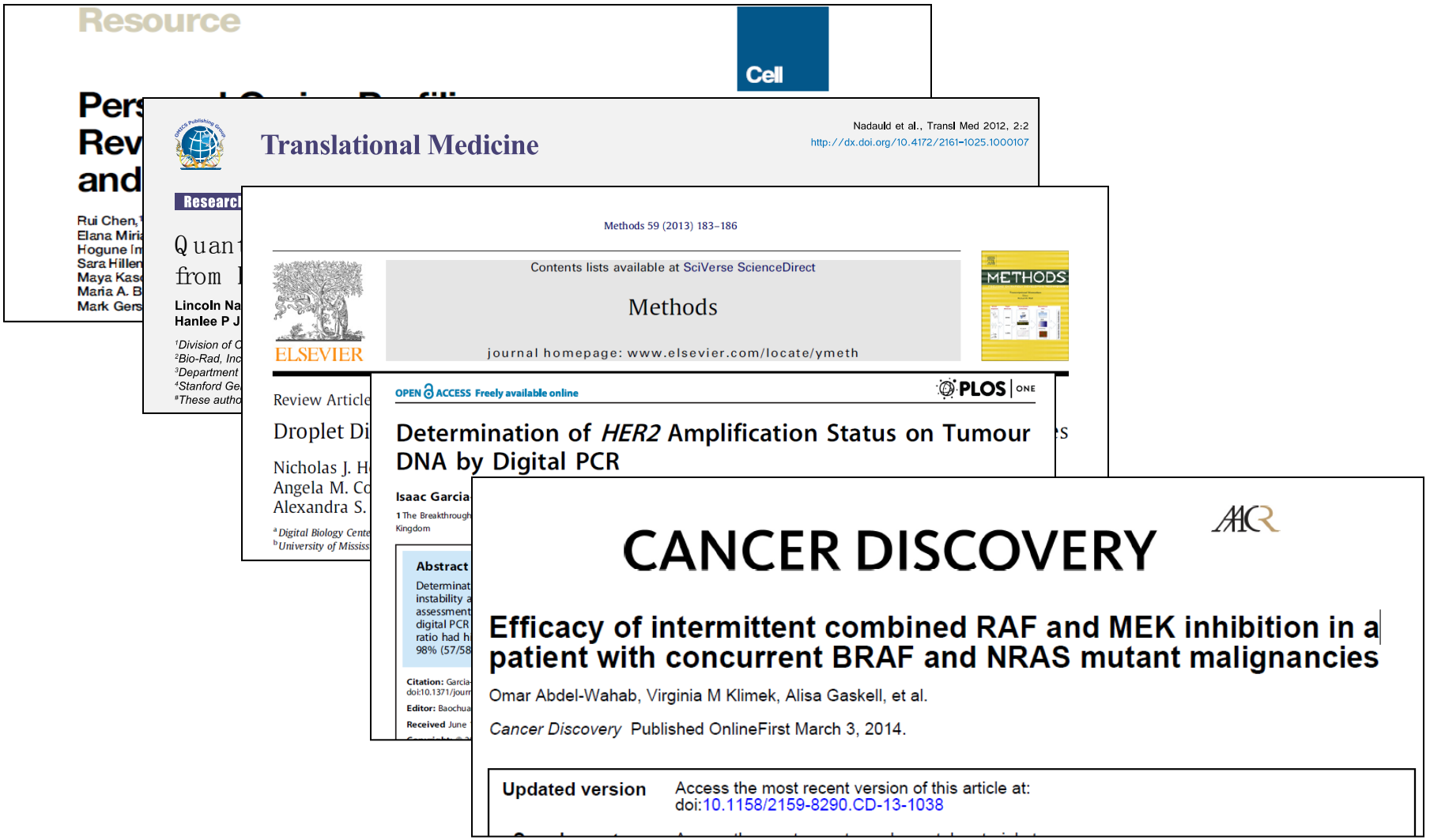
WT only

1% MT

KRAS(G12A) fractional abundance (% Mutant)

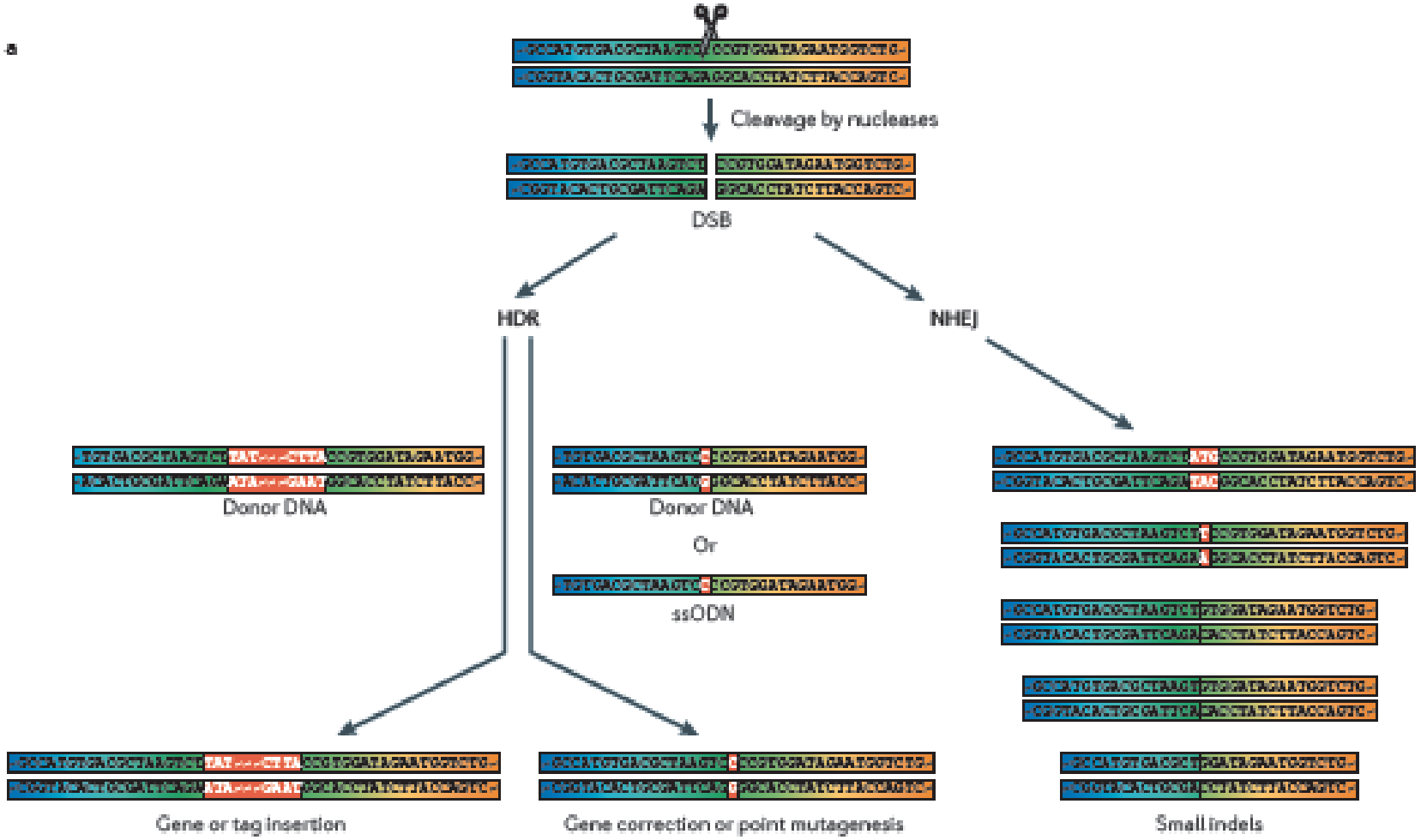


Examples in the literature



Genome Editing Experiments

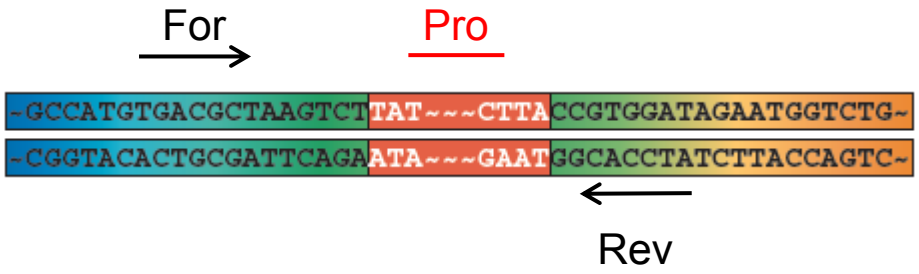
Pathways for genome editing



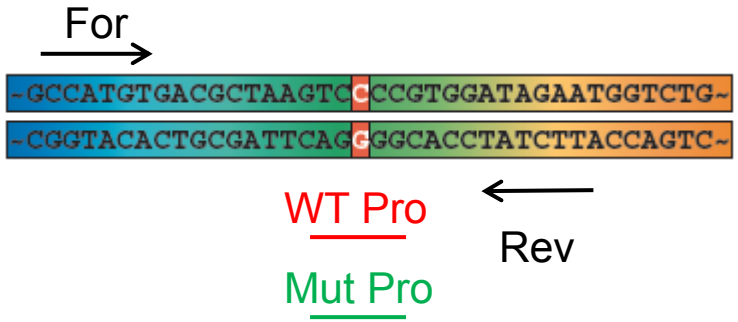
BIO-RAD

Quantification of genome editing events by ddPCR (1)

- HDR (Homologous Directed Repair)
  - Gene or tag insertion: creation of new sequence

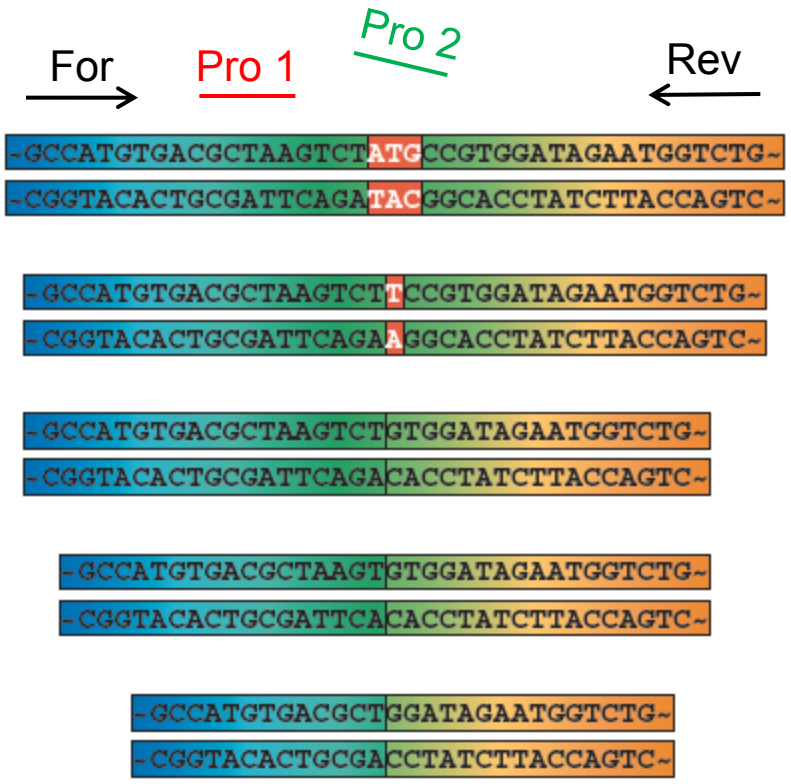


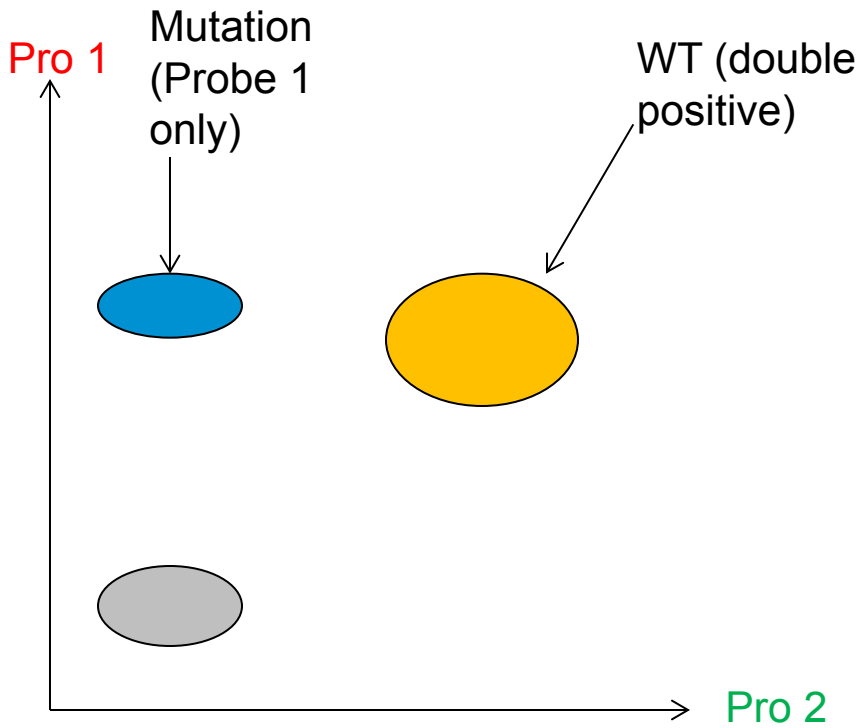
  - Gene correction or point mutagenesis: rare mutation detection



Quantification of genome editing events by ddPCR (2)

- NHEJ (Non Homologous End Joining): loss of signal on one of the 2 WT probes





Haplotyping

What is linkage?

- When two genomic loci are physical connected to one another

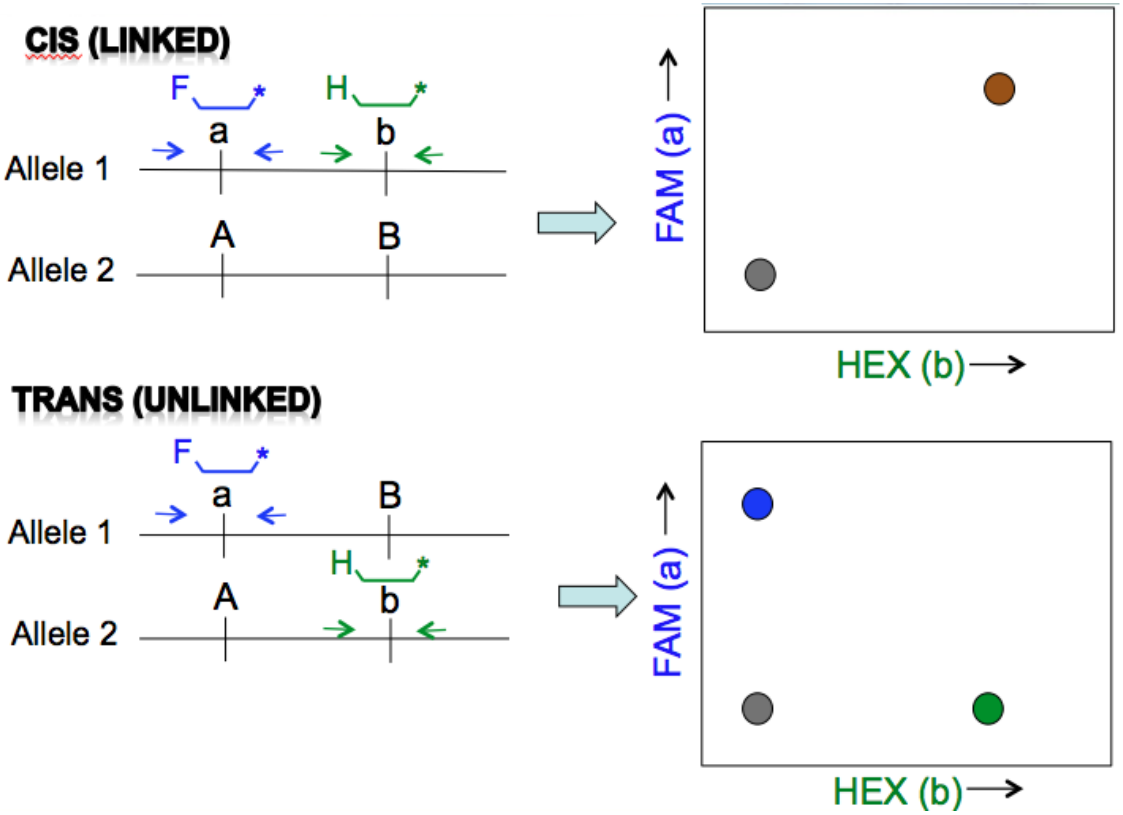
Linked

versus

Not Linked

Who is interested in linkage and why is it important?

- Lifescience researchers and labs performing molecular diagnostics*
  - Cis/Trans configured genomic variants



E.g. CFTR, (c.350G>A & 5T allele)

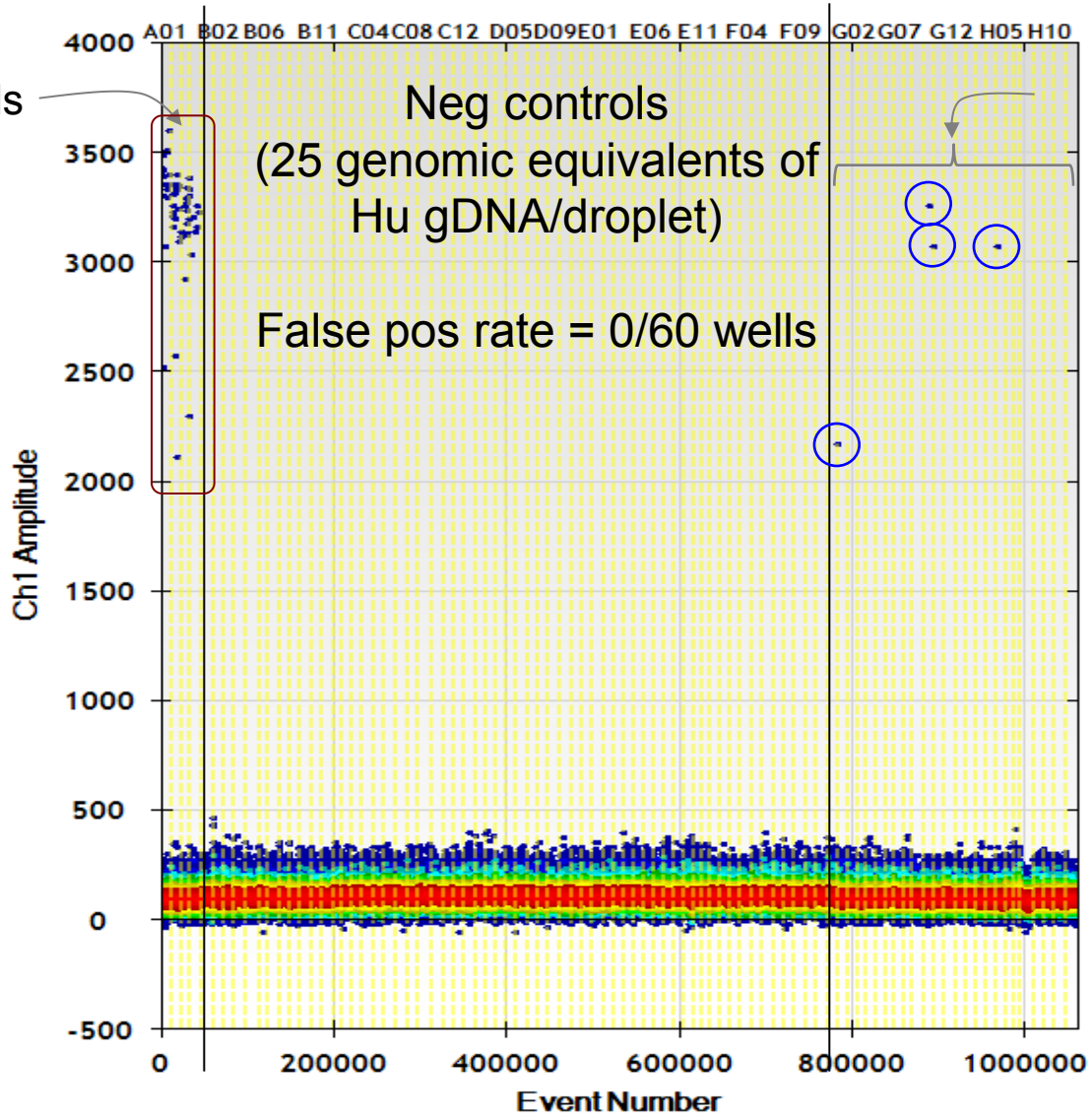
Diseases influenced by compound heterozygosity in single genes

1. Cystic Fibrosis
2. Cerebral palsy
3. Deafness
4. Turcot's syndrome
5. Chondrodysplasias
6. Hyperphenylalaninaemia
7. Blistering skin
8. Charot-Marie-Tooth neuropathy
9. Haemachromatosis
10. Miller syndrome
11. Mediterranean fever
12. Paraganglioma
13. Ataxia-telangiectasia
14. Glycogen storage type II
15. Fructose-1,6-bisphosphatase.

The list hampered by the lack of tools to easily determine phase

Additional Applications

Rare Species Detection: HHV6 in human gDNA (1 in 1.7M)



Pos controls

Neg controls
(25 genomic equivalents of Hu gDNA/droplet)

False pos rate = 0/60 wells

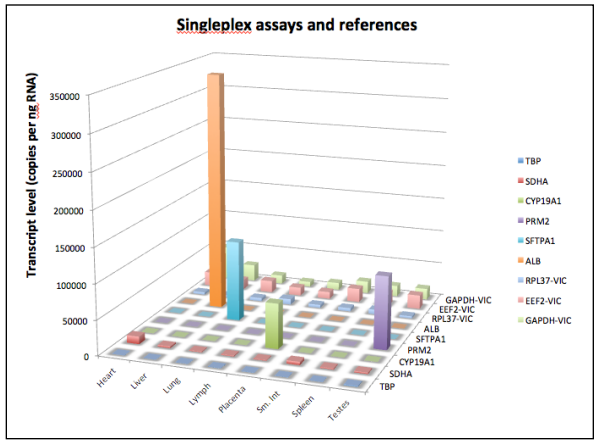
HHV6 spike-in

4 HHV6 positives in ~8M human genomes
(4.5 expected)

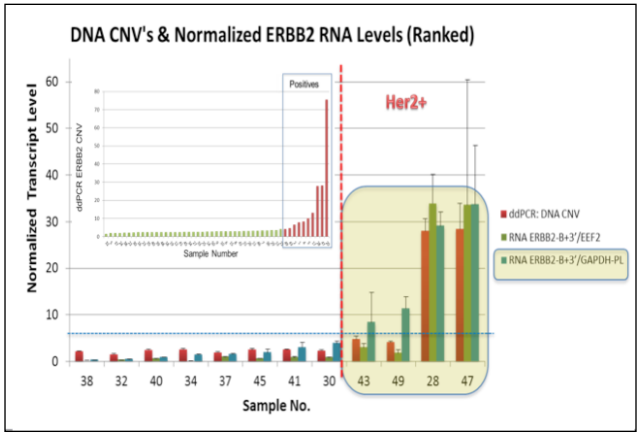
BIO-RAD

Gene expression applications

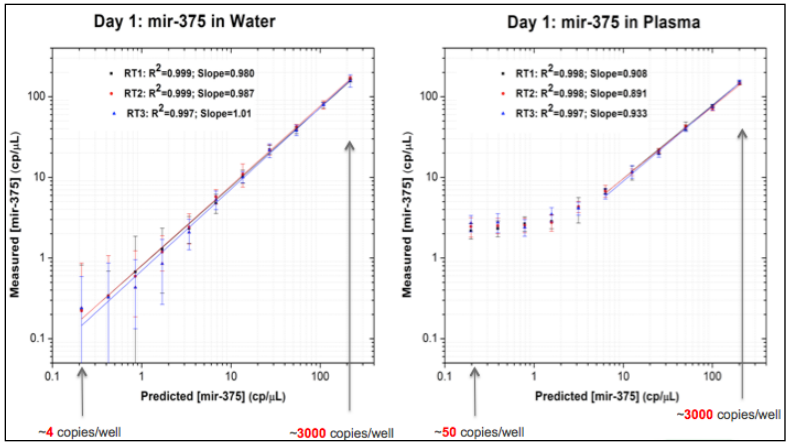
Tissue-specific Gene Expression



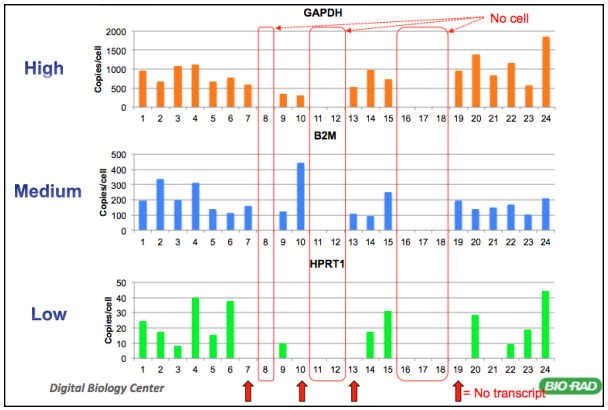
Her2 mRNA in FFPE samples



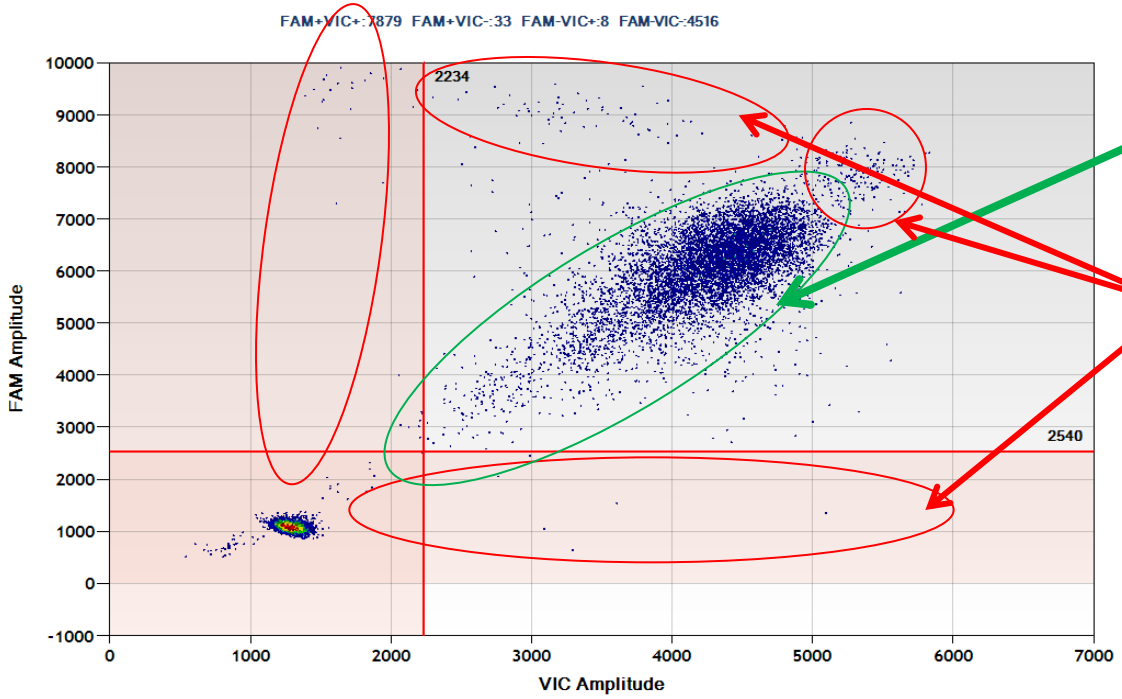
miRNA's in plasma



Single-cell transcript detection



NGS insert size, quantity, and quality



Well-formed fragments, will make **good clusters**

Ill-formed fragments or adaptor-adaptor, **cannot make clusters**

Combination of insert sizes enables detection of the widest range of structural variant types, essential for accurately identifying more complex rearrangements

Telomerase repeat amplification protocol (TRAP)

Make cDNA

STEP 1. Addition of Telomeric Repeats By Telomerase

TS-Telomerase Product

5' – TS | Telomeric Repeats

AATCCGTCGAGCAGAGTT ag ggttag ggttag ggttag ggttag – 3'

+

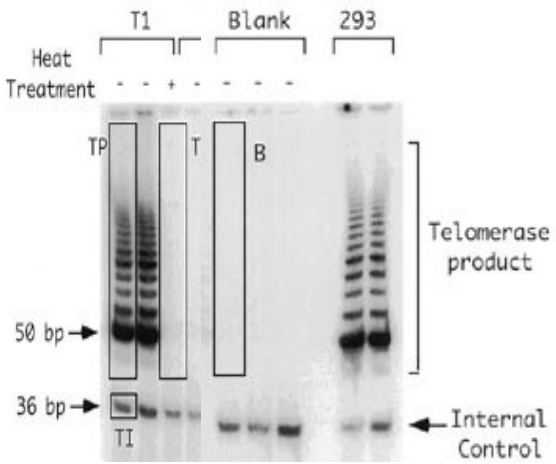
ag ggttag ggttag ggttag ggttag ggttag – 3'

+

ag ggttag ggttag ggttag ggttag ggttag $(ggttag)_n$ – 3'

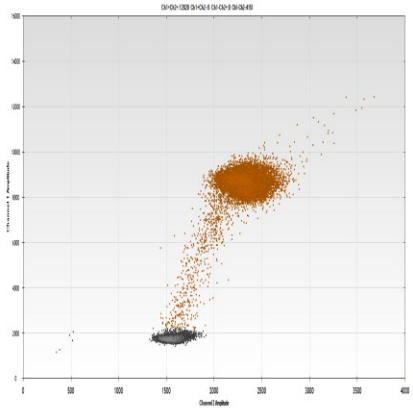
Perform PCR

STEP 2. Amplification of TS-Telomerase Product By PCR



(Kim *et al.* 1997)

100 cells/rxn



What are researchers doing with ddPCR

- Mainstream Applications
  - Detection and Quantitation
  - Rare Mutation Detection
  - Copy Number Quantitation
  - Gene Expression
  - NGS Library Quant

- Additional ddPCR Applications
  - Allele Specific Gene Expression
  - MicroRNA
  - Methylation Studies
  - Haplotyping
  - TRAP Assay (Telomerase)
  - Genome Editing

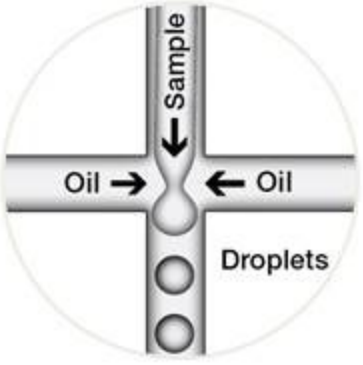
Additional applications

- Water treatment testing
- Waterborne viruses and pathogen testing
- Asian Carp population studies
- Cow Mastitis
- Malaria Mosquito sexing
- Canine mammary Carcinoma
- Fetal ccfDNA
- …

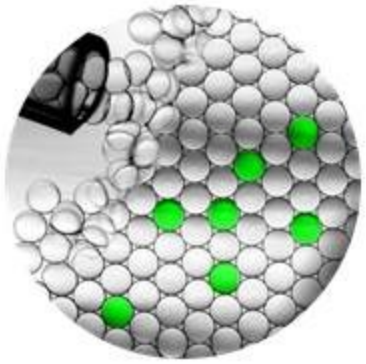
What is the work involved?

Droplet digital PCR workflow

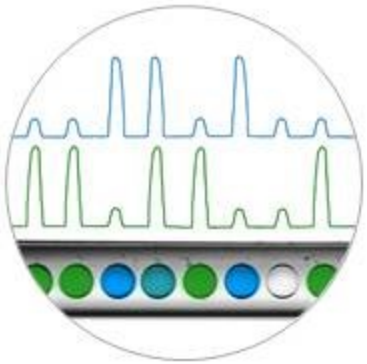
- Partition reagents and sample into 20,000 droplets
- Perform PCR on thermal cycler
- Count droplets with a positive PCR product (fluorescent) and a negative PCR product
- Digital readout provides concentration of target DNA



Make Droplets



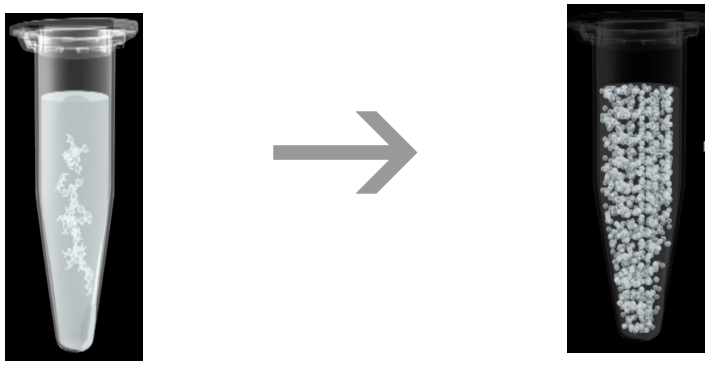
PCR Droplets

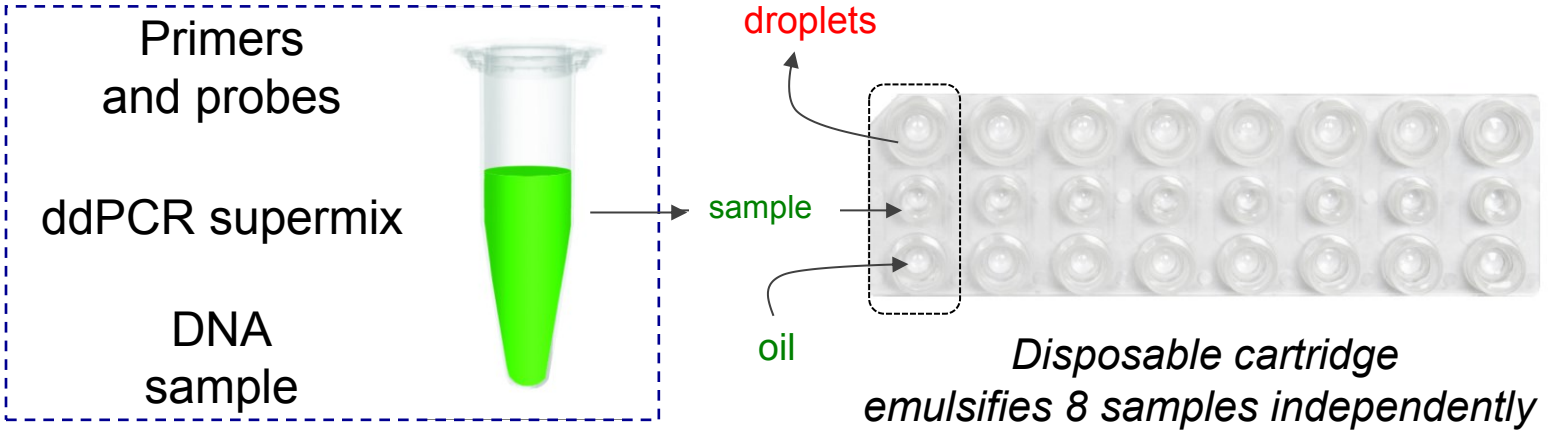


Read Droplets

Results

Preparing sample and reagent mixture



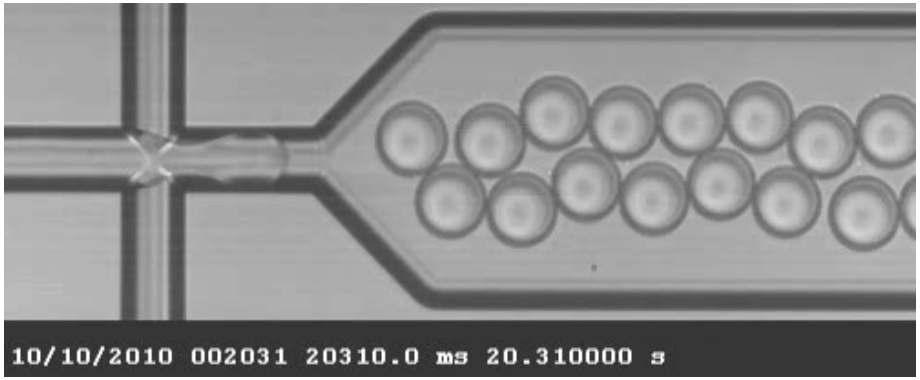


Generating droplets

96 well Reaction Plate

QX200™ Auto DG Droplet Generator

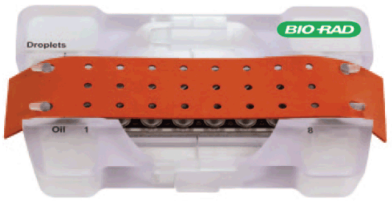
96 well Droplet Plate

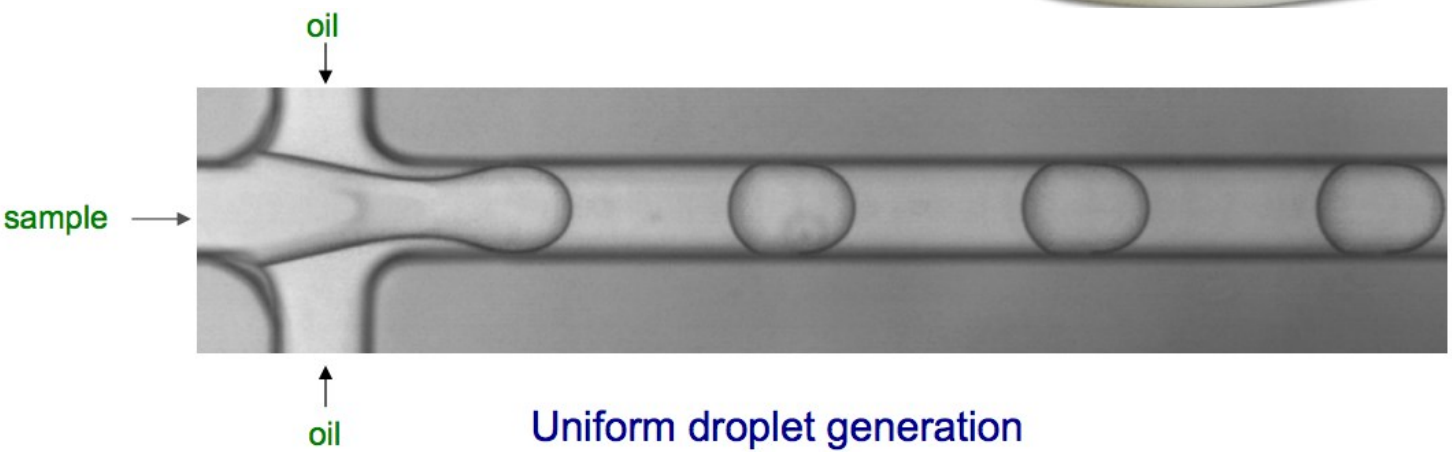


Amplifying droplets

Making droplets

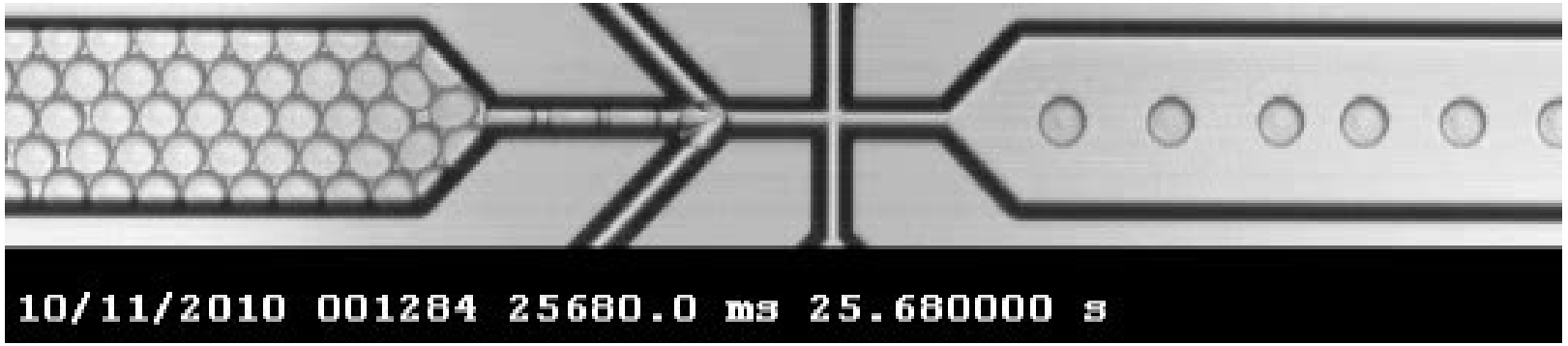
- Place loaded cartridge into QX200 Droplet Generator
- Generate 20,000 droplets per sample, 2 ½ min for 8 samples



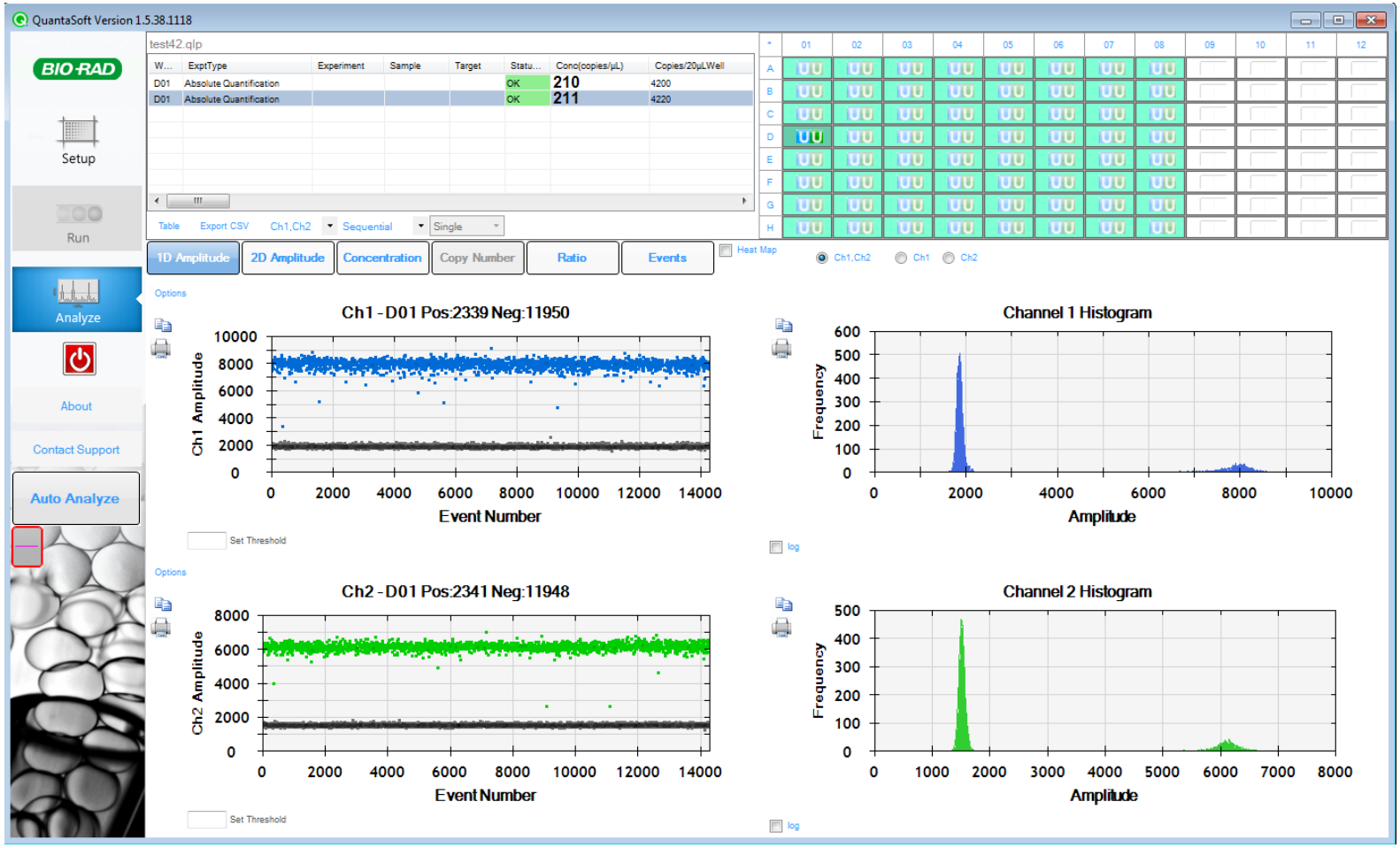


Reading droplets

QX200™ Droplet Reader



Software calculates target copies



Additional workload (96 samples)

QX200™ Droplet Generator

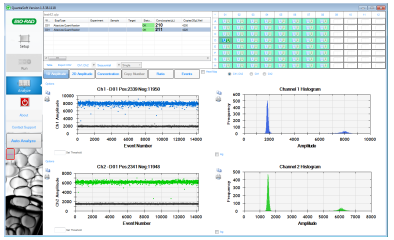
40 minutes

QX200™ Auto DG Droplet Generator

Less than 5 minutes

Labor involved

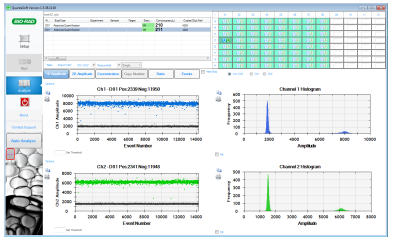
96 well Reaction Plate



- Total process time from PCR reaction plate to results – approx 5 hours (96 wells).
- Total hands on time – less than 45 minutes.
- Staggered processing allows for 3 plates in an 8 hour work day (4 with an overnight run).
- Analysis time – approx equivalent to 96 well qPCR plate.

Labor involved

96 well Reaction Plate



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- Staggered processing allows for 3 plates in an 8 hour work day (4 with an overnight run).
- Analysis time – approx equivalent to 96 well qPCR plate.

Conclusion

- Technological advantages
  - Absolute Quantitation independent of an external reference
  - High resolution quantitative and detection
  - Affordable cost per result with minimal labor.

- Common Applications
  - Nucleic acid quantitation and detection (viral, pathogen, GMO, etc..)
  - Copy Number Variation analysis
  - Rare Mutation Abundance
  - High resolution Gene expression
  - Proximity studies (phasing)
  - NGS library quant
  - …

- Additional ddPCR Applications
  - Allele Specific Gene Expression
  - MicroRNA
  - Methylation Studies
  - Haplotyping
  - TRAP Assay (Telomerase)
  - Genome Editing

Thank you!

Thank you!

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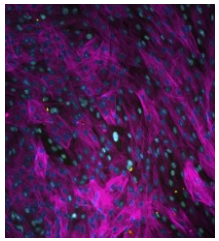

November 13, 2014
10:00 AM, 3:00 PM EST
John Pulliam, Ph.D., *Field Application Scientist, ATCC*
3D Tissue Modeling

Thank you for joining today!
Please send additional questions to tech@atcc.org