

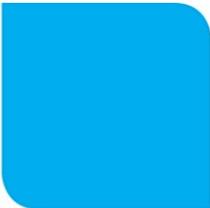


Droplet Digital PCR

Precise Counting of Targeted Nucleic Acids has Never Been Easier

Francisco Bizouarn

Global Digital Applications Specialist
Digital Biology Center



About ATCC®

- Founded in 1925, ATCC® is a non-profit organization with headquarters in Manassas, VA
- World's premiere biological materials resource and standards development organization
- ATCC® collaborates with and supports the scientific community with industry-standard products and innovative solutions
- Broad range of biomaterials
 - Cell lines
 - Microorganisms
 - Native & synthetic nucleic acids
 - Reagents



ATCC® Molecular standards

- ATCC® Genuine Nucleics represents the largest and most diverse array of native, synthetic, and certified reference materials for use in:
 - Molecular-based assays
 - Quality control
 - Establishing sensitivity, linearity, and specificity during assay validation or implementation
 - Validating or comparing test methods
 - Benchmarking critical assay performance during development and validation for regulatory submissions and production lot release



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



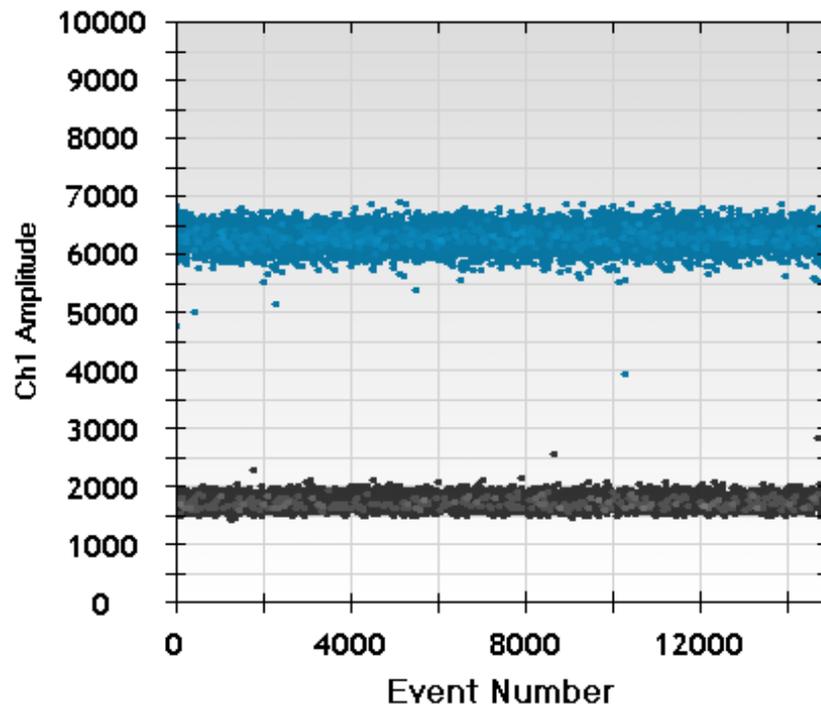
Nanodroplet PCR reactions
are independent, single
amplification events



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

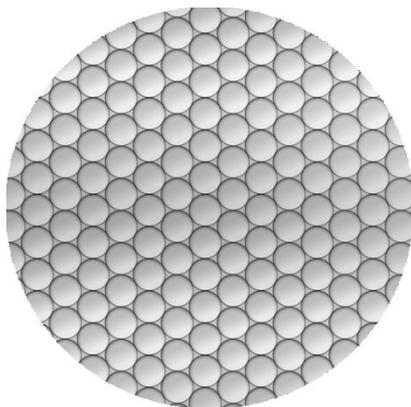


Each positive counted as 1

Each negative counted as 0

Counting positives to estimate target concentration

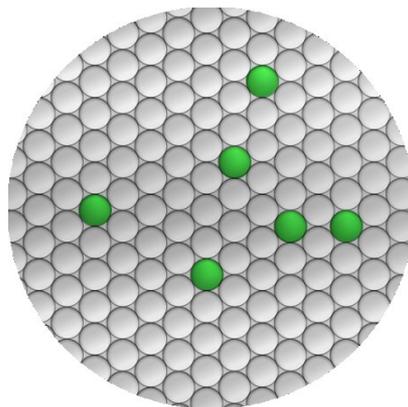
Sample 1



NO
targets

p=0 positive/143 total

Sample 2

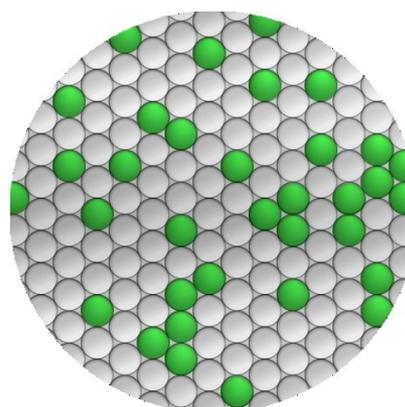


Low
concentration

p=6/143

Poisson corrected
6.2/143

Sample 3

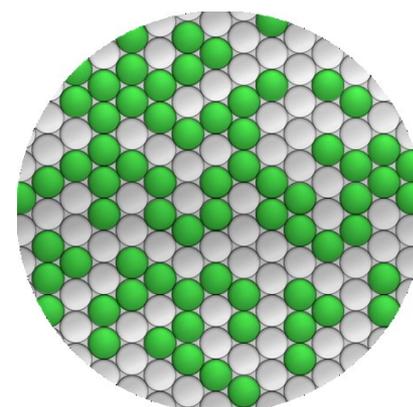


Medium
concentration

p=34/143

Poisson corrected
38/143

Sample 4



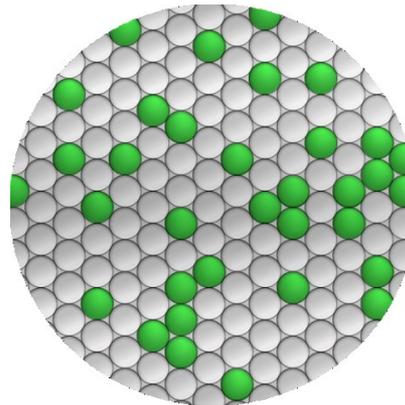
High
concentration

p=70/143

Poisson corrected
96/143

Increased number of partitions

Sample 3



Medium
concentration

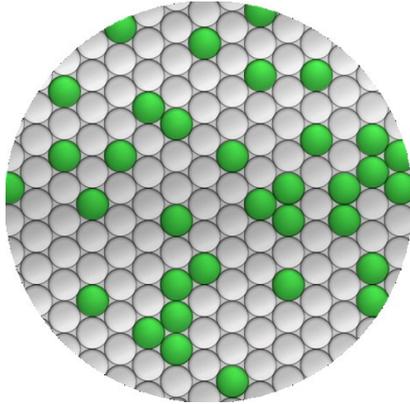
$$p=34/143$$

Poisson corrected

38

Increased number of partitions

Sample 3

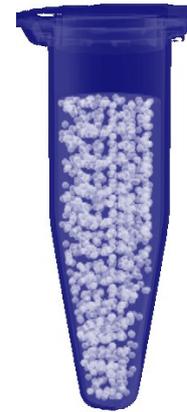


Medium
concentration

$$p=34/143$$

Poisson corrected
38

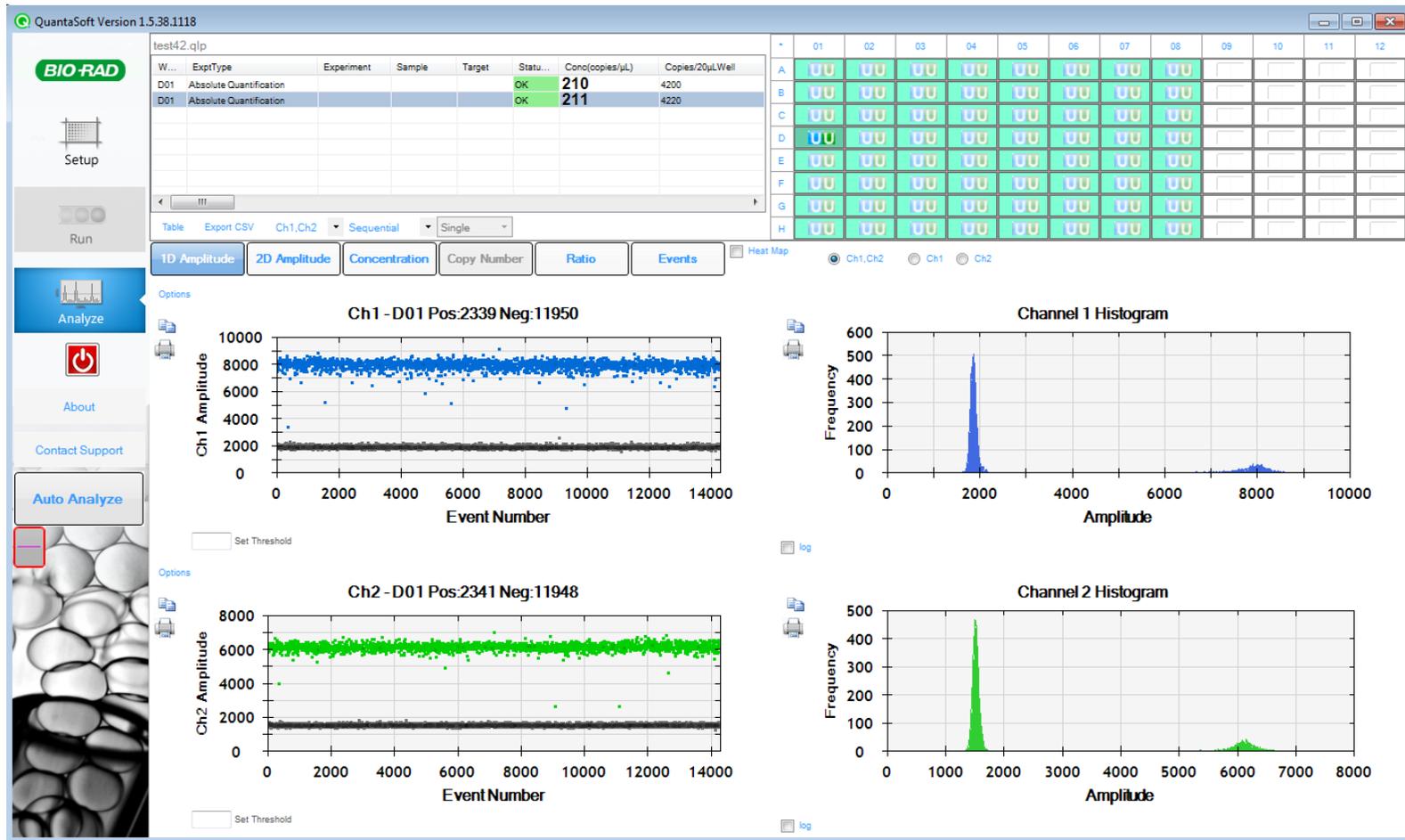
Well A3



$$P=16\,076/17\,451$$

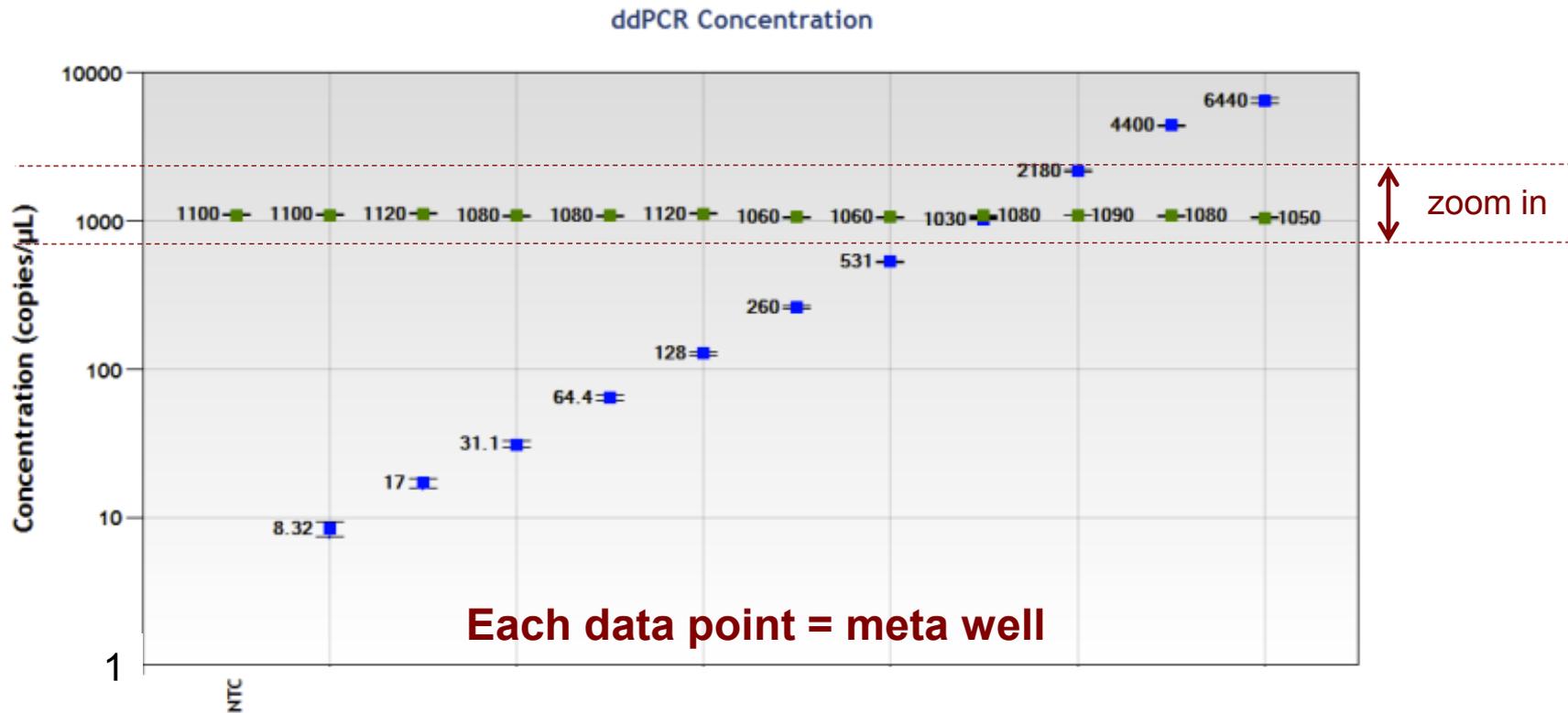
Poisson corrected
55 800

Software calculates number of target molecules



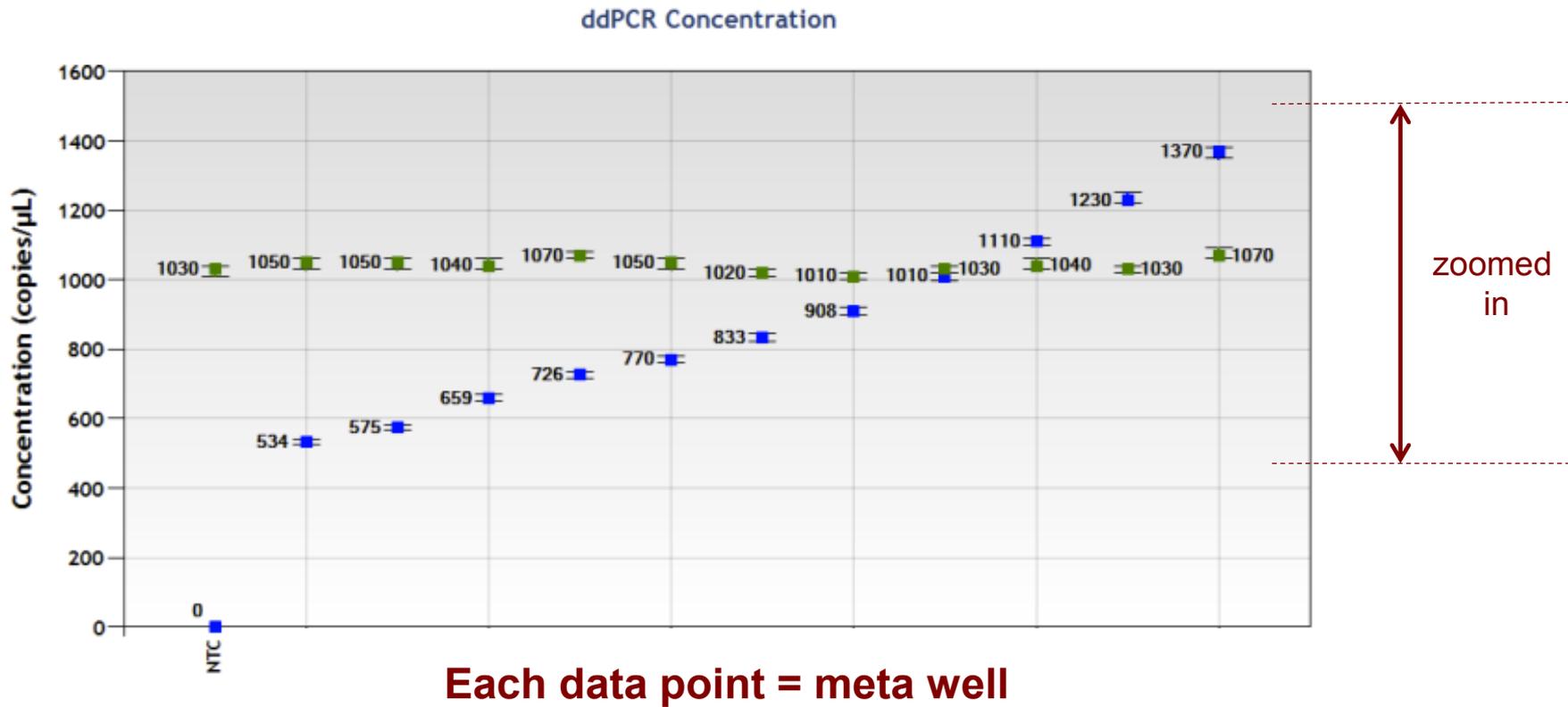
What kind of results can we expect
from Digital PCR?

4 merged wells of each 2-fold serial dilution



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

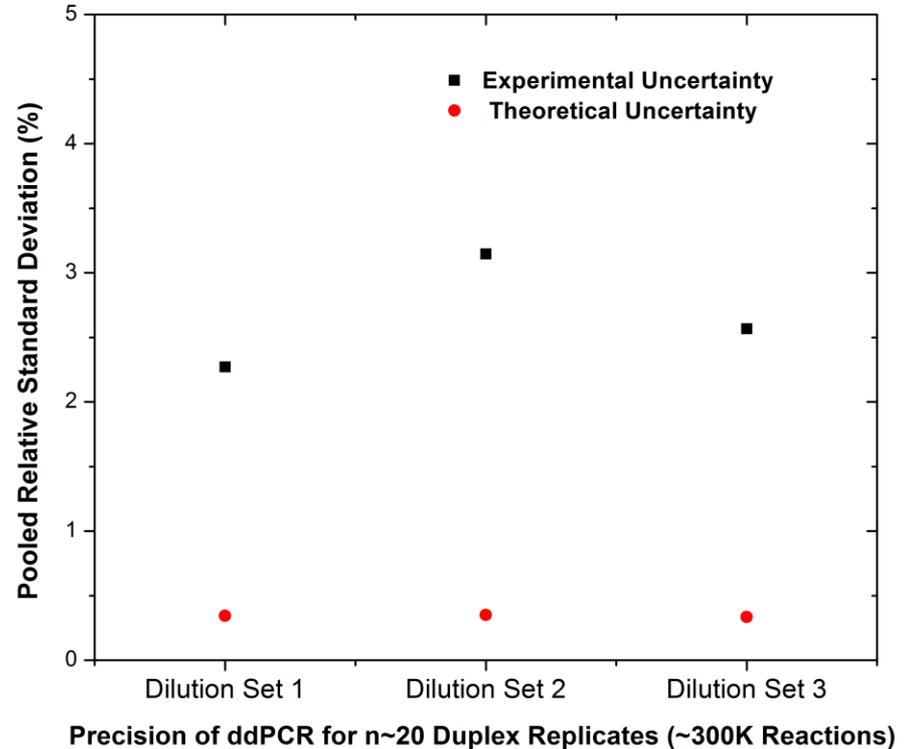
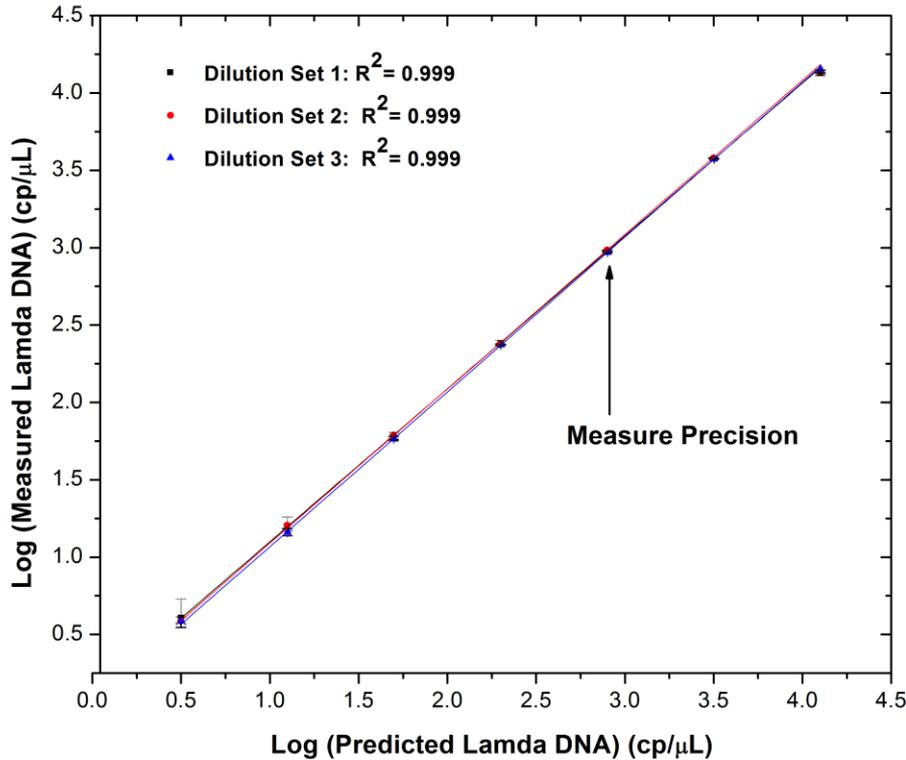
Excellent precision from 10% dilutions



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

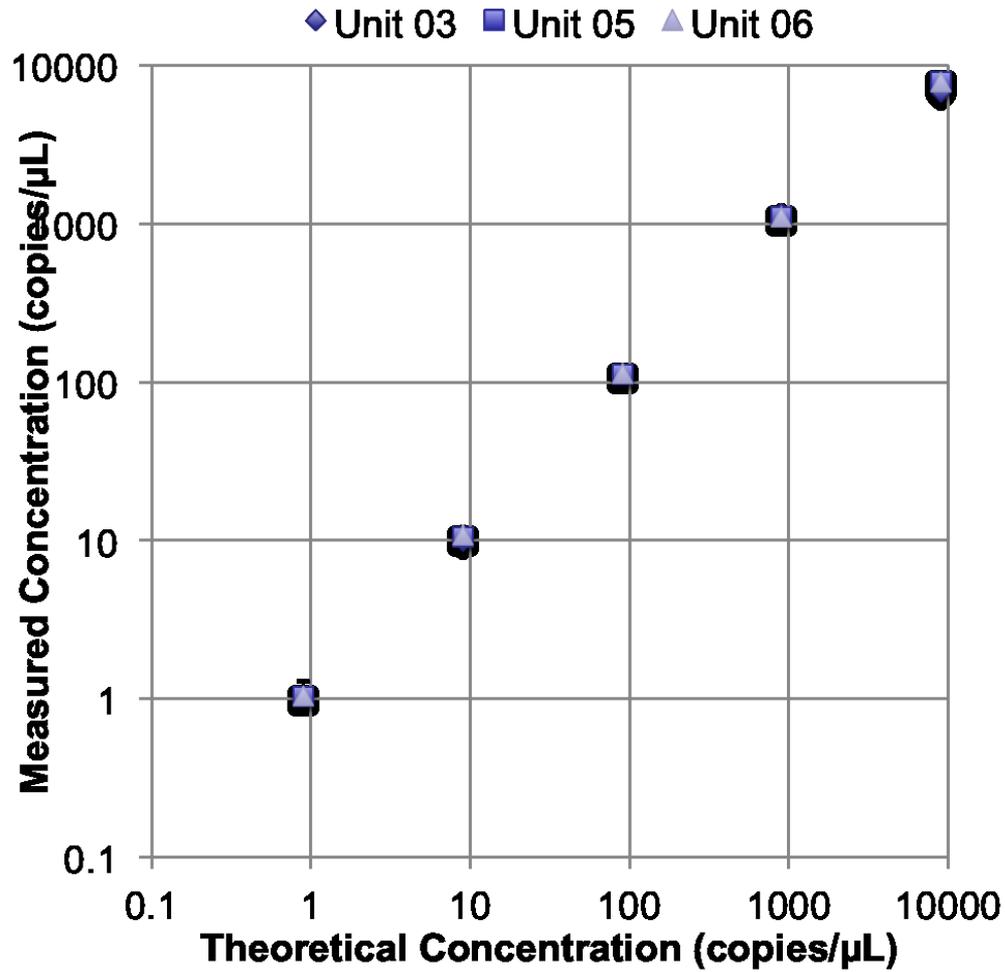
Precision independently verified and observed,
+/- 1.5% uncertainty over theoretical value

ddPCR Performance over Theoretical Dynamic Range for a 20,000 droplet Assay



Gravimetric Experiments Conducted at [National Measurement Institute, NSW \(Australia\)](#)

Excellent reproducibility and linearity across concentrations and instruments



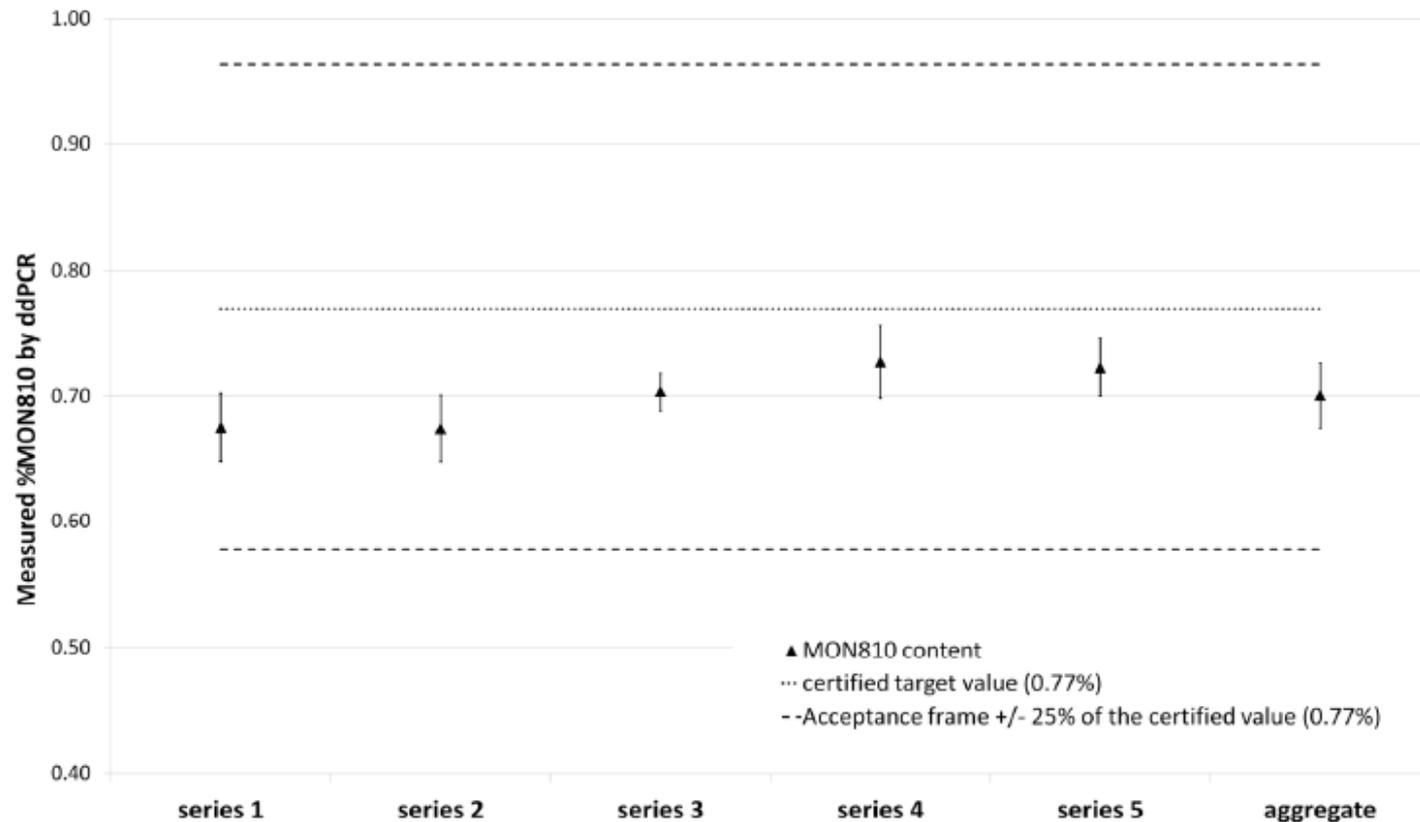
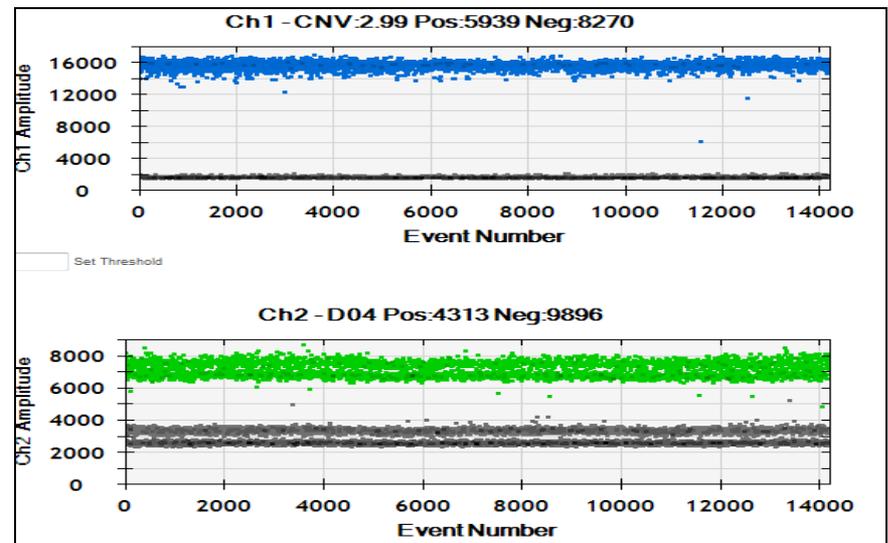
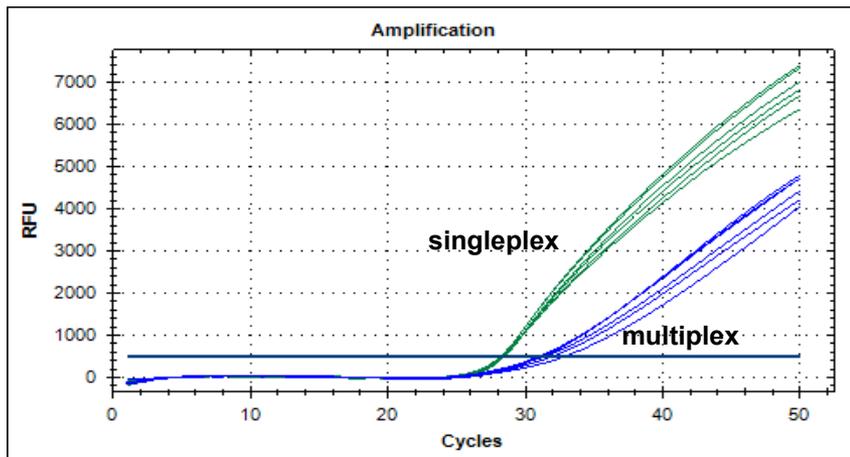
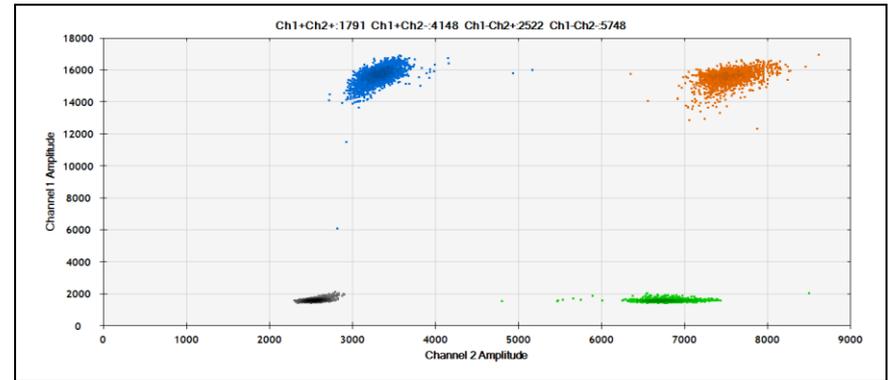
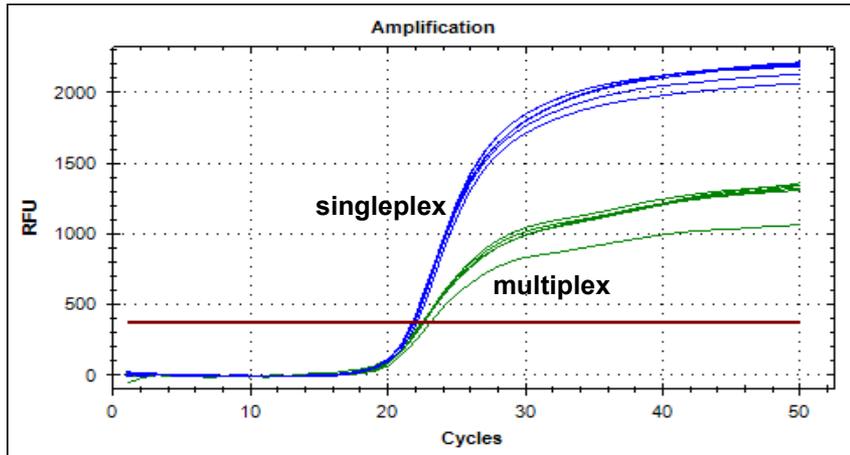
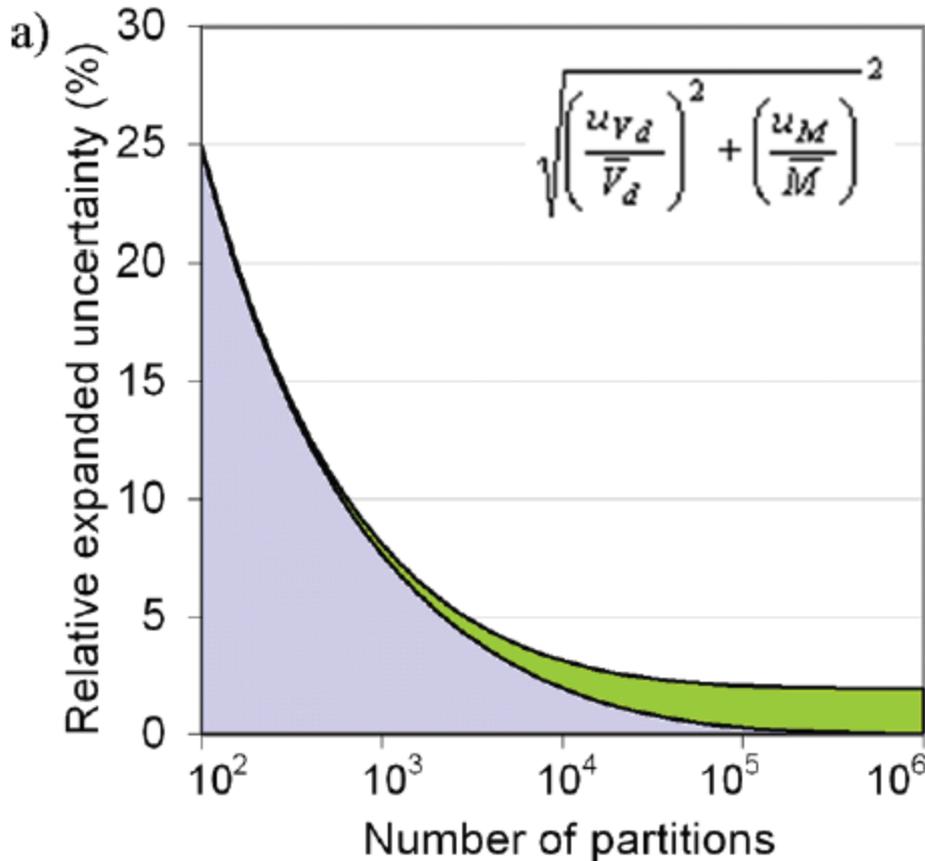


Figure 2. Repeatability results of the ddPCR duplex assay. MON810 content measured by ddPCR in five series of seven replicates. The aggregate represents the sum of the five series. The target certified MON810 content (0.77%) is indicated by a dotted line. Acceptance criterion for repeatability is $\pm 25\%$ 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



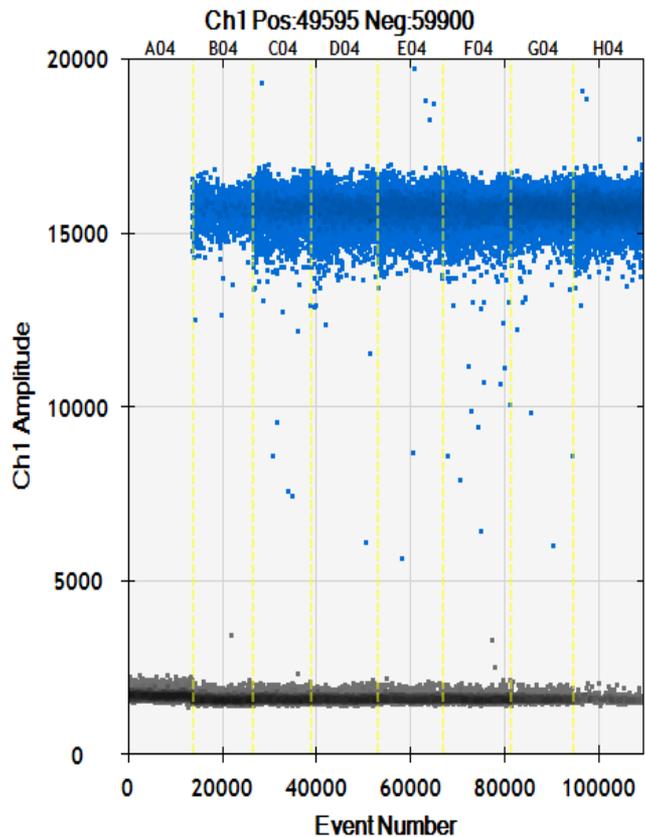
Why use 20 000 partitions?



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

The NEW ENGLAND JOURNAL of MEDICINE

The NEW ENGLAND JOURNAL of MEDICINE

Clinical Chemistry 60:5
000-000 (2014)

Molecular Diagnostics and Genetics

Anal Bioanal Chem
DOI 10.1007/s00216-013-7546-1

RESEARCH PAPER

Evaluation of droplet digital PCR for characterizing plasmid

ORIGINAL ARTICLE

Use of Graft-Derived Cell-Free DNA as an Organ Integrity Biomarker to Reexamine Effective Tacrolimus Trough Concentrations After Liver Transplantation

Michael Oellerich, MD,* Ekkehard Schütz, MD,† Philipp Kanzow,* Jessica Schmitz,*‡ Julia Beck, PhD,† Otto Kollmar, MD,‡ Frank Streit, PhD,* and Philip D. Walson, MD*

Background: Immunosuppressant therapeutic ranges for transplant patients have traditionally been established by indirect clinical means. However, “liquid biopsy” methods measuring graft-derived cell-free DNA (GcfDNA) in blood directly interrogate donor organ integrity. This study was performed to determine whether GcfDNA quantification could be used to reexamine minimally effective trough tacrolimus

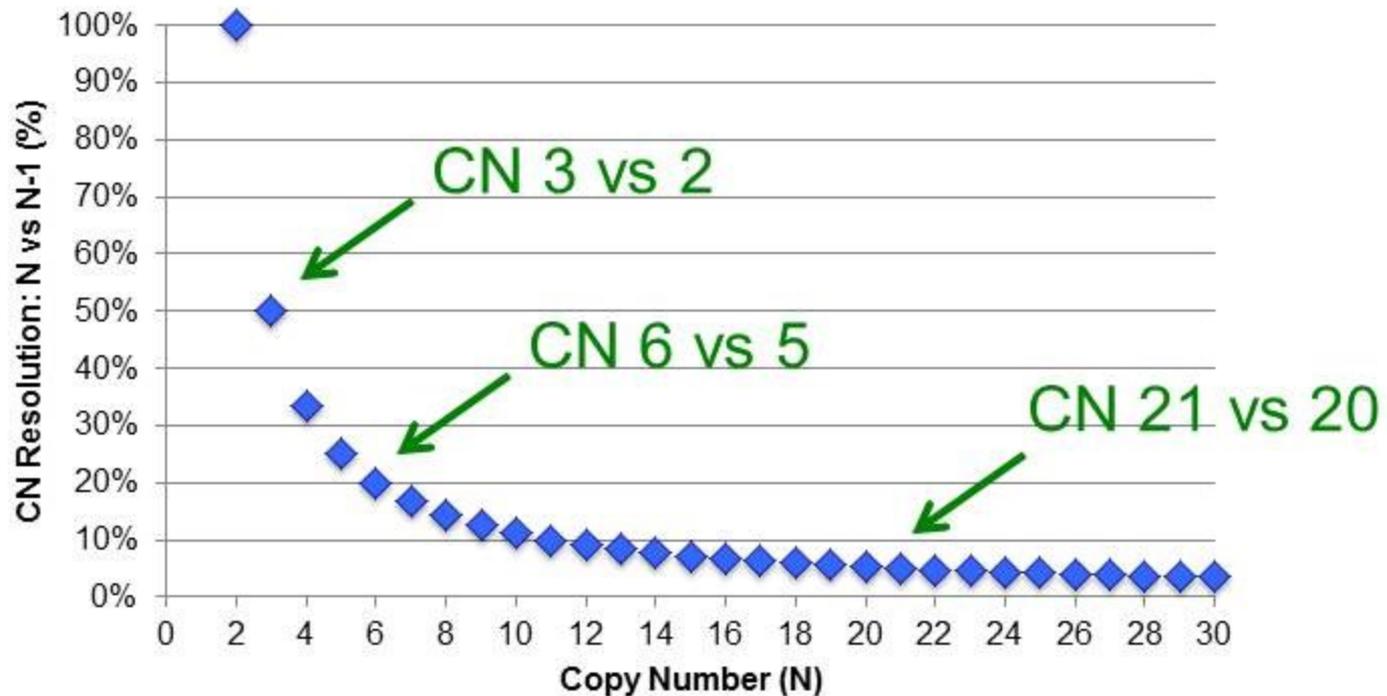
trough Tacro concentrations after LTx. It would probably be useful to do so also for other immunosuppressant drugs and after other solid organ transplants. The method might be especially useful to detect graft injury during immunosuppressant dose minimization strategies.

Key Words: graft-derived cell-free DNA, tacrolimus TDM, liver transplantation, graft injury

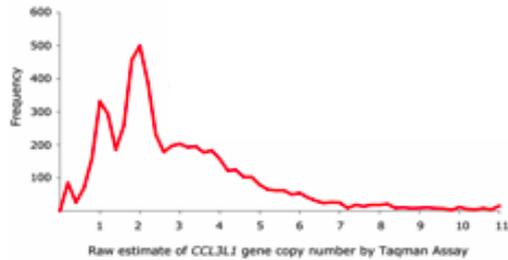
Copy Number Variation

Copy Number Variation: What is the challenge?

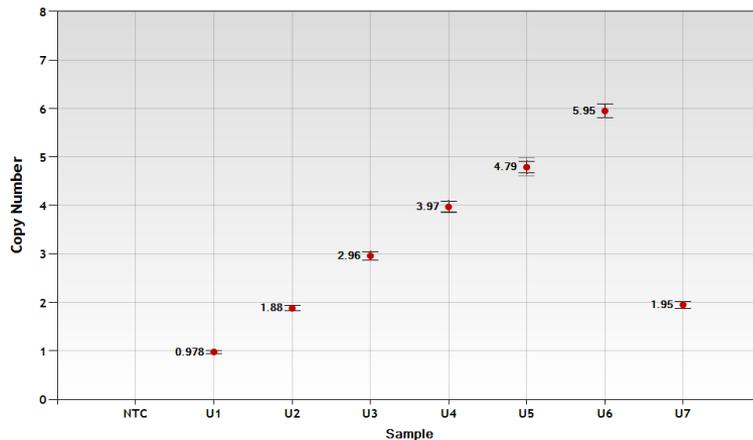
Homogeneous Samples: Discrimination between consecutive copy number states is more difficult at higher order copy number.



Challenge



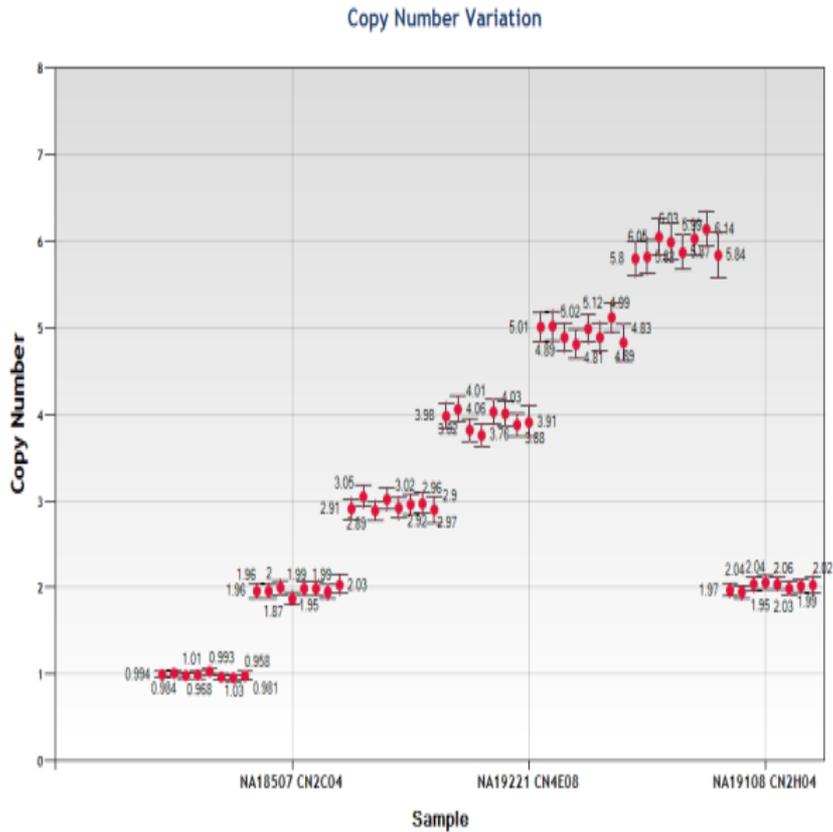
Gonzales et al., 2005.



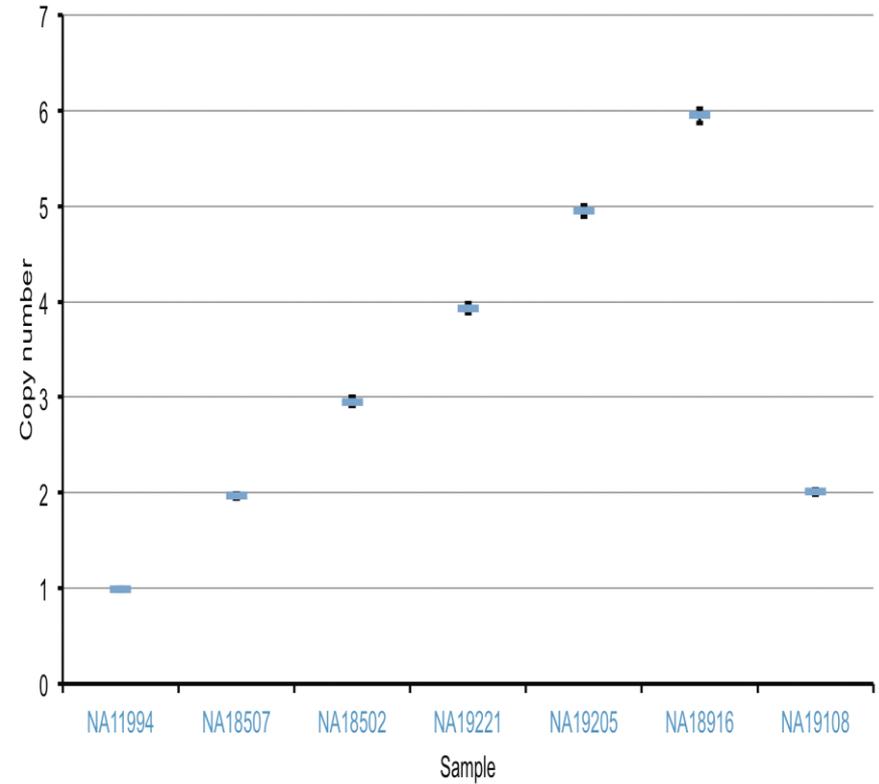
- Techniques such as qPCR can differentiate 1 from 2 from 3 copies with relative ease when using robust assays and reasonable amounts of template.
- Accurate quantification at higher levels (ex 5 from 6 copies) can prove difficult. The difference in $C(q)$ is small and these values need to be normalized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

Measuring copy number for MRGPRX1

ddPCR individual wells

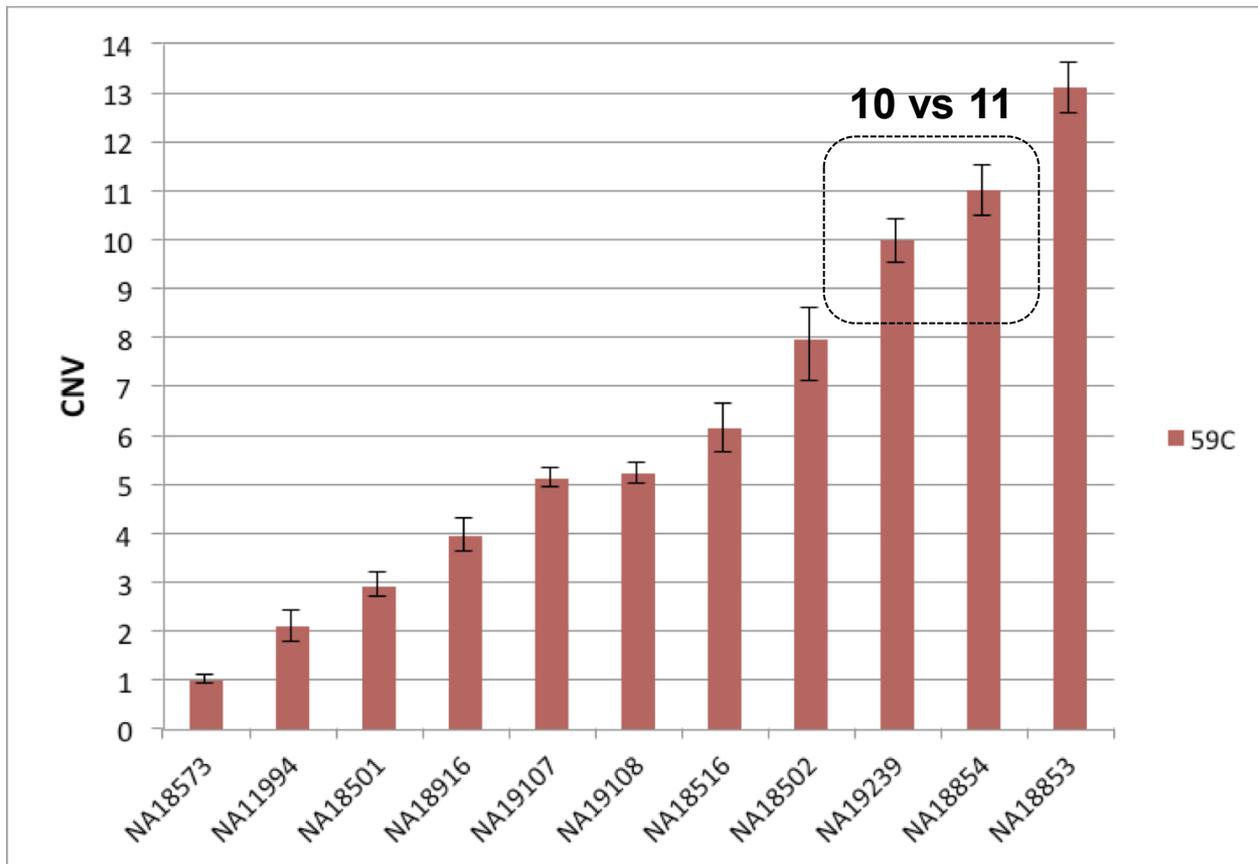


ddPCR merged wells



Higher CNV level discrimination

CCL3L1 Copy Number Analysis of 11 HapMap samples

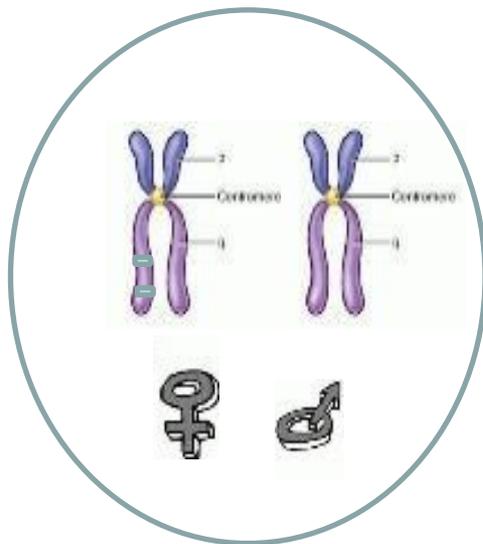


- Merged duplicate wells w/ 16.5ng DNA each – 95% CI's

Can we tell if gene copies are on different chromosomes?

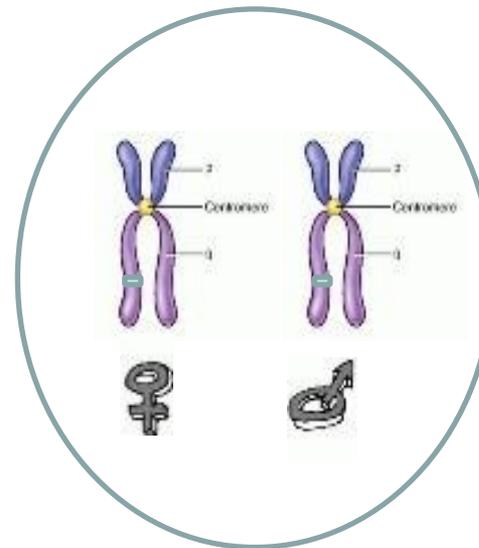
Problem: determine if a normal-seeming CNV=2 is a deletion carrier:

Deletion Carrier



VS

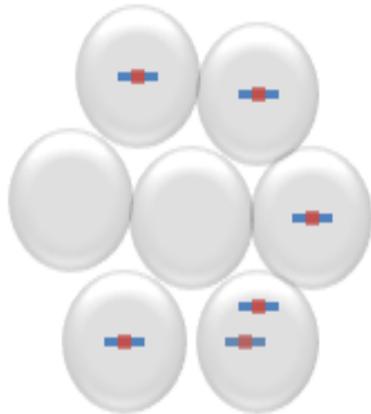
Normal



Approach: compare CNV estimates with and without restriction digestion.

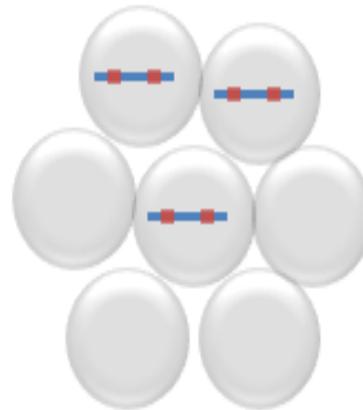
Can we tell if gene copies are on different chromosomes?

With restriction digestion



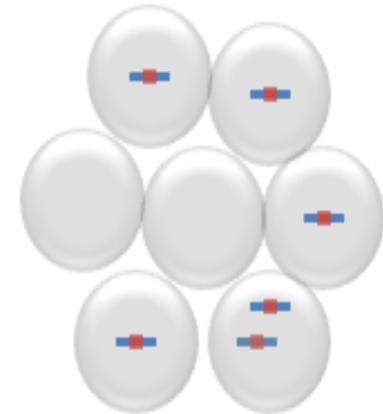
No restriction digestion

Two tandem copies:



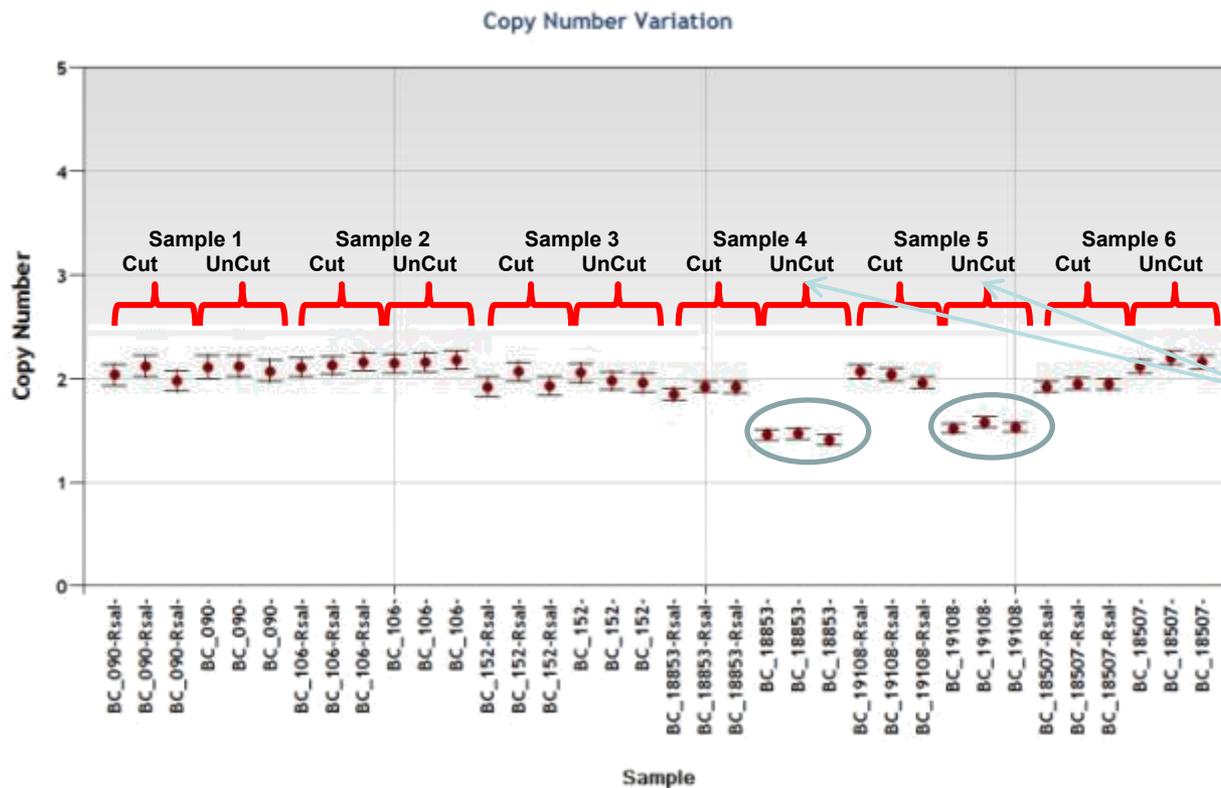
Expect lower CNV estimate

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

LETTERS

nature
genetics

Clinical Cancer Research

ACR

Stru
17q2
Non-ii
Digita
Linda M
Heidrun Ge
Clin Cance



Translational Medicine

Nadav et al. Trans Med 2017, 7:7
<http://dx.doi.org/10.4172/2151-1025.1000007>

Research Article

Open Access

Quantitative and Sensitive Detection of Cancer Genome Amplifications from Formalin-Fixed Paraffin-Embedded Tissue by Droplet Digital PCR

Lincoln Nadav
Hanlee P Ji^{1,4*}

LETTER

doi:10.1038/nature11629

¹Division of Oncology
²Bio-Rad, Inc., Pleasanton, CA, USA
³Department of Pathology
⁴Stanford Genome Center
^{*}These authors contributed equally and significantly to the work

Somatic mutations reveal

Alexej Abyzov¹
Anthony F. Fernandez¹
Nathaniel E. Calafatis¹
Alexander Eckert¹

METHOD

Open Access

Killer-cell Immunoglobulin-like Receptor gene linkage and copy number variation analysis by droplet digital PCR

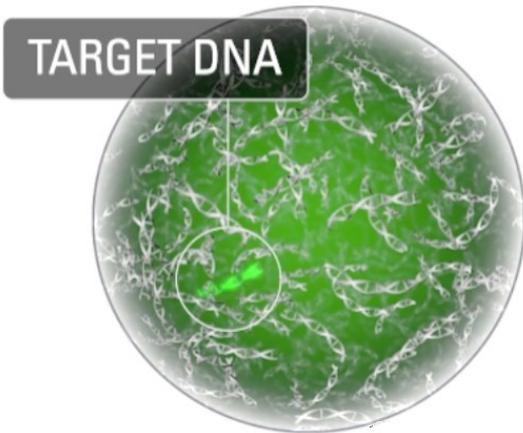
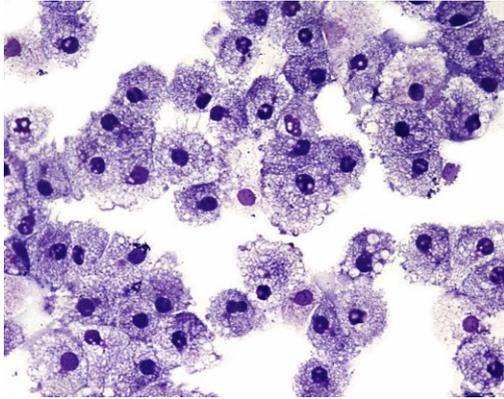
Chrissy Roberts^{1*}, Wei Jiang^{2,3}, Jyothi Jayaraman^{2,3}, John Trowsdale^{2,3}, Martin J Holland¹ and James A Traherne^{2,3}

Abstract

The Killer-cell Immunoglobulin-like Receptor (KIR) gene complex has considerable biomedical importance. Patterns of polymorphism in the KIR region include variability in the gene content of haplotypes and diverse structural arrangements. Droplet digital PCR (ddPCR) was used to identify different haplotype motifs and to enumerate KIR copy number variants (CNVs). ddPCR detected a variety of KIR haplotype configurations in DNA from well-characterized cell lines. Mendelian segregation of ddPCR-estimated *KIR2DL5* CNVs was observed in Gambian families and CNV typing of other KIRs was shown to be accurate when compared to an established quantitative PCR method.

Rare Mutation Detection

Rare mutation detection challenge

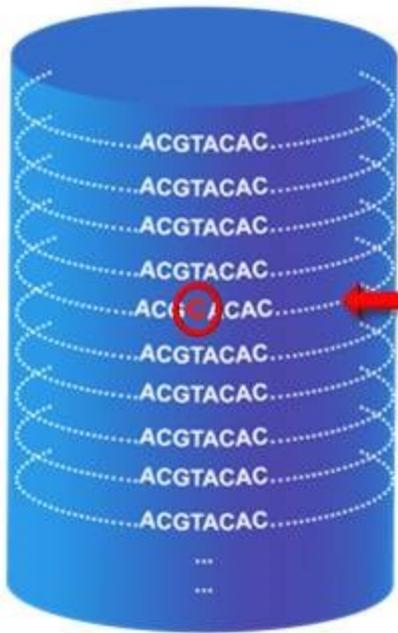


- Growing set of somatic mutations are of key importance for diagnostics, prognostics, and therapeutics
 - Kinases (serine, tyrosine)
 - Phosphatases
- Biggest application is in clinical diagnostics
 - Body fluids
 - Whole blood, serum, plasma, urine
 - Peripheral Blood Mononuclear Cells (PBMCs)
 - Biopsies 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

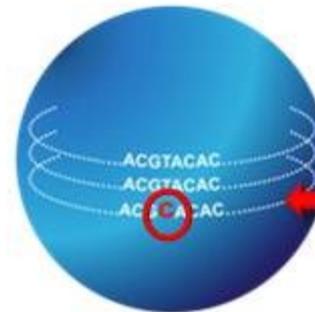
Bulk Sample – 20 μ L

40,000 wildtype molecules
40 mutant molecules



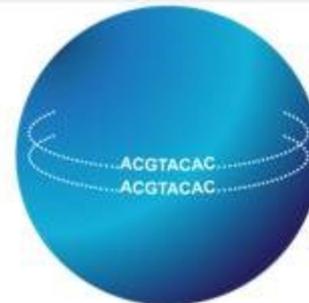
Mutant abundance 0.1%

Partitioned Sample – 20,000 \times 1nL



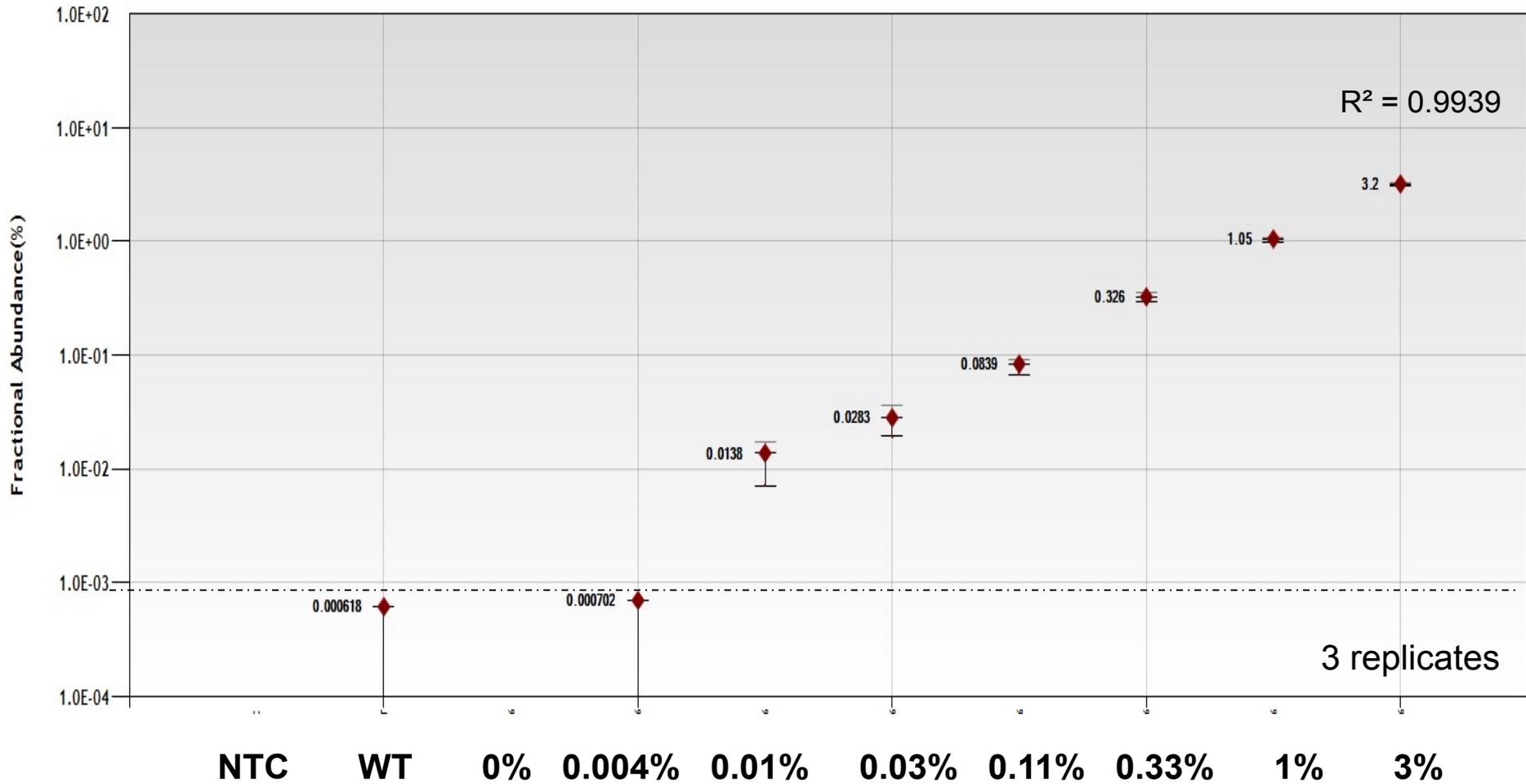
40 droplets w/ mutant

Mutant abundance 33%



19,960 droplets w/o mutant

KRAS(G12A) fractional abundance (% Mutant)



Examples in the literature

Resource

Cell

Personal
Review
and

Rui Chen,¹
Elana Miri,
Hogune In,
Sara Hillen,
Maya Kas,
Maria A. B,
Mark Gers



Translational Medicine

Nadauld et al., Transl Med 2012, 2:2
<http://dx.doi.org/10.4172/2161-1025.1000107>

Research

Quantitative
from

Lincoln Na
Hanlee P J

¹Division of C
²Bio-Rad, Inc
³Department
⁴Stanford Ge
⁵These auth

Methods 59 (2013) 183–186



ELSEVIER

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth



Review Article

Droplet Di

Nicholas J. H
Angela M. C
Alexandra S.

^aDigital Biology Cente
^bUniversity of Mississ

OPEN ACCESS Freely available online

PLOS ONE

Determination of *HER2* Amplification Status on Tumour DNA by Digital PCR

Isaac Garcia
¹The Breakthrough
Kingdom

Abstract

Determinat
instability a
assessment
digital PCR
ratio had h
98% (57/58

Citation: Garcia
doi:10.1371/jour

Editor: Baochua

Received June

CANCER DISCOVERY

ACR

Efficacy of intermittent combined RAF and MEK inhibition in a patient with concurrent BRAF and NRAS mutant malignancies

Omar Abdel-Wahab, Virginia M Klimek, Alisa Gaskell, et al.

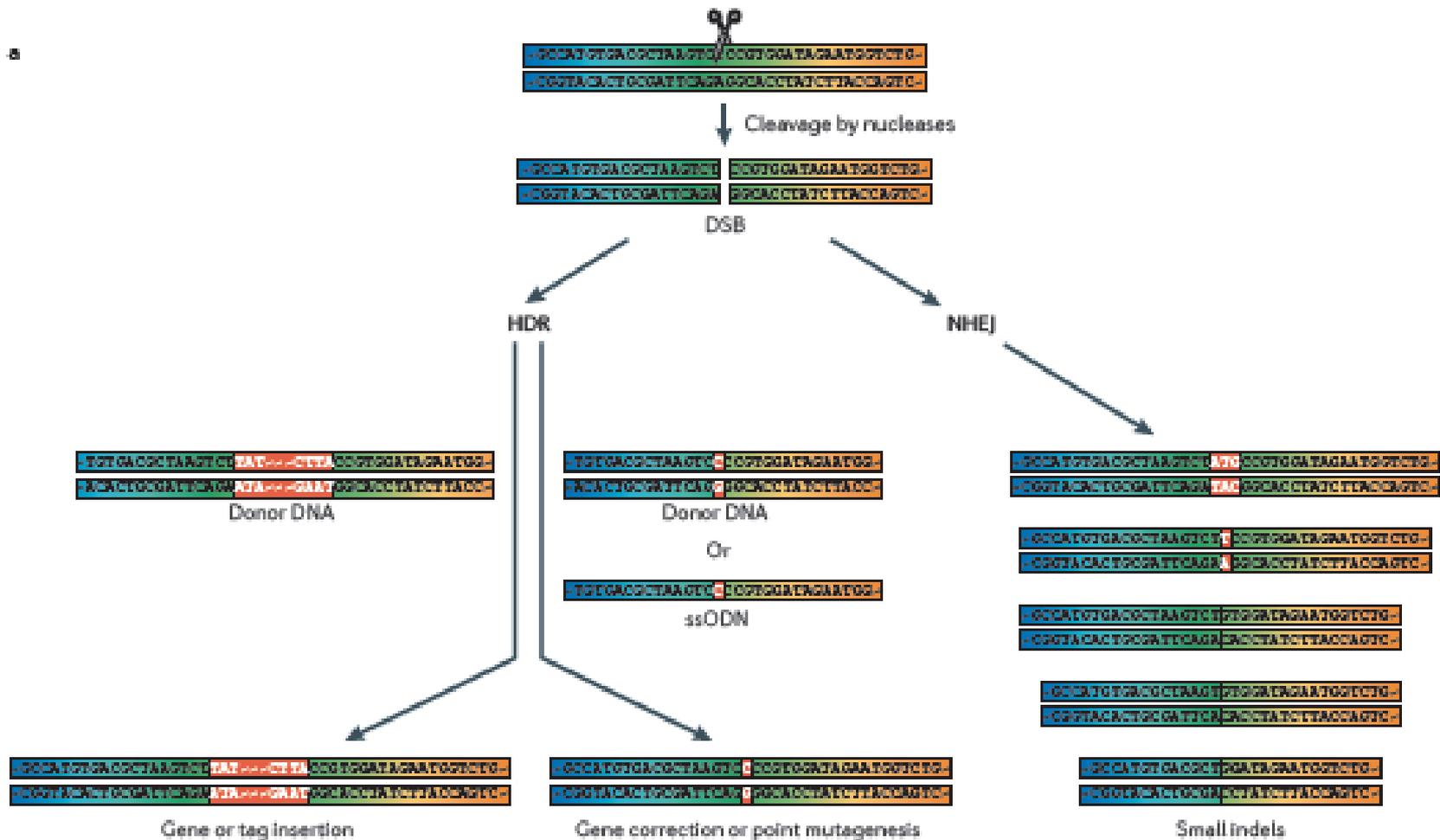
Cancer Discovery Published OnlineFirst March 3, 2014.

Updated version

Access the most recent version of this article at:
doi:[10.1158/2159-8290.CD-13-1038](https://doi.org/10.1158/2159-8290.CD-13-1038)

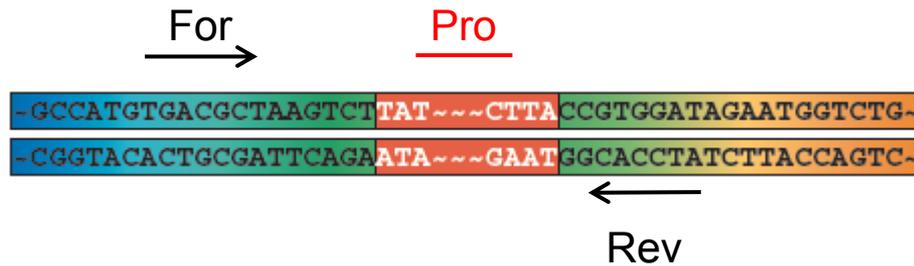
Genome Editing Experiments

Pathways for genome editing

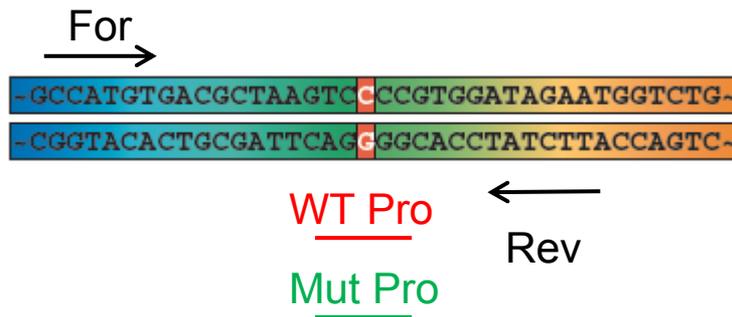


Quantification of genome editing events by ddPCR (1)

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

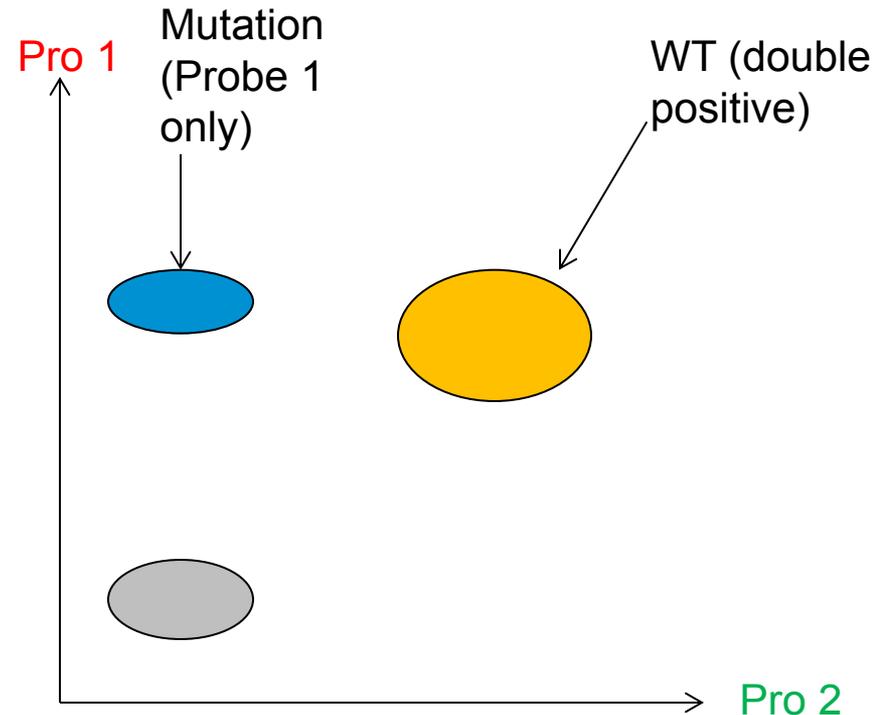
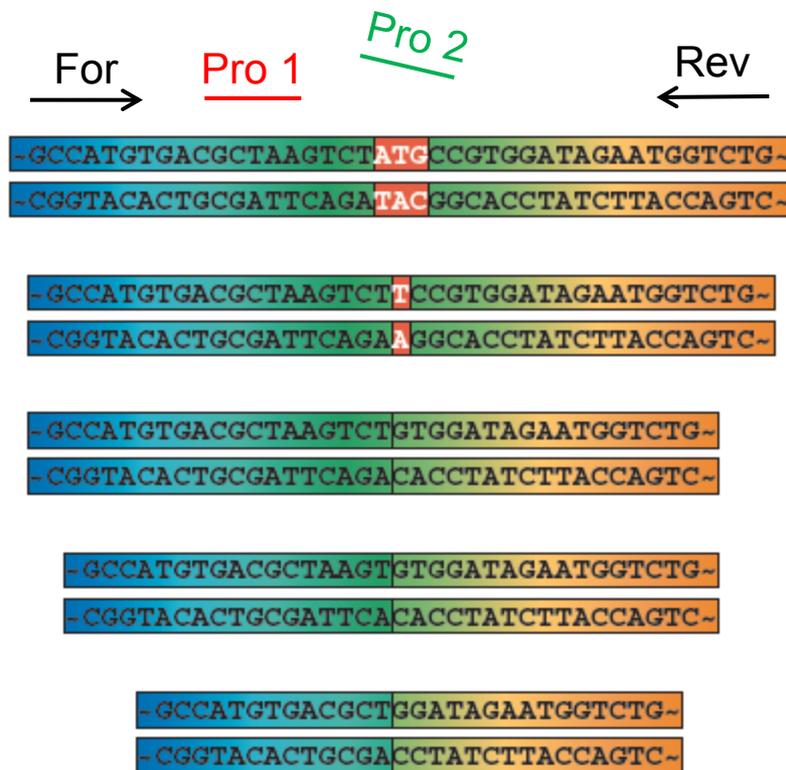


- Gene correction or point mutagenesis: rare mutation detection



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

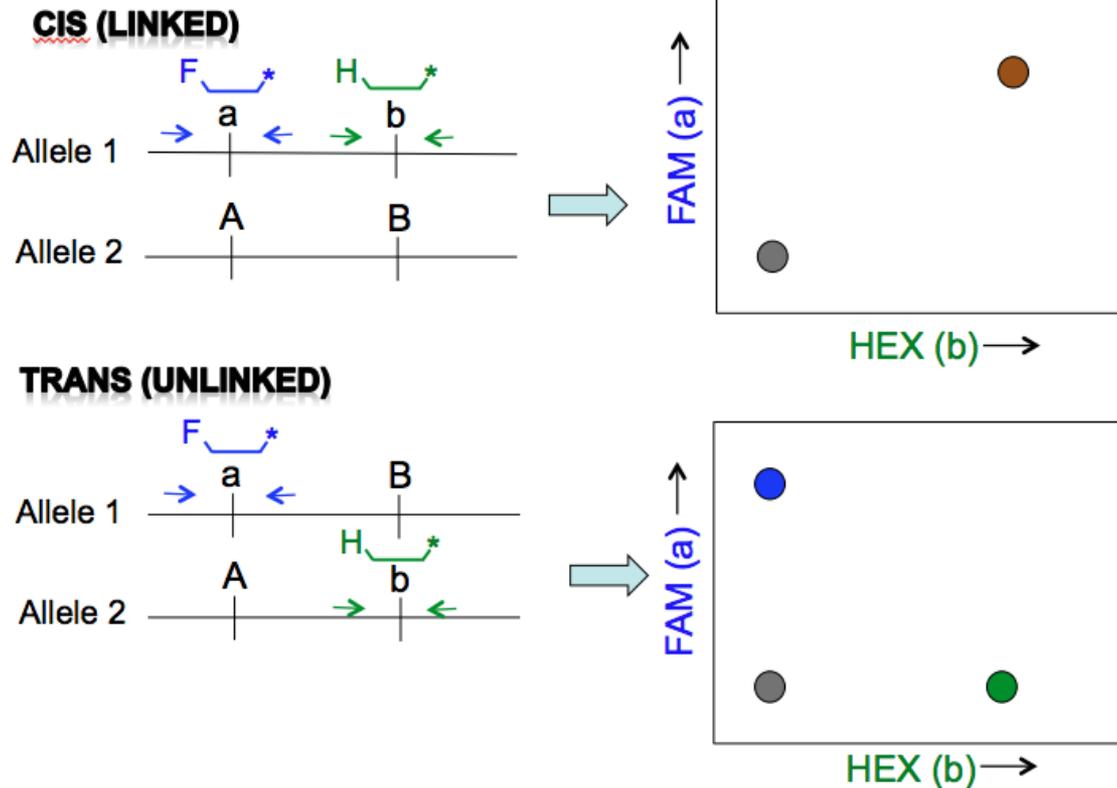
versus



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)



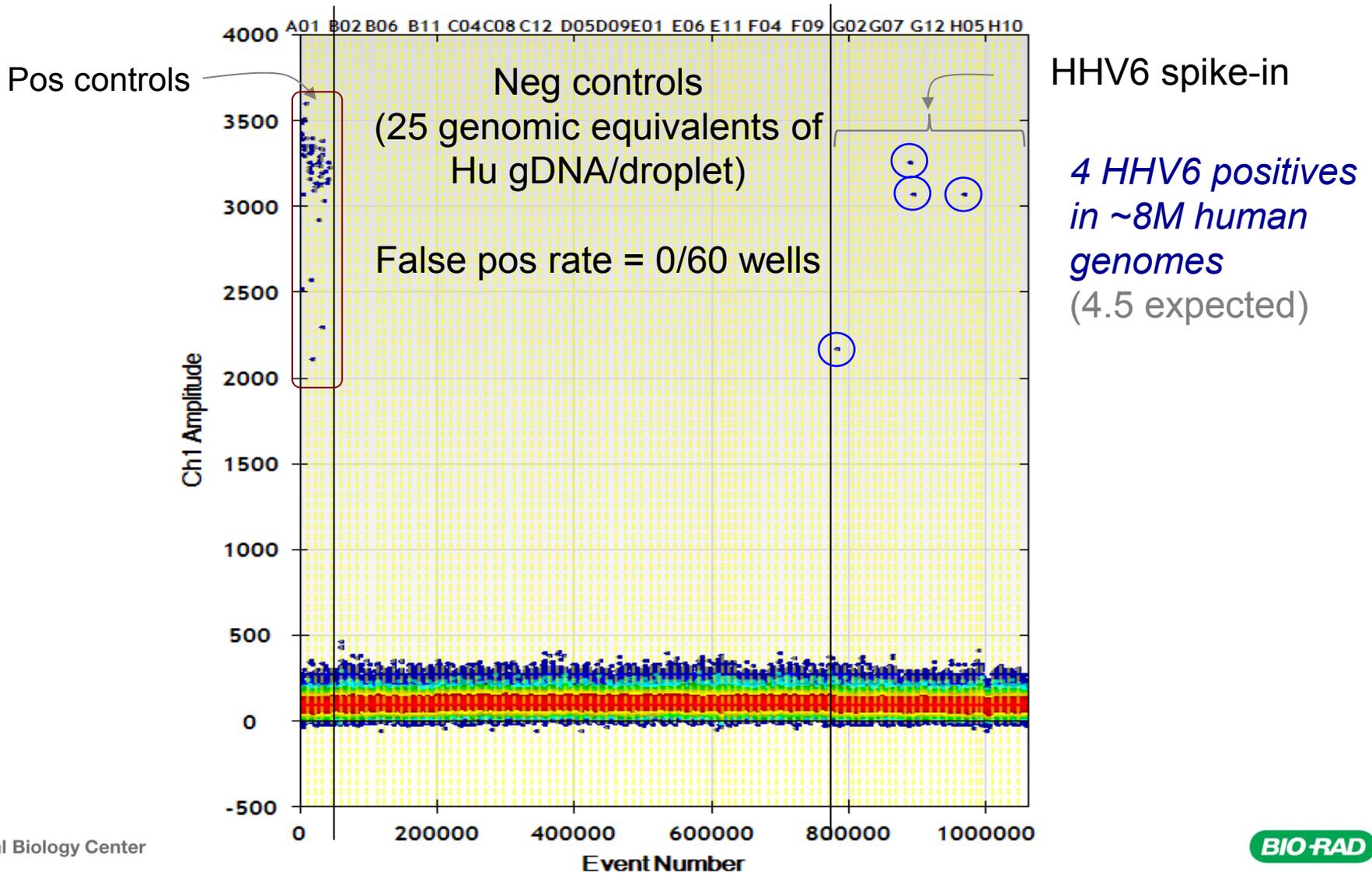
Diseases influenced by compound heterozygosity in single genes

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

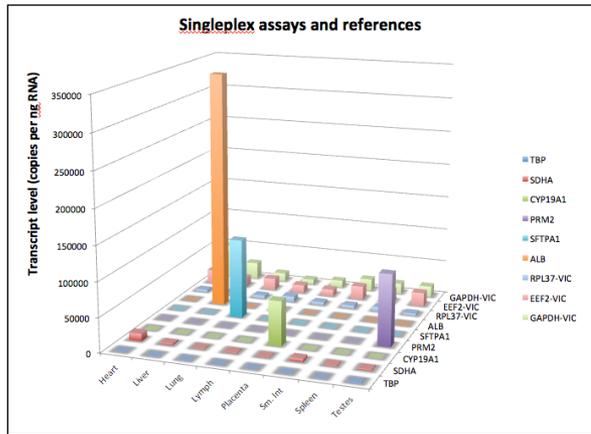
Additional Applications

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

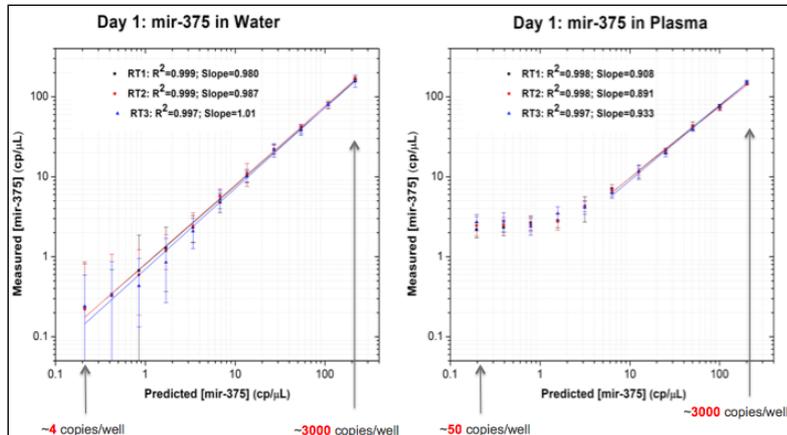


Gene expression applications

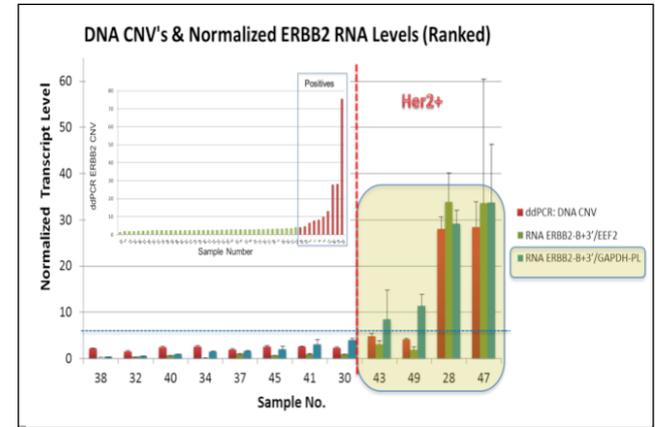
Tissue-specific Gene Expression



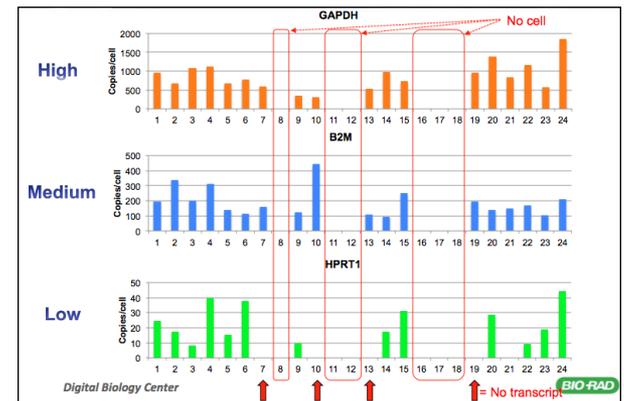
miRNA's in plasma



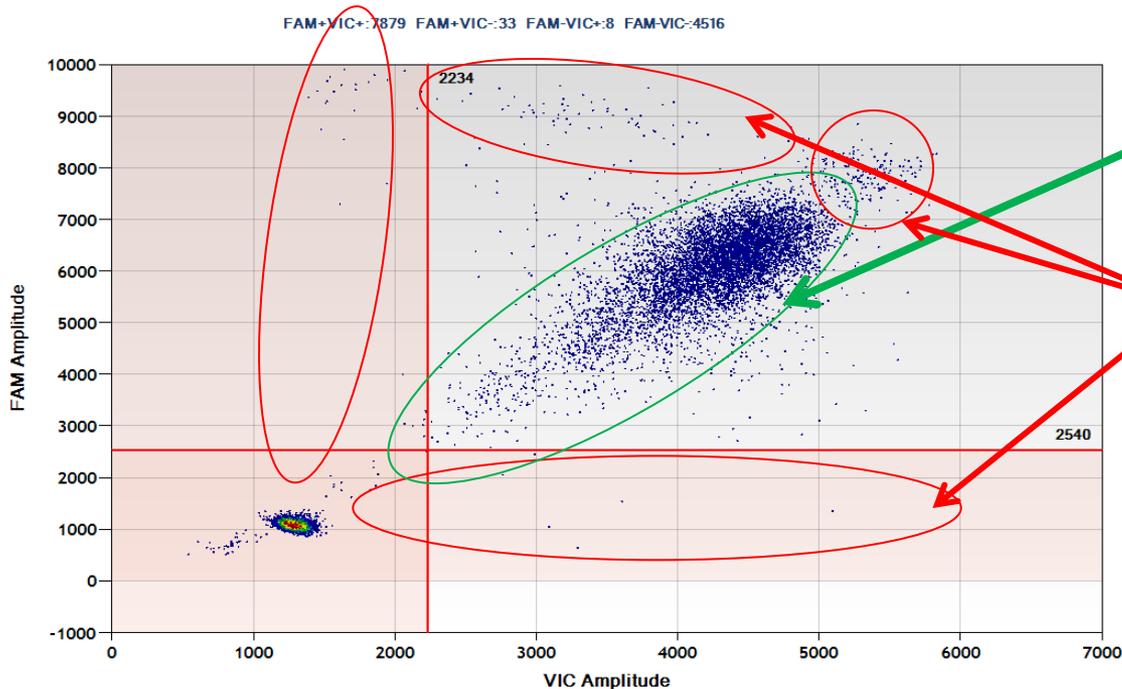
Her2 mRNA in FFPE samples



Single-cell transcript detection



NGS insert size, quantity, and quality



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

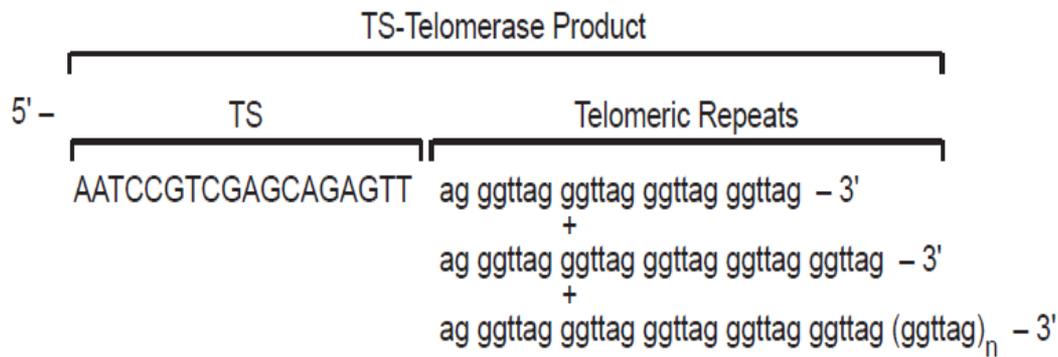
Ill-formed fragments or
adaptor-adaptor, **cannot
make clusters**

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)

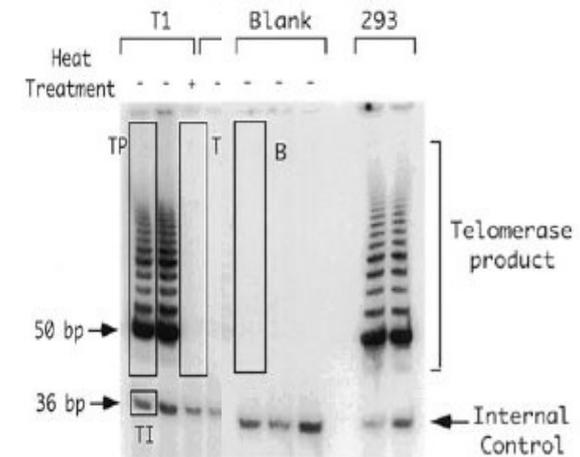
Make cDNA

STEP 1. Addition of Telomeric Repeats By Telomerase



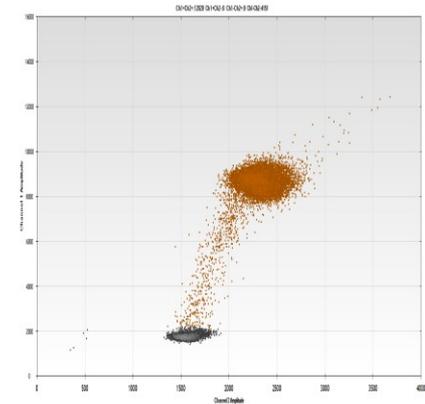
Perform PCR

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

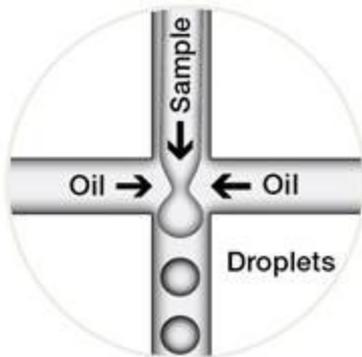
Additional applications

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

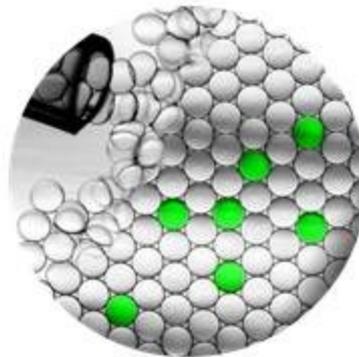
What is the work involved?

Droplet digital PCR workflow

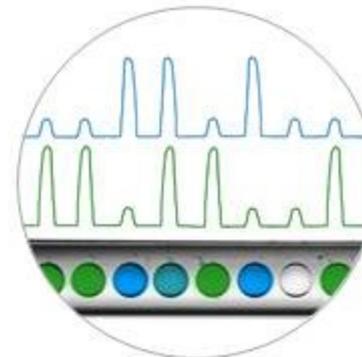
- Partition reagents and sample into 20,000 droplets
- Perform PCR on thermal cycler
- Count droplets with a positive PCR product (fluorescent) and a negative PCR product
- Digital readout provides concentration of target DNA



Make Droplets



PCR Droplets

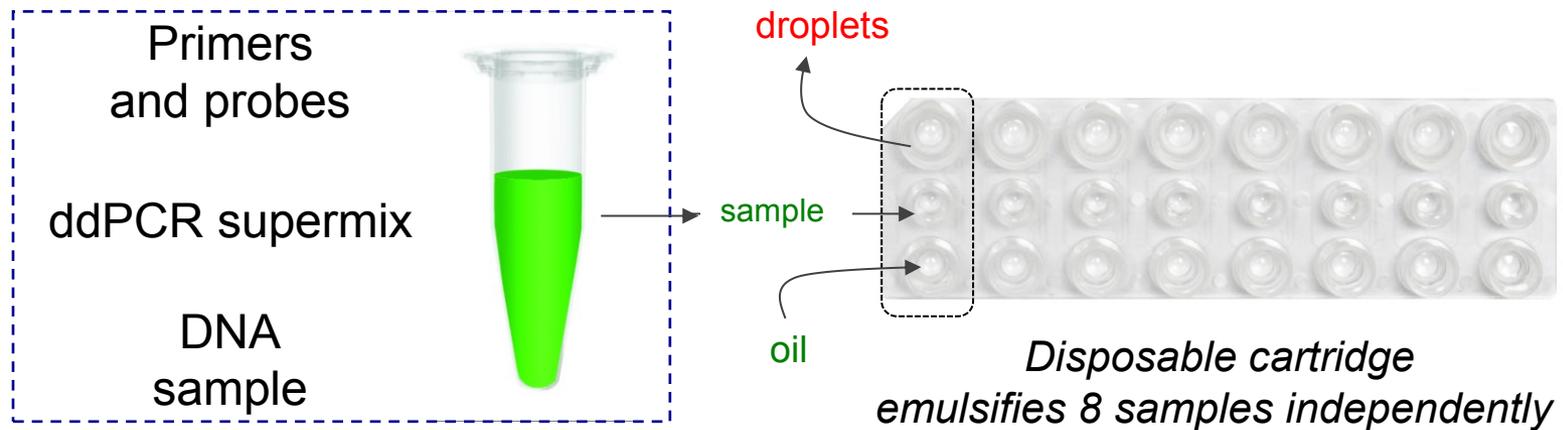
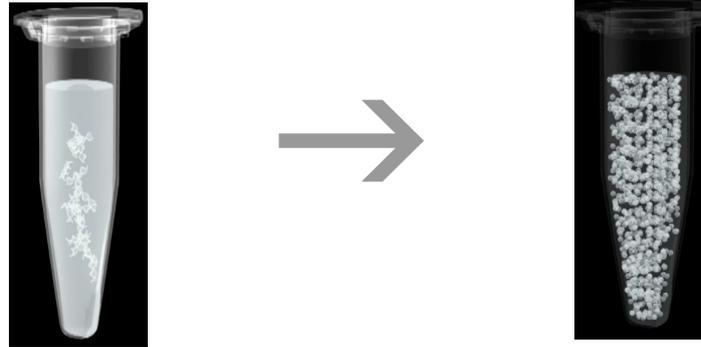


Read Droplets

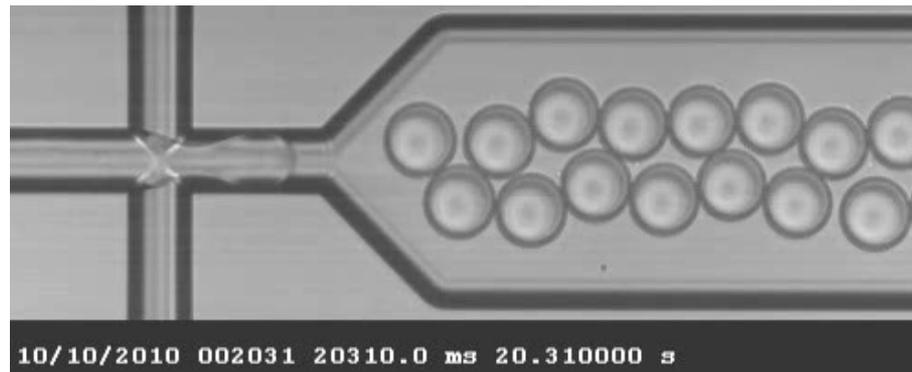


Results

Preparing sample and reagent mixture



Generating droplets

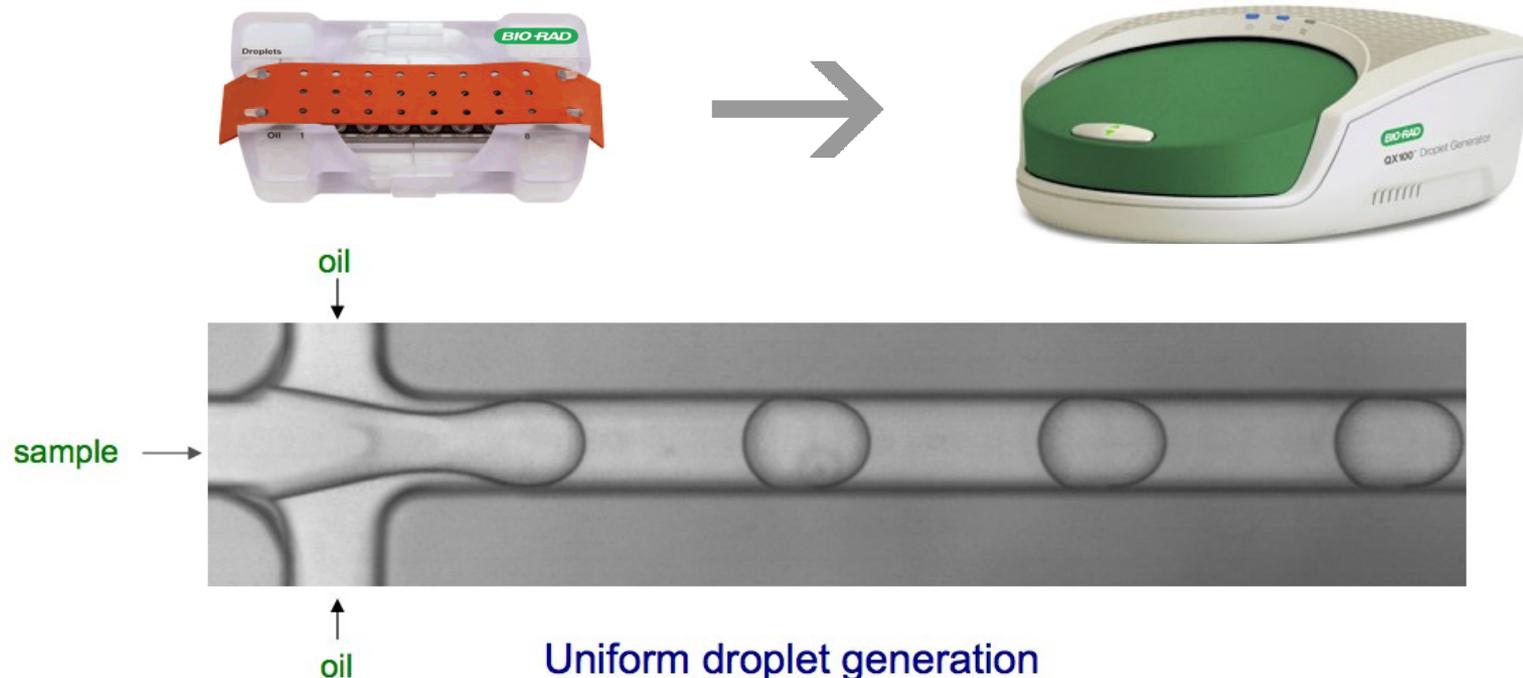


Amplifying droplets



Making droplets

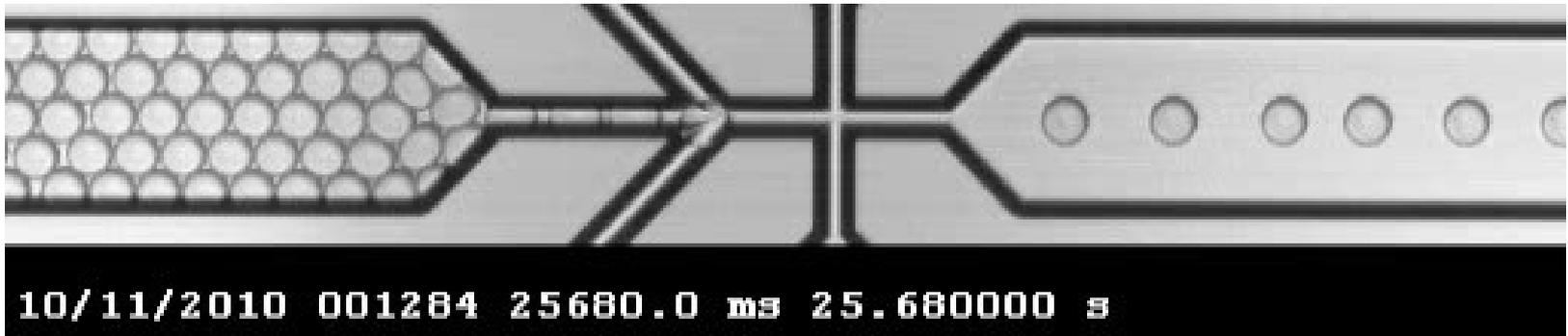
- Place loaded cartridge into QX200 Droplet Generator
- Generate 20,000 droplets per sample, 2 ½ min for 8 samples



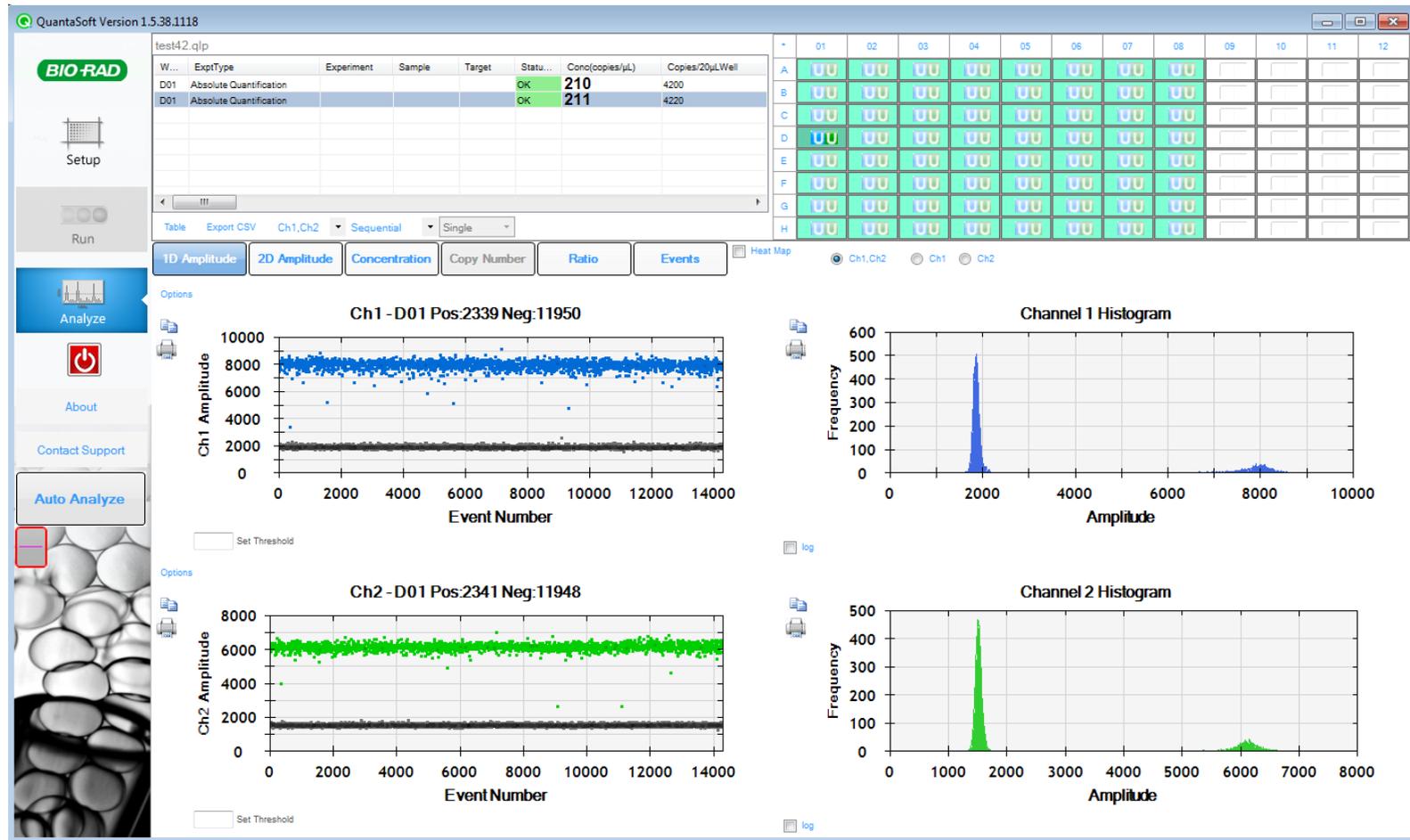
Reading droplets



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



- Total process time from PCR reaction plate to results – approx 5 hours (96 wells).
- Total hands on time – less than 5 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.

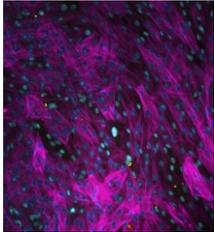
- Technological advantages
 - Absolute Quantitation independent of an external reference
 - High resolution quantitative and detection
 - Affordable cost per result with minimal labor.
- Common Applications
 - Nucleic acid quantitation and detection (viral, pathogen, GMO, etc..)
 - Copy Number Variation 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