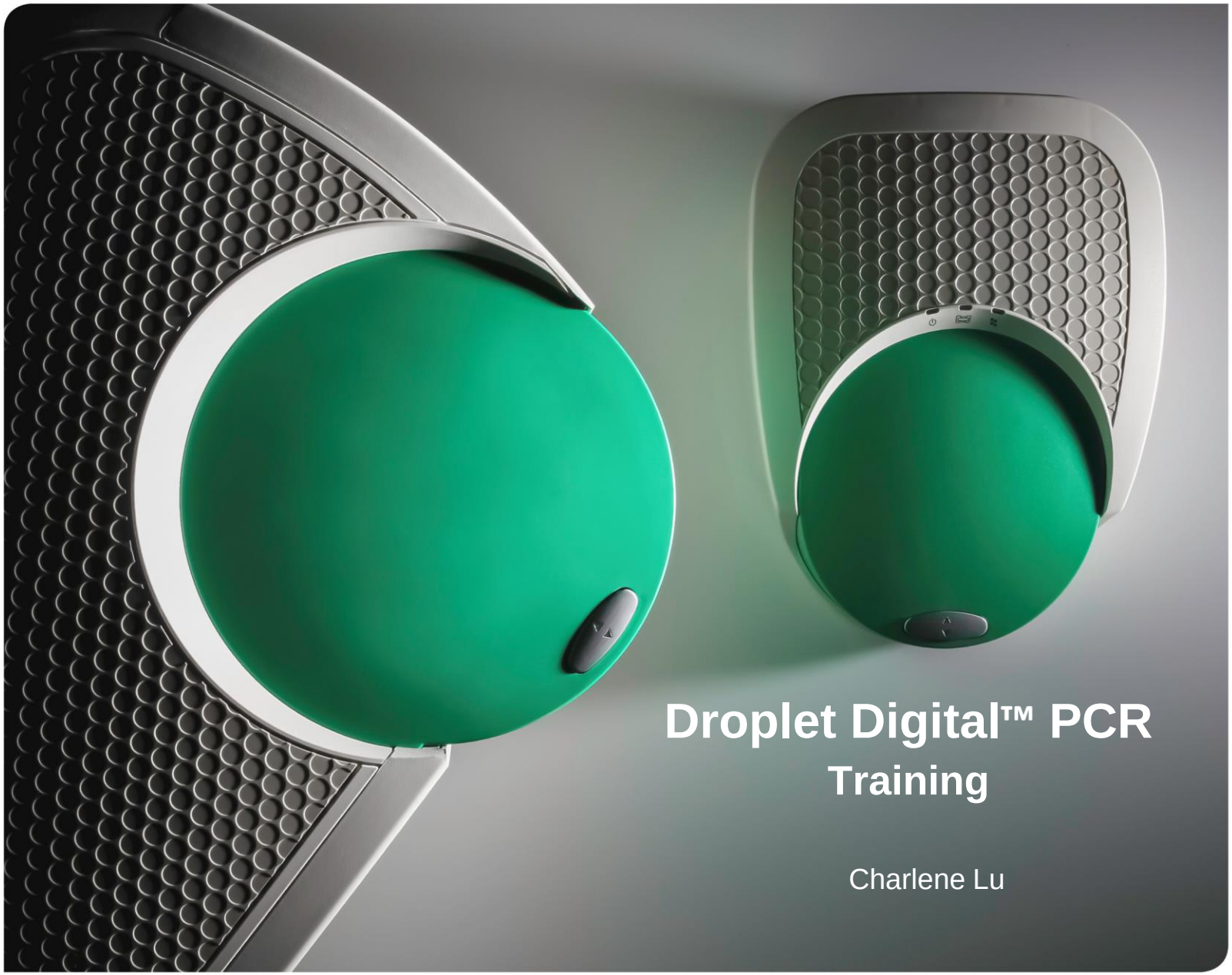


Droplet Digital™ PCR
Training

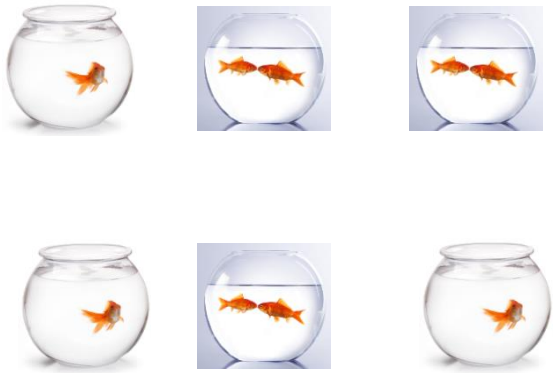
Charlene Lu

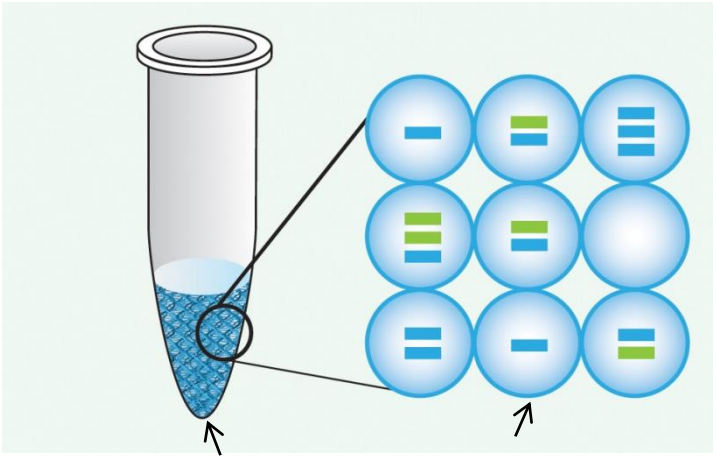
Outline

- What is digital PCR?
- What's Bio-Rad digital PCR?
- How is Droplet Digital PCR (ddPCR™) performed on the QX200™ Droplet Digital PCR systems
- Applications updated based upon QX100/QX200 Published data
  - Copy Number Variation
  - Rare Event Mutation detection
  - Gene Expression
- Summary

What is Digital PCR?

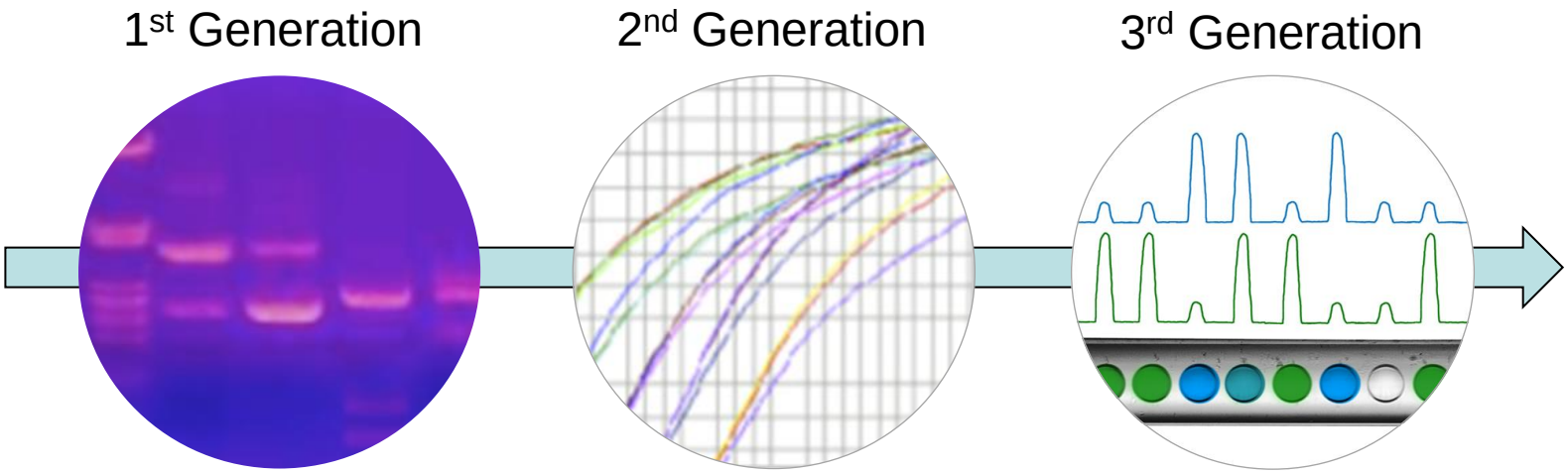
PCR reaction that is partitioned.





Many thousands
of discrete measurements

Generations of PCR



Gel Electrophoresis (qualitative)

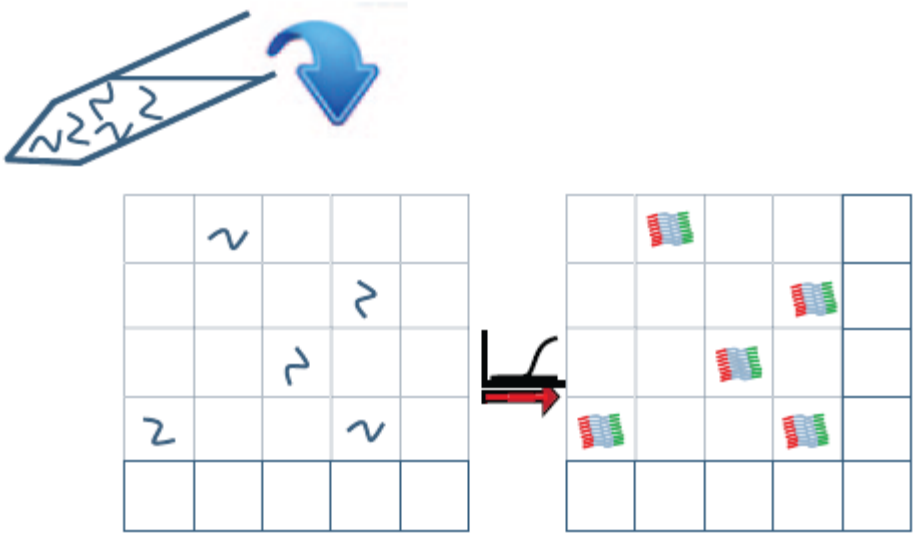
Real-Time PCR (indirect quantification)

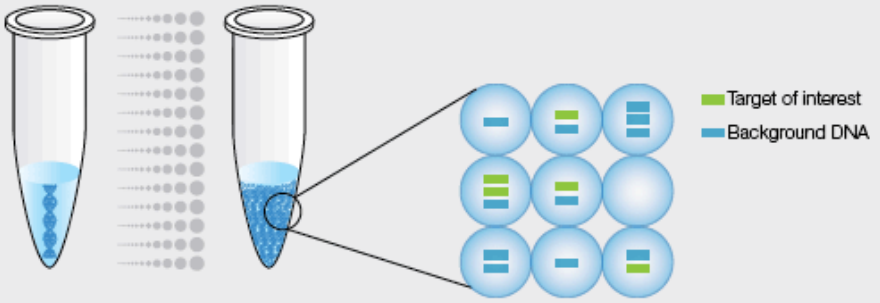
Droplet Digital PCR (absolute quantification)

- End point (0's or 1's)
- Less sensitive to PCR efficiency
- No standard curve
- More tolerant to PCR inhibitors

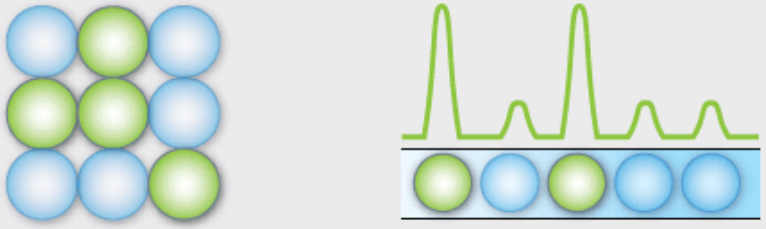
ddPCR improves precision, sensitivity and reproducibility

dPCR Principle





The sample is partitioned into 20,000 droplets, with target and background DNA randomly distributed among the droplets.



After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target DNA was present or not present after partitioning. Each droplet provides an independent digital measurement.

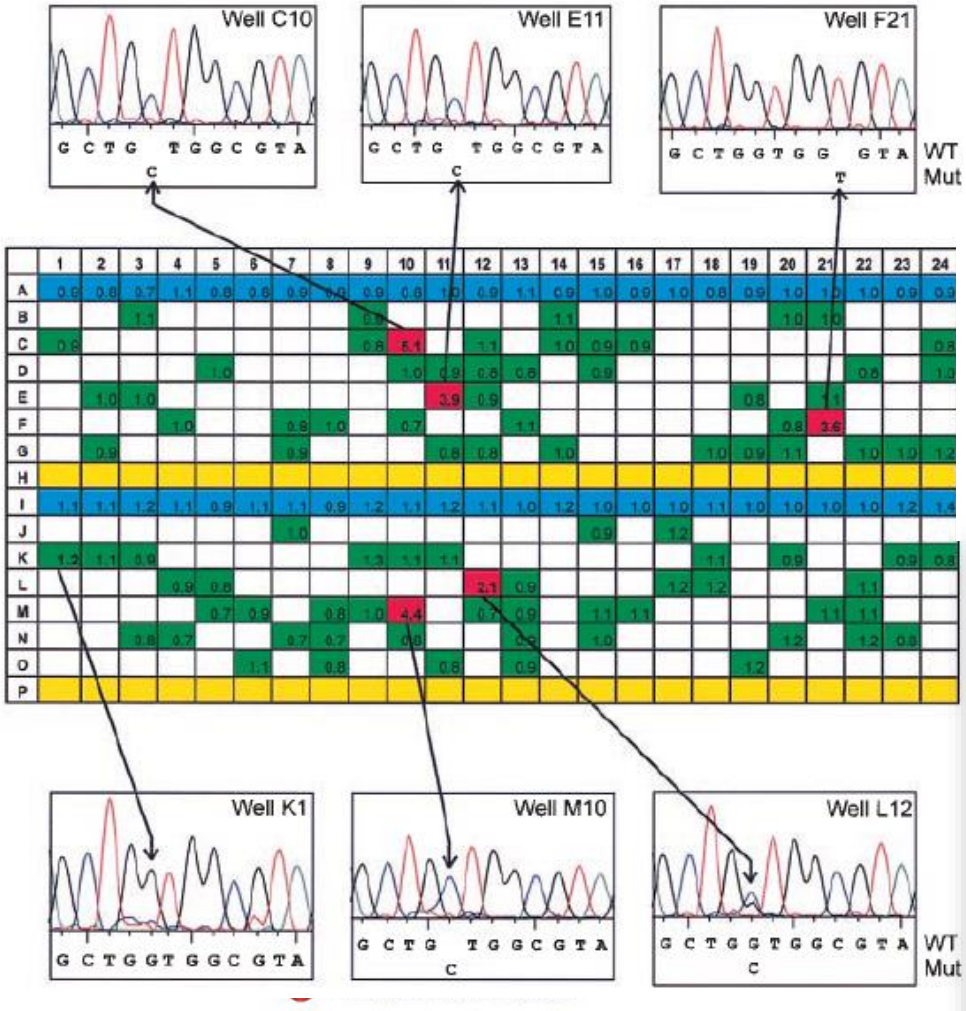
"X" target copies

Positive and negative droplets are counted for the sample and the software calculates the concentration of target DNA as copies per microliter.

- End point (0's or 1's)
- Less sensitive to PCR efficiency
- No standard curve
- More tolerant to PCR inhibitors

ddPCR improves precision, sensitivity and reproducibility

The 1st Paper about dPCR



- Mutant allele (KRAS)
- Wild type allele
- No amplification
- Positive control
- Negative control

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9236–9241, August 1999
Genetics

Digital PCR

BERT VOGELSTEIN* AND KENNETH W. KINZLER

The Howard Hughes Medical Institute and the Johns Hopkins Oncology Center, Baltimore, MD 21231

Contributed by Bert Vogelstein, June 9, 1999

Bert Vogelstein (born 1949) is a Howard Hughes Medical Institute investigator[1] at The Johns Hopkins University. He clarified the role of the gene p53, which repairs DNA in dividing cells and destroys the cell if its DNA cannot be repaired. Damaged p53 is responsible for half of all cancers. More recently, his group sequenced the DNA of human breast and colon cancer, identifying genes which are mutated in each cancer.

Vogelstein developed the concept that some genes, such as TP53, KRAS, and APC are involved in cancer with great frequency, in close to 100% of some cancers; he called these genes "mountains." But thousands of genes are involved in cancer but are found at very low frequency, under 5%; he called these genes "hills." Collectively, however, the hills are also required for most cancers.

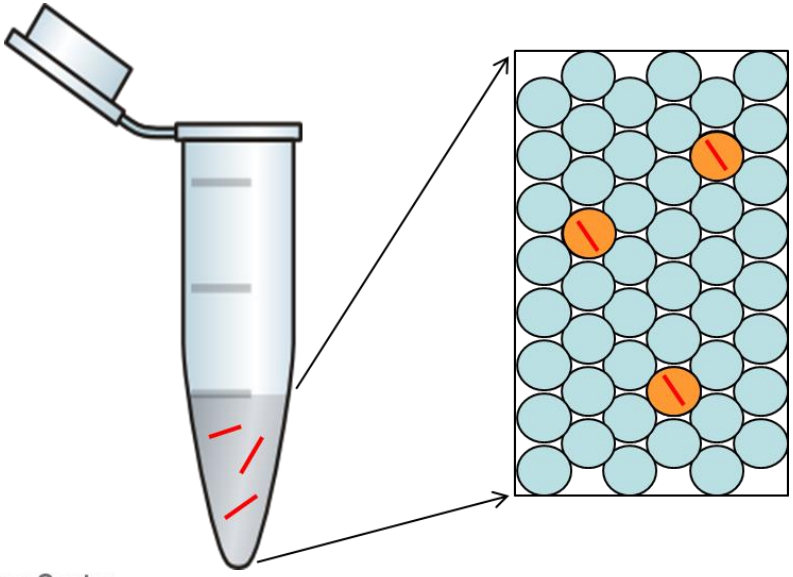
He found that while the number and complexity of these thousands of genes might be bewildering, most of them can be grouped into twelve critical pathways, such as apoptosis, DNA damage control, invasion, cell cycle signaling, KRAS signaling, and TGF-beta signaling.

How does Partitioning Enable Molecular Counting? One Partition — One Reaction, One Data Point

One measurement

Nanodroplet PCR reactions are independent, single amplification events

Many thousands of discrete measurements



- Every partition is an individual reaction vessel
- End-point PCR
- If a partition has a target molecule it will be read as a positive
- If a partition has no target molecules it will be read as a negative
- In quantitative PCR (qPCR) — time course, quantification cycle (Cq) or threshold cycle (C_T), standard curves

Droplet Digital PCR Work Flow

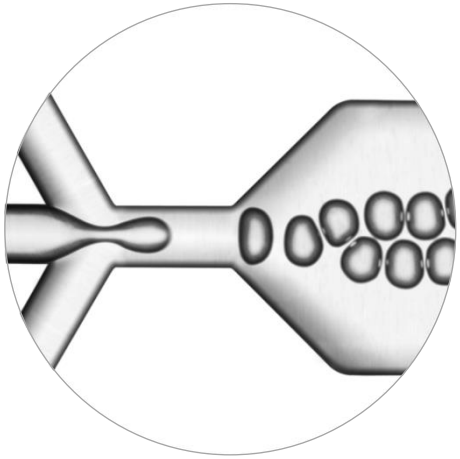
Workflow of Droplet Digital PCR

Primers and optional probes

ddPCR supermix

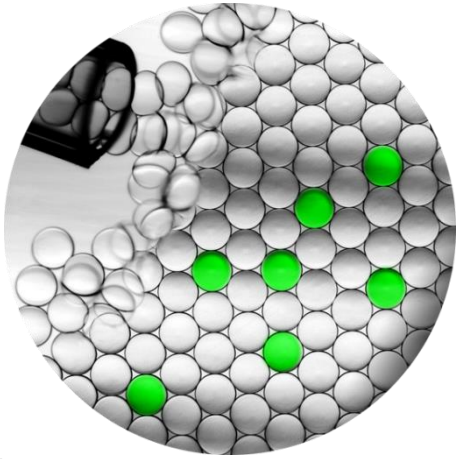
sample

1. Make Droplets



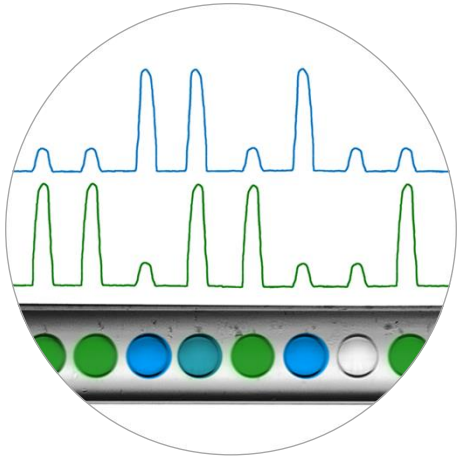
Droplet Generator

2. Cycle Droplets

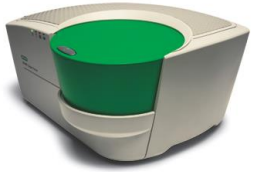


Bulk PCR Thermal Cycler

3. Read Droplets



Droplet Reader

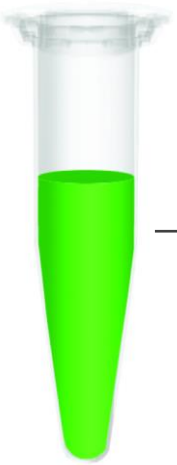


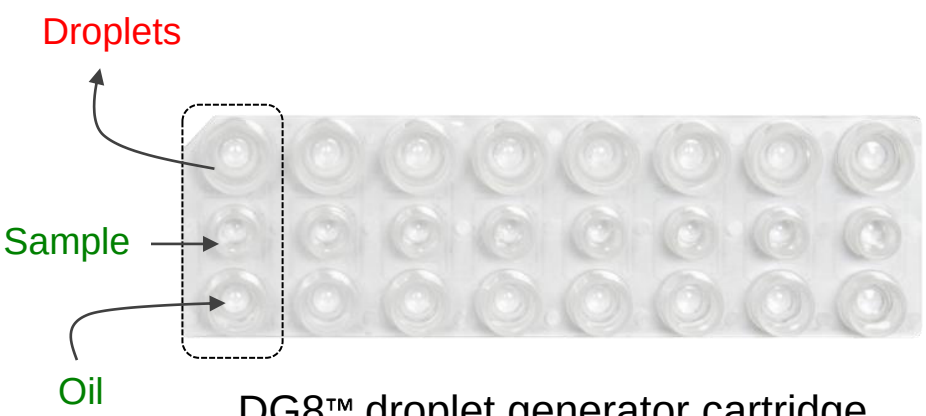
- Readout: copies/µl
- Dynamic range: 1–100,000 copies/well (~330 ng human genomic DNA)

Prepare Sample and Reagent Mixture

Prepare samples exactly the same as qPCR or PCR

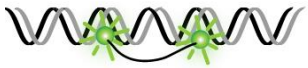
Primers
and probes
+
Bio-Rad ddPCR
supermix
+
DNA or RNA
sample

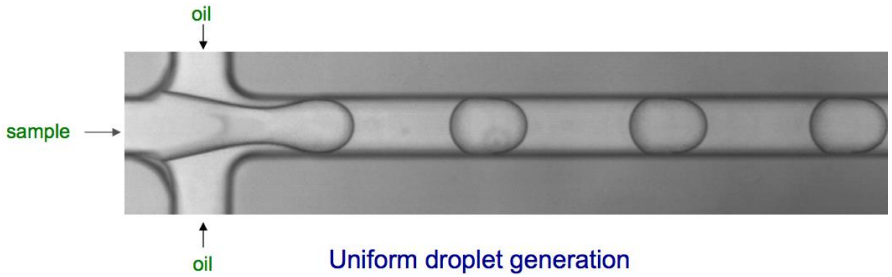




DG8™ droplet generator cartridge
emulsifies eight samples independently

Compatible with probes
(FAM and HEX/VIC) or EvaGreen





Uniform droplet generation

- Bio-Rad ddPCR supermix specific for the droplet chemistry

1. Insert DG8 cartridge and Add Sample

Inserting the DG8 cartridge into the cartridge holder.

- ✓ All 8 sample wells must contain sample or 1x control buffer and 8 oil well s must contain droplet generation oil.
- ✓ Add 20ul sample before add oil
- ✓ Avoid bubbles

Transfering sample to the sample wells (middle row) of the DG8 cartridge. Hold the pipet tip at a 15° angle and at the bottom of the well (middle and right panels); do not dispense sample onto the wall or side of the well.

BIO-RAD

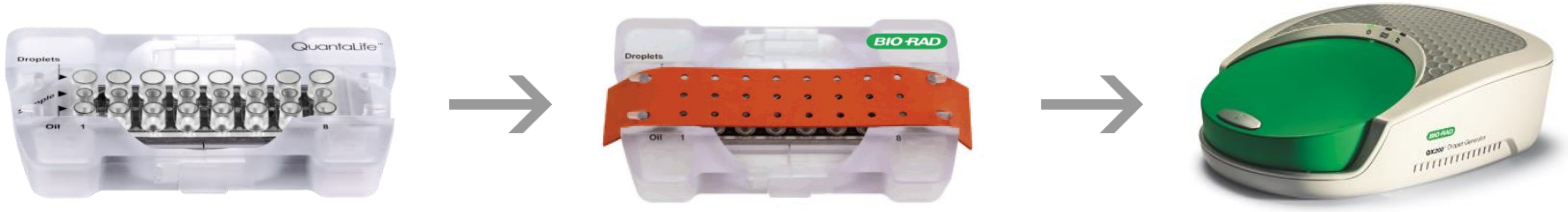
2. Add oil and place the gasket

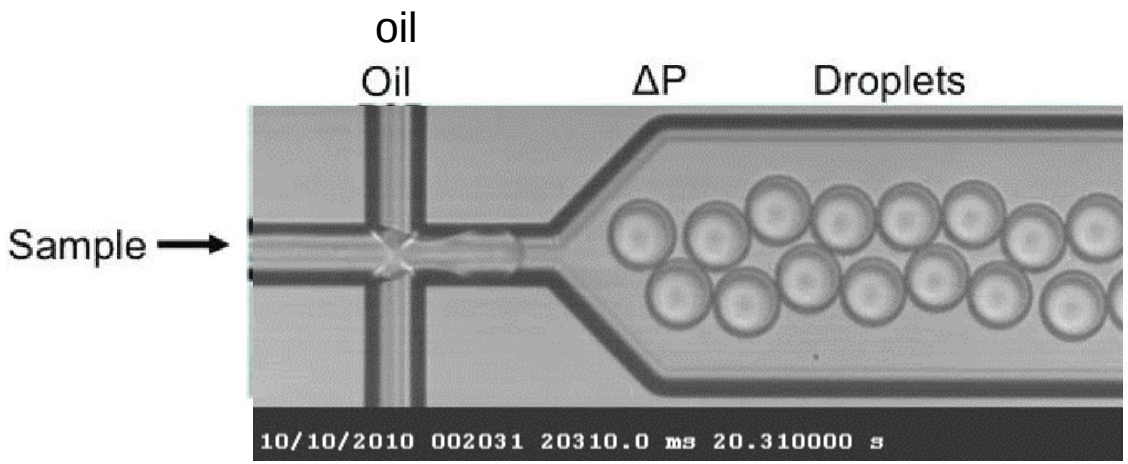
- ✓ Add 70ul generation oil (check oil type)

- ✓ Hook the gasket

Droplet Generation by Flow Focusing

- Place loaded cartridge into QX200 Droplet Generator
- Generate 20,000 droplets per sample, 2 ½ min for 8 samples
- Average droplet size is 0.91 nl volume (125 micron diameter)





Uniform droplet generation

BIO-RAD

3. Transfer the droplets and seal the plate

Aspirating droplets from the DG8 cartridge.

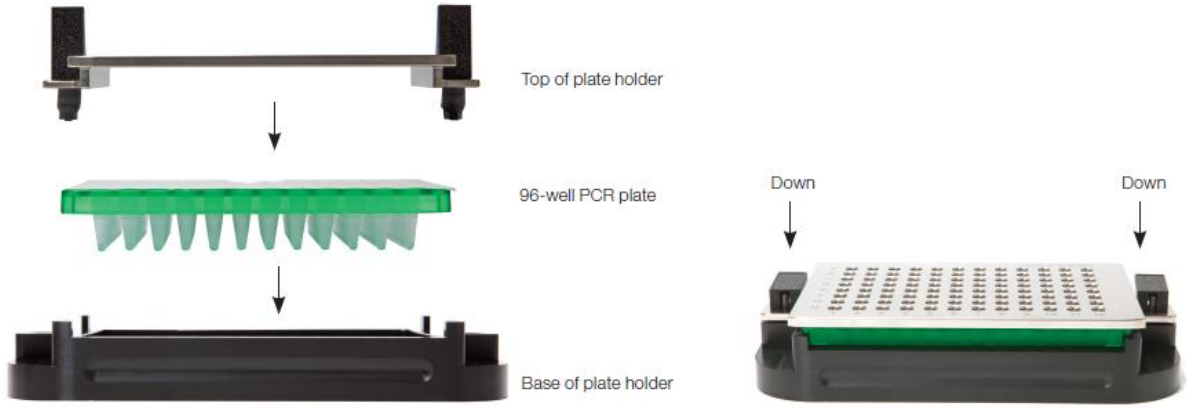
Dispensing droplets into a 96-well PCR plate.

- ✓ Place holder on a flat surface
- ✓ Position pipet tips at a 30-45^{o} angel
- ✓ Slowly draw 40ul of droplets into the pipet tips (~5 sec)

- ✓ Position the pipet tip along the side of the well and near the bottom of well
- ✓ Slowly dispense the droplets (~5 sec)

- ✓ 180^{o}C 5sec

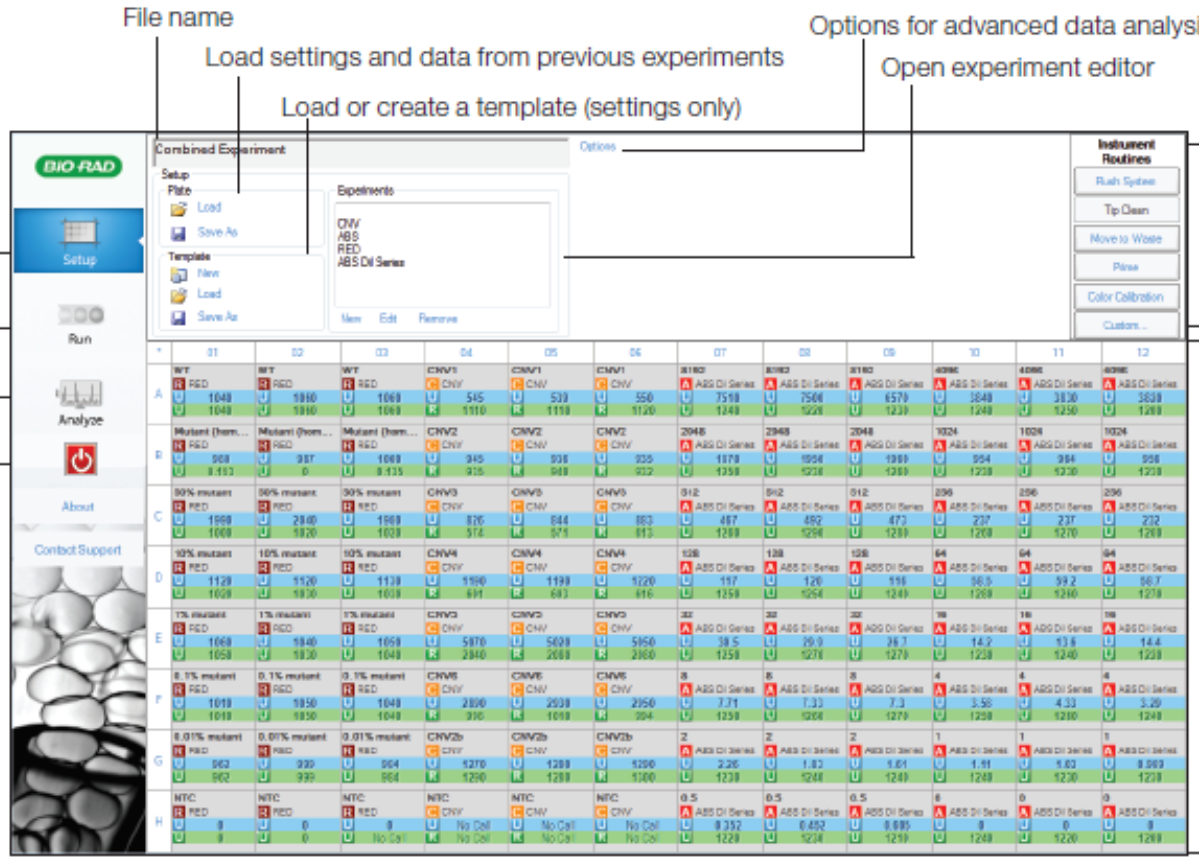
4. Place the holder into the reader



Placing the 96-well plate into the plate holder.

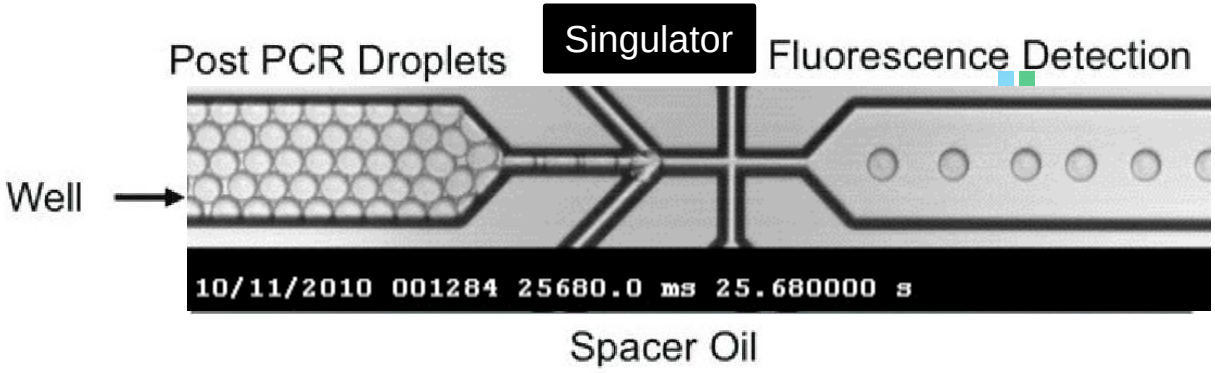
Placing the plate holder into the droplet reader.

5. Quantasoft software setup interface

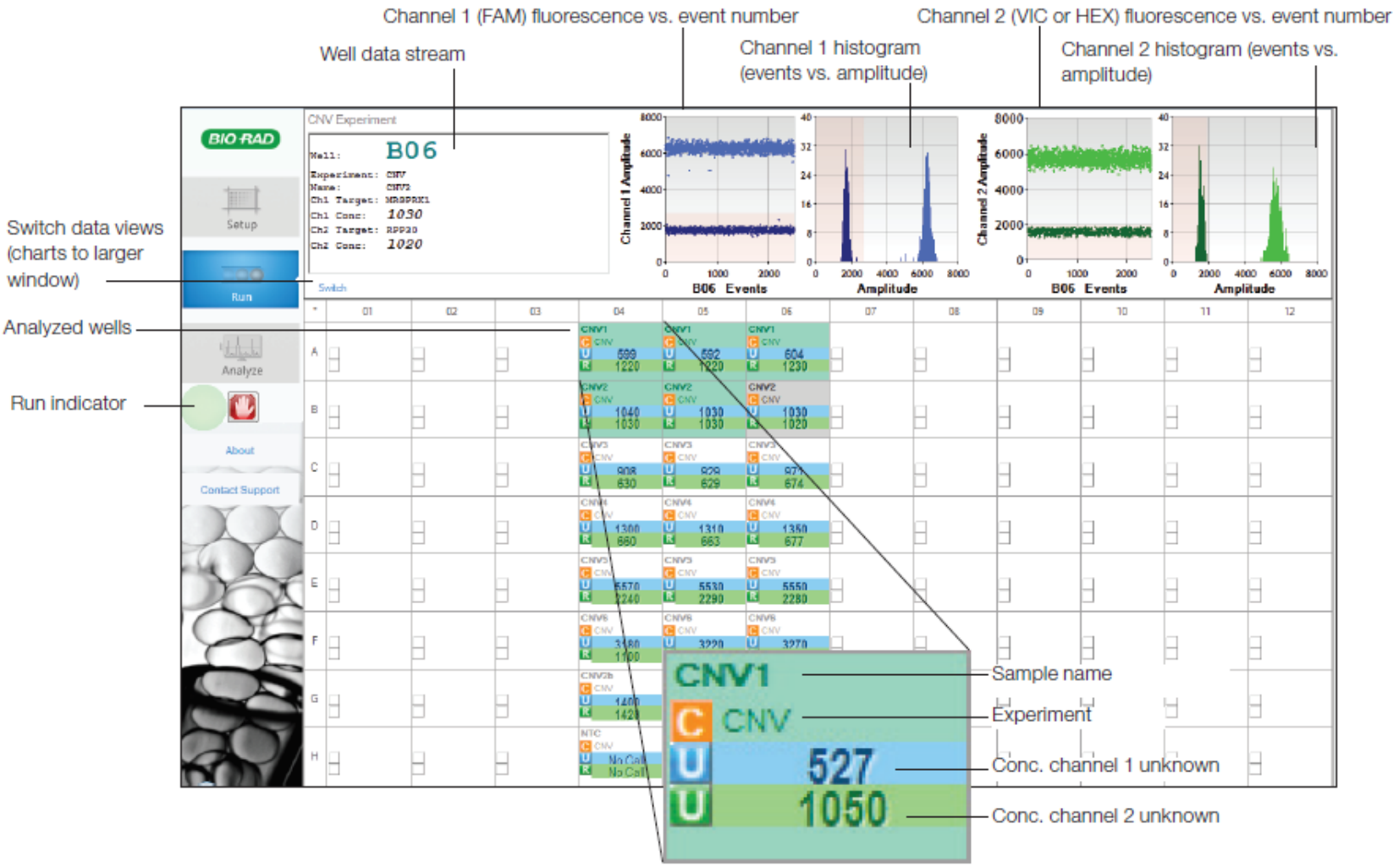


Droplet Reading

- Autosampler of QX200 Droplet Reader processes each sample independently
- Droplets stream single-file past the optical detector (32 wells/hr in 2 color)



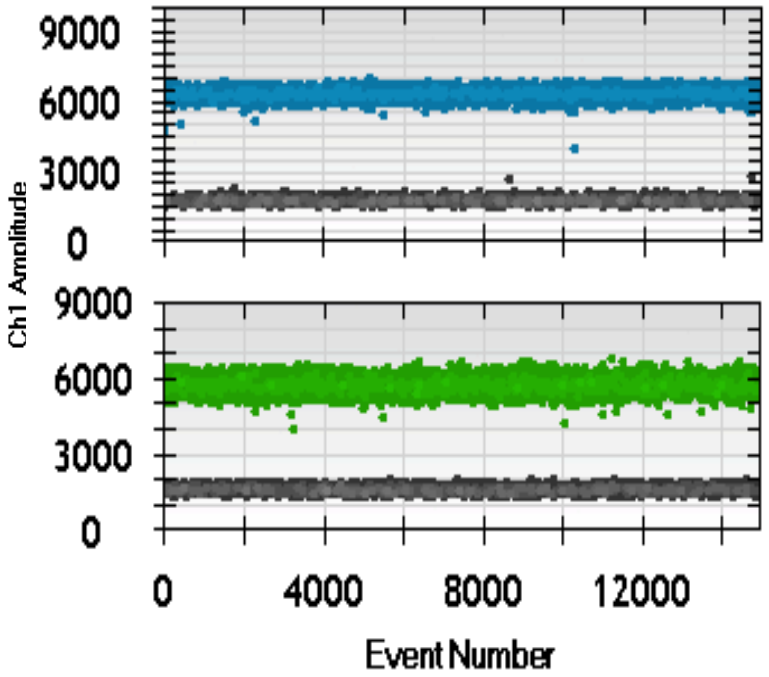
Quantasoft software interface

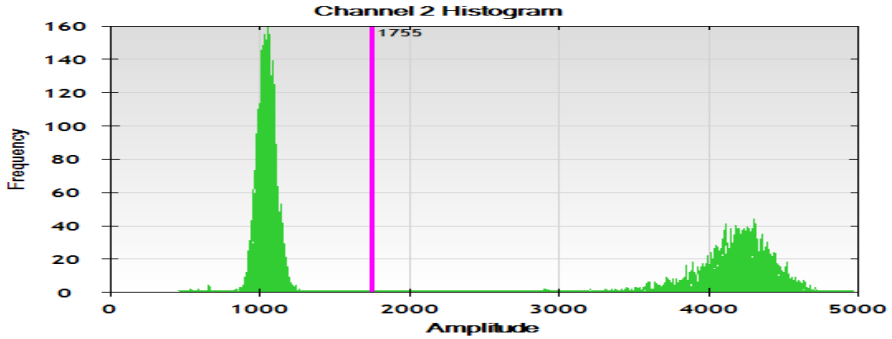


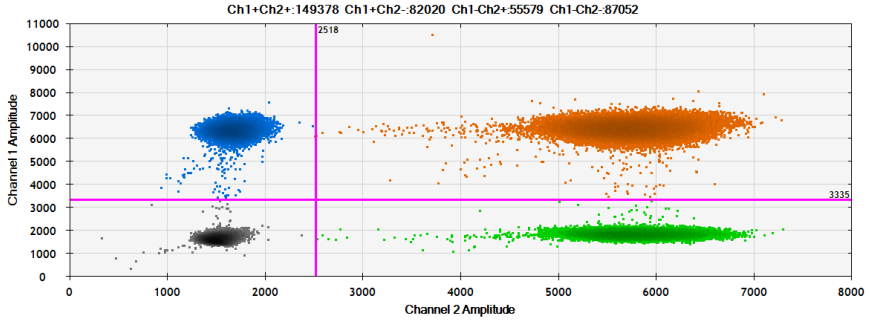
Interface during an active run. Data for both detector channels are shown for the well being read.

Droplet Fluorescence Converted to a Digital Signal

- Positive droplets contain at least 1 copy of target DNA (cDNA)
- Positive droplets have increased fluorescence vs. negative droplets
- QuantaSoft™ software measures the number of positive and negative droplets per fluorophore per sample

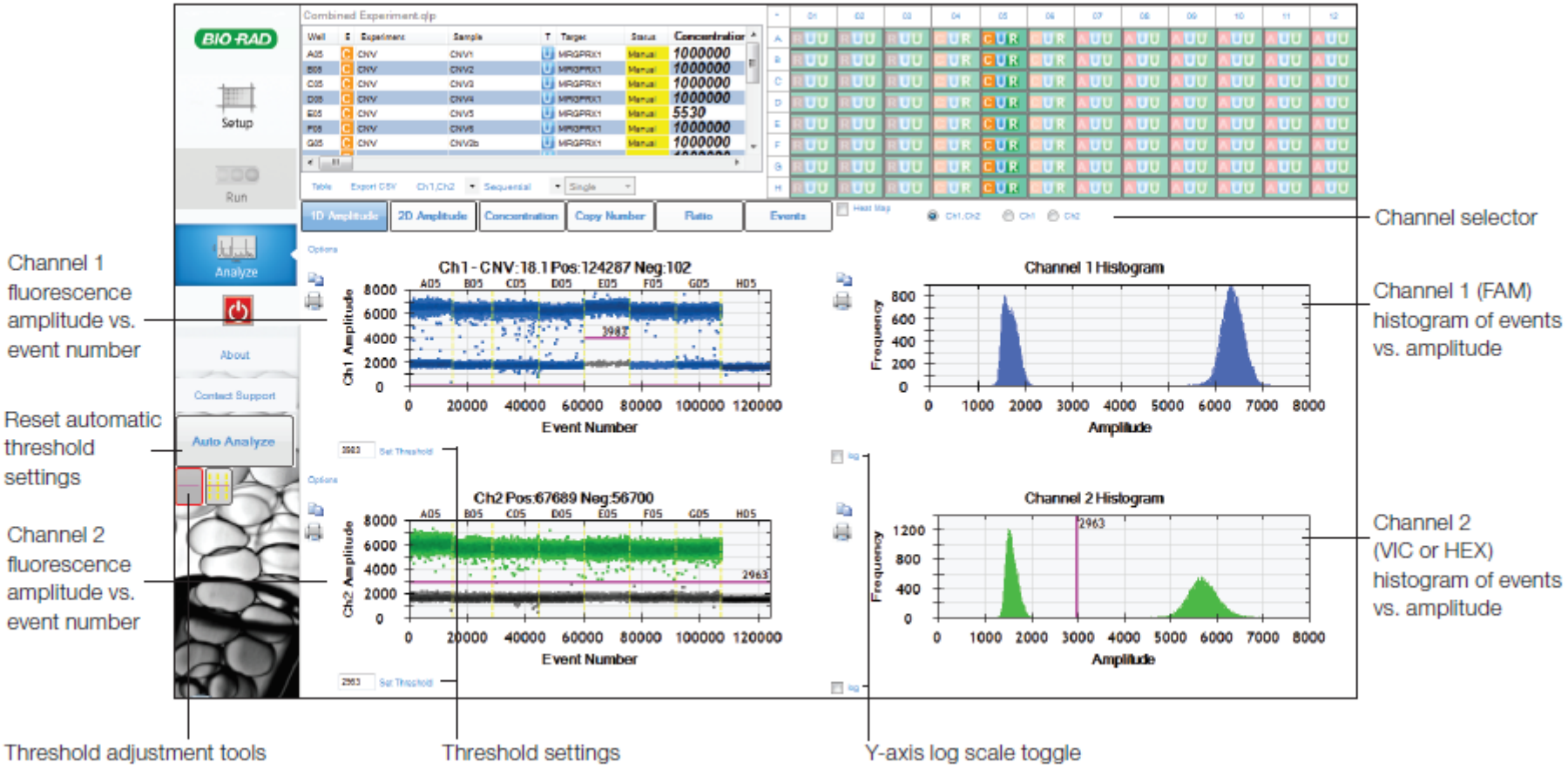




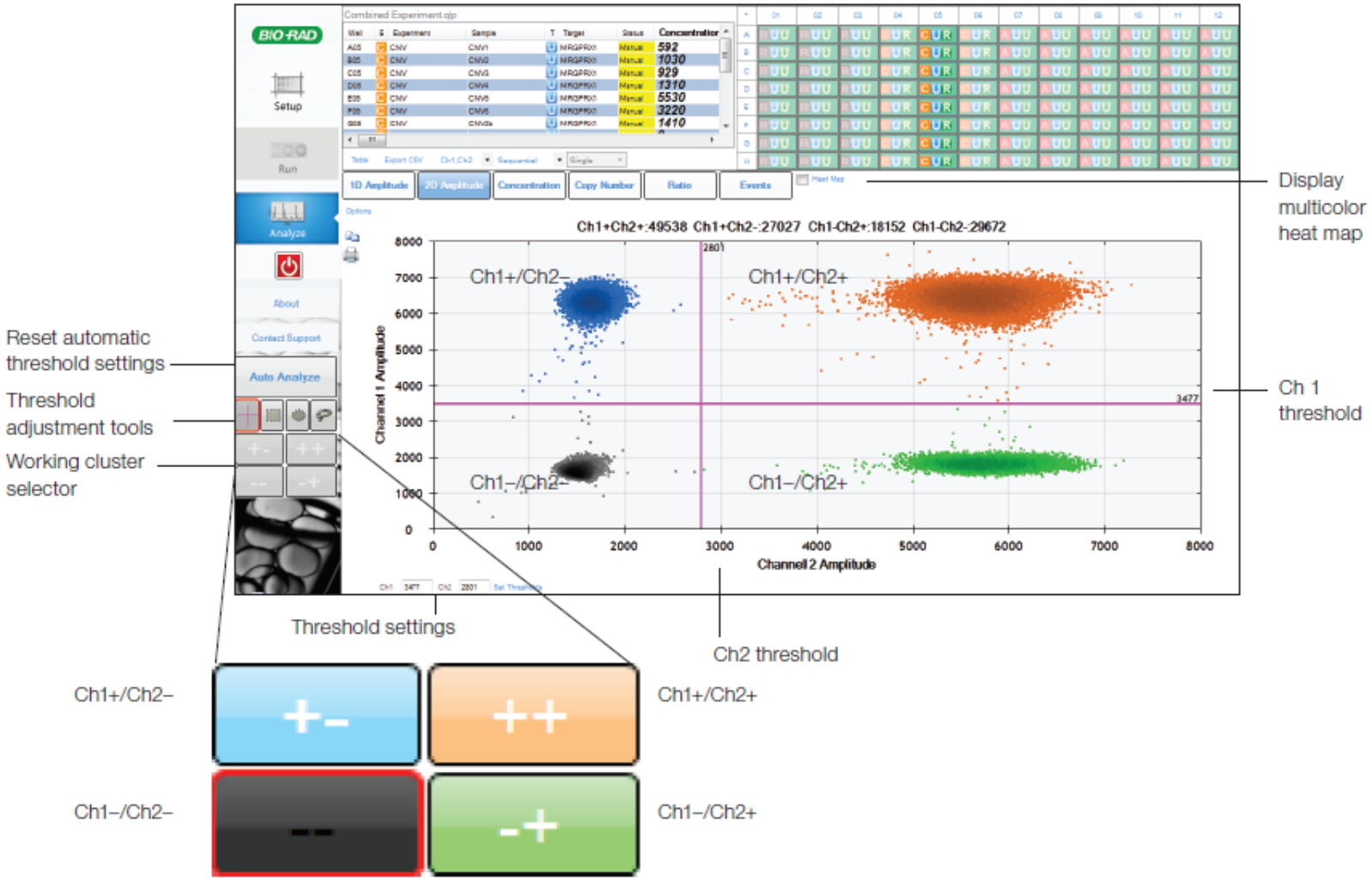


2-D Cluster Plot

6. Analysis


Channel 1 fluorescence amplitude vs. event number
Reset automatic threshold settings
Channel 2 fluorescence amplitude vs. event number
Threshold adjustment tools
Threshold settings
Y-axis log scale toggle
Channel selector
Channel 1 (FAM) histogram of events vs. amplitude
Channel 2 (VIC or HEX) histogram of events vs. amplitude
Ch1 - CNV:18.1 Pos:124287 Neg:102
Ch2 Pos:67689 Neg:56700
Channel 1 Histogram
Channel 2 Histogram
Event Number
Amplitude
Frequency
Setup
Run
Analyze
About
Contact Support
Auto Analyze
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Events

6. Analysis


Reset automatic threshold settings
Threshold adjustment tools
Working cluster selector
Display multicolor heat map
Ch 1 threshold
Threshold settings
Ch2 threshold
Ch1+/Ch2–
Ch1+/Ch2+
Ch1–/Ch2–
Ch1–/Ch2+
Ch1+Ch2+:49538 Ch1+Ch2-:27027 Ch1-Ch2+:18152 Ch1-Ch2-:29672
Channel 1 Amplitude
Channel 2 Amplitude
Setup
Run
Analyze
About
Contact Support
Auto Analyze
1D Amplitude
2D Amplitude
Concentration
Copy Number
Ratio
Events

6. View Concentration Data

Select channel(s) to display

Y2 Axis options:

None — no Y2 axis

Ch2 — display Ch2 concentration

CNV — display copy number (CNV analysis only)

Ratio(a/b) — ratio (a/b) of unknown:reference

Fractional Abundance (a/a+b) — ratio (a/[a+b]) of unknown:reference; abundance

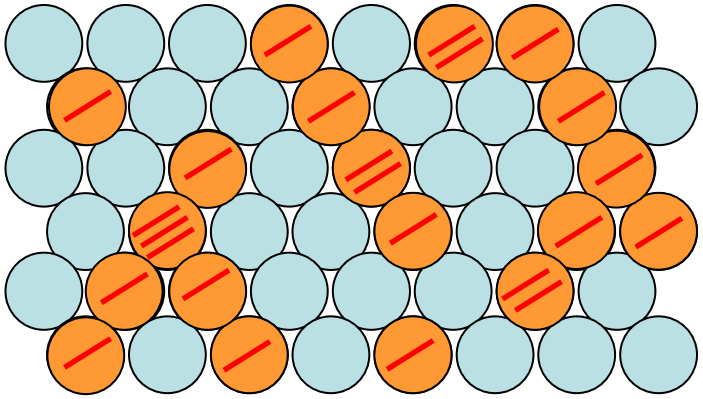
Inverse — inverse of ratio or fractional abundance

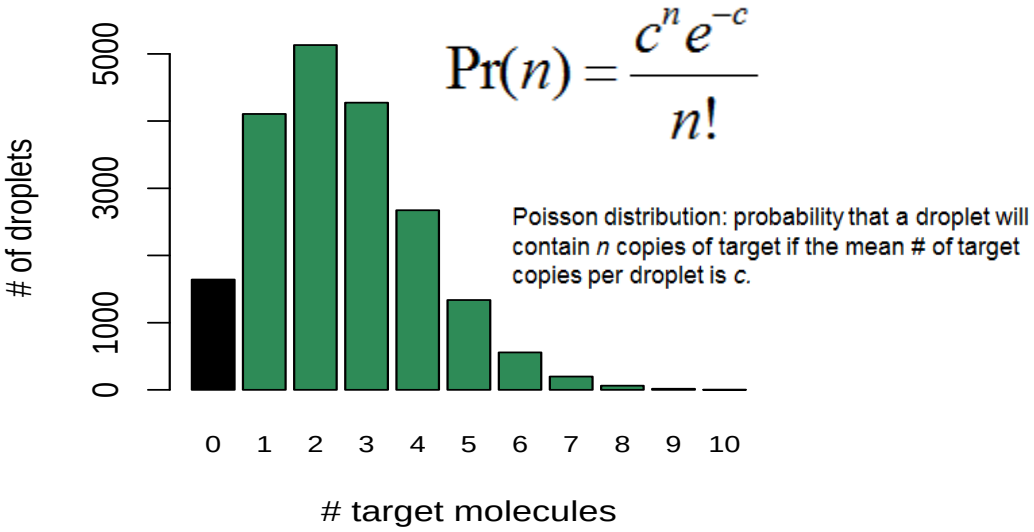
Toggle y-axis log scale

Display data from single wells, merged data, or both

Toggle x-axis values and well, name, or label display

Beyond Limiting Dilution: High Target Concentration (with ddPCR)





Siméon Denis Poisson
(1781-1840)

"X" target copies

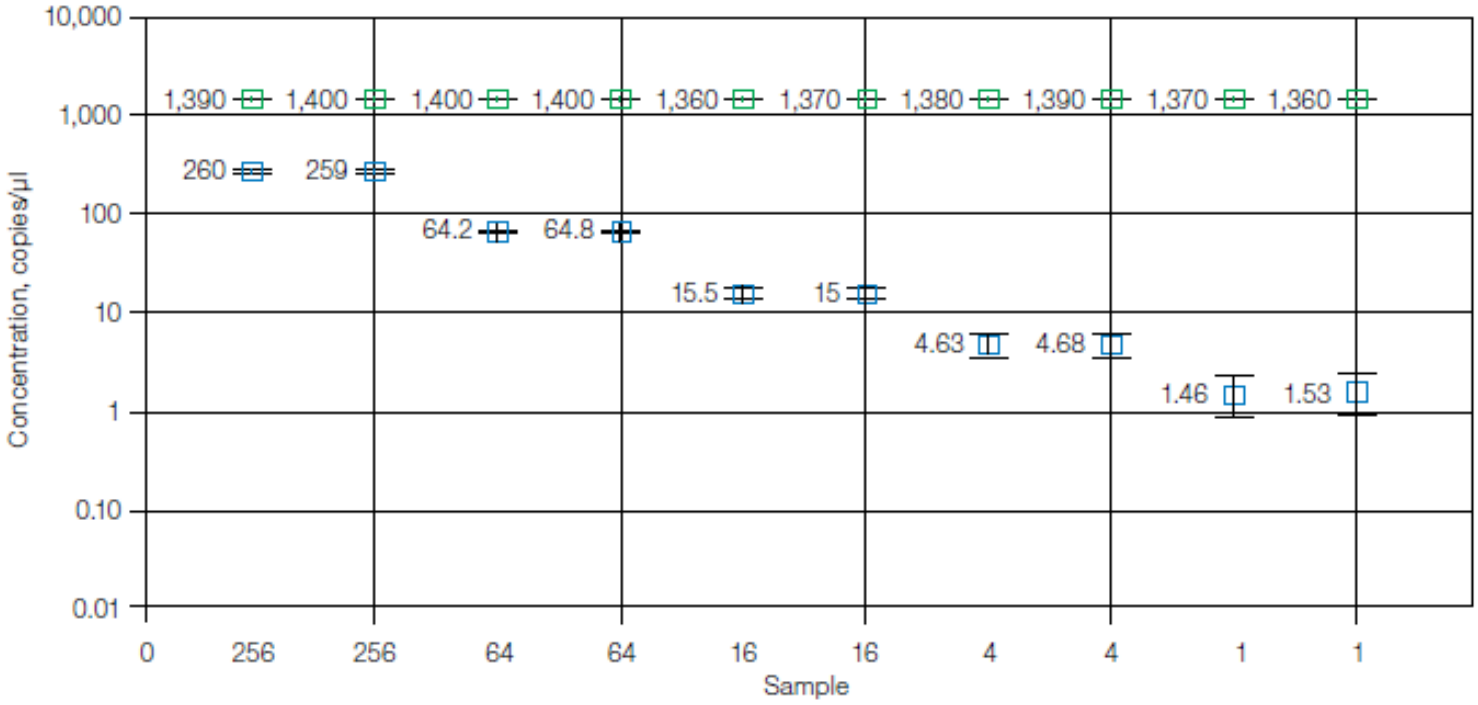
Modeling as Poisson

Copies per droplet = –ln (1 – p)

where p = fraction of positive droplets **and** (1 – p) = fraction of negative droplets.

Note: At 1 target copy/droplet, 37% negatives!

Excellent Reproducibility and Linearity Across Concentrations and Instruments

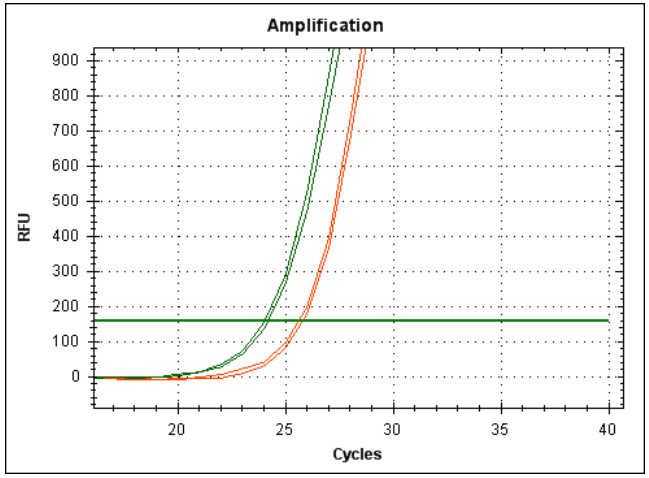


- Readout: copies/µl
- Dynamic range: 1–100,000 copies/well (~330 ng human genomic DNA)

Benefits of Droplet Digital PCR

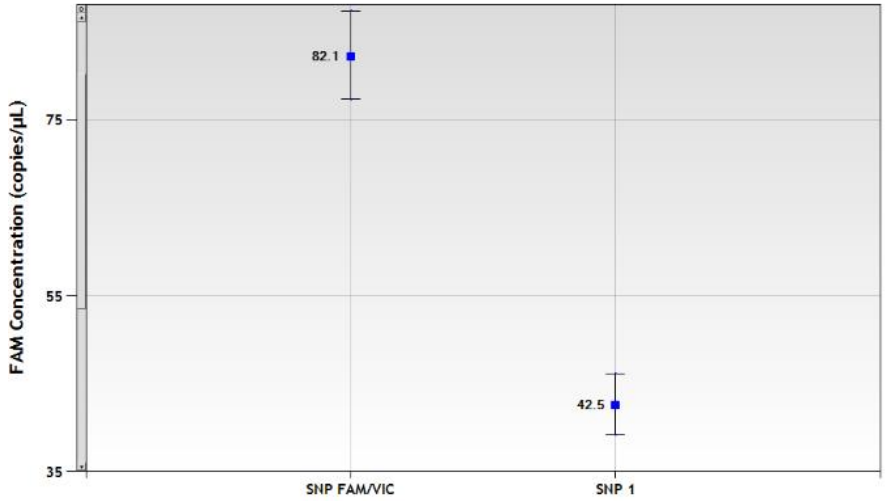
Detecting Small Fold Changes

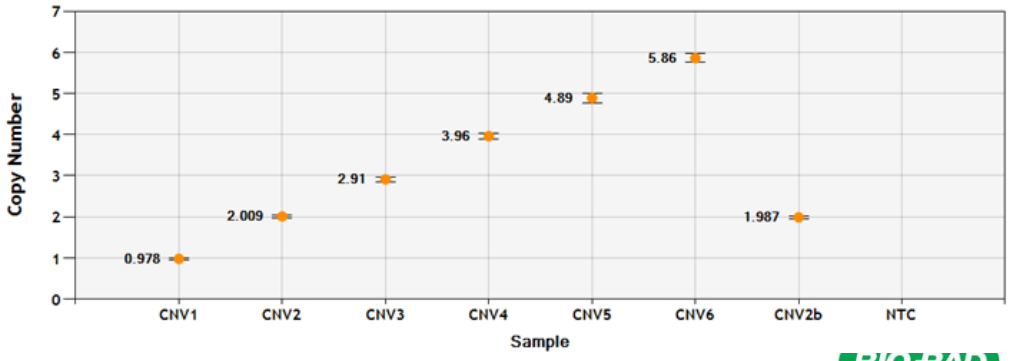
A 2-fold change measured by real-time PCR



Content	Cq	Cq Std. Dev
Unkn-14	26.99	0.110
Unkn-14	26.84	0.110
Unkn-15	25.47	0.115
Unkn-15	25.31	0.115

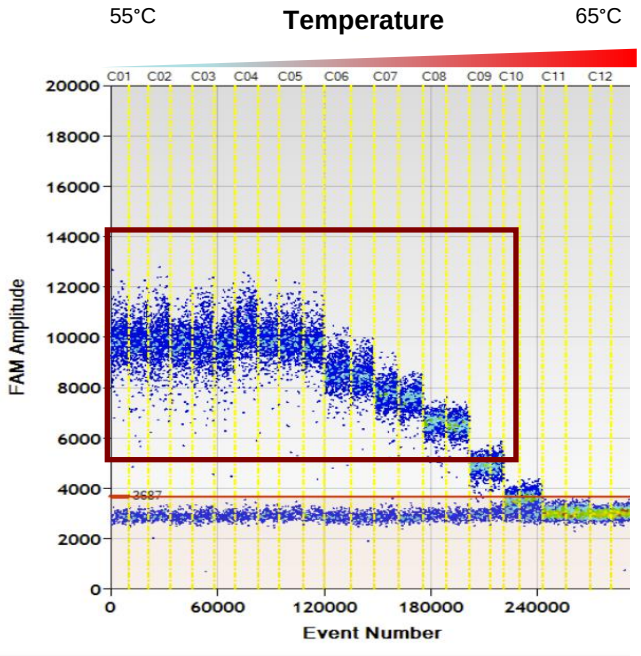
A 2-fold change measured by ddPCR

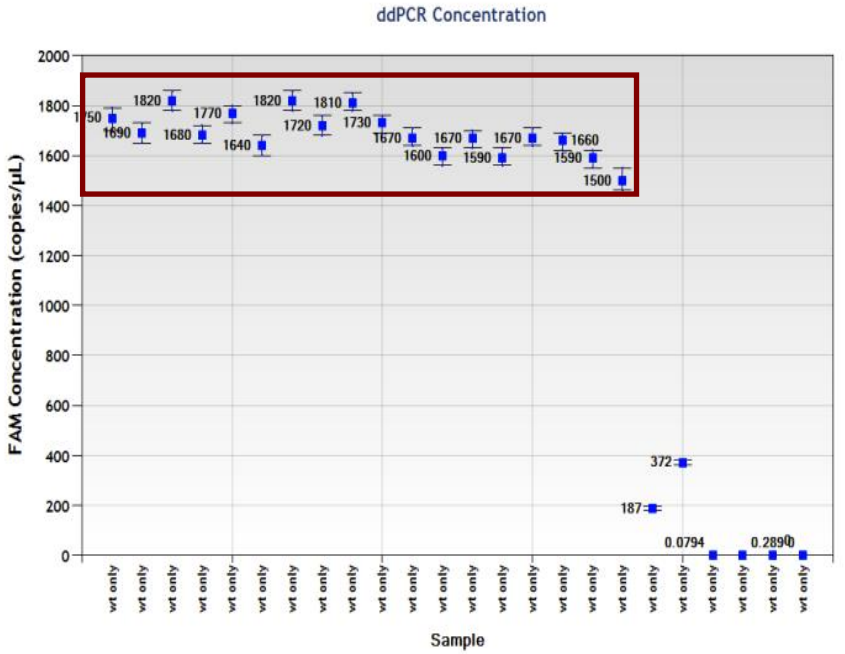




PCR efficiency has a minimal impact on ddPCR™

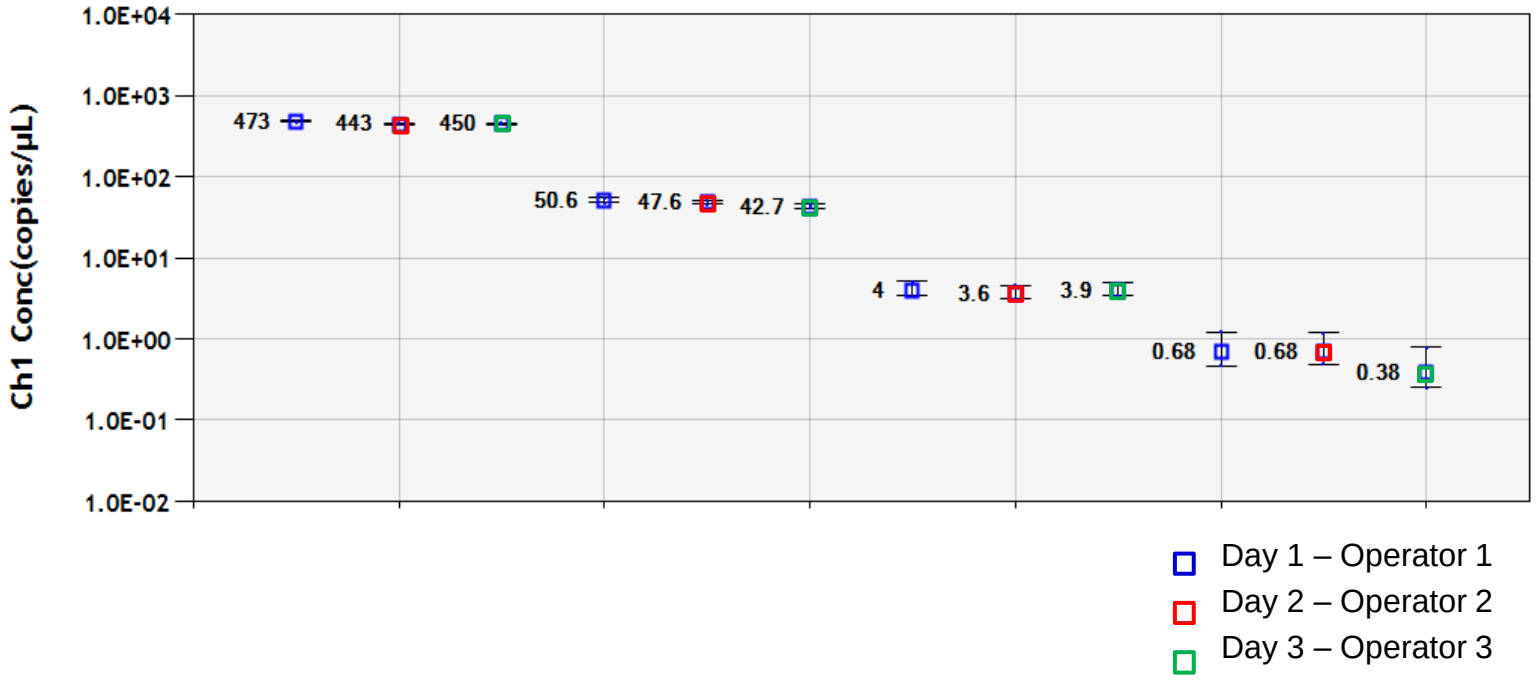
- Thermal gradient mimics different PCR efficiencies
- Same concentration measured across different annealing temps





ddPCR™ reproducibility

- ✓ Reproducibility trial – 3 operators in 3 different Korean laboratories using the same assay and samples obtained a high degree of data uniformity

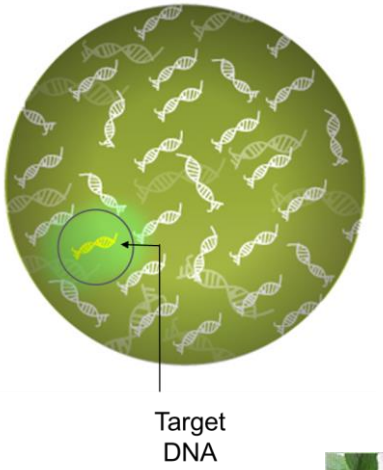


Applications of Droplet Digital PCR

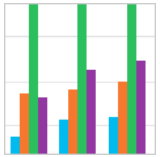
Applications of ddPCR

- **Cancer Biomarker Studies and Copy Number Variation**
  - Measure varying degrees of cancer mutations, detect rare DNA target copies, and resolve copy number variation states with superior sensitivity and resolution.

- **Pathogen Detection**
  - Employ the extreme precision of the QX200 System to quantify small fold changes in target DNA or RNA molecules in pathogen detection and monitoring.



- **Next Generation Sequencing**
  - Perform accurate quantification and qualification of NGS libraries
  - NGS data validation



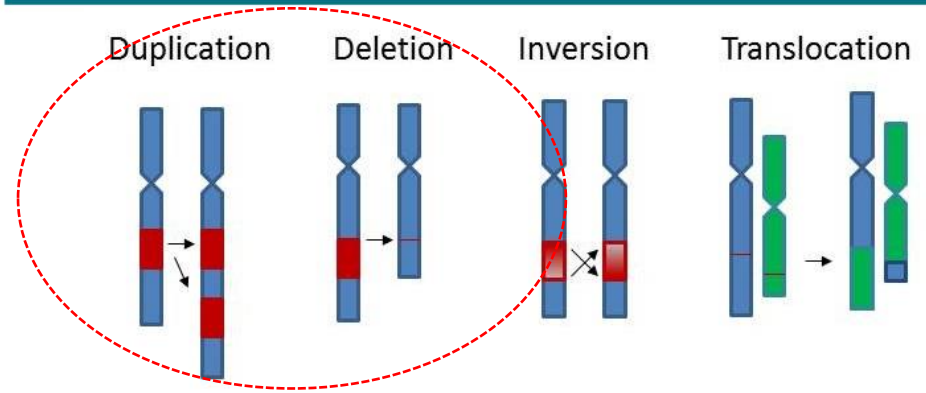
- **Gene Expression Analysis**
  - Achieve reliable and reproducible measurements of small fold changes for low abundance of mRNA and microRNA and RNA variants

- **Food Testing**
  - Perform routine evaluation of genetically modified organisms (GMO) using validated ddPCR methods.

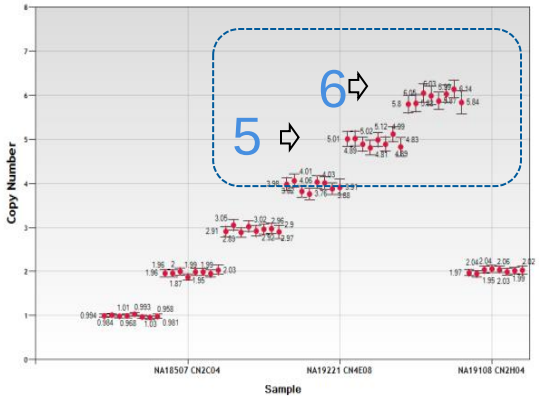
- **Environmental Monitoring**
  - Test a wide variety of environmental samples like soil and water

BIO-RAD

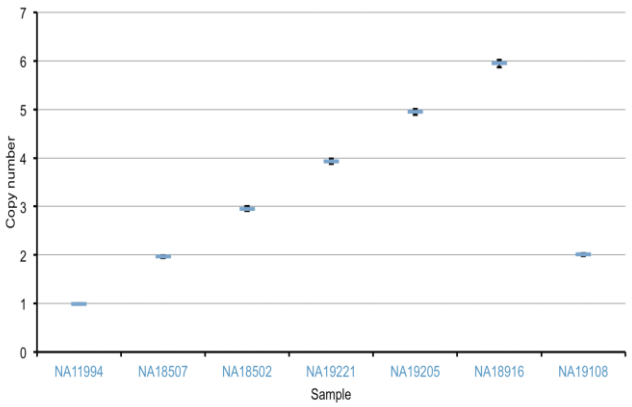
Copy Number Variation (CNV's)



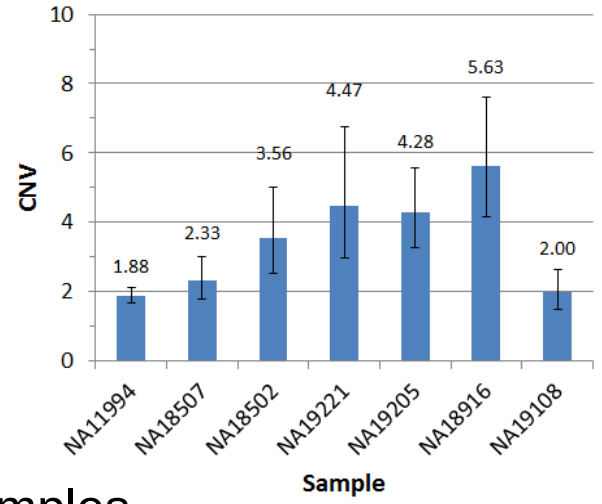
ddPCR individual wells



ddPCR merged wells



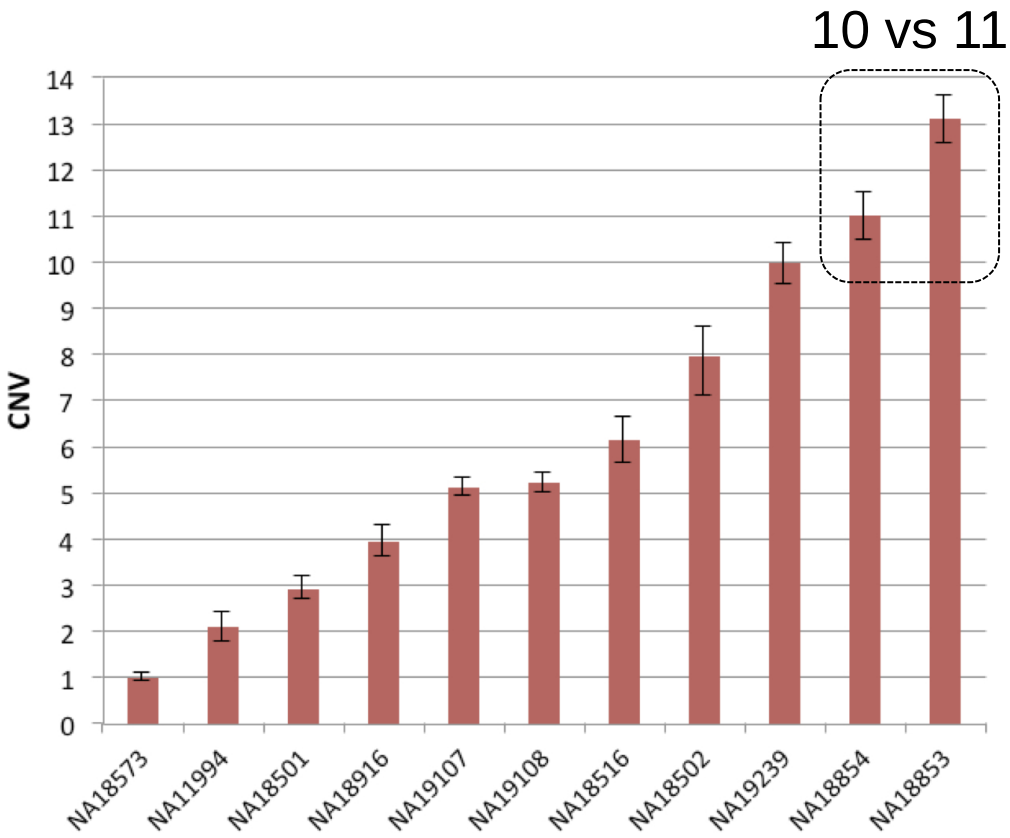
real-time PCR (8 wells)



- Measure *MRGPRX1* gene copies from various samples

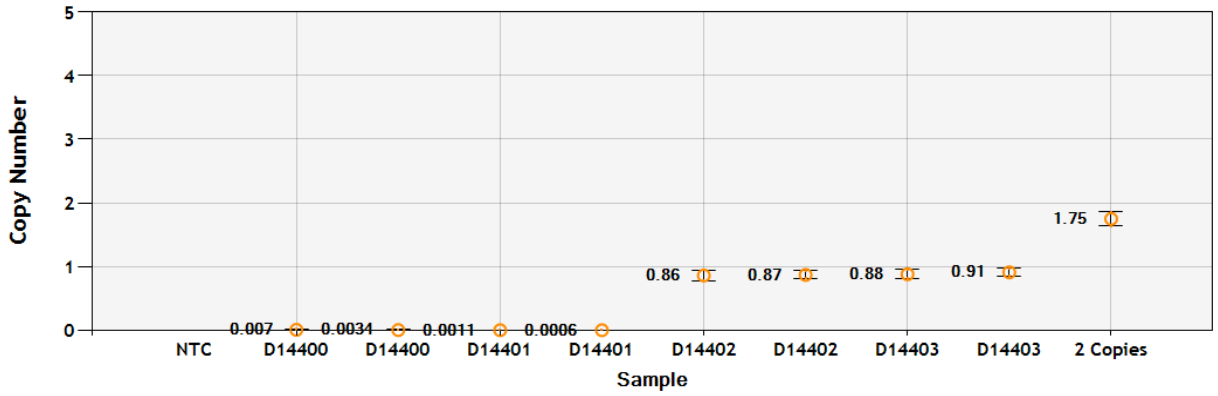
Higher CNV Level Discrimination

Resolution of 10 vs. 11 copies of CCL3L1 from HapMap samples

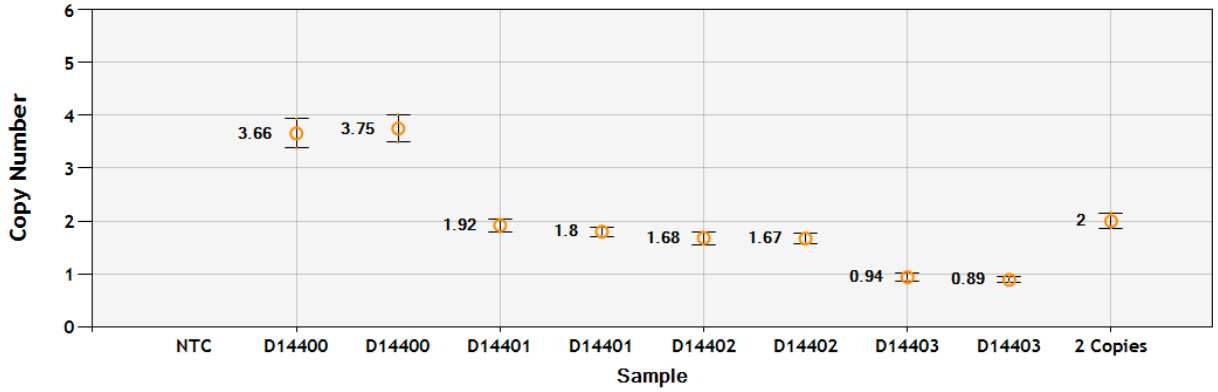


SMN1, SMN2 Copy Number Determinant Kit

SMN1

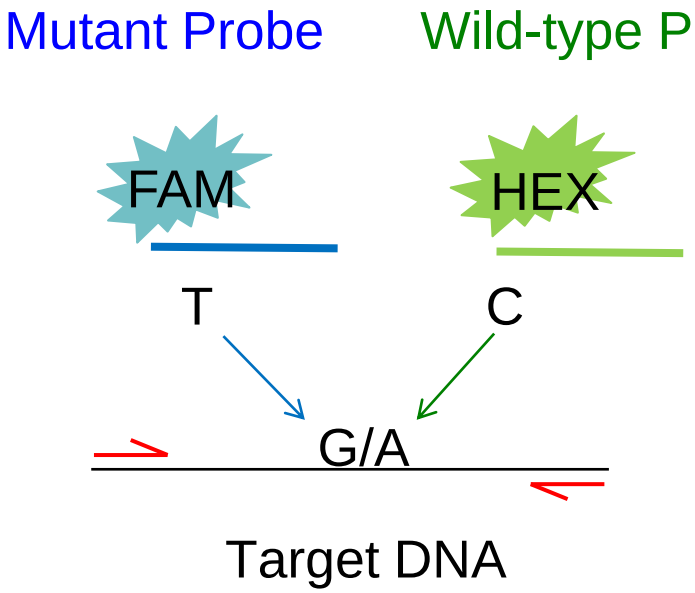


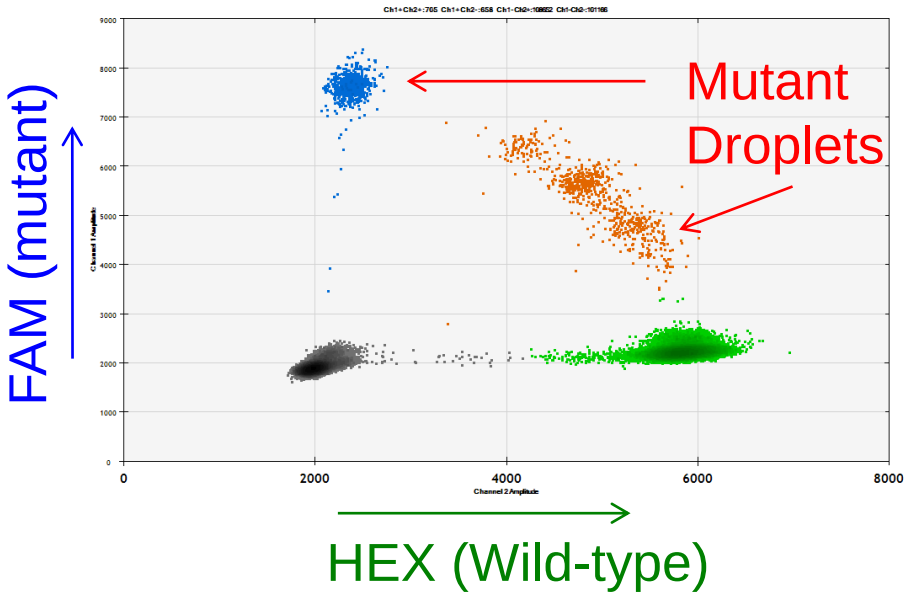
SMN2



Rare Event Detection Assays

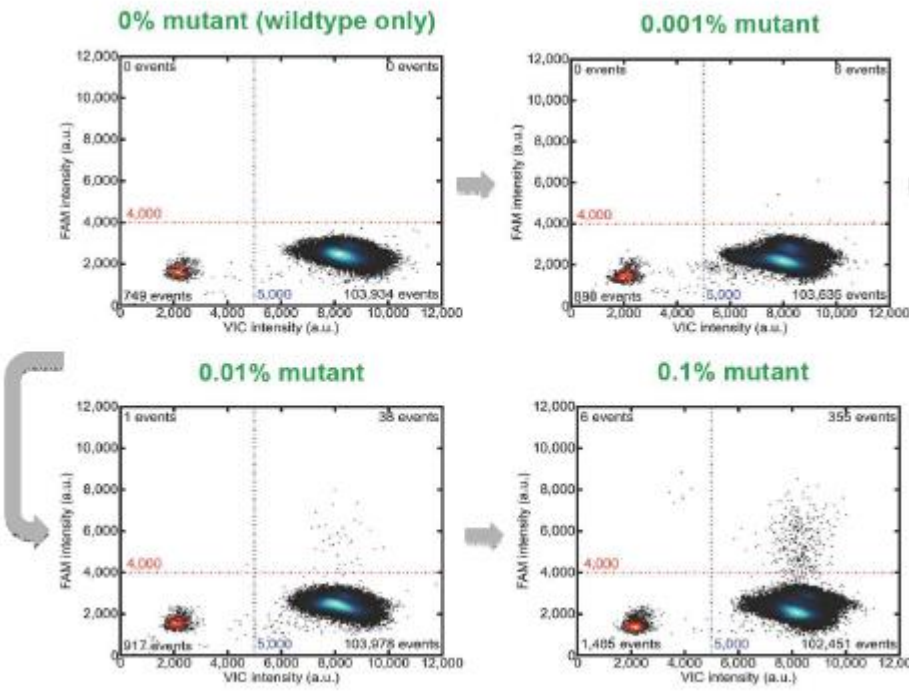
Rare mutation detection assays share common primers while the probes are SNP-dependent

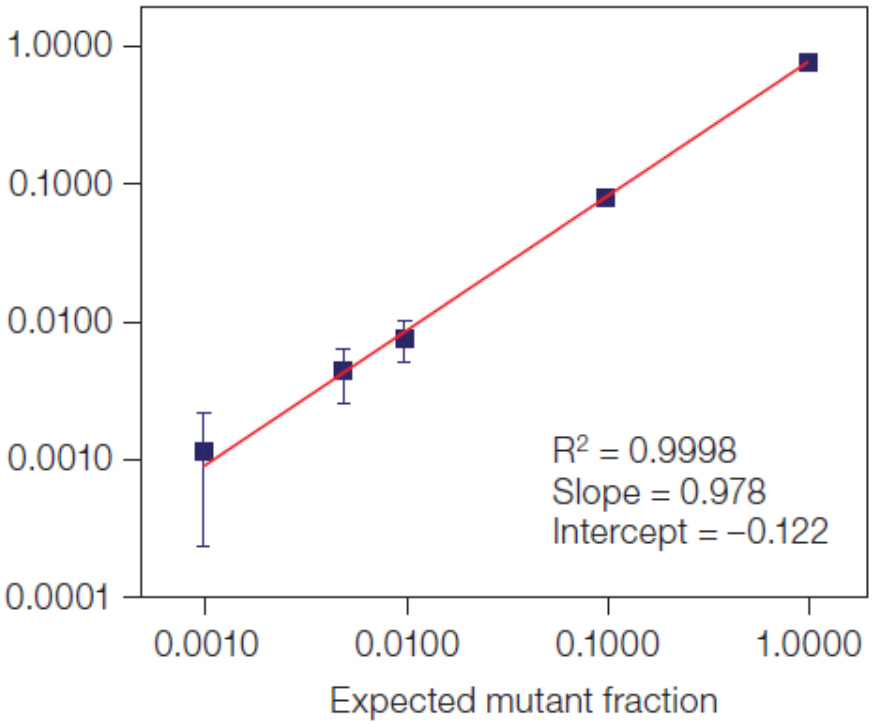




Rare Event Detection

BRAF V600E ddPCR Detected Down to 1/100,000

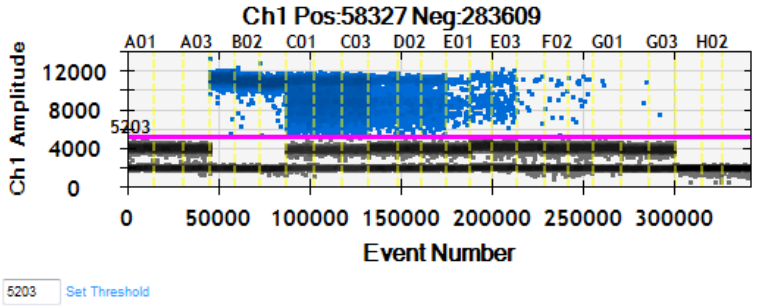


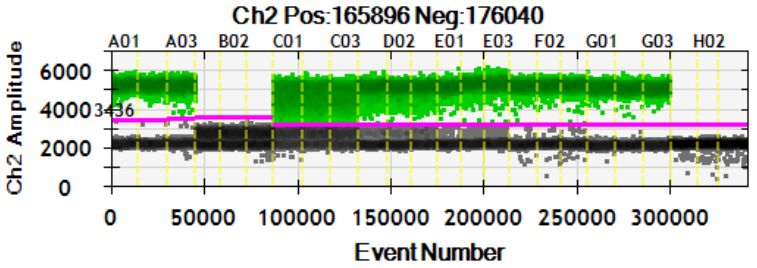


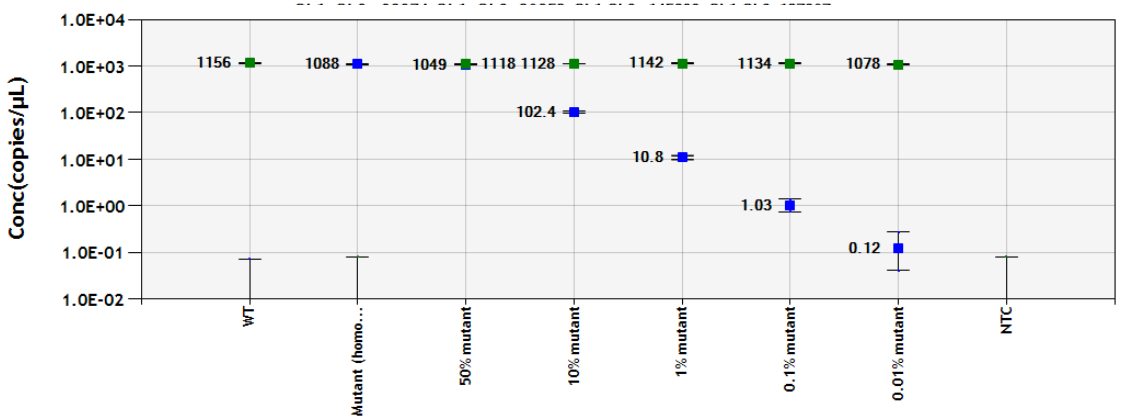
qPCR detects *BRAF V600E* down to 2.0%

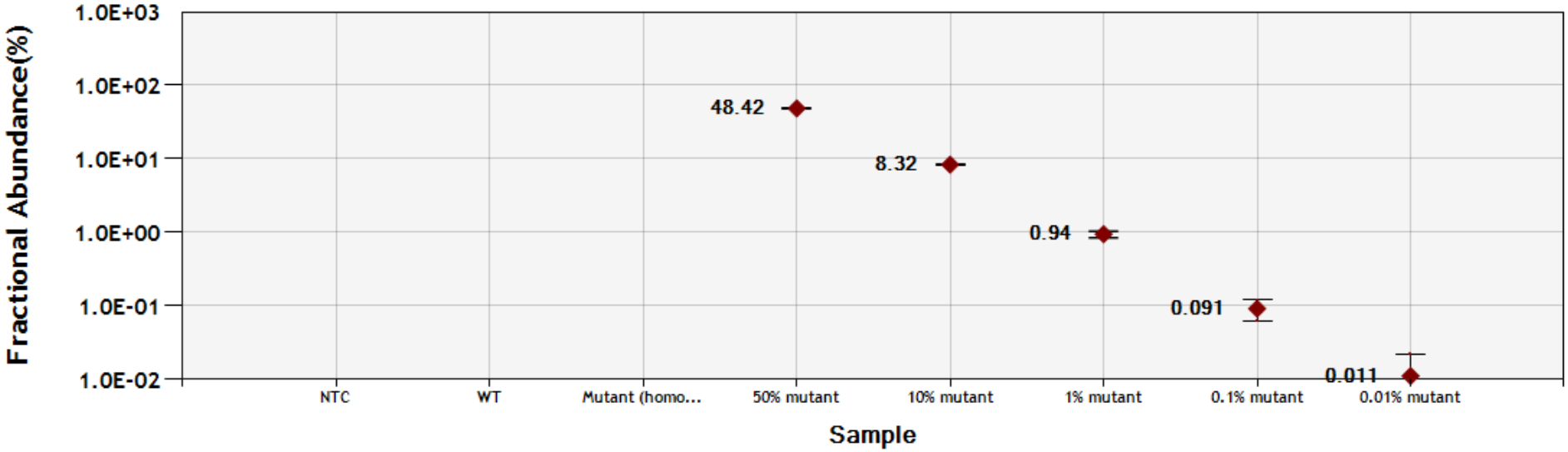
BIO-RAD

BRAF *V600E* detection by ddPCR sensitivity *0.01%*





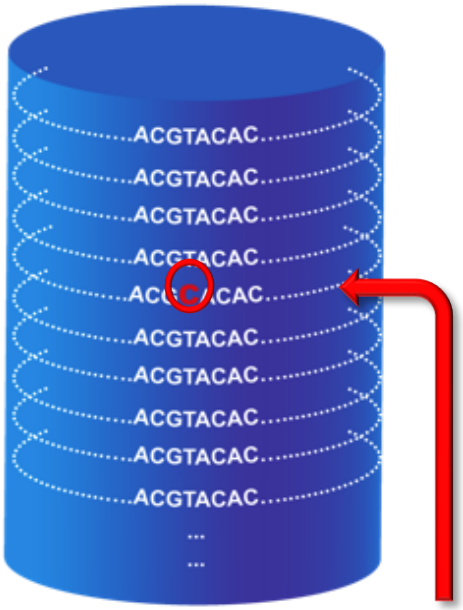




Droplet Partitioning Increases Mutant Abundance

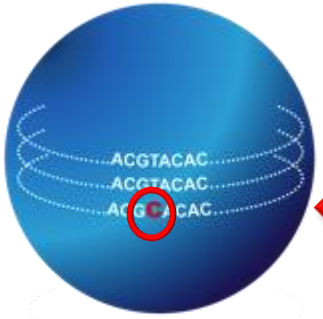
qPCR Reaction — 1 x 20 µl

40,000 wild-type molecules
40 mutant molecules



Mutant abundance: 0.1%

ddPCR Partitioned Reaction — 20,000 x 1 nl

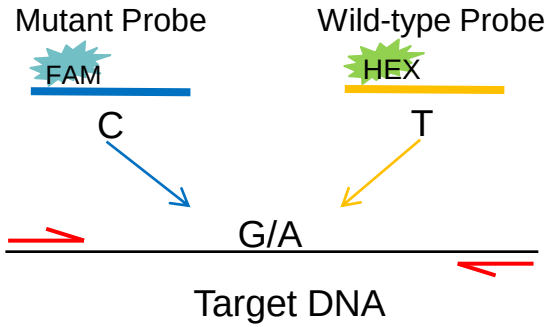


40 droplets with mutant

Mutant abundance: 33%

19,960 droplets
without mutant

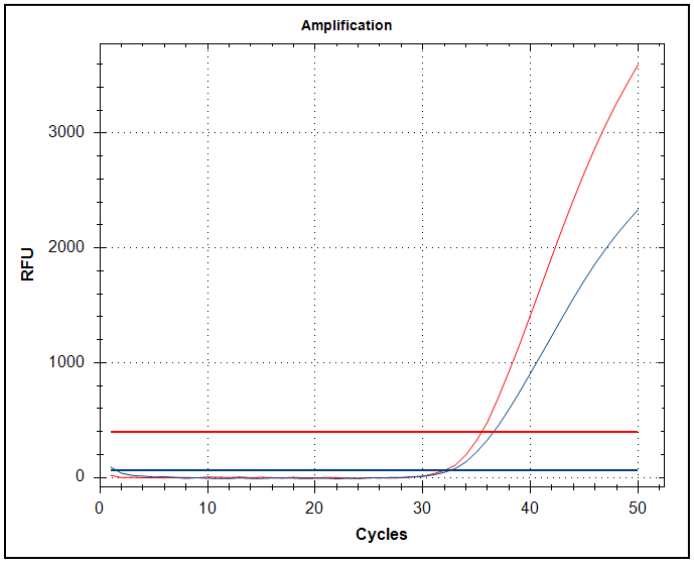
ddPCR assays are more easy to design and validate



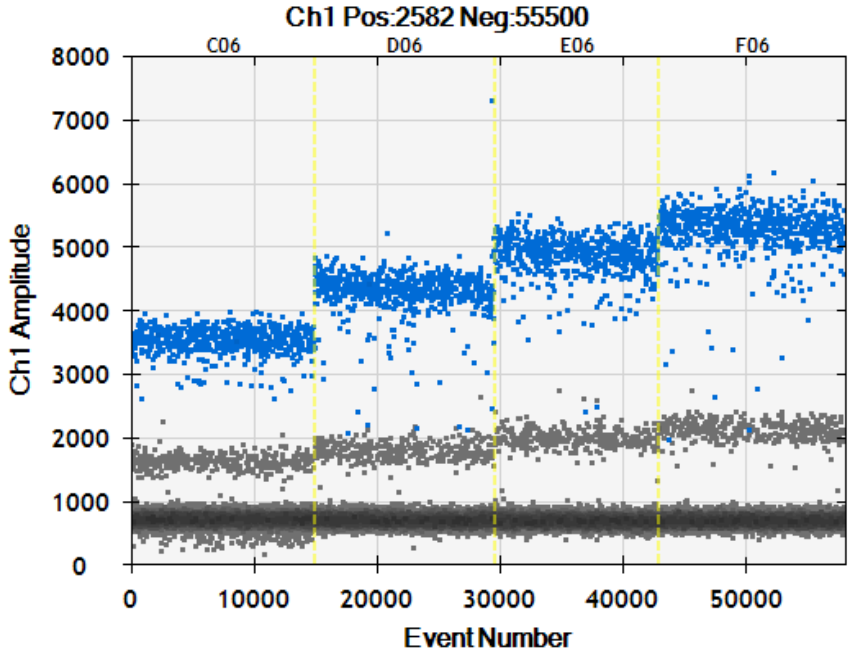
More Tolerance to:

1. Non-specific binding
2. Primer dimer
3. PCR inhibitor

Non-specific cross reaction

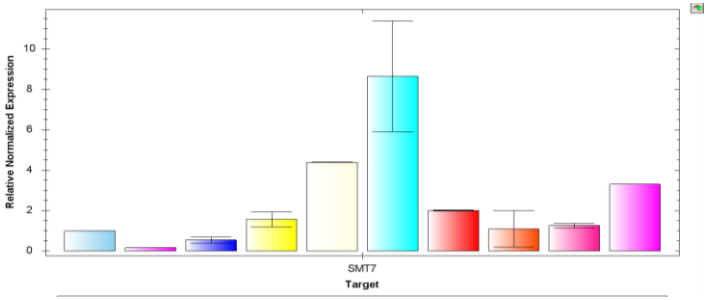


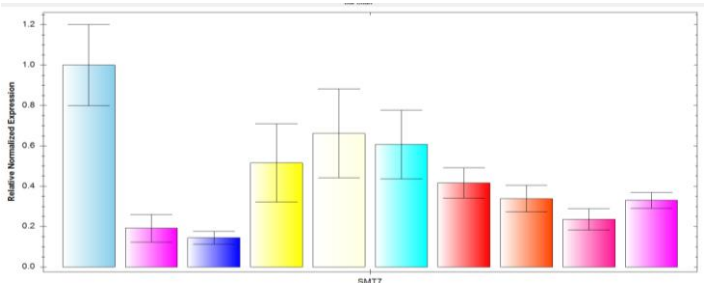
qPCR

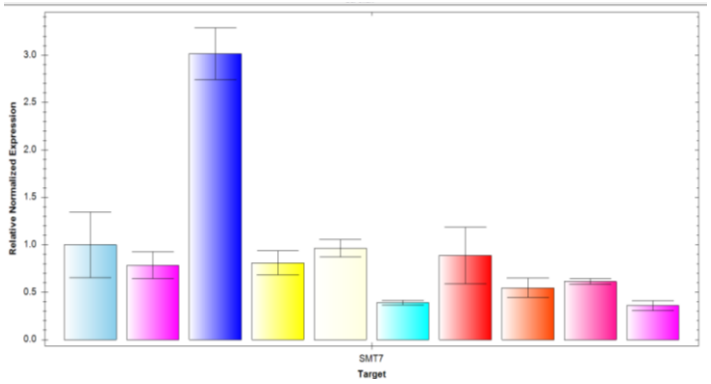


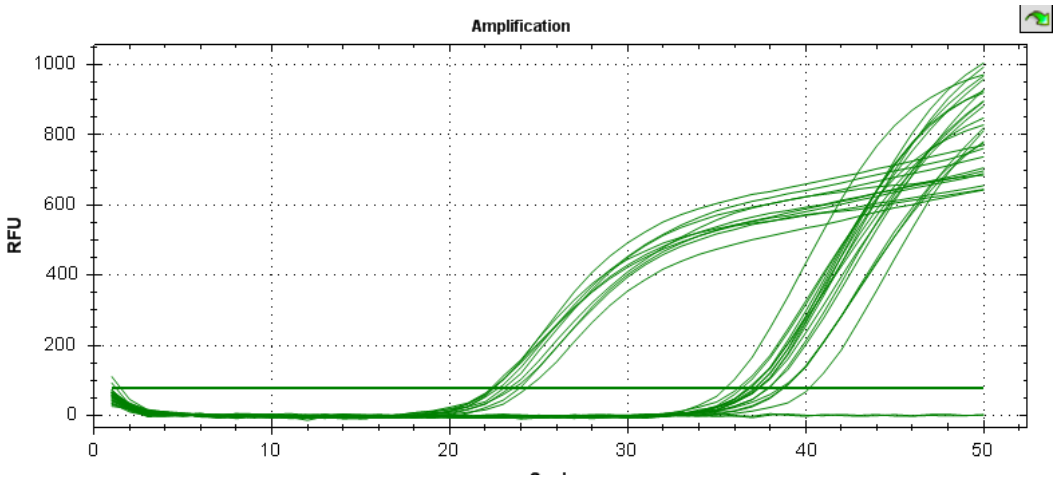
ddPCR

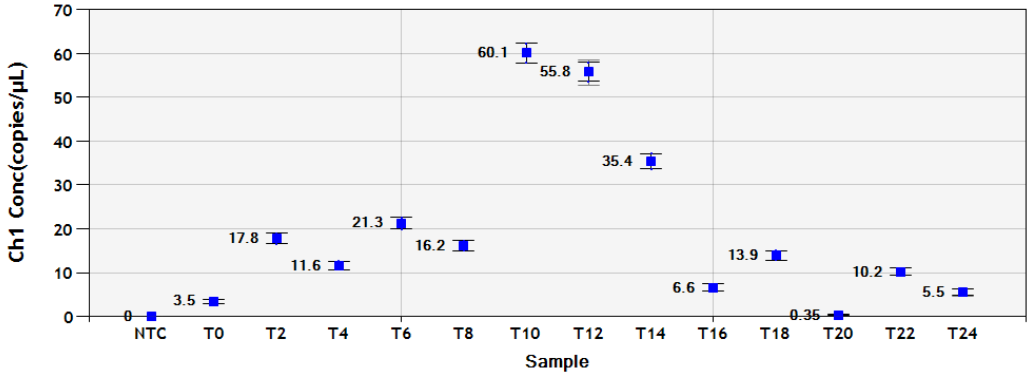
Gene Expression Q-PCR VS ddPCR







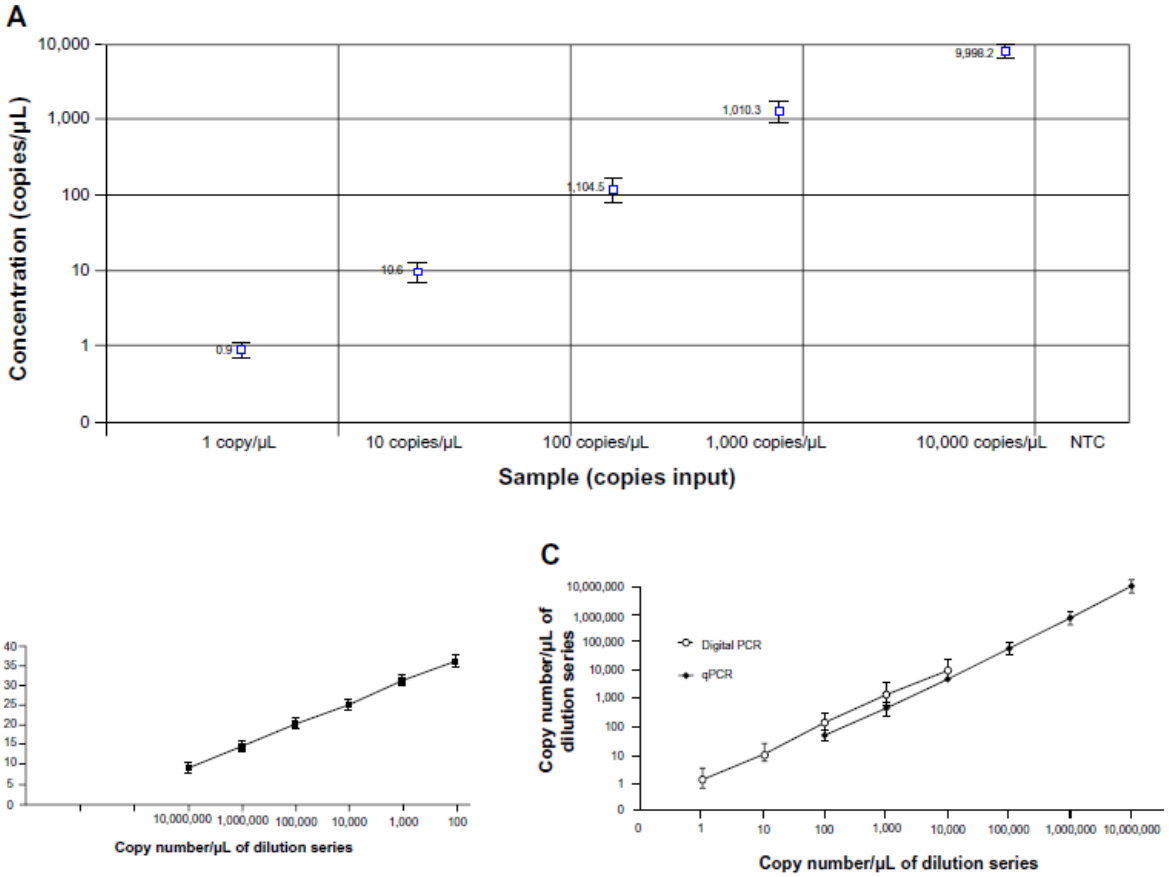




BIO-RAD

Gene expression

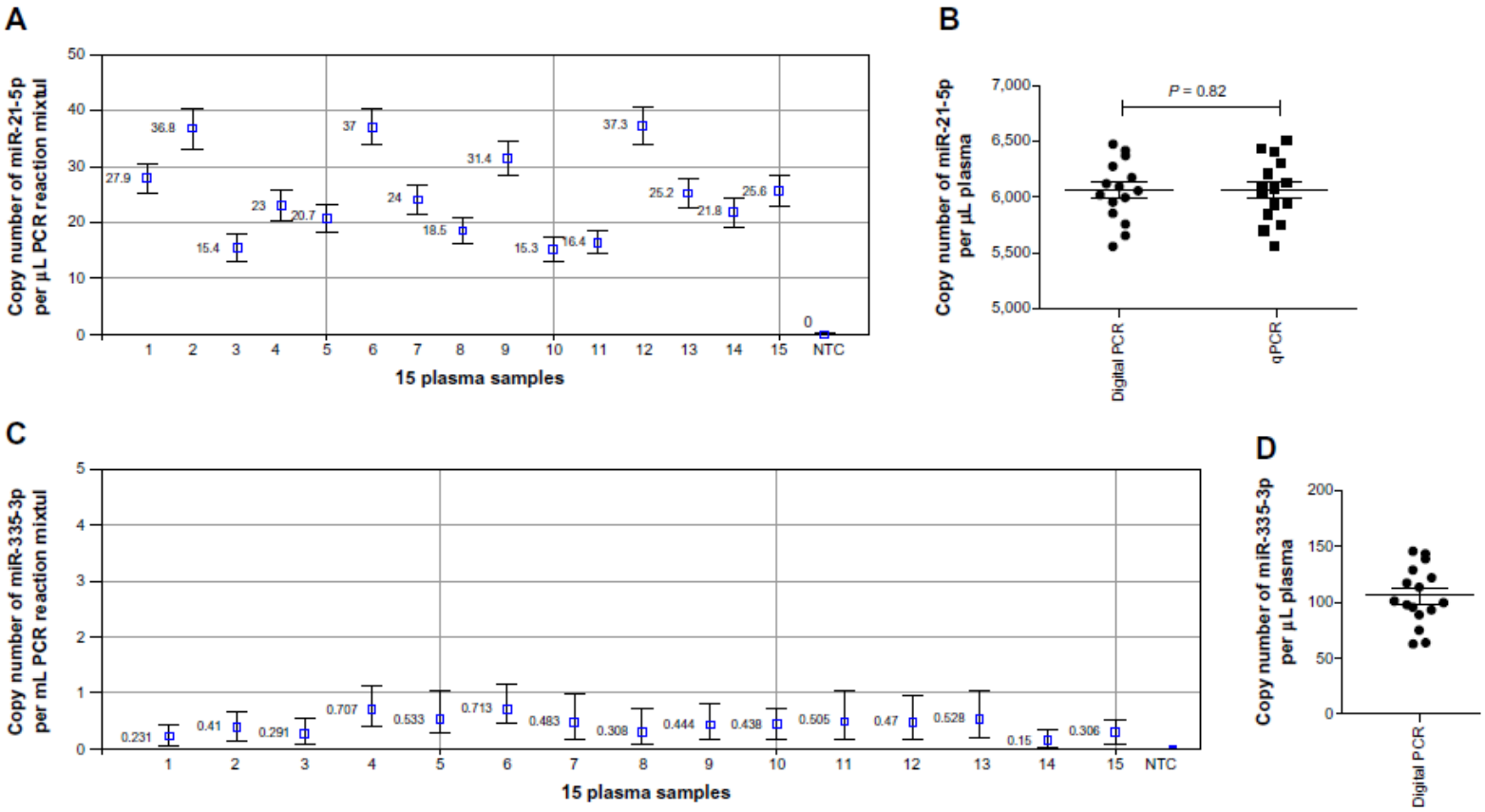
Quantification of Plasma miRNAs by Digital PCR for Cancer Diagnosis



Overall, digital PCR had a narrower dynamic range.

However, the lowest copy number detected by digital PCR was significantly lower (1 copy per µL of input) than that by qPCR (approximately 100 copies per µL of input)..

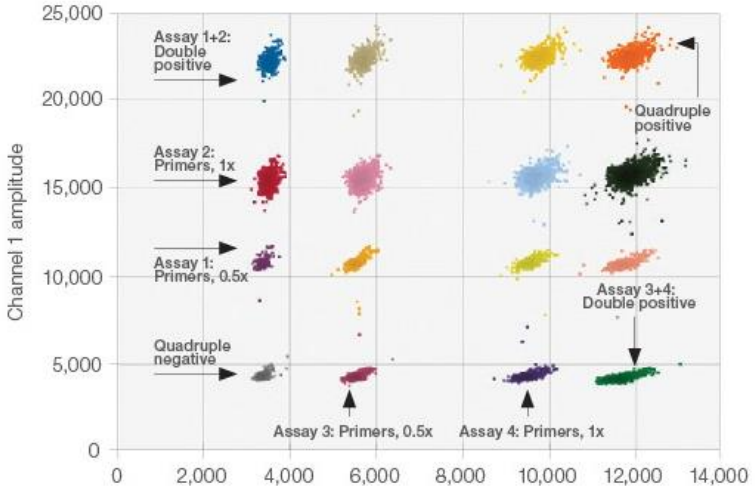
Quantification of Plasma miRNAs by Digital PCR for Cancer Diagnosis



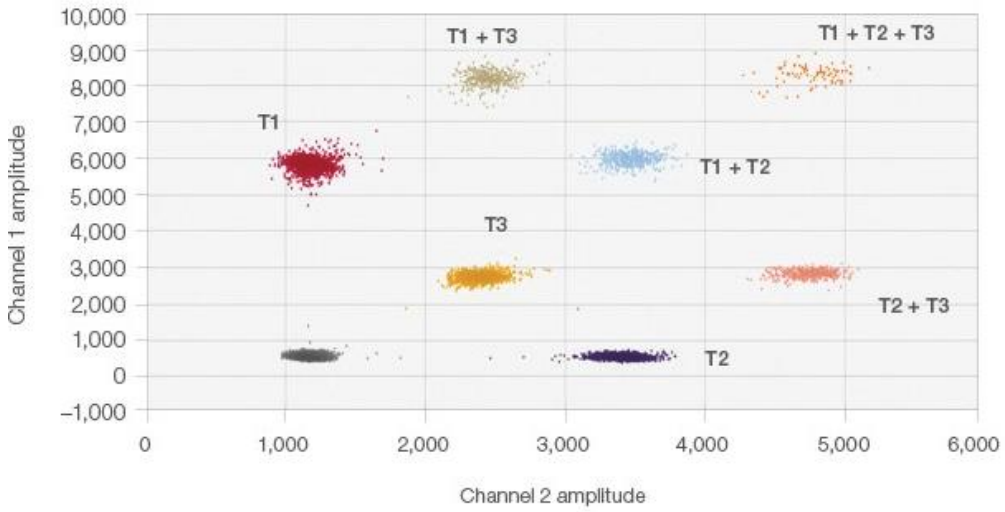
Therefore, digital PCR rather than qPCR might reliably and sensitively measure the copy number of miR-335–3p that has endogenous low-level expression in plasma.

Expanded Droplet Digital PCR Multiplexing Capability

- Amplitude Multiplex Droplet Digital PCR



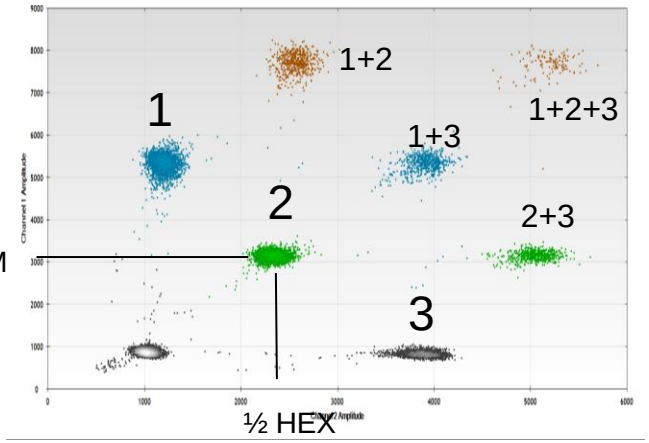
- Probe-Mixing Multiplexing Droplet Digital PCR



BIO-RAD

Multiplexing in 2 channels overview

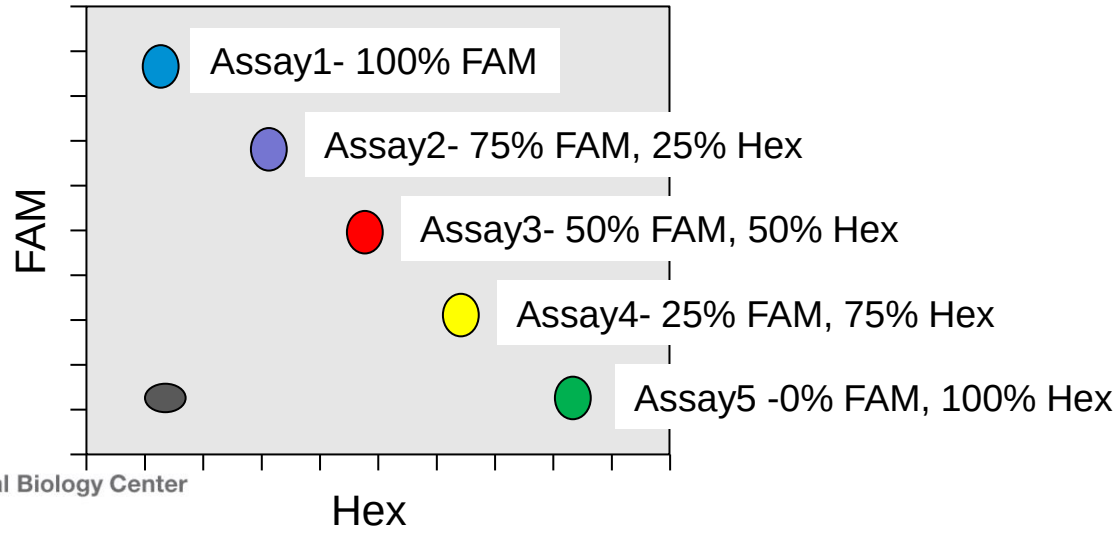
Concept of probe mixing - triplex



Double, Triple positives

3plex	Assay mixing
1	100% FAM, 0% Hex
2	50% FAM, 50% Hex
3	0% FAM, 100% Hex

Theoretical clustering -5plex



5plex	Assay mixing
1	100% FAM, 0% Hex
2	75% FAM, 25% Hex
3	50% FAM, 50% Hex
4	25% FAM, 75% Hex
5	0% FAM, 100% Hex

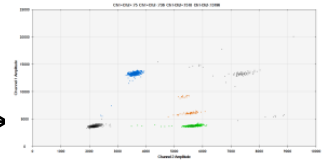
Single Cell Analysis Using ddPCR

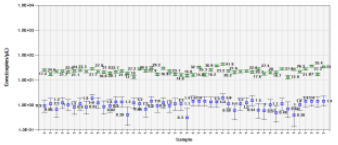
Simplified workflow completed in less than 1 workday

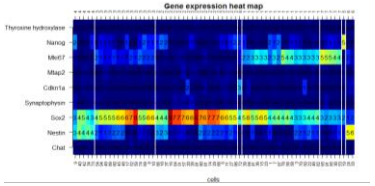
S3 cell sorter

QX200
Droplet Digital PCR

Data
analysis







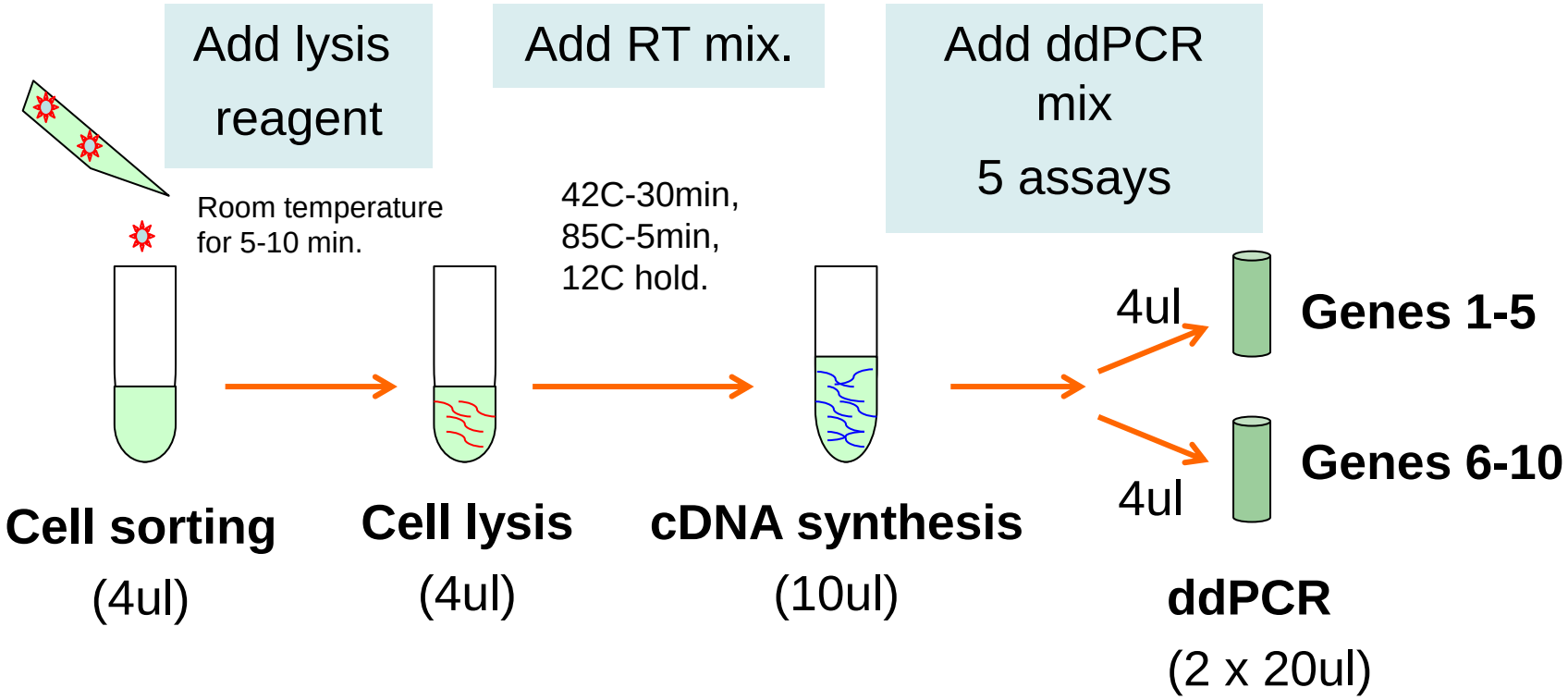
Single cells

Cell culture

PrimePCR Assays

Single Cell Analysis Using ddPCR

Measure 10 genes per single cell with no pre-amp



5-plexing with probe mixing

Purified RNA

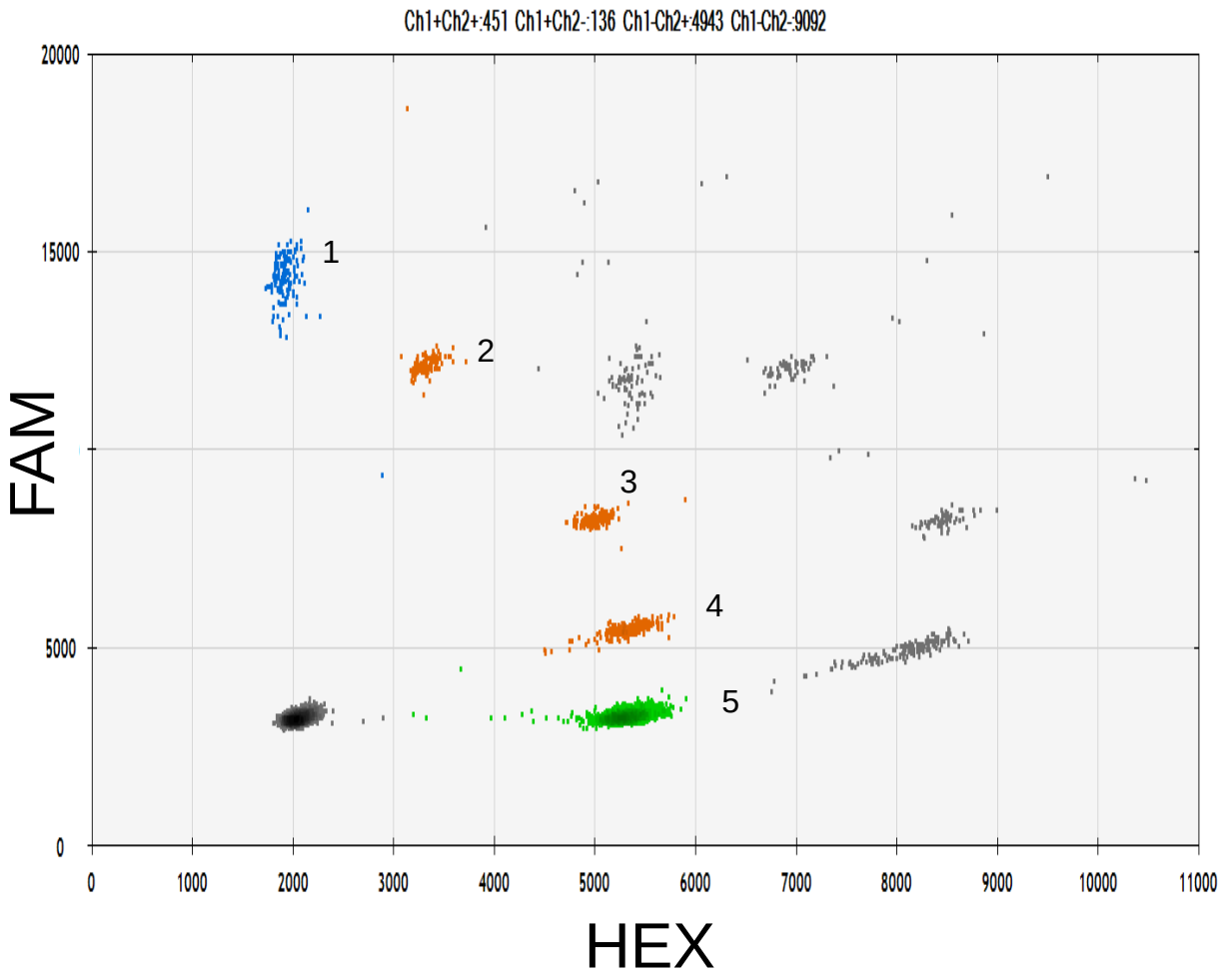
↓ RT reagents

RT reaction

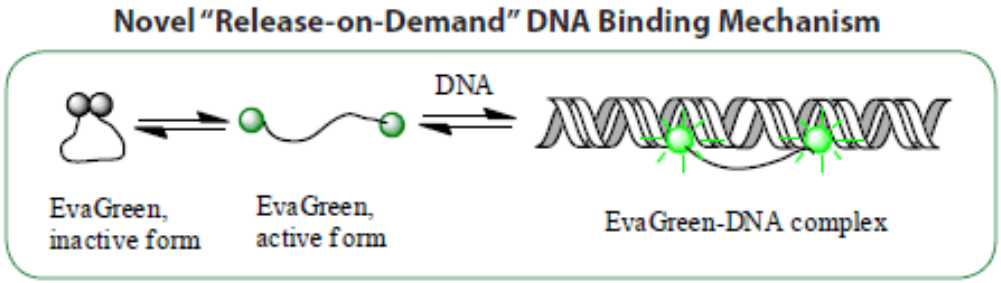
↓

5-plex ddPCR

5plex	Gene	Assay mixing
1	Hoxa1	100% FAM
2	Oct4	75% FAM, 25% Hex
3	Nestin	50% FAM, 50% Hex
4	Sox2	25% FAM, 75% Hex
5	RPLP0	100% Hex



QX200 System Enables dsDNA Detection Capability with EvaGreen (without TaqMan probes)



- No preference for GC- or AT-rich sequence
- Less PCR inhibition than SYBR® Green and lower tendency to cause nonspecific amplification
- Tolerated at a higher concentration, which enables a brighter signal
- Good stability
- Safety
  - Dye is impenetrable to both latex gloves and cell membranes
  - Dye is noncytotoxic and nonmutagenic at concentrations used in the laboratory

High Sensitivity Detection and Quantitation of DNA Copy Number and Single Nucleotide Variants with Single Color Droplet Digital PCR

analytical chemistry

Article

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High Sensitivity Detection and Quantitation of DNA Copy Number and Single Nucleotide Variants with Single Color Droplet Digital PCR

Laura Miotke,[‡,†] Billy T. Lau,[‡,§,†] Rowza T. Rumma,[‡,†] and Hanlee P. Ji*[,‡,§]

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[§]Stanford Genome Technology Center, Stanford University, Palo Alto, California, 94304 United States

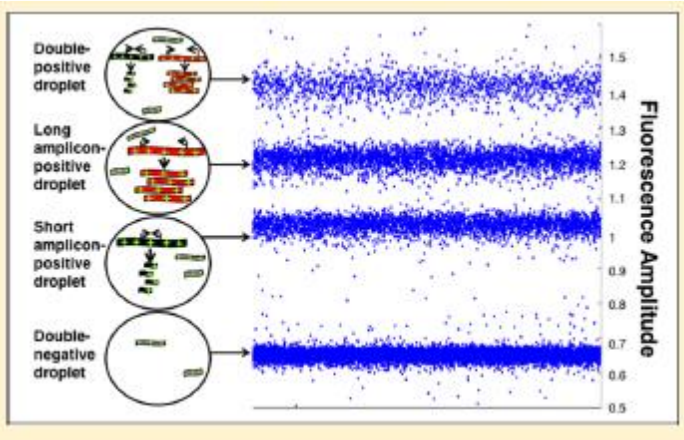
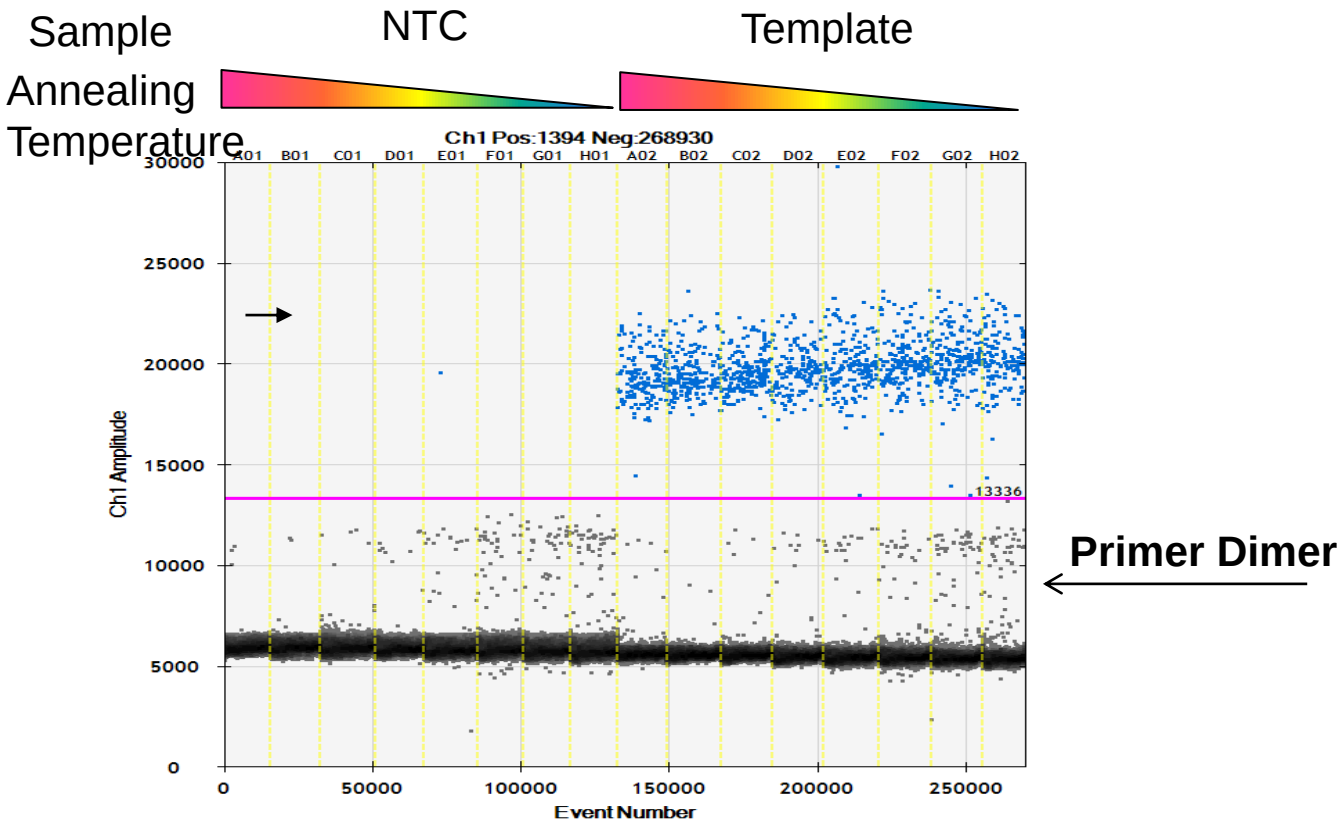


Table 2. Comparison between EvaGreen and TaqMan Mutation Quantification Methods on Control Template DNA

template	source	% mutant	
		Evagreen	TaqMan
18507	normal diploid DNA	0.01	0.00
Human Male Control	normal diploid DNA	0.00	0.00
HT29	cancer cell line	25.38	25.68
LS411N	cancer cell line	67.48	66.36
168B	patient-benign	0.00	0.00
168M	patient-malignant	25.30	27.13

Temperature Gradient: EvaGreen

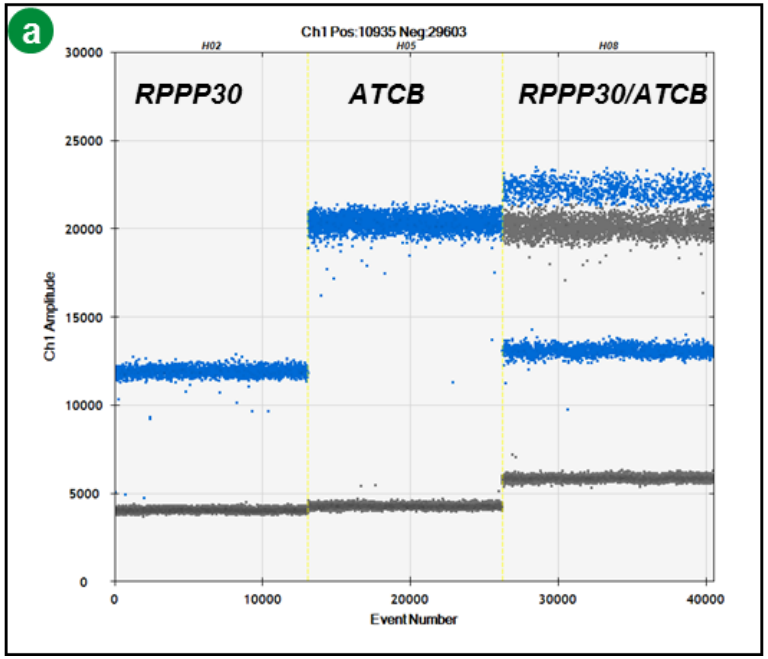
- Primer dimers can be visualized with EvaGreen chemistry
- Primer dimer frequency increases at lower annealing temperature and are visible in NTCs

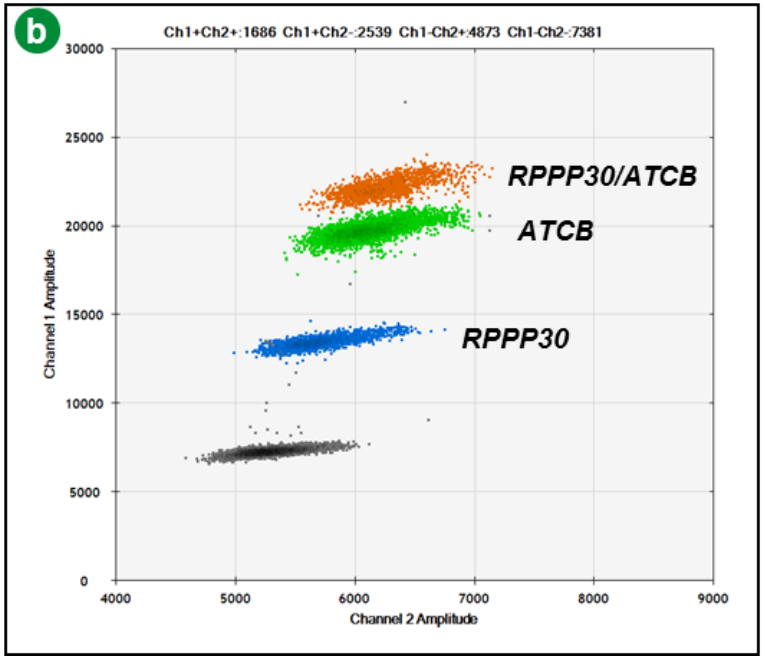


BIO-RAD

Multiplexing with EvaGreen

Multiplexing with EvaGreen by varying amplicon length





RPP30 amplicon = 62 base pairs
ACTB amplicon = 137 base pairs

Selection Criteria for ddPCR Supermixes

Applications and Considerations	ddPCR Supermix for Probes	* ddPCR Supermix for Probes (no dUTP)	* QX200 ddPCR EvaGreen Supermix	One-Step RT-ddPCR Kit for Probes
Suitable for UNG decontamination protocols	✓	–	–	–
Compatible with validated PrimePCR ddPCR mutation detection assays	•	✓	–	–
Compatible with validated PrimePCR ddPCR copy number assays	•	✓	–	–
Compatible with PrimePCR gene expression primer assays	–	–	✓	–
ddPCR library quantification kit for Illumina TruSeq	–	✓	–	–
ddPCR library quantification kit for Ion Torrent	–	✓	–	–
Amplification in droplets for downstream sequencing	–	✓	–	–
Double-stranded DNA detection	–	–	✓	–
Absolute quantitation of target RNA molecules	–	–	–	✓

✓ Recommended supermix
• Compatible supermix

PrimePCR™ ddPCR™ Assays

- Designed for digital PCR, fully validated assays
- Can be used on the QX200 ddPCR system
- Universal cycling conditions and primer/probe design strategy
- Universal restriction enzyme strategy for copy number assays
- World class design & manufacturing expertise

Mutation Detection Assays

- Targets for most frequent mutations in COSMIC
- >2500 target with matching wild type assays

BRAF		EGFR		HRAS		KRAS	
V600E	P367R	T790M	L858R	G12V	G13R	G12D	G12S
V600V	F595S	E746 A750	L858M	G12A	G13S	G12A	G13D
S616F	T599I	G719D	H835L	G12R	Q61K	G12V	G13A
D494N	G460G	L747S	E709A	G13V	Q61H	G12C	K5N
K601N	I326T	L861R	P848L	G13C	H27H	G12R	A146T

Copy Number Assays

- All high-value targets in Cancer & Neuro
- >700 target assays + 2 reference assays

EIF2C1

AP3B1

APC	AR	ARID1A	ATM	BIRC2	BRCA1	BRCA2	CCND1	CCND2	CCNE1	CDK4
CDK6	CDKN1A	CRKL	CSMD1	DCUN1D1	DEFB119	EGFR	ERBB3	FGFR1	FGFR2	FOXO1
GAB2	GRB2	HMGA2	IGF1R	IRS2	JUN	KDM6A	KIT	KRAS	MAGI3	MAP2K4
MDM2	MELK	MET	MTAP	MYB	MYC	MYCN	NCOA3	NCOR1	ORAOV1	PARK2
PDGFRA	PIK3CA	PPP2R1A	PSEN1	PTEN	RB1	REL	RPS6KB1	SHH	SKP2	SLIT2
		SMAD4	TERT	TSC1	TSC2	WHSC1L1	WISP1	YAP1		

You can use our look up tool to see if we have it or not:
www.bio-rad.com/primepcr_lookup.

Digital Biology Center

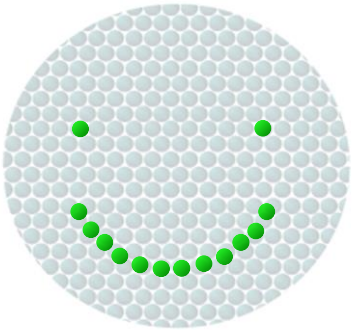
BIO-RAD

Publication Database

https://www.bio-rad.com/ddPCR/publications

Best Reasons to use ddPCR

- **Sensitivity**
  - 10-1000x fold improvement over qPCR
  - Works with FFPE blood and tissues samples
- **Absolute Quantification**
  - Quantify gene expression more precisely
  - No Standard Curve
- **Precision**
  - Measure more subtle differences in expression or mutation
  - Detect structural variants in cancers
- **High Throughput**
  - Maximum 4 x 96 per day
  - Save $ and Time



Q&A

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