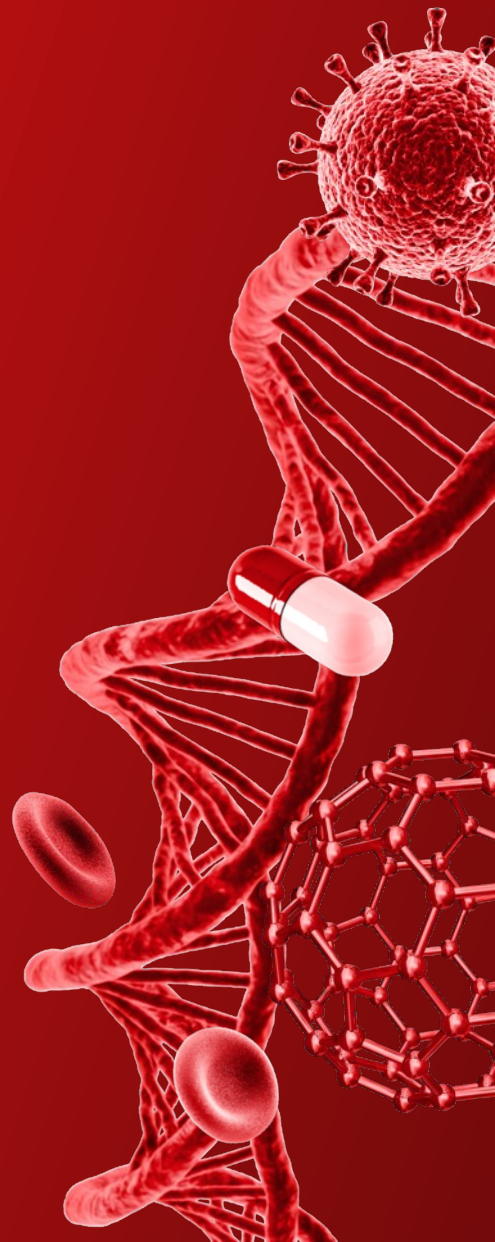
Welcome

The presentation will start shortly

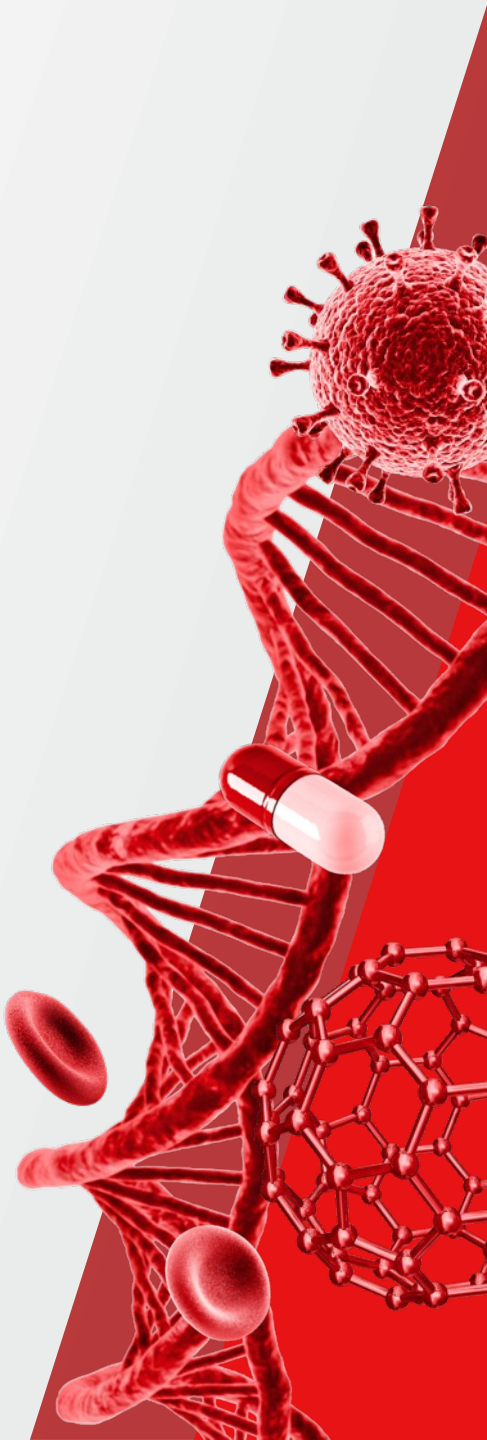


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Introduction to Digital PCR (dPCR)

QuantStudio Absolute Q™ Instrument SmartStart Orientation

Anton Borissenko
Field Applications Scientist



The world leader in serving science

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Agenda

1 What is digital PCR?

2 Real-Time PCR compared to digital PCR

3 Poisson distribution

4 Key Performance attributes of digital PCR

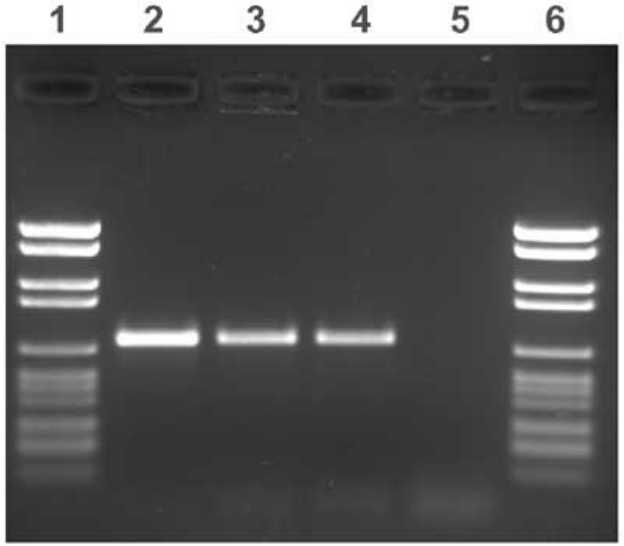
5 dPCR Applications Overview

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What is Digital PCR (dPCR)?

Digital PCR is a method of quantifying nucleic acid targets without standard curves by dividing the bulk reaction into thousands of smaller, independent reactions.

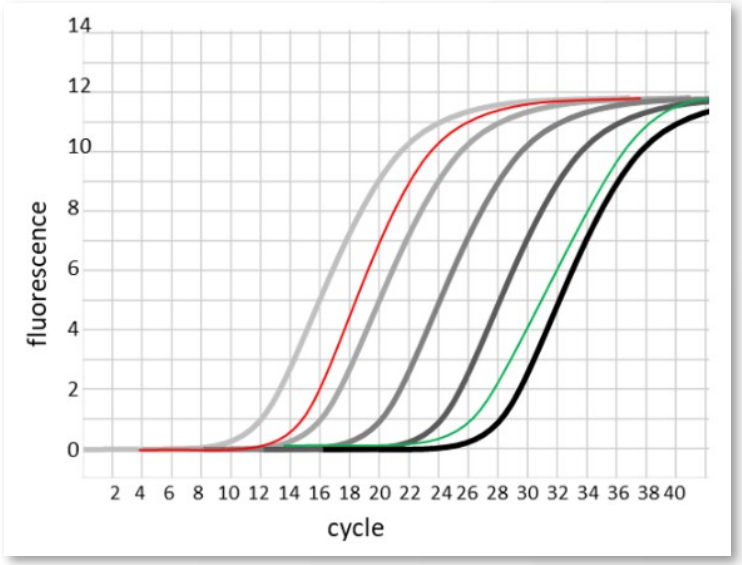
Traditional PCR



Endpoint

Qualitative / Semi-quantitative

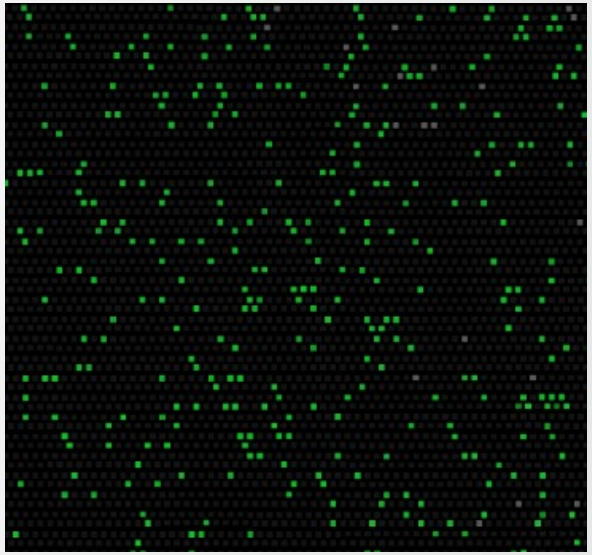
Quantitative PCR (qPCR)



Real-time analysis

Relative quantification

Digital PCR (dPCR)



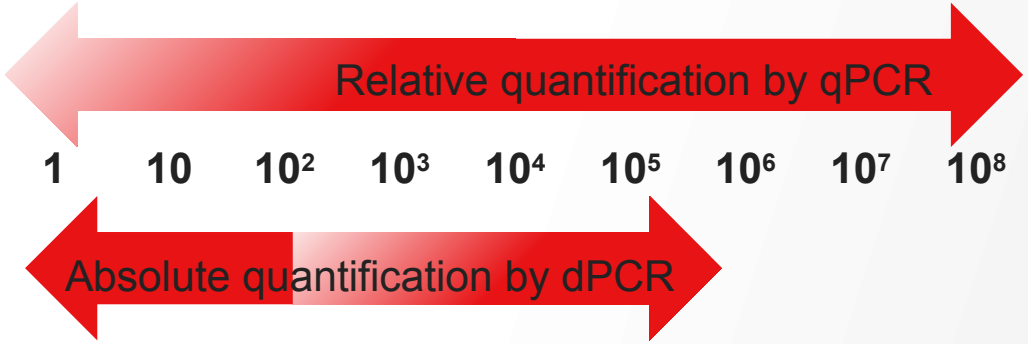
Endpoint

Absolute quantification

chambers → copies/uL

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digital PCR compared to real-time PCR



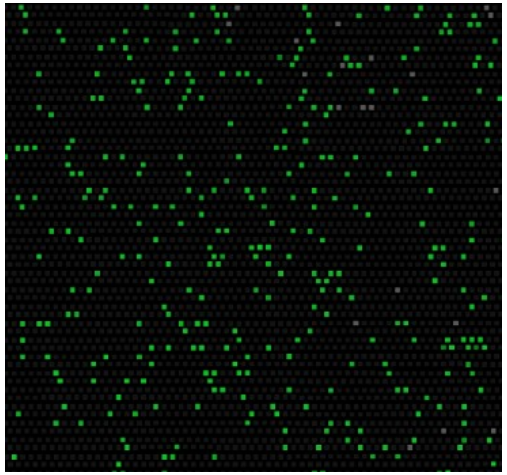
Digital PCR:

Absolute quantification

Quantifying low-abundance targets

High precision to monitor small changes

Fewer samples / targets



Quantify standards

dPCR is **complementary** to qPCR

Assay development

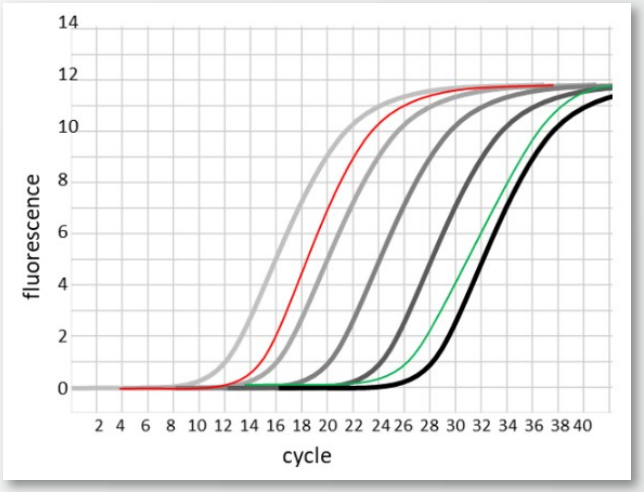
Real time PCR:

Relative quantification

Larger dynamic range

Qualitative presence/absence

Higher-throughput analysis



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digital PCR compared to real-time PCR

Digital PCR Strengths

Absolute quantification of target(s)

Improved accuracy and precision over qPCR

No standard curve required

More reproducible from lab to lab
(directly measure target concentration)

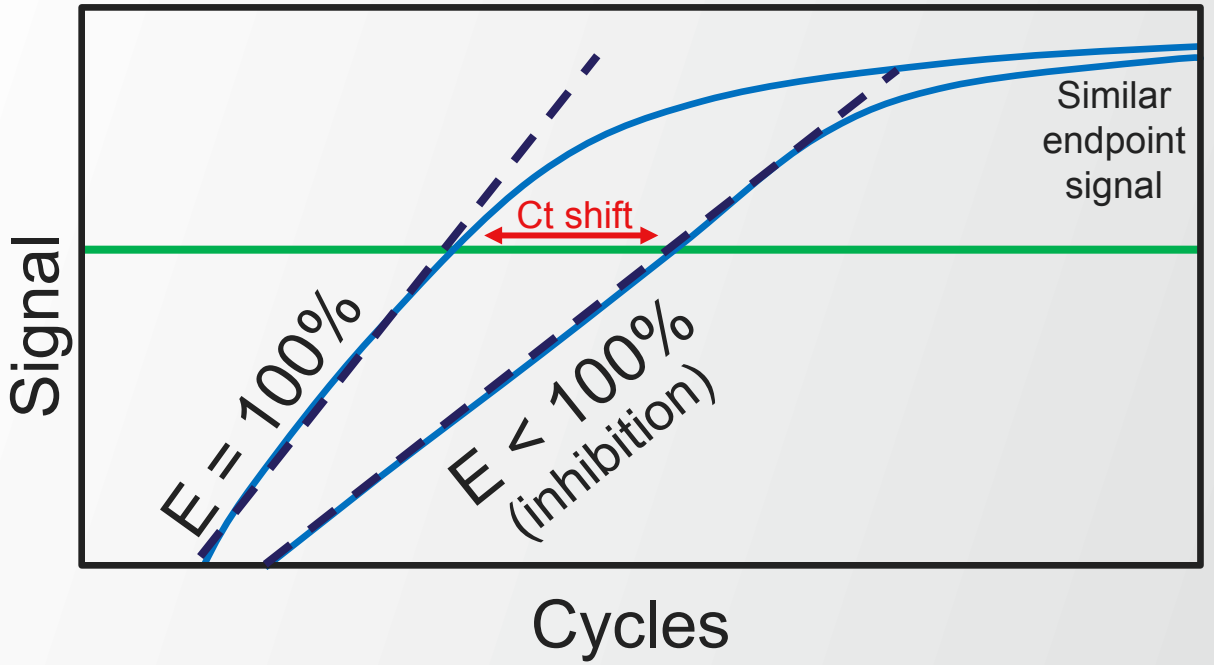
Reduced impact of inhibitors

Endpoint analysis of data post-amplification

Detecting lower concentrations

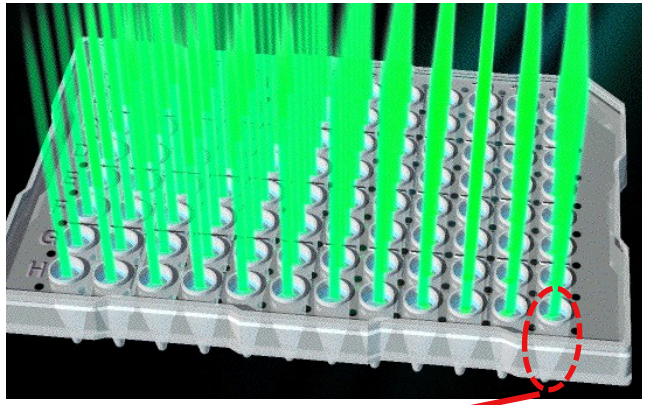
Provides higher statistical power to quantify low concentration samples

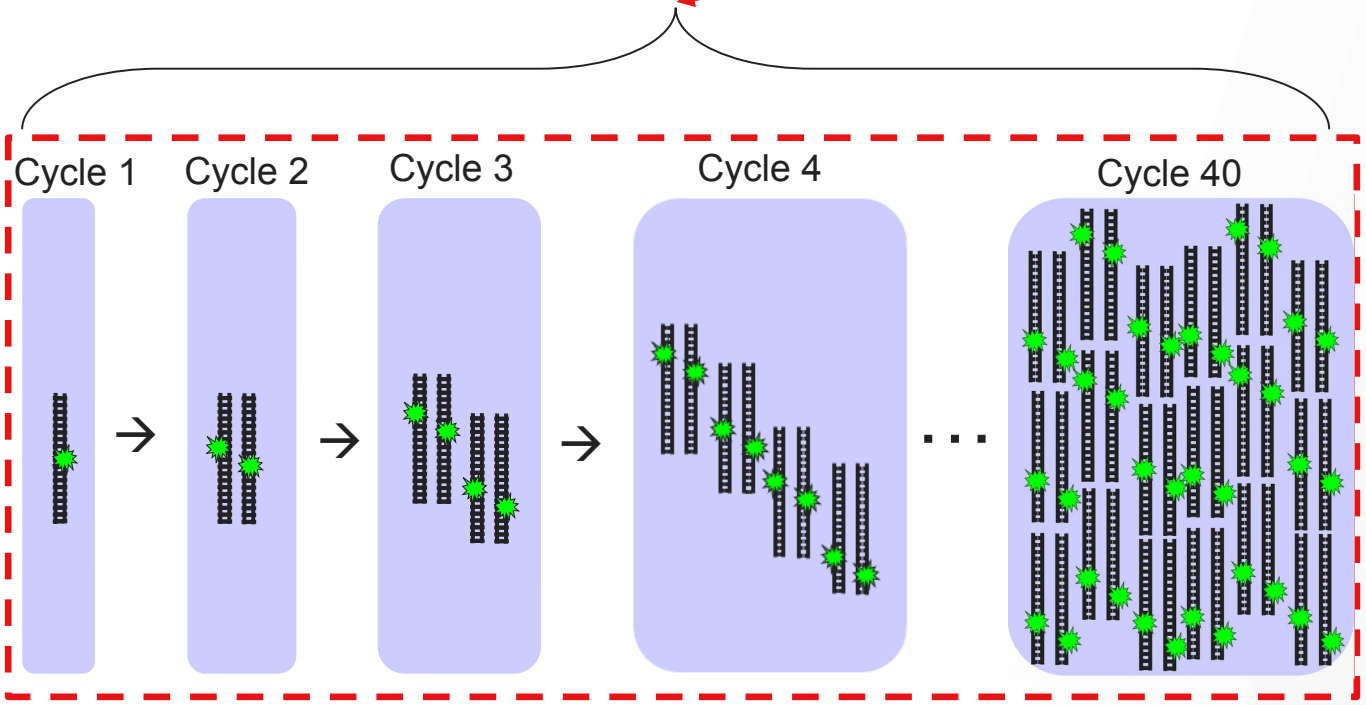
- Digital PCR does not rely on PCR efficiency
- Endpoint assessment of reactions (positive vs negative)
- Easier to multiplex with digital PCR



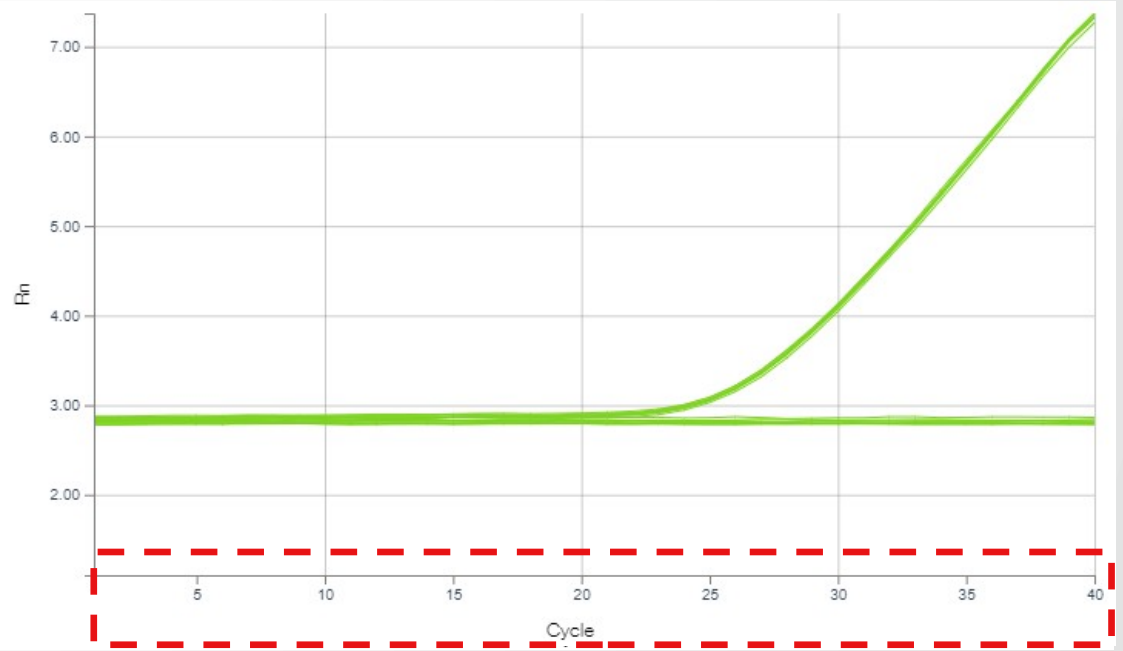
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Data generation in Real-Time PCR



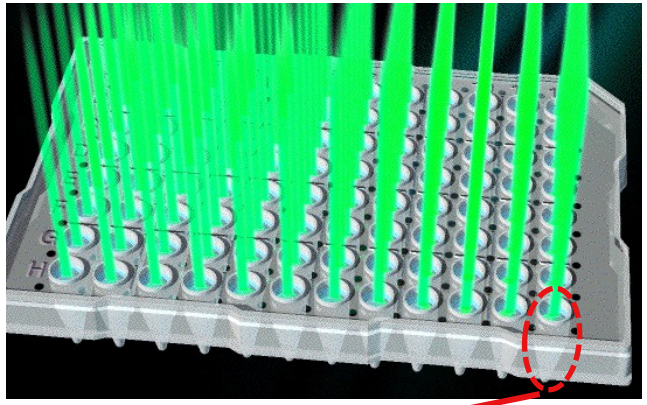


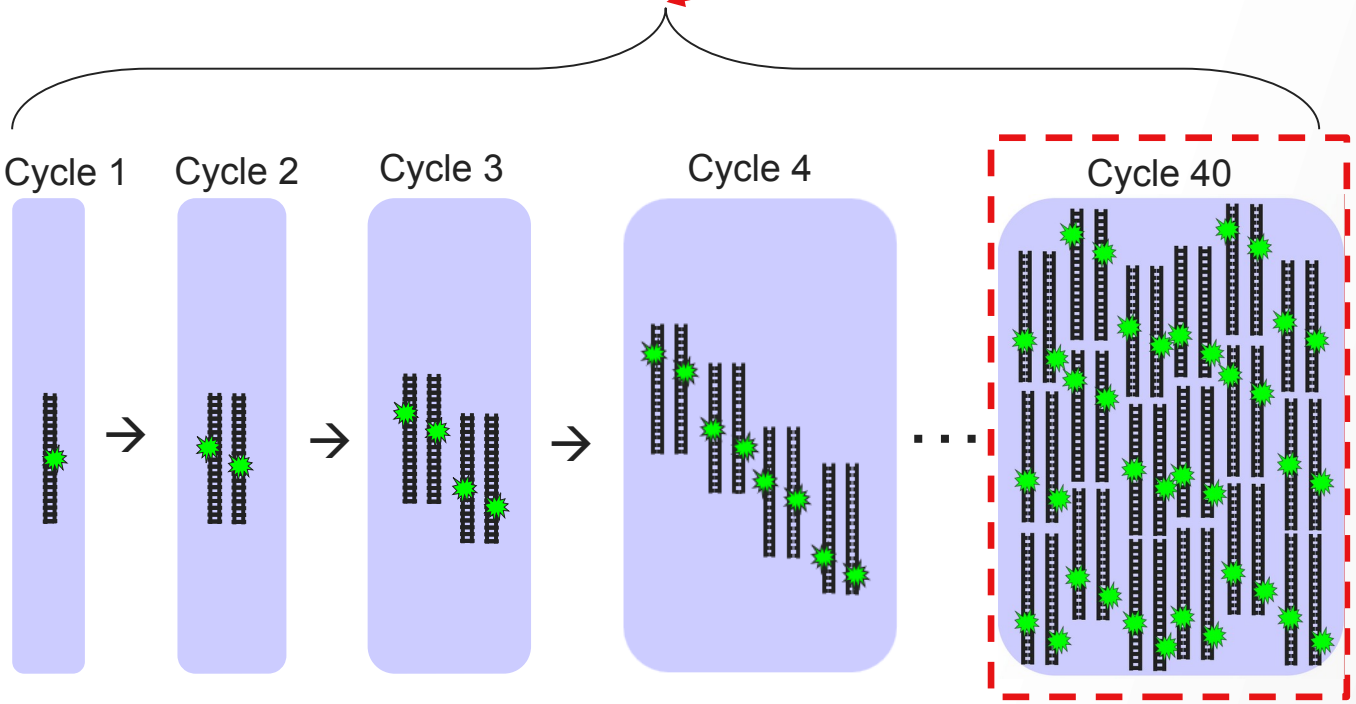
- Fluorescence is measured throughout PCR reaction (i.e., in real-time)
- Provides insight about relative concentrations
- Can ‘see’ reaction dynamics



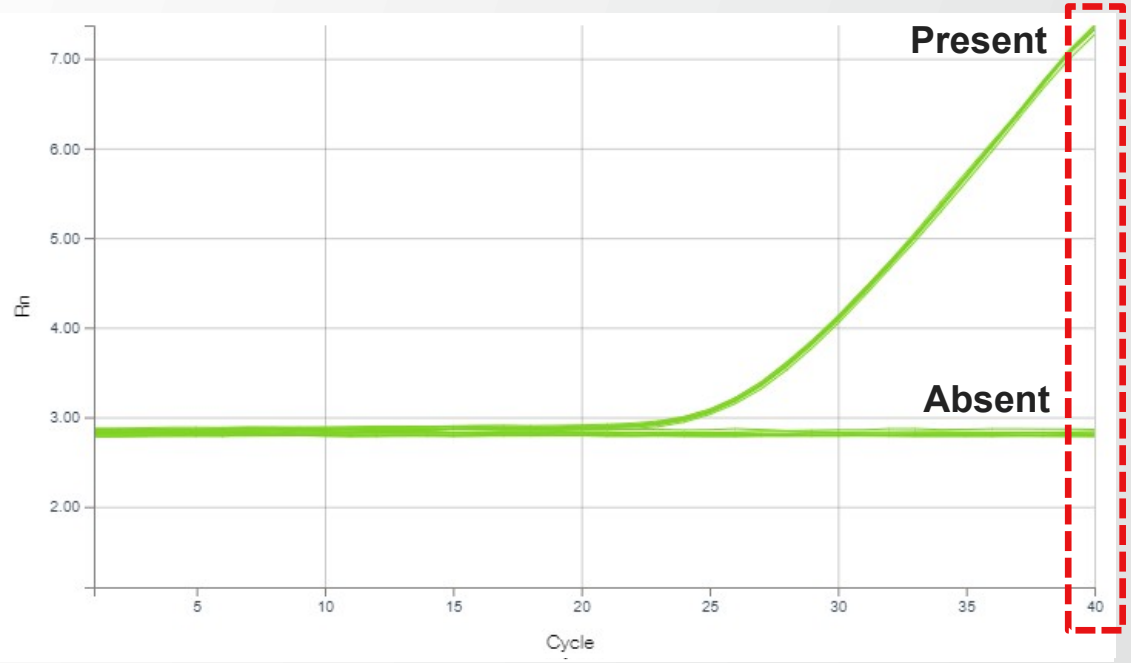
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Real time Data vs Endpoint Data



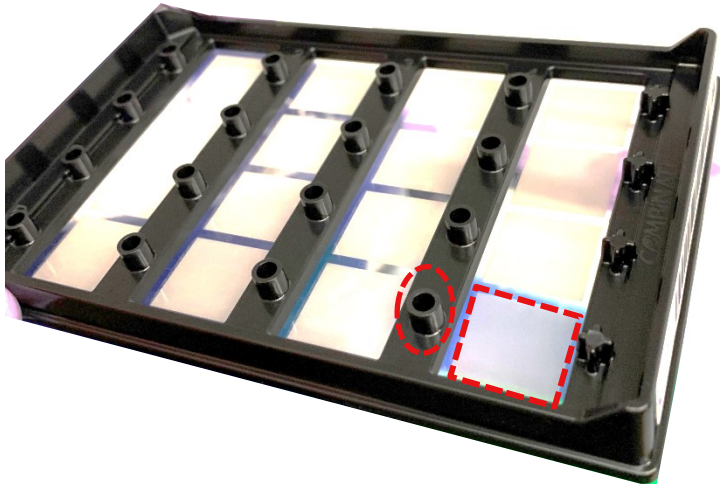


- To discern if target was simply present or absent from a reaction, only need the endpoint data, not the entire amplification curve

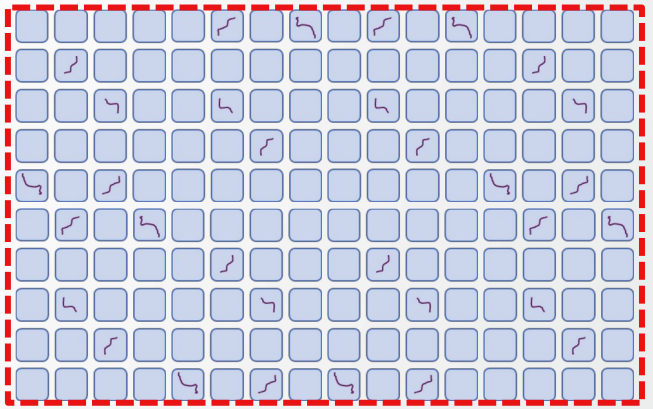


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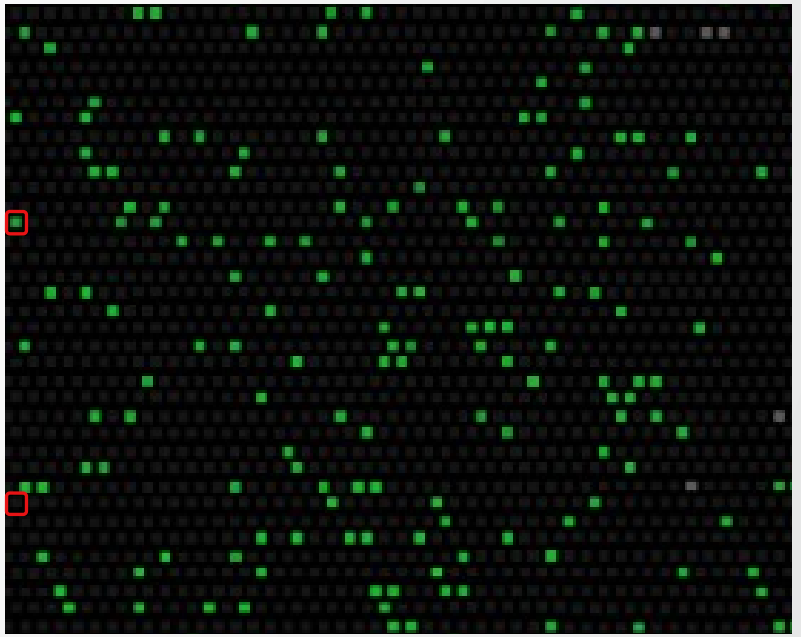
Data generation in Digital PCR



Separate into **MANY** sub-reactions



Endpoint measurement of fluorescence



Cycle 1

Target present

Target absent

PCR Cycling

Cycle 40

High signal

Low signal

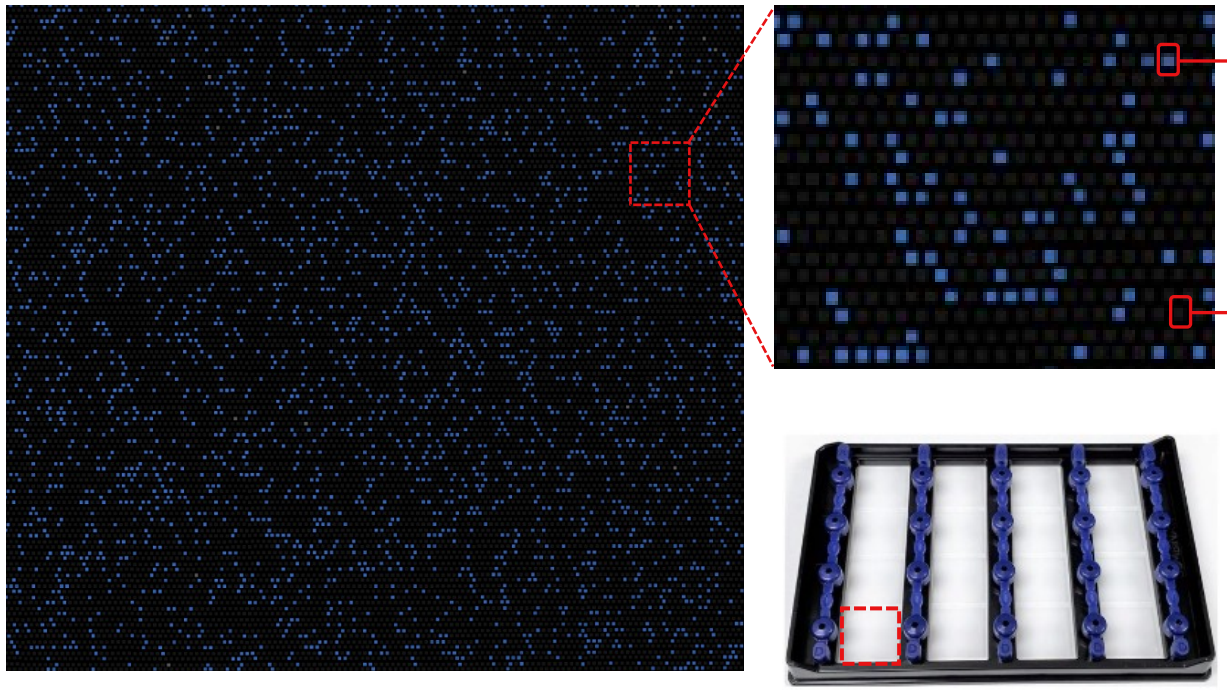
Higher target concentration → More positive reactions

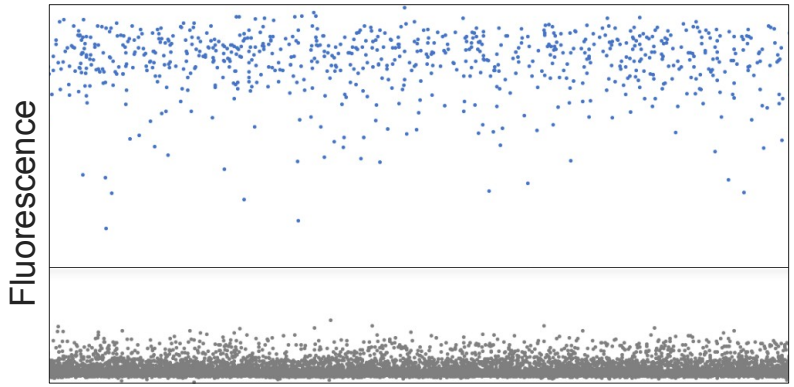
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Digital PCR Leverages Simple Counting for Quantification

- Microchambers with target present distinguished from microchambers without target
- Number of high & low fluorescence microchambers used to determine the **absolute quantity** of the target

20,480 microchambers per array





High fluorescence (target present)

Low fluorescence (target absent)

Positives	Total	**Conc. cp/uL**
3,630	20,466	**451.96**
Number of positive microchambers	Number of microchambers accepted	Absolute concentration of target in reaction

But… 3,630 positives / 9 uL would equal "*403 cp/uL*"…?

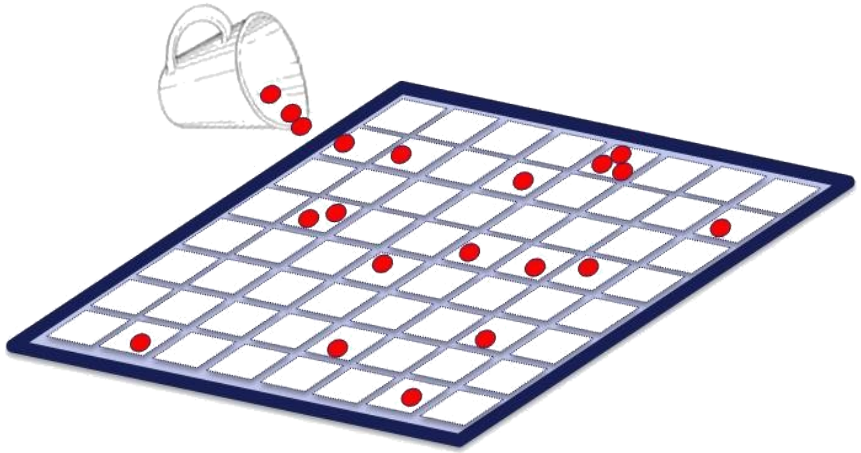
Different answer because 1 fluorescent chamber ≠ 1 molecule

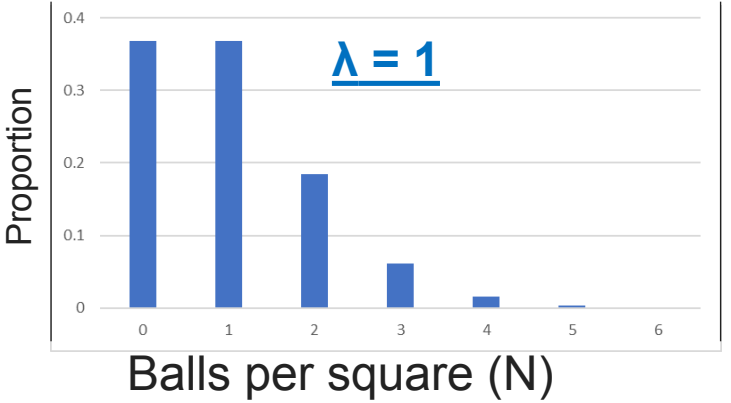
Need to use "Poisson Statistics"

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Poisson Distribution example

- Randomly disperse balls into grid
- *Average "concentration"* of 1 ball per square ("λ")
- We don't expect to see exactly 1 ball within each square
- Instead, expect to see 0, 1, 2, 3,… balls per square

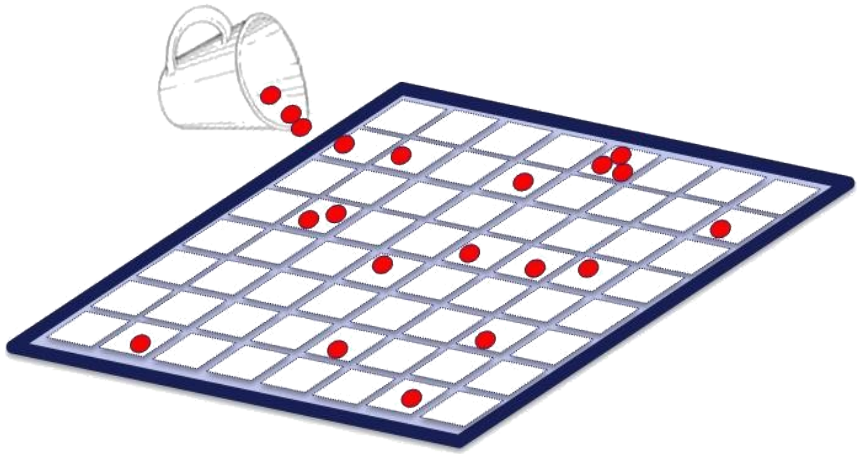


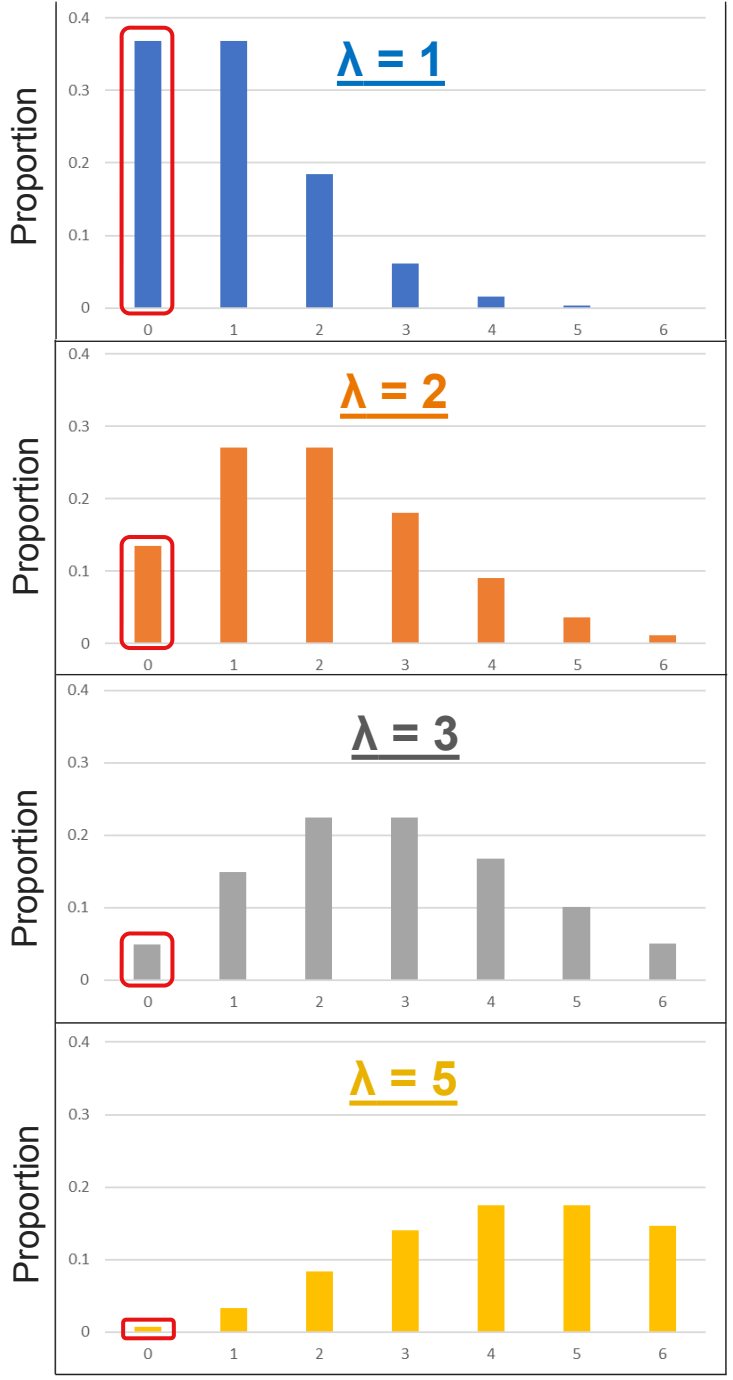


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Poisson Distribution example

- Distribution of balls per square changes based on λ
- Proportion of empty (N = 0) decreases with increasing λ

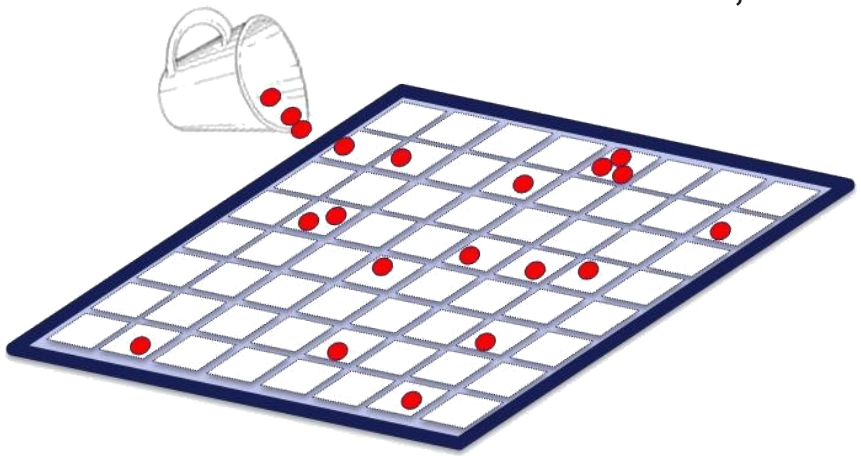


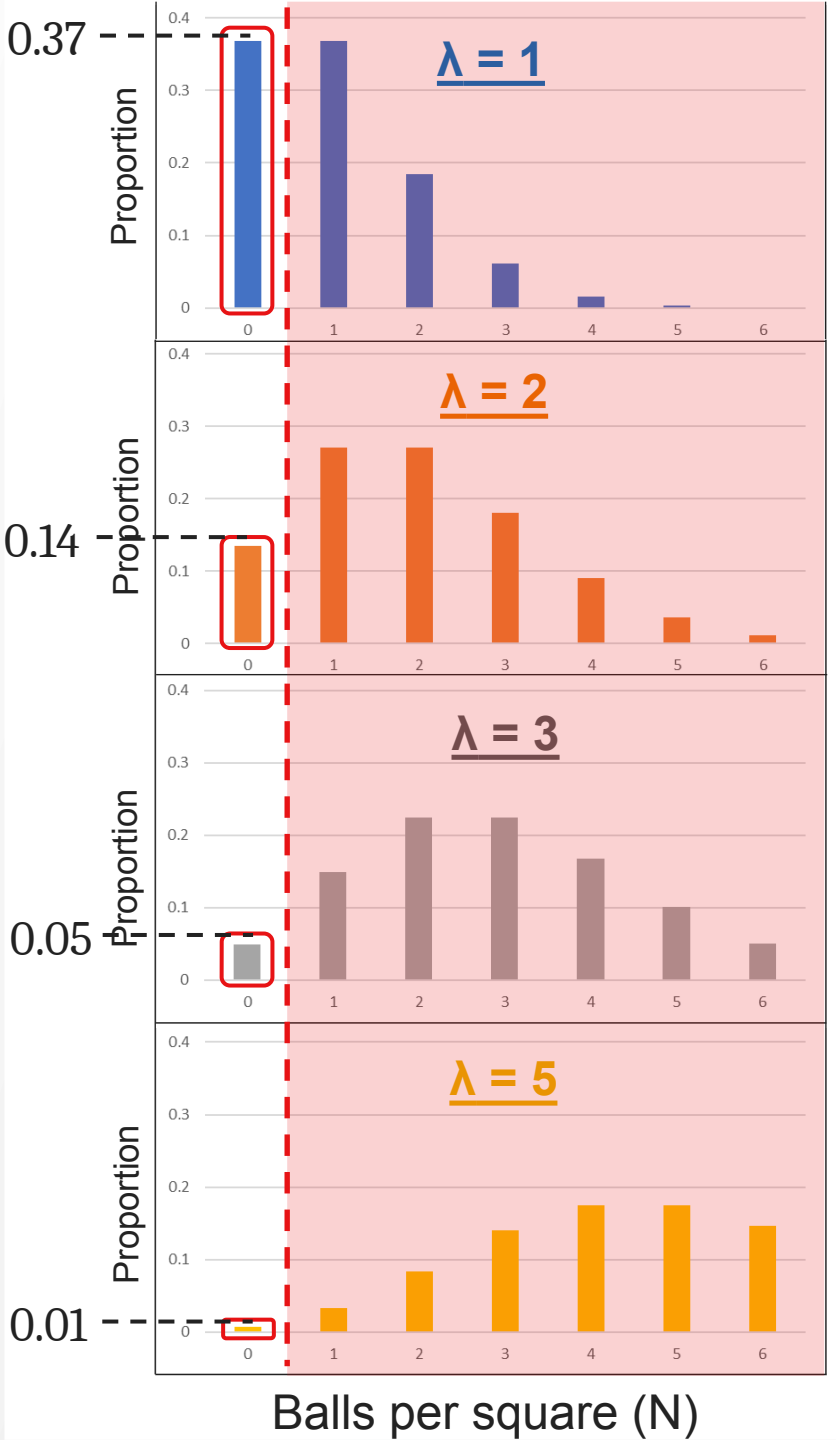


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Poisson Distribution example

- Distribution of balls per square changes based on λ
- Proportion of empty (N = 0) decreases with increasing λ
- In dPCR we only distinguish **empty** versus **not-empty**
  (N = 0) **(N ≥ 1)**
- We are “blind” to the distribution, but can identify the empty

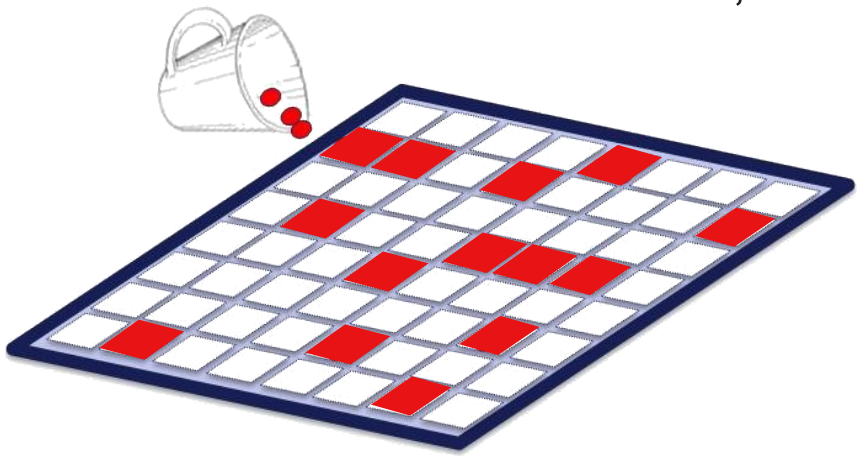




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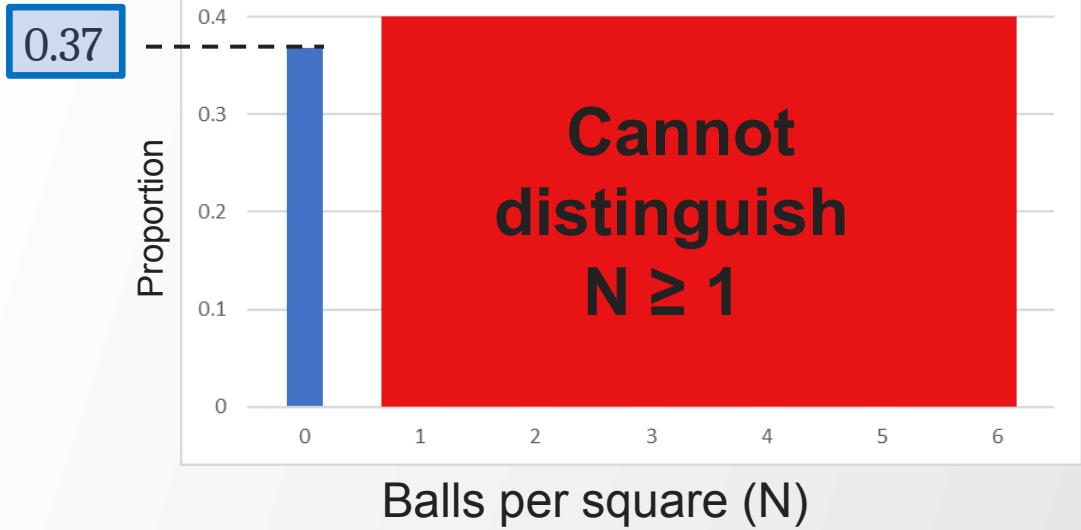
Poisson Distribution example

- Distribution of balls per square changes based on λ
- Proportion of empty (N = 0) decreases with increasing λ
- In dPCR we only distinguish **empty** versus **not-empty**
  (N = 0) **(N ≥ 1)**
- We are "blind" to the distribution, but can identify the empty



- Measure the proportion of empty to estimate λ value
- In dPCR, convert λ to concentration:

$$\frac{1\,\text{copy}}{\text{microchamber}} \times \frac{\text{microchamber}}{432\,\text{pL}} \times \frac{10^6\,\text{pL}}{1\,\text{uL}} = \mathbf{2{,}315\ cp\ /\ uL}$$



Calculating balls per square (λ)

$$\lambda = -\ln(\textit{Prop empty})$$

$$\lambda = -\ln(0.37)$$

$$\lambda = 1\,\text{ball/square}$$

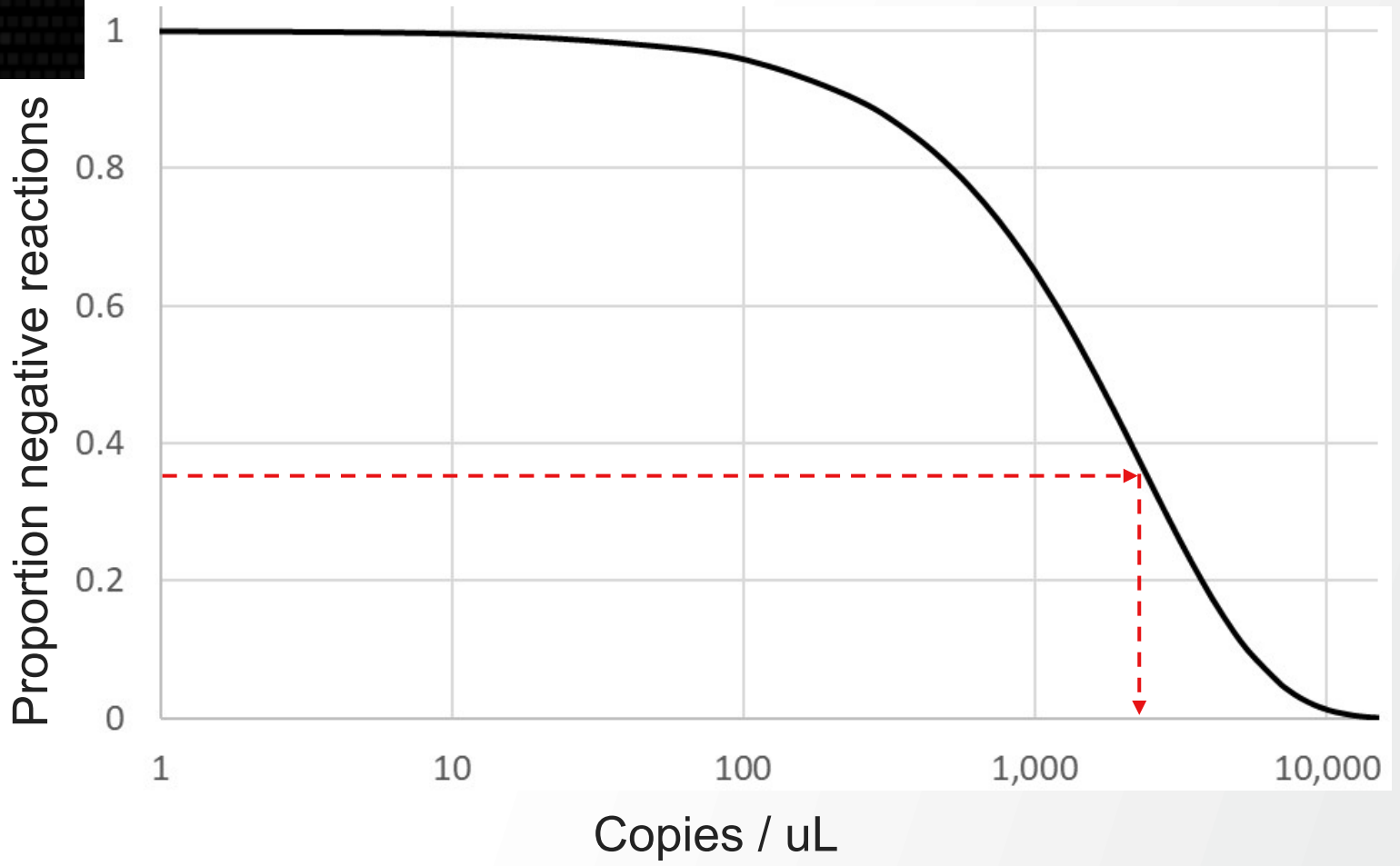
Key takeaway:
There is a direct relation between the *proportion of empty* microchambers, and the *concentration* of target

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Negative Reactions and Average Copies per Reaction

The Poisson Statistics describes relationship between negative reactions and concentration





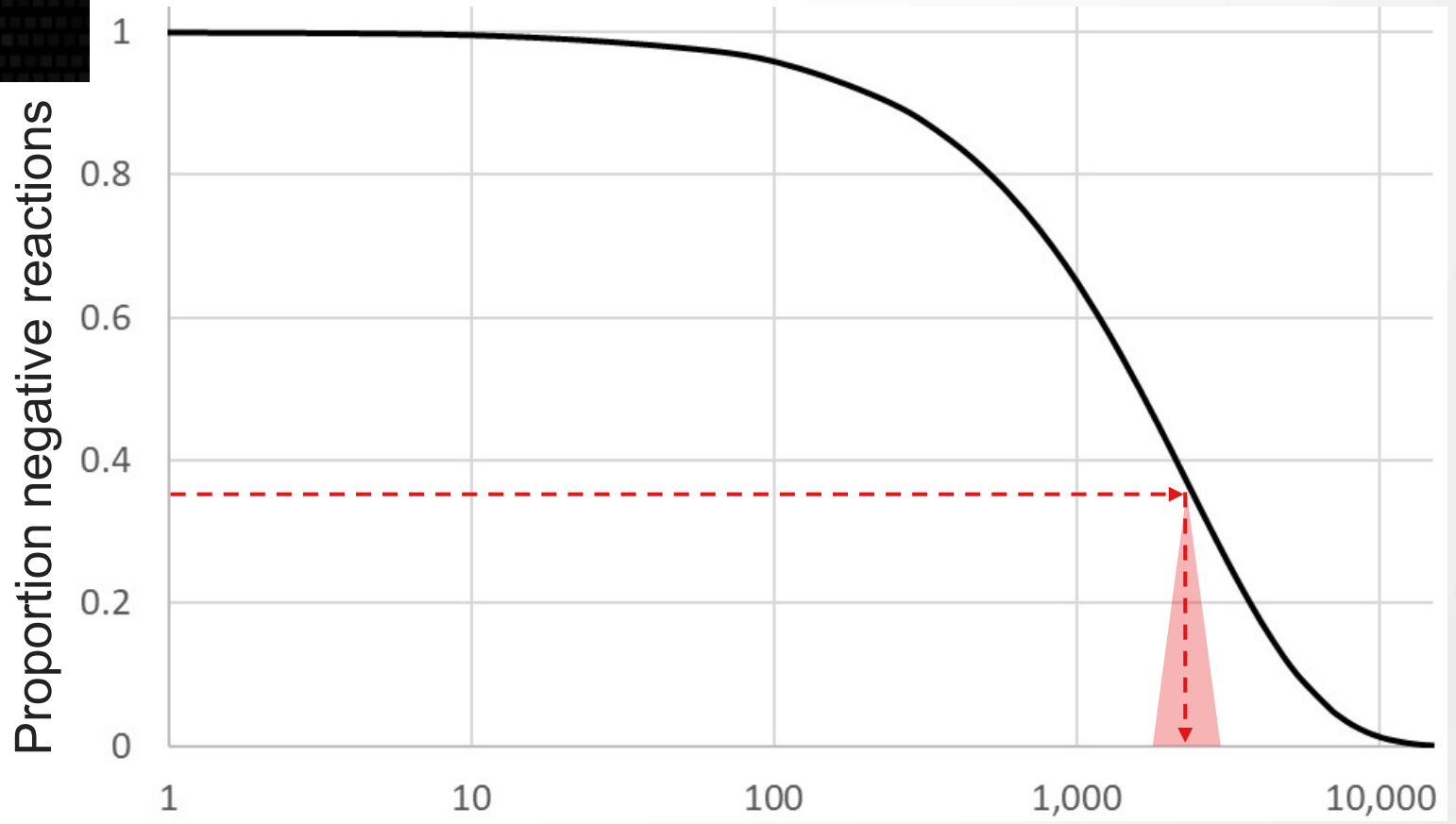


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Calculating Precision for dPCR Results

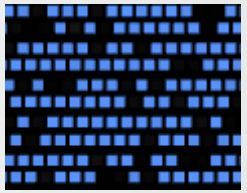
The Poisson Statistics describes relationship between negative reactions and concentration





Cp/uL	95% C.I.
2,176	(39.2,40.0)

Precision
1.84%



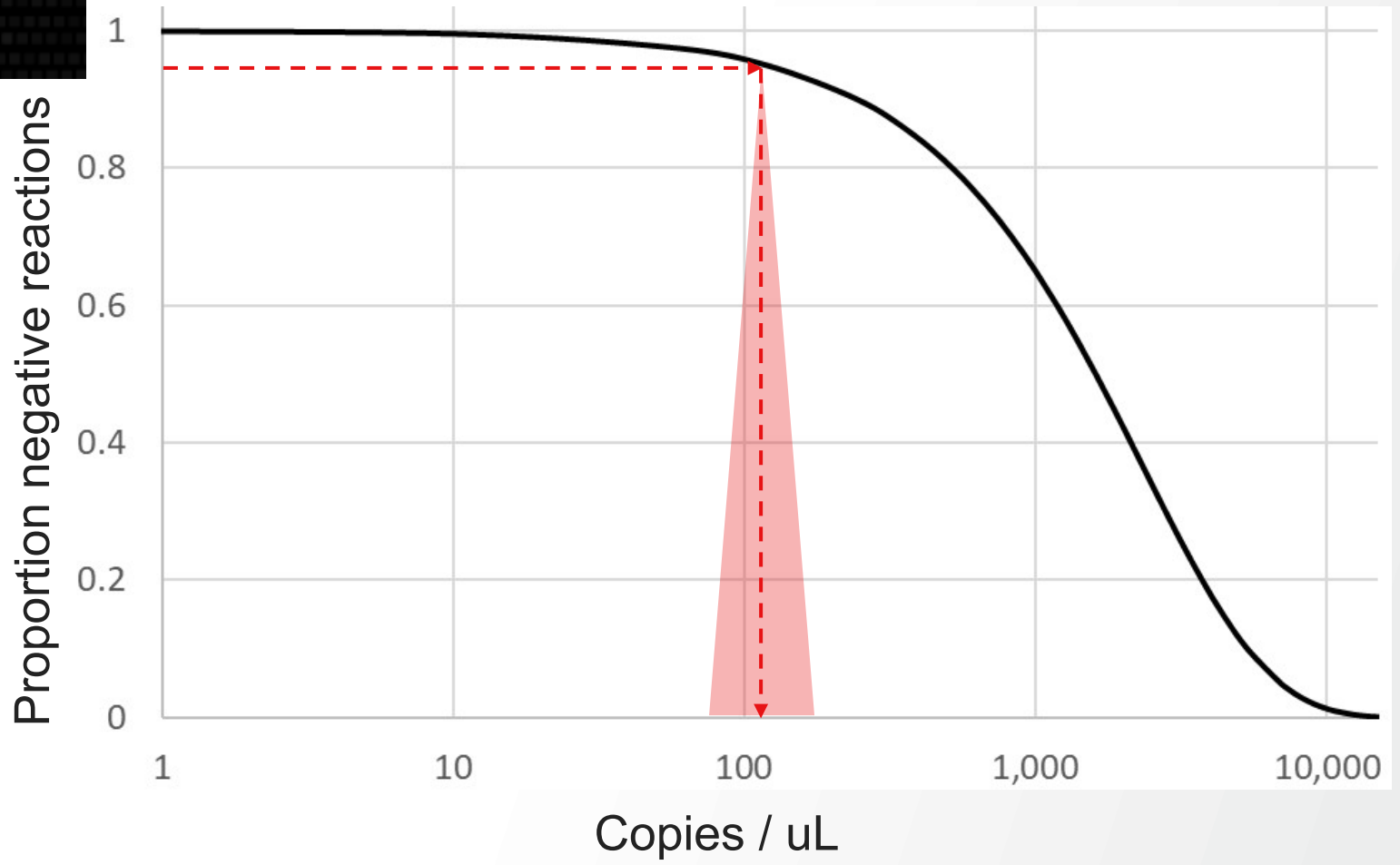
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Precision defined as: 100% * (Upper 95% Confidence Interval) / Concentration

Calculating Precision for dPCR Results

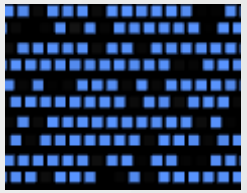
The Poisson Statistics describes relationship between negative reactions and concentration





Cp/uL	95% C.I.
125	(7.26,7.71)

Precision
6.15%



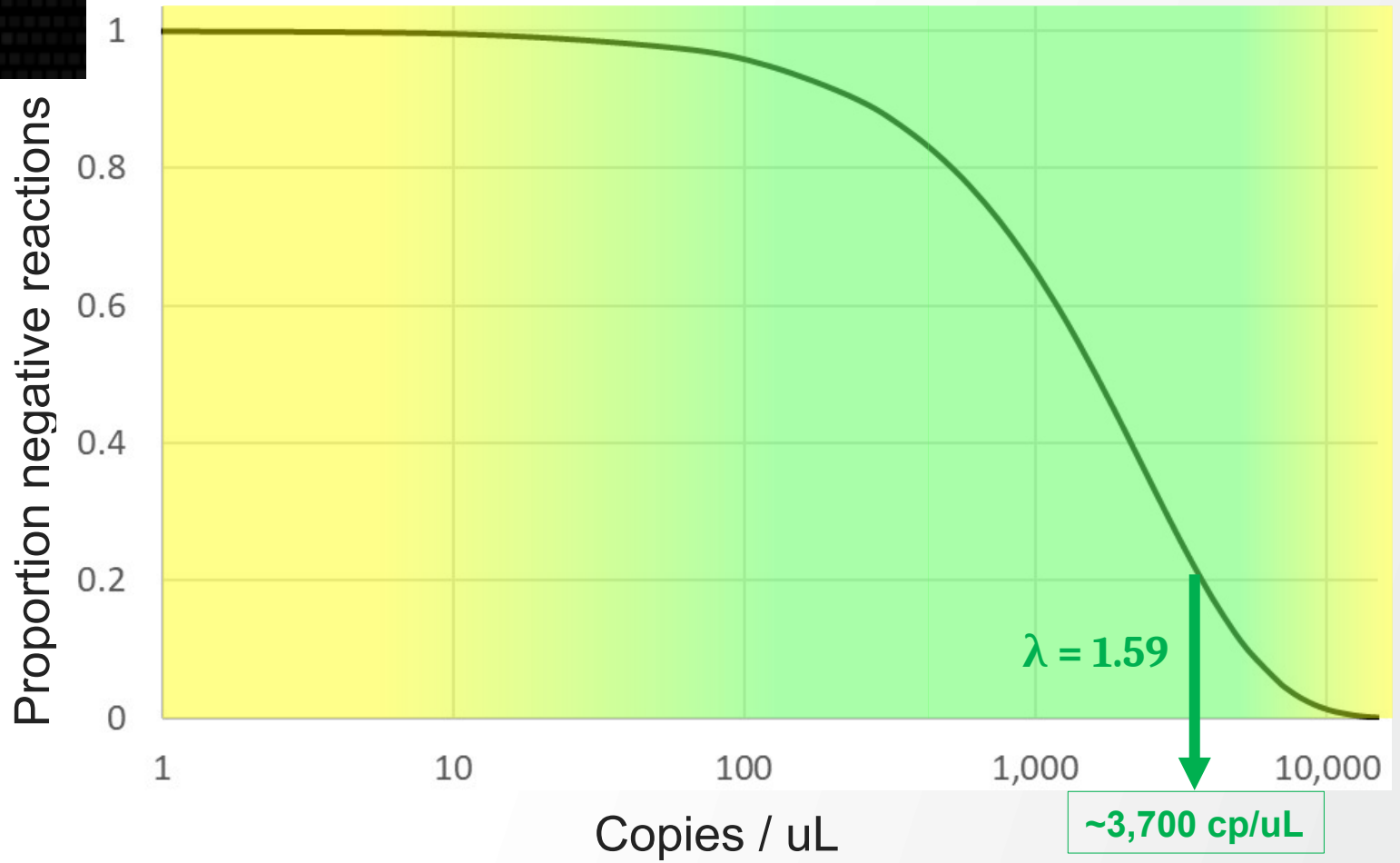
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Precision defined as: 100% * (Upper 95% Confidence Interval) / Concentration

Calculating Precision for dPCR Results

The Poisson Statistics describes relationship between negative reactions and concentration

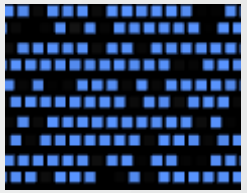




"sweet spot"
(optimal precision)

Cp/uL	95% C.I.
3,691	62.3,63.4

Precision
1.72%



Precision defined as: 100% * (Upper 95% Confidence Interval) / Concentration

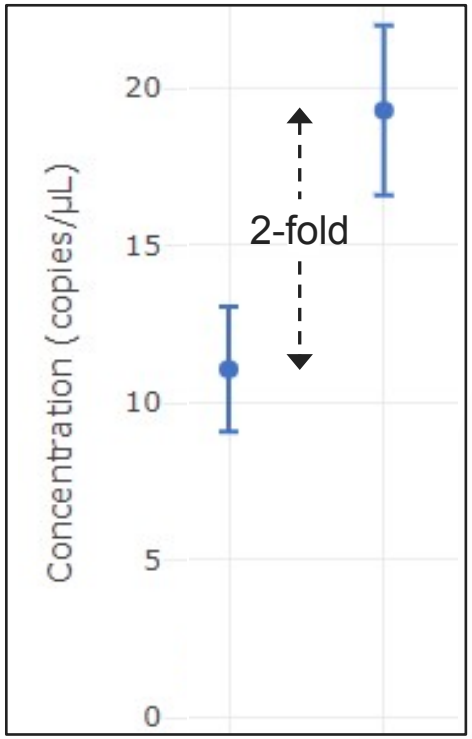
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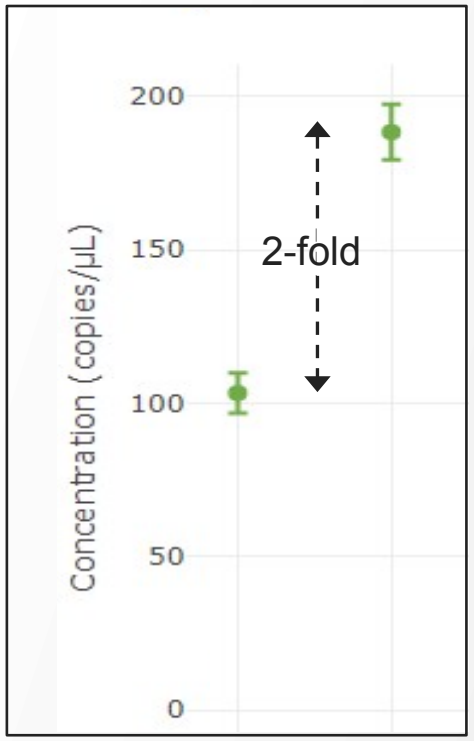
Precision levels

What precision level is “acceptable”?

….it depends!
What precision does ***your*** experiment need?

Lower resolution comparison





Higher resolution comparison

Notice the size of the error bars relative to the difference of the two estimates

Ability to reliably and accurately detect smaller differences requires better precision

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Estimating target abundance

Less precision

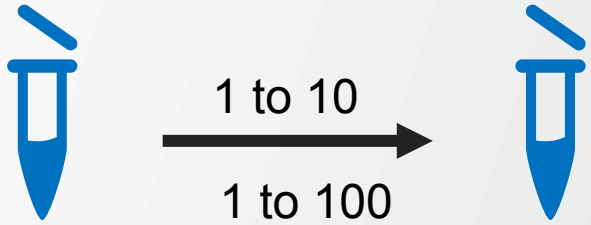
Ct range		Copies
40	35	1
39	34	2
38	33	4
37	32	8
36	31	16
35	30	32
34	29	64
33	28	128
32	27	256
31	26	512
30	25	1024
29	24	2048
28	23	4096
27	22	8192
26	21	16384
25	20	32768
24	19	65536
23	18	131072
22	17	262144
21	16	524288
20	15	1048576
19	14	2097152

"Sweet spot"

Above dynamic range

- Precision is directly related to concentration
- Can use Ct values to roughly approximate concentration of target
- For genomic DNA targets, can approximate target abundance from DNA concentration

  (Based on genome size and copies per genome)
- If no, or limited, prior knowledge of target abundance, can run multiple dilutions of sample



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Common dPCR applications and research areas

dPCR applications



Absolute quantification



Copy number variation



Rare target detection



Gene expression analysis



Molecular integrity (linkage analysis)



Drop off assay

Common dPCR use cases



Validate Standards



Cancer research



Cell and gene therapy development



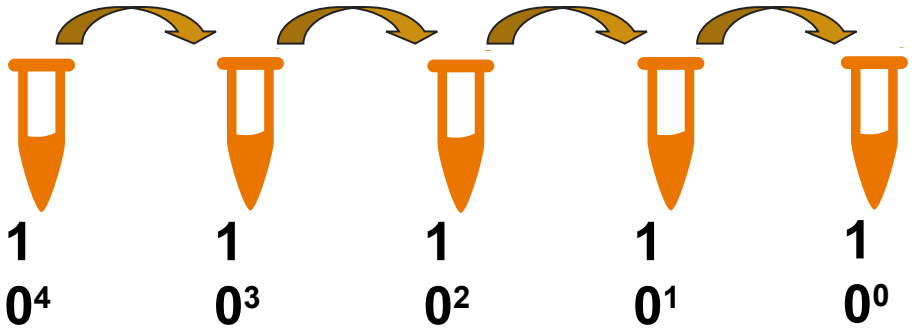
Water and environmental testing

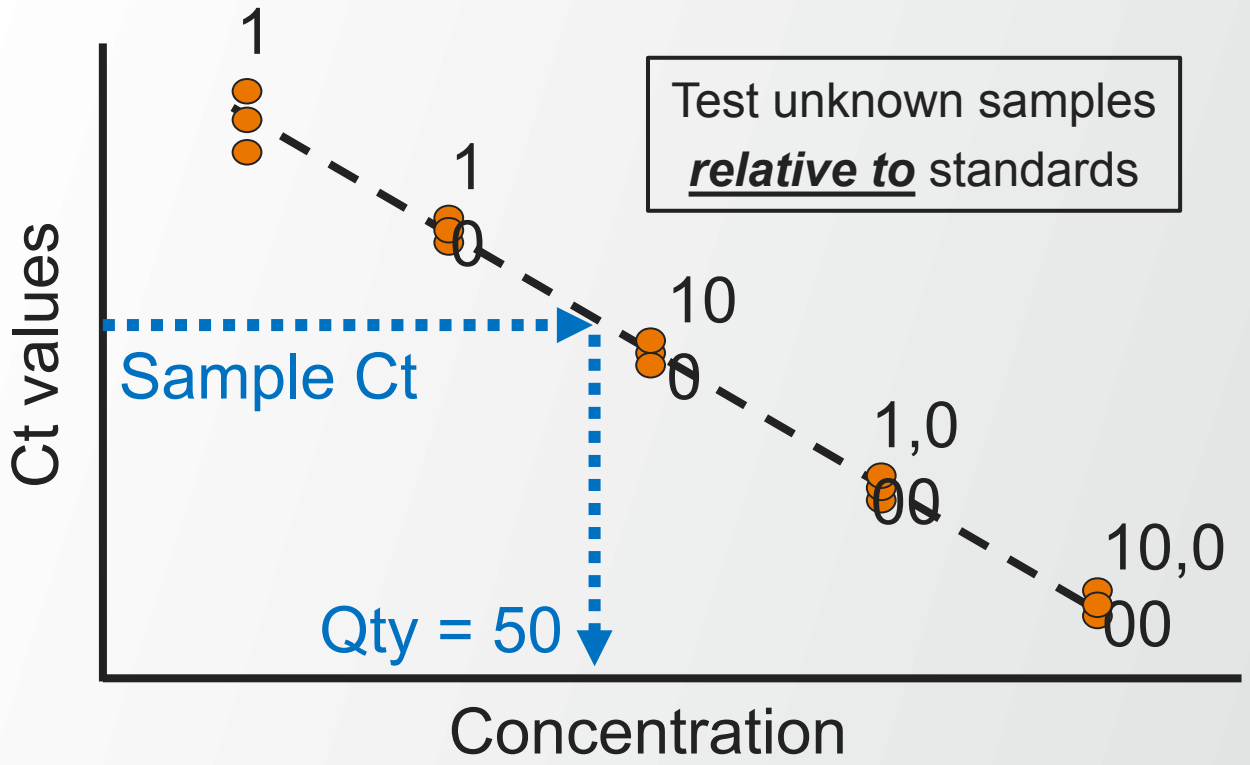
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Absolute Quantification

- dPCR is and accurate and efficient method for determining absolute concentrations of nucleic acids
- Absolute Quantification can be performed using qPCR, but includes potential hurdles…

	1	2	3	4	5	6	7	8	9	10
A	S 1E4	S 1E4	S 1E4	S 1E3	S 1E3	S 1E3	S 100	S 100	S 100	S 10
B	S 1	S 1	S 1	U Sample 1	U Sample 1	U Sample 1	U Sample 2	U Sample 2	U Sample 2	U Sample 3
C	U Sample 4	U Sample 4	U Sample 4	U Sample 5	U Sample 5	U Sample 5	U Sample 6	U Sample 6	U Sample 6	U Sample 7

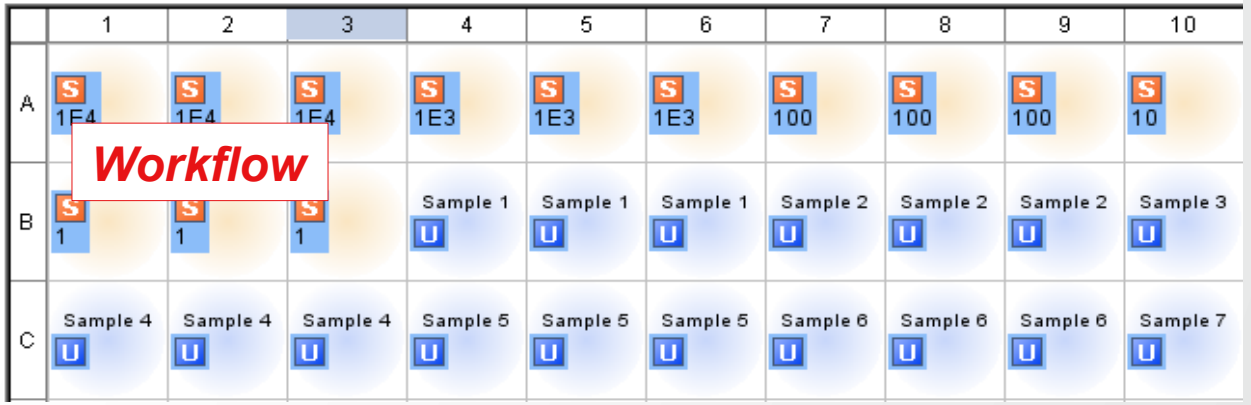


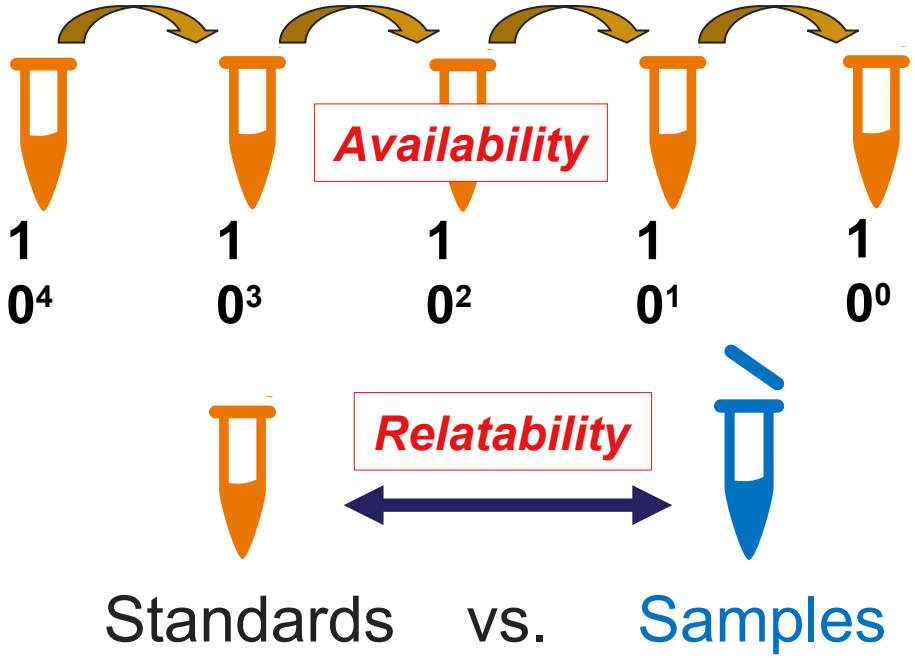


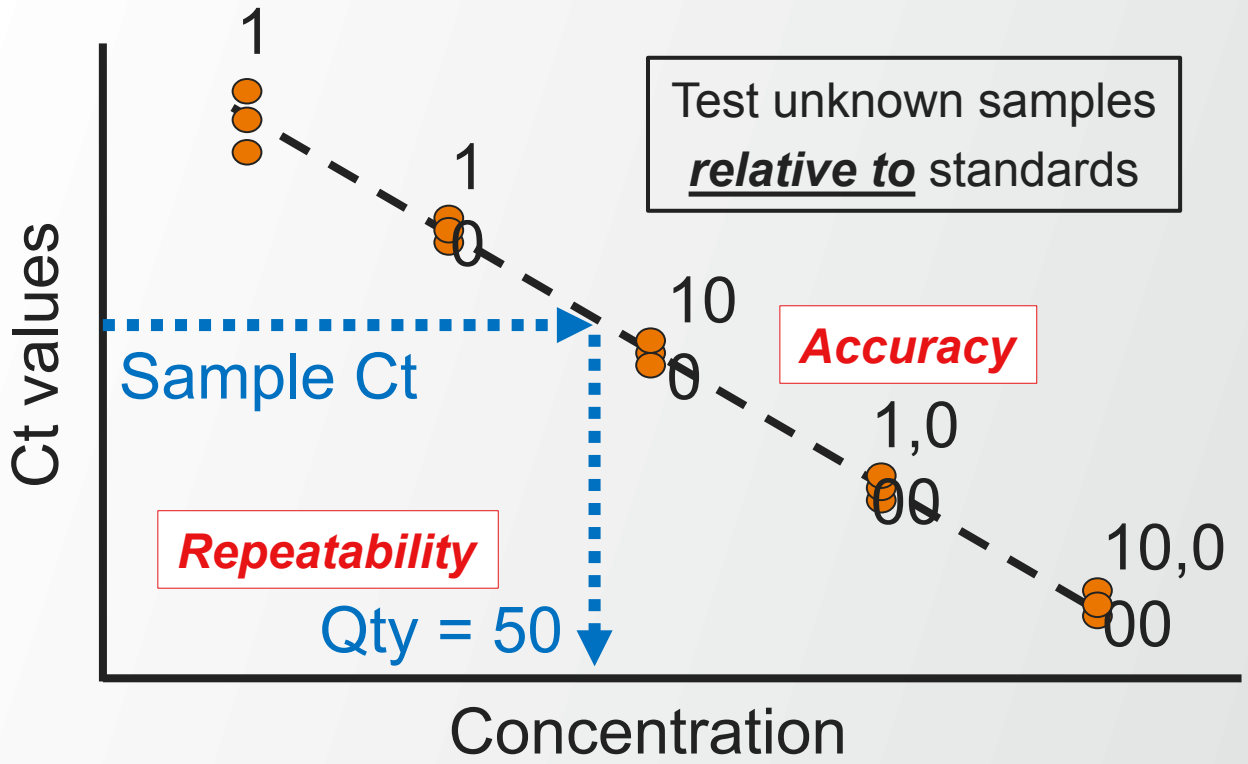
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Absolute Quantification

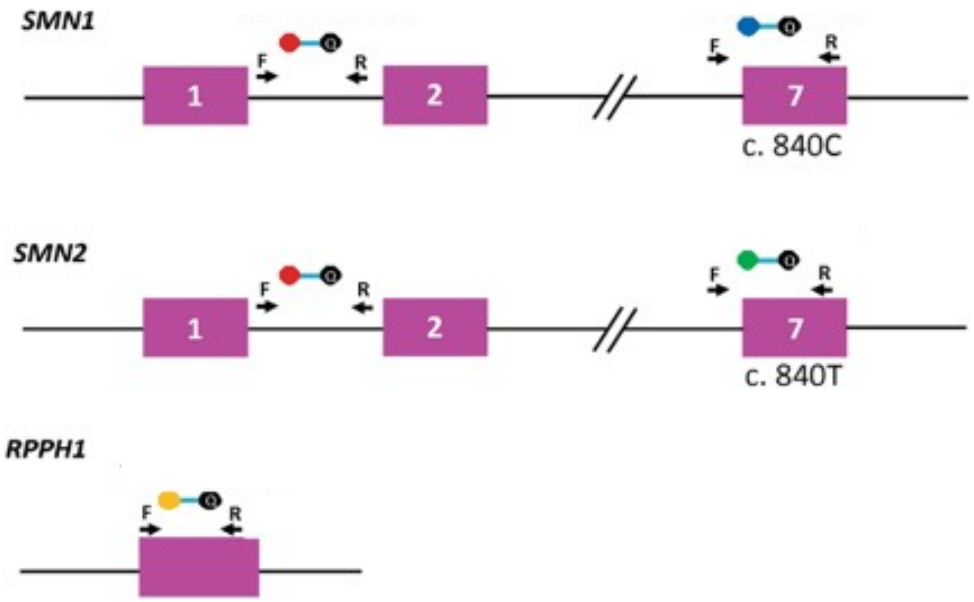
Drawbacks of standard curves:

- **Relatability:** Standard curve data can be misleading if reference does not adequately mimic samples
- **Availability:** Reference material may not be readily available or easy to generate
- **Accuracy**: Orthogonal testing of reference material is recommended to confirm the concentration of reference material
- **Repeatability:** Real-time PCR may not yield acceptable precision or reproducibility
- **Workflow:** Generation of high-quality standard curves is somewhat laborious, expensive, and requires some technical expertise

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Copy Number Variation

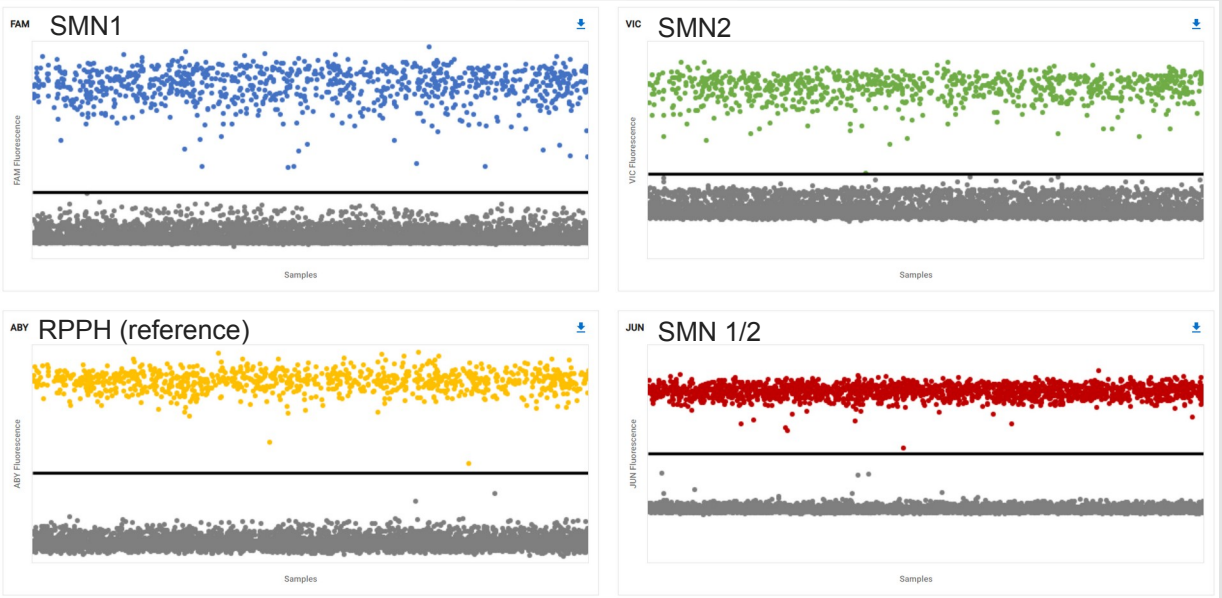
- dPCR is an ideal fit for CNV experiments due to high precision
  - Better precision allows more accurate discernment between copy number values
- Real-time PCR data may not provide sufficient resolution (e.g. 7 versus 8 copies)



*Note: For dPCR, sample may require endonuclease treatment if repeated sequences are physically linked

FAM

Target	Conc. cp/µL	SD	CV%	95%CI	Precision %	Lambda(cp/Rxn)	Lambda_95%CI	Positives	Copy #	CNV_95%CI
TARGET 1	1349.05	1674.12	124.096	11.059,11.268				6693	1.97	0.06,0.06
TARGET 1	3981.77			67.530,68.695	1.725	1.72013	0.02917,0.02968	16704	1.92	0.05,0.05
TARGET 1	3938.42			67.148,68.313	1.735	1.70140	0.02901,0.02951	16443	1.95	0.05,0.05



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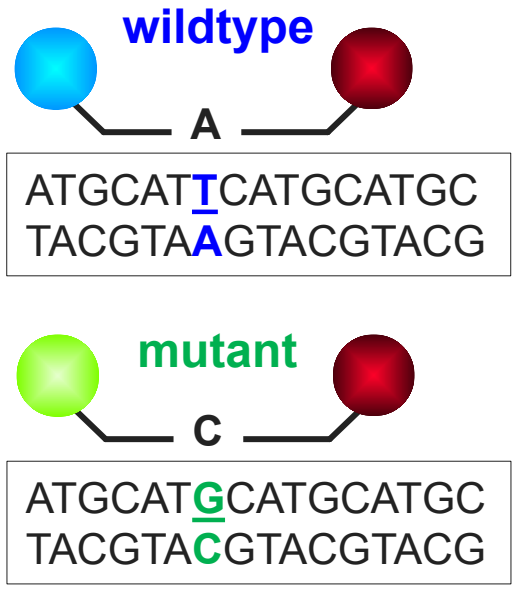
Rare target detection – SNP assays

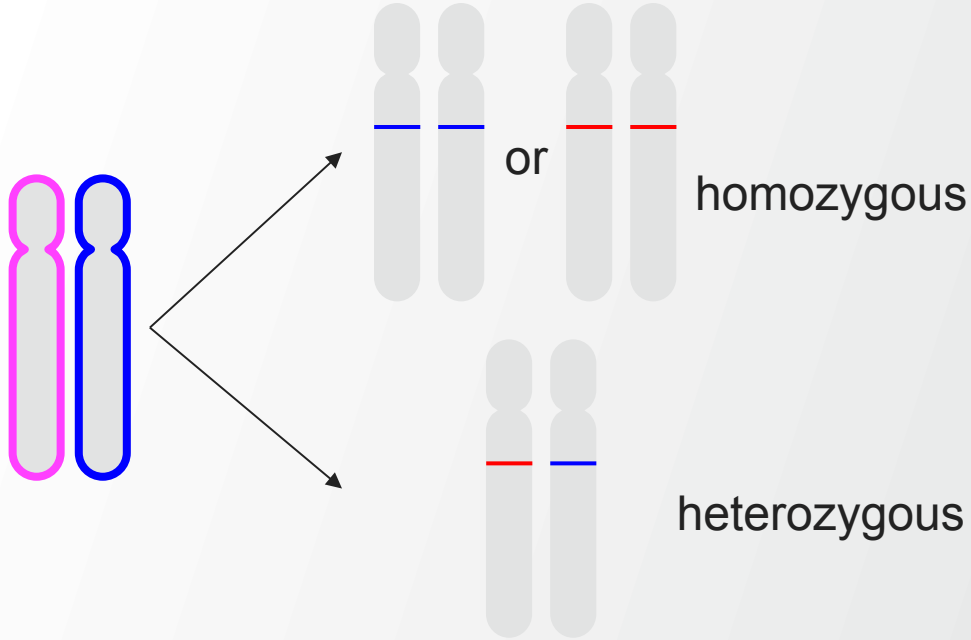
Common primers

- Forward primer
- Reverse primer

Allele specific probes (e.g.,)

- 1 VIC® labeled probe
- 1 FAM labeled probe





- Genotyping assays are often used with qPCR for *qualitative* assessment
- With dPCR, same assay design can *quantitatively* assess **rare targets**

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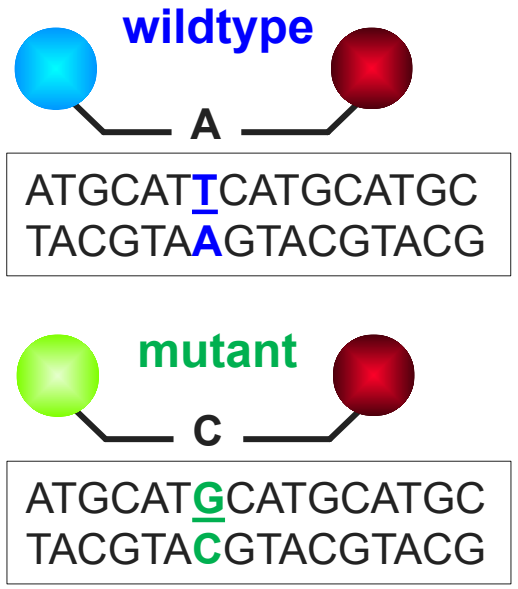
Rare target detection – SNP assays

Common primers

- Forward primer
- Reverse primer

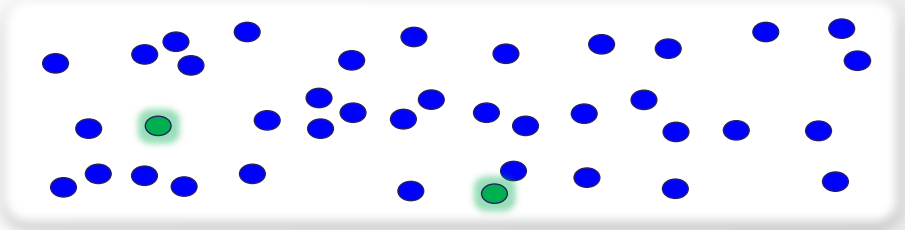
Allele specific probes (e.g.,)

- 1 VIC® labeled probe
- 1 FAM labeled probe



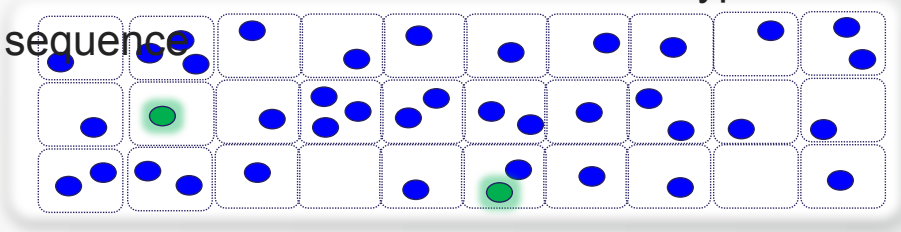
Mutant difficult to detect within abundant wildtype

1:1000 Bulk reaction (qPCR)

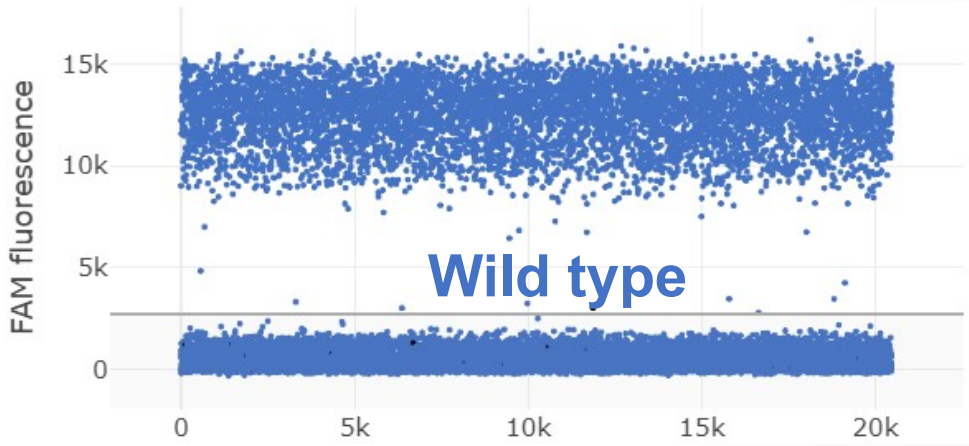


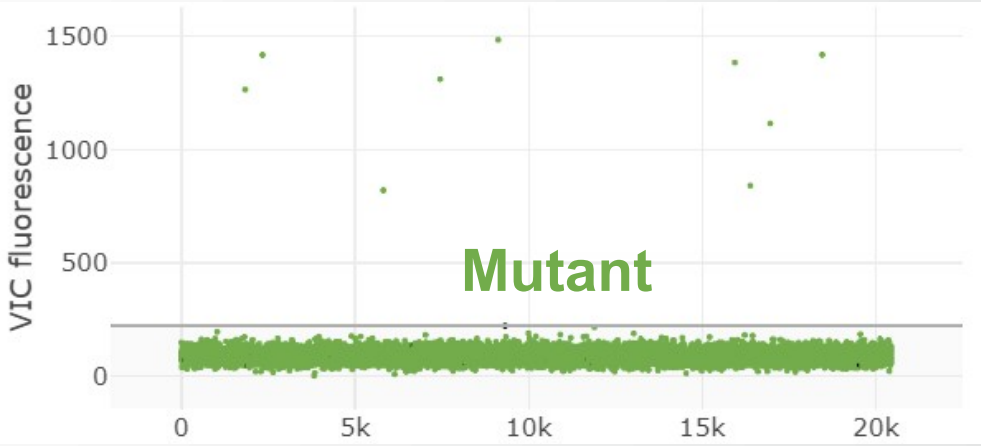
Mutant chambered with 0,1,2 of wildtype sequence

1:1 Micro-reactions (dPCR)



Mutant Allele Frequency = 0.1%





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Rare target detection – Use case

Rare-target quantification on the QuantStudio Absolute Q dPCR System

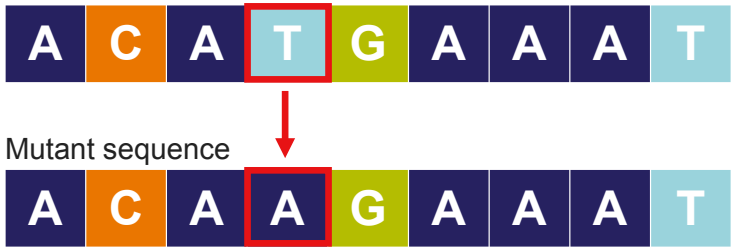
Leveraging Absolute Q Liquid Biopsy dPCR Assays

Thermo Fisher offers a library of predesigned assays

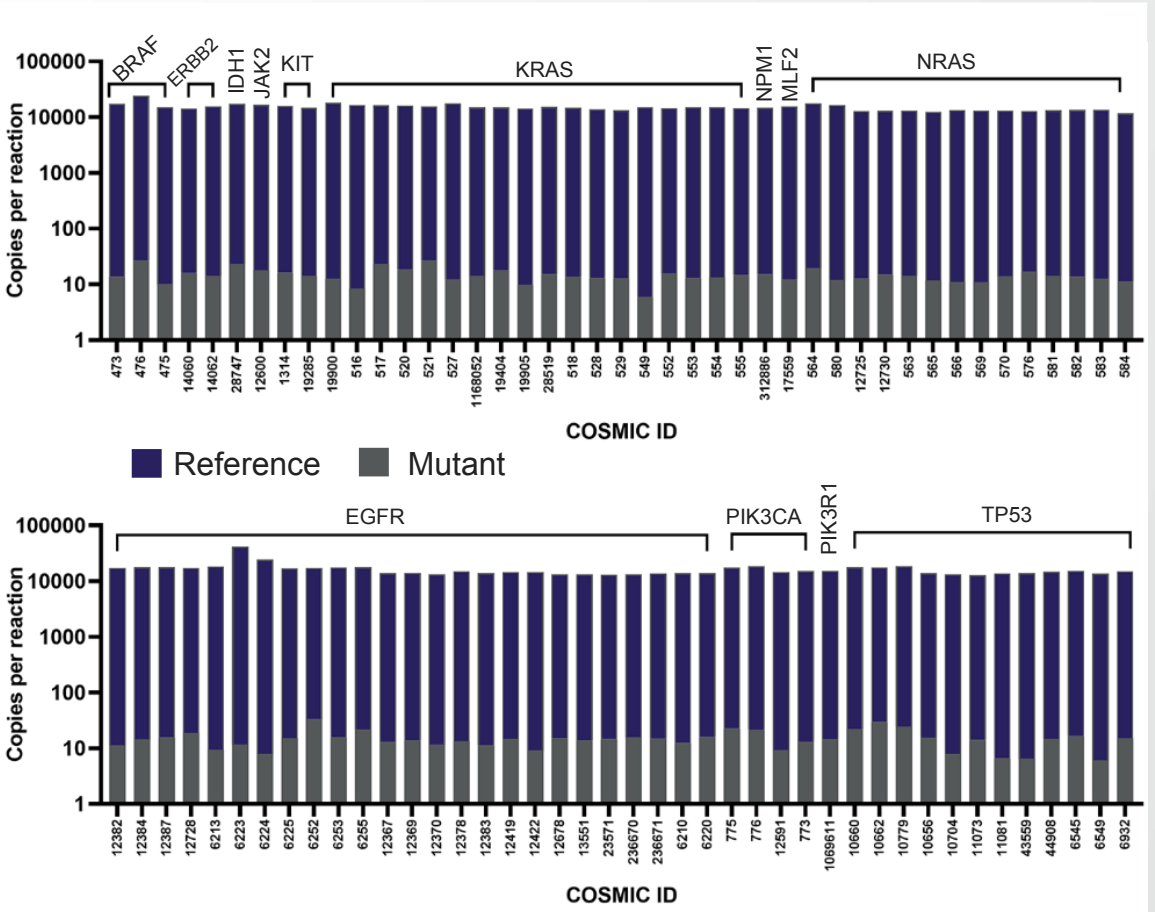
- Many commonly studied mutations
- Wet-lab tested to quantify *at least* 0.1% mutant frequency
- Can also design and create custom assays for your target

Example hotspot mutation *BRAF* p.V600E

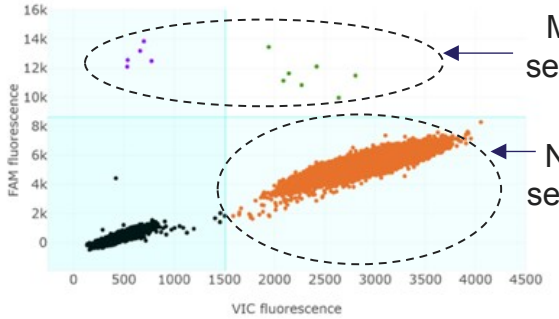
Normal sequence (wild type)







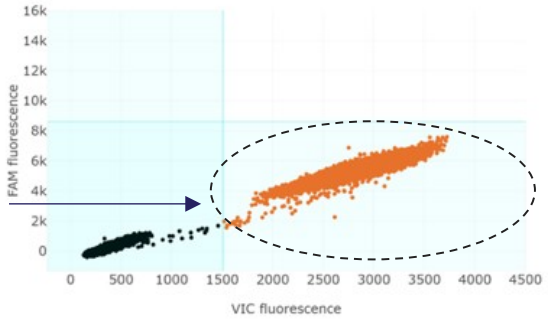
0.1% Mutant allele frequency



Mutant sequence

Normal sequence

Reference sequence control

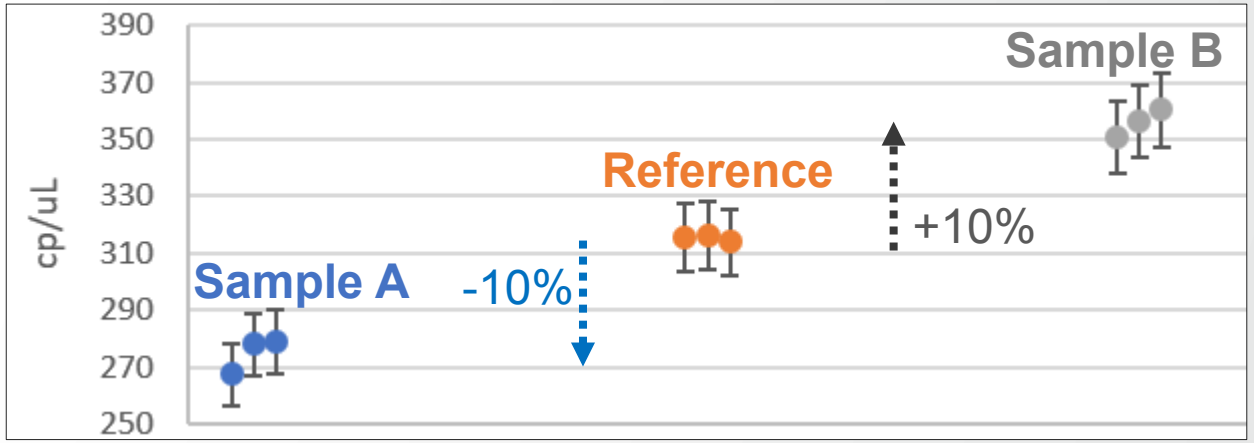


[More Information: Absolute Q liquid biopsy assays]

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Gene expression (Relative Quantification)

- Digital PCR great fit for gene expression studies when:
  - Target is low abundance (high Ct values from qPCR often have low precision)
  - Interested in quantifying small changes



- Gene expression data still needs to be normalized
  - Could be normalized by physical sample mass (*but may not be precise enough for small changes*)
  - Generally recommend use of Endogenous Control / Reference gene (*just like for qPCR*)
  - Multiplexed assays provide best precision (*eliminates variation introduced by pipetting*)
- Conversion of absolute quantities into relative quantities:
  - qPCR RQ uses delta delta Ct
  - dPCR RQ uses ratio of ratios of Concentrations

$$RQ = \frac{C_goi / C_endo}{C_goi / C_endo}$$

Treated sample

Reference

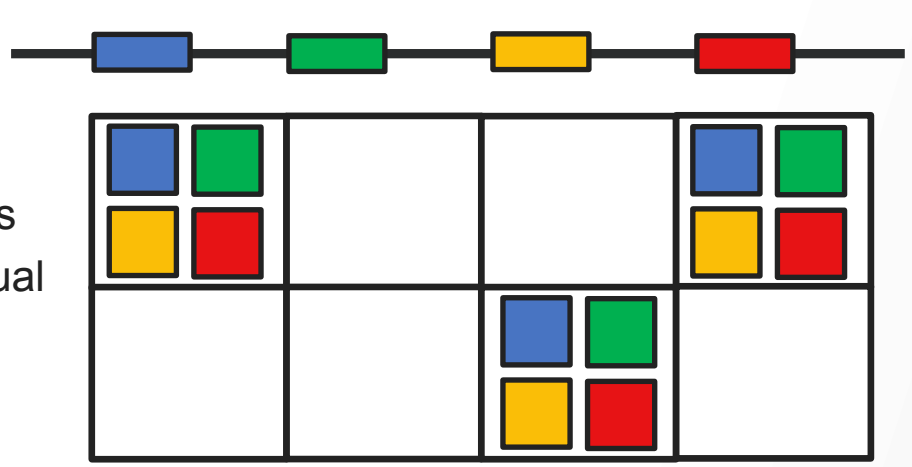
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Molecular integrity

- Asks: “are targeted locations on same *physical molecule*?”
- Assays targeting different regions of molecule (e.g., viral genome, chromosome, RNA)
- Analyze data based on chambers being positive for multiple targets

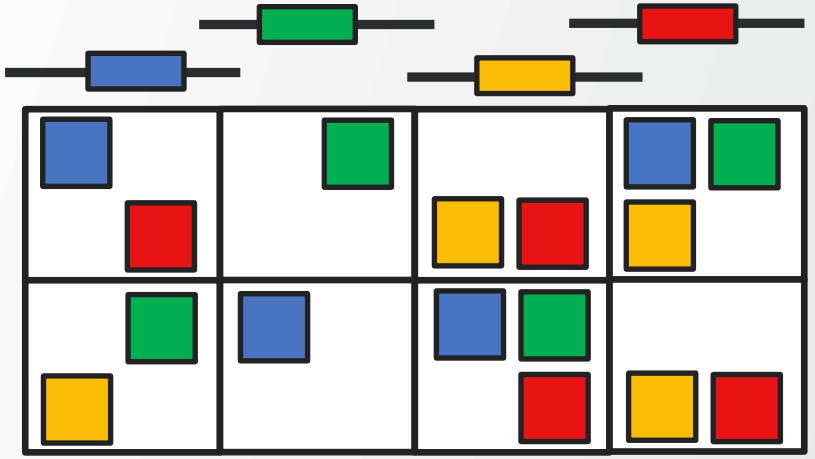


Intact molecule



All/multiple targets present in individual chambers

Fragmented molecule



Random dispersion of targets across chambers

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Drop off assay

- Asks: “What proportion of sequences are altered?”
- One probe targeting edit/mutation site and probe for a nearby reference site
- Detection and quantification of modified sequences (*without the need to know the modification*)
- E.g., screen for range of mutations, evaluate CRISPR editing

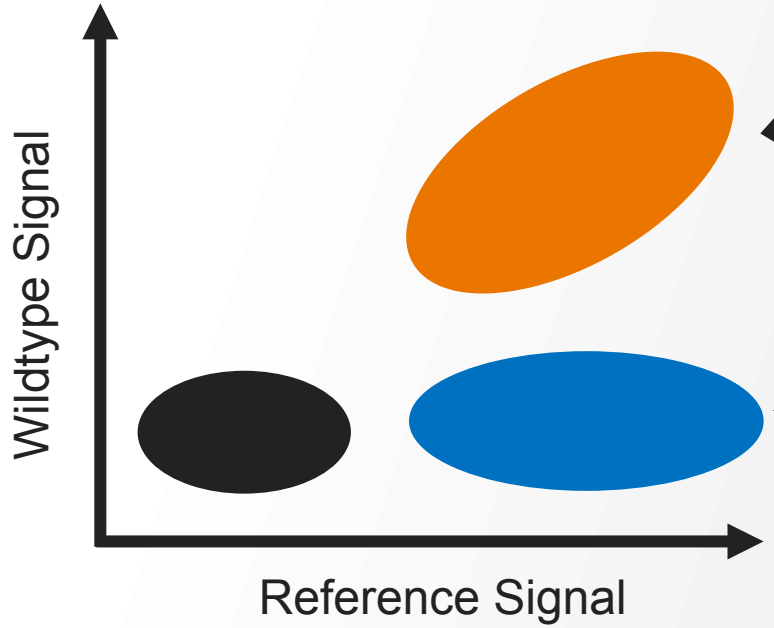
Assay

Reference probe:
Identify all copies

Wildtype probe:
Identify WT copies



Data

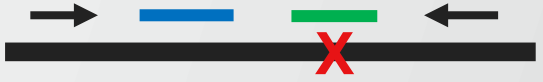


Interpretation

WT: Signal from both probes



Edit/Mutation:
Reference probe only



X: Change in sequence

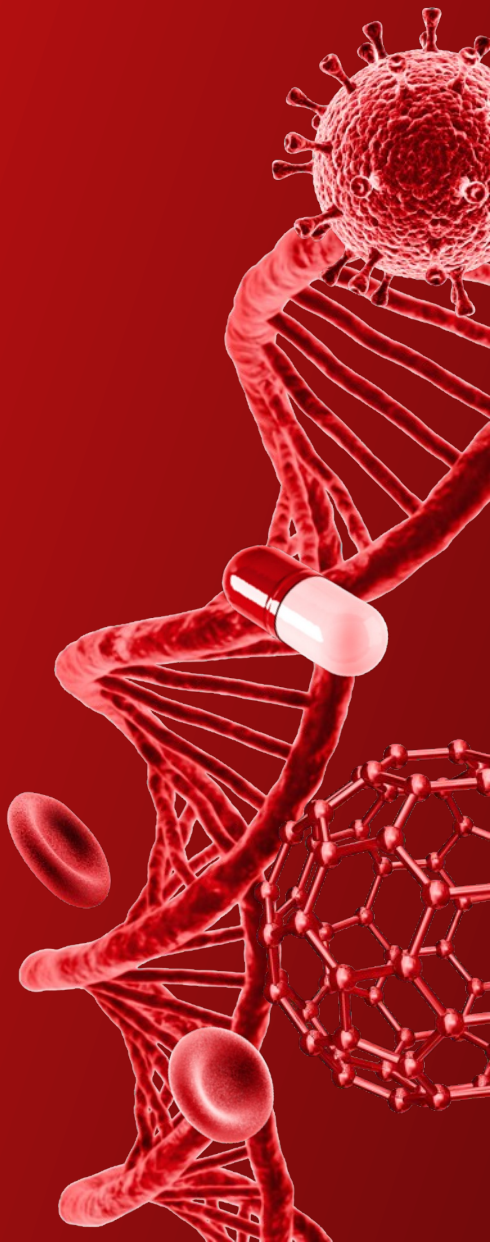
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Questions



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Thank You



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Need Help?

qPCR Application Support Center

Real-Time PCR Learning Center

Technical Support:

1-800-955-6288, option 2, 1, 2

TAC (Instrument Support):

1-800-955-6288, option 3, option 1

Insert Name Here

Insert Email Here



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Need Help?

qPCR Application Support Center

Real-Time PCR Learning Center

ONE NUMBER:

00-800-5345-5345

#2 Instrument Service; #3 Application Support

EMAIL

eurotech@thermofisher.com



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