Workshop Training Series

Real-Time PCR and Droplet Digital PCR

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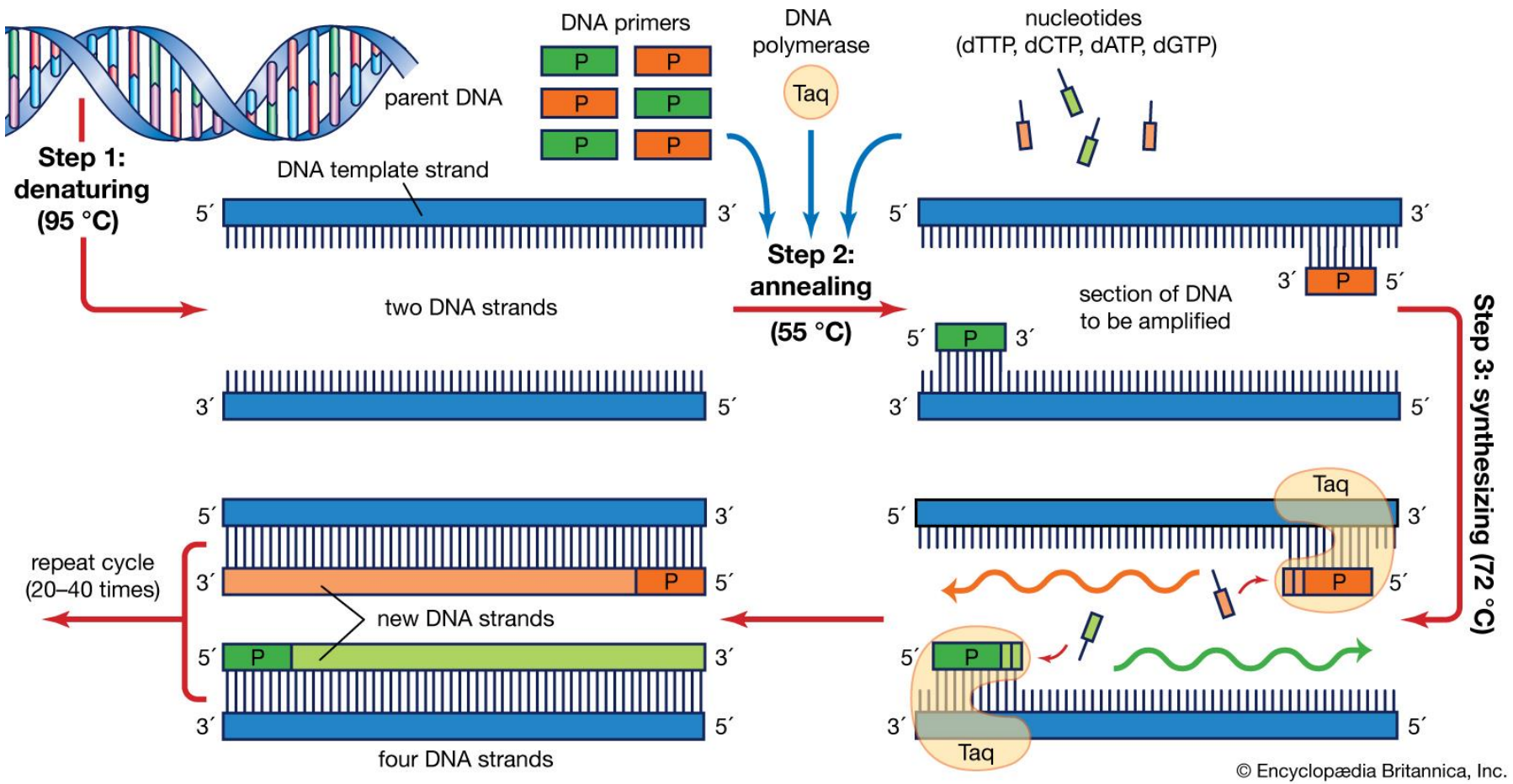
PCR – A simple idea

In vitro method for enzymatically synthesizing DNA

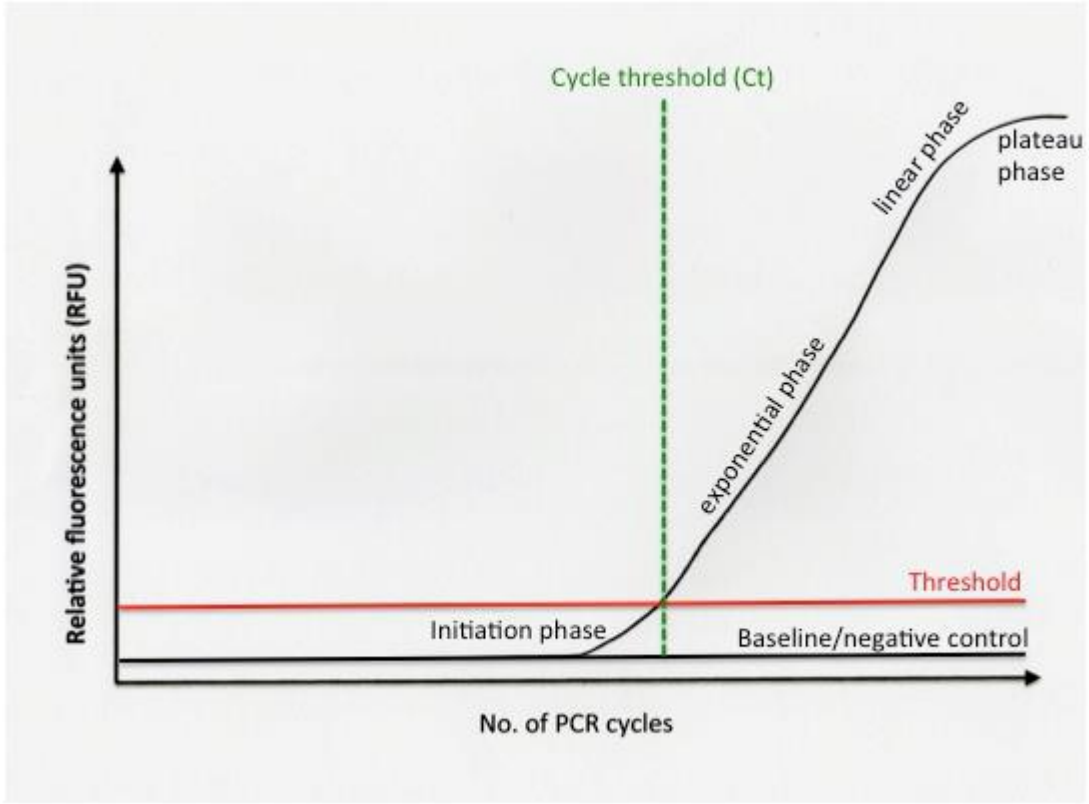
- Polymerase Chain Reaction: Kary Mullis (1983)
- The reaction uses two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified
- A repetitive series of cycles gives exponential accumulation of a specific DNA fragment
  - Template denaturation
  - Primer annealing
  - Extension of annealed primers by the polymerase
- The number of target DNA copies doubles every PCR cycle (20 cycles $\Rightarrow 2^{20} \approx 10^6$ copies of target)

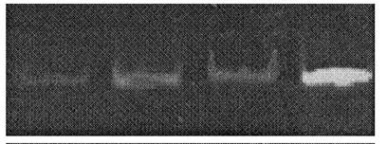
Principle of PCR



https://www.britannica.com/science/polymerase-chain-reaction

Three Phases of PCR

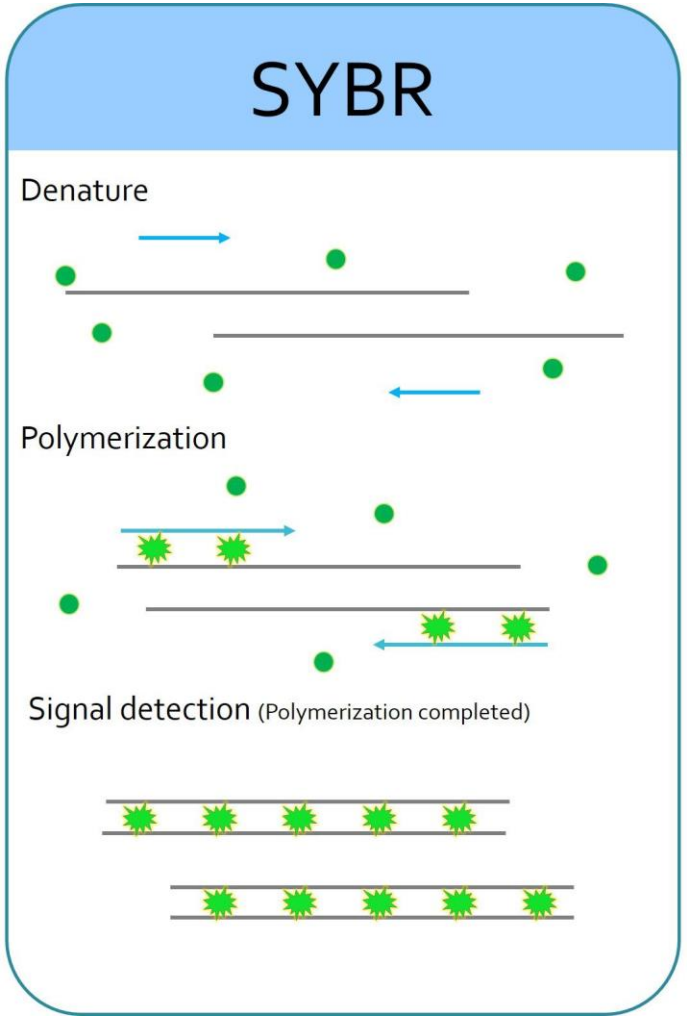


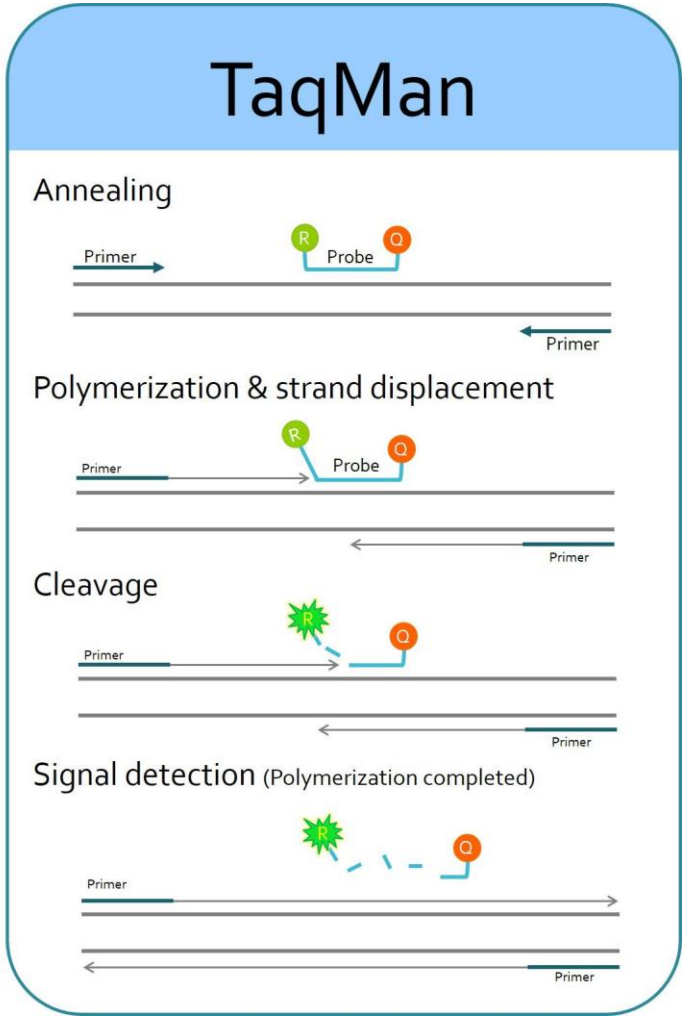


How to Quantify DNA Using Realtime PCR

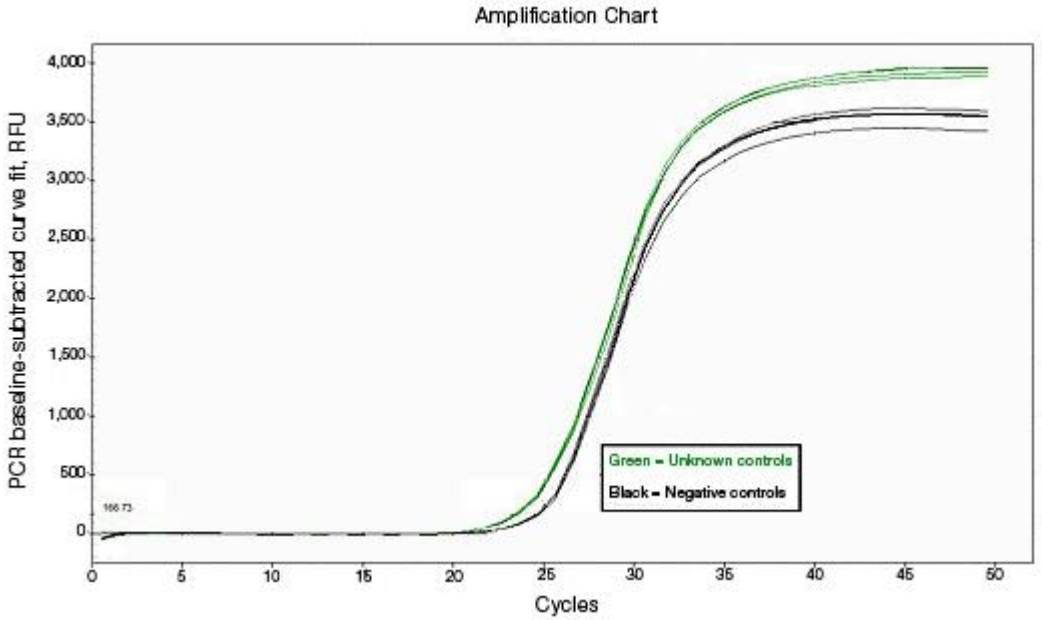
- **Fluorescence signal is measured every cycle. (signal positively correlated with amount of PCR product).**
- **Amplification curves have three phases: exponential, linear and plateau phases.**
- **A cycle threshold (Ct) is the cycle number in exponential phase where the signal intensity cross the threshold of detection.**
- **Ct is negatively correlated with the amount of templates and used to indirectly represent the amount of original templates**
- **Comparison with standard curve gives quantification.**

SYBR VS TaqMan



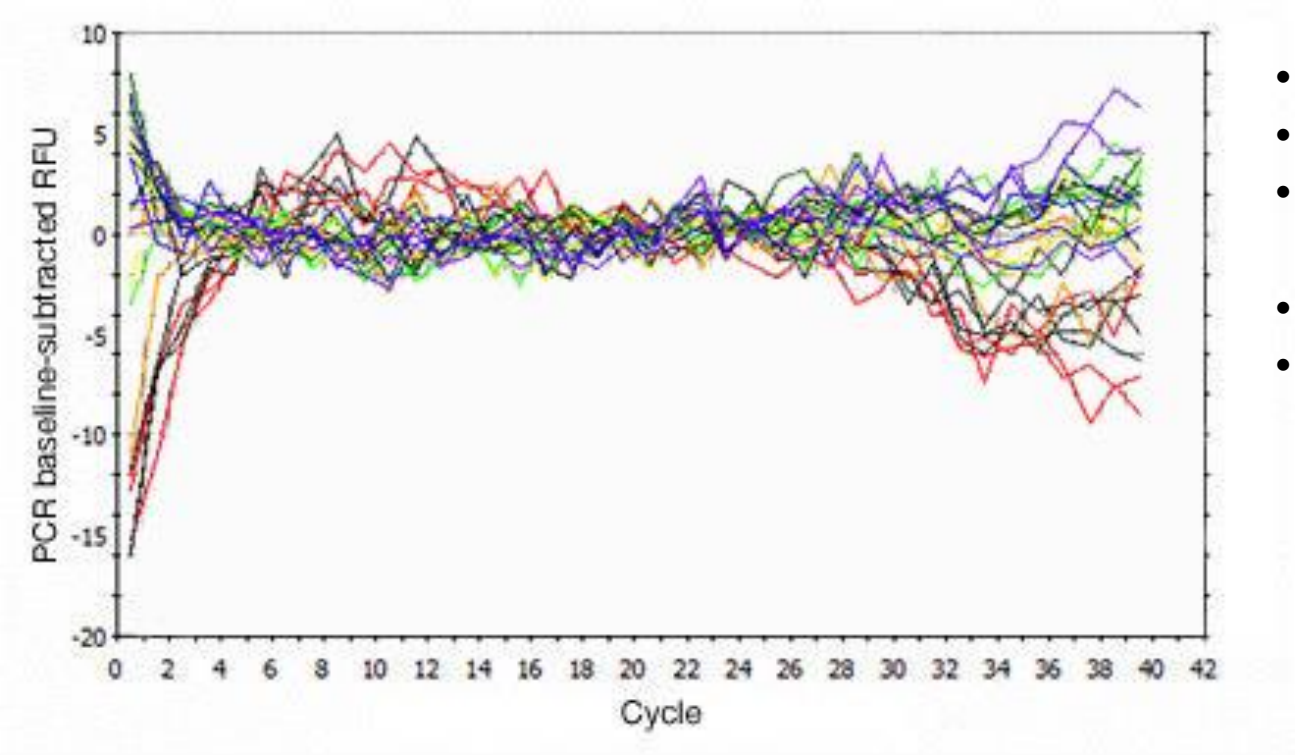


Signal in Negative Control



Primer-Dimer Formation
Unspecified amplification
Contamination of Reaction components
Amplification of Genomic DNA

Troubleshooting



- **Inhibitor Present**
- **Non-Optimized Buffer Composition**
- **Non-Optimized Thermal Cycling Conditions**
- **Degraded templates or primers**
- **Probe Quality Issues**

Droplet Digital PCR and Real-Time PCR

Bio-Rad QX200 ddPCR system

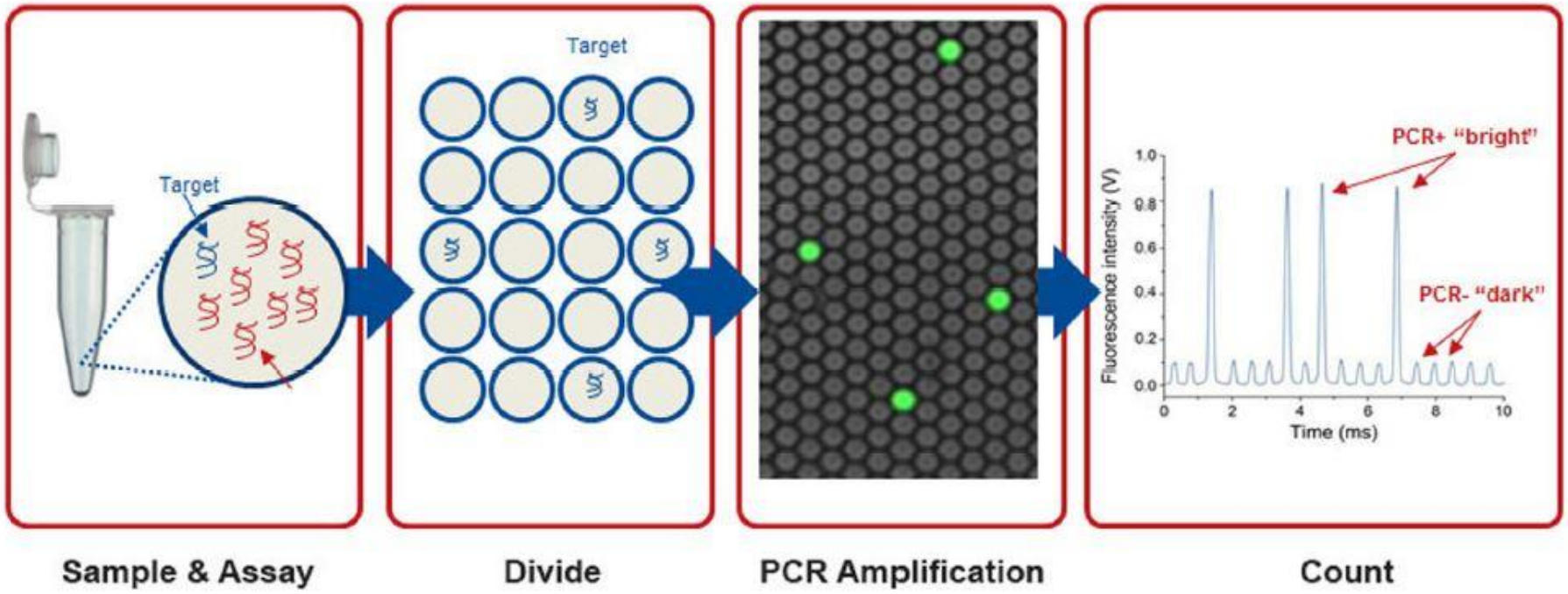
CFX Connect™ Real-Time PCR Detection System

How ddPCR Work?

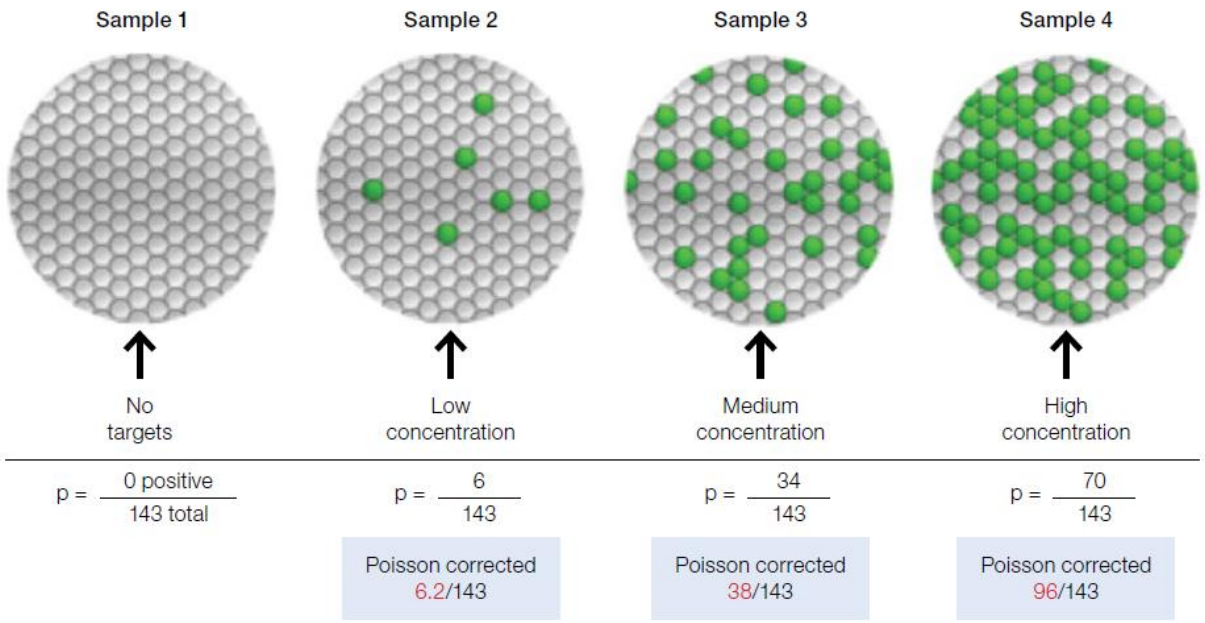
- **Digital Droplet PCR (ddPCR) is an end-point measurement that enables you to quantify nucleic acids without the use of standard curves and independent of reaction efficiency.**
- **In ddPCR, the sample is separated into a large number of partitions (droplets).**
- **The PCR is carried out as regular PCR but the template in each partition is amplified independently.**
- **The PCR product is read using a droplet reader to determine the fraction of positive partitions.**

Why Digital?

- **Theoretically, the sample and qPCR assay mixture are distributed into a very large number of compartments (droplet), such that there is either zero or one target molecule present in any individual reaction. The number of positive reaction is the amount of template.**

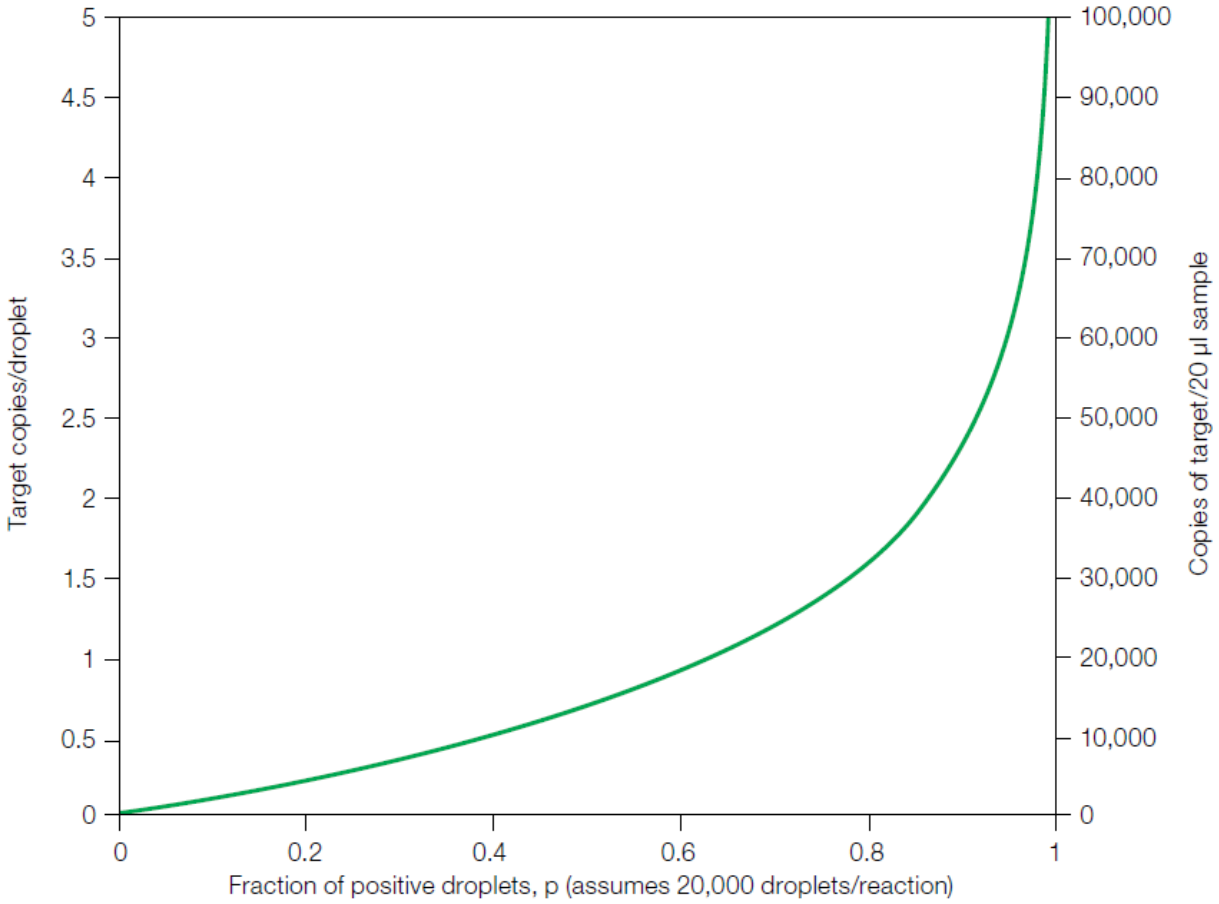


Droplet Digital PCR-How Does It Work?



- **Practically, more than one target molecule may be co-located in the same compartment.**
- **The number of the molecules in a compartment follows a Poisson distribution.**
- **The number of target molecules in compartments can be calculated using Poisson statistics**

Estimating target concentration



- **The ideal droplet number is 20,000 per action.**
- **The concentration of target molecules can be determined by the production of 20,000 and the number of targets per droplet.**

Benefits of Droplet Digital PCR

- **Unparalleled precision — the massive sample partitioning afforded by ddPCR enables small fold differences in target DNA sequence between samples to be reliably measured**
- **Increased signal-to-noise — enrich for rare targets by reducing competition that comes from high-copy templates**
- **Removal of PCR efficiency bias — error rates are reduced by removing the amplification efficiency reliance of PCR, enabling accurate quantification of targets**
- **Simplified quantification — a standard curve is not required for absolute quantification**

Thank you!