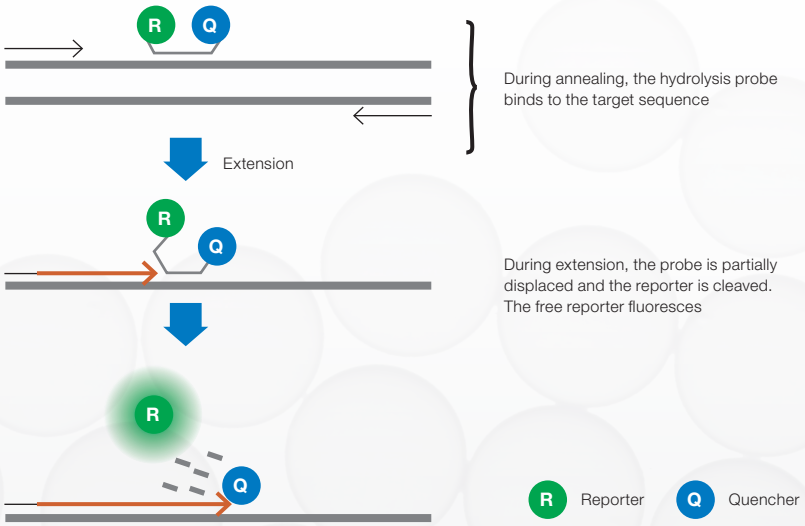
TIPS, TRICKS & BEST PRACTICES

Droplet Digital PCR

DESIGNING PRIMERS AND PROBES FOR ddPCR

- Design amplicons that are 50-200bp long
- Design primers that have a GC content of 40-60%
- Avoid sequences with long (3+) repeats of a single base
- For most assays your primers should have a melting temperature (T_m) of 60° and your probe should be between 64° and 67°C. For SNP detection and gene editing, a primer T_m of 55° and probe T_m of 56°C should be utilized instead
- Check the sequences for 3' complementarity to avoid primer dimers
- Place Gs and Cs at the 3' end of primers (GC clamp) when possible
- Probes should not have a G at the 5' end
- Probes should be designed between the two primers, the sequence must not overlap the primers but may sit directly beside them
- Probes may be labeled with FAM, HEX, or VIC fluorophores. Probes should be quenched with any non-fluorescent quencher



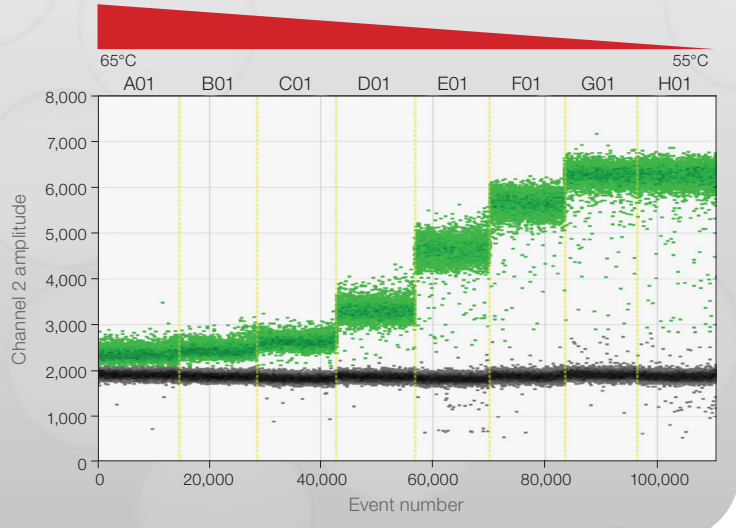
Primer3Plus is a handy open-access option for assay design (Untergasser et al. 2007). We recommend the following settings:

Concentration of monovalent cations:	50
Concentration of divalent cations:	3.8
Concentration of dNTPs:	0.8
Salt correction formula:	SantaLucia
Product size ranges:	40-100 100-150 150-200
Primer/Probe Tms	60°/64°-67° or 55°/56°C

ASSAY OPTIMIZATION

In many cases ddPCR assays will work well under standard conditions predicted by design software algorithms. However, if positive droplets do not separate well from the negative population, or significant rain is seen, assays can be optimized.

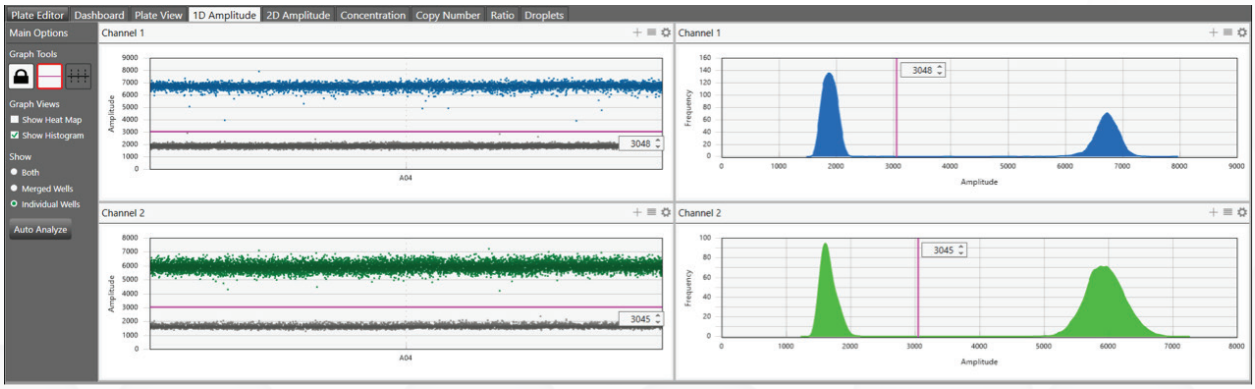
- Optimize the annealing temperature for PCR using a gradient, spanning a few degrees above the predicted primer Tm, and several degrees below. Typically, testing a 10°C span is sufficient
- Annealing time may be increased, especially for amplicons >250bp and low efficiency reactions
- If using genomic DNA, utilize a restriction enzyme to ensure maximum accessibility
- Ensure sample is not overloaded with too much DNA
- Always include relevant positive and negative controls, including a no-template control for each primer/probe set



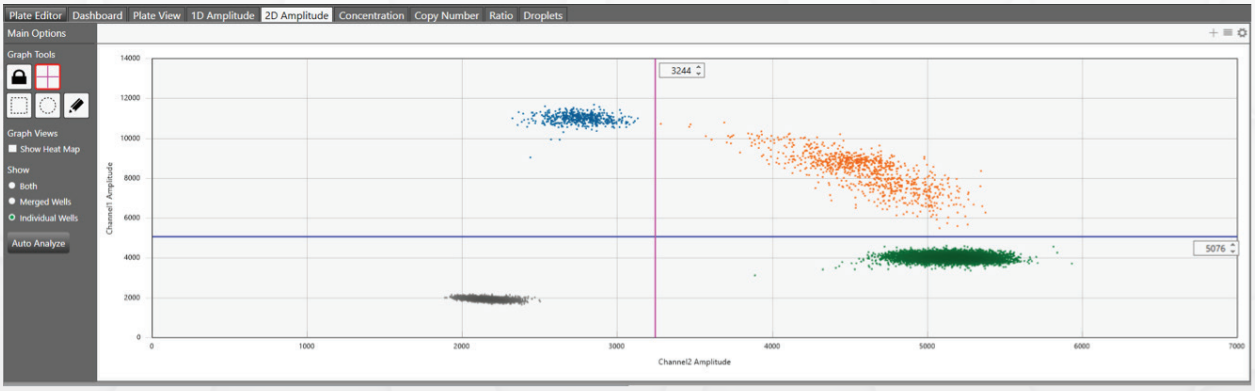
DATA ANALYSIS

Highlight single or multiple wells by highlighting wells on plate map

Set thresholds in 1D plot by selecting single or multiple threshold button  , then clicking on droplet plot.



Alternatively, set thresholds in 2D plot using crosshair or pencil lasso function  . 2D plot is favored for mutation detection assays.

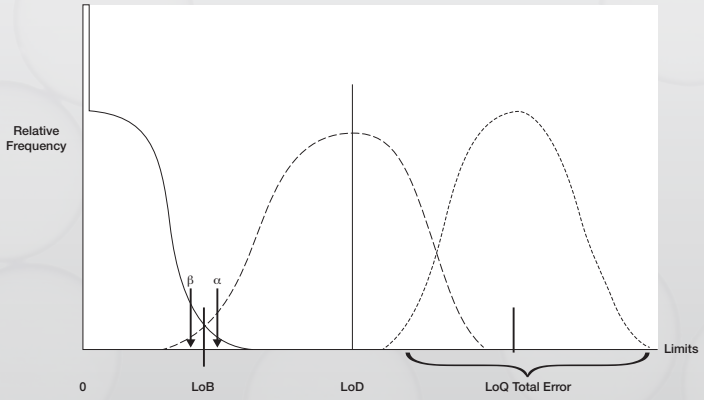


QuantaSoft calculates Concentration in copies/ul. Ratio, Copy Number and Fractional Abundance can be viewed in their respective tabs, depending on experiment type.

LIMIT OF DETECTION

Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantitation (LoQ) are concepts that are utilized to describe the lowest concentration of a target that can be reliably measured.

- LoB is the highest measurement result when replicates of a blank sample containing no target are tested
  - LoB = mean blank + 1.645(SD blank)
- LoD is the lowest concentration of the target that can be reliably detected but not necessarily quantified. LoD is calculated by utilizing the measured LoB and testing replicates with a low concentration of the target
  - LoD = LoB + 1.645(SD low concentration of target)
- LoQ is the lowest concentration of the target that can be quantitatively determined with predefined acceptable precision. The LoQ may be equivalent to the LoD or higher but never lower than the LoD



Clinical and Laboratory Standards Institute. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline, Second Edition. CLSI document EP17-A2. Wayne, PA USA: CLSI; 2012

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