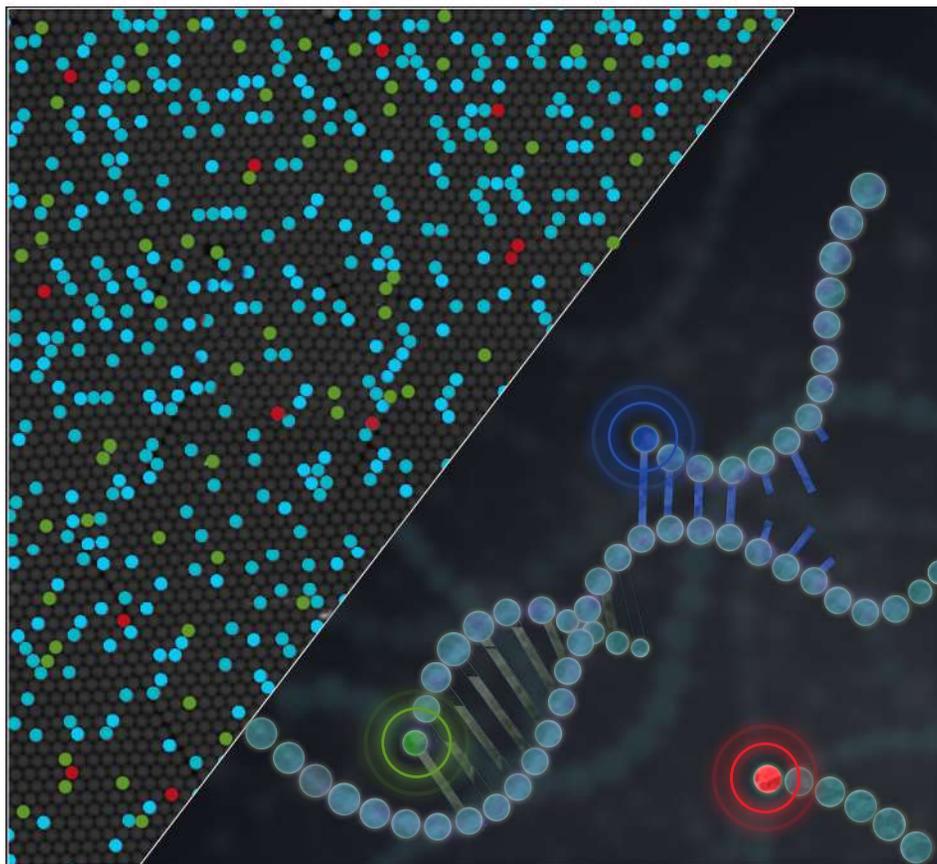




How to Quantify Drop-Off digital PCR assays with Crystal™ Miner



How to interpret Drop-Off digital PCR assays using Crystal Miner

Digital PCR enables the Mutant Allelic Fraction (MAF) of a drop-off mutant to be quantified with high sensitivity using only two detection channels:

- A 1st channel to detect the **Drop-Off Probe** that spans the mutation site but is uniquely complementary to the wild-type sequence (e.g. «Blue Detection Channel» on the vertical axis in Fig. 1)
- A 2nd channel to detect the **Reference Probe** that hybridizes adjacent to the mutation site and is complementary to both the mutant and wild-type alleles (e.g. «Green Detection Channel» on the horizontal axis in Fig. 1)

In the presence of a wild-type allele, both the drop-off and reference probes will hybridize with their target, leading to a double positive signal (**turquoise population** in Fig. 1). Whereas in the presence of a mutant allele, even a single nucleotide mutation is enough to destabilize the hybridization of the drop-off probe so that only the reference probe anneals to its target leading to a single positive signal (**green population** in Fig. 1).

For more information on Drop-off assay design, see our Application Note “Drop-off Crystal™ Digital PCR detects *NRAS*, *KRAS* & *EGFR* mutations” at <https://www.stillatechnologies.com/application-notes/>

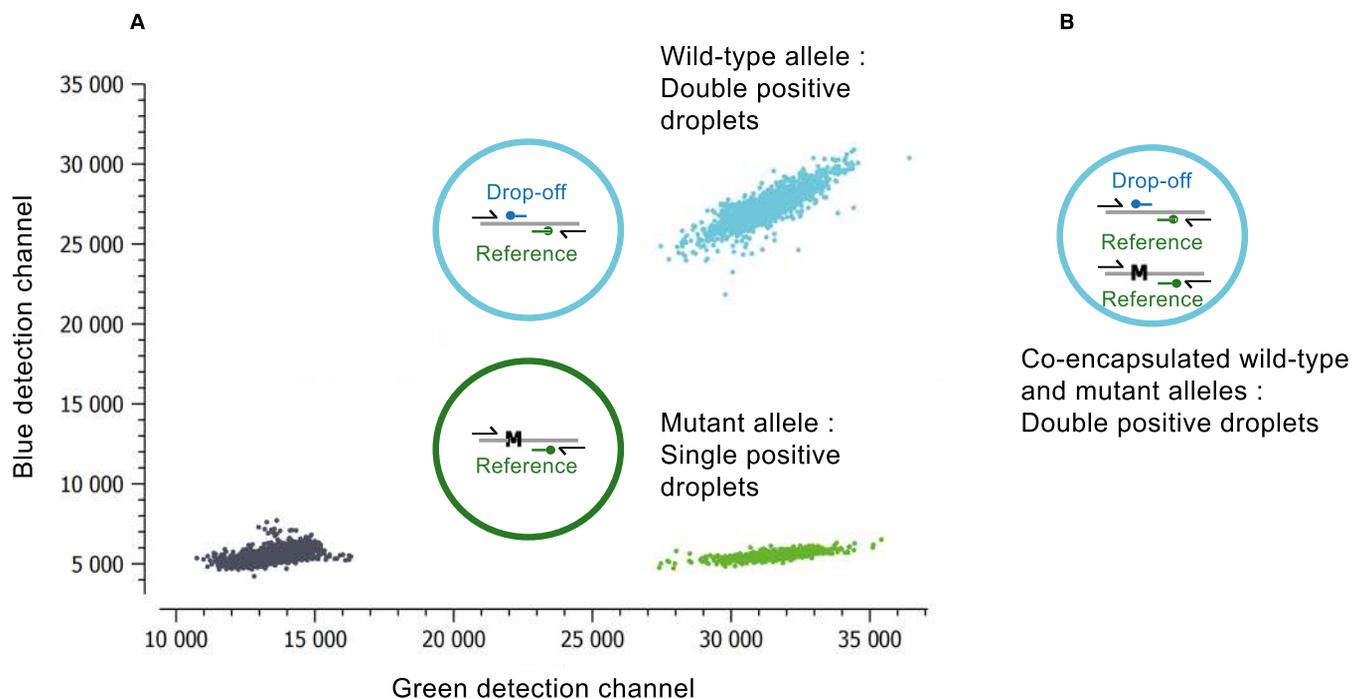


Figure 1: A. Crystal™ Miner 2D dot-plot of a drop-off assay showing three clusters: double-positive droplets (**turquoise**) containing wild-type alleles, single positive droplets (**green**) containing mutant alleles, and double negative droplets (**black**). B. During droplet generation, a fraction of mutant alleles and wild-type alleles can be randomly co-encapsulated within the same droplet rendering the droplet double positive wild-type and mutant (**turquoise**). Although the proportion of double-positive droplets containing mutant alleles cannot be measured, it is expected to be similar to the proportion of non-double-positive droplets (**green** or **black**) containing mutant alleles (**green**). M, mutation.

Step-by-step method to quantify Drop-Offs using Crystal Miner

The wild-type concentration C_{WT} can be directly derived from the proportion P_{WT} of droplets that are double-positive (whether they are positive or negative in a 3rd detection channel). However, the drop-off mutant concentration C_{Mut} must be derived from the proportion P_{Mut} of droplets that confidently contains mutant alleles. Due to the potential for wild-type and mutant allele co-encapsulation, double-positive droplets are ambiguous droplets for mutant quantification. As such, P_{Mut} can only be determined by excluding the double-positive droplets and thus restricting the mutant positive population to the droplets that are positive for the Reference Probe but negative for the Drop-Off Probe (whether they are positive or negative in a 3rd detection channel).

To quantify Drop-off populations using Crystal™ Miner, follow the steps below:

1. Define the three droplet populations: mutant positive, mutant negative, wild-type positive

- Go to ANALYZE DATA > Plots & Populations > 2D dot plot
- Select the “Polygon” type for the thresholding mode, then select your well(s) of interest in the left-hand panel
- In the 2D dot plot showing the “Drop-Off Detection Channel” on the vertical axis and the “Reference Detection Channel” on the horizontal axis (double-click on the axes to modify their Channel attributions if needed), draw a polygon around each of the following three droplet populations of interest:
 - Mutant positive population, discounting double positives (e.g. “EGFR Del 19 Mut” in Fig. 2)
 - Mutant negative population, discounting double positives (e.g. “EGFR Del 19 Mut - Negatives” in Fig. 2)
 - Wild-type positive population, i.e. the double positives (e.g. “EGFR Del 19 WT” in Fig. 2)

To draw a polygon: press and hold the Ctrl key and right-click to create each vertex, then release the Ctrl key before the last right-click to close the polygon. You can then name the droplet population contained in the polygon and choose its color.

2. Specify the negative population associated with the mutant positive population

- Go to ANALYZE DATA > Plots & Populations > Population Editor
- In the “Population” window on the right: select the mutant positive population, then click on “EDIT”, expand the option “Need to define specific negative droplets” and click on “Edit Negatives”
- In the “Zones List” window in the middle: check the box corresponding to the mutant negative population in the “Negative” column (e.g. the zone “2” in Fig. 2)
- In the “Population” window on the right: click on “APPLY”

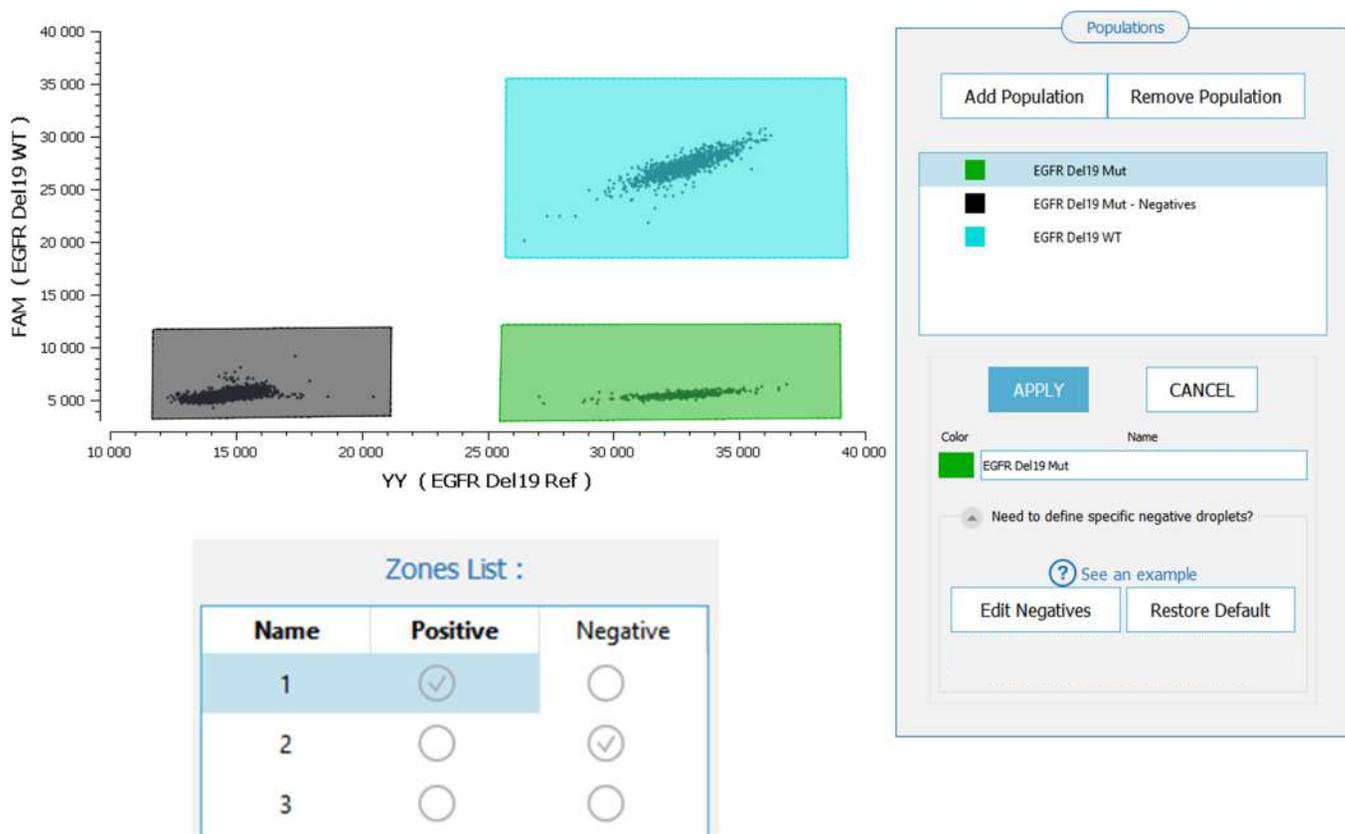


Figure 2: Example of Crystal™ Miner’s display for a digital PCR experiment targeting the drop-off mutant *EGFR* Del19 using two channels (channel 1: reference probe with YY fluorophore; channel 2: drop-off probe with FAM fluorophore). In the case where the 3rd detection channel is used, for example to detect an unrelated mutation or an internal control, the polygon zones will project in the 3rd dimension so they may contain droplets that are negative or positive for the 3rd detection channel. The use of a 3rd channel will in no way impact reliable drop-off quantification.

3. Calculate the MAF of a drop-off mutant

- Go to VIEW RESULTS > Result Table and get the drop-off mutant concentration C_{Mut} and the wild-type concentration C_{WT} automatically estimated by Crystal Miner
- Go to EXPORT and export all result data
- Deduce the MAF of drop-off mutant by applying the following formula: $MAF_{dropoff} = C_{Mut} / (C_{WT} + C_{Mut})$

Notes:

The relative loss of Limit of Detection (LOD) for a drop-off mutant compared to the LOD of a point mutant increases exponentially with the expected wild-type concentration (e.g. LOD at 95% confidence level raises from 0.2 to 0.27 cp/μL for a wild-type concentration $C_{WT}=500$ cp/μL in the loaded sample of one Sapphire chip well).

If the double-positive droplets are not excluded and instead counted as mutant negative droplets, the MAF of the drop-off mutant will be underestimated almost linearly according to the expected wild-type concentration C_{WT} and with a nearly constant factor for any $C_{Mut} \leq 100$ cp/μL (e.g. underestimation around 25% for $C_{WT}=500$ cp/μL in the loaded sample). In addition, keeping the double-positive droplets would neither decrease the relative uncertainty of the measured drop-off mutant concentration nor the LOD of the drop-off mutant.

Technical Note Highlights



By using the Naica™ Crystal Miner advanced Population Editor feature, ambiguous droplets can be discounted ensuring accurate quantification of drop-off mutations



Excluding ambiguous droplets avoids underestimating the concentration of the drop-off mutant without decreasing the concentration measurement precision



Because only two detection channels are required to estimate a drop-off mutant allelic fraction, the third detection channel can be used, for example, to add an internal amplification control or to simultaneously quantify the MAF of a point mutation (see the Application Note “Drop-off Crystal™ Digital PCR detects *NRAS*, *KRAS* & *EGFR* mutations” at <https://www.stillatechnologies.com/application-notes/>).



To learn more about digital PCR, please visit Stilla Technologies' Learning Center at www.gene-pi.com

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