Guidelines for Classifying Droplets in Rare Mutation Quantification Experiments in the KRAS Interlab Study

These guidelines were prepared to assist in the data analysis for the detection and quantification of the KRAS G12D mutation. As part of the experimental design, the following controls were included: a non-template control (NTC), three negative controls (wild-type only DNA input at a level comparable to the test samples) and a positive control (a sample with enough mutant and wild-type DNA to create a double-positive cluster of greater than 100 droplets). For more complete explanations of experimental design, execution and analysis, refer to Bio-Rad’s “Rare Mutation Detection Best Practices Guidelines” (Bulletin #6628).

1. Guidelines for Droplet/Well QC
   a. Select all wells containing the same assay and display droplets using the 1D fluorescence amplitude plot (Figure 1).
   b. Examine the double-negative droplet bands (grey) across all wells and reject any wells ("Bad") that have a significant difference in amplitude from the norm. These wells evidence poor droplet generation - possibly due to poor handling or mixing of samples - and are likely to give aberrant concentration values.
   c. Wells with less than 10,000 accepted droplets should also be rejected as evidence of poor droplet generation and they may also give aberrant quantification.
   d. Individually inspect each well to confirm acceptable droplet quality (i.e. no significant number of droplets lying along a 45° line (Figure 2) and clusters are reasonably well defined with no 'tails').
   e. If positive or negative control wells are not represented due to above exclusion criteria, caution should be exercised in drawing conclusions from the test samples.

2. Guidelines for Droplet Classification
   a. In the 2D droplet fluorescence amplitude view with all wells selected, classify the droplets into the four clusters (Figure 3). This can be achieved by auto analysis using 'combined wells' or manually classifying the droplets using one of the QuantaSoft classification tools (e.g. cross-hair threshold or lasso). QuantaSoft uses this classification to provide concentration measurements for both mutant and wild-type targets, which are then used to arrive at the fractional abundance of the mutant. N.B. as the mutant DNA concentration increases there will be an increase in droplets in the MT+WT double-positive cluster.
   b. In the 2D fluorescence amplitude plot, with all wells highlighted, set the amplitude scale to 'fixed' for easy subsequent comparison between individual wells.
c. **Visualize the NTC wells** to determine cleanliness of the reagents and workflow. In a clean reaction, there should ideally be no positive droplets. More than the occasional mutant positive droplet (blue or orange) will impact the limit of detection and should be investigated for root-cause.

d. **Visualize the negative control (wild-type only) wells** to establish the specificity of the reactions. Assuming a clean NTC, any droplets in the negative control that are classified as mutant-positive will increase the specificity of the reactions. Reclassifying these to be negative will increase specificity at the potential risk of a decrease in sensitivity. Rare single nucleotide mutation detection assays are associated with a low level false positive rate (FPR) < 0.05% (Appendix E, Bulletin #6628). Therefore, it is advised to set the FAM threshold (wild-type) based on the FAM fluorescence of the majority of positive droplets in the wild-type only control. This can be facilitated using the heatmap view.

e. **Visualize the positive control wells** to confirm the reaction worked as desired. Confirm by inspecting the single-well 2D droplet plots that the classification does not significantly misclassify true mutant-positive droplets (orange and blue) as wild-type only (green) or as double-negative droplets (grey), respectively, resulting in false negatives. If a change to the threshold is made, return to the negative control wells to confirm the altered classification has not adversely affected the specificity of the assay.

f. If the controls do not perform acceptably, caution should be exercised in drawing conclusions from these data.

3. **Sample Data Analysis**

a. If all the above quality control metrics are satisfied, proceed to analyze the test samples.

b. **Examine apparent mutant positive wells.** If there is not very clear separation between the double positive cluster (orange) and the wild-type only cluster (green), the experimenter should use the positive and negative controls to set a combined-well droplet classification across all wells that best separates the adjacent clusters. As the aim of the inter-laboratory study is assessment of the reproducibility of quantification of KRAS G12D (in copies/µL or expressed as fractional abundance) rather than detection of presence/absence, the experimenter must determine what impact threshold setting has on the G12D concentration and fractional abundance of the positive and negative controls and unknown samples - whether to sacrifice sensitivity in favor of specificity or to sacrifice specificity in favor of sensitivity (Figure 4). The optimum FAM threshold ensures that false positives are minimized in the negative control while ensuring that G12D concentration values are maximized in the positive control. The impact of adjusting droplet classification can also be assessed by viewing the concentration values in the QuantaSoft Table or Concentration chart.

![Figure 4: Raising the FAM threshold line may increase specificity and decrease sensitivity. Lowering the threshold may increase sensitivity but would reduce specificity.](image-url)