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Drop-off Crystal Digital PCR™ for *NRAS, KRAS* and *EGFR* mutations

Samples for clinical cancer research, diagnostics and therapeutic monitoring can be limited in quantity. In this Application Note, accurate detection of the most prevalent *KRAS*, *NRAS* and *EGFR* sequence mutations, insertions and deletions using three internally controlled three-color multiplex drop-off assays was done using the Naica™ system. A drop-off assay is a cost- and time-efficient alternative for the simultaneous detection of clinically relevant alterations within a genomic interval.

How to design and quantify using a drop-off assay

A major advantage of drop-off digital PCR is the single-assay detection of multiple proximal genetic lesions (including deletions, insertions and nucleotide substitutions) within a short genomic interval. The simplest version of a drop-off assay includes two TaqMan™ probes targeting the same amplicon: a drop-off probe that spans the mutation hotspot but is uniquely complementary to the wild-type sequence, and a reference probe that hybridizes adjacent to the mutation site and is complementary to both the mutant and the wild-type alleles. In the presence of a wild-type allele, both the drop-off and reference probes will hybridize with their targets, leading to a double-positive signal. By contrast, 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 simple positive signal.

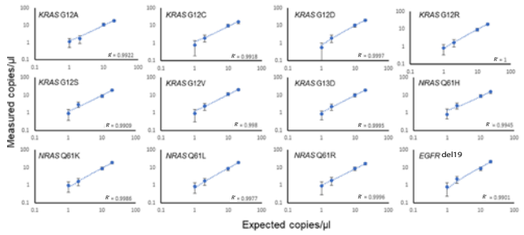

Figure 1 | Reliability of drop-off assays for the detection of the seven and four most prevalent *KRAS* exon 12–13 and *NRAS* exon 3 mutations, respectively, and *EGFR* exon 19 deletions. The mutations were detected with a 95% confidence level in serial dilutions ranging from 5% to 0.25% mutant DNA at final concentrations down to 1 copy/µl in a 25 µl PCR mixture. All assays were performed in a reaction background of 104 copies of wild-type DNA and 400 copies of the internal positive control DNA (ΦX174 bacteriophage). $N = 3$ replicates for each dilution point. The displayed confidence intervals are the means of the theoretical confidence intervals accounting for sampling and partitioning error at a 95% confidence level.

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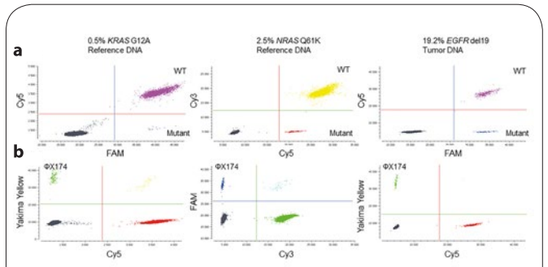

Figure 2 | 2D dot plots of the triplex drop-off assays. a. Crystal Miner-generated 2D dot plots of the *KRAS*, *NRAS* and *EGFR* drop-off digital PCR assays in triplex experiments. Commercial DNA (*KRAS* and *NRAS*), as well as DNA derived from frozen tumor samples (*EGFR*), was used. WT, wild type. **b**, Dot plots displaying the signal obtained in the third color channel for each assay using the ΦX174 DNA internal positive-control detection assay.

Drop-off assays detect *KRAS*, *NRAS* and *EGFR* hotspot mutations

In clinical settings, a set of predictive genetic markers is routinely monitored to track therapy efficacy. For example, in non-small-cell lung cancer, the presence of deletions in the epidermal growth factor (*EGFR*) exon 19 confers sensitivity to first-generation tyrosine kinase inhibitors. Moreover, in colorectal carcinoma, *KRAS* and *NRAS* proto-oncogene mutations are strong indicators of resistance to anti-EGFR antibodies. Using drop-off assays and the three-color multiplexing capacity of the Naica system, three internally controlled drop-off digital PCR assays were designed to detect the most prevalent *KRAS* exon 12, *NRAS* exon 3 and *EGFR* exon 19 sequence alterations (Figs. 1 and 2). The addition of an internal control allows the straightforward evaluation of assay robustness and the identification of PCR inhibition, which can occur as a result of sample impurity.

As multiple mutations are known to occur in *KRAS* and *NRAS* hotspots and several deletions or insertions of varying lengths have been described in *EGFR* exon 19, the use of drop-off assays allows rapid and cost-efficient simultaneous screening of a variety of clinically relevant genetic alterations using a limited number of probes. If desired, once a drop-off assay has identified a sample as mutant, subsequent assays using sequence-specific probes can be employed to determine the exact mutant alleles existing within the DNA sample.

APPLICATION NOTE HIGHLIGHTS

- Drop-off digital PCR assays enable the simultaneous detection of multiple mutations occurring at genomic hotspots.
- Drop-off assays allow rapid and cost-efficient screening of a variety of genetic alterations using a limited number of probes.
- Using Crystal Digital PCR™, we designed and validated three internally controlled drop-off assays for the detection of seven *KRAS* mutations, four *NRAS* mutations and a range of *EGFR* exon 19 deletions or insertions commonly monitored in clinical practice.

NATURE METHODS | JULY 2020