Detection of Rare Mutant Alleles within a Background of Wild-Type Sequences Using the QX100™ Droplet Digital™ PCR System

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Introduction

Droplet Digital PCR (ddPCR™) enables extremely sensitive and precise detection and analysis of nucleic acids. In ddPCR, a standard 20 µl qPCR assay is partitioned into 20,000 water-in-oil microdroplets, or "microreactors." Following PCR, each droplet can be counted as positive or negative, depending on the presence or absence of the target DNA. By exploiting Poisson sampling statistics, an absolute measure of concentration can be obtained. In essence, the fluorescence signal from qPCR is converted from an analog signal into a digital one, thereby removing the need for standard curves and allowing the determination of target copies on an absolute scale with high precision.

One application that harnesses the power of ddPCR is rare event detection (RED), where a biomarker exists within a background of a highly abundant counterpart that differs by only a single nucleotide. This detection is particularly relevant to studies of biomarkers associated with the onset and progression of cancers (Gormally et al. 2006) because associated mutations can be used to determine specific courses of therapeutic intervention (Molinari et al. 2011, Li et al. 2008, Soverini et al. 2011). Although a variety of methods exist for mutation analysis, many have poor selectivity and fail to detect mutant sequences with abundances of less than 1 in 100 wild-type sequences (Scott 2011, Benlloch et al. 2006, Whitehall et al. 2009). Methods with better selectivity and sensitivity for somatic mutations are thus needed.

Through the dilution effect of ddPCR, the relative abundance of a mutant target with respect to the wild-type gene is increased dramatically in a given partition (Figure 1). The partitioning further reduces competitive effects and thereby increases the specificity and sensitivity of the qPCR, yielding accurate and precise RED.

In this technical note, using the *BRAF V600E* mutation as a model assay, we demonstrate a significant increase in sensitivity over qPCR (>100-fold) that can be achieved in the detection of mutant alleles of clinically relevant biomarkers using the QX100 ddPCR system.

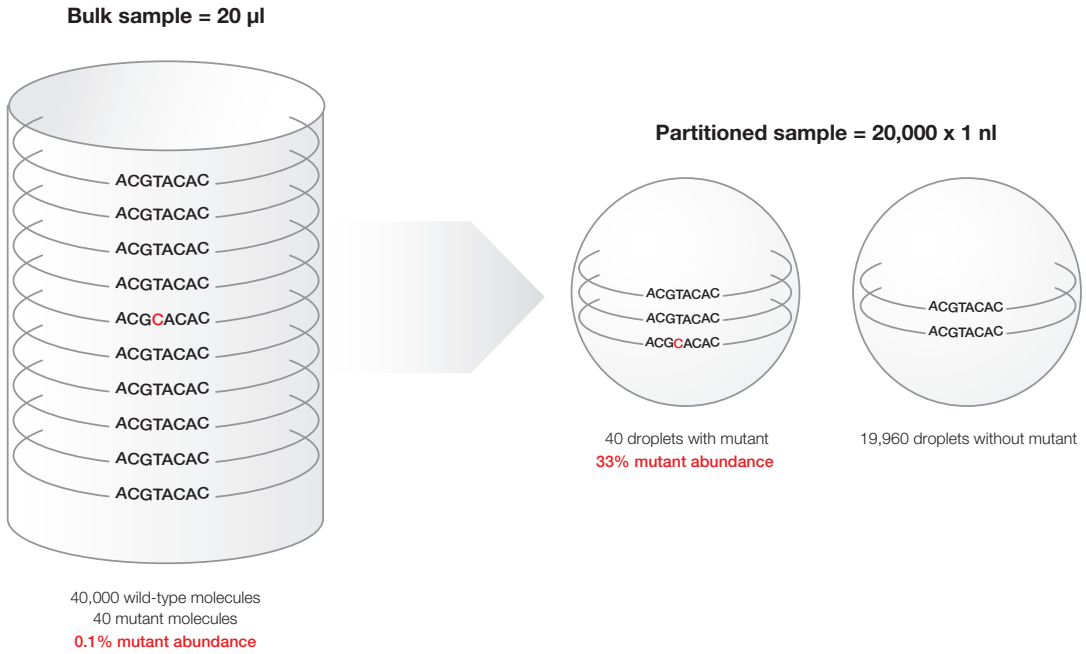

Fig. 1. Partitioning minimizes competition between target sequence and highly abundant background DNA.

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Materials and Methods

Sources of Cell Lines and Genomic DNA

RKO (CRL-2577) cell lines harboring the *BRAF V600E* mutation were obtained as frozen stocks from the American Type Culture Collection (ATCC). Genomic DNA (gDNA) was prepared from cell line material using a commercially available kit (DNeasy Blood & Tissue kit, QIAGEN) according to the manufacturer's suggested protocol. Estimates of DNA concentration were obtained spectrophotometrically by measuring the OD_{260} (NanoDrop 1000, Thermo Fisher Scientific Inc.). For human gDNA, 33 ng/µl corresponds to ~10,000 genome equivalents per microliter (GE/µl). Background human gDNA (female; Promega Corporation) was used as wild-type gDNA.

ddPCR Assay Design

The QX100 ddPCR system permits the use of TaqMan hydrolysis probe chemistry in combination with the ddPCR supermix for probes (Bio-Rad Laboratories, Inc.), detecting up to two fluorophores per reaction. This system enables the simultaneous detection and quantification of both the mutant and wild-type alleles from a single sample using a duplex assay format. Standard design rules should be employed in the design of TaqMan probes and primer sets; alternatively, online primer design software such as Primer3 can be utilized. In general, primers should be designed to have melting temperatures (T_m) of ~60°C (1 M NaCl, 1 µM concentration) and should be within 2°C of each other. Furthermore, the probe must have a T_m 5–10°C higher than the primer T_m. Finally, the mutant site should be positioned near the middle of the probe sequence and five to seven bases away from the minor groove binder (MGB) moiety, if utilized. All assay designs should be validated with a temperature gradient to ensure the highest specificity between the mutant and wild-type clusters (Figure 2). The optimal annealing temperature is defined by the following empirical criteria:

1. The mutant probe exhibits no false positives in the wild-type–only sample.
2. The relative distance between the FAM-only (mutant) and VIC-only (wild-type) clusters is maximal.

For the *BRAF V600E* mutation assay (Benlloch et al. 2006), the following primers were obtained desalted from Integrated DNA Technologies: Fwd 5'-CTACTGTTTTCCTTTACTTACTACACCTCAGA-3'; Rev 5'-ATCCAGACAACTGTTCAAACTGATG-3'. TaqMan MGB probes were obtained as a custom design from Life Technologies: 5'-FAM-TTGGTCTAGCTACAGTGAAAT-MGB-3' (mu); 5'-VIC-TTGGTCTAGCTACAGAGAAAT-MGB-3' (wt).

Rare Event Detection

To enable the ultrasensitive detection of mutant targets, very high loads of DNA are required. However, for ddPCR, when amounts of intact human DNA exceeding 66 ng (20,000 GEs) per 20 µl reaction are introduced, the accompanying increase in solution viscosity reduces the average droplet volume, negatively affecting the accuracy of DNA quantitation. To mitigate this effect, gDNA must be fragmented by restriction digestion using an enzyme that cuts around the amplicon(s) of interest. Once fragmented, the human gDNA concentration can exceed 1 µg/20 µl reaction without affecting droplet formation.

Wild-type and *BRAF V600E* gDNA samples were digested with MseI (New England BioLabs Inc. [NEB]) for 1 hr at 37°C in 1× NEB4 buffer supplemented with 0.1 mg/ml of bovine serum albumin (BSA). Following digestion, a dilution series of digested *BRAF V600E* gDNA was prepared in a high constant background (2,000–5,000 GE/µl in the final ddPCR mix) of wild-type DNA. Up to 2 µl of this material is needed to assemble a ddPCR mix as described above to limit the perturbation of Mg^{2+} concentrations.

Comparison of ddPCR and Real-Time PCR

Two sets of 20 µl PCR mixtures in quadruplicate were assembled from 2× ddPCR supermix for probes, 20× stocks of each assay (900 nM for each primer and 250 nM probe), and 2 µl of MseI-digested gDNA in a wild-type background of 2,000 GE/µl. Each quadruplicate PCR mixture was then converted into droplets for analysis via the QX100 ddPCR system or interrogated via qPCR using the CFX96™ real-time PCR detection system (Bio-Rad). Concentration calls from Quantasoft™ software and quantification cycle (Cq) values from CFX Manager™ software (both from Bio-Rad) were exported as CSV files and graphically summarized using MicroCal Origin 8.5 (OriginLab Corporation).

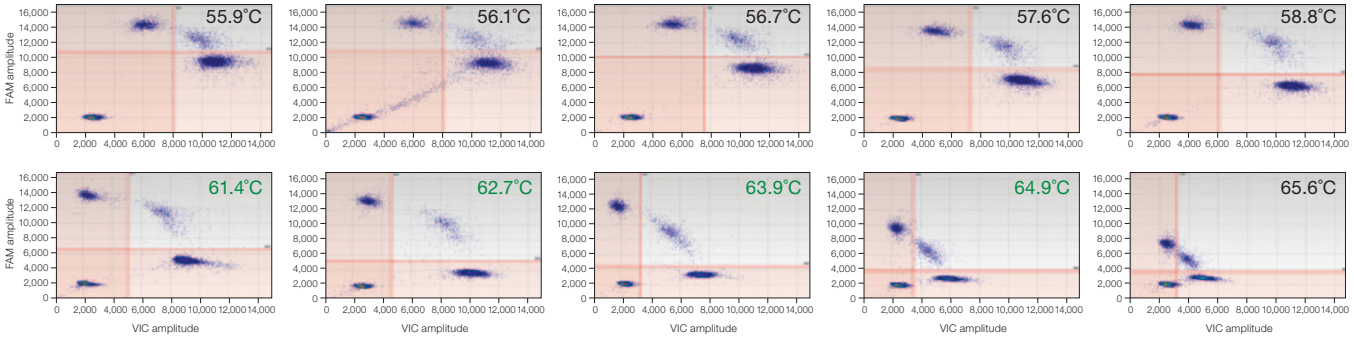

Fig. 2. Representative 2-D cluster plots for annealing temperature optimization of the *BRAF V600E* assay with ddPCR. Twelve samples of RKO genomic DNA were cycled across a range of annealing temperatures as shown. Data display signal amplitudes of droplets in both the FAM (y-axis) and VIC (x-axis) channels. Optimal annealing temperatures (in green) occur where there is minimal cross talk between fluorescence channels and fluorescence amplitudes are maximal.

Results and Discussion

The prognostic value of any TaqMan-based mutation assay is limited by its sensitivity, which is defined by the ability of the mutant TaqMan probe to preferentially bind to the mutant target sequence over the wild-type sequence and vice versa. Cross-reactivity between probes, however, is unavoidable and can be exacerbated when the wild-type species is present in vast excess relative to the mutant. This is the case in the assessment of minimal residual disease following therapeutic intervention or in the routine monitoring of mutant load. While this challenge has led to the development of a variety of alternative strategies that selectively inhibit wild-type amplification, it places an additional burden of design and cost on the user and prevents the simultaneous interrogation of both mutant and wild-type alleles in the same reaction.

To test the efficacy of ddPCR for increasing the specificity and sensitivity of qPCR for accurate and precise RED, the performance of TaqMan chemistry was assessed with the QX100 ddPCR platform and compared with standard qPCR conditions (Figure 3). A titration series of gDNA harboring the *BRAF V600E* mutation into a background of wild-type DNA was assayed under both systems. When interrogated with qPCR, TaqMan assays for the mutant lose the ability to detect input mutant fractions below 1%, the point at which the Cq values plateau at ~28 cycles (right panel, Figure 3A), due to cross-hybridization of the mutant probe with wild-type gDNA. Although this point defines the limit of detection (LOD) for this assay, its limit of quantification (LOQ), defined in qPCR by the highest Cq value that falls within the linear range of a Cq vs. input ratio plot (Bustin et al. 2009), is observed to be at a 2% mutant fraction.

In contrast, when this same titration series is assayed under identical conditions but partitioned and analyzed using the QX100 system, both the LOD and LOQ are improved up to 1,000-fold (Figure 3C). In particular, a high degree of linearity is observed down to 0.01% mutant fraction (R^2 = 0.9987; Figure 3C, left). When the background wild-type gDNA is increased to 5,000 GE/µl, as little as 0.001% mutant fraction can be detected (Figure 3C, right). Moreover, the linearity of the assay at this level of sensitivity is preserved (R^2 = 0.9998), defining the LOQ/LOD of this assay under ddPCR as 0.001% versus 2% under qPCR. Finally, because the QX100 provides direct counts of the number of target molecules in the sample, standard curves are not required.

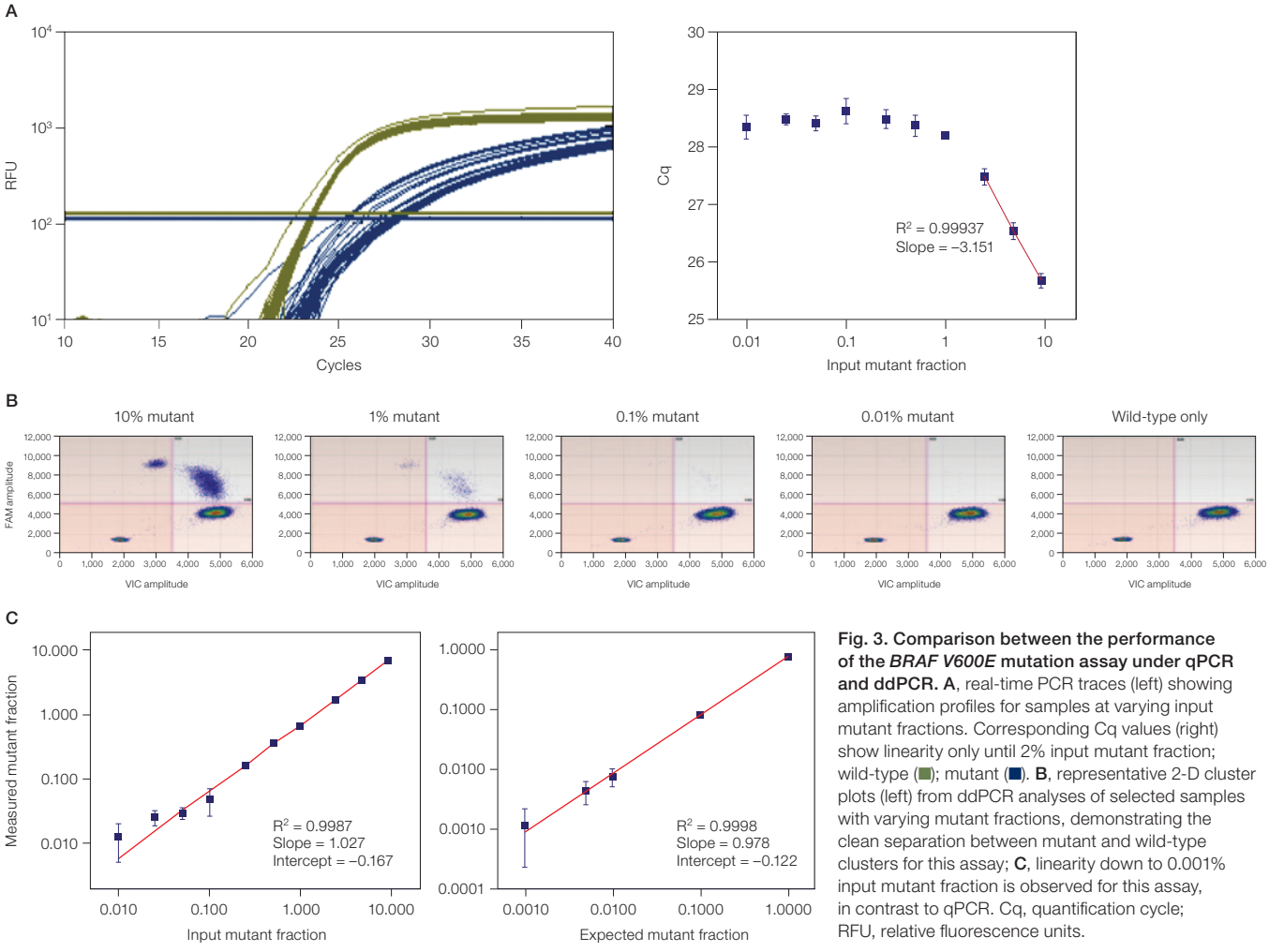

Fig. 3. Comparison between the performance of the *BRAF V600E* mutation assay under qPCR and ddPCR. A, real-time PCR traces (left) showing amplification profiles for samples at varying input mutant fractions. Corresponding Cq values (right) show linearity only until 2% input mutant fraction; wild-type (■); mutant (■). **B**, representative 2-D cluster plots (left) from ddPCR analyses of selected samples with varying mutant fractions, demonstrating the clean separation between mutant and wild-type clusters for this assay; **C**, linearity down to 0.001% input mutant fraction is observed for this assay, in contrast to qPCR. Cq, quantification cycle; RFU, relative fluorescence units.

In principle, reaction partitioning via ddPCR with the QX100 system should mitigate the impact of cross-reactivity and increase the sensitivity of TaqMan assays performed under standard qPCR conditions. Thus, partitioning of the PCR reaction alone, by diluting the competing wild-type DNA away from the mutant, mitigates the competitive effects of high background on mutant species detection and yields improved detection limits over standard TaqMan assays.

Conclusions

The QX100 ddPCR platform offers a simple and robust means to increase the precision and sensitivity of TaqMan assays for rare event detections. Not only are direct counts of template molecules within a sample possible (Pinheiro et al. 2012), but partitioning mitigates the effects of target competition, greatly improving the discriminatory capacity of assays that differ by only a single nucleotide. Here, the benefits for clinically relevant assay targets are demonstrated through the *BRAF V600E* mutation assay, where the LOD improved >100-fold when performed in ddPCR. This platform should prove invaluable to the molecular diagnostics field in general and to the cancer field in particular for the routine assessment of mutation status. Combined with the high accuracy achieved through the use of tens of thousands of partitions, ddPCR represents a unique platform for extremely precise and sensitive measurements of target molecules.

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