Droplet Digital PCR: Multiplex Screening of *KRAS* Mutations in Cell-Free DNA Colorectal Cancer Samples

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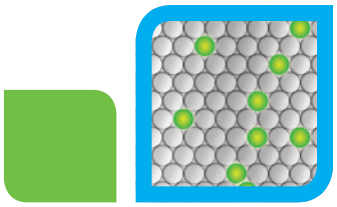

Droplet Digital PCR

Bulletin 6679

Abstract

Mutations in the *KRAS* gene that lead to its constitutive activation have been identified in 24–43% of colorectal cancer (CRC) tumors and are common in other tumor types, such as pancreatic, lung, and thyroid cancers, and myeloid leukemia. The majority of activating mutations in CRC tumors occur in codons 12 (~82%) and 13 (~17%) of exon 2 of the *KRAS* gene. The presence of activating mutations has been shown to be predictive of a negative response to anti–epidermal growth factor receptor (EGFR) therapy.

Targeted therapies in many cancers have allowed unprecedented progress in the treatment of disease. However, routine implementation of genomic testing is limited due to: 1) limited amounts of input samples (pg–ng range), 2) challenges in detecting mutational loads below 5%, 3) diagnostic evaluation and turnaround, and 4) cost. To optimize therapy strategies for personalized care, it is therefore critical to rapidly screen patient samples for the presence of multiple *KRAS* mutations. We have developed a ddPCR *KRAS* Screening Multiplex Kit to screen actionable *KRAS* mutations using Droplet Digital PCR (ddPCR). The ability to screen for multiple *KRAS* mutations down to 0.2% in a single well reduces the likelihood that mutations will be missed in poor-quality cell-free DNA and formalin-fixed, paraffin-embedded (FFPE) samples with limited amounts of amplifiable DNA.

Materials and Methods

- 12 mutant CRC patient plasma samples (mCRC; 6 female, 6 male, average age 52 years, 52 draws) and 12 normal plasma (12 female) samples were purchased (Conversant Bio and ProMedDx LLC, respectively). Five mCRC tissue samples were classified as *KRAS* mutation positive by the vendor, but not tested as plasma. Additional samples were provided by the Janku laboratory at MD Anderson Cancer Center. Samples were prepared using standard protocols and the QIAamp Circulating Nucleic Acid Kit (QIAGEN)
- Droplet Digital PCR (QX200 Droplet Digital PCR System) was performed on 1–8.75 µl sample per well using either the ddPCR *KRAS* Screening Multiplex Kit (Bio-Rad, catalog #1863506) or validated PrimePCR ddPCR Mutation Assays for 1 of 7 individual *KRAS* mutations (*G12D, G12V, G13D, G12A, G12C, G12R, G12S,* Bio-Rad)
- Positive mutation references were from Horizon Diagnostics, and negative controls were wild-type–only from Promega Corporation (female genomic DNA [gDNA]). Statistical significance was determined using 95% confidence intervals

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Results

Multiplex detection results are shown in Figures 1–5.

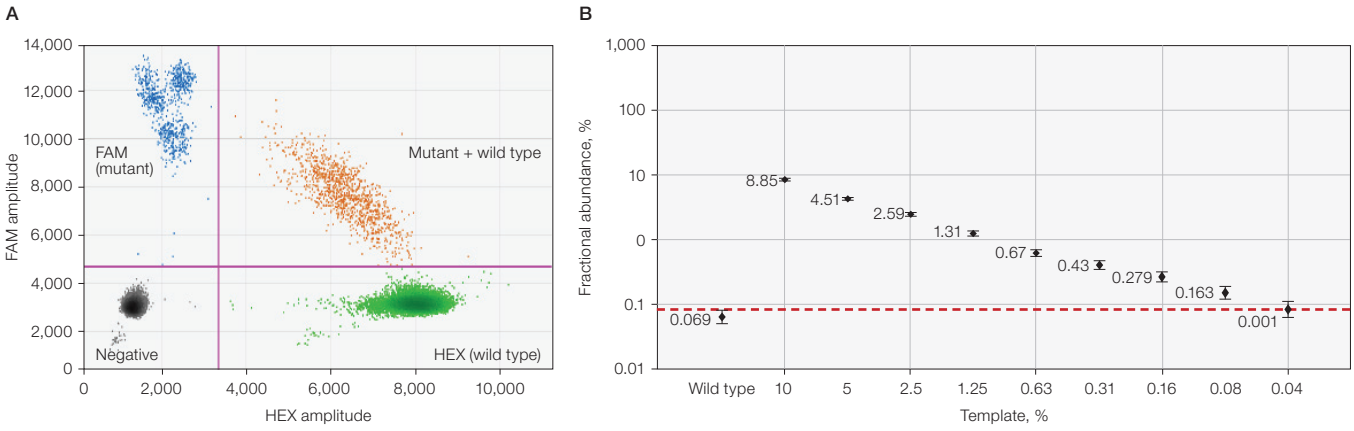

Fig. 1. Multiplexed single-well detection of seven actionable *KRAS* mutations. A, 2-D scatter plot of mixed *KRAS* gDNA; **B**, fractional abundance dilution series data using a *G12D* gDNA template and two wells.

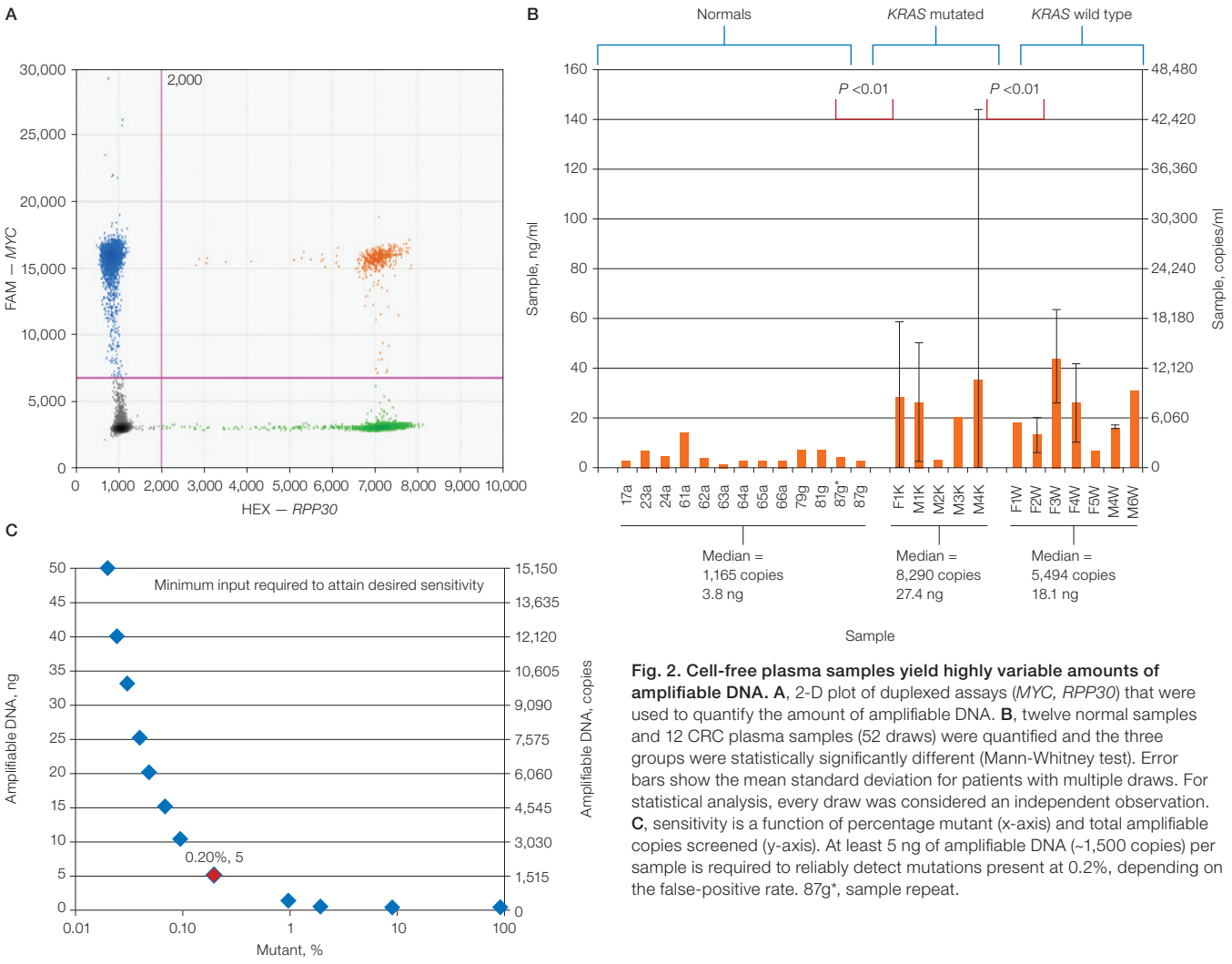

Fig. 2. Cell-free plasma samples yield highly variable amounts of amplifiable DNA. A, 2-D plot of duplexed assays (*MYC, RPP30*) that were used to quantify the amount of amplifiable DNA. **B**, twelve normal samples and 12 CRC plasma samples (52 draws) were quantified and the three groups were statistically significantly different (Mann-Whitney test). Error bars show the mean standard deviation for patients with multiple draws. For statistical analysis, every draw was considered an independent observation. **C**, sensitivity is a function of percentage mutant (x-axis) and total amplifiable copies screened (y-axis). At least 5 ng of amplifiable DNA (~1,500 copies) per sample is required to reliably detect mutations present at 0.2%, depending on the false-positive rate. 87g*, sample repeat.

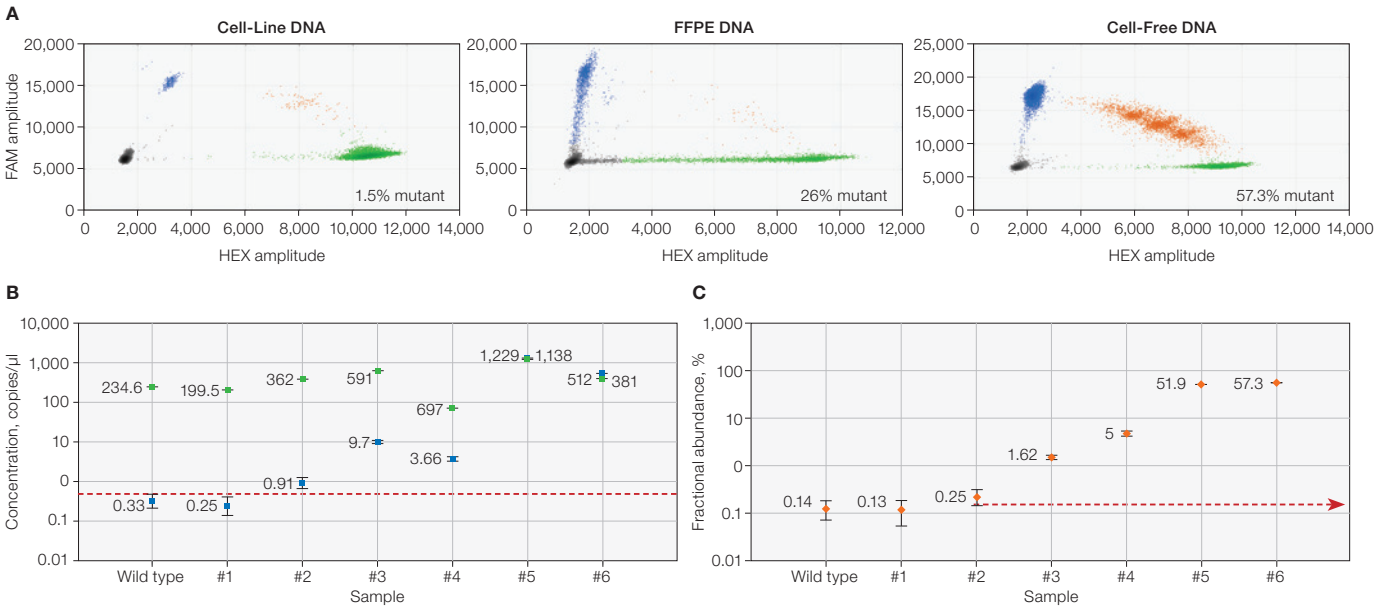

Fig. 3. Screening cell-line, FFPE, and cell-free plasma DNA samples with the ddPCR *KRAS* Screening Multiplex Kit. A, 2-D plots of ddPCR *KRAS* Screening Multiplex Assays applied to cell-line, FFPE, and cell-free DNA samples from patients from the MD Anderson Cancer Center (MDACC; Janku laboratory); **B**, concentration plot and **C**, fractional abundance plot (percentage mutant) of wild-type *KRAS* (■), mutant *KRAS* (■), and percentage mutant (■) from six MDACC cell-free plasma DNA samples, demonstrating the range of percentage mutant detection.

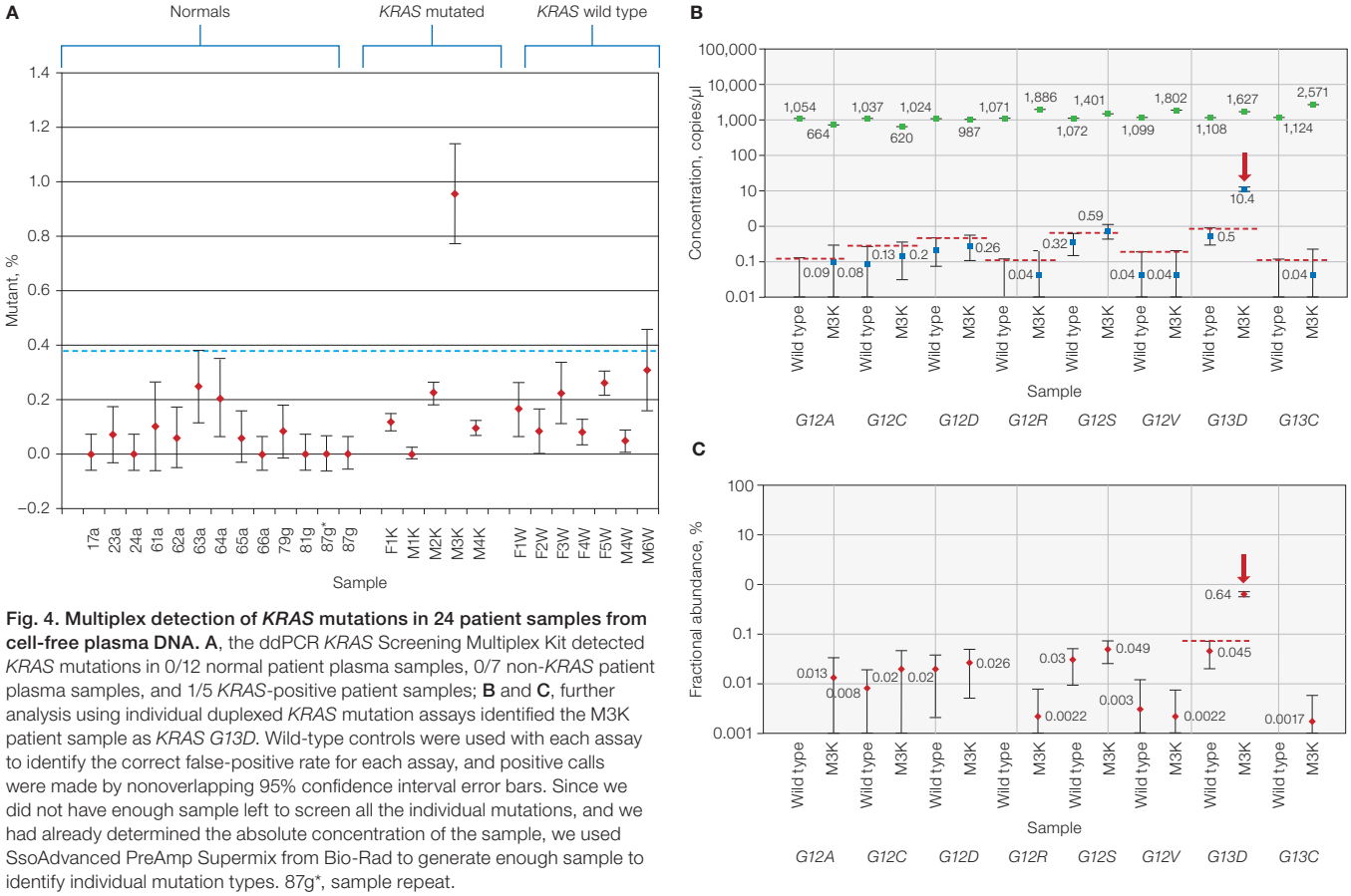

Fig. 4. Multiplex detection of *KRAS* mutations in 24 patient samples from cell-free plasma DNA. A, the ddPCR *KRAS* Screening Multiplex Kit detected *KRAS* mutations in 0/12 normal patient plasma samples, 0/7 non-*KRAS* patient plasma samples, and 1/5 *KRAS*-positive patient samples; **B** and **C**, further analysis using individual duplexed *KRAS* mutation assays identified the M3K patient sample as *KRAS G13D*. Wild-type controls were used with each assay to identify the correct false-positive rate for each assay, and positive calls were made by nonoverlapping 95% confidence interval error bars. Since we did not have enough sample left to screen all the individual mutations, and we had already determined the absolute concentration of the sample, we used SsoAdvanced PreAmp Supermix from Bio-Rad to generate enough sample to identify individual mutation types. 87g*, sample repeat.

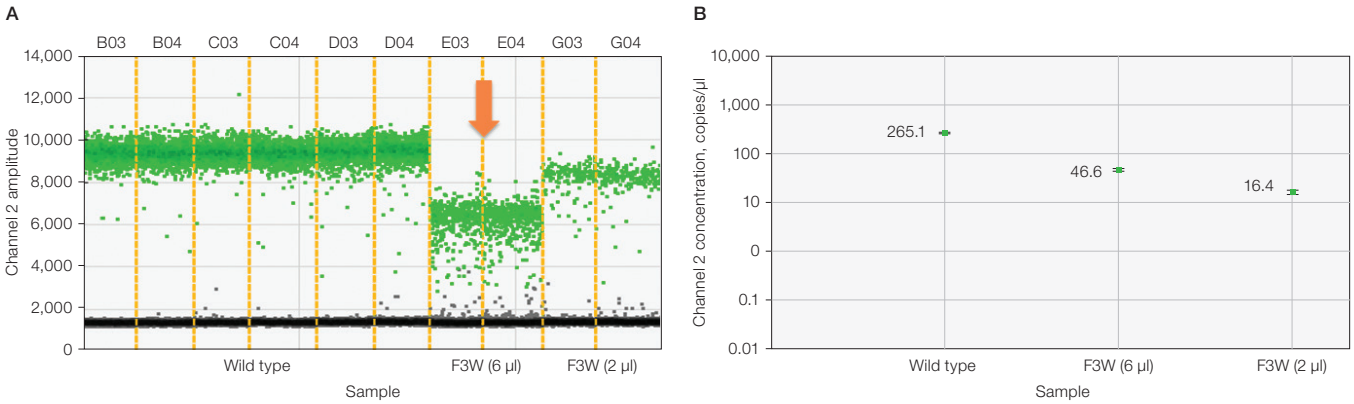

Fig. 5. ddPCR enables visualization of PCR inhibition from FFPE and cell-free plasma DNA samples. A, 1-D plot allows visualization and troubleshooting of PCR inhibition. For sample F3W, inhibitors are present (6 µl load), impacting positive fluorescence amplitudes. Loading less sample (2 µl) allows better amplification. **B**, regardless of sample load, the end-point quantification is the same.

Conclusions

- Droplet Digital PCR is an inexpensive method for the absolute quantification of minimal amounts of cell-free and FFPE DNA, for both mutation detection and quantification
- The amplifiable amount of cell-free DNA is significantly different between samples from normal patients and patients with cancer, and between *KRAS* mutant and *KRAS* wild-type samples from patients
- Using Bio-Rad's kit we have demonstrated sensitive and precise detection down to 0.2% for multiple actionable *KRAS* mutations in cell-free plasma DNA samples from patients with colorectal cancer
- The kit allows screening of a large volume of patient samples in a minimal amount of time
- Easy visualization of data enables rapid identification of PCR inhibition, either by poor assay design, sample inhibitors, poorly optimized conditions, or template degradation

Visit **bio-rad.com/ddPCRKRASmutations** for more information.

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