Droplet Digital PCR: Multiplex Detection of *KRAS* Mutations in Formalin-Fixed, Paraffin-Embedded Colorectal Cancer Samples

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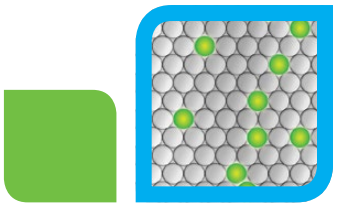

Droplet Digital PCR

Bulletin 6578

Abstract

Targeted therapies in many cancers have allowed unprecedented progress in the treatment of disease. However, routine implementation of genomic testing is constrained due to: 1) limited amounts of sample (pg–ng range) per biological specimen, 2) diagnostic turnaround time and workflow, 3) cost, and 4) difficulties in detection of mutational loads below 5%. *KRAS* is mutated in approximately 40% of colorectal cancers (CRCs). The majority of mutations affect codons 12, 13, and 61 and indicate a negative response to anti–epidermal growth factor receptor (EGFR) therapy. To optimize therapy strategies for personalized care, it is critical to rapidly screen patient samples for the presence of multiple *KRAS* mutations.

We have developed a multiplexing strategy to screen seven actionable *KRAS* mutations in colorectal cancer samples using digital PCR. This panel includes *KRAS* point mutations with individual frequencies higher than 1% and covers 98% of *KRAS* mutant colorectal cancers (Faulkner et al. 2010, unpublished data). No preamplification step is required. This *KRAS* screening assay was used to quantify *KRAS* mutational load in a panel of formalin-fixed, paraffin-embedded (FFPE) samples from patients with advanced metastatic colorectal cancer. *KRAS* mutations present at <1% fractional abundance were detected in multiple samples. This sensitive and inexpensive method reduces the risk of contamination and can be easily implemented for rapid, routine screening of cancer samples.

Materials and Methods

- 16 mCRC (7 female, 9 male, average age 64 years) and 4 grossly normal colon (2 female, 2 male, average age 65 years) FFPE blocks were purchased (Advanced Tissue Services). mCRC samples were classified as *KRAS* mutation positive by the vendor. Samples were prepared using standard protocols (QIAGEN)
- Droplet Digital PCR (ddPCR; QX200 Droplet Digital PCR System) was performed on 1–5 µl per sample per well using either a multiplexed *KRAS G12/G13* Assay or validated PrimePCR ddPCR Mutation Assay for one of seven 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 are shown in Figures 1–4.

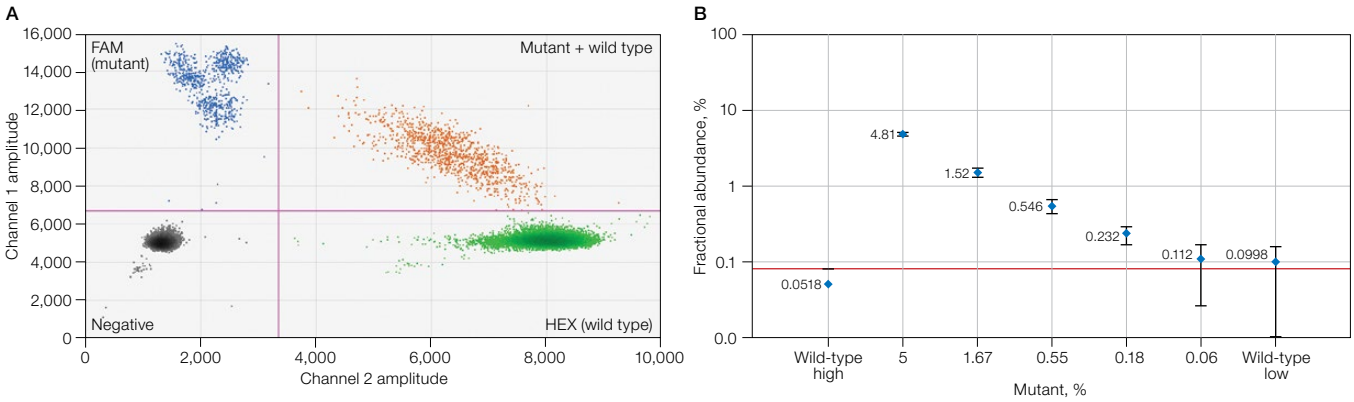


Fig. 1. Multiplexed single-well detection of seven actionable *KRAS* mutations. A, 2-D scatter plot; **B**, fractional abundance dilution series data.

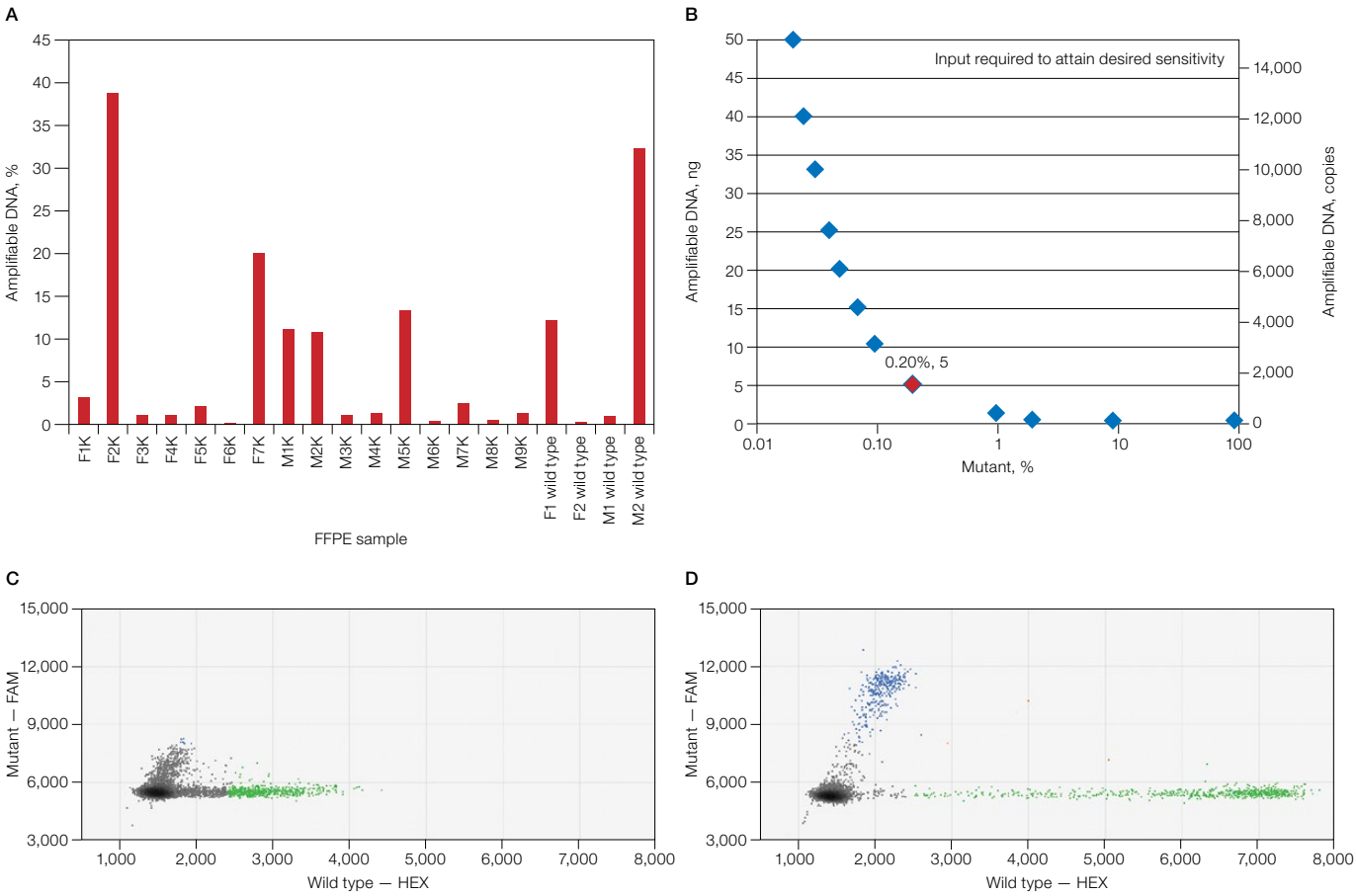
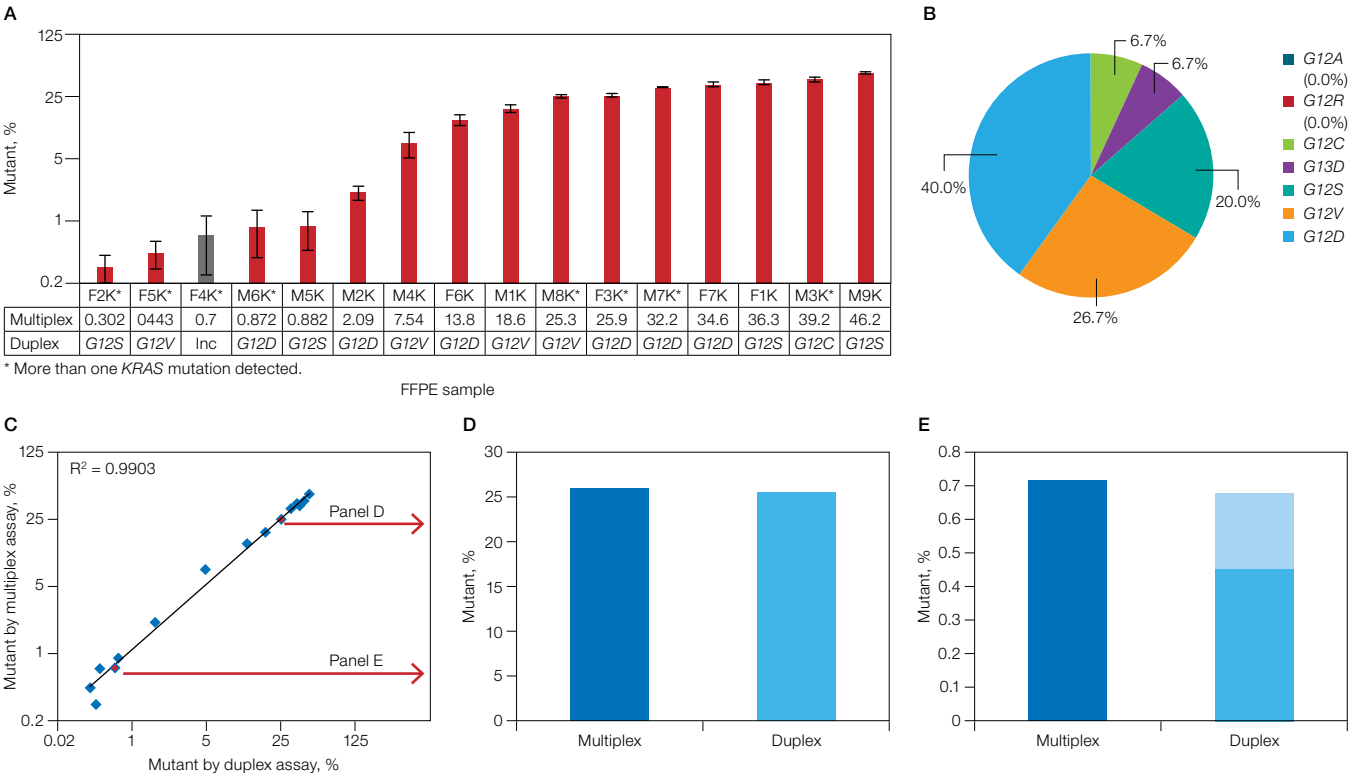


Fig. 2. FFPE samples yield low and variable amounts of amplifiable DNA. Duplexed reference assays (*AP3B1, EIF2C1*) were used to estimate the fraction of sample that could be PCR amplified. Eleven of 16 mCRC samples had <5% amplifiable material (**A**). Sensitivity is a function of percentage mutant (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% (**B**). 2-D scatter plots allow visualization and troubleshooting of PCR inhibition. For sample F7K, 20% of material is amplifiable, but the inhibitors present (5 µl loading) impact positive amplitudes (**C**). Loading less of sample F7K (2 µl) allows better amplification (**D**).



	F2K*	F5K*	F4K*	M6K*	M5K	M2K	M4K	F6K	M1K	M8K*	F3K*	M7K*	F7K	F1K	M3K*	M9K
Multiplex	0.302	0443	0.7	0.872	0.882	2.09	7.54	13.8	18.6	25.3	25.9	32.2	34.6	36.3	39.2	46.2
Duplex	*G12S*	*G12V*	Inc	*G12D*	*G12S*	*G12D*	*G12V*	*G12D*	*G12V*	*G12V*	*G12D*	*G12D*	*G12D*	*G12S*	*G12C*	*G12S*

* More than one *KRAS* mutation detected.

FFPE sample

Fig. 3. Detection and quantification of *KRAS* mutations. Quantification of *KRAS* mutational load across 16 mCRC FFPE samples was determined by a multiplex screening assay. Six samples had a mutation fractional abundance of <3%. Seven individual *KRAS* mutation assays were used to identify the dominant or sole *KRAS* mutation picked up by the initial screen (**A**). Consistent with the catalogue of somatic mutations in cancer (COSMIC) database, the majority of *KRAS* mutations (>80%) in these mCRC samples contained either *G12D, G12V, G13D*, or *G12S* (**B**). Quantification of *KRAS* mutational load by either a multiplex screening assay (y-axis) or individual mutation assay duplexes (x-axis) is tightly correlated (**C**). Two examples illustrate how *KRAS* mutational burden can be attributed to a single mutation (*G12D*, sample F3K) (**D**), or to more than one mutation (*G12D* plus *G12S*, sample M6K) (**E**). Inc, inconclusive.

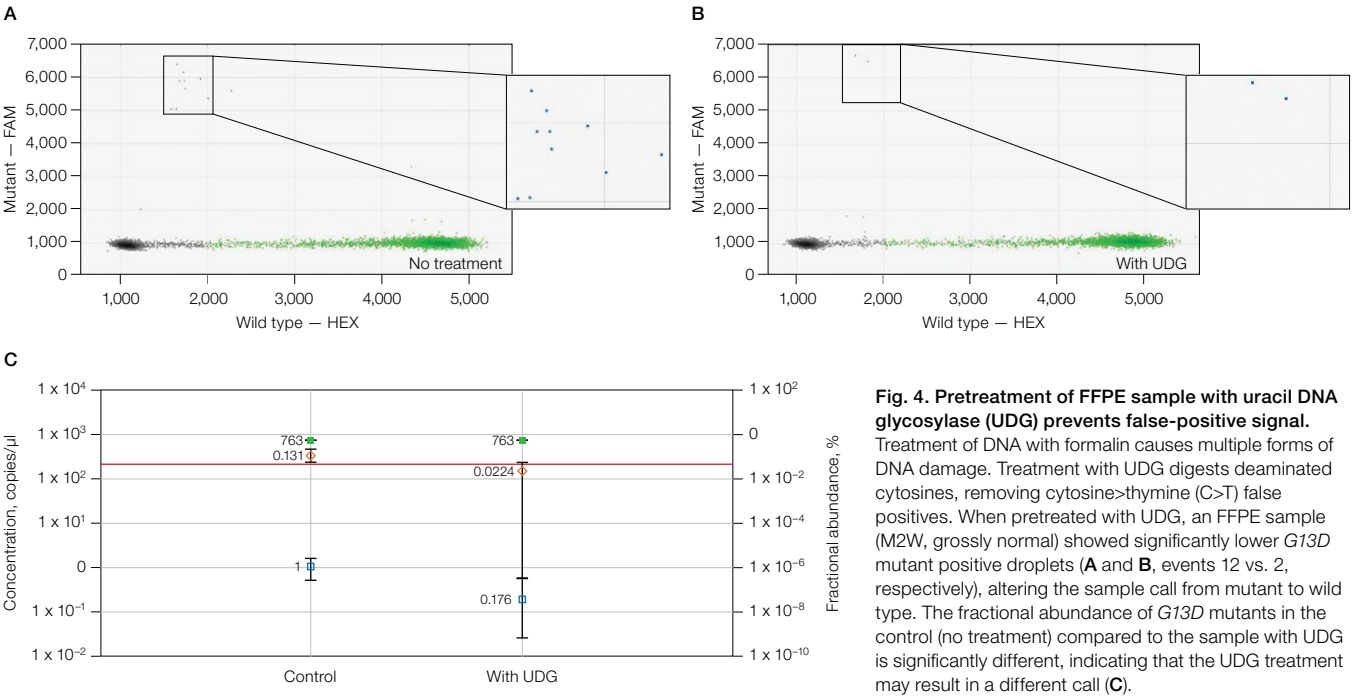


Fig. 4. Pretreatment of FFPE sample with uracil DNA glycosylase (UDG) prevents false-positive signal. Treatment of DNA with formalin causes multiple forms of DNA damage. Treatment with UDG digests deaminated cytosines, removing cytosine>thymine (C>T) false positives. When pretreated with UDG, an FFPE sample (M2W, grossly normal) showed significantly lower *G13D* mutant positive droplets (**A** and **B**, events 12 vs. 2, respectively), altering the sample call from mutant to wild type. The fractional abundance of *G13D* mutants in the control (no treatment) compared to the sample with UDG is significantly different, indicating that the UDG treatment may result in a different call (**C**).

Conclusions

- We have demonstrated sensitive and precise detection (less than 1%, single reaction) of multiple actionable *KRAS* mutations in FFPE samples from patients with colorectal cancer
- Concordance between duplex- and multiplex-based detection is excellent
- Droplet Digital PCR provides a simple and robust workflow for mutation detection of patient samples in a rapid and cost-effective manner
- UDG treatment of FFPE DNA reduces the false positives generated by deaminated C>T transitions caused by formalin fixation

References

Do H and Dobrovic A (2012). Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil- DNA glycosylase. Oncotarget 3, 546–558.

Faulkner NE et al. KRAS mutation analyses of more than 16,500 colorectal carcinomas. Poster presented at: 2010 ASCO-NCI-EORTC Annual Meeting on Molecular Markers in Cancer; October 18–20, 2010; Hollywood, FL [unpublished data].

Visit bio-rad.com/web/ddPCRKRAS for more information.

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