Multiplex detection of KRAS mutations in colorectal cancer FFPE and cfDNA samples using droplet digital PCR

BIO-RAD

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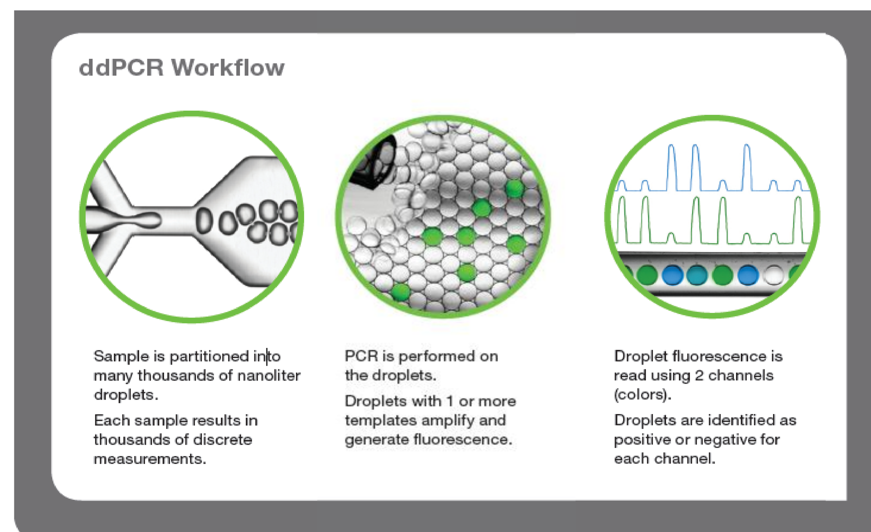
Introduction

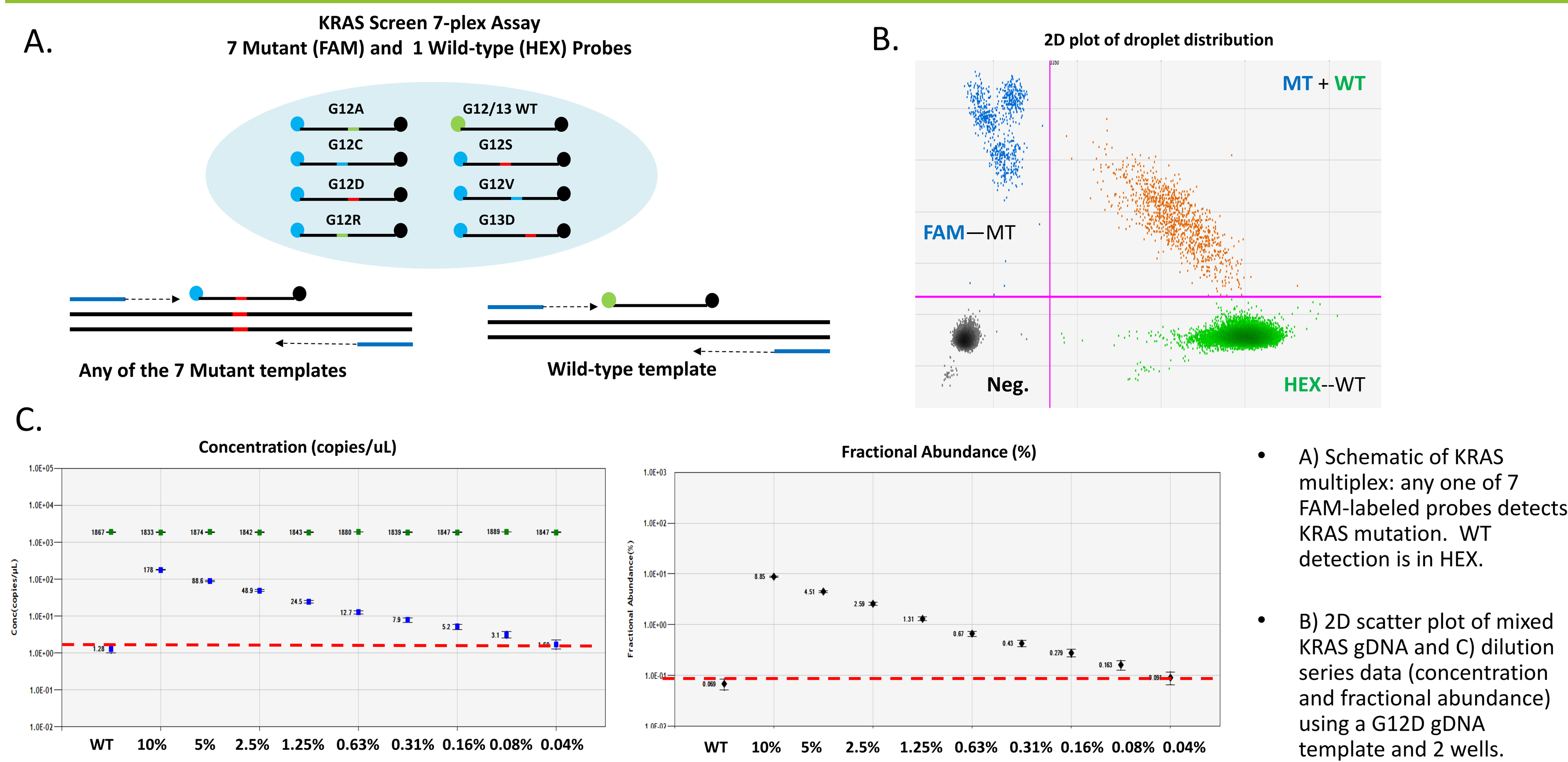
Targeted therapies in many cancers have allowed unprecedented progress in the treatment of disease. However, routine implementation of genomic testing is limited due to: 1) difficulties in detection of mutational loads below 5%, 2) limited amounts of sample (pg-ng range) per biological specimen, 3) diagnostic turnaround time and workflow, and 4) cost. KRAS is mutated in approximately 40% of colorectal cancers, and KRAS mutations are predictive of a negative response to αEGFR therapy. 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 multiplexing strategy to screen clinically-actionable KRAS mutations using digital PCR. No pre-amplification step is required. This sensitive and inexpensive method reduces the risk of contamination and can be easily implemented in molecular diagnostic laboratories for rapid, routine screening of cancer patients.

Materials and Methods



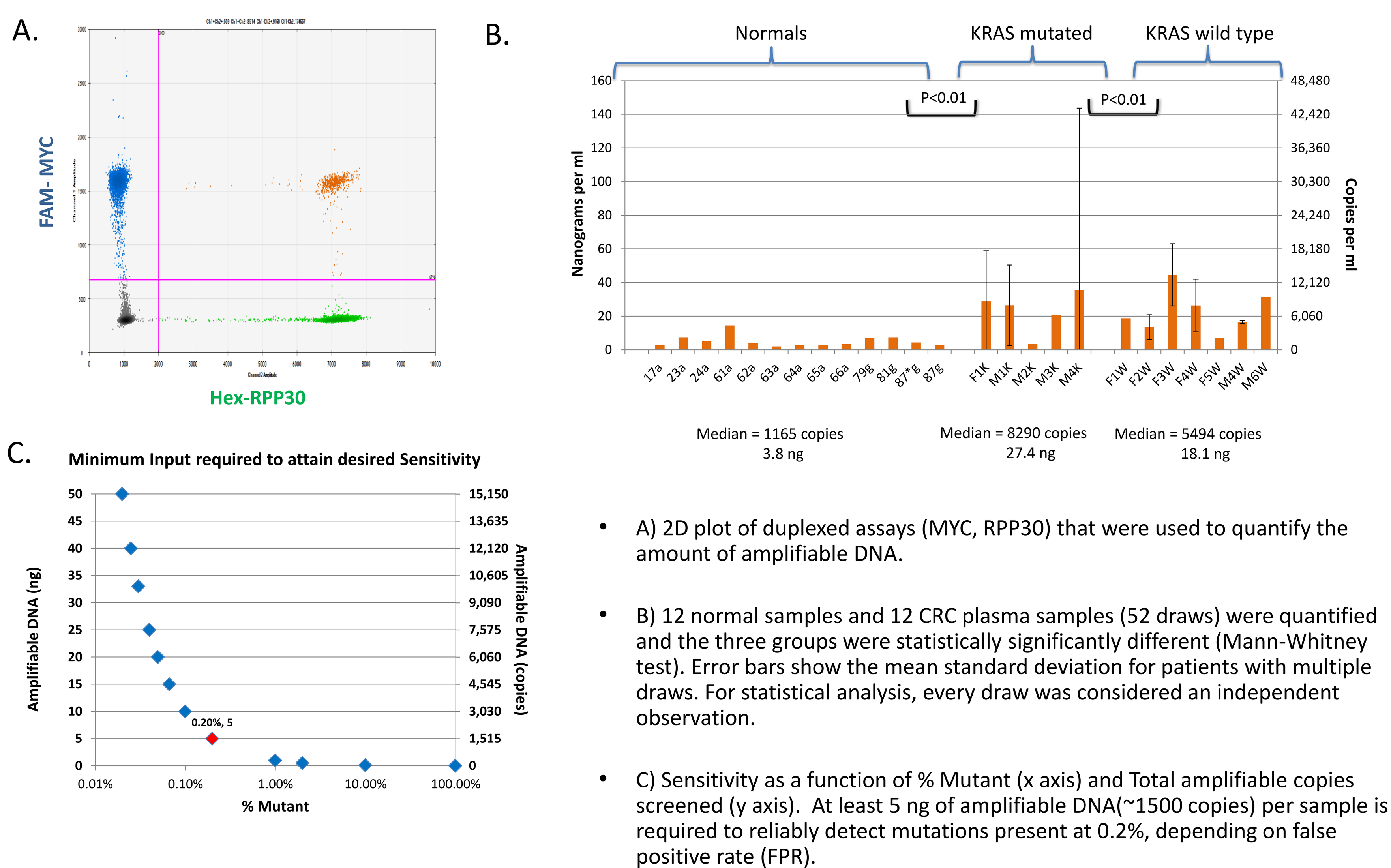
- 12 mCRC patient plasma samples (6 female, 6 male, average age 52 years, 52 draws) and 12 normal plasma (12 female) samples were purchased (ConversantBio, Huntsville, AL) and (ProMedDx, Lafayette, LA) respectively. Five mCRC tissue samples were classified as KRAS mutation-positive by the vendor, but not tested as plasma. Additional samples (Fig.3) were provided by the Janku lab at MDACC. Samples were prepared using standard protocols (Qiagen CNA kit).
- ddPCR was performed on 1 – 8.75 uL per sample per well using either the KRAS Screening Multiplex for ddPCR or validated PrimePCR ddPCR mutation assays for one of seven individual KRAS mutations (G12D, G12V, G13D, G12A, G12C, G12R, G12S, Bio-Rad Laboratories)
- Positive mutation references were from Horizon Diagnostics, and negative controls were wild type-only from Promega (female gDNA) . Statistical significance was determined using 95% confidence intervals.

Figure 1: Multiplexed single-well detection of 7 actionable KRAS mutations



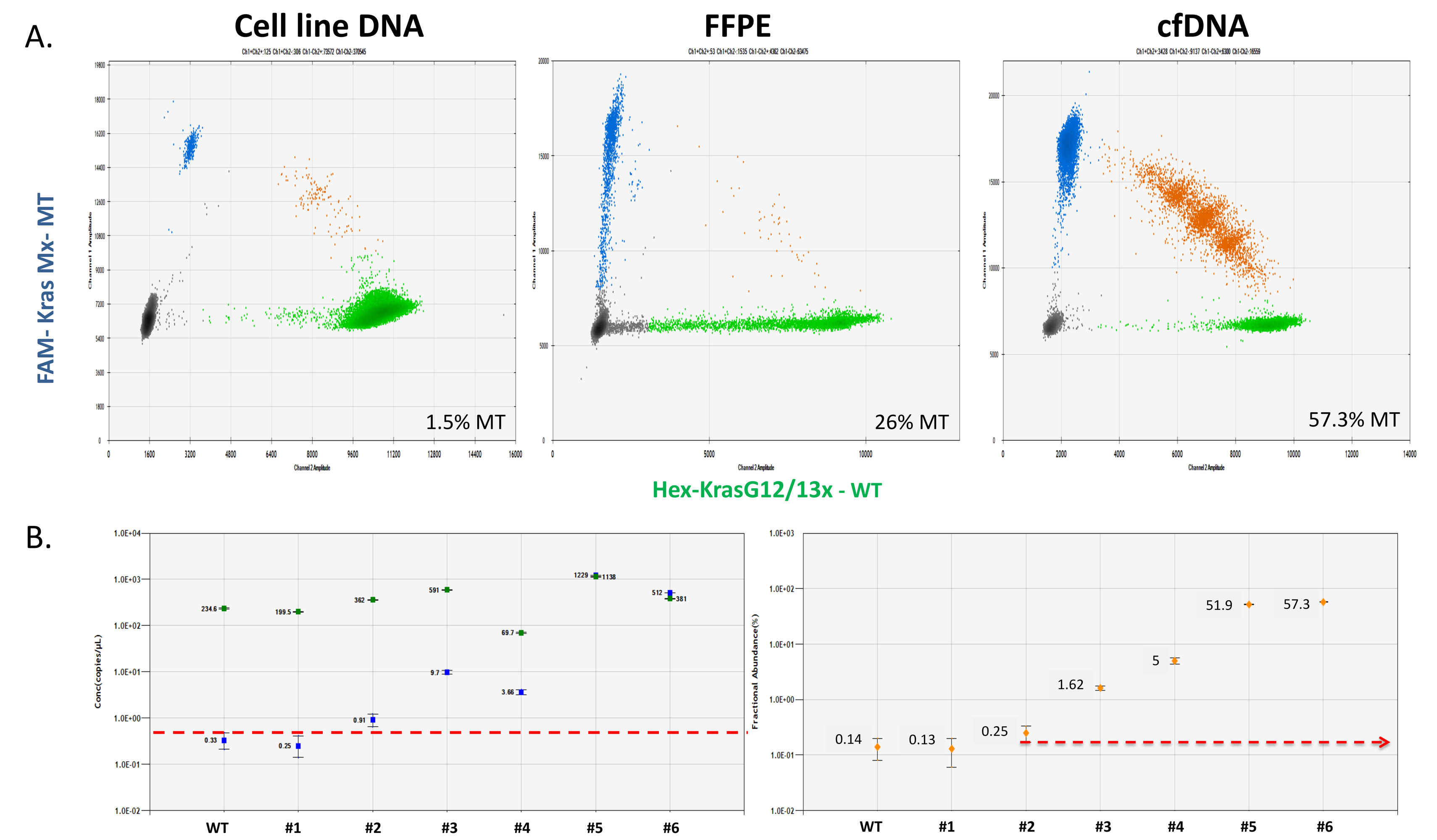
- A) Schematic of KRAS multiplex: any one of 7 FAM-labeled probes detects KRAS mutation. WT detection is in HEX.
- B) 2D scatter plot of mixed KRAS gDNA and C) dilution series data (concentration and fractional abundance) using a G12D gDNA template and 2 wells.

Figure 2: Cell-free plasma samples yield highly variable amounts of amplifiable DNA



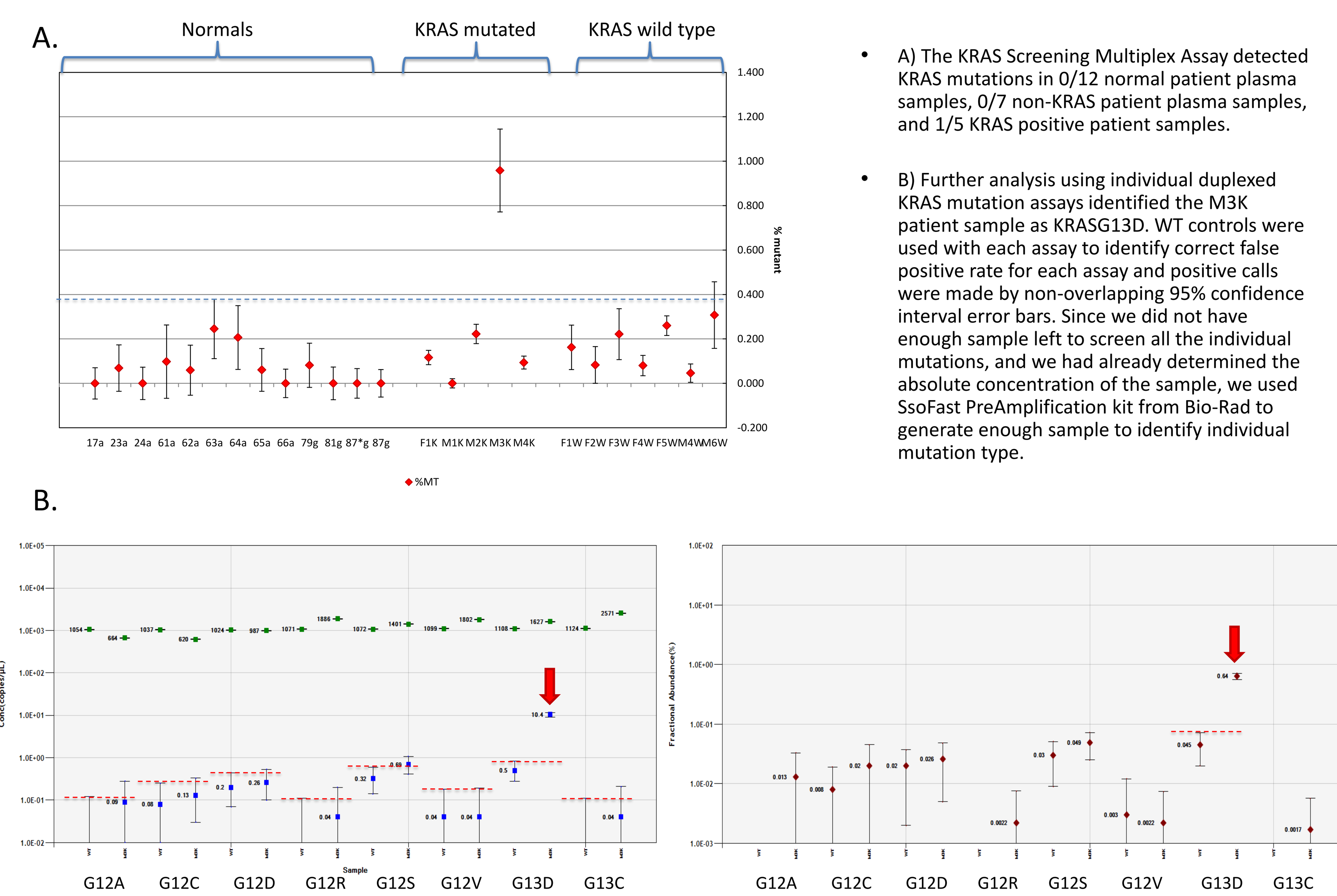
- A) 2D plot of duplexed assays (MYC, RPP30) that were used to quantify the amount of amplifiable DNA.
- B) 12 normal samples and 12 CRC plasma samples (52 draws) were quantified and the three groups were statistically significantly different (Mann-Whitney test). Error bars show the mean standard deviation for patients with multiple draws. For statistical analysis, every draw was considered an independent observation.
- C) Sensitivity as a function of % Mutant (x axis) and Total amplifiable copies screened (y axis). At least 5 ng of amplifiable DNA(~1500 copies) per sample is required to reliably detect mutations present at 0.2%, depending on false positive rate (FPR).

Figure 3: KRAS Screening Multiplex assay on cell line DNA, FFPE, and cell-free plasma DNA



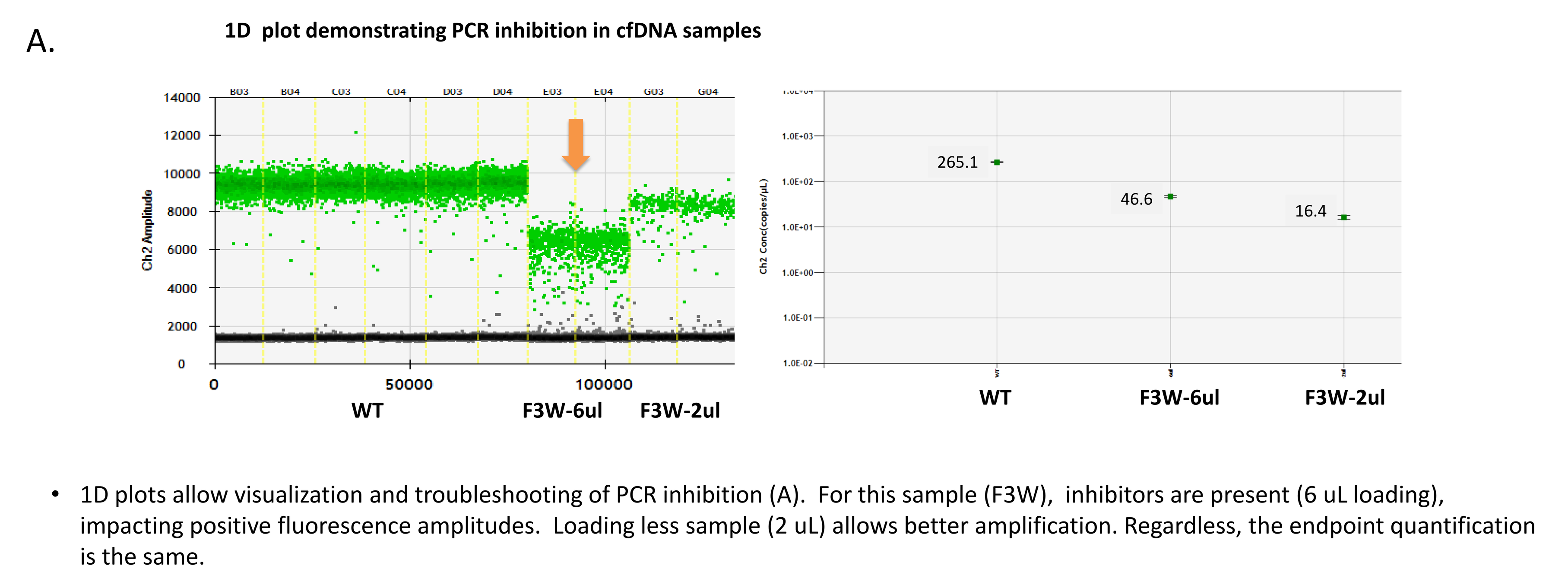
- A) 2D plots of KRAS Screening Multiplex assay on cell line DNA, FFPE, and cfDNA from MD Anderson Cancer Center patients .
- B) Concentration plot and Fractional Abundance plot (%mutant) of wildtype KRAS (**green**), mutant KRAS (**blue**), and % mutant (**orange**) from 6 MDACC cfDNA plasma samples, demonstrating range of %MT detection.

Figure 4: Multiplex detection of KRAS mutations in 24 patient samples from cell-free plasma DNA



- A) The KRAS Screening Multiplex Assay 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) Further analysis using individual duplexed KRAS mutation assays identified the M3K patient sample as KRASG13D. WT controls were used with each assay to identify correct false positive rate for each assay and positive calls were made by non-overlapping 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 SsoFast PreAmplification kit from Bio-Rad to generate enough sample to identify individual mutation type.

Figure 5: ddPCR enables visualization of PCR inhibition from FFPE and cell-free plasma DNA samples



- 1D plots allow visualization and troubleshooting of PCR inhibition (A). For this sample (F3W), inhibitors are present (6 uL loading), impacting positive fluorescence amplitudes. Loading less sample (2 uL) allows better amplification. Regardless, the endpoint quantification is the same.

Conclusions

- Droplet digital PCR is a inexpensive method to quantify, absolutely, minimal amounts of FFPE and cfDNA, both for quantification and mutation detection.
- The amplifiable amount of cfDNA is significantly different between normal and cancer patient samples, and between KRAS mutant and KRAS wild type patient samples.
- We have demonstrated sensitive and precise detection down to 0.25% for multiple actionable KRAS mutations in cfDNA plasma samples from colorectal cancer patients.
- Droplet digital PCR provides a simple and robust workflow to screen a large volume of patient samples in a minimal amount of time.
- 1D & 2D plots of droplets enables rapid identification of PCR inhibition, either by poor assay design, sample inhibitors, poorly optimized conditions, or template degradation.

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