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

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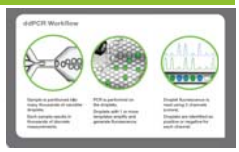
Introduction

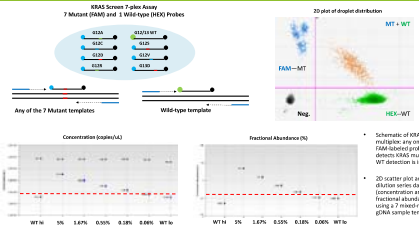
Targeted therapies in many cancers have allowed unprecedented progress in the treatment of disease. However, routine implementation of genomic testing is limited due to: 1) difficulties in detection of mutational loads below 5%, 2) limited amounts of sample (pg-ng range) per biological specimen, 3) diagnostic turnaround time and workflow, and 4) cost. KRAS is mutated in approximately 40% of colorectal cancers, and KRAS mutations are indicative 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 was 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



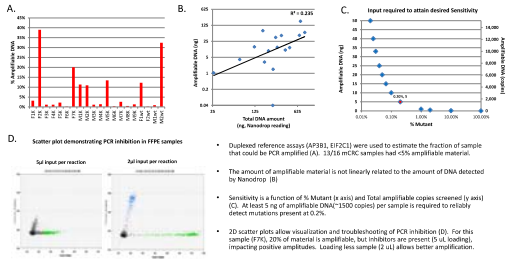
- 16 mCRC (7 female, 9 male, average age 64 years) and 4 grossly normal colon (2 female, 2 male, average age 65 years) FFPE blocks were purchased (Advanced Tissue Services, Phoenix, AZ). mCRC samples were classified as KRAS mutation-positive by the vendor. Samples were prepared using standard protocols (Qiagen).
- ddPCR was performed on 1 – 5 uL per sample per well using either a multiplexed KRAS G12/G13D assay 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 wildtype-only from Promega (female gDNA) . Statistical significance was determined using 95% confidence intervals.

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



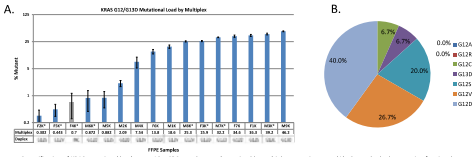
- Schematic of KRAS multiplex: any one of 7 FAM-labeled probes detects KRAS mutation. WT detection is in HEX.
- 2D scatter plot and dilution series data (concentration and fractional abundance) using a 7 mixed-mutation gDNA sample template

Figure 2: FFPE samples yield low and variable amounts of amplifiable DNA



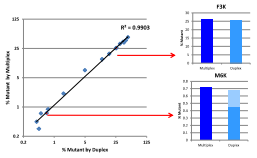
- Duplexed reference assays (AP3B1, EIF2C1) were used to estimate the fraction of sample that could be PCR amplified (A). 13/16 mCRC samples had <5% amplifiable material.
- The amount of amplifiable material is not linearly related to the amount of DNA detected by Nanodrop (B)
- Sensitivity is a function of % Mutant (x axis) and Total amplifiable copies screened (y axis) (C). At least 5 ng of amplifiable DNA(~1500 copies) per sample is required to reliably detect mutations present at 0.2%.
- 2D scatter plots allow visualization and troubleshooting of PCR inhibition (D). For this sample (FFK), 20% of material is amplifiable, but inhibitors are present (5 uL loading), impacting positive amplitudes. Loading less sample (2 uL) allows better amplification.

Figure 3: Multiplex detection of KRAS mutations in 16 mCRC FFPE samples



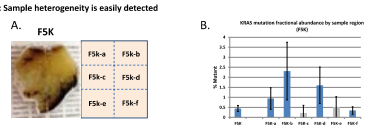
- Quantification of KRAS mutational load across 16 mCRC samples was determined by multiplex screening assay (A). 6 samples had a mutation fractional abundance of <3%. Seven individual KRAS mutation assays were used to identify the dominant or sole KRAS mutation picked up by the initial screen.
- Consistent with public databases (COSMIC), the majority of KRAS mutations (>80%) in these mCRC samples contained either G12D, G12V, G13D, or G12S (B).

Figure 4: Correlation of quantified KRAS mutational load by multiplex and single KRAS assays



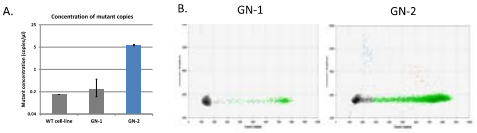
- Quantification of KRAS mutational load by either multiplex screening assay (y axis) or individual mutation assay duplexes (x axis) is tightly correlated.
- Two examples illustrate how KRAS mutational burden can be attributed to a single mutation (G12D, sample F3K), or to more than one mutation (G12D plus G12S, sample M6K)

Figure 5: Sample heterogeneity is easily detected



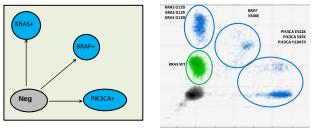
- Two FFPE blocks per patient were acquired from the vendor. In this example, mCRC sample F5K, block 1, was prepared in totality and analyzed for KRAS mutation by multiplex ("F5K", B). Block 2 from F5K was first cut into 6 sections (A, F5k-a through F5K-f), and these sub-sections were prepared and analyzed in parallel.
- Percentage Mutant plot (B) for F5K subsamples indicates that amongst significantly positive subsections (blue), a difference in mutational load is detectable.

Figure 6: KRAS mutations detected in grossly normal FFPE samples



- Grossly normal samples were analyzed for the presence of KRAS mutations (A). Grossly normal FFPE sample 2 (GN-2) had a significant level of KRAS mutation present as compared to grossly normal FFPE sample 1 (GN-1) or wild-type Promega DNA. Detection of KRAS mutation was confirmed using single duplexed assays (data not shown).
- 2D scatter plots (B) showing the presence of statistically-significant levels of mutant (FAM positive, blue and orange) droplets in GN-2 but not in GN-1. Analysis of two additional grossly normal samples was inconclusive (data not shown).

Figure 7: Multiplex screening of additional actionable EGFR pathway mutations in a single well



- Example plot showing a screening strategy for the most prevalent KRAS (G12D, G12V, G13D), BRAF (V600E), and PIK3CA (E542K, E545K, H1047R) mutations using a single well.
- This screening assay detects the majority of mutations found in mCRC samples
- We are investigating higher order multiplexing in one well.

Conclusions

- We have demonstrated sensitive and precise detection (less than 1%, single reaction) of multiple actionable KRAS mutations in FFPE samples from colorectal cancer patients
- Concordance between duplex- and multiplex-based detection is excellent
- Precision and sensitivity of the measurements reveal significant spatial heterogeneity of the samples
- Droplet-digital PCR provides a simple and robust workflow for mutation detection in clinical patient samples
- Future work will extend the number of targets per reaction, optimize the workflow and sample compatibility to deliver on the promise of NextGen Digital technologies in precision medicine and research

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