Single molecule droplet-based digital PCR for high sensitivity detection of cancer biomarkers

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Introduction

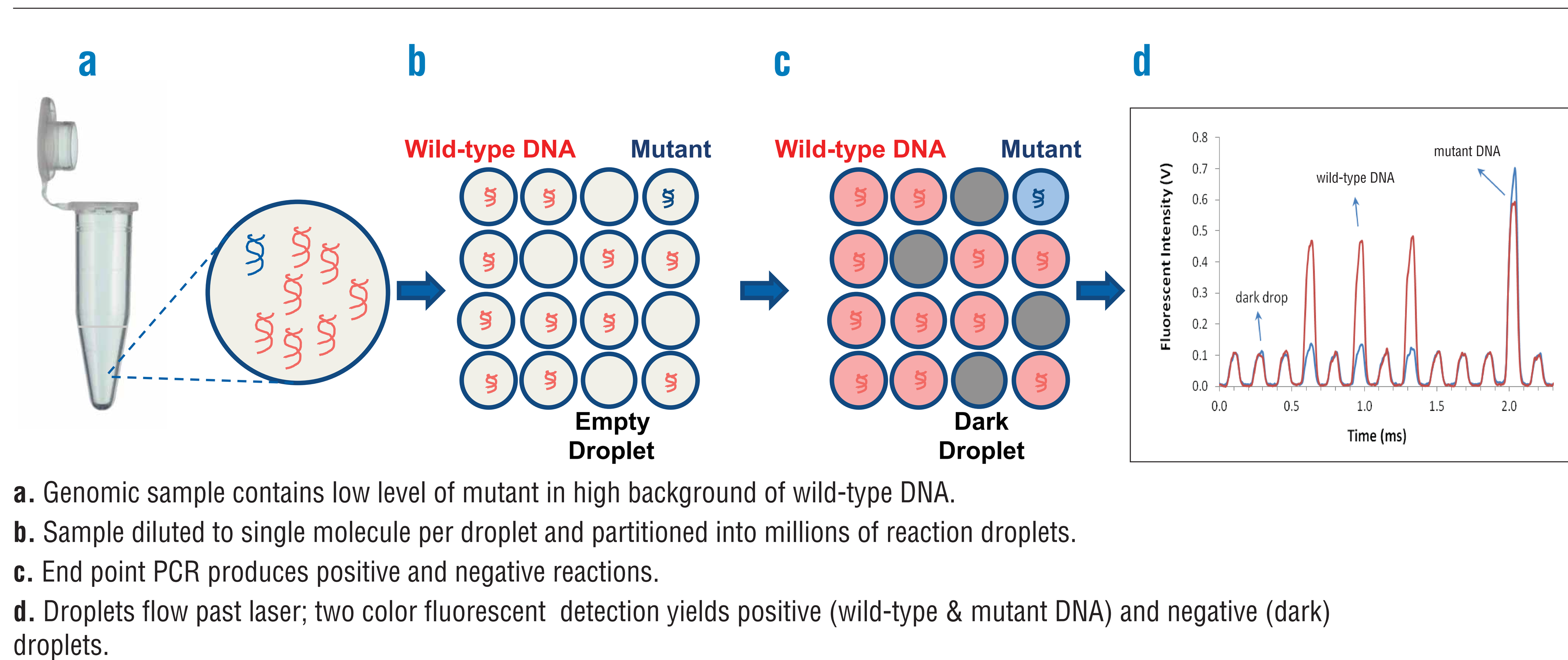
Somatic mutations within tumoral DNA are highly specific biomarkers that distinguish cancer cells from normal cells. The biomarkers are likely to be useful for the diagnosis, prognosis, treatment, and follow-up of patients, but use of these biomarkers in clinical oncology requires a highly sensitive strategy that discriminates tumor-specific mutations in a large excess of non-mutated DNA. Digital PCR (dPCR) is an attractive alternative to qPCR due to its superior sensitivity and absolute quantification of target molecules. The RainDance RainStorm™ droplet-based microfluidics method utilizes up to 10 million reactions to enable detection of rare mutations in a background of wild-type DNA at levels better than 1 in 200,000.[1] The combination of superior sensitivity, unprecedented multiplexing, and flexibility in experiment design provide a powerful genomic analysis platform for new research in cancer including rare variant detection, absolute quantitation of biomarkers, tumor profiling, and the ability to monitor residual disease.

KRAS mutations are found at high rates in several cancers, including colon cancer. The RainDance digital PCR technology is being used to screen colorectal tumor samples from a collection of patients with the goal of correlating the identity and level of one or more KRAS mutation with known clinical outcomes. This system will also be used to validate tumor characterization achieved with next-generation sequencing and Sanger sequencing, and to monitor patient-specific biomarkers for detecting minimal residual disease and disease recurrence.

What is Digital PCR?

Digital PCR is an absolute method of quantifying DNA. Template DNA is diluted to single molecule occupancy per reaction. Using millions of droplets as reaction chambers and end-point amplification, positive PCR reactions are counted as a direct measurement of the number of DNA molecules originally present. Fluorogenic probes are commonly used to discriminate PCR(+) and (-) reactions, and Poisson statistics account for the possibility of multiple molecules occupying the same reaction. Unlike qPCR, standard curves are not necessary.

Figure 1

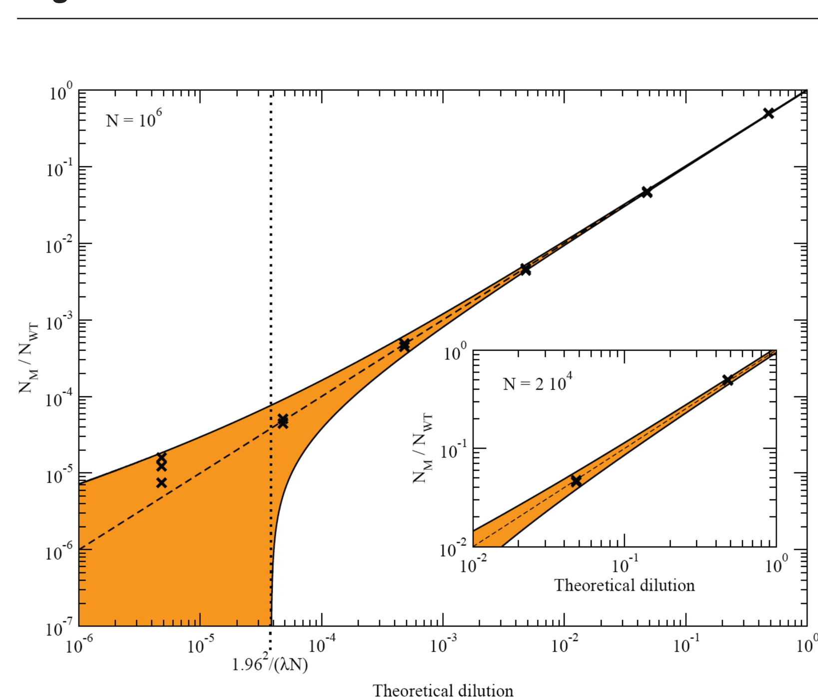


a. Genomic sample contains low level of mutant in high background of wild-type DNA.
b. Sample diluted to single molecule per droplet and partitioned into millions of reaction droplets.
c. End point PCR produces positive and negative reactions.
d. Droplets flow past laser; two color fluorescent detection yields positive (wild-type & mutant DNA) and negative (dark) droplets.

Superior Sensitivity for Detecting Rare Mutations

Superior sensitivity provides potential for non-invasive diagnostic applications including use of cell-free mutant DNA in blood samples.

Figure 2

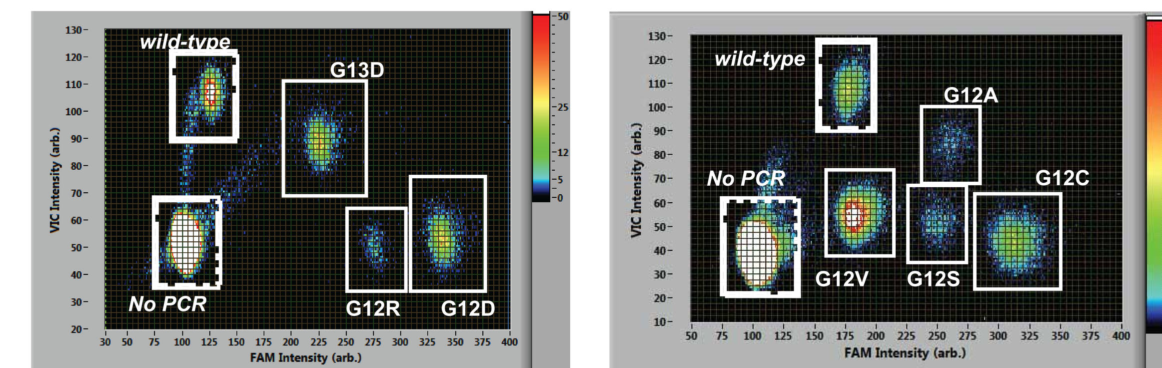


Serial dilutions of mutant KRAS DNA were prepared by adding wild-type KRAS DNA over five logs of concentration to demonstrate the sensitivity of the digital PCR method. The figure reveals detection of the mutant at a level of 1:200,000 in wild-type KRAS DNA within the 95% confidence intervals (represented by the orange shading).

Unprecedented Multiplexing Capability With Droplet-Based dPCR

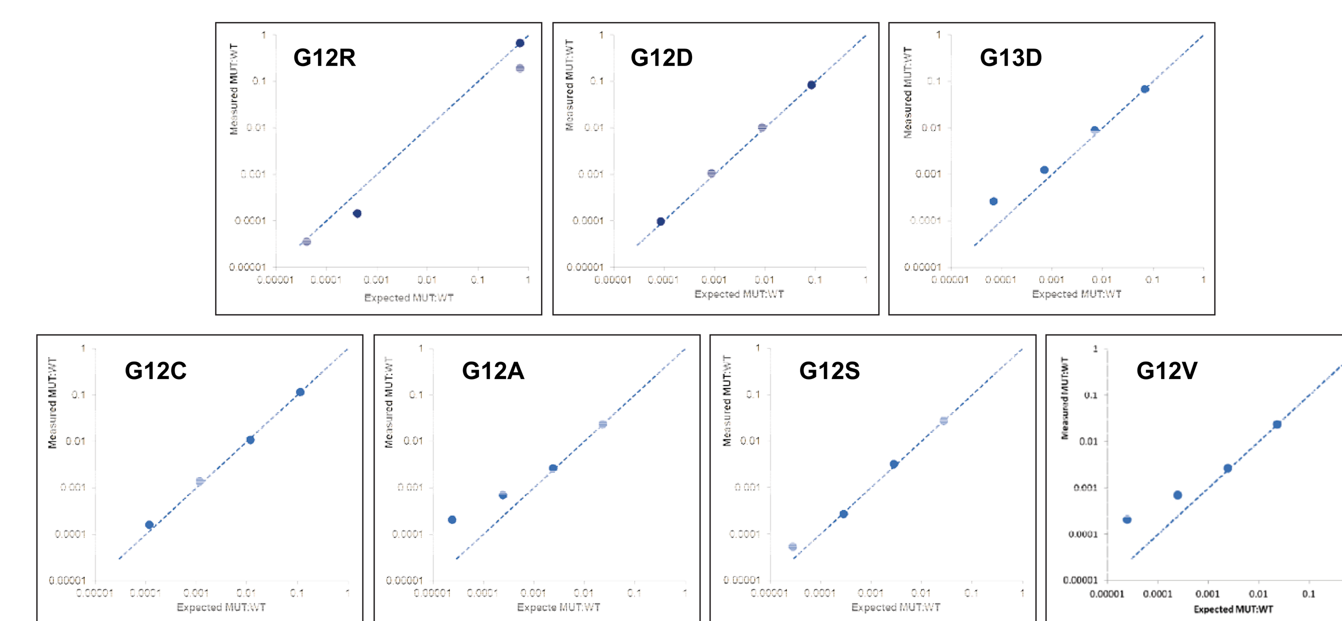
A new method for differentiating targets on the basis of end-point fluorescence intensity was developed by varying the concentration of the fluorescent probes.[2] To demonstrate, two panels containing multiple TaqMan® assays for KRAS SNP mutations were assembled. By adjusting concentrations of the probes for each individual assay, 3 or 4 KRAS mutations were measured simultaneously with just VIC and FAM fluorophores. This approach can be expanded to higher plex levels, and it enables detection, identification, and measurement of multiple possible mutations from a single DNA sample.

Figure 3



TaqMan assays for each of the seven most frequent mutations of KRAS were assembled into two multiplex panels by mixing mutation-specific VIC and/or FAM TaqMan probes with a single wild-type (VIC) probe and a single pair of PCR primers in each panel. The heat-map histograms reveal that concentrations of probes were tuned to enable discrimination of droplets containing no amplifiable fragments, wild-type KRAS DNA, or a DNA fragment with a unique KRAS mutation.

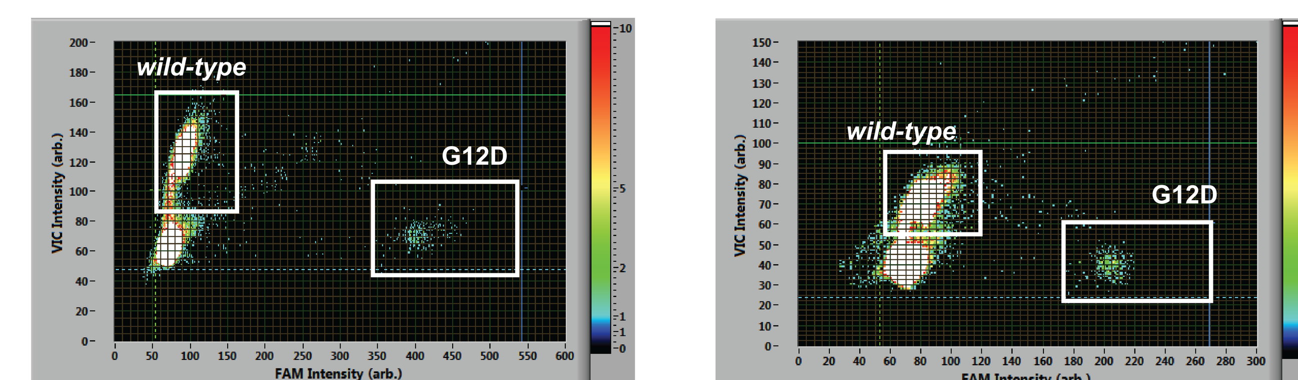
Figure 4



DNA isolated from one of seven tumor cell-lines was mixed with wild-type DNA to prepare serial dilutions over four logs of mutant-to-wild-type DNA ratio. The samples were then analyzed with the appropriate multiplex digital PCR panel. The results indicate that each mutation is detectable across the range of concentrations.

Figure 5

DNA was isolated from plasma of a patient with metastatic colorectal cancer. The DNA sample was split and analyzed by a duplex digital PCR assay and a multiplex digital PCR assay. The results from the two analyses are the same. In both cases — duplex or multiplex — digital PCR reveals that approximately 0.5% of the circulating KRAS DNA is the G12D mutation.



Duplex detection
(Assays: G12D and KRAS wild-type DNA)

~700 ng DNA isolated from plasma
0.58% G12D mutant in wild-type DNA

Multiplex detection
(Assays: G12D, G12R, G13D, and KRAS wild-type DNA)

~550 ng DNA isolated from plasma
0.54% G12D mutant in wild-type DNA

Conclusions

- Droplet-based microfluidics is a versatile and universal format to perform nucleic acids analysis.
- Very low levels of mutant DNA can be detected in a high background of wild-type KRAS DNA.
- The detection of target sequences is quantitative even for high dilutions.
- Different rare sequences can be detected in multiplex experiments.
- Mutant KRAS DNA can be detected in circulating cell-free DNA from blood.

References

[1] D. Pekin, Y. Skhiri, J. Baret, D. Le Corre, L. Mazutis, C. Ben Salem, F. Millot, A. El Harrak, J. B. Hutchison, J.W. Larson, D.R. Link, P. Laurent-Puig, A.D. Griffiths and V. Taly, Lab on a Chip, 2011, 11, 2156-2166.

[2] Q. Zhong, S. Bhattacharya, S. Kotsopoulos, J. Olson, V. Taly, A.D. Griffiths, D.R. Link and J.W. Larson, Lab on a Chip, 2011, 11, 2167-2174.

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