

Quantitative detection of circulating tumor DNA by droplet-based digital PCR.

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Summary

Droplet-based digital PCR is used to detect and quantify the seven most frequent *KRAS* mutations in circulating tumor DNA of patients with advanced colorectal cancer.

- The droplet-based digital PCR method is versatile and two modes of analysis are demonstrated:
 - o **Duplex** analysis enables sensitive detection of wild-type DNA plus one *KRAS* or *BRAF* mutation.
 - o **Multiplex** analysis enables simultaneous detection of wild-type DNA plus 3 or 4 *KRAS* mutations.
- Detection of rare sequences is highly sensitive compared to the same TaqMan assay in bulk (10% LLOD bulk vs 0.0005% LLOD droplets).
- Biomarkers detection is quantitative: the fraction of mutated DNA in patient samples ranges from 0.1% to 42%.
- Results from circulating tumor DNA analysis match the tumor DNA characterization in most cases, and discordant results reveal need for further studies.



Background

Circulating tumor DNA (ctDNA) is present in plasma of individuals with advanced cancers.¹ ctDNA is a prognostic marker for patients with colorectal cancer (CRC) and it might also be used for predicting the response to targeted therapy. For example, mutations in *KRAS* indicate which patients will fail to respond to specific therapies (cetuximab, panitumumab).² Although ctDNA is characterized by the presence of a somatic mutation, direct quantitative detection through a simple workflow of such mutant DNA is not feasible by current technologies because the ratio of ctDNA to wild-type DNA can be as low as 1/10,000.

This study describes the use of droplet-based digital PCR for detection and quantification of one of the seven most frequent *KRAS* mutations in ctDNA from plasma of patients with advanced colorectal cancer. Furthermore, we demonstrate that multiplex digital PCR enables testing samples for different mutations simultaneously.

Abstract

By segregating individual target DNA molecules into millions of aqueous droplets acting as independent microreactors, our procedure allows for extremely precise, sensitive, and fast quantification of mutated genes. The sensitivity of the procedure was confirmed by measuring 1/200,000 dilution of *KRAS*-mutated cell-line DNA in a background of wild-type DNA. Furthermore, plasma of more than 50 patients with metastatic CRC were tested. Our procedure enabled detection of the target mutation and the wild-type DNA, and thus, measurement of the total amplifiable DNA. DNA concentration in the plasma samples varied by two orders of magnitude and was not correlated with the proportion of mutated DNA, which varied from 42% to 0.1%. By using a duplex analysis to detect either of the two most frequent mutations of *KRAS* (G12D and G13D) and the wild-type DNA, the expected mutation (known by primary tumor characterization) was detected in 16 out of the 19 samples. Two samples had a low amount of amplifiable DNA leading to an inconclusive result. Five samples, positive for the G13D mutation, were also tested for the G12D mutations and were negative. Moreover, we tested 54 plasma samples from patients with metastatic cancer with known *KRAS* status (mutated or not) in a multiplex format allowing the simultaneous analysis of the seven most frequent mutations of *KRAS* or the wild-type sequence. Among the mutated samples, 13 out of the 19 mutated ones were positive and 32 of the non mutated ones were negative.

Our results demonstrate that our digital PCR method enables non-invasive detection of *KRAS* in plasma of patients with metastatic CRC with high sensitivity and high specificity. We anticipate that the method will be employed in multiple applications in the clinic, including diagnosis, cancer recurrence monitoring, and treatment management.

The approach

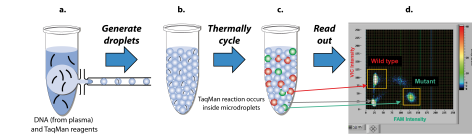


Figure 1: Droplet-based PCR approach^{3,4}.
 a. Patient sample containing low level of mutant sequences in a high background of wild-type sequences.
 b. Sample diluted to single molecule per droplet and partitioned into millions of reaction droplets.
 c. End point PCR produces positive and negative reactions.
 d. Droplets flow past laser; two color fluorescent detection yields positive (wild type and mutant) and negative (dark) droplets.

Detecting mutant and wild-type alleles

TaqMan reaction in droplets. As a proof of principle, gDNA extracted from different cell-lines was tested. The amplification of mutant DNA gives green-fluorescent droplets while the amplification of wild-type DNA gives red-fluorescent droplets⁵. The emulsion is then reinjected onto a microfluidic chip, the droplets are spaced by oil, and the fluorescent signal of each droplet is analyzed. Droplets can also be analyzed with confocal microscopy

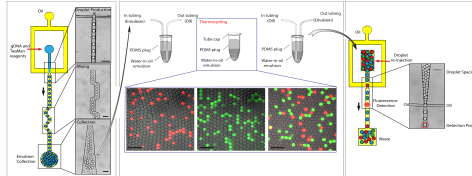


Figure 2: An aqueous phase containing the gDNA, PCR reagents and TaqMan® probes specific for the wild-type and mutant genes is emulsified within a microfluidic device. The emulsion is collected and thermocycled. During DNA amplification, the TaqMan® probes are cleaved and the corresponding fluorophores are released. The TaqMan® probes recognizing wild-type alleles carried a red-fluorescent fluorophore and TaqMan® probes specific for the mutant sequences carried a green fluorophore.

A highly sensitive and quantitative procedure. To demonstrate that genes bearing a somatic mutation can be detected within a large excess of wild type sequences, gDNA from a heterozygous cell-line bearing a *KRAS* mutation, was serially diluted into gDNA containing the wild-type *KRAS* gene. By analyzing 10⁶ fluorescent droplets, even the results obtained for the highest dilution (1/200,000) fall within the 95% confidence interval⁶. As a comparison, when using the same probes and same cell-lines, we previously demonstrated a sensitivity of 10%². The method is thus, both sensitive and quantitative, the sensitivity being limited only by the number of droplets analyzed.

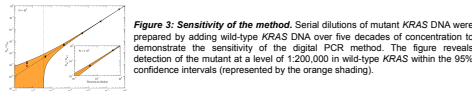


Figure 3: Sensitivity of the method. Serial dilutions of mutant *KRAS* DNA were prepared by adding wild-type *KRAS* DNA over five decades of concentration to demonstrate the sensitivity of the digital PCR method. The figure reveals detection of the mutant at a level of 1/200,000 in wild-type *KRAS* within the 95% confidence intervals (represented by the orange shading).

The approach

Unprecedented Multiplexing Capability With Droplet-Based dPCR
 Multiplexing enables development of assays for biomarker panels at reduced cost and sample consumption, increased throughput and the potential for built-in assay controls. Conventional qPCR has limited multiplexing capability due to spectral overlap of fluorescent probes. A new method for differentiating targets on the basis of fluorescence intensity was developed by varying the concentration of the fluorescent probes⁷. To demonstrate, results of a 4- and 5-plex TaqMan® dPCR copy number assay for *KRAS* mutations were measured simultaneously with just VIC and FAM fluorophores. This approach can be expanded to higher plex levels.

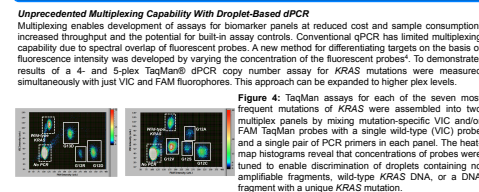
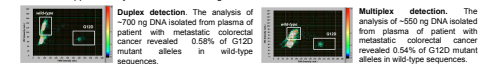


Figure 4: TaqMan assays for each of the seven most frequent mutations of *KRAS* were assembled into two multiplex panels by mixing mutation-specific VIC and/or FAM TaqMan probes with a single wild-type (VIC) probe and a single pair of PCR primers in each panel. The heatmaps reveal that concentrations 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.

Sensitivity detection of *KRAS* alleles in the multiplex procedure.
 Figure 5: DNA isolated from one of seven tumor cell-lines was mixed with wild-type DNA to prepare serial dilutions over four decades of mutant-to-wild-type ratio. Each of the samples were analyzed with the appropriate multiplex digital PCR panel. The results indicate that each mutation is detectable across the range of concentrations.

Detection of *KRAS* G12D mutation in DNA isolated from plasma- comparison between duplex and multiplex procedures.

Figure 6: 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 both both the presence of the G12D (the mutation that have been highlighted previously in the corresponding tumor) and wild-type sequences. 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.



Quantitative detection of circulating tumor DNA

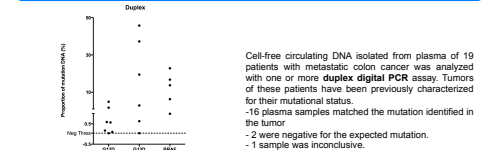


Figure 7: Proportion of mutated DNA observed in the plasma samples tested with duplex droplet-based digital PCR for the different targeted mutations.

- Cell-free circulating DNA isolated from plasma of 19 patients with metastatic colon cancer was analyzed with one or more duplex digital PCR assays. Tumors of these patients have been previously characterized for their mutational status.
- 16 plasma samples matched the mutation identified in the tumor
- 2 were negative for the expected mutation.
- 1 sample was inconclusive.

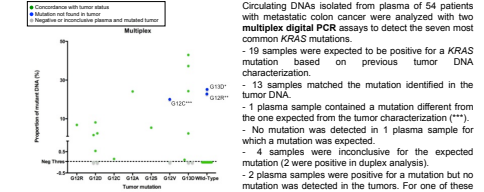


Figure 8: Proportion of mutated DNA observed in the plasma samples tested with multiplex droplet-based digital PCR for the different targeted mutations.

- Circulating DNAs isolated from plasma of 54 patients with metastatic colon cancer were analyzed with two multiplex digital PCR assays to detect the seven most common *KRAS* mutations.
- 19 samples were expected to be positive for a *KRAS* mutation based on previous tumor DNA characterization.
- 13 samples matched the mutation identified in the tumor DNA.
- 1 plasma sample contained a mutation different from the one expected from the tumor characterization (**).
- No mutation was detected in 1 plasma sample for which a mutation was expected.
- 4 samples were inconclusive for the expected mutation (2 were positive in duplex analysis).
- 2 plasma samples were positive for a mutation but no mutation was detected in the tumors. For one of these samples⁸), the initial tumor sample contained less than 15% of tumor cells and interestingly the patient had a progressive disease at the first evaluation under cetuximab. The other (**) was not evaluated for the number of tumor cells due to the size of the biopsy.

References

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