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Multiplex Picodroplet Digital PCR to Detect *KRAS* Mutations in Circulating DNA from the Plasma of Colorectal Cancer Patients

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BACKGROUND: Multiplex digital PCR (dPCR) enables noninvasive and sensitive detection of circulating tumor DNA with performance unachievable by current molecular-detection approaches. Furthermore, picodroplet dPCR facilitates simultaneous screening for multiple mutations from the same sample.

METHODS: We investigated the utility of multiplex dPCR to screen for the 7 most common mutations in codons 12 and 13 of the *KRAS* (Kirsten rat sarcoma viral oncogene homolog) oncogene from plasma samples of patients with metastatic colorectal cancer. Fifty plasma samples were tested from patients for whom the primary tumor biopsy tissue DNA had been characterized by quantitative PCR.

RESULTS: Tumor characterization revealed that 19 patient tumors had *KRAS* mutations. Multiplex dPCR analysis of the plasma DNA prepared from these samples identified 14 samples that matched the mutation identified in the tumor, 1 sample contained a different *KRAS* mutation, and 4 samples had no detectable mutation. Among the tumors samples that were wild type for *KRAS*, 2 *KRAS* mutations were identified in the corresponding plasma samples. Duplex dPCR (i.e., wild-type and single-mutation assay) was also used to analyze plasma samples from patients with *KRAS*-mutated tumors and 5 samples expected to contain the *BRAF* (v-raf murine sarcoma viral oncogene homolog B) V600E mutation. The results for the duplex analysis matched those for the multiplex analysis for *KRAS*-mutated samples and, owing to its higher sensitivity, enabled detection of 2 additional samples with low levels of *KRAS*-mutated DNA. All 5 samples with *BRAF* mutations were detected.

CONCLUSIONS: This work demonstrates the clinical utility of multiplex dPCR to screen for multiple mutations simultaneously with a sensitivity sufficient to detect mutations in circulating DNA obtained by noninvasive blood collection.

Somatic genetic alterations in most cancers represent molecular signatures that are valuable for prognosis *(1)* and treatment management *(2, 3)*. For example, *KRAS*[8] (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene mutations, which are present in 40% of colorectal adenocarcinomas, are predictive markers of nonresponse to anti–epidermal growth factor receptor (anti-EGFR)[9] antibodies *(4–6)*, and *BRAF* (proto-oncogene B-Raf, serine/threonine-protein kinase B-Raf) mutations have been shown to be associated with poor prognosis *(7)*.

The evaluation of patient blood samples (i.e., liquid biopsies) for genetic alterations is particularly attractive. Upon cell death, tumors release DNA that can be detected in blood, as well as in other body fluids, such as lymph, stool, and urine *(2, 8–10)*. Such circulating tumor DNA (ctDNA) has been associated with a variety of malignancies. Identification and quantification of ctDNA can be used to evaluate response to

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[8] Human genes: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *BRAF*, proto-oncogene B-Raf, serine/threonine-protein kinase B-Raf.
[9] Nonstandard abbreviations: EGFR, epidermal growth factor receptor; ctDNA, circulating tumor DNA; mCRC, metastatic colorectal cancer; dPCR, digital PCR; qPCR, quantitative PCR; 5FU, 5-fluorouracil; LOB, limit of blank; MGB, minor groove binder (probe); CAST, competitive allele-specific hydrolysis TaqMan (probe).

treatment and to monitor disease recurrence. Additionally, it can provide real-time assessment of the mutation status without having to rely on archival samples from the primary tumor or the need for invasive biopsies of metastatic sites *(9, 11)*. The ability to use of ctDNA also raises the possibility of screening and early diagnosis before a cancer becomes clinically detectable *(12)*.

The moderate sensitivity of mutation-detection methods currently used in clinical practice has limited the detection of ctDNA. Conventional methods demonstrate sensitivity thresholds of approximately 1%, but ctDNA may represent only a small fraction of the total circulating DNA. In early cancers, this fraction may be <0.01% *(13)*. Until recently, ctDNA detection was based on detecting a single molecular target per sample. Increasing the clinical relevance of ctDNA requires analysis tools that are highly sensitive and capable of efficient multiplexing so that multiple mutations can be detected without prior knowledge of the alteration *(14)*.

High sensitivity can be achieved with digital PCR (dPCR) *(15, 16)*, which is based on the compartmentalization and amplification of single DNA molecules [for a comparison of commercially available approaches, see *(17)*]. A DNA sample is distributed among many compartments such that each compartment contains, statistically, either no copies or only a single copy of the target DNA. After the PCR, counting the compartments with a fluorescence signal at the end point reveals the number of copies of target DNA. The sensitivity of dPCR is limited only by the number of molecules that can be amplified and detected (i.e., the number of PCR-positive compartments) and the false-positive rate of the mutation-detection assay.

One dPCR approach is based on compartmentalization of DNA into droplets *(18)*. A water-in-oil emulsion provides a flexible format for parallel amplification of millions of individual DNA fragments *(19, 20)*. Droplet microfluidics systems are used to make, manipulate, and analyze nanoliter to picoliter droplets *(18, 21)*, which enable simple dPCR work flows that produce highly sensitive mutation detection within complex DNA mixtures *(22, 23)*. For example, the detection of 1 mutant *KRAS* gene among 200 000 wild-type *KRAS* genes has been demonstrated for genomic DNA from tumor cell lines *(22)*. Other examples of emulsion-based dPCR for highly sensitive mutation detection have recently been described *(23–25)*.

The ability to detect multiple mutations in parallel has also been demonstrated with picodroplet-based dPCR *(22, 26)*. For true multiplexing—in which all droplets contain multiple molecular-detection assays—optimal performance is achieved by minimizing the number of droplets with multiple copies of the target, because each of the colocalized targets may not amplify and/or the end point fluorescence signal from a droplet with multiple targets may not be readily distinguished from droplets with other targets. In short, multiplex dPCR of high sensitivity requires the sample DNA to be compartmentalized at the level of a single target molecule by distributing the sample among the maximum number of compartments, which is achieved by creating and processing the smallest feasible droplet volume.

This report describes the first demonstration of multiplex emulsion-based dPCR applied to detecting mutations in ctDNA prepared from clinical plasma samples. We describe the use of picodroplet dPCR for detecting and quantifying the 7 most common mutations in the *KRAS* oncogene. We applied 2 assay panels to ctDNA from patients with metastatic colorectal cancer (mCRC). Results from the multiplex dPCR analysis of plasma samples are compared with quantitative PCR (qPCR) characterization of matched tumor samples. Furthermore, results obtained with duplex dPCR (i.e., detection of only 1 mutation per assay) are compared with those for the multiplex analysis.

Materials and Methods

PATIENTS

Fifty mCRC patients in the CETRAS study approved by the Ile-de-France ethics committee number 2 (CPP Ile-de-France 2 2007–03–01-RCB 2007-A00124–49 AFSSAPS A70310–31) were included in this study. All patients signed an informed-consent form. The mean (SD) age was 63 (10.7) years, and the male/female sex ratio was 1.66. All patients received an anti-EGFR–based therapy. The therapy consisted of a combination of cetuximab and irinotecan; a combination of cetuximab with a 5-fluorouracil (5FU)- and irinotecan-based chemotherapy regimen; a combination of cetuximab with 5FU and an oxaliplatin-based chemotherapy regimen; or a panitumumab monotherapy in 61%, 27%, 7%, and 5% of the cases, respectively. The 7 most frequent mutations in *KRAS* codons 12 and 13 *(27)* and the *BRAF* V600E mutation were assessed in the primary tumors as previously described *(5, 28)*.

TUMOR SAMPLE PREPARATION

Tumors were snap-frozen after resection. Each tumor was reviewed by a pathologist (J.F.E.) and tumor cell content was assessed by hematoxylin-eosin-safran staining. Of the tumor samples, 44% contained >60% tumor cells, 24% contained 40%–60% tumor cells, 14% had <40% tumor cells, and 18% of the samples had a biopsy too small to permit tumor cell quantification. DNA was extracted with the QIAamp DNA Mini Kit (Qiagen) and eluted in 50 μL of Buffer AE (in the

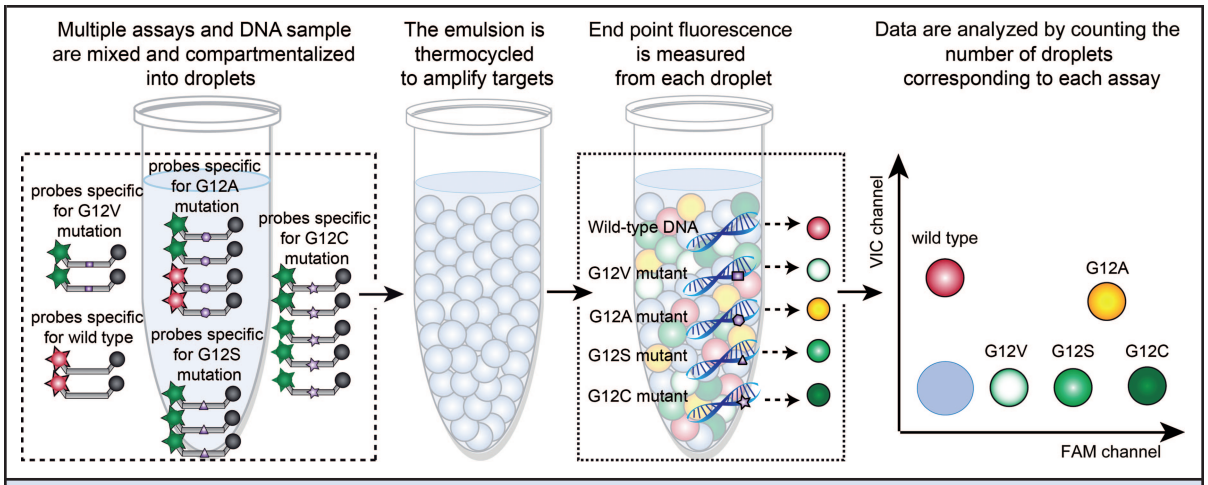

Fig. 1. Multiplex analysis of circulating tumor DNA: example of the 5-plex assay.

An aqueous phase containing TaqMan® assay reagents and genomic DNA is emulsified. Probes specific for the 4 screened mutations and the wild-type sequence are present at varying concentrations. After thermal cycling the droplets' end point fluorescence depends on the initial concentration of the probes, allowing the identification of the target sequences.

Qiagen kit). DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

PLASMA SAMPLE PREPARATION

Eight milliliters of blood were collected into EDTA-containing tubes before the anti-EGFR therapy. Plasma samples were separated from the cellular fraction by centrifugation at 3000*g* at 4 °C and stored at −80 °C. Before extraction, plasma samples were centrifuged for 10 min at 3000*g*. Plasma DNA was extracted with the QIAmp Circulating Nucleic Acid Kit (Qiagen). DNA was quantified by SYBR Green I real-time PCR of the gene encoding 18S rRNA *(29)*. Reactions were performed in a 10-μL reaction volume, which consisted of 1 μL extracted DNA, 0.05 μmol/L each of the forward primer (5′-CGGCTACCACATCCAAGGAA-3′) and the reverse primer (5′-GCTGGAATTACCGCGGCT-3′), and 1× SYBR Green I Master Mix (Applied Biosystems). Amplifications were carried out with an ABI Prism 7900 Sequence Detection System (Applied Biosystems) as follows: 15 min at 95 °C and 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Data were analyzed with SDS Software (version 2.0; Applied Biosystems). A standard calibration curve was valid for DNA input concentrations up to 25 ng/μL (calibration points: 0 pg/μL, 0.25 pg/μL, 2.5 pg/μL, 25 pg/μL, 2.5 ng/μL, 25 ng/μL).

GENOMIC DNA PREPARATION, ASSAYS DESIGNS, MICROFLUIDICS PROCEDURES, AND DATA ANALYSIS

See the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue11.

Results

MULTIPLEX dPCR TO DETECT THE 7 MOST COMMON *KRAS* MUTATIONS

An efficient multiplex assay is required to detect multiple mutations simultaneously in a single experiment while consuming a minimum of the patient sample *(18)*. Fig. 1 describes the use of multiplex dPCR in millions of picoliter droplets to measure the ratio of mutant to wild-type genes in biological samples. This schema is derived from the methods described earlier *(22, 26)*, and the approach has 3 distinct steps: (*a*) All reagents, TaqMan® probes (see Table 1 in the online Data Supplement), and primers are combined in a multiplex reaction with the sample DNA before droplets are formed. The mixture is emulsified in a fluorinated carrier oil containing a fluorosurfactant to generate 5 million precisely sized 5-pL droplets. (*b*) The emulsion is thermally cycled for PCR amplification and probe hydrolysis when an amplifiable target is present. (*c*) Finally, the end point fluorescence signal(s) [i.e., the fluorescence intensities of VIC (red) and/or 6-carboxyfluorescein (green)] of each individual droplet are measured. By limiting the amount of input DNA, predominantly only a single molecule or no target molecules are contained within any droplet before the PCR. As previously demonstrated, the end point fluorescence intensity can be tuned by varying the concentration and the nature of the TaqMan probe, which enables identification and counting of droplets containing each unique amplifiable target *(26, 30)*. The different populations of droplets appear as distinct clusters in a 2-dimensional histogram.

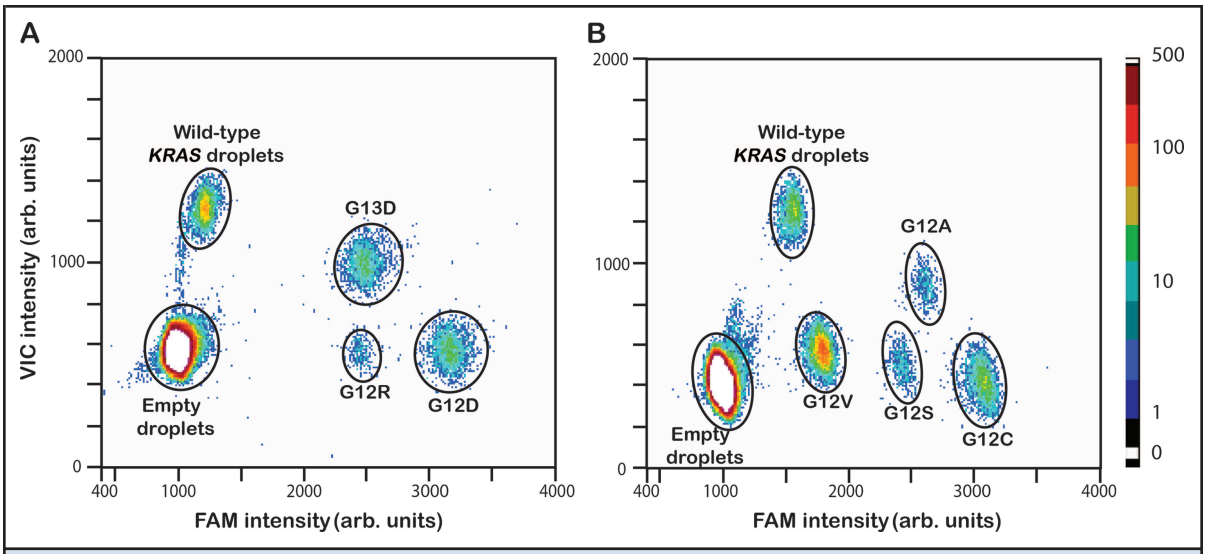

Fig. 2. Multiplex assays for mutant *KRAS* analysis. Two-dimensional histogram of the 4-plex (A) and the 5-plex assay (B).
Fragmented genomic DNA extracted from cell lines was encapsulated in droplets and submitted to the procedure described in Fig. 1. FAM, 6-carboxyfluorescein; arb. units, arbitrary units.

Assays for each of the targeted *KRAS* mutations were assembled into 2 multiplex panels (a 4- and 5-plex dPCR assay) by mixing mutation-specific VIC and/or 6-carboxyfluorescein TaqMan probes with a single wild-type (VIC) probe and a single pair of PCR primers in each panel. As Fig. 2 shows, the concentrations of the probes were tuned to discriminate between droplets containing no amplifiable DNA targets, droplets containing wild-type *KRAS* DNA, and droplets containing DNA with a unique *KRAS* mutation. The 4-plex panel revealed the presence of G12D, G12R, or G13D *KRAS* mutations, and the 5-plex panel contained probes for the G12A, G12C, G12S, and G12V mutations. To improve probe discrimination, we included nonfluorescent blockers consisting of 3′-phosphate oligonucleotides in each reaction (see Supplemental Methods in the online Data Supplement). In short, the 4-plex assay contains blockers against mutated *KRAS* sequences targeted in the 5-plex panel and the 5-plex assay contains blockers against mutated *KRAS* sequences targeted in the 4-plex panel.

To demonstrate the dynamic interval of the multiplex procedure, we mixed DNA isolated from each of the 7 tumor cell lines with wild-type DNA to prepare serial dilutions over 4 logarithms of mutant concentrations. Each dilution was analyzed separately with the appropriate multiplex panel (see Fig. 1 in the online Data Supplement). As a general characterization of all assays, the measured concentration of DNA matches the anticipated concentration over the range of 10% to 0.01%. For some assays, the measured concentration is higher than the expected concentration at the lowest concentration. Such results are due to the counting of false-positive droplets and define the limit of detection.

The limit of blank (LOB) is the primary characteristic of an assay that determines the limit of detection, and the LOB is defined by the frequency of positive droplets measured in wild-type samples or in controls with no DNA present. Additional results and discussion of the LOB measurement are included in the Supplemental Methods in the online Data Supplement. Our finding from this work is that the rate of false-positive droplet events does not depend on the total amount of DNA (see Figs. 2 and 3 in the online Data Supplement). Therefore, the LOB cannot be expressed as a definitive mutant allele percentage for each assay. Rather, the LOB was determined for each assay as a finite number of false events of mutant droplets detected per analysis. According to wild-type DNA controls, the number of false-positive droplet events (i.e., the LOB) for each of the 7 *KRAS* assays is: 3 for G12R, 5 for G12D, 3 for G12C, 3 for G12A, 3 for G12S, 7 for G12V, and 7 for G13D.

MULTIPLEX ANALYSIS OF CIRCULATING TUMOR DNA IN PLASMA FROM A PATIENT WITH mCRC

To demonstrate the possibility of detecting and quantifying tumor DNA directly from the plasma of patients with mCRC, we performed a multiplex analysis of 50 plasma samples. Table 1 summarizes the findings of this analysis. According to qPCR analysis, amplifiable DNA concentrations in the amplified plasma samples

Table 1. Duplex and multiplex analysis of plasma samples of patients with *KRAS*- or *BRAF*-mutated primary tumor.[a]

			Multiplex *KRAS* analysis		Duplex *KRAS* analysis	
Sample	Concentration, ng/mL of plasma	Tumor mutation	Mutation	Mutant DNA, %	Mutation	Mutant DNA, %
1	12	G12D	G12D	0.65	G12D	0.59
4	24	G12D	G12D	2.52	G12D	5.81
7	14	G12D	**NM[b]**	—	G12D	0.17
8	53	G12C	G12C	0.16	G12C	0.18
9	201	G12A	G12A	24.09	G12A	24.79
10	89	G13D	G13D	42.99	G13D	45.78
11	1466	G12D	G12D	0.53	G12D	0.57
13	32	G13D	G13D	1.27	G13D	0.63
17	472	G13D	G13D	2.46	G13D	2.92
22	8	G12V	**G12C**	**17.14**	**G12C**	**14.52**
23	755	G13D	G13D	37.20	G13D	37.25
25	32	G12S	G12S	5.41	G12S	7.49
26	196	G13D	G13D	18.02	G13D	19.48
28	207	G12D	G12D	8.14	G12D	7.27
29	790	G13D	**NM**	—	G13D	0.04
39	56	G13D	**NM**	—	**NM**	—
40	510	G12D	G12D	1.61	G12D	1.72
41	26	G12R	G12R	6.85	G12R	6.36
48	18	G12V	**NM**	—	**NM**	—
12	3539	V600E	NM	—	V600E	13.72
30	507	V600E	NM	—	V600E	6.27
42	237	V600E	NM	—	V600E	22.85
45	19	V600E	NM	—	V600E	0.99
46	279	V600E	NM	—	V600E	13.72
Neg.	7–1840	NM	NM	—	NM	—
20	178	NM	**G13D**	**25.2**	**G13D**	**27.04**
21	18	NM	**G12R**	**22.9**	**G12R**	**18.28**

[a] Results that do not match results of tumor characterization are highlighted in yellow and indicated in boldface.
[b] NM, nonmutated; Neg., analysis of plasma samples of patients characterized as NM in both their tumor (qPCR) and their plasma (multiplex and duplex digital PCR).

varied from 0.33 ng/μL to 283 ng/μL. Fig. 3A shows that the dPCR assessment of amplifiable DNA concentration matched the qPCR data. Furthermore, Fig. 3B reveals that the distributions of amplifiable DNA concentrations were similar for samples with *KRAS* or *BRAF* mutations identified in the tumor DNA (red) or for samples with no *KRAS* or *BRAF* mutation identified in the tumor DNA (green). The combined distribution (blue) shows that 50% of the samples had <100 ng of amplifiable DNA per milliliter of plasma. Finally, dPCR analysis revealed that the proportion of mutant DNA ranged from 0.16% to 43% (corresponding to 0.08–38 ng of mutant DNA per milliliter of plasma), but the proportion of mutated DNA did not correlate with the total amount of circulating plasma DNA (Fig. 3C).

For each patient sample, the expected mutation status was determined from the primary tumor DNA via conventional TaqMan assays with TaqMan MGB (minor groove binder) probes *(5, 28)*. *KRAS* or *BRAF* mutations were found in 24 tumor samples, with the mutation types distributed as presented in Table 1. Of the 19 plasma samples for which a *KRAS* mutation was identified in the tumor, 14 were positive for the same mutation in plasma as that assessed with the multiplex dPCR assay. Five samples were negative for the ex-

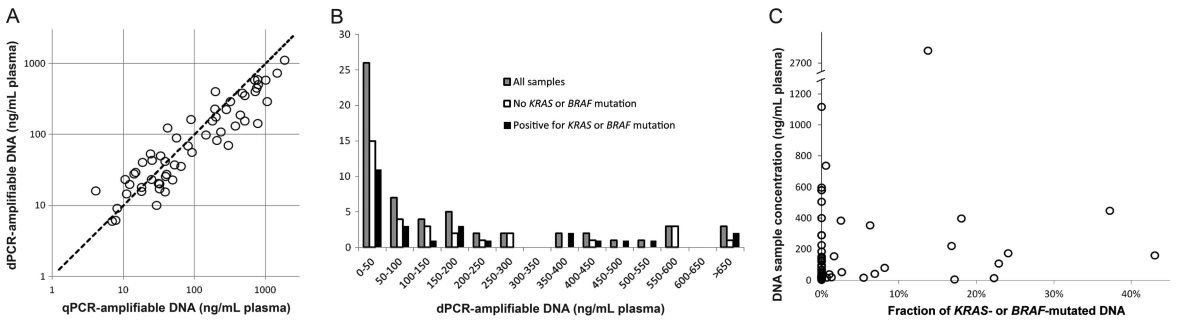

Fig. 3. (A), Correlation between the quantity of amplifiable DNA determined by microfluidics multiplex dPCR and that obtained by bulk qPCR.

(B), Distribution of amplifiable DNA concentrations in the 50 analyzed plasma samples (gray), samples with *KRAS*- or *BRAF*-mutated tumor (black), and *KRAS* or *BRAF* wild-type tumor (white). (C), Analysis of the proportion of mutant sequence and the total amount of *KRAS* sequences observed in the multiplex droplet PCR assay.

pected mutation. One sample was negative for the expected mutation (G12V), but it appeared positive for a different mutation (patient 22, G12C at 17% of the total circulating DNA). For this patient, retesting of the tumor DNA with the multiplex assays revealed that the sample was positive only for the G12V mutation (see Table 2 in the online Data Supplement). Of the 26 samples expected to test negative, 2 were positive for a *KRAS* mutation (one for G13D and one for G12R at 25% and 23% of the total circulating DNA, respectively). DNA extracted from the 2 tumors was retested in the multiplex assays, and negative results were obtained for all 7 *KRAS* mutations. Finally, the results of *KRAS* multiplex assays of 5 plasma samples from patients with tumors containing *BRAF* mutations were negative for all 7 *KRAS* mutations.

DUPLEX ANALYSIS OF CIRCULATING DNA IN PLASMA FROM PATIENTS WITH mCRC

To confirm the results obtained with multiplex dPCR, we conducted additional analyses of many of the plasma samples with a duplex dPCR approach, in which only 2 molecular targets are detected in each experiment (i.e., wild type and a given mutant sequence). Table 1 summarizes the results of the duplex dPCR analysis for these samples. Table 3 in the online Data Supplement and Table 1 reveal that the results of the duplex dPCR analyses were completely concordant with those of the multiplex *KRAS* analyses (see also Fig. 4), with the exception of 2 samples that were scored negative in the multiplex analysis and positive with the duplex procedure (samples 7 and 29 with 0.17% and 0.04% mutant sequences, respectively). Furthermore, the sample obtained from patient 22 was negative for the tumor mutation (G12V), but was positive for the G12C mutation, a result that confirms the multiplex analysis. Duplex analyses of ctDNA from patients 20 and 21, which were nonmutated in their respective tumors but mutated in plasma according to the multiplex analysis, confirmed the results of the multiplex analysis but were not consistent with the nonmutated status anticipated from the tumor DNA characterization. Results obtained for these 3 plasma samples were also confirmed with competitive allele-specific hydrolysis TaqMan (CAST) probes, as described earlier *(29)*. Results obtained for samples 20 and 21 were also confirmed by next-generation sequencing with the Ion Torrent PGM technology (with 30% G13D alleles for sample 20 and 19% G12R alleles for sample 21; see Supplemental Materials in the online Data Supplement). The 5 plasma samples from patients with *BRAF* V600E mutant tumors all tested positive in the duplex assay (the fraction of mutated *BRAF* DNA varied from 1% to 23% of the total circulating DNA). Finally, the proportions of ctDNA measured by multiplex and duplex dPCR analyses were highly correlated ($r^2 = 0.99$; Fig. 5 and Table 1).

To verify the specificity of the dPCR assay, we also tested 8 plasma samples that were positive for a *KRAS* mutation, for a mutation that was different from the one identified in the tumor DNA. Furthermore, we tested 7 plasma samples from patients with nonmutated tumor DNA in a duplex assay for one of the 8 targeted mutant sequences. The results of each assay were negative (see Table 3 in the online Data Supplement).

Discussion

We report the quantitative analysis of circulating DNA and mCRC via multiplex and duplex picodroplet dPCR assays. Our results suggest that a liquid biopsy

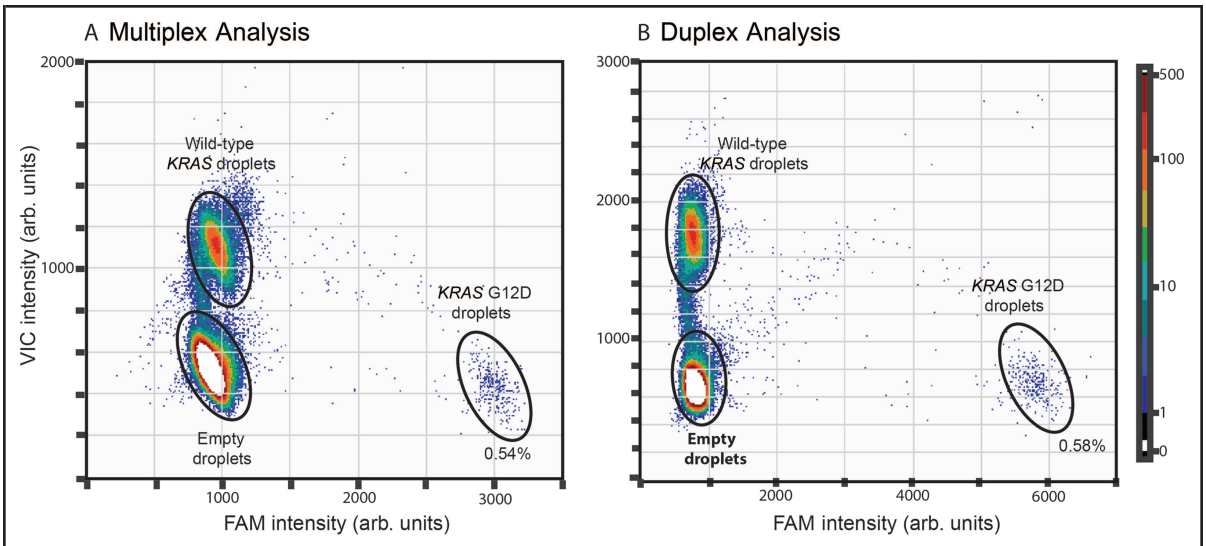

Fig. 4. Analysis of an identical sample with duplex and multiplex assays.

(A), Multiplex analysis of DNA isolated from a plasma sample of patient 11 (approximately 550 ng) with mCRC reveals 0.54% of G12D mutant alleles. (B), Duplex analysis of DNA isolated from a plasma sample of patient 11 (approximately 700 ng) with mCRC reveals 0.58% of G12D mutant alleles. FAM, 6-carboxyfluorescein; arb. units, arbitrary units.

(i.e., blood draw) is a feasible alternative to a solid-tissue biopsy for detecting specific mutations.

The concentration of DNA circulating in the plasma ranges from 0 to 100 ng/mL, with a mean of 30 ng/mL for healthy individuals *(31)*. The results of this study reveal that the concentration of amplifiable circulating DNA ranges from 10 to 1000 ng/mL for most plasma samples. In contrast to previous results *(32)*, we observed no correlation between the quantities of total circulating DNA and ctDNA. Interestingly, our results demonstrate the efficient amplification of low DNA amounts in plasma and that the proportion of ctDNA can be high, even with low quantities of starting material. The ctDNA concentration varied from 0.1% to 43% of the total circulating DNA, which is consistent with recent studies that used BEAMing *(11, 33)*.

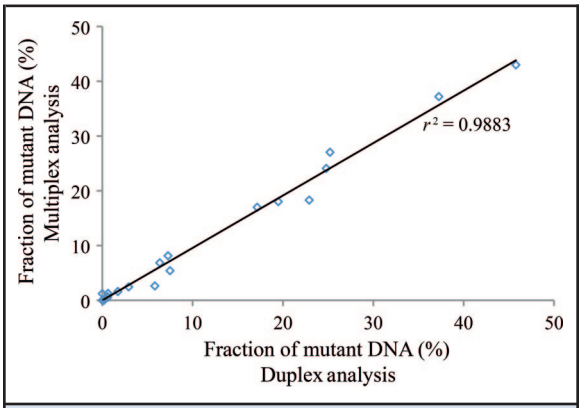

Fig. 5. Comparison of results obtained by multiplex and duplex analyses.

Compilation of the analysis of 19 plasma samples from patients with primary tumors mutated for *KRAS* and the results for the analysis of the 2 plasma samples from patients for whom a mutation was identified in the plasma but presented a nonmutated primary tumor (samples 20 and 21).

The mutational status of the patients was determined from samples of primary tumors and plasma collected before the start of anti-EGFR therapy. Plasma samples from patients with *KRAS*-mutated tumors showed a concordance in mutation identity of 74% and 84% for the multiplex and duplex formats, respectively. One plasma sample contained a *KRAS* mutation that differed from that of the tumor (17% of G12C), leading to an overall concordance of 78% and 89% for the multiplex and duplex assays, respectively. The difference between the mutation found in primary tumor and that found in plasma at progression can be explained by initial heterogeneity of the tumor. A subclone might be selected during tumor evolution, owing to the selection pressure produced by chemotherapy. The 5 plasma samples from patients with *BRAF*-mutated tumors were tested in a duplex format, and all showed mutation identity, further increasing the overall concordance to 92%.

The specificity of the *KRAS* multiplex procedure was tested with 26 plasma samples from patients with nonmutated tumors and with 5 plasma samples from patients with *BRAF*-mutated tumors. All but 2 samples were classified as nonmutated by multiplex analysis. These samples were reproducibly positive with a high proportion of *KRAS*-mutated DNA (25.1% or 25.2% G13D and 21.6% or 22.9% G12R measured in 2 independent experiments and confirmed by duplex dPCR analysis), results that render contamination unlikely. The nonmutated status of these 2 tumors was confirmed with the multiplex procedure. Interestingly, the sample from one of the patients contained <15% tumor cells. This patient demonstrated progressive disease at the first evaluation after starting cetuximab therapy, which began after the patient became refractory to conventional chemotherapy. The other case was not evaluated for the number of tumor cells, owing to the limited size of the biopsy and because the patient was treated with a combination of cetuximab, irinotecan, and 5FU as a first-line therapy. The observed tumor response may have been due to the combination of 5FU and irinotecan. The differences between the mutational status of the tumor and that of the plasma observed for these patients could also be explained by selection of a minority subclone of the primary tumor that has metastasized. Discrepant results between primary and metastatic sites in *KRAS* mutational status have rarely been observed *(34, 35)*. Such information could enhance the pertinence of performing liquid biopsies of multimetastatic patients.

dPCR has multiple advantages over conventional approaches *(18)*. In particular, its sensitivity is limited by the number of compartments that can be analyzed, the false-positive rate of the mutation-detection assay, and the amount of amplifiable DNA *(36)*. The TaqMan dual-probe assays used had previously been validated for clinical purposes with conventional qPCR *(4, 22)*, with sensitivities of 10%–20%. This level of sensitivity is impractical for plasma samples, in which tumor DNA may represent only a small fraction of the total circulating DNA. Only 7 of the 24 positive samples had >10% tumor DNA, and just 13 of the 24 samples had >1% tumor DNA.

Picodroplet dPCR is especially well-suited for multiplex assays. The key distinction of picodroplet dPCR is that each PCR-positive droplet arises from a single target molecule. Therefore, multiple and distinct concentrations of fluorescent probe(s) can be used to indicate the identity of each PCR-positive droplet. In this study, we converted the TaqMan assays to a multiplex format *(26)*, which allowed detection and quantification of multiple *KRAS* mutations in patient samples.

Detecting the 7 most common *KRAS* mutations is particularly challenging for hybridization assay specificity, because the 7 mutations are all located in 2 adjacent codons, with the mutations spanning 5 consecutive nucleotides. We overcame this limitation by developing 2 panels for assaying 3 or 4 *KRAS* mutants.

The challenge with multiplex dPCR is that the limit of detection for some assays is sometimes compromised by poor discrimination of the end point signal from other clusters in a 2-dimensional histogram. The limited separation of clusters leads to false positives—producing lower sensitivity. For example, the fluorescence cluster associated with the assay for G12V in the 5-plex *KRAS* panel is located immediately adjacent to the cluster associated with droplets without PCR target (i.e., "empty droplets"). Furthermore, poor sample quality and/or occasional emulsion degradation during thermal cycling tend to introduce spurious droplet events—"noise"—near the empty droplet cluster. Consequently, the LOB for G12V is relatively high. We have found that for assay elements that are influenced more severely by spurious droplet events, duplex dPCR provides a better separation of clusters and more definitive identification of true-positive droplet events, especially for low concentrations of mutant copies. A potential clinical work flow may include multiplex dPCR to screen for multiple mutations and to use duplex dPCR for quantify specific target mutations.

Development of targeted therapies has improved survival prospects for cancer patients, but the efficacy of therapies is often compromised by the emergence of resistant mutations *(37)*. The use of "liquid biopsies" for treatment triage offers multiple advantages. First, analyzing circulating DNA from plasma (or other body fluids) obviates reliance on invasive biopsies or tissue archives, which may be of poor quality or difficult to obtain *(38)*. Furthermore, ctDNA is likely to be a homogeneous representation of all tumor DNA, and its analysis could enable detection of cancer subclones that would otherwise be missed because of tumor heterogeneity *(39)*. In addition to the quality of molecular analysis at diagnosis, the simplicity of multiplex dPCR should improve patient follow-up by facilitating serial blood testing for: (*a*) evaluating treatment efficacy by monitoring the quantity of ctDNA, (*b*) detecting the selection of mutant subclones before clinical resistance occurs *(40)*, and (*c*) detecting disease recurrence.

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