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Comparison of digital PCR systems for the analysis of liquid biopsy samples of patients affected by lung and colorectal cancer

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ABSTRACT

Background and aims: Highly sensitive technologies are available for the molecular characterization of solid tumors, including digital PCR (dPCR). Liquid biopsy, based on the analysis of cell-free DNA (cfDNA), is often used to assess EGFR or RAS alterations in lung and colorectal cancers. Our study aimed to compare the results of two different dPCR platforms for the detection of mutations in cfDNA.

Methods: Plasma samples from lung and colorectal cancer patients collected as per routine procedures have been tested. cfDNA Was extracted from plasma, and samples were screened on the droplet digital PCR (ddPCR, Bio-Rad) and solid dPCR QIAcuity (Qiagen).

Results: A total of 42 samples were analyzed, obtained from 20 Non-Small Cell Lung Cancer (NSCLC) patients carrying an EGFR or a KRAS mutation on tissue at diagnosis, and from 22 samples of colorectal cancer (CRC) patients, 10 of which presenting a KRAS mutation. EGFR mutation detection was 58.8% for ddPCR and 100% for dPCR ($\kappa = 0.54$; 95% CI, 0.37–0.71), compared to tissue results. The detection rate for RAS mutations was 72.7% for ddPCR and 86.4% for dPCR ($\kappa = 0.34$; 95% CI, 0.01–0.68), compared to tissue results.

Conclusions: This study showed moderate agreement between dPCR and ddPCR. Sampling effect or threshold settings may potentially explain the differences in the cfDNA data between the two different platforms.

1. Introduction

The evolution of technologies for molecular analysis has increased the knowledge of tumor biology, allowing a better strategy for the management of cancer patients. Cell-free DNA (cfDNA), detected in plasma and other body fluids, represents a valuable source of tumor-derived DNA as an alternative to tissue samples [1] and its analysis has been widely investigated in recent years [2].

The detection of EGFR or RAS mutations in cell-free tumor DNA (cfDNA) from plasma has been demonstrated to be feasible and easily repeatable [3]. Its implementation in clinical practice allows a better management of patients to select the appropriate treatment, and to monitor tumor dynamics during therapies [4].

The introduction of highly sensitive techniques, including different digital PCR platforms, and the use of technologies allowing the analysis of multiple genes instead of a single target (i.e. Next Generation Sequencing – NGS), opened new scenarios in terms of comprehension of tumor heterogeneity and therapeutic strategies [5]. NGS can simultaneously detect an extensive range of genomic alterations, including point mutations, copy number alterations (CNA), translocations, and fusions in multiple genes. However, its introduction in clinical practice is facing issues due to its long turnaround time and costs. On the contrary, a targeted approach by dPCRs allows the evaluation of a limited number of known and selected biomarkers, with higher sensitivity, limited cost, and shorter turnaround time. The request for a liquid biopsy test is highly frequent in clinical practice, considering that it is minimally invasive, it collects nucleic acids deriving from all metastatic sites and representative of tumor heterogeneity, it can be repeated

Abbreviations: PCR, Polymerase Chain Reaction; dPCR, digital PCR; ddPCR, droplet digital PCR; cfDNA, cell-free DNA; NGS, Next Generation Sequencing; CNA, Copy Number Alterations; TAT, Turnaround Time; sdPCR, solid digital PCR.

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multiple times during patient treatment, allowing a molecular monitoring of tumor dynamics [6]. Therefore, the dPCR target approach seems to be close to clinical needs, in terms of 1) sensitivity, 2) turnaround time, and 3) data interpretation [7].

Recently, different dPCR technologies have been released in search of ever greater sensitivity and shorter turnaround time. Nowadays, three types of dPCR platforms are available: 1) the BEAMing (Beam, Emulsion, Amplification, Magnetics) dPCR, 2) the droplet digital PCR (ddPCR), and 2) the solid digital PCR (sdPCR). In the ddPCR system, partitions are composed of thousands (~20,000) of droplets in a water-in-oil emulsion [8]. In the sdPCR system the partitions are depicted by thousands of physical wells (~20,000) on a solid support (chip), thus preventing the emulsion and droplets breaking, which can affect the performance of the analysis [2]. In both ddPCR and sdPCR systems, single DNA molecules are released inside the droplets or the wells according to the Poisson distribution [2]. The analysis is based on fluorescence detection and the results are acquired by measuring the number of positive and negative partitions, obtaining both qualitative information and absolute quantification of even a limited number of mutated alleles in a background of wild-type alleles. The present data were aimed to compare two different dPCR platforms for ctDNA analysis: the droplet digital PCR (BioRad) and the QIAcuity sdPCR (Qiagen). No data which compare the performance of these two methods on clinical plasma samples are available. Previously, comparisons between BEAMing and dPCR platforms have been performed [9–11], demonstrating a concordance between these technologies. Our testing cohort was derived from clinical routine practice comprising patients with NSCLC and CRC.

2. Materials and methods

Liquid biopsy samples of patients with Non-Small Cell Lung cancer (NSCLC) and Colorectal Cancer (CRC) collected as per routinary procedures were tested.

Twelve ml of blood were collected in EDTA tubes and centrifuged at 1,900 × g for 10 min at 4 °C within 2 h after drawing to collect plasma, which was stored at − 80 °C until analysis. Plasma samples were centrifuged at 1,900 × g for 15 min to remove cellular debris. cfDNA Was extracted from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA).

Samples were analysed on both platforms using the ddPCR Mutation Assay (BioRad®, Hercules, CA) with FAM fluorophore mutant probes and HEX fluorophore wild-type probes. For KRAS and NRAS multiplex tests were used: ddPCR™ KRAS G12X/G13X Screening Kit (Bio-Rad, Cat Number: 1863506), ddPCR™ NRAS G12X/G13X Screening Kit (Bio-Rad, Cat Number: 12001627), ddPCR™ NRAS Q61X Screening Kit (Bio-Rad, Cat Number: 12001006).

ddPCR Mutation Assays were used for BRAF p.V600E (c.1799 T > A), EGFR p.L858R (c.2573 T > G), and EGFR p.T790M (c.2369C > T) testing. EGFR exon 19 deletions were analysed by the screening kit (Bio-Rad, Cat Number: 12002392); a custom EGFR assay was used for p.C797S (c.2390G > C/T > A) resistance mutation.

2.1. ddPCR analysis

PCR reactions were assembled into individual wells (96-well PCR plate) according to the following protocol: 4 μL of cfDNA, 1.25 μL primer/probe assay (FAM), 1.25 μL 20X primer/probe assay (HEX), 12.5 μL 1X ddPCR Super Mix, 6 μL DNase/RNase-free water (total volume: 25 μL). Samples were partitioned into ~ 20,000 uniform nanoliter-sized droplets in a 96-well PCR plate using the AutoDG Instrument (Bio-Rad). After partitioning and plate sealing, PCR were run in a standard thermal cycler according to the following protocol: 95 °C for 10 min, 94 °C for 30 s, 55 °C for 1 min, 98 °C for 10 min; all ramp increments were 2 °C/sec. A fluorescence intensity threshold of 3,000 was set as a cut-off point; the result was considered valid when at least one droplet was above the threshold level. Non-template controls were run with every assay. Bio-Rad QX-200 was used for data analysis using the QuantaSoft package version 1.7.4.0917 (Bio-Rad), which measures the number of positive versus negative droplets for both fluorophores (FAM/HEX); their ratio is then adjusted to a Poisson distribution to determine the copy number of the target molecule, as copies per milliliter (copies/mL), in the input reaction. No false-positive mutant partitions were observed.

2.2. sdPCR analysis

PCR reactions were assembled into individual wells (96-well PCR plate) according to the following protocol: 4 μL of ctDNA, 1 μL primer/probe assay (FAM), 1 μL 20X primer/probe assay (HEX), 10 μL 4X Probe PCR Master Mix (Qiagen), 24 μL DNase/RNase-free water (total volume: 40 μL). The contents of each well were transferred into the wells of a 26 k 24-well nanoplate (Qiagen), in which one reaction mix is distributed over 4 wells and separated into approximately 26,000 partitions. The nanoplate were sealed using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kit (Qiagen). The following conditions were used for the PCR reaction: 95 °C for 2 min, 95 °C for 15 s, 55 °C for 30 min. After PCR, the amplification target was acquired by measuring the fluorescence in all positive partitions. Poisson statistics was used to calculate the average amount of target DNA per partition. The total amount of target DNA in all partitions of a well was calculated by multiplying the amount of average target DNA per partition with the number of valid partitions. The QIAcuity Software Suite analyzes the mutation frequencies in target samples as absolute quantification of copies of each wild-type and mutant target present in samples, and by determining the mutation frequency in each sample. Table 1 reports the volume of reagents needed for both the techniques.

2.3. Comparison cohort and statistical analysis

Patients were considered suitable for the comparison if results for all the mutations tested with both platforms were available. As a primary outcome, comparisons were performed on the overall agreement of the mutations.

The secondary outcome was the agreement between specific mutations in each gene.

Agreement between the two platforms was calculated with the Cohen κ and the concordance correlation coefficient (Pearson). All statistical calculations were performed with MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2014).

3. Results

A total of 42 samples was analyzed, 20 obtained from NSCLC patients and 22 from CRC patients. Ten CRC patients were carriers of a RAS mutation on tissue at diagnosis and 12 were RAS wild-type. Seventeen NSCLC patients were carriers of an EGFR activating mutation and 3 patients were carriers of a KRAS mutation. Fourteen samples were analyzed before starting treatment and 28 samples were analyzed on-treatment.

The comparison was performed in all patients with data obtained on

Table 1
Details of the volumes of reagents used for each platform.

Reagent	ddPCR	sdPCR
PCR Master Mix	12.5 μL	10 μL
20X primer/probe assay (FAM or FAM + HEX)	1.25 μL	1 μL
20X primer/probe assay (HEX)	1.25 μL	1 μL
H_2O	6 μL	24 μL
Sample (cfDNA)	4 μL	4 μL
Total volume	25 μL	40 μL

both platforms. ddPCR detected an EGFR mutation in 58.8% (10/17) of NSCLC patients, whereas sdPCR detected EGFR mutations in 100% (17/17) of cases. There was a moderate agreement between EGFR mutation status by ddPCR and sdPCR, with a Cohen κ 0.54 (95% CI, 0.37–0.71) and a concordance correlation coefficient of 0.990. Discrepant results were found in 41.2% (7/17) of cases (Fig. 1A).

Considering RAS, both the ddPCR and sdPCR detected a mutation in 100% (3/3) of NSCLC patients. In CRC samples, ddPCR detected a RAS mutation in 59.1% (13/22), whereas sdPCR analysis resulted in 68.2% (15/22) of positive detection. Concerning the BRAF in CRC samples, ddPCR detected the p.V600E mutation in 1 case, whereas dPCR detected p.V600E mutation in 3 cases.

Regarding the overall detection of RAS mutations, ddPCR revealed them in 72.7% (16/22) of samples compared to 86.4% (19/22) with sdPCR (Fig. 1B), with a proportion of discordant cases of 18.2% (4/22), a Cohen κ of 0.34 (95% CI, 0.01–0.68) and a concordance correlation coefficient of 0.694.

Considering the 12 discordant cases, the overall quantification of detected variants was found to be close to the threshold of 100 copies/mL (Fig. 2). In particular, discordant cases detected with only sdPCR were EGFR mutations (p.L858R, p.T790M, p.C797S) in seven NSCLC cases, RAS mutations (p.G12X/G13X, p.Q61X) in 3 CRC patients, and BRAF p.V600E mutation in 2 CRC cases. In concordant cases, the overall quantification expressed in copies/mL ranged up to 103,000 and 65,600 for ddPCR and sdPCR, respectively.

The agreement between different mutations within EGFR, RAS and BRAF genes was evaluated. A complete agreement was found for exon 19 deletions of EGFR (Cohen κ = 1) and a moderate agreement (Cohen κ = 0.39) for EGFR p.T790M and p.C797S mutations (Fig. 3A). Concerning the specific RAS and BRAF mutations, there was a low agreement for RAS p.G12X/G13X and p.Q61X mutations (Cohen κ = 0.32), and a moderate agreement (Cohen κ = 0.5) for BRAF p.V600E mutation (Fig. 3B).

Fig. 4 depicts the output graphics of a discordant CRC case for RAS p.G12X mutation with a cfDNA low abundance (copies/mL) analyzed with ddPCR and sdPCR.

4. Discussion

It has been demonstrated that cfDNA analysis is an emerging tool to be used in patients with advanced cancer [12]. Quality and quantity of cfDNA is strongly influenced by disease characteristics such as tumor biology, disease burden and location, and number of metastatic sites. Low disease burden, brain or bone metastases typically characterize low cfDNA-shedding tumor, which can be therefore responsible for false negatives [2]. The low amount of cfDNA in the circulation needs highly sensitive techniques to provide reliable detection in clinical samples.

In the present analysis, we found a weak agreement between the two

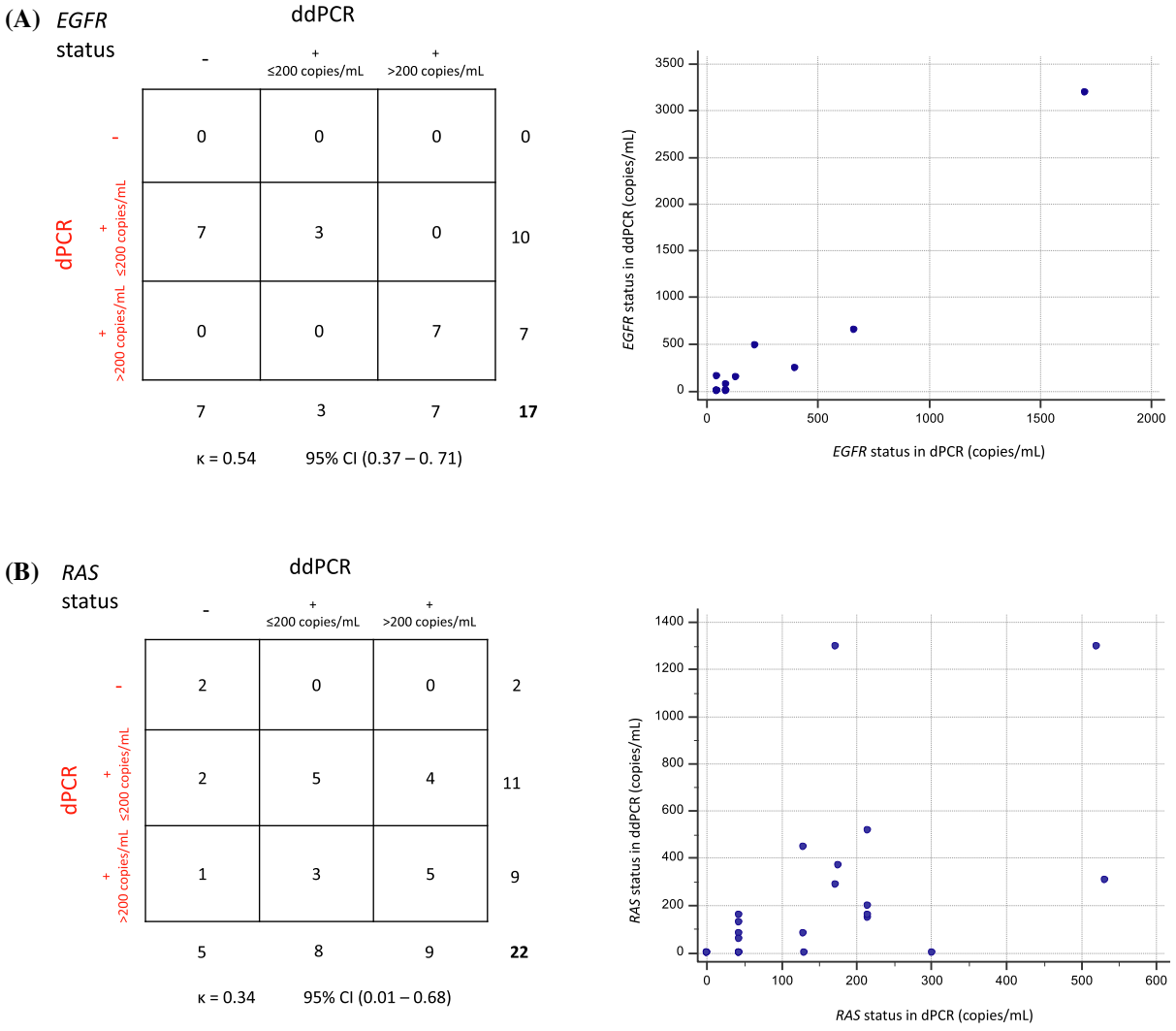

Fig. 1. Contingency tables for EGFR (A) and RAS (B) mutation status by patients from ddPCR and sdPCR (dPCR), with the agreement of mutational status shown in the right panel.

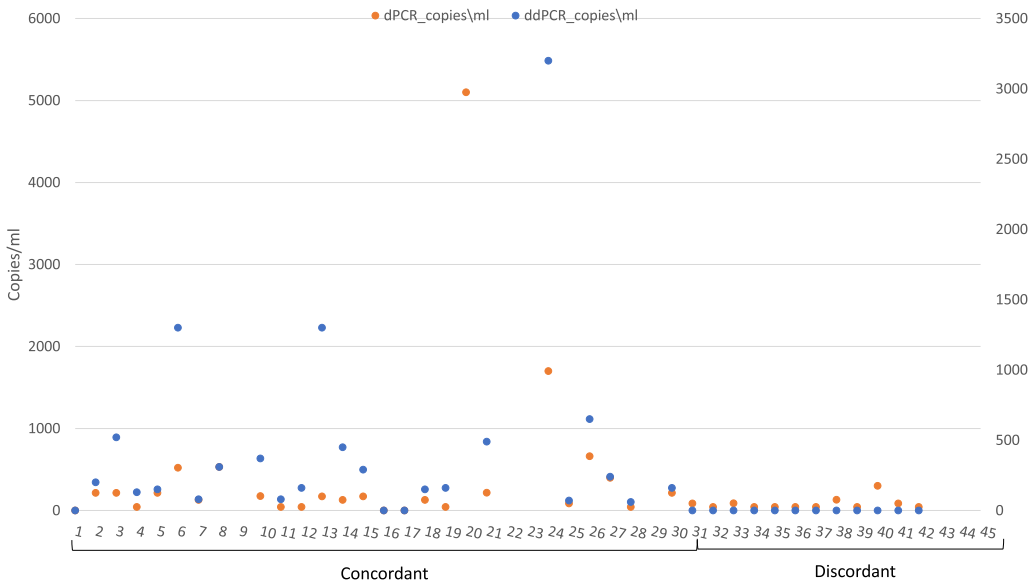

Fig. 2. Overall concordant and discordant cases for EGFR, RAS, and BRAF status. Digital PCR results are reported as copies/mL.

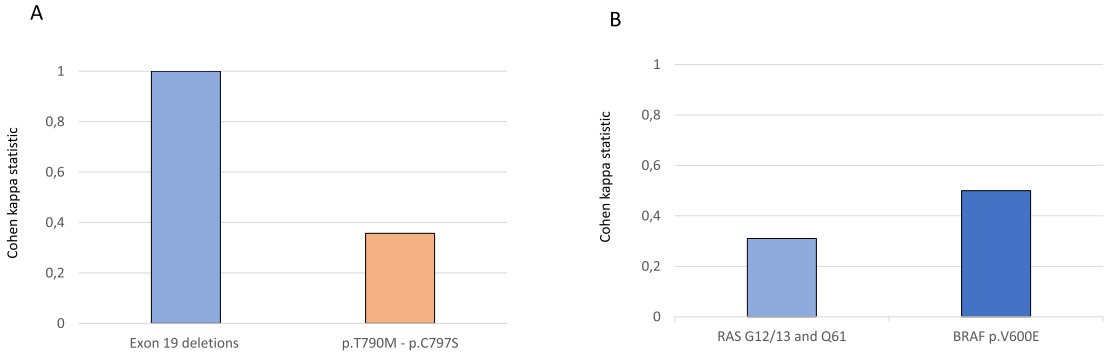

Fig. 3. Cohen Kappa statistic for individual EGFR (A), RAS, and BRAF mutations (B).

systems as concerns EGFR or RAS mutations (κ = 0.54, and 0.34, respectively, Fig. 1) with a moderate proportion of discordant cases (18–40%). Concordance was higher for mutations in both genes with higher variant allele fraction (VAF). It is important to notice that discordances in analysis that may be used to select a pharmacological treatment, is serious issue. To date, a limited small set of data comparing different platforms are available and it is challenging to evaluate stochastic effects.

O'Leary et al. evaluated the comparison of BEAMing and ddPCR in cfDNA plasma samples from patients enrolled in phase-3 PALOMA-3 trial. The authors found a close agreement between both platforms as for any ESR1 or PIK3CA mutation analyzed (K = 0.9 and 0.87, respectively) [10]. Recently, a cross-platform comparison of three distinct molecular methods (quantitative PCR, digital PCR, and NGS) for mutation detection in cfDNA samples from 55 NSCLC patients has been performed. A concordance of 96% when testing for EGFR p.T790M has been found, with a different limit of detection for identification of mutations (>0.1% and > 0.2% for ddPCR and NGS, respectively) [13]. Similarly, Garcia et al. evaluated the performance of three technologies, including dPCR for RAS mutation detection in cfDNA. The authors showed a higher sensitivity and specificity for BEAMing technology with respect to ddPCR. Conversely, it has been demonstrated that ddPCR is adequate for the detection of somatic mutations in NSCLC and CRC [14,15]. Iwama et al. showed a better performance of dPCR and a higher frequency for EGFR activating mutation detection in cfDNA at baseline, and at the time of disease progression than NGS (81.3% and 100% versus 71.9% and 60.0%, respectively) [15]. Similarly, a meta-analysis investigated the accuracy of high sensitivity detection of KRAS mutations in cfDNA samples from CRC patients. Comparison between dPCR and NGS demonstrated higher sensitivity (81%), diagnostic odds ratio (29.18), and AUC of summary ROC curve (0.9067) of dPCR vs. NGS (65%, 14.61, and 0.8574), suggesting a greater accuracy of digital platform [16].

Despite clinically relevant thresholds still need to be established, an accurate quantitative detection of actionable mutations may help with disease evaluation and monitoring response to treatment. One potential bias is that the definition of threshold levels, above or below which a sample is considered positive or negative, is dependent on the operator; therefore, it is fundamental to acquire adequate experience to interpret the results, especially borderline ones, considering the above mentioned pre-analytical and analytical challenges.

One limitation of dPCR vs. sequencing-based methods, is that dPCR only detects known mutations, thus precluding the possibility to identify new mutations. Moreover, some reagents such as ethanol or paraffin can interfere with the oil-in-water emulsion and the analysis may therefore fail. Despite these limits, dPCR is a valid technique and, due to its capability of highly-sensitive quantification of mutated alleles, it provides the possibility to monitor patients and their treatment response over time [17].

Our findings have some limitations since this study compares only two digital platforms for EGFR and RAS mutations, and includes a

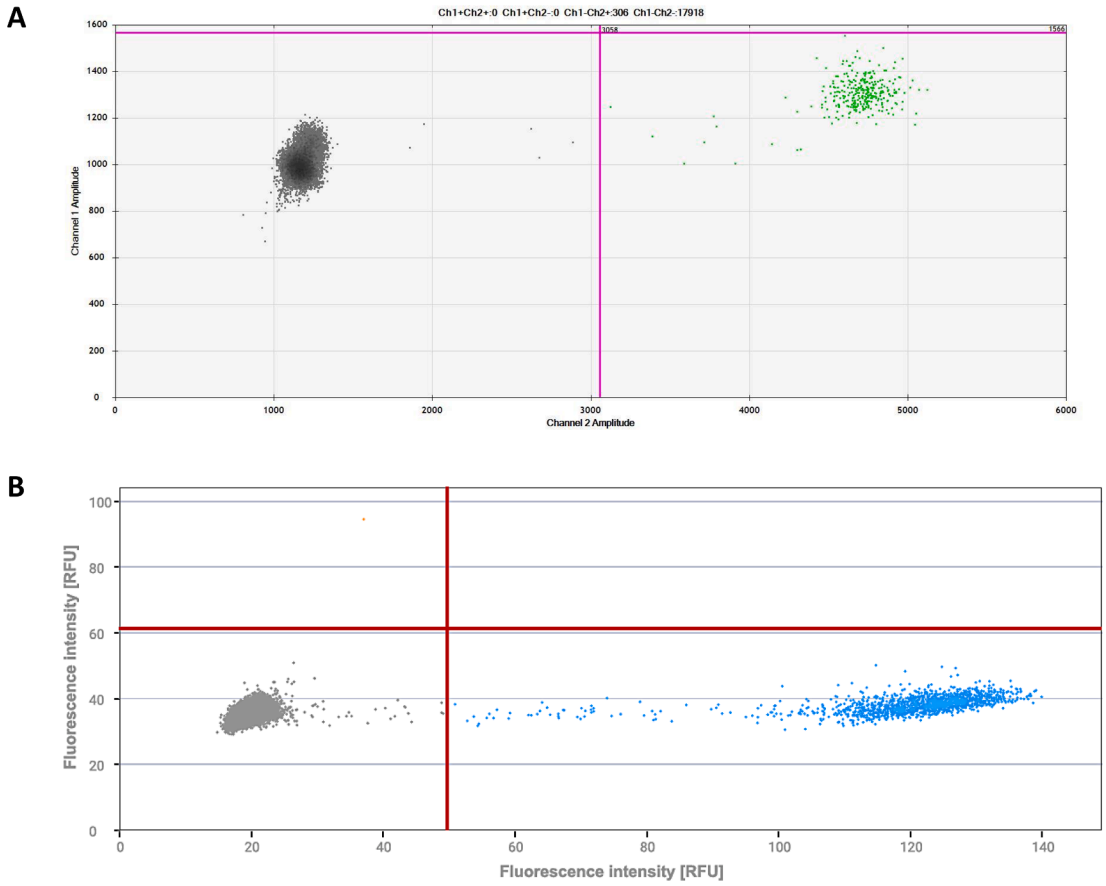

Fig. 4. Graphic output of ddPCR (A) and sdPCR (B) of one discordant case using two-dimensional distribution of droplets/partitions. X-axis indicates the HEX signal generated by the presence of WT RAS sequences. Y-axis indicates the FAM signal generated by the presence of mutant RAS sequences. Fluorescence-positive droplets are distinguished into HEX single-positive (green - ddPCR or light blue - sdPCR), and FAM single-positive (orange - dPCR). The light blue or green dots represent the wild-type sequences, whereas the orange dot in (B) represents a mutated allele (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

moderately small cohort of patients. However, our cohort resulted as one of the most extensive groups of patients analyzed for cfDNA RAS and EGFR alterations in cross-platform comparison.

5. Conclusions

Our results show that dPCR platforms, including ddPCR and sdPCR, used for EGFR, RAS and BRAF mutational analysis present sufficient levels of agreement for clinical testing, but a discordance between the two techniques was found. Moreover, our data show that sdPCR has a higher sensitivity than ddPCR allowing the detection of a larger number of mutated patients, even with low cfDNA abundance.

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Author contributions

All authors read and approved the final manuscript.

CRediT authorship contribution statement

Stefania Crucitta: Formal analysis, Investigation, Writing – original draft. **Martina Ruglioni:** Formal analysis, Investigation, Writing – original draft. **Claudia Novi:** Formal analysis, Investigation. **Mascia Manganiello:** Formal analysis, Investigation. **Roberta Arici:** Formal analysis, Investigation. **Iacopo Petrini:** Investigation, Writing – review & editing. **Eleonora Pardini:** Investigation. **Federico Cucchiara:** Formal analysis, Investigation. **Federica Marmorino:** Investigation, Writing – review & editing. **Chiara Cremolini:** Investigation, Writing – review & editing. **Stefano Fogli:** Writing – review & editing. **Romano Danesi:** Conceptualization, Writing – review & editing, Visualization. **Marzia Del Re:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MDR consultant/speaker: Astellas, Astra Zeneca, Celgene, Novartis, Pfizer, Bio-Rad, Janssen, Sanofi-Aventis, Roche, Lilly, MSD and Ipsen. C. C. reports personal fees from Roche, Amgen, Bayer, and Sevier; research founding from Merck Serono; and consulting/advisory role with Roche, Bayer, Amgen. SF received support for travel expenses from Novartis, Teva, Roche, BMS, Lilly and Ipsen. RD consultant/speaker: Ipsen, Novartis, Pfizer, Sanofi Genzyme, AstraZeneca, Janssen, Gilead, Lilly, and EUSA Pharma. All other authors report no competing interests.

Data availability

Data will be made available on request.

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