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Non-invasive profiling of *KRAS* mutations in lung cancer using droplet digital polymerase chain reaction

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Contributions: RK, performed the experiments, analyzed the data, prepared the original draft; SNK, performed the experiments, interpreted the results, and contributed to draft preparation; RhK, performed the experiments, analyzed the data, prepared the original draft; RV, obtained samples and provided clinical data; SA, helped in data interpretation; GC, supervised the study, helped in result analysis, reviewed and edited the original draft; AC, conceptualized the study, obtained funding and other resources, analyzed the data, edited the draft manuscript; GBH, obtained samples and provided clinical data.

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Abstract

With rising cases of lung cancer worldwide, liquid biopsies are becoming increasingly popular as clinically relevant potential non-invasive alternatives to tissue-based biopsies. The principle of partitioning utilized by the droplet digital polymerase chain reaction (ddPCR) makes it a highly sensitive technique for detecting rare tumor-derived mutations in blood. The presence of *KRAS* mutations is a negative prognostic marker for tyrosine kinase inhibitor (TKI) therapy in lung cancer; hence, profiling of major *KRAS* mutations before treatment is very crucial for the success of TKI therapy. This study was aimed at profiling three major *KRAS* mutations, namely G12D (GGT→GAT), G12V (GGT→GTT), and G13D (GGC→GAC) in lung cancer patients using ddPCR. ddPCR assays that rely on probe-based chemistry were standardized for *KRAS* G12D, *KRAS* G12V, and *KRAS* G13D mutations using cfDNA extracted from the patient's blood. To determine the concordance, blood-derived cfDNA and tumor DNA were compared using ddPCR. A positivity rate of 81.67% for *KRAS* mutations was observed in the cohort analyzed. *KRAS* mutations in the cfDNA from blood were effectively detected by ddPCR even at low fractional abundance. Moreover, a comparison of blood-derived cfDNA and tumor-derived genomic DNA-based analysis revealed a concordance of 66.67%, suggesting tumor heterogeneity as the probable reason for the lack of total concordance between the data. This study highlights the usefulness of ddPCR as a prospective clinical tool in oncology and liquid biopsy using blood cfDNA. It can be considered a better alternative to tissue biopsies and mutation profiling of candidate genes, particularly those that are linked to therapeutic response to TKIs.

Key words: cell-free DNA, droplet digital PCR, liquid biopsy, lung cancer.

Introduction

Lung cancer has been the major cause of a huge number of cancer-associated deaths worldwide [1]. Histologically, lung cancer is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC represents a substantial percentage (85%) of the cases and is further categorized into squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma [2]. The common underlying cause for tumorigenesis in lung cancer is the disruption of the Epidermal Growth Factor signaling pathway with *Epidermal Growth Factor Receptor* (*EGFR*) and *Kirsten Rat Sarcoma Viral Oncogene Homolog* (*KRAS*) being the most frequent oncogenic drivers [3].

The KRAS protein is a membrane-bound, signal-transducing guanosine triphosphatase (*GTPase*) functioning as a GDP/ GTP binary control [4]. Cancer-linked variations in *KRAS* are somatic in nature [5]. The hotspot variations in *KRAS* occur at exons 2 and 3 with 98% in glycine residues at codon 12 or 13 (exon 2) [6]. Transversions in *KRAS* are more frequent than transitions with transversions commonly occurring in smokers and transitions in never-smokers [7,8]. Transition mutations at the second position in codon 12 or 13 (GGT → GAT or GGC → GAC) result in an amino acid change from glycine to aspartate, and a transversion mutation in codon 12 at the second position (GGT → GTT) results in an amino acid substitution of glycine by valine. *KRAS* G12C is the most common variation and is seen in 41% of lung cancer cases. *KRAS* G12V, *KRAS* G12D, and *KRAS* G13D account for 19.35%, 11.12%, and 2.88% of lung cancer cases, respectively [9].

The primary mode for targeted therapy in lung cancer is the administration of Tyrosine Kinase Inhibitors (TKIs) against cell surface EGF receptors [10]. *KRAS* mutations are considered negative prognostic factors for TKI therapy as KRAS is a downstream effector in the EGFR signaling pathway.

The common approach to profile tumor mutations involves Next-generation Sequencing (NGS) of tumor DNA. Though extremely useful, this approach is invasive, cost-intensive, time-consuming, and unfeasible to monitor tumor evolution. This has led to a shift in attention to liquid biopsy, which refers to the detection of variations in cellular components such as DNA, RNA, proteins, and exosomes, released from tumor entities, in body fluids such as blood, urine, saliva, CSF, etc. [11].

Cell-free DNA (cfDNA) refers to DNA free from cellular boundaries. As cells undergo cell death, fragments of DNA are released into the bloodstream. Cancer patients are known to have higher

amounts of cfDNA in the bloodstream [12,13]. Substantial data indicates that the detection of alterations in cfDNA from plasma is both feasible and repeatable, particularly the *KRAS* variations [14]. In lung cancers, blood represents an ideal repository of tumor-derived information and hence is the most widely used source of cfDNA in liquid biopsy. Blood can be collected from the patient with minimum invasion, thereby serving as a painless alternative to tissue biopsies.

To detect low concentrations of cfDNA, ultra-sensitive tools are a necessity. The Droplet Digital PCR (ddPCR) which works on the principle of partitioning is a highly sensitive and accurate technology, capable of detecting even a single copy of an altered DNA. This tool provides quantitative, real-time, non-invasive monitoring of mutant alleles at negligible concentrations in plasma [15]. This study focused on non-invasive profiling of *KRAS* mutations in a cohort of lung cancer patients in southern India.

Materials and Methods

Selection of study participants

Twenty patients clinically and histologically diagnosed with lung cancer, at K S Hegde Charitable Hospital, Mangalore, India, and above the age of 18, were included in this study. Patients who were already under treatment for lung cancer, those who had prior history of malignancy and those with lung cancer but not willing to participate in the study were excluded. The details of the patients (including gender, smoking status, cancer type and EGFR mutation status) are given in *Supplementary Table 1*. The ethical clearance was obtained from the Institutional Ethics Committee, NUCSER (INST.EC/2022-23/004).

Collection and processing of samples

Peripheral blood (10 ml) was collected from each patient in EDTA-coated vacutainers after obtaining informed consent, and plasma was separated within two hours of collection. In brief, blood samples were centrifuged at 1800g for 10 minutes at 4°C and the plasma was collected and stored at -80 °C until further use. Matched-tumor samples were collected during routine diagnostic biopsy or surgical removal and were stored at -80°C.

Extraction of cell free DNA and genomic DNA

To extract cfDNA, 1 ml of plasma was centrifuged at 16000g for 10 minutes at 4°C to remove any debris. cfDNA was extracted using QIAamp® MinElute® ccfDNA Mini Kit (Qiagen, Germany) as per the manufacturer's instructions. The quantity of the extracted cfDNA was measured using

Qubit™ dsDNA BR Assay Kit (Thermo Fischer Scientific, USA) in a Qubit 4 fluorometer (Thermo Fischer Scientific, USA). The extracted cfDNA was stored at -80°C till further use. Genomic DNA from the tumor tissue was extracted using a commercially available kit (Nucleospin® Tissue XS kit, Macherey-Nagel, Germany), quantified using a Nanophotometer (Implen, Germany) and stored at -80°C till further use.

Preparation of wild-type and mutant plasmids for the ddPCR assays

A 222bp region of the *KRAS* gene, encompassing exon 2 was amplified and cloned into a pDrive vector to obtain wild-type plasmids. Plasmids harboring the target mutations (*KRAS* G12D*, KRAS* G12V*, KRAS* G13D) were generated using site-directed mutagenesis (New England Biolabs, USA). Primer details are given in Tables 1 and 2. These plasmids were used as positive controls.

Development of the ddPCR assay

The validated primer-probe combinations of three variations namely G12D (Assay ID-dHsaCP2500596 and Assay ID- dHsaCP2000002), G12V (Assay ID-dHsaCP2500592 and Assay ID- dHsaCP2000006) and G13D (Assay ID- dHsaCP2500598 and Assay ID- dHsaCP2000014) were procured from Bio-Rad Laboratories, USA. These were used for standardization of copy number and annealing temperature of the assays along with ddPCR supermix for probes (no dUTP, Bio-Rad, USA).

For copy number validation, 20,000, 10,000, and 5,000 copies of control plasmids (KRAS wild-type and KRAS mutant plasmids separately) were used. A gradient ddPCR was employed to standardize the annealing temperature that showed the most efficient separation between the positive and the negative droplets.

The assays for *KRAS* mutations were carried out in QX200 Droplet Digital System (Bio-Rad, USA) as per the protocol outlined for rare-event detection. Briefly, 20µl reaction mix comprised 10 µl of 2X ddPCR Supermix for probes (no dUTP), 1 µl each of wild and mutant type 20X primer/ probe mix, and 5ng of cfDNA or 25ng of tumor DNA. For every run, positive control (5,000 copies each of wild-type DNA and mutant DNA in separate wells) and No Template Control (NTC) were used. Cycling conditions employed were 95°C for 10 minutes (1 cycle), 94°C for 30 seconds and 52°C (G12D) / 50°C (G12V, G13D) for 60 seconds (40 cycles), 98°C for 10 minutes and 30°C for 5 minutes (1 cycle) and 4°C hold indefinitely. The blue and green droplets were considered positive for the mutant and wild-type sequences, respectively. Samples with six or more blue droplets in the FAM channel or a fractional abundance of 1% were considered positive for the mutation.

The results of the cfDNA-based ddPCR analysis for five random samples were compared with the corresponding tumor tissue DNA-based ddPCR analysis data.

Statistical analysis

The quantification of FAM and HEX positive droplets were done using the QuantaSoft™ software (Bio-Rad Laboratories, USA) based on Poisson statistics. The fractional abundance was calculated as the ratio of mutant droplets and total positive droplets and the concentrations were expressed in absolute values as copies/µl.

Results

Standardization of the ddPCR assays

Standardization of copy number for the positive control for all three mutation detection assays was conducted. As shown in Figure 1A, a reduction in the number of positive droplets was observed with a corresponding decrease in the amount of input DNA for both the mutant plasmid (Figure 1A upper panel) and for the WT plasmid (Figure 1A, lower panel). There was no saturation of the droplets as evident from the presence of negative droplets (represented as black droplets, below the magenta threshold line) in all the concentrations tested. Based on the results obtained an input of 5,000 copies for each plasmid was considered as sufficient for input positive controls and the same copy number was used for the successive runs (Figure 1A, upper and lower panel). The annealing temperature was optimized for all the three variations (*KRAS* G12D, *KRAS* G12V and *KRAS* G13D) by gradient ddPCR with WT and mutant plasmids. A representative image of a gradient ddPCR run with four different annealing temperatures is shown in Figure 1B with mutant plasmid in the upper panel and the WT plasmid in the lower panel with a constant copy number of 5000 copies per reaction. As shown in Figure 1B, the assays showed increasingly better separation between the negative and the positive droplets with decreasing annealing temperatures, both for mutant (blue droplets, upper panel) and wild type alleles (green droplets, lower panel). Based on these assays, the annealing temperature for *KRAS* G12D assay was optimized at 52°C whereas for *KRAS* G12V and *KRAS* G13D, the optimized temperature was 50 °C.

Screening of cfDNA samples for KRAS mutations using ddPCR

A total of twenty plasma samples were screened for three *KRAS* variations namely *KRAS* G12D, *KRAS* G12V, and *KRAS* G13D. The ddPCR assays revealed that 14 patients harbored all three

variations, 2 patients had two variations namely G12V and G13D, 3 patients harbored only the G12D variation and 1 patient did not harbor any of the three mutations. Interestingly, 5 out of 20 patients were negative for at least two activating *EGFR* variations [15]. Representative images of cfDNA-ddPCR *KRAS* variation analysis are shown in Figure 2. The median mutant copies/µl for *KRAS* G12D, *KRAS* G12V, and *KRAS* G13D mutations were 11.5 copies/µl, 12.4 copies/µl, and 8.3 copies/µl respectively in the cfDNA samples (*Supplementary Table 2*).

Screening of tumor samples for KRAS variations by ddPCR

Genomic DNA extracted from tumor (tDNA) of five patients was analyzed for all three mutations under study using ddPCR and the results were compared with the data from matched- cfDNA analysis. All the five tDNA samples were positive for *KRAS* G12D variation. Two tumor samples harbored the *KRAS* G12V mutation and three harbored the *KRAS* G13D mutation. Representative images of tDNA-ddPCR analysis are shown in Figure 3 (A-F).

Discussion and Conclusions

KRAS has been on the radar of lung cancers for decades as pathogenic variations in this oncogene are associated with poor survival rates and therapy response [16]. It has been a tough candidate to target due to its small size, smooth surface, and high affinity to GTP [17]. However, scientists have developed therapeutic strategies to target *KRAS*-driven cancers, hence effective variation profiling is crucial.

The gold standard to detect pathogenic variations in the clinical setting involves tissue biopsy, followed by NGS [18]. Tissue biopsies are painful, and physically and emotionally hard on patients. In certain cases, the inaccessibility of the tumor sites makes sample collection difficult. Over the years, liquid biopsy has developed into a reliable alternative to tissue biopsy based on the evidence that genetic perturbations can be detected in cfDNA, exosomal DNA, and DNA from circulating tumor cells (CTCs) isolated from body fluids [19]. Isolation of cfDNA is less demanding and it can be completely automated. Conversely, the gold standard for exosomal DNA extraction involving ultracentrifugation does not qualify as a high-throughput procedure [20]. Considering the sensitivity for variation detection in cfDNA and CTCs, cfDNA fares better [21]. The potential of obtaining cfDNA with minimal invasiveness, application of high-throughput techniques, and ease of repeated sampling makes liquid biopsy with cfDNA an appealing choice for routine testing in cancer management. The sample partitioning concept employed in the third-

generation ddPCR increases the sensitivity of rare variation detection in cfDNA many folds, particularly in a background of a large pool of WT DNA.

In this study, profiling of three *KRAS* variations that occur in 36% of lung cancer cases worldwide was carried out in plasma-derived cfDNA obtained from 20 lung cancer patients [9]. The concentration of cfDNA in the blood of cancer patients ranges from 0-5 ng to greater than 1000 ng per ml of plasma [13]. In our samples, the concentration of the majority of cfDNA samples ranged from 2-10.8 ng/µl which amounts to 60-324ng of cfDNA per ml of plasma. Three samples had a higher concentration of 291ng, 324ng, and 936ng of cfDNA /ml of plasma.

The cfDNA-ddPCR analysis employed in this study revealed that 85% of the patients were positive for *KRAS* G12D, whereas 80% were positive for *KRAS* G12V and *KRAS* G13D variations. One sample did not harbor any of the three variations. Interestingly, a staggering 70% of the study cohort harbored all three variations. An earlier study by Aggarwal et al., [22], proclaimed that south India had a high incidence of *EGFR* variations and not *KRAS* mutations, which is not in compliance with the result of the present study. However, the earlier study employed ARMS technique, which is less sensitive than ddPCR. The high positivity observed in our study could be due to the small sample size that was screened. The five samples negative for at least two activating *EGFR* variations harbored *KRAS* variations indicating the mutual exclusivity of these variations in these samples. It has long been believed that KRAS variations cannot co-occur with *EGFR* variations and vice versa. A study by Arun *et al.,* revealed that synthetic lethality is the underlying basis for mutual exclusivity of these two genes in lung adenocarcinomas [23]. However, cases of co-occurrence of *EGFR* and *KRAS* variations in lung tumors have been reported in many studies [24-26].

To determine if cfDNA truly represents the variational landscape of tumor DNA, the cfDNA-ddPCR data was compared with tDNA-ddPCR data for five samples. Concordance was observed in 66.67% of the cases. In 33.3% of the cases, the variations detected in cfDNA were not seen in the matched tDNA. The absence of the same variations in tDNA might be due to tumor heterogeneity. Tumor heterogeneity refers to the epigenetic, genetic, and chromosomal differences in the cells of a tumor or between primary and secondary tumors. It is one of the major contributing factors to drug resistance in lung cancers [27]. Relatively recent research by Visser et al., [28], indicated that ddPCR could pick additional mutations through cfDNA-ddPCR, which is consistent with the results of this study.

Another important observation in this study was the ability of the method to detect mutations in extremely low percentage of fractional abundance. As shown in supplementary data, three

samples had a fractional abundance of less than 5% for *KRAS* G12D variation and one sample had a fractional abundance of 1.3% for *KRAS* G13D variation. On the other hand, the tDNA of four samples had a fractional abundance of less than 3% for *KRAS* G12D variation, and three had a fractional abundance of less than 5% for *KRAS* G13D variation. It is most likely that these variations would evade detection through NGS, as NGS correctly determines variations with at least 5% Variant Allele Frequency [29]. However, the ddPCR method employed here could pick up these variations further highlighting the sensitivity of the technique.

Lung tumor samples are usually obtained via needle biopsies and hence the amount of tissue that is available for downstream analysis is extremely small and becomes insufficient to extract enough DNA for sequencing [30]. Although lung biopsies are associated with a high success rate of 88-97%, this drops significantly for lesions smaller than 1.5 cm. In addition, biopsies are usually associated with complications such as pneumothorax, pulmonary bleeding, and hemoptysis [31]. Thus, these inherent disadvantages further justify the need for alternative techniques, ideally those that employ non-invasive methods.

The prevalence of *KRAS* mutations varies across regions. For instance, western countries have reported highmutation rates of 23-33% [32], whereas studies conducted in Chinese cohort reported a *KRAS* positivity rate in the range of 10-12.1% [33-35]. Johan et al. reported a KRAS prevalence of 38% in adenocarcinomas and 28% in NSCLC groups in the Swedish cohort [36]. A study by HCG Cancer Centre, Bangalore reported a positivity rate of 34.09% of *KRAS* variations. Generally, *KRAS* variations vary between 3% and 19% in Asian cohorts [37]. In our study, the *KRAS* positivity rate was 81.67%, which seems to be quite high compared to the other studies. Although the small sample size in our study could be a major factor for such a high percentage positivity, it should also be noted that none of the earlier studies employed ddPCR and this technique has high sensitivity and accuracy compared to other methods of variant detection. The advent of ddPCR has revolutionized molecular diagnosis making it possible to detect rare variations, particularly those that interfere with therapeutic outcome, with extremely high sensitivity and accuracy. This study represents one of the first studies conducted in a South Indian cohort that used ddPCR to detect *KRAS* mutations in cfDNA from plasma of the patients. Although the positivity percentage was very high, the results of this study can not be generalized for the entire population and such assays must be carried out using a bigger size of the cohort to truly indicate the prevalence of *KRAS* mutations in lung cancer. Nevertheless, despite the small sample size, the results of this study clearly indicate that non-invasive profiling of *KRAS* variations using cfDNA from the blood could be an effective alternative to tissue biopsies in lung cancer patients.

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Online supplementary material:
Supplementary Table 1. Details of the patient samples analyzed in this study.
Supplementary Table 2. Droplet digital polymerase chain reaction results for KRAS G12D, G12V and G13D mutation analysis of cfDNA. MC/µl- Mutant copies/ microliter, FA- Fractional Abundance (%)

Table 1. Details of the primers used for amplification of the 222bp *KRAS* gene fragment.

Primer	Sequence (5′-3′)	Annealing Temperature
Forward Primer	GTATTAACCTTATGTGTGTGACA	52°C
Reverse Primer	GTCCTGCACCAGTAATATGC	

Table 2. Details of the primers used in site-directed mutagenesis to generate KRAS-Mutant plasmids.

Primer	Sequence (5′-3′)	Annealing temperature
KRAS G12D Forward	GTTGGAGCTGATGGCGTAGG	66°C
KRAS G12D Reverse	TACCACAAGTTTATATTCAGTCATGGTG	
KRAS G12V Forward	GTTGGAGCTGTTGGCGTAGGC	64°C
KRAS G12V Reverse	TACCACAAGTTTATATTCAGTCATGGTG	
KRAS G13D Forward	GGAGCTGGCGACGTAGGCAAG	66°C
KRAS G13D Reverse	AACTACCACAAGTTTATATTCAGTCATGG	

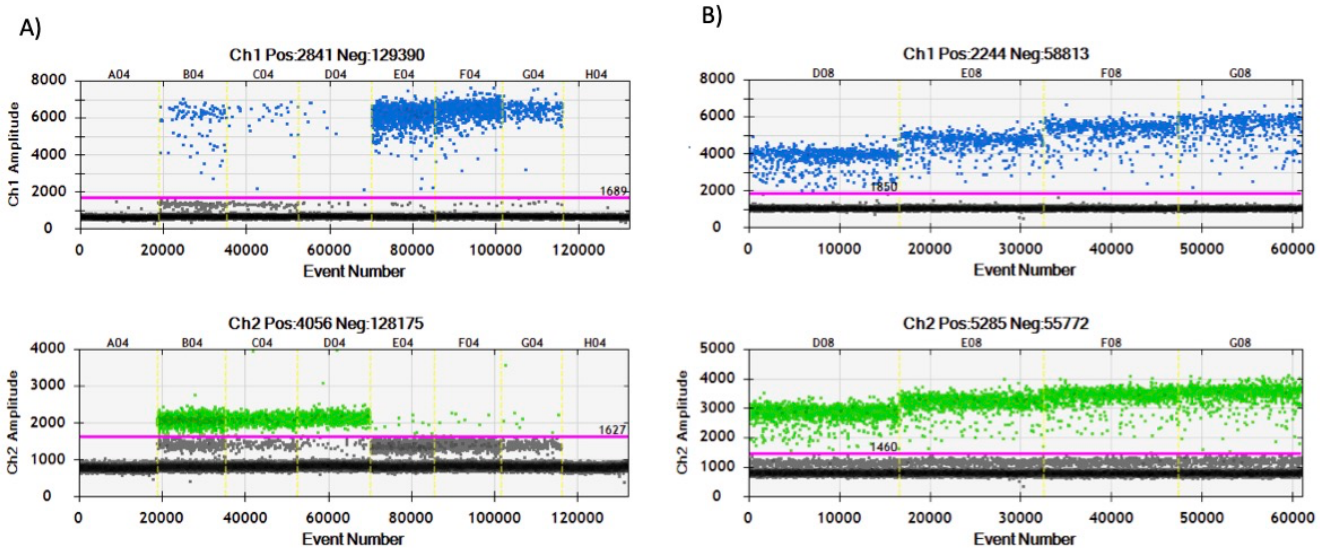

Figure 1. Standardization of the KRAS ddPCR assays. A) Representative image of 1D plot for standardization of copy number for positive control for KRAS wild-type and KRAS mutant plasmids. Lane A04 is the NTC, Lanes B04, C04 and D04 indicate 20,000, 10,000, 5,000 copies of KRAS wild-type plasmid respectively. Lanes E04, F04, G04 and H04 indicate 20,000, 10,000 and 5,000 copies of KRAS mutant plasmid and NTC respectively. The blue droplets indicate mutant copies and the green droplets indicate the wild-type copies. The pink line indicates the threshold and the black droplets are considered to be negative; B) representative image of 1D plot for standardization of annealing temperature for KRAS variation detection ddPCR assay. Lanes D08, E08, F08 and G08 are the corresponding positive controls at 4 different temperatures: 53°C, 51.9°C, 51°C and 50.3°C, respectively.

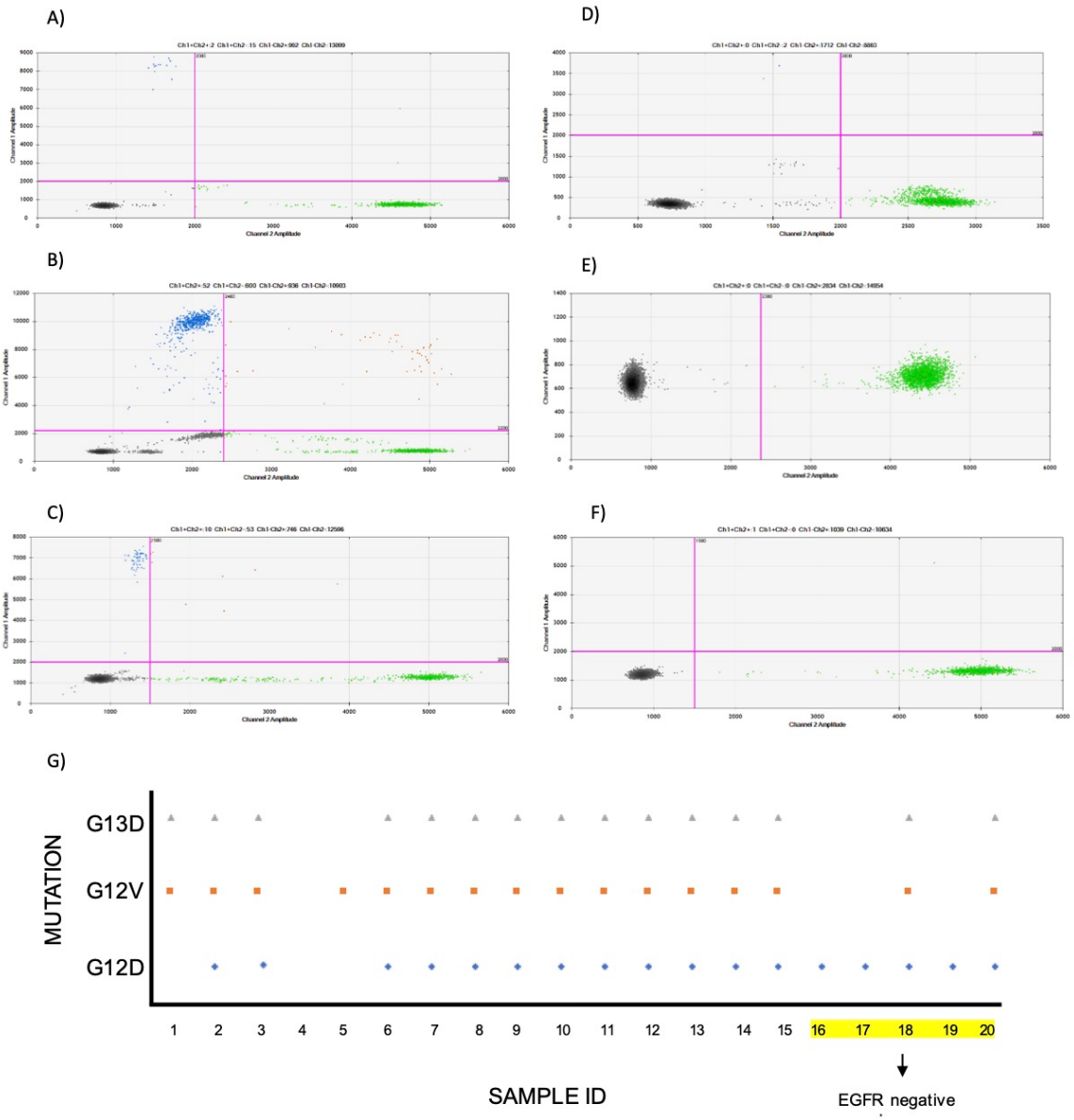

Figure 2. Screening for *KRAS* mutations in cfDNA by ddPCR. A-C) Representative images of 2D plots of plasma samples from patients positive for *KRAS G12D, KRAS G12V* and *KRAS G13D* variations by cfDNA-ddPCR; D-F) 2D plot of a *KRAS G12D, KRAS G12V* and *KRAS G13D* mutation negative cfDNA sample through ddPCR analysis. The blue droplets indicate droplets that contained mutant allele whereas green droplets indicate the droplets with the WT allele. The orange droplets represent the droplets that contain both mutant and WT alleles. The pink line is the threshold and the black droplets indicate the droplets without DNA (negative); G) Plot depicting the variation status of the screened lung cancer cfDNA samples through ddPCR. A sample positive for a variation is indicated by symbols namely blue rhombus (G12D), orange square (G12V) and grey triangle (G13D). Absence of these symbols indicates sample the sample is negative for that target variation.

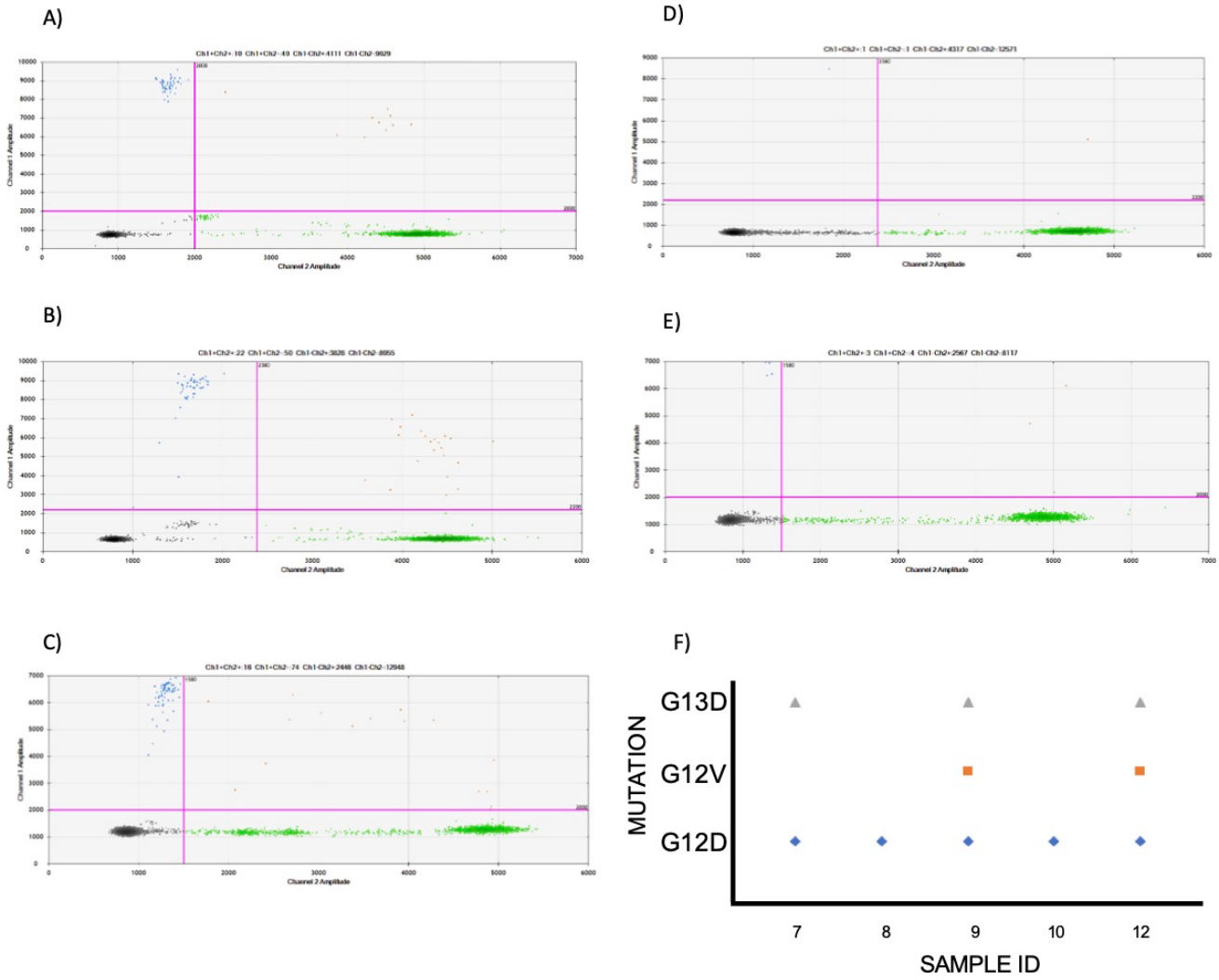

Figure 3. Screening for *KRAS* mutations in tumor DNA by ddPCR. A-C) Representative images of 2D plots of ddPCR assays using tumor DNA samples positive for *KRAS G12D* (A), *KRAS G12V* (B) and *KRAS G13D* (C) variation respectively; D,E) Representative images of 2D plots of ddPCR assays using tumor DNA samples negative for *KRAS mutations*. The blue droplets indicate droplets that contained mutant allele whereas green droplets indicate the droplets with the WT allele. The orange droplets indicate the droplets that contained both mutant and WT alleles. The pink line is the threshold and the black droplets indicate the droplets without DNA (negative). F: Plot depicting the variation status of the screened lung cancer tDNA samples through ddPCR. A sample positive for a variation is indicated by symbols namely blue rhombus (G12D), orange square (G12V) and grey triangle (G13D). Absence of these symbols indicates sample negative for that target variation.