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Development and Analytical Performance of Multiplex Droplet Digital PCR Assays for the Detection of PIK3CA Mutations in Metastatic Breast Cancer Patients

Dana Leng Hui Chan[1], Valerie Yue Ming Heong[2], Wey Cheng Sim[1], Ching Kit Chen[3] and Liuh Ling Goh[1*]

[1]*Molecular Diagnostic Laboratory, Tan Tock Seng Hospital, Singapore*

[2]*Department of Medical Oncology, Tan Tock Seng Hospital, Singapore*

[3]*Department of Paediatrics, National University of Singapore, Singapore*

Correspondence should be addressed to Liuh Ling Goh, Molecular Diagnostic Laboratory, Block 203, CDC 2, 11 Jalan Tan Tock Seng, Tan Tock Seng Hospital, 308433, Singapore

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ABSTRACT

The detection of PIK3CA mutations in patients with metastatic HR-positive and HER2-negative breast cancer is important for targeted therapy. We describe the development and validation of a multiplex assay using droplet digital PCR (ddPCR) for the simultaneous and sensitive detection of 11 mutations in exons 8, 10 and 21 of the PIK3CA gene from DNA extracted from plasma and tissue. Using synthetic DNA spike-in controls, the assay was optimized in two separate reaction wells by varying the combinations and concentrations of primer-probe pairs. The performance parameters were then assessed with 21 known clinical samples and 8 Horizon controls. A perfect concordance rate of 100% was achieved with 100% specificity in wild-type samples and a verified limit-of-detection (LOD) of 2.8 to 26 copies of mutant per well. This lab developed assay was then offered to eligible patients for testing on plasma at no cost through an access program. Thirty-six patients were enrolled in 3 months with a positive rate of 22.2% (8/36) in plasma. This study supports the use of this multiplex assay in clinical laboratory and demonstrates the value of cell-free DNA testing when tissue is unavailable.

KEYWORDS

PIK3CA; Breast cancer; Droplet digital PCR; Liquid biopsy; Circulating tumor DNA

INTRODUCTION

The phosphoinositide 3-kinases (PI3Ks) are a family of enzymes that is recruited upon growth factor receptor activation and produces phosphoinositide lipids through phosphorylation of the 3' hydroxyl group [1]. Several classes of PI3Ks exist with class I PI3Ks being the most widely studied due to its prevalence in cancer [2]. Class I PI3Ks are heterodimeric proteins that consist of a catalytic subunit and a regulatory subunit [2]. Within class I PI3Ks, there is a further subdivision into class IA and IB based on the catalytic and regulatory subunits. Class IA consists of

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three enzymes, PI3Kα, PI3Kβ, and PI3Kδ [3]. An important catalytic subunit component of PI3Kα is the phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA). Activating somatic missense mutations of the PIK3CA gene that increase the kinase activity of the PI3Kα protein have been identified in tumor tissues and have been linked to many different human cancers [4], including hormone receptor positive (HR+) breast cancer [5].

An isoform-specific PI3Kα inhibitor, Alpelisib, in combination with fulvestrant has received FDA approval in May 2019 for men and postmenopausal women with HR+/HER2-, PIK3CA-mutated advanced or metastatic breast cancer following progression while on or after treatment with an endocrine-based therapy. The efficacy of alpelisib was studied in the SOLAR-1 trial (NCT02437318) where patients with the above profile experienced prolonged overall survival (OS) compared to the placebo group. The median OS was prolonged by 7.9 months versus fulvestrant alone and with data additionally suggested a survival benefit in various patient subgroups with no new safety concern [6,7]. PIK3CA mutations that were considered for trial enrollment in SOLAR-1 included C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R, and H1047Y, and were reported elsewhere [8,9].

Digital PCR, most notably droplet digital PCR (ddPCR), is a powerful technology for sensitive and accurate mutation detection. DdPCR works by partitioning reaction to around 20,000 uniformed size droplets for a massive parallel PCR reaction. After endpoint PCR amplification, each droplet is read individually and assigned as positive or negative depending on the presence or absence of the target sequence, respectively. The Poisson law, together with other proprietary algorithms, are then factored in to compute the precise DNA concentration with high sensitivity and reliability, without the need of calibration curves [10]. With good optimization it is possible to develop discriminatory multiplex ddPCR assays that enable very rapid and cost-effective monitoring for several mutations in serial plasma samples [11].

The purpose of this study is to develop a sensitive ddPCR multiplex screening assay for the detection of 11 PIK3CA mutations together with exon 15 for internal nucleic acid extraction and PCR control. The analytical performance of this assay was evaluated and tested on clinical plasma and tumor samples for clinical use. In addition, the assay was subsequently prospectively used on 36 patients to inform clinical practice. The results obtained from this real-world clinical testing on HR+/HER2- advanced or metastatic breast cancer patients are also presented.

MATERIALS AND METHODS

DNA Extraction from Blood and Plasma Samples

Synthetic DNA (gBlock, IDT) spike-in, horizon controls and known clinical samples were used in the validation. For clinical samples, cell free DNA (cfDNA) was extracted from 4 mL blood samples using QIAamp Circulating Nucleic Acid Kit (Qiagen) and DNA from 5 FFPE sections was extracted using QIAamp® DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's instructions. The yield of DNA was quantified using Qubit dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (ThermoFisher Scientific). A total of 36 HR+/HER2- advanced or metastatic breast cancer patients underwent prospective plasma PIK3CA testing by ddPCR in Molecular Diagnostic Laboratory of Tan Tock Seng Hospital (TTSH) from October 2021 to January 2022. The test results extracted from laboratory information system were retrospectively reviewed in this study.

PIK3CA Assays

The 11 PIK3CA mutations evaluated in this study were C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R, and H1047Y. The evaluation also included an internal control for Exon 15 of the

PIK3CA allele. Assays for 10 of the 12 targets were predesigned and ordered from Bio-Rad Laboratories PrimePCR™ with the exception for E545A and Exon 15 which were custom designed (Table S1). All assays employed the hydrolysis probe chemistry with appropriate fluorescence probe as indicated (Table S1). Predesigned and custom designed assays came in a concentrated ready-to-use primer-probe mix (9 µM primer and 5 µM probe). Primer and probe sequences for E545A and Exon 15 are available in Table S2. Custom assays were designed using the publicly available software Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with the sequences of the PIK3CA gene (NG_012113.2) for E545A mutation and a short sequence of Exon 15 of PIK3CA. Targeted sequence were analyzed for gene specificity using Primer-BLAST. gBlock Gene Fragment (IDT) were designed for all 12 targets for positive controls (Table S1).

PIK3CA Multiplexing Assays and Droplet Digital PCR Workflow

The analyses of the 12 targets were based on two distinct reactions detecting 6 targets each. The volume used for each target is indicated in Table 1. For each reaction, apart from the above defined primer-probe mix assays, the ddPCR mixture consisted of 4x ddPCR Multiplex Supermix for probes (Bio-Rad), and 40 ng of DNA extracted from FFPE, in a total volume of 24 µL. For cfDNA, the sample volume was maximized as the concentration in these samples were typically low. Positive, negative and no-template (nuclease-free water) controls were systematically used for each experiment. The droplet generation was performed in the QX200 Droplet Generator (Bio-Rad) using 20 µL of the ddPCR mixture and 70 µL of the droplet generation oil (Bio-Rad). An average of 20,000 droplets were generated per well. In case of less than 10,000 droplets were generated per well, the sample was repeated.

Droplets were transferred into a 96-well plate for the thermal cycling amplification and sealed using the PX1 PCR Plate Sealer (Bio-Rad). The PCR protocol on a C1000

Touch Thermal Cycler (Bio-Rad) was as follows: 37°C for 30 min, 95°C for 10 min followed by 40 cycles of denaturation at 94°C for 30 s, 55°C for 1 minute, with a final 10 minutes at 98°C. After end-point PCR amplification, fluorescence signals were quantified by the QX200 Droplet Reader (Bio-Rad) and data were analyzed using the QuantaSoft software v.1.7.4 (Bio-Rad). Positive and negative controls served as a guide to call markers.

	Assay 1		Assay 2	
Signals	**Target**	**µL/reaction**	**Target**	**µL/reaction**
FAM	H1047Y	1.5	E542K	1.0
	H1047L	1.2	Q546E	0.5
	H1047R	1.0	C420R	0.5
	E545K	1.2	E545D	0.8
	E545G	1.0	Q546R	1.0
HEX	Exon 15	0.3	E545A	1.0

Table 1: Multiplex assay primer-probe combination and volume used in a 24 µL reaction.

RESULTS

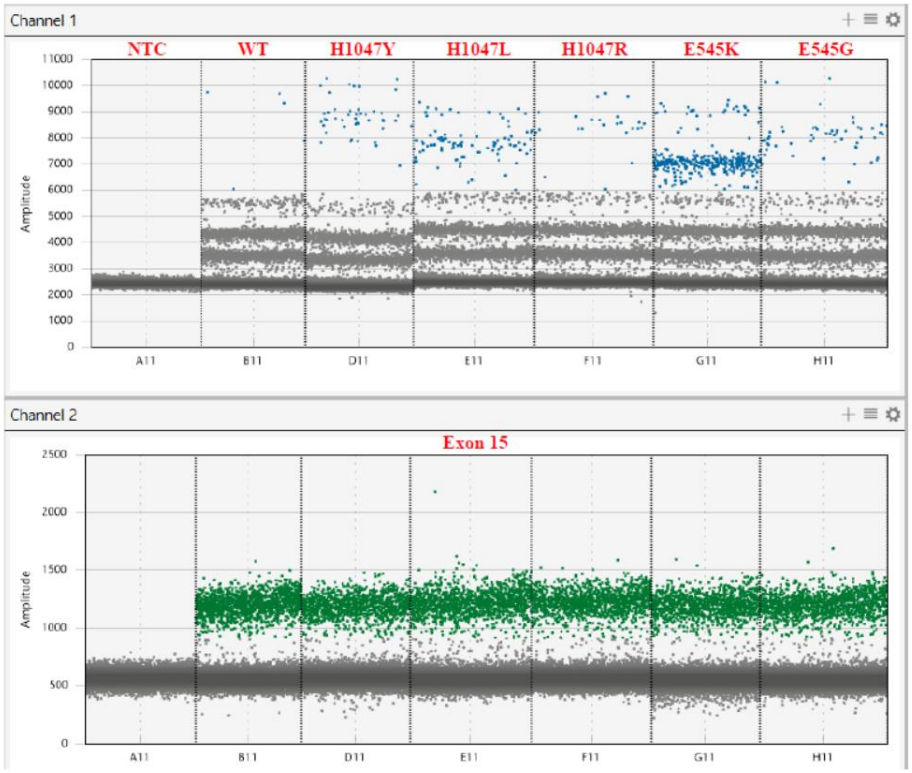
Sample characteristics

A total of 46 samples were used to evaluate for known actionable PIK3CA mutations in this study. Eleven of which were synthetic DNA (gBlock) spike-in positive controls, 8 were Horizon reference controls and 21 were known clinical samples. Among the Horizon and clinical samples, 16 were FFPE DNA and 13 were cfDNA. This study included clinical samples with various cancer types, 9 of them had non-small cell lung cancer (NSCLC), 4 with glioma, 4 with colorectal cancer, 3 with breast cancer, and 1 with bladder cancer. The concentration of DNA input from FFPE samples for the PIK3CA assay ranged from 17.5 ng to 507.6 ng, with an average of 93.7 ng, while concentration of cfDNA input ranged from 2.2 ng to 30.2 ng, with an average of 10 ng.

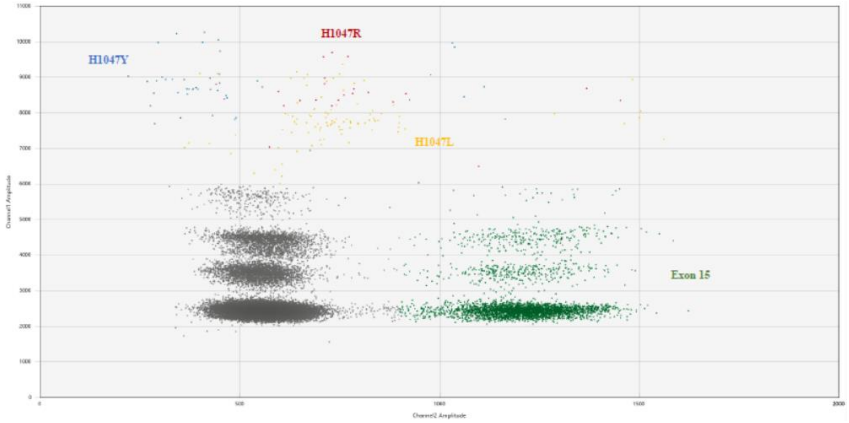
Multiplex assay optimization

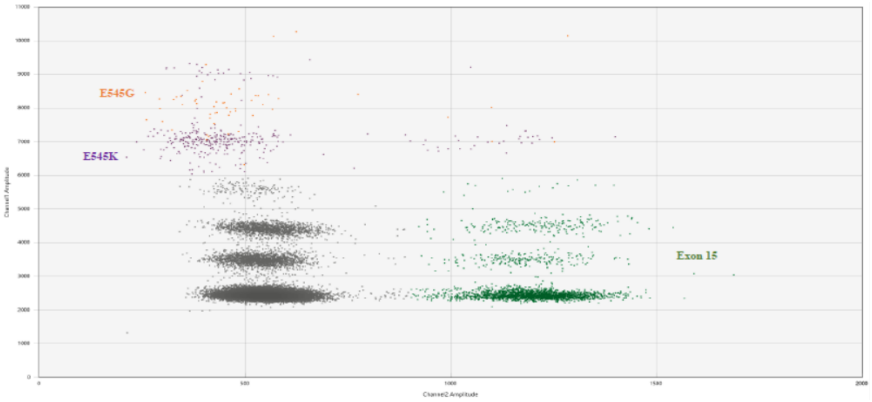
Developing the assay in 2 separate reaction wells allowed the efficient use of limited specimen, faster turnaround

time, and reduced running cost. The combination of the primer-probe pairs in the 2 separate reactions are shown in Table 1. Assay 1 shows multiplex amplification and detection of H1047Y, H1047L, H1047R, E545K, E545G (FAM conjugated) and Exon 15 (HEX conjugated). A subsequent analysis included probes for E542K, Q546E, C420R, E545D (FAM conjugated) and E545A (HEX conjugated). High probe-primer concentrations were observed to increase fluorescence and background events. Optimization was performed to identify the optimal primer-probe concentration to clearly distinguish positive from negative thresholds. Figure 1 and Figure 2 show the quality of the signals obtained with the established concentrations in both assays 1 and 2, respectively.



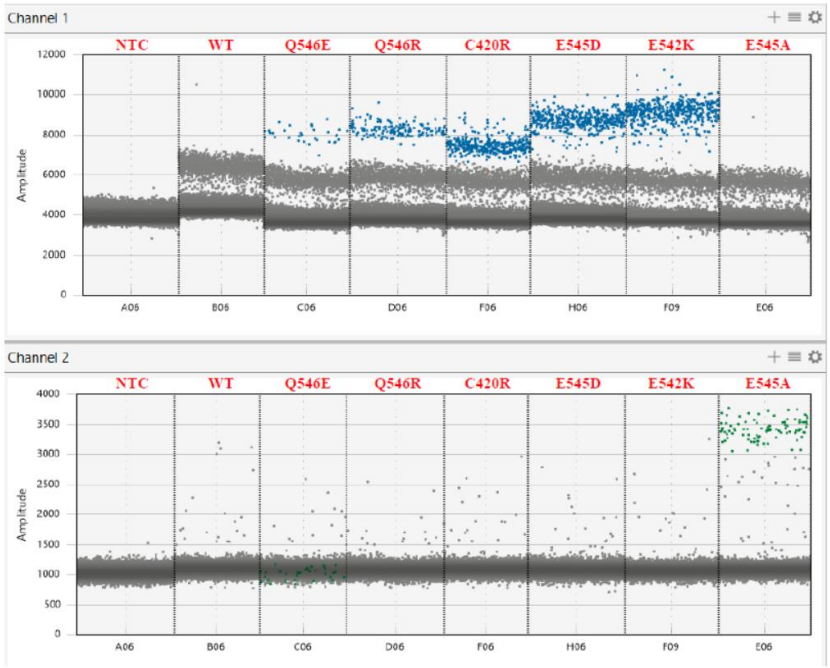
(A)



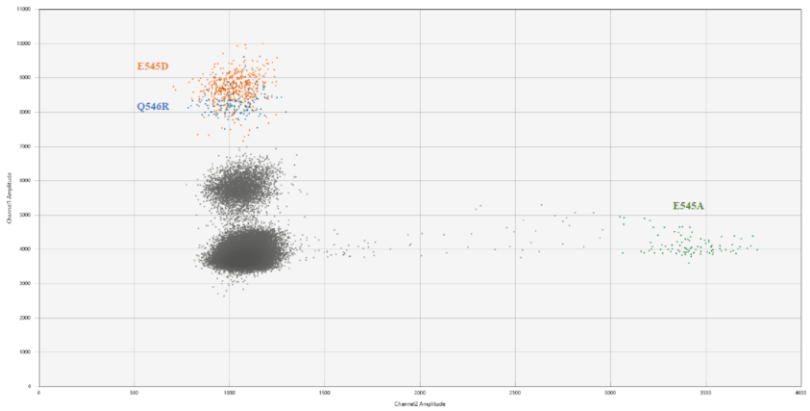


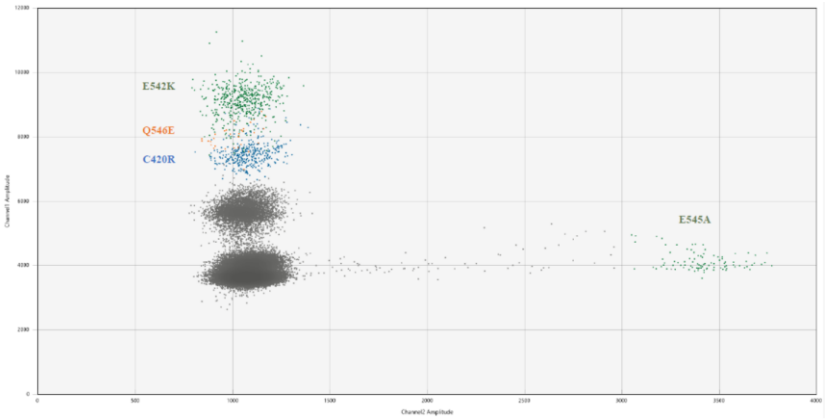
(B)

Figure 1: Signal quality and clustering of mutant populations in assay 1. (A) One-dimensional plots of individual gBlock. Channel 1 depicts FAM signal while channel 2 depicts HEX signal. **(B)** Individual gBlock was used to determine the location of each mutation cluster in the two-dimensional (2D) plots. The 2D plots represent merging of different wells in respective multiplex assay.



(A)





(B)

Figure 2: Signal quality and clustering of mutant populations in assay 2. **(A)** One-dimensional plots of individual gBlock. Channel 1 depicts FAM signal while channel 2 depicts HEX signal. **(B)** Individual gBlock was used to determine the location of each mutation cluster in the two-dimensional (2D) plots. The 2D plots represent merging of different wells in respective multiplex assay.

Distinct separation of the negative (empty droplets, without template), wild-type (WT, Exon 15), and mutant signals were observed and are shown in the one-dimensional (1D) plots (Figure 1A and 2A) while the clustering of different target regions are defined in the two-dimensional (2D) plots (Figure 1B and 2B). The identity of each mutant population was determined by running assays with individual spike-in mutant positive control (data not shown). The genes when analysed individually were easily distinguishable from WT or NTC controls (Figures 1A and 2A). However, when analysed together, the mutant signals tended to coincide in the same cluster and were indistinguishable (Figures 1B and 2B). Only E545A in HEX channel of assay 2 was clearly identifiable and well defined.

Hence, this test was unable to individually identify the PIK3CA variants, but provides a qualitative analysis.

Limit of blank and analytical sensitivity

The detection capability of the assay was evaluated by its limit of blank (LOB) and Limit of detection (LOD). LoB is defined as the upper boundary on wild-type DNA sample measurements. Next, specificity was examined by the occurrence of false positive among previously known PIK3CA wild-type samples. A total of 14 known PIK3CA wild-type clinical samples (6 FFPE DNA and 8 cfDNA) were analyzed for assays 1 and 2 (Table 2). FAM positive droplets ranged from 1 to 4 were observed in samples with higher input.

The background noise was more evident in FFPE than plasma specimens (Table 2). A cut-off of minimum 5 positive droplets was implemented to call a sample positive for PIK3CA mutation. With this criterion, the specificity of the assay is 100%.

The optimum DNA input was determined to minimize background noise. CfDNA has minimal impact on background noises due to their low concentration which ranged from 2.2 ng to 30.2 ng from 4 mL of blood. However, FFPE DNA with input ranging from 17.5 ng to 507.6 ng was optimized at 40 ng of input per reaction as higher input contributed to complex clustering issues (data not shown).

Limit of detection is defined as the lowest concentration of mutant DNA in a background of wild-type DNA at which a mutant sample will provide a positive mutation result. The LOD of ddPCR for PIK3CA mutation was determined using E545K (assay 1) and E542K (assay 2) gBlocks at 100, 50 and 10 copies (Table 3). The detection limit was

established at 2.8 mutant copies per reaction for assay 1 (H1047Y/L/R, E545K/G) and 26 mutant copies per reaction for assay 2 (Q546E/R, C420R, E545A/D, E542K).

No.	Assay	Sample ID	Specimen type	Expected results	ddPCR	FAM droplets	HEX copies/20µL	Conc (ng/µL)	Input (ng/µL)
1	1	Clinical sample 5	FFPE	PIK3CA Negative	PIK3CA Negative	4	2980	10.00	40.00
2		Clinical sample 8		PIK3CA Negative	PIK3CA Negative	4	10200	13.40	120.60
3		Clinical sample 9	cfDNA	PIK3CA Negative	PIK3CA Negative	0	468	0.29	2.29
4		Clinical sample 10		PIK3CA Negative	PIK3CA Negative	0	5680	2.16	30.24
5		Clinical sample 11		PIK3CA Negative	PIK3CA Negative	1	2980	0.95	13.24
6	2	Clinical sample 13	FFPE	PIK3CA Negative	PIK3CA Negative	2	-	6.96	27.84
7		Clinical sample 14		PIK3CA Negative	PIK3CA Negative	2	-	10.00	40.00
8		Clinical sample 15		PIK3CA Negative	PIK3CA Negative	3	-	20.00	80.00
9		Clinical sample 16		PIK3CA Negative	PIK3CA Negative	0	-	10.00	40.00
10		Clinical sample 17	cfDNA	PIK3CA Negative	PIK3CA Negative	0	-	0.16	2.18
11		Clinical sample 18		PIK3CA Negative	PIK3CA Negative	3	-	1.28	17.92
12		Clinical sample 19		PIK3CA Negative	PIK3CA Negative	0	-	0.63	8.79
13		Clinical sample 20		PIK3CA Negative	PIK3CA Negative	0	-	0.87	12.18
14		Clinical sample 21		PIK3CA Negative	PIK3CA Negative	1	-	0.24	3.36

Table 2: Determination of limit of blank and analytical specificity using known clinical samples.

	Copies/20µL	
Dilution	**Assay 1**	**Assay 2**
100 copies	82	46
50 copies	20	38
10 copies	2.8	26

Table 3: Determination of limit of detection (analytical sensitivity) using synthetic DNA.

Accuracy and concordance

To evaluate the accuracy of this PIK3CA mutation test, 21 clinical samples (12 FFPE DNA and 9 cfDNA), 11 gBlocks and 8 Horizon reference standards (4 FFPE and 4 cfDNA) were tested (Table 4). The clinical samples, previously confirmed on next-generation sequencing (NGS) or mass spectrometry platform, were from different cancer types including NSCLC, breast cancer, glioma, colorectal cancer and bladder cancer. The concordance rate was 100%, demonstrating the accuracy of this lab developed test (Table 4).

Reproducibility is assessed by testing samples covering both positive and negative mutations by different operators and runs performed on different days. The precision of inter-run concordance was also demonstrated to be at 100% (data not shown).

No.	Assay	Sample ID	Specimen type	Cancer type	Previous test platform	Expected results	Expected VAF (%)	ddPCR	Concordance
1	1	Neat Horizon FFPE	FFPE	-	-	E545K and H1047R	E545K: 9.00 H1047R: 17.50	PIK3CA positive	✓
2		1% Horizon FFPE		-	-	E545K and H1047R	-	PIK3CA positive	✓
3		0.5% Horizon FFPE		-	-	E545K and H1047R	-	PIK3CA positive	✓
4		0.1% Horizon FFPE		-	-	E545K and H1047R	-	PIK3CA positive	✓
5		Clinical sample 1		Bladder	NGS	E545K	7.14	PIK3CA positive	✓
6		Clinical sample 2		Breast	NGS	E545K	40.23	PIK3CA positive	✓
7		Clinical sample 3		CRC	NGS	E542K (Assay 2)	31.97	PIK3CA negative	✓
8		Clinical sample 4		Breast	NGS	H1047R	34.87	PIK3CA positive	✓
9		Clinical sample 5		Glioma	-	PIK3CA negative	-	PIK3CA negative	✓
10		Clinical sample 6		CRC	Mass spectrometry	E545K	-	PIK3CA positive	✓

11		Clinical sample 7		CRC	Mass spectrometry	E545K	-	PIK3CA positive	✓
12		Clinical sample 8		CRC	NGS	PIK3CA negative	-	PIK3CA negative	✓
13		H1047Y gBlock	gBlock	-	-	H1047Y	-	PIK3CA positive	✓
14		H1047L gBlock		-	-	H1047L	-	PIK3CA positive	✓
15		H1047R gBlock		-	-	H1047R	-	PIK3CA positive	✓
16		E545K gBlock		-	-	E545K	-	PIK3CA positive	✓
17		E545G gBlock		-	-	E545G	-	PIK3CA positive	✓
18		5% Horizon cfDNA	cfDNA	-	-	E545K and endogenous H1047R	-	PIK3CA positive	✓
19		1% Horizon cfDNA		-	-	E545K and endogenous H1047R	-	PIK3CA positive	✓
20		0.5% Horizon cfDNA		-	-	E545K and endogenous H1047R	-	PIK3CA positive	✓
21		0.1% Horizon cfDNA		-	-	E545K and endogenous H1047R	-	PIK3CA positive	✓
22		Clinical sample 9		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
23		Clinical sample 10		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
24		Clinical sample 11		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
25		Clinical sample 12		NSCLC	Mass spectrometry	PIK3CA negative	-	PIK3CA negative	✓
26	2	Clinical sample 5	FFPE	Glioma	-	PIK3CA negative	-	PIK3CA negative	✓
27		Clinical sample 8		CRC	NGS	PIK3CA negative	-	PIK3CA negative	✓
28		Clinical sample 3		CRC	NGS	E542K	31.97	PIK3CA positive	✓
29		Clinical sample 13		Breast	NGS	PIK3CA negative	-	PIK3CA negative	✓
30		Clinical sample 14		Glioma	-	PIK3CA negative	-	PIK3CA negative	✓
31		Clinical sample 15		Glioma	-	PIK3CA negative	-	PIK3CA negative	✓
32		Clinical sample 16		Glioma	-	PIK3CA negative	-	PIK3CA negative	✓
33		H1047R gBlock	gBlock	-	-	H1047R (Assay 1)	-	PIK3CA negative	✓
34		E542K gBlock		-	-	E542K	-	PIK3CA positive	✓
35		Q546E gBlock		-	-	Q546E	-	PIK3CA positive	✓
36		C420R gBlock		-	-	C420R	-	PIK3CA positive	✓
37		E545D gBlock		-	-	E545D	-	PIK3CA positive	✓
38		Q546R gBlock		-	-	Q546R	-	PIK3CA positive	✓
39		E545A gBlock		-	-	E545A	-	PIK3CA positive	✓
40		5% Horizon cfDNA	cfDNA	-	-	E545K and endogenous H1047R (Assay 1)	-	PIK3CA negative	✓
41		Clinical sample 9		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
42		Clinical sample 17		NSCLC	Mass spectrometry	PIK3CA negative	-	PIK3CA negative	✓
43		Clinical sample 18		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
44		Clinical sample 19		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
45		Clinical sample 20		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓
46		Clinical sample 21		NSCLC	NGS	PIK3CA negative	-	PIK3CA negative	✓

Table 4: Determination of assay accuracy using clinical samples, commercial reference controls and synthetic DNA.

PIK3CA testing and detection rate

We subsequently prospectively tested this assay on clinical samples. The results trend for samples received between October 2021 to January 2022 were reviewed. There was a total of 36 blood samples with HR+/HER2- metastatic breast cancer received during this duration. Sufficient DNA were successfully extracted from all samples for analysis with a turn-around time for results generation of approximately 7 working days making this assay suitable for prospective testing in a clinical setting. Overall, 22.2% (8/36) of the samples tested positive for a *PIK3CA* mutation. Among these, one sample harbored double mutations with positive droplets in both assays 1 and 2. Overall, the positivity rate in assay 1 was higher (66.7%) than assay 2 (33.3%).

DISCUSSION

The SOLAR-1 trial showed that the addition of a specific PI3K inhibitor (alpelisib) to a backbone of fulvestrant provided statistically significant and clinically meaningful progression-free survival (PFS) benefit in *PIK3CA*-mutated, *HR+ HER2-* metastatic breast cancer (BC) [8]. This established alpelisib as the new treatment standard for patients with metastatic HR positive, HER2 negative breast cancer harbouring a *PIK3CA* mutation as second line therapy. This now also requires evidence of the presence of *PIK3CA* mutations in tumor cells by either tissue or plasma testing before alpelisib treatment. The *therascreen*® *PIK3CA* test (QIAGEN Manchester, Ltd.) used in SOLAR-1 is a FDA approved companion diagnostic for alpelisib that detects 11 *PIK3CA* hotspot mutations using real-time qualitative PCR [12]. Here, we described an alternative lab developed assay that detects the 11 *PIK3CA* mutations using ddPCR with the objective for more sensitive and accurate detection in liquid biopsies.

Plasma testing for *PIK3CA* mutation is appropriate when no primary or metastatic breast tumor tissue is available for molecular testing. Several studies have shown that molecular alterations present in cancer cells can be detected non-invasively in circulating tumor DNA (ctDNA) to select targeted therapy, track tumor dynamics, and to monitor the emergence of drug resistance [13,14]. The ddPCR platform was favored for its high sensitivity that enables the detection of low abundance somatic mutation in ctDNA. It is capable for the detection and absolute quantification of point mutations up to 0.01% in limited sample types [15]. It also enables the flexibility in multiplexing to reduce costs and increase sample throughput. In our assay validation we demonstrated high specificity and sensitivity with Horizon controls and clinical samples that were previously tested across different platforms. The detection limit was established at 2.8 mutant copies per reaction for assay 1 (H1047Y/L/R, E545K/G) and 26 mutant copies per reaction for assay 2 (Q546E/R, C420R, E545A/D, E542K). The assay demonstrated 100% concordance with known samples and is deemed satisfactory for testing in both tissue and plasma specimens.

PIK3CA mutations in tumor specimens occurred in approximately 40% of patients in the SOLAR-1 study. We observed a positive rate of about 22% in plasma specimens of patients enrolled in an access program for alpelisib (n = 36). Among the approximately 80% tested negative in plasma specimen, about 60% of results will be true negatives. The lower positivity rate in plasma compared to tissue specimens was expected due to low or insufficient shedding of ctDNA [16,17]. Repeat testing with tumor tissue is recommended when plasma is tested negative.

A recent study evaluating the distribution of *PIK3CA* mutations in breast cancer reported that H1047R (35%),

E545K (17%), E542K (11%), N345K (6%), and H1047L (4%) represent 73% of all *PIK3CA* mutations detected. [18]. Although we cannot differentiate the individual mutation detected using our multiplex assay, a similar distribution of PIK3CA mutations was noted. Among those tested positive, 66.7% harboured a mutation in assay 1 which includes H1047R, E545K and H1047L. This report also brought to our attention that N345K, which is not part of our assay, represented 5.5% of all PIK3CA mutations in the analyzed dataset. This mutation confers a gain of function and has been shown to increase sensitivity to PI3K inhibitors in preclinical models [19,20]. As N345K is likewise not captured by *therascreen*® panel, its clinical benefit from alpelisib is currently uncertain.

One patient was observed to harbour double *PIK3CA* mutations. The frequency of patients with double mutations could be higher as we cannot differentiate the mutations in the same multiplex assay. Preclinically, double compound *PIK3CA* mutations result in increased PI3K activity and seem to predict for increased sensitivity to PI3K alpha-specific inhibitors compared to single hotspot mutants in both preclinical models and also in selected patients with BC treated in early phase 1 trials [21]. Hence, the targeted genotyping approach is a major limitation of our assay.

CONCLUSION

This study presents the feasibility of two multiplexed ddPCR assays for rapid, easy, cost-effective, and simultaneous assessment of *PIK3CA* mutations in clinical samples. To the best of our knowledge, this is the first multiplex ddPCR assays for the detection of *PIK3CA* mutations in both tissue and plasma of metastatic breast cancer patients. These data are useful and serve as a reference for other laboratories interested to develop their assays for the routine diagnosis of *PIK3CA* mutations in HR+/HER2- advanced or metastatic breast cancer patients.

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REFERENCES

1. Katso R, Okkenhaug K, Ahmadi K, et al. (2001) Cellular function of phosphoinositide 3-kinases: Implications for development, immunity, homeostasis, and cancer. Annual Review of Cell and Developmental Biology 17(1): 615-675.
2. Jean S, Kiger AA (2014) Classes of phosphoinositide 3-kinases at a glance. Journal of Cell Science 127(5): 923-928.
3. Vadas O, Burke JE, Zhang X, et al. (2011) Structural basis for activation and inhibition of class I phosphoinositide 3-kinases. Science Signaling 4(195): re2-re2.
4. Zhao L, Vogt PK (2008) Class I PI3K in oncogenic cellular transformation. Oncogene 27(41): 5486-5496.
5. Anderson EJ, Mollon LE, Dean JL, et al. (2020) A systematic review of the prevalence and diagnostic workup of PIK3CA mutations in HR+/HER2–metastatic breast cancer. International Journal of Breast Cancer 2020: 3759179.
6. Chang DY, Ma WL, Lu YS (2021) Role of Alpelisib in the treatment of PIK3CA-mutated breast Cancer: Patient selection and clinical perspectives. Therapeutics and Clinical Risk Management 17: 193-207.
7. André F, Ciruelos EM, Juric D, et al. (2021) Alpelisib plus fulvestrant for PIK3CA-mutated, hormone receptor-positive, human epidermal growth factor receptor-2–negative advanced breast cancer: Final overall survival results from SOLAR-1. Annals of Oncology 32(2): 208-217.

8. André F, Ciruelos E, Rubovszky G, et al. (2019) Alpelisib for PIK3CA-mutated, hormone receptor–positive advanced breast cancer. New England Journal of Medicine 380(20): 1929-1940.
9. Khoury K, Tan AR, Elliott A, et al. (2020) Prevalence of Phosphatidylinositol-3-Kinase (PI3K) pathway alterations and co-alteration of other molecular markers in breast cancer. Frontiers in Oncology: 1475.
10. Hindson BJ, Ness KD, Masquelier DA, et al. (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Analytical Chemistry 83(22): 8604-8610.
11. Rowlands V, Rutkowski AJ, Meuser E, et al. (2019) Optimisation of robust singleplex and multiplex droplet digital PCR assays for high confidence mutation detection in circulating tumour DNA. Scientific Reports 9(1): 1-13.
12. QIAGEN (2019) therascreen PIK3CA RGQ PCR kit instructions for use (Handbook).
13. Nawroz H, Koch W, Anker P, et al. (1996) Microsatellite alterations in serum DNA of head and neck cancer patients. Nature Medicine 2(9): 1035-1037.
14. Siravegna G, Mussolin B, Venesio T, et al. (2019) How liquid biopsies can change clinical practice in oncology. Annals of Oncology 30(10): 1580-1590.
15. Palacín-Aliana I, García-Romero N, Asensi-Puig A, et al. (2021) Clinical utility of liquid biopsy-based actionable mutations detected via ddpcr. Biomedicines 9(8): 906.
16. Malapelle U, Pisapia P, Rocco D, et al. (2016) Next generation sequencing techniques in liquid biopsy: Focus on non-small cell lung cancer patients. Translational Lung Cancer Research 5(5): 505-510.
17. Said R, Guibert N, Oxnard GR, et al. (2020) Circulating tumor DNA analysis in the era of precision oncology. Oncotarget 11(2): 188-211.
18. Martínez-Sáez O, Chic N, Pascual T, et al. (2020) Frequency and spectrum of PIK3CA somatic mutations in breast cancer. Breast Cancer Research 22(1): 1-9.
19. Dogruluk T, Tsang YH, Espitia M, et al. (2015) Identification of variant-specific functions of PIK3CA by rapid phenotyping of rare mutations. Cancer Research 75(24): 5341-5354.
20. Gymnopoulos M, Elsliger MA, Vogt PK (2007) Rare cancer-specific mutations in PIK3CA show gain of function. Proceedings of the National Academy of Sciences 104(13): 5569-5574.
21. Vasan N, Razavi P, Johnson JL, et al. (2019) Double *PIK3CA* mutations in cis increase oncogenicity and sensitivity to PI3Kα inhibitors. Science 366(6466): 714-723.