Heliyon 10 (2024) e37873

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Development and validation of a multi-marker liquid bead array assay for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations in single circulating tumor cells (CTCs)

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ARTICLE INFO

Keywords:
Liquid biopsy
CTCs
PIK3CA
ESR1
Breast cancer
Single cell analysis
Liquid bead array

ABSTRACT

Background: *PIK3CA* and *ESR1* mutations are associated with progression and therapy resistance in metastatic breast cancer (MBC). CTCs are highly heterogeneous and their analysis at single cell level can provide unique information for mutational profiling and the existence of different subclones related to tumor progression. We have developed a novel multi-marker liquid bead array assay based on combination of an enzymatic mutation enrichment method, multiplex PCR-based assay, and liquid bead array technology for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations in liquid biopsy samples. We focus on single CTCs, however the assay can be used for bulk CTC and ctDNA analysis.

Materials and methods: Single CTCs were isolated from an ER+/HER2+ MBC patient from CellSearch® cartridges using the VyCAP Puncher System and subjected to whole genome amplification followed by nuclease-assisted minor-allele enrichment with probe-overlap (NaME-PrO) enrichment. The assay was validated for analytical sensitivity and specificity for the simultaneous detection of *PIK3CA* (*E545K, E542K, H1047R, H1047L*) and *ESR1* (*Y537S, Y537C, Y537N, D538G, L536H*) mutations in single CTCs, while its clinical performance was evaluated on 22 single CTCs and three single white blood cells (WBCs).

Results: The developed multi-marker liquid bead array assay is novel, highly specific and sensitive for both mutation panels. The assay can reliably detect mutation-allelic-frequencies (MAFs) as low as 0.1 %. The presence of *PIK3CA* and *ESR1* mutations was detected in 13.6 % and 72.7 % of single CTCs, respectively. The developed assay is sample-saving since it requires only 2 μL of amplified DNA to check for nine hotspot *PIK3CA* and *ESR1* mutations in a single cell. The developed liquid bead array assay (Luminex, US), based on a 96 microwell plate format, enables the simultaneous analysis of 96 single cells.

Conclusions: The developed novel multi-marker liquid bead array assay for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations in single CTCs is highly specific, highly sensitive, high-throughput, and sample-, cost-, and time-saving. This multi-marker liquid bead array assay can be extended to detect up to 100 mutations in many genes at once and can be applied for bulk CTC and ctDNA analysis.

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https://doi.org/10.1016/j.heliyon.2024.e37873
Received 20 June 2024; Received in revised form 10 August 2024; Accepted 11 September 2024
Available online 12 September 2024

1. Introduction

During the last ten years, targeted therapies consist a major part of breast cancer therapeutic approaches. Endocrine therapies targeting the estrogen and progesterone receptors have been fundamental in systemic treatment for hormone receptor-positive breast cancer. Additionally, the discovery of HER2 overexpression has spurred the development of multiple HER2-targeted agents which have significantly transformed the treatment of HER2-positive breast cancer [1]. Additional *PIK3CA* and *ESR1* mutations are the most thoroughly studied biomarkers in metastatic breast cancer (MBC), as they are associated with progression and resistance to therapy [2, 3]. *PIK3CA* mutations occur in up to 40 % of HR+/HER2- MBC patients [4] and are mainly located in exon 9 (mostly E545K, E542K) and exon 20 (mostly H1047R) [2]. *ESR1* mutations are a common cause of acquired endocrine resistance in ER+/HER2- MBC patients,

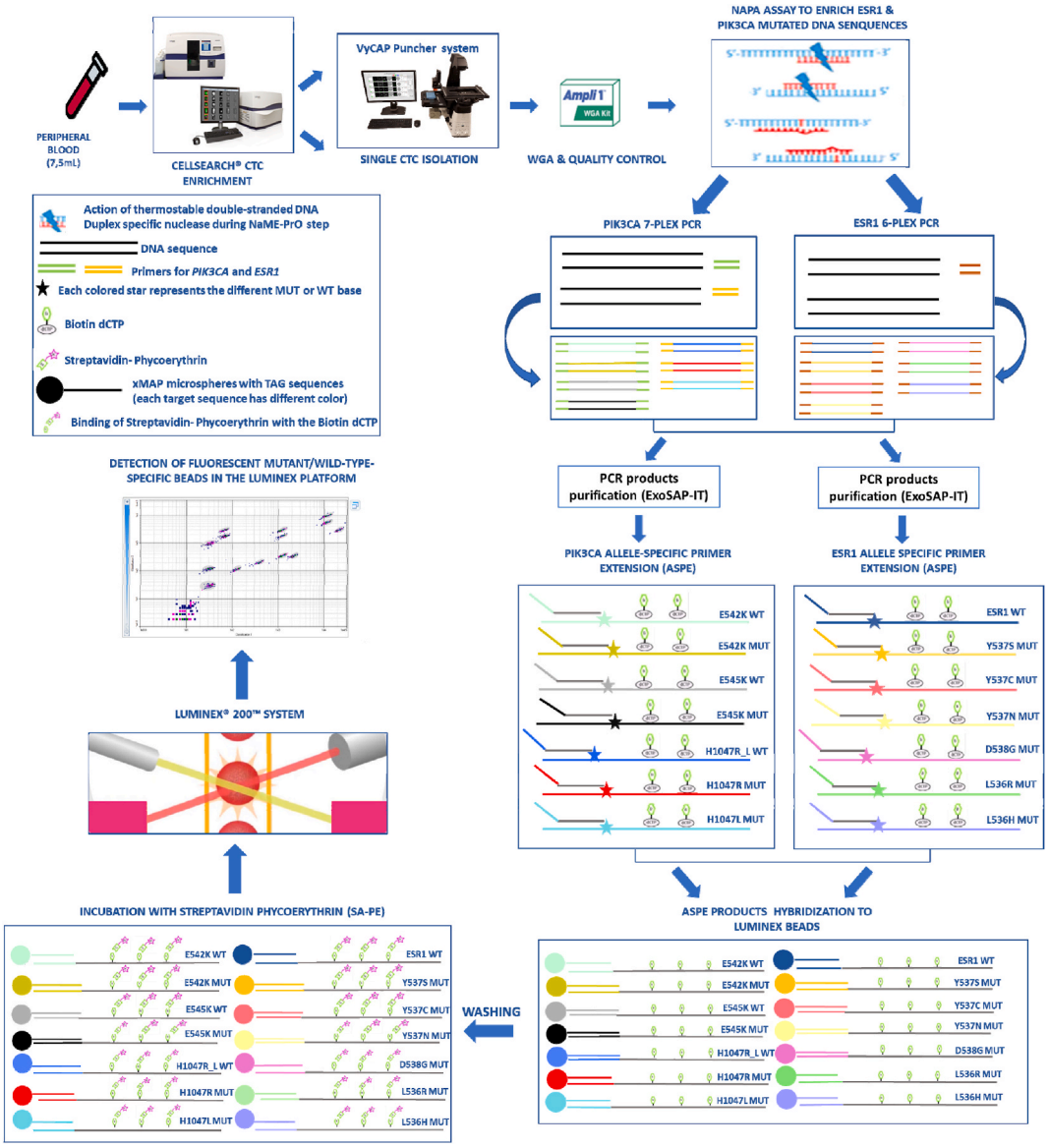


Fig. 1. Schematic outline of the developed multi-marker liquid bead array methodology for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations. Single circulating tumor cells (CTCs) are isolated from the CellSearch® cartridges using the VyCAP Puncher System and subjected to whole genome amplification (WGA). The NaME-PrO step is applied to the amplified DNA samples to enrich *ESR1* and *PIK3CA* mutated DNA sequences and then the nuclease-assisted minor-allele enrichment with probe-overlap (NaME-PrO) products are amplified by two separate multiplex PCR reactions for *PIK3CA* and *ESR1* followed by two separate multiplex Allele Specific Primer Extension (ASPE) reactions for *PIK3CA* and *ESR1* mutation panels. The products are then mixed and hybridized against fluorescent microspheres carrying specific capture probes in-silico designed for each PCR product and incubated with streptavidin–phycoerythrin (SA-PE) and analyzed using the Luminex xMAP system. NAPA: NaME-PrO-assisted ARMS, MUT: mutated, WT: wild-type, 7-plex PCR: multiplex PCR which amplifies seven targets, 6-plex PCR: multiplex PCR which amplifies six targets.

with most of them localized in the ligand-binding domain (most commonly D538G, Y537S, Y537N, Y537C or E380Q) [5,6].

Liquid biopsy (LB) is now a well-established approach to follow-up cancer patients in regular time intervals through minimally invasive analysis in peripheral blood or other biological fluids [7–9]. The clinical utility of LB in breast cancer has been clearly shown in many clinical studies so far [10–16]. Cell-free DNA (cfDNA) and circulating tumor cells (CTCs) are the major LB analytes and their analysis at the DNA mutation, DNA methylation and gene expression level, provides important information at the prognostic and predictive level [17,18].

CTCs are characterized by a high heterogeneity and their understanding at a single-cell resolution reveals unique information that is normally obscured by bulk cell analysis [19]. Single-cell CTC analysis offers a new perspective for understanding the biological process of tumors by revealing in detail tumor heterogeneity [20,21]. Downstream molecular analysis of single CTCs requires first enrichment and single cell isolation. More and more commercially available single-cell isolation and analysis technologies have been developed, the choice of which depends mainly on cell loss, study cost per CTC and workflow complexity [19]. Single-cell genomics allows detection of somatic mutations in each CTC that may not be detectable in a bulk CTC analysis and reveals sub-clonal populations of CTCs with different mutational profiles associated with disease progression and drug resistance [22]. Detection of these clones is critical to understanding tumor progression and its impact on clinical outcome. Several studies have shown the presence of *PIK3CA* and *ESR1* mutations in individual CTCs [23–33].

The detection of mutations in CTCs or plasma-cfDNA requires highly sensitive technologies. This is partly due to the small amount of mutant DNA amidst the excessive amount of wild-type (WT) DNA from normal cells. Thus, assays with high sensitivity and high specificity are required. We have previously shown that the use of PCR-based assays in combination with pre-PCR mutation enrichment methods such as nuclease-assisted minor-allele enrichment with probe-overlap (NaME-PrO) can improve the detection of tumor-related, low-level mutations in blood and increase the frequency of low mutant allele frequences (MAF) mutations, making them detectable by routine diagnostic procedures [34]. In addition, next-generation sequencing (NGS)-based methods allow a comprehensive description of mutation profiles [35]. Our group has developed and validated a variety of PCR-based methods for the detection of *PIK3CA* [36–38] and *ESR1* [39] mutations. However, the main limitation of single PCR-based assays for mutation analysis in LB components is the amount of sample required and the relatively small number of mutations that can be analyzed.

Luminex xMAP technology is based on liquid bead array analysis that enables high-throughput multiplexing in a variety of protein and nucleic acid-based assays. The xMAP technology is based on the use of spectrally defined fluorescent beads that are coated with monoclonal antibodies against specific analytes, or specific DNA oligonucleotides. Using this technology, multiplex quantitative assays such as enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridization assays can be performed saving time and precious clinical sample [39]. Moreover, it is possible to develop customized multiplex assays using this technology [40]. There are a plethora of applications using Luminex xMAP technology, including single nucleotide polymorphism (SNP) genotyping, genetic disease screening, gene expression profiling, human leukocyte antigens (HLA) DNA typing, vaccine development, screening for protein biomarkers for Alzheimer's disease, CTC analysis, and pathogen detection [41–43].

Up to now, there are no highly sensitive and cost-effective methodologies for the simultaneous detection of *ESR1* and *PIK3CA* hotspot mutations in single cells. In this study, we present the development and analytical validation of a novel highly sensitive and specific multi-marker liquid bead array assay for the simultaneous detection of *PIK3CA* (*E545K, E542K, H1047R, H1047L*) and *ESR1* (Y537S*, Y537C, Y537N, D538G, L536H*) mutations in single CTCs. The developed multi-marker liquid bead array assay is based on the combination of a) an enzymatic mutation enrichment method, b) a multiplex PCR-based assay and liquid bead array technology. The developed assay is novel and is characterized by high analytical sensitivity and specificity. The assay requires a minimal amount of sample an important feature for liquid biopsy analysis, is characterized by cost efficiency, and high throughput potential that minimizes analysis time.

2. Material and methods

An outline of the developed assay is presented in Fig. 1.

2.1. OLigonucleotides

The assay is designed for the detection of four hotspot mutations in the *PIK3CA* gene (*E545K, E542K, H1047L and H1047R*) and five hotspot mutations in *ESR1* (*Y537S, Y537C, Y537N, D538G, L536H*) (Fig. 1). The PCR and Allele Specific Primer Extension (ASPE) primers were carefully designed in silico to avoid the formation of stable secondary primer structures (e.g. hairpins, dimers, cross-dimers) and false primer sites. For this purpose, Primer Premier 5.00 software (Premier Biosoft, CA, USA) was used (Supplementary Table 1, Supplementary Table 2). The ASPE primers are in silico designed to target individually all wild-type and mutant alleles. The sequences of all ASPE primers include at their 5′-end additional 24-mer oligonucleotides as "tags" for each individual ASPE primers (Supplementary Table 2). In the same way, all xTAG beads include the reverse complements to these TAGs as anti-TAGs specific for each sequence (Supplementary Table 3) (Luminex Corp., USA).

The analytical validation of the developed assay was performed using specific oligonucleotides for each mutation and WT sequence, as given in Supplementary Table 4. For *PIK3CA* mutations, genomic DNA (gDNA) from MCF7 cell line, T47D cell line and peripheral blood mononuclear cells (PBMCs) from five healthy donors (HD), were used as positive controls for the NM_006218.4:c.1633G > A: E545K, NM_006218.4:c.3140A > G: H1047R, and WT controls respectively. For *ESR1* mutations there were no available cell lines. All oligos, synthetic oligonucleotides for each mutation (gBlocks), and the corresponding synthetic WT oligonucleotides were synthesized by IDT (Coralville, IA, USA).

2.2. Patients and samples

Peripheral blood (PB) samples were collected at four time points (TP#) during the follow-up period (Fig. 2) [13] of an ER+/HER2- MBC patient. This patient was a 52-year-old premenopausal woman who was diagnosed in 2010 with ER + breast cancer. After five years of initial diagnosis, liver metastasis was confirmed. In May 2020, the patient was diagnosed with carcinomatous meningitis and after a few months she died [13]. A signed informed consent form was obtained from the patient to participate in the study. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Medical Ethical Committee of the General Hospital of Heraklion, Crete, Greece (Ethical Allowance: 8756/23-6-2014).

DNA extraction from PBMCs isolated from peripheral blood of 5 HDs was performed using TRIZOL-LS according to the manufacturer's instructions (Thermo Fisher Scientific, USA).

2.3. Single CTC isolation

7.5 ml of peripheral blood sample was collected and were processed in the CellSearch® System (Menarini, Silicon Biosystems S.p.A.) for CTC enumeration according to the manufacturer's recommendations. Following CellSearch analysis, single cells were isolated from each CellSearch® cartridge using the VyCAP Puncher System (VyCAP B.V., Enschede, Netherlands) according to the company's protocol. Before the isolation of the single cells using the Puncher System in order to be sure for the single CTC recovery, the microchip with the trapped single cells in its wells was imaged at four fluorescence channels (DAPI, FITC, PE and APC) using a fluorescence microscope. All immunofluorescent signals were analyzed automatically, and wells which contain a WBC ($DAPI^+/CK^-/CD45^+$) or a CTC ($DAPI^+/CK^+/CD45^-$) were pre-selected automatically and the user finally selected the cells for punching after verification.

2.4. Whole genome amplification (WGA) of single CTCs

All single cells obtained from the four different time points (TP#1: 76mo, TP#2: 103mo, TP#3: 108mo, TP#4: 118mo) were subjected to WGA using the Ampli1™ WGA kit (Menarini Silicon Biosystems S.p.A) according to the manufacturer's instructions. The quality of the amplified DNA was tested using the VyCAPQC-Mix (VyCAP BV, Enschede, The Netherlands) together with the QIAGEN Multiplex PCR Plus Kit Mastermix (Qiagen, Germany) according to the VyCAP protocol and by amplification of a WT region in exon 20 of the *PIK3CA* gene [44]. The amplified DNA samples were stored at −20 °C until further analysis. From the total number of the recovered single cells with good quality at the four different time points we selected 22 single CTCs and three WBCs for the downstream analysis.

2.5. NaME-PrO step

The NaME-PrO step was applied to the amplified DNA samples (diluted 1:10), as previously described [37,39] using the NaME-PrO oligonucleotide probes specifically designed for the *ESR1* and *PIK3CA* WT target regions, to eliminate the WT DNA sequences against the mutant DNA targets [37,39]. The NaME-PrO products were placed on ice or were stored at −80°C till the subsequentanalysis steps.

2.6. Multiplex PCR

PIK3CA and *ESR1* multiplex PCR amplification was performed with 2 μl NaME-PrO product in a final volume of 25 μl. The reaction consisted of 12.5 μl Master Mix, 2.5 μl Q Solution (Multiplex PCR Kit, Qiagen, Germany), 0.08μM of each primer (Suppl.Table S1). PCR was performed in a Mastercycler® epgradient (Eppendorf, Germany). The final PCR conditions were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 30 s. Samples were then stored at 4 °C until use. Each run included a PCR negative control containing no target, a WT sample (single WBC) and a positive control.

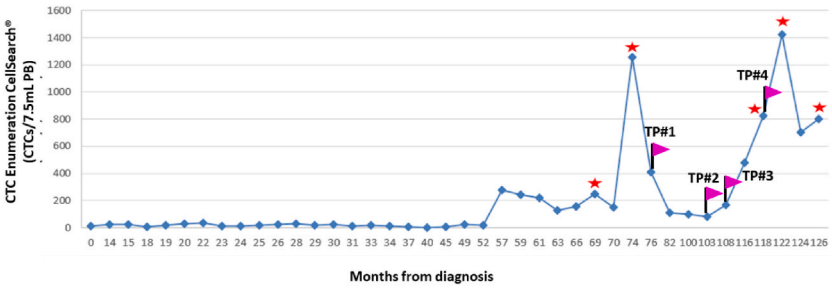


Fig. 2. Time points for mutation analysis of single CTCs during the timeline of the patient's disease in relation to CTC enumeration (CTCs/7.5 mL PB) based on CellSearch® (red stars represent the time of progression disease, purple flags represent the four different timepoints (TP#) of analysis TP#1: 76 months (mo), TP#2: 103mon, TP#3: 108mon, TP#4: 118mon).

2.7. ExoSAP-IT treatment

To remove the unincorporated PCR primers and dNTPs, 2 μL of ExoSAP-IT (Applied Biosystems, ThermoFisher Scientific, USA) was added to 5 μl of each multiplex PCR product and mixed. The mixture was then incubated at 37 °C for 20 min, followed by 15 min at 80 °C to inactivate the enzymes.

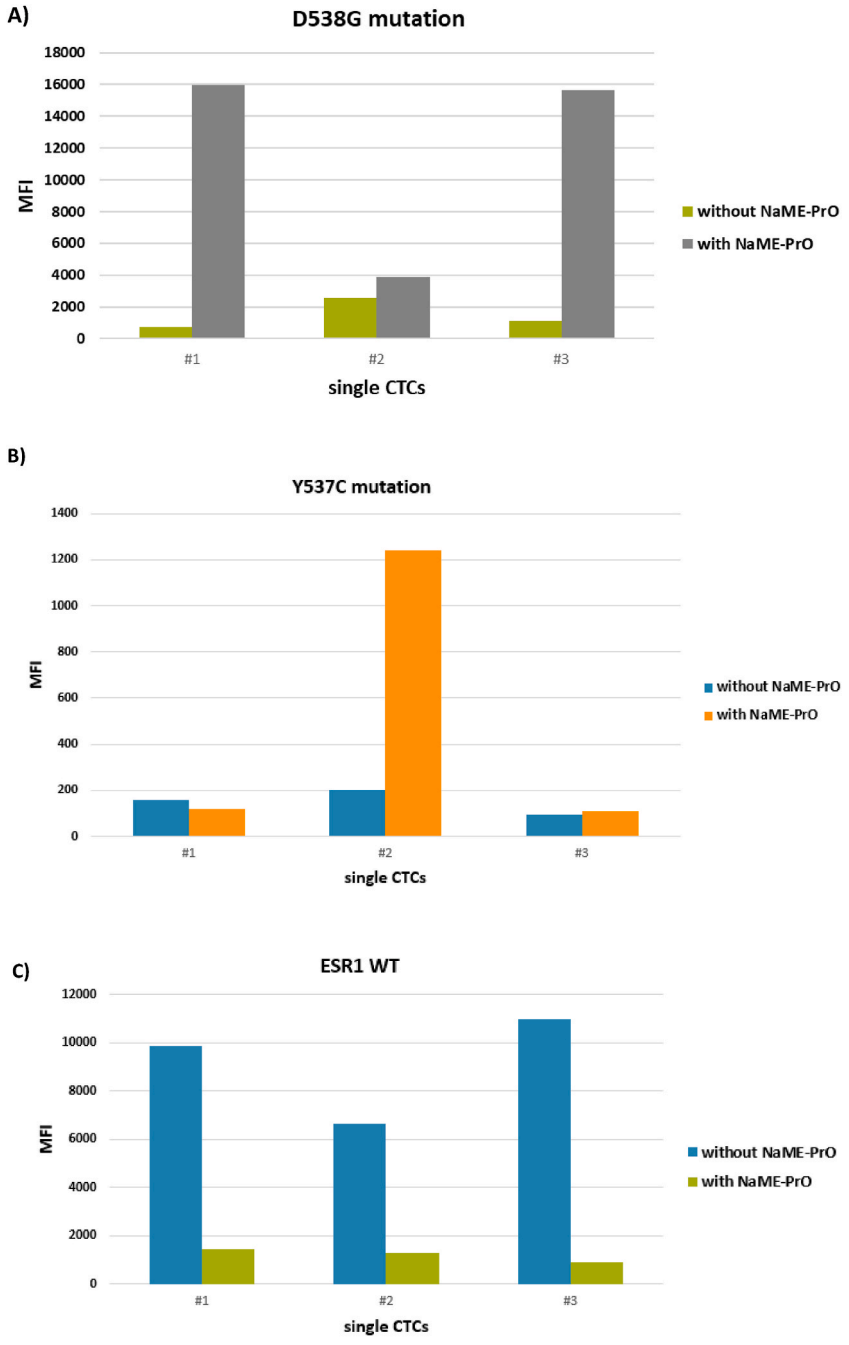


Fig. 3. MFI signals of the coupled microspheres corresponded to A) D538G, B) Y537C as well the C) ESR1 WT sequences with and without the NaME-PrO step for three single CTCs. Without the NaME-PrO enrichment A), B) the MFI signal of the coupled microspheres corresponding to the D538G and Y537C mutations (olive-green and blue bars, respectively) were remarkably low as well C) the MFI of the coupled microspheres corresponding to the WT sequences were significantly high (blue bars) compared to the same CTCs analyzed involving the NaME-PrO step.

2.8. ASPE

The ASPE reaction used here follows the protocol recommended by Luminex (http://www.luminexcorp.com/support/protocols/xtag_protocols.html).Briefly, each 20 μL multiplex ASPE mixture reaction contained 5 μL or 3 μl of Exo-SAP-treated multiplex PCR product of *PIK3CA* products or *ESR1* products respectively, 0.0375 U/μl of *Tsp* DNA polymerase (Invitrogen, USA), 375 nM ASPE primer mixture for all each mutation (Suppl.Table S2), 5μM dATP/dTTP/dGTP and biotin-dCTP (Invitrogen, USA), 1.25 mM $MgCl_2$ and 1xPCR buffer included with the Platinum Tsp enzyme (Invitrogen, USA). Thermocycling was performed for *PIK3CA* at 95 °C for 3min, 30 cycles of 94 °C for 30s, 60 °C for 1min and 72 °C for 30s, and for *ESR1* at 95 °C for 3min, 30 cycles of 94 °C for 30s, 67 °C for 1min and 72 °C for 30s. ASPE reactions were performed separately for the *PIK3CA* and *ESR1*. Samples were kept at 4 °C until use. In each run, a negative control of the ASPE reaction was added.

2.9. Coupling of capture probes with the carboxylated beads

Coupling of capture probes with the carboxylated beads Suppl.Table S3) was performed as previously described [41,45,46]. The capture probe-bead conjugates were stored at 4°C in the dark and for each run a fresh microsphere mix containing all the conjugates was prepared.

2.10. HYbridization

A microsphere mixture containing 2500 Luminex xTAG beads from each of 13 bead sets was prepared. The microsphere mixture was centrifuged at 11,600 rpm for 2min. The supernatant was removed, and the pellet was resuspended to 100 of each microsphere set per μl in 2X Tm Hybridization buffer (0,4M NaCl, 0,2M Tris pH 8,0, 0,16 % Tween-20, dH_2O). Then, 25 μL of the bead mixture was added per well with 5 μL of ASPE products, followed by 20 μL of dH2O for a total volume of 50 μL. The products and bead mixture were denatured at 96 °C for 90s followed by hybridization at 37 °C for 60min. The coupled microspheres were centrifuged at 5800 rpm for 3min, and the supernatant was removed. The pellet of coupled microspheres was then resuspended in 75 μl of 1X Tm hybridization buffer (1:2 dilution of 2X Tm). The washing step was repeated a total of two times. For each sample, microspheres were resuspended in 75 μl reporter solution (1X Tm hybridization buffer with 2 μg/ml streptavidin-conjugated phycoerythrin) and incubated at 37 °C for 15min. Hybridization of the *PIK3CA* and *ESR1* mutation panels was performed separately.

2.11. Bead analysis

We used 96-well microliter plates to resuspend the coupled microspheres and then analyzed all samples in a Luminex® 200 instrument (Luminex Corporation, USA). The sample volume was set at 50 μl, and the flow rate was 60 μl/min. The instrument settings were set to read a minimum of 100 events per bead population, mean fluorescence intensities (MFIs) were computed, and analysis was completed in less than 60s for each sample.

3. Results

3.1. Optimization of the assay conditions

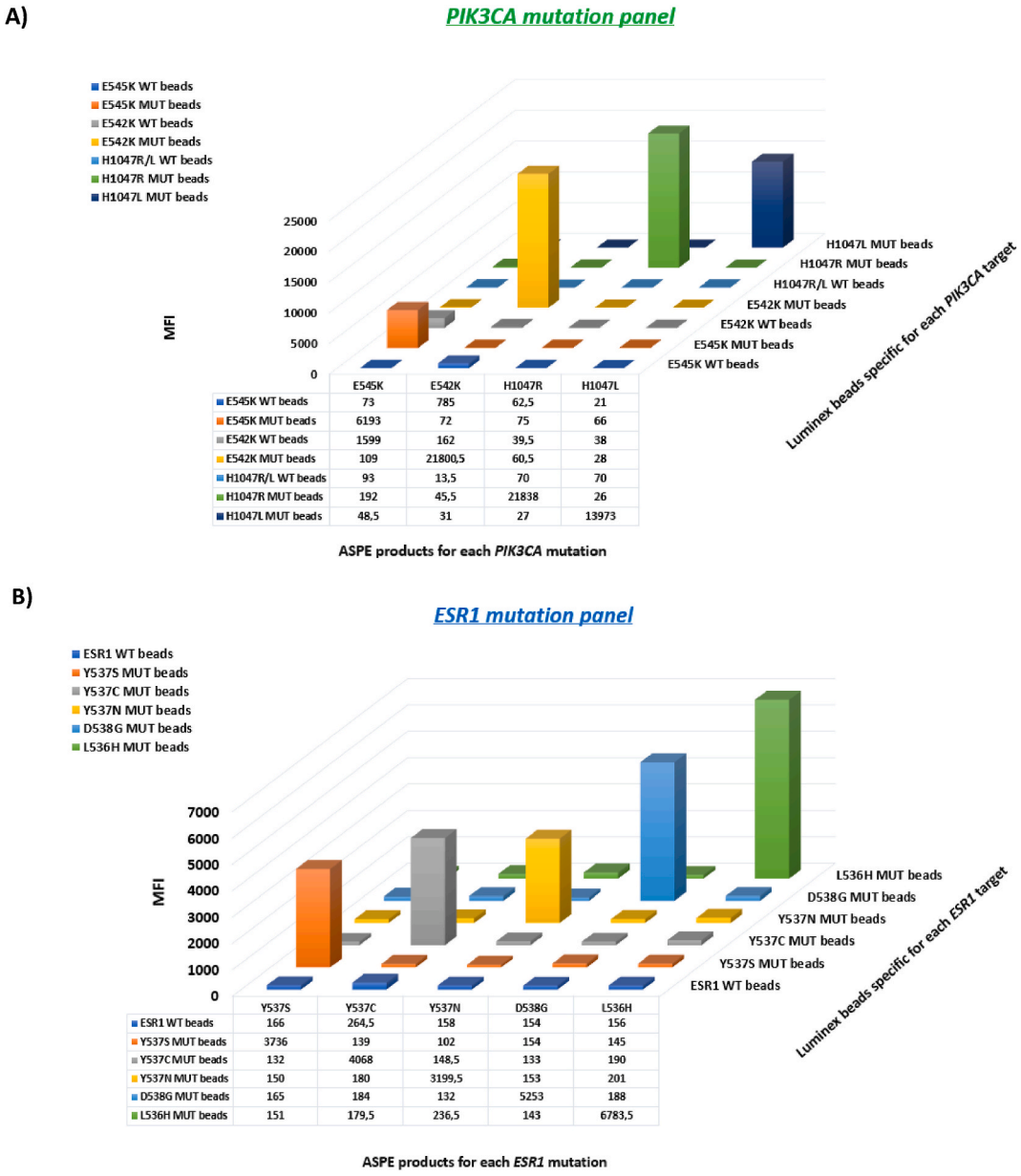
For the assay optimization, MCF7 and T47D cell lines were used as positive controls for the E545K and H1047R mutations respectively, and synthetic oligonucleotides for each mutation of *PIK3CA* and *ESR1* and the corresponding synthetic WT oligonucleotides and WT controls (PBMCs from healthy donors and single WBCs. Multiplex PCR and the ASPE experimental conditions were first optimized; the temperature programs, the number of cycles, the concentration of primers and ASPE sequences, the dNTPs and the $MgCl_2$ concentration as well as the volume of ExoSAP-IT treated PCR product used in the ASPE reaction (Supplementary Figs. 1 and 2). Duration of the hybridization step was also optimized (Supplementary Fig. 2).

We evaluated whether the inclusion of the NaME-PrO step would improve the sensitivity of our assay. Towards this, we first analyzed three single CTCs without the NaME-PrO step. According to our results, the MFI of the coupled microspheres corresponding to the WT sequences were significantly high, while the signals for the D538G mutation were remarkably low when compared to the same CTCs analyzed involving the NaME-PrO step. More specifically, using NaME-PrO enrichment, a significant decrease in the MFI signals of the coupled microspheres corresponding to the WT sequences was observed in comparison to the same samples analyzed without prior NaME-PrO enrichment (Fig. 3). The MFI signal of the coupled microspheres corresponding to the D538G mutation was also remarkably increased. Following the same procedure for the detection of Y537C mutation, it is important to note that one of the three individual CTCs was positive for the Y537C mutation only after NaME-PrO enrichment (Fig. 3). In Fig. 3 it is clear that the three single CTC tested are different in terms of *ESR1* mutations. CTC #1 and CTC #3 are positive for D538G, while CTC #2 is positive only for Y537C. This information is evident only after NaME-PrO step, and this is the justification of this step in the assay.

3.2. Analytical validation of the developed assay

3.2.1. Analytical specificity

The analytical specificity of the developed assay was evaluated by using synthetic oligonucleotides for E545K, E542K, H1047R and H1047L hotspot mutations of *PIK3CA* (*PIK3CA* panel) and for Y537S, Y537C, Y537N, D538G and L536H hotspot mutations of *ESR1* (*ESR1* panel). We estimated the analytical specificity for the *PIK3CA* and *ESR1* mutation panels separately. The ASPE products of multiplex PCR reactions performed in the presence of all ASPE sequences were hybridized in the presence of all six microsphere sets for the *PIK3CA* panel and seven microsphere sets for the *ESR1* panel, with capture probes ('anti-TAG' sequences) coupled to all microsphere sets. The assay was highly specific for both *PIK3CA* and *ESR1* panels, as we could detect each individual mutation in the presence of all oligos (Fig. 4A).

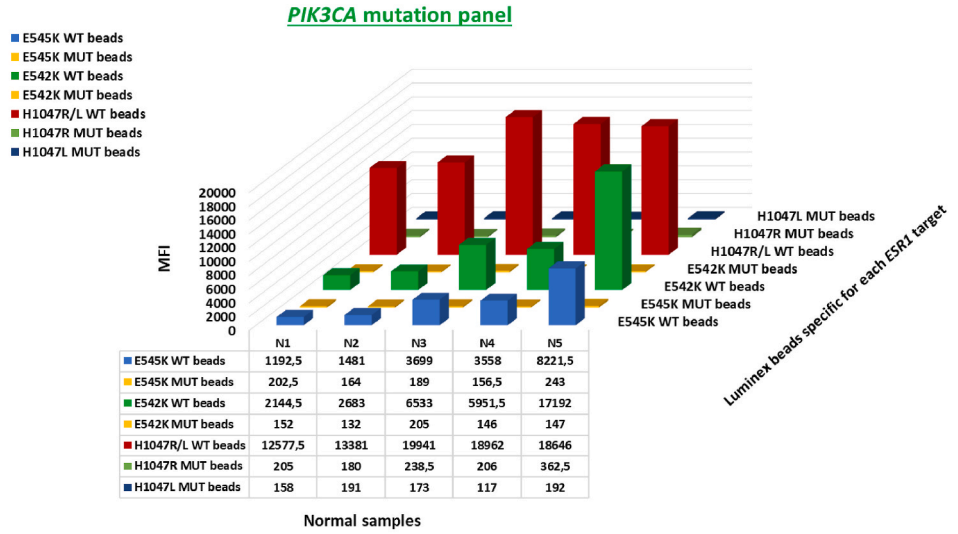


	E545K	E542K	H1047R	H1047L
E545K WT beads	73	785	62,5	21
E545K MUT beads	6193	72	75	66
E542K WT beads	1599	162	39,5	38
E542K MUT beads	109	21800,5	60,5	28
H1047R/L WT beads	93	13,5	70	70
H1047R MUT beads	192	45,5	21838	26
H1047L MUT beads	48,5	31	27	13973

	Y537S	Y537C	Y537N	D538G	L536H
ESR1 WT beads	166	264,5	158	154	156
Y537S MUT beads	3736	139	102	154	145
Y537C MUT beads	132	4068	148,5	133	190
Y537N MUT beads	150	180	3199,5	153	201
D538G MUT beads	165	184	132	5253	188
L536H MUT beads	151	179,5	236,5	143	6783,5

Fig. 4. A), B) Analytical specificity: each individual mutation was detected in the presence of all oligos for both *PIK3CA* and *ESR1* panels and C), D) Clinical specificity: using DNA samples isolated from PBMCs of 5 HDs, MFI signals were detected only for the microsphere sets which corresponded to the WT sequences of the target regions of both genes for the *PIK3CA* mutation panel and *ESR1* mutation panel of the developed multiplex liquid bead array methodology. The tables on each diagram contain the MFI signals for each microsphere set.

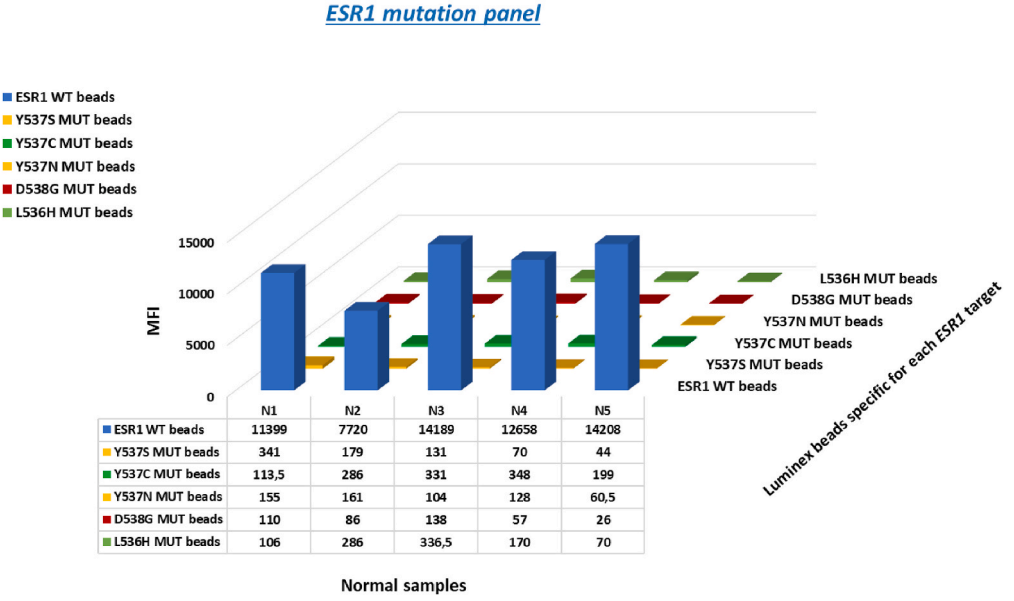
C)



	N1	N2	N3	N4	N5
E545K WT beads	1192,5	1481	3699	3558	8221,5
E545K MUT beads	202,5	164	189	156,5	243
E542K WT beads	2144,5	2683	6533	5951,5	17192
E542K MUT beads	152	132	205	146	147
H1047R/L WT beads	12577,5	13381	19941	18962	18646
H1047R MUT beads	205	180	238,5	206	362,5
H1047L MUT beads	158	191	173	117	192

Normal samples

D)



	N1	N2	N3	N4	N5
ESR1 WT beads	11399	7720	14189	12658	14208
Y537S MUT beads	341	179	131	70	44
Y537C MUT beads	113,5	286	331	348	199
Y537N MUT beads	155	161	104	128	60,5
D538G MUT beads	110	86	138	57	26
L536H MUT beads	106	286	336,5	170	70

Normal samples

Fig. 4. (*continued*).

The specificity of the assay was also evaluated using five gDNA samples extracted from PBMCs of HDs. MFI signals were detected only for the microsphere sets which corresponded to the WT sequences of the mutated target regions of *PIK3CA* and *ESR1* (Fig. 4B).

3.2.2. Analytical sensitivity

The analytical sensitivity of the *PIK3CA* panel was determined by using serial dilutions of gDNA from the MCF7 and T47D cell lines for E545K and H1047R mutations respectively in WT gDNA at ratios of 10 %, 1 %, 0.5 %, 0.1 % and 0 % (WT) of mutant DNA sample. The analytical sensitivity of the *ESR1* panel was estimated by serial dilutions of synthetic oligonucleotide sequences containing each of the five *ESR1* mutations with synthetic WT oligonucleotide sequences at ratios of 5 %, 1 %, 0.5 %, 0.1 % and 0 % (WT). All experiments to estimate the analytical sensitivity of the assay were performed in triplicate. Based on MFI signals of different mutant ratios and comparing them with the corresponding signals of the WT (0 %), the limit of detection (LOD) for E545K was 0.5 % and for H1047R was 0.1 % (Fig. 5A). Similarly, the LOD for *ESR1* mutations was 0.1 % for Y537S, Y537N and L536H mutations and 0.5 % for Y537C and D538G (Fig. 5B).

3.2.3. Inter-assay precision

Inter-assay precision of the Multiplex Liquid Bead Array Assay for the two mutation panels expressed as CV% for MFI values, at two

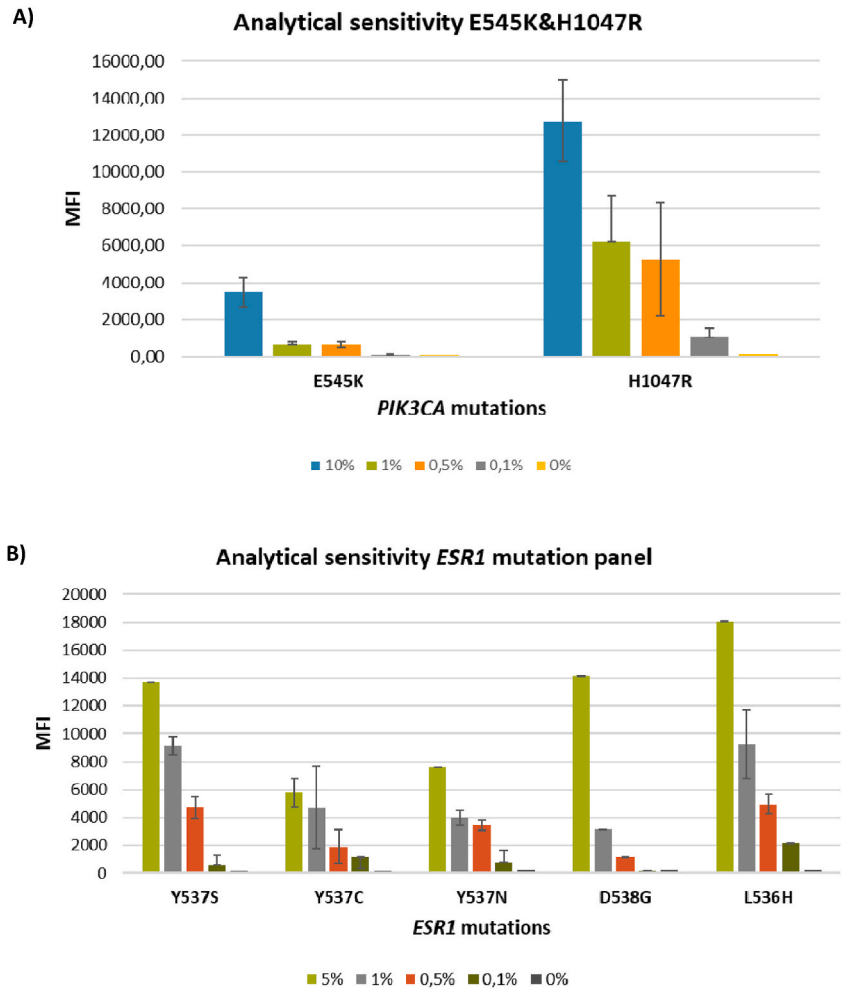


Fig. 5. Analytical sensitivity of the developed multiplex liquid bead array methodology A) for E545K and H1047R *PIK3CA* mutations as well B) for *ESR1* mutation panel. Based on the MFI signals, the developed assay could reliably detect for E545K up to 0.5 % (mean MFI signal 627.83) of mutant DNA sample from MCF7 cell line and for H1047R up to 0.1 % (mean MFI signal 1046.67) of mutant DNA sample of T47D. As for *ESR1* mutation panel, based on the MFI signals the assay could reliably detect up to 0.1 % of dilution of synthetic mutant oligonucleotide sequences with synthetic WT oligonucleotide sequences for Y537S (mean MFI signal 585.25), Y537N (mean MFI signal 786.00) and L536H (mean MFI signal 2122) mutations and up to 0.5 % of dilution of synthetic mutant oligonucleotide sequences with synthetic WT oligonucleotide sequences for Y537C (mean MFI signal 223.00) and D538G (mean MFI signal 137.00).

		Single CTCs																					
Mutation analysis		TP#1	TP#2	TP#3				TP#4															
		#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22
PIK3CA mutations	E545K	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	●	▲	▲	▲	▲	▲	▲	▲	▲
	E542K	▲	▲	▲	▲	▲	▲	▲	▲	▲	●	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
	H1047R	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
	H1047L	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
ESR1 mutations	Y537S	▲	▲	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	●	▲	▲	▲	●	▲	▲	▲	▲	▲
	Y537C	▲	▲	▲	▲	▲	●	▲	▲	▲	●	▲	▲	▲	▲	▲	▲	▲	▲	▲	●	●	●
	Y537N	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
	D538G	▲	●	●	▲	●	●	●	▲	●	▲	▲	●	●	●	●	▲	●	▲	●	▲	▲	●
	L536H	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲

● positive
▲ negative

Fig. 6. *PIK3CA* and *ESR1* mutations in single CTCs at four different time points (TP#) of analysis (TP#1: 76mon, TP#2: 103mon, TP#3: 108mon, TP#4: 118mon) using the developed multiplex bead array assay. A positive sample for a mutation is presented with reddish-purple circle and a negative sample is presented with green triangle.

different concentration levels (1 % and 10 %) ranged between 3.7 % and 14.9 %, and is shown in the Supplementary data, as Supplementary Table 5.

3.3. Simultaneous detection of PIK3CA and ESR1 hotspot mutations in single CTCs

We applied the developed liquid bead array assay in 22 single CTCs and 3 single WBCs of an ER+/HER2- MBC patient that were isolated from CellSearch® cartridges using the VyCAP Puncher system. More specifically, we analyzed 1 single CTC from TP#1: 76mo, 1 single CTC from TP#2: 103mo, 4 single CTCs from, TP#3: 108mo, and 16 single CTCs from TP#4: 118mo. First, we analyzed three single WBCs to estimate the specificity of the developed assay for both hotspot mutation panels. MFI signals of the coupled microspheres corresponded to WT.

As is shown in Fig. 6, in TP#1, no mutations were detected in one single cell studied, while at TP#2 D538G was detected. This mutation was detected at all timepoints thereafter, TP#3, TP#4, indicating the presence of this clone in the CTCs. Moreover, at TP#3, Y537S and Y537N mutations were also detected in CTCs, and the same mutations were also detected in TP#4. *PIK3CA* hotspot mutations E545K, E542K, H1047R were detected only at TP#4. In total, the *PIK3CA* E545K and H1047R mutations were detected in 1/22 (4.5 %) and E542K mutation in 2/22 (9.1 %). Both E542K and H1047R mutations were detected in one CTC.*ESR1* mutations were detected in 16/22 (72.7 %) of the single CTCs. More specifically, the Y537S was detected in 3/22 (13.6 %), the Y537C in 5/22 (22.7 %) and the D538G mutation was detected in 16/22 (59.1 %) of the single CTCs. Five out of 22 single CTCs were positive for two *ESR1* mutations. It should be mentioned that *PIK3CA* and *ESR1* mutations were detected in three single CTCs. We have already reported the presence of *PIK3CA* hotspot mutations (E545K and H1047R) and *ESR1* mutations (D538G, Y537C, Y537S, Y537N) in this patient, not only in CTC (bulk analysis), but also in plasma-cfDNA [11].

4. Discussion

New therapies such as PI3K inhibitors (alpelisib) and selective estrogen receptor degraders (elacestrant) based on the mutation profile of ER+/HER2+ breast cancer patients are currently entering clinical practice. Detection of *PIK3CA* mutations is now in routine clinical practice as the U.S. Food and Drug Administration (FDA) has approved alpelisib in combination with fulvestrant as a therapy for patients with advanced or metastatic hormone-positive, HER2-negative breast cancer [47]. Recently, the FDA approved elacestrant as targeted therapy for ER-positive, HER2-negative, *ESR1*-mutated advanced or metastatic breast cancer with disease progression after at least one line of endocrine therapy using the Guardant360 CDx assay as a companion diagnostic [48].

The detection of *PIK3CA* and *ESR1* hotspot mutations may help to improve our understanding of therapy resistance and clonal evolution in breast cancer patients. For the detection of these mutations, especially in liquid biopsy samples, the development of multiplex highly sensitive and specific assays is very important. Up to now there are studies focused either on the detection of *PIK3CA* mutations in single cells, mainly based on classic PCR-based methods like Specific-To-Allele PCR in single cells in intact archived tissues [32], Sanger cycle sequencing [33], or *ESR1* mutations using NGS [29]. However, there are no methodologies for the simultaneous detection of *ESR1* and *PIK3CA* hotspot mutations in single cells.

In the present study, we developed and analytically validated a novel liquid bead array assay for the simultaneous detection of nine *PIK3CA* and *ESR1* hotspot mutations (E545K, E542K, H1047R, H1047L, Y537S, Y537C, Y537N, D538G, L536H) based on the combination of a) mutation enrichment method, b) multiplex PCR and ASPE with liquid bead array technology, requiring a very small amount of sample. In this study we have chosen to analyze single CTC from one single patient, for whom we have all the clinical information [13], however, the methodology presented can be used further for single CTC mutation analysis in many samples. The developed test is sample-saving since it requires only 2 μL of amplified DNA to check for nine hotspot *PIK3CA* and *ESR1* mutations in a single cell. The Luminex technology used, is based on a 96 microwell plate format, enabling the simultaneous analysis of 96 clinical samples, eg material from 96 single cells using the developed assay. Moreover, the Liquid Bead array technology has the capacity to extend the number of mutations tested up to 100 in this instrumentation (LUMINEX 200) or 500 in latest technology instruments.

The developed liquid bead array assay is characterized by a high sensitivity and specificity for all individual mutations. Due to its high sensitivity and specificity, it can be used as a cost-effective, time- and sample-saving assay, for the simultaneous detection of hotspot *PIK3CA* and *ESR1* mutations in all types of liquid biopsy samples such as single CTCs, bulk CTCs and plasma cfDNA. LODs reported here of 0.1 % and 0.5 % are already very low, even in terms of digital PCR. These variations in LODs could be due to sampling errors (Poisson effect), since precision is always worse at very low concentrations. Concerning specificity, it is well known that WGA kits have several limitations depending on the underlying method, including allele dropout (ADO). ADO is a common phenomenon in WGA kits that affects PCR efficiency and may lead to false positive results. This phenomenon also occurs in PCR-based targeted sequencing. To verify the specificity of our method, before analyzing the individual CTCs, we analyzed single WBCs showing a normal genotype. As shown in our results, no mutations were detected in the five single white blood cells analyzed.

We wanted to show the potential of this methodology for single CTC analysis and especially its ability to show differences in single CTCs in the same patient during time. To achieve this, we had analyzed available stored enriched-CTC samples through CellSearch System and particularly from ER+/HER2-metastatic breast cancer patients. We prefer not to analyze bulk CTC or ctDNA samples since this can be the goal of a future clinical study using a lot of samples very well documented. Our choice to evaluate the performance of this assay in single CTCs was not random, as we wanted to show that the developed assay can reliably detect the mutations at the single cell level. According to our results, the developed liquid bead array assay can successfully detect *PIK3CA* and *ESR1* mutations at the single cell level. The addition of the NaME-PrO step prior to multiplex PCR for the *PIK3CA* and *ESR1* target regions was crucial, as we observed that the addition of NaME-PrO significantly decreased the MFI signal of the coupled microspheres corresponding to the WT

sequences, while the MFI signal of the coupled microspheres corresponding to the mutant sequences was enhanced.

Using the developed liquid bead array assay, the presence of *PIK3CA* mutations was detected in 13.6 % of analyzed single CTCs. More specifically, the E545K mutation was detected in 4.5 % of the single CTCs, as was the H1047R mutation. The E542K mutation was detected in 9.1 % of single CTCs. Longitudinal analysis of bulk CTCs and cfDNA samples during the follow-up period of this patient has shown the presence of E545K and H1047R mutations [13]. The results of this analysis have shown a high heterogeneity between CTCs from the same patient at different time points and that not all single cells carried *PIK3CA* mutations. These results are consistent with those of the study by Pestrin et al. in which heterogeneity between single CTCs was found in two patients with regard to the detection of *PIK3CA* mutations [24].

We did not detect any mutation in a single CTC at TP#1, (76 months after diagnosis). However, D538G was detected in one single CTC at TP#2, (103 months after diagnosis). At TP#3, (108 months after diagnosis), Y537S, Y537C and D538G were detected in 3/4 single CTCs tested. At TP#4 (118 months after diagnosis), the vast majority of single CTCs tested were found positive for *ESR1* mutations (Y537S, Y537C and D538G). At the same timepoint, *PIK3CA* (E545K, E542K and H1047R) mutations were also detected in three single CTCs while none of the mutations were detected in other three single CTCs. These results showed the presence of different CTC subpopulations not only at different timepoints but also at the same timepoint. *PIK3CA* mutations were detected 118 months since diagnosis in three single CTCs when most of the single CTCs were positive for *ESR1* mutations. At this timepoint, the patient showed a progression disease and after eight months the patient received Alpelisib based on the detection of *PIK3CA* mutations in ctDNA and CTCs [13]. Our results highlight the importance of single cell analysisto detect tumor mutation heterogeneity.

In addition, 72.7 % of single CTCs were found positive for *ESR1* mutations, the vast majority of which were Y537C (22.7 %) and D538G (59.1 %). These results demonstrate the heterogeneity of single CTCs for the detection of *ESR1* mutations, while data from the analysis of bulk CTCs and cfDNA samples at different time points during the patient's follow-up period showed the presence of the specific mutations at the time she received endocrine therapy and the disease progressed [13]. More than one *ESR1* mutation was detected in five single CTCs, which is consistent with the study by Paolillo et al. in which two *ESR1* mutations were detected in a single CTC of an ER + metastatic breast cancer patient [30]. The presence of *PIK3CA* and *ESR1* mutations in single CTCs in one patient demonstrates the existence of heterogeneity within the same patient due to the presence of different subclonal populations. In addition, positive *PIK3CA* and *ESR1* mutations were found in three individual CTCs, which is consistent with the results of the study by Bingham et al. [27].

This is the first time that the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations is reported in single CTCs using combination of mutation enrichment method, multiplex PCR and ASPE with liquid bead array technology. Most methods use the Luminex Technology System with the ASPE step for the detection of SNPs. Lang et al. have developed a bead-based multiplex assay using the ASPE step and the Luminex xMAP system for genotyping CDKN2A/CDK4 variants in melanoma-prone families [49]. There are also studies on the simultaneous detection of multiple mutations of different genes with ready-to-use kits using Luminex technology in tissue samples that do not contain the ASPE step for amplification of mutated sequences [50–52]. In comparison to the methods described so far, we could mention as strengths of the method the multiplex format, the use of a minute amount of sample, and the high sensitivity, due to the inclusion of the NaME-PrO step, while as limitations we could mention that the method needs more hands-on time and includes more steps. In this study we focused on the development of the Luminex bead array assay for the simultaneous detection of *PIK3CA* and *ESR1* mutations in single CTCs. The assay can be used downstream of any isolation system for single CTCs. We used the VyCAP system, and we decided to analyze only CTCs that passed the quality control in terms of the molecular tests that we used, even if more CTCs were recovered at each time point. It is not correct to mention that only a single CTC was recovered, it was a single CTC analyzed. The method can reveal heterogeneity if more CTCs are analyzed.

The entire process of the developed assay is up to now not fully automated. Single cell isolation can be performed in specific instrumentation dedicated to CTCs. All the other steps like WGA step quality control of the products, NaME-PrO step, multiplex PCR, ExoSAP-IT treatment and ASPE reaction require hands on work in the lab. The liquid bead array analysis and consequently the computation of MFI signals is fully automated, using the Luminex 200 system.

In summary, we developed and validated a novel multiplex assay based on the combination of multiplex PCR and ASPE with liquid bead array technology for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations (E545K, E542K, H1047R, H1047L, Y537S, Y537C, Y537N, D538G, L536H). Our main goal was to demonstrate the potential of the liquid bead array approach for the simultaneous detection of mutations in different genes in single CTCs.

5. Conclusions

In this study, we present for the first time a novel, reliable, highly specific and sensitive multi-marker liquid bead array assay for the simultaneous detection of *PIK3CA* and *ESR1* hotspot mutations in single CTCs. This assay is high-throughput, sample-, cost-, and time-saving, and can be further expanded to include additional gene mutation panels by performing the analysis independently and selecting the panels of interest. The assay can also be used for the detection of hotspot mutations in bulk CTCs and plasma-cfDNA samples. Our next goal is to extend the capacity of the Liquid Bead array technology by increasing the number of mutations tested up to 100 in the near future.

Funding

This project was supported by the Stavros Niarchos Foundation within the framework of a grant to Evi Lianidou and the National and Kapodistrian University of Athens, grant number 16785. Part of this work was supported by Luminex Corporation.

CRediT authorship contribution statement

Dimitra Stergiopoulou: Writing – review & editing, Writing – original draft, Validation, Methodology. **Vassilis Georgoulias:** Resources. **Athina Markou:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Conceptualization. **Evi Lianidou:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to acknowledge the important support of this project by the Stavros Niarchos Foundation. We would like to thank Luminex Corporation and in particular Dr. Sheryl Dunbar for providing part of Luminex reagents and support in conducting this study. We would like to thank Prof. L. Alexopoulos (National Technical University of Athens) for kindly providing technical support. We would like to express our deep thanks to the late breast cancer patient who donated her peripheral blood for this molecular analysis at different time points, in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37873.

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