Evaluation of a Mass Spectrometry-Based PIK3CA Mutation Assay for Predictive Breast Cancer Therapeutic Decision Making

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Introduction

Alterations of PI-3 kinases (PIK3) in cancer represent a novel drug-targetable biomarker for multiple solid tumors. One such drug, alpelisib, in combination with fulvestrant, has been found to increase clinical response to treatment and prolong progression-free interval in advanced breast cancer patients, whose tumours exhibit putative gain-of-function mutations in the major PI3K gene, *PIK3CA*. 14 different PIK3CA mutations were evaluated in the SOLAR1 clinical trial exploring the treatment of breast cancer with alpelisib/fulvestrant; C420R, E542K, E545A, E545D, E545G, E545K, E545X, Q546E, Q546R, Q546X, H1047L, H1047R, H1047X, H1047Y. Identification of these variants is necessary for the selection of patients who might benefit from alpelisib/fulvestrant therapy. We describe the development and validation of a simple, highly sensitive assay for the identification of potential alpelisib-responsive cases of primary breast cancer.

Methods and Materials

Tumour Formalin Fixed Paraffin Embedded (FFPE) DNA extraction and Control samples

Full microtome sections from 61 archival FFPE tissue samples with known PIK3CA mutational status were deparaffinized and DNA was extracted using the QIAmp DNA FFPE kit (Qiagen) and processed using a Qiacube extractor. Unless otherwise stated, 10 ng of genomic DNA was used in each subsequent analysis. FFPE samples of normal incidental lymph node were used as a negative control. For LOD determination (VAF and input DNA) and to assess rare PIK3CA mutations not represented in available tissue specimens, the following commercial controls were used: Seraseq Tumor Mutaion DNA Mix v2 AF2 (Sercare #0710-0203), Oncoref Genomic DNA Reference Standard PIK3CA p.E545A/p.E545G/p.C420R/p.E546K (AccuRef # Ase-6056G-1, ASEW-6055G-1, ASE-6060G-1, ASE-6057G-1), and SensID PIK3CA-11 mutations 12.5% AF cfDNA (SensеID SID-000099).

ClearSEEK™ PIK3CA Mutational Analysis via MassARRAY® System

PIK3CA mutation analysis was performed using a custom ClearSEEK panel on the MassARRAY System (Agena Bioscience, San Diego, CA, USA), which employs matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for variant detection (MALDI-TOF-MS). Primers designed for initial PCR (polymerase chain reaction) amplification of mutation hotspots in PIK3CA, with specific mutations detected by single nucleotide extension probes. Probes were prepared using the MassARRAY Assay Design Version 3.1 software (Agena Bioscience, San Diego, CA, USA). PCR reactions were performed as per manufacturer's recommendations.

ClearSEEK chemistry is a wild-type (WT) terminator-depleted chemistry designed to only generate extension terminator signal in a mutant DNA specimen. This allows for quantification of a mutation down to a very low variant allele frequency (VAF) as the analytical window is not dominated by the wild-type allele. To confirm wild-type sample amplification an extension primer for each wild type PCR amplicon is included in the panel. Following the PCR reaction, SAP addition, and ClearSEEK extension reaction, the samples were desalted by ion exchange resin treatment, spotted onto SpectroCHIP® Arrays (Agena Bioscience, San Diego, CA), analyzed by mass spectrometry, and ultimately interpreted on TYPER v4.0 software (Agena Bioscience, San Diego, CA).

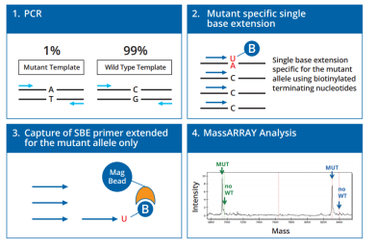

Figure 1: Principles of ClearSEEK wildtype suppression for low variant allele detection.
Capture probes towards mutant alleles after single base pair extension reduce background wildtype single, increasing allele LOD. (https://agenabio.com/wp-content/uploads/2017/03/51-20053R2.0_UltraSEEK_Reagents_Brochure_0317_WEB.pdf)

Results

Table 1: PIK3CA ClearSeek detectable mutations

p.C420R c.1258T>C	p.E545A c.1634A>C	p.Q546K c.1636C>A	p.H1047Y c.3139C>T
p.E542K c.1624G>A	p.E545G c.1634A>G	p.Q546E c.1636C>G	p.H1047N c.3139C>A
p.E542Q c.1624G>C	p.E545V c.1634A>T	p.Q546P c.1637A>C	p.H1047P c.3140A>C
p.E545K c.1633G>A	p.E545D c.1635G>C	p.Q546R c.1637A>G	p.H1047R c.3140A>G
p.E545Q c.1633G>C	p.E545D c.1635G>T	p.Q546L c.1637A>T	p.H1047L c.3140A>T

Table 2: Clinical Validation Characteristics

	True Positive	False Positive	False Negative	True Negative	Sensitivity	Specificity
VAF 2.0%	171	0	4	18	97.7%	100.0%
VAF 1.0%	191	0	25	18	88.4%	100.0%
VAF 0.5%	202	0	55	18	78.6%	100.0%

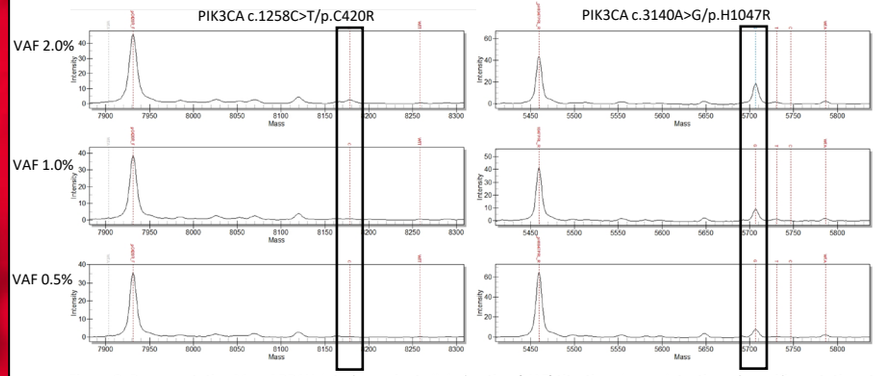

Figure 2: Representative MassARRAY data for variant allele fraction (VAF) titration shows detection of specific mutations to as low as 0.5% mutational burden.
M/Z data shows detection of p.C420R and p.H1047R to a VAF of 2.0% and 0.5%, respectively. (C420R n=3, H1047R n=7)

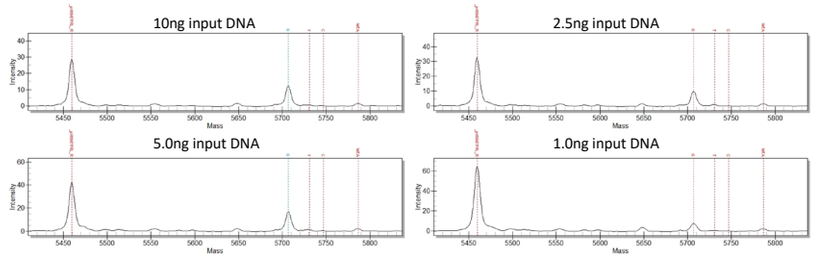

Figure 3: Representative MassARRAY data for input DNA titration reliably detects mutations with as little as 1.0-2.5ng of starting material.
M/Z data shows specific detection of the PIK3CA c.3140A>G/p.H1047R mutation (VAF 2.0%) at 10, 5.0, 2.5, and 1.0 ng of input DNA (representative data, n=4).

Results Continued

Table 3: Mutation Specific Sensitivity (at 10ng DNA input)

Mutation	Sensitivity at VAF=2%	Sensitivity at VAF=1%	Sensitivity at VAF=0.5%
C420R	100 (3/3)	0 (0/3)	0 (0/3)
E452K	100 (3/3)	0 (0/3)	0 (0/3)
E545K	100 (7/7)	57 (4/7)	14 (1/7)
E545D	100 (3/3)	0 (0/3)	0 (0/3)
E545G	100 (3/3)	100 (3/3)	100 (3/3)
E545A	100 (3/3)	0 (0/3)	0 (0/3)
Q546R	100 (3/3)	0 (0/3)	0 (0/3)
Q546E	100 (3/3)	0 (0/3)	0 (0/3)
H1047R	100 (7/7)	100 (7/7)	100 (7/7)
H1047L	100 (3/3)	100 (3/3)	0 (0/3)
H1047Y	100 (3/3)	100 (3/3)	0 (0/3)

Conclusions

In the SOLAR-1 trial, efficacy of alpelisib was largely associated with cases of breast cancer tumors bearing one of 14 different PIK3CA mutations: C420R, E542K, E545A, E545D, E545G, E545K, E545X, Q546E, Q546R, Q546X, H1047L, H1047R, H1047X, H1047Y versus tumours without PIK3CA mutations. Thus, identification of these particular sequence variants has an outcome validated in a clinical trial.

In this study, we describe and validated a comprehensive PIK3CA assay which detects all 20 actionable mutations responsive to alpelisib in a single sample. Using a combination of previously tested FFPE derived DNA samples and commercial controls, we have demonstrated a 97% sensitivity and 100% specificity for this panel at 10ng of input DNA to a lower limit of 2% VAF. This corresponds to an initial estimated tumor percentage in FFPE samples to ~4-5% tumor burden, making this assay appropriate for small core biopsies of breast tumors or potentially low cellularity fine needle aspirates of axillary lymph nodes. For the most common PIK3CA mutations, DNA input can be decreased to 2.5 to 5.0 ng of starting material, while still maintaining a reliable LOD to 2% VAF.

MassArray technology has been available for clinical use for almost a decade and is the main platform for the Molecular Pathology Laboratory in Calgary, Alberta, Canada. In our experience it is a robust, highly sensitive and extremely cost-effective platform for our diagnostic needs. In our laboratory context, cost-effectiveness, reliability, analytical sensitivity, turnaround times and simplicity of informatics needs are crucial criteria and discovery research is not a priority for our funding stakeholders, hence the MassArray platform is ideal for our needs. The need for an enhanced assay for PIK3CA mutations that would have an analytical sensitivity to match our other assays of 2-3% neoplastic cell frequency led us to work with Agena Bioscience to develop and validate this new assay.

Our data show that this assay is highly accurate with a 100% concordance to known PIK3CA mutations identified by other methods and has a high analytical sensitivity of 2% allele frequency. In our laboratory, the new assay has an estimated cost of less than 50% of the cost of NGS and can typically be performed with turnaround times of 2-3 days. We suggest that this assay may be the preferred method to identify clinically important PIK3CA mutations in breast cancer specimens in a routine molecular pathology diagnostic laboratory.

Acknowledgements
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