Evaluation of a Screening Tool to Detect Clinically Actionable DNA Variants in Lung Cancer Patients

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INTRODUCTION

Genomic testing for actionable DNA variants in non-small cell lung carcinoma (NSCLC) is performed on patients with Stage III/IV disease. Core needle biopsies or fine needle aspirates (FNAs) provide limited material that must primarily be used for morphologic diagnosis. This study was undertaken to explore genotyping that may be more rapid and cost-effective than standard Next Generation Sequencing (NGS) procedures. We used a multiplexed PCR/single base extension (SBE) and matrix-assisted, laser-desorption/ionization time of flight (MALDI-TOF) based analytical approach and detected low allele fraction variants in nine NSCLC cases that had previously been analyzed by a cancer "hotspot" NGS-based laboratory developed test (LDT).

METHODS

DNA from NSCLC patients was purified from formalin-fixed paraffin-embedded tissues, fine needle aspirates, and pleural fluids and tested by NGS with 300 ng DNA input per sample. For a subset of nine selected specimens, 20 ng DNA, on average, was re-analyzed using single multiplex PCR and SBE based iPLEX® High Sensitivity (HS) Lung Panel (Agena Bioscience, San Diego, CA). The panel is designed to interrogate 70 variants across five NSCLC-relevant genes: BRAF (4 variants), EGFR (46), ERBB2 (2), KRAS (14), and PIK3CA (4) (**Figure 1**; **Table 1**). Variant detection was performed on a MALDI-TOF based MassARRAY® System (Agena Bioscience). A positive control, Seraseq® Tumor Mutation DNA Mix v2 AF 4 HC (Seracare, Milford, MA), detailed in **Table 2**, a wild-type cell line DNA, and a "no template control" (NTC) were included in each run.

RESULTS

All overlapping variants that had been observed by the NGS-based LDT with variant allele frequencies (VAF) ranging from 0.75% to 74% were confirmed by the iPLEX HS Lung Panel at 100% concordance. Five specimens were positive for six EGFR activating mutations: L858R, E746_A750del (2), L861Q, S768I, and the rare V774M variant; and one specimen harbored the 1st and 2nd-generation EGFR TKI resistance mutation, T790M. KRAS mutations (G12C, G12F, G12V) and one BRAF V600E mutation (**Table 3** and **Figure 2**) were observed in three specimens. All nine expected mutations from the Seraseq positive control were detected. No false positive results were observed. The wild-type cell line DNA and NTC control results were in range.

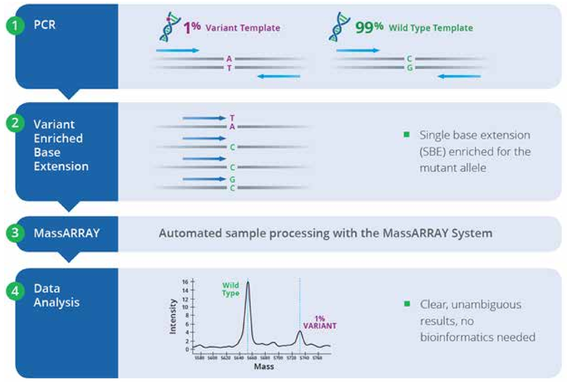

Figure 1: Following sample preparation, multiplexed PCR is performed (1), using at least 1 ng of input DNA. After PCR, remaining free deoxynucleotides are dephosphorylated using shrimp alkaline phosphatase (not shown). During the single base extension (SBE) reaction (2), a site-specific primer is extended by one of the nucleotides, which terminates the extension of the primer and forms extension products of different masses depending on the sequence analyzed. Increased sensitivity for the iPLEX HS reaction is achieved by modifying the ratio of the nucleotide mix in the extension reaction to favor the mutant alleles, increasing their detection rate. The wild type nucleotide is still present at a limited concentration, providing relative quantification and internal quality control for the assay (4).

iPLEX HS Lung Panel		
Gene	**Coverage***	**# of Variants***
BRAF	Codons 469 (exon 11), 594, 600 (exon 15)	4
EGFR	Exon 19 indels, exon 20 insertions and substitutions across exons 18, 19, 20 and 21	46
ERBB2	Exon 20 insertions	2
KRAS	Codons 12, 13 (exon 2) and 61 (exon 3)	14
PIK3CA	Codons 542, 545 (exon 9) and 1047 (exon 20)	4
Total Variants		**70**

Table 1: Variants across 5 NSCLC-relevant genes contained in the iPLEX HS Lung Panel that facilitates variant detection as low as 1% allele frequency from poor quality and degraded samples such as FFPE tissue, fine needle aspirates, core needle biopsies, smears, pleural fluid and cytology blocks. *Complete variant list available at agenabio.com

Gene	Nucleotide	Amino Acid
BRAF	c.1799T>A	p.V600E
EGFR	c.2236_2250del15 c.2310_2311insGGT c.2573T>G c.2369C>T	p.E746_A750delELREA p.D770_N771insG p.L858R p.T790M
ERBB2	c.2324_2325ins12	p.A775_G776insYVMA
KRAS	c.35G>A	p.G12D
PIK3CA	c.1633G>A c.3140A>G	p.E545K p.H1047R

Table 2: List of the 9 out of 40 unique multiplexed variants across 28 genes included in Seraseq® Tumor Mutation DNA Mix v2 AF 4 HC reference standard at 4% allele fraction that are overlapping with the iPLEX HS Lung Panel.

Sample	Sample Type	Tissue Type	Tumor %	Tumor AF	Gene	AA changes	NT change	VAF %
1	FFPE	Lower lobe, left lung	80%	52%	*EGFR*	p.L858R	c.2573T>G	41.6%
2	FFPE	right supraclavicular lymph node	30%	87%	*EGFR*	p.L861Q	c.2582T>A(p.Leu861Gln)	26.4%
3	FNA	bronchial upper lobe mass	15%	5%	*EGFR*	p.E746_A750del	c.2235_2249del19	0.75%
4	FFPE	left lingula	60%	26%; 46%	*EGFR*	p.S768I; p.V774M	c.2303G>T; c.2320G>A	15.6%; 27.6%
5	FFPE	lymph node	<20%	26%; 43%	*EGFR*	p.E746_A750del; p.T790M	c.2236_2250del19; c.2369C>T	5.2%; 8.6%
6	pleural fluid	pleural fluid	90%	60%	*KRAS*	p.G12F	KRAS c.34_35delinsTT	54.0%
7	FNA	bronchial	80%	92%	*KRAS*	p.G12V	c.35G>T	73.6%
8	FFPE	peri aortic nodule	10% to 20%	5%	*BRAF*	p.V600E	c.1799T>A	<1.0%
9	FFPE	rectum biopsy	35%	11%	*KRAS*	p.G12C	c.34G>T	3.9%

Table 3: List of variants initially detected by NGS and confirmed by the iPLEX HS Lung Panel. Percent variant allele frequencies (VAF %) as determined by NGS ranging from 74% in sample 7 to below 1% in samples 3 and 8. With tumor fraction varying from 10 to 90% the allele frequency in the tumor was calculated (Tumor AF %).

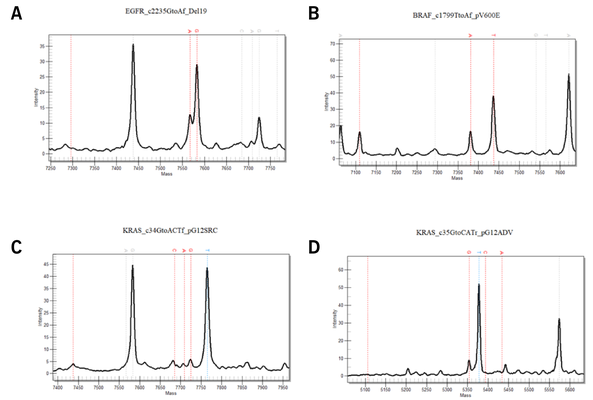

Figure 2: Example mass spectra for iPLEX HS Lung Panel from the MassARRAY with specific signal indicated by red and blue labels; **A)** Sample 3 EGFR p.E746_A750del at 0.75% on NGS, **B)** Sample 8 BRAF p.V600E at <1%, **C)** Sample 6, the complex compound substitution KRAS G12F (KRAS c.34_35delinsTT) which is correctly detected as KRAS p.G12C and **D)** p.G12V. The mass spectra represent MassARRAY raw data and allele calls are being made by the automated iPLEX HS report. All variants were detected and called with high significance. The other large peaks in the spectra represent results from different assays in the same multiplex reaction (with gray peak labels).

CONCLUSIONS

In this small comparison study, clinically relevant variants that had been reported using an NGS-based LDT were observed with 100% concordance with the iPLEX® HS Lung Panel with MassARRAY® System. The variants detected can potentially inform clinical decisions for NSCLC patients. The MassARRAY system provides an alternative for molecular pathology laboratories interested in implementing somatic mutation detection and generation of actionable results from limited sample input in a single day, with minimal hands-on and analysis time and at a low cost per reportable.