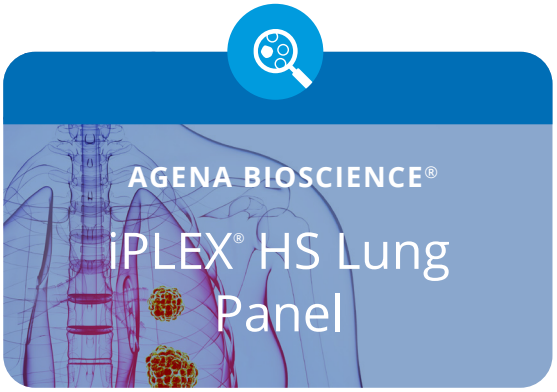
Reliable and Comprehensive Detection of Somatic Variants in Non-Small Cell Lung Cancer Specimens

INTRODUCTION

Identifying low frequency variants in tumor tissue samples requires tools that offer both high sensitivity detection and efficient use of starting material. Low-level variants are difficult to detect due to tumor heterogeneity and may easily be missed when the analytical sensitivity of the detection assay is >5%. In addition, the use of less invasive methods to obtain tumor tissue results in less sample material available for molecular testing. Labs typically reject 20%-30% of tumor tissue samples due to insufficient tumor content for next generation sequencing (NGS).[1]



The ideal technology for the detection of low-frequency somatic variants would enable detection of multiple variants at as low as 1% variant allele frequency, have a quick turnaround time with easy data analysis, and have a low cost. Next generation sequencing (NGS) assays are well suited for the discovery of new variants that may have clinical utility in the future. However, due to long turnaround times and the high tumor content required for detection of low frequency variants, NGS is better suited as a secondary follow-up test when no clinically relevant targets are detected. Real-time PCR (RT-PCR) assays are quick and not as expensive as NGS however, they require a much higher level of input DNA to achieve the required sensitivity and coverage across multiple genes and variants.

In this white paper, we present the MassARRAY® System, powered by iPLEX® HS chemistry, as an ideal solution for detecting somatic variants from non-small cell lung cancer tissue specimens. Using minimal DNA input of 20 ng, the majority of the >90 variants (57 assays) are detectable at <2.5% variant allele frequency (VAF). The MassARRAY System uses a simple, PCR-based single-day workflow with easy data analysis, making it ideally suited for targeted variant detection and orthogonal validation studies.

Agena® BIOSCIENCE

iPLEX® HS LUNG PANEL V2

The iPLEX HS Lung Panel v2 facilitates the detection of >90 variants implicated in non-small cell lung cancer. Poor quality and degraded samples such as formalin-fixed, paraffin-embedded (FFPE) tissue and cytology blocks with low tumor content have been successfully used as sample sources for this assay.[2]

Table 1: Variants detected by the iPLEX HS Lung Panel v2

Gene	Coverage
BRAF	Exon 11 - codon 469; Exon 15 - codons 594, 600
EGFR	Exon 19 and 20 indels and substitutions across exons 18, 19, 20, and 21
ERBB2	Exon 20 insertions
KRAS	Exon 2 – codons 12, 13; Exon 3 – codon 61
PIK3CA	Exon 9 – Codons 542, 545; Exon 20 – codon 1047

IPLEX HS CHEMISTRY

The iPLEX HS chemistry consists of single global PCR amplification of a set of pre-defined loci harboring the variants of interest. After the removal of unincorporated dNTPs, a sequence specific primer extension step is performed. Increased sensitivity for the iPLEX HS reaction is achieved by modifying the ratio of the nucleotide mix in the extension reaction to favor the variant alleles, increasing their detection rate. The wildtype nucleotide is still present at a limited concentration, providing relative quantification and internal quality control for the assay. The extension products are desalted, transferred to a SpectroCHIP® Array and then loaded into the MassARRAY Analyzer and detected using time-of-flight measurements (Figure 1). The Somatic Variant Report rapidly provides an automated, easy to interpret readout of variants detected within each sample.

With multiplexing of >90 variants per sample using short amplicons of 80 – 120 bp, iPLEX HS chemistry is ideally suited for detection of somatic variants from FFPE tissue, core needle biopsies, fine needle aspirates, and cytology smears.

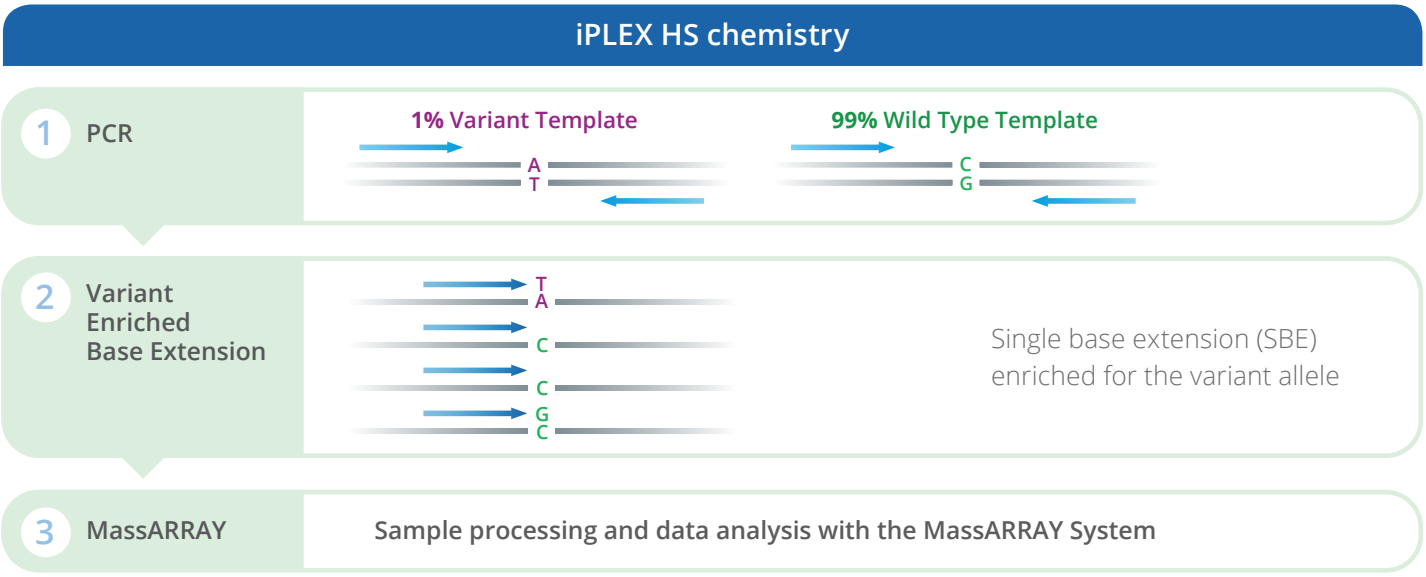


Figure 1: Somatic variant detection with iPLEX HS chemistry on the MassARRAY System

SOMATIC VARIANT REPORT

The Somatic Variant Report enables easy analysis of the results from the iPLEX HS Lung Panel v2. The user can specify the z-score, peak intensity type (Area, Signal to noise ratio [SNR], height), and minimum values to be used as cutoffs for the detection of variants. Determining the z-score cutoff that gives the required sensitivity and specificity is crucial for confident calling of the data. As you decrease z-score, sensitivity increases, and specificity decreases. The opposite is true as you raise the z-score cutoff.

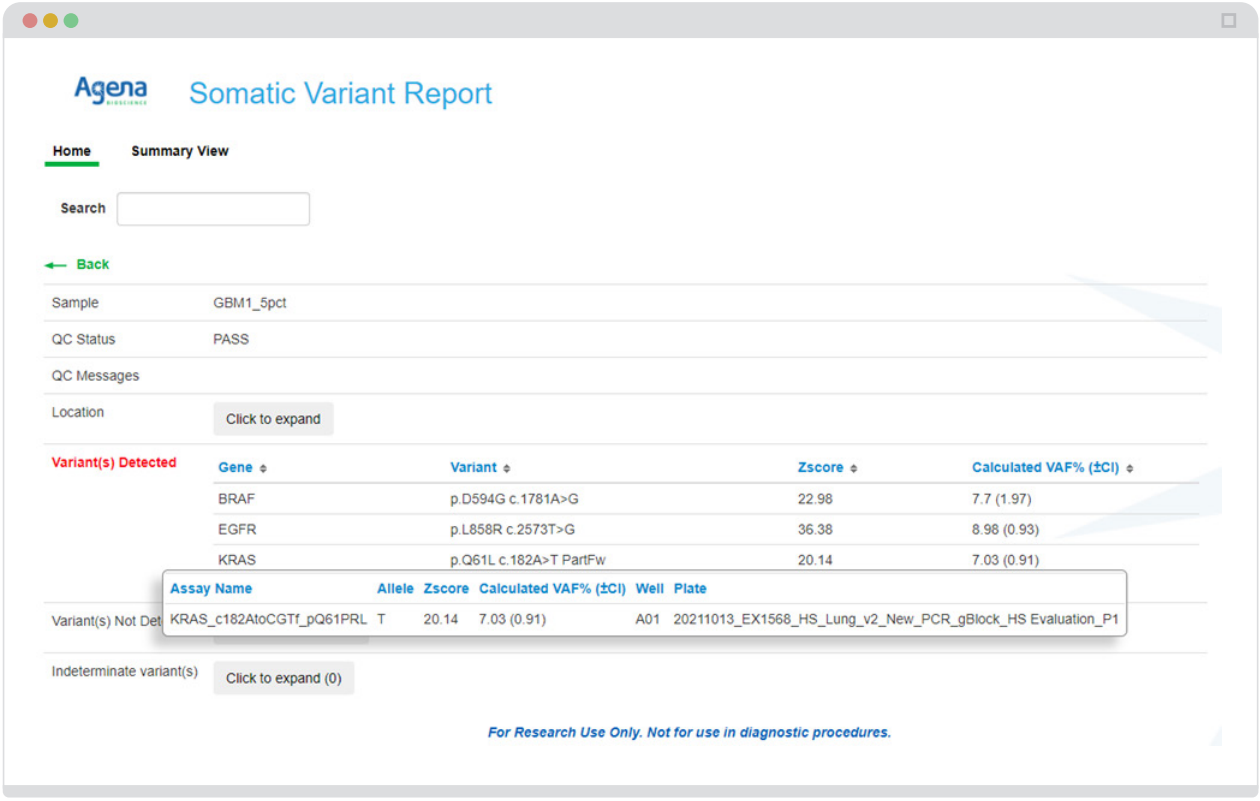


Figure 2: Example output from Somatic Variant Report showing variants detected, z-score, and variant allele frequency

LIMIT OF DETECTION (LOD) STUDY

Samples Tested

Sensitivity and specificity assessments were performed for every assay using a contrived model system. The model consisted of synthetic double-stranded constructs known as gBlocks™ from Integrated DNA Technologies (IDT). Each construct contains a single variant present in the iPLEX HS Lung Panel v2. To maximize data, up to three gBlocks were mixed into a single sample with a background of high molecular weight wild-type genomic DNA. gBlocks and genomic DNA were individually quantified for copy number by digital droplet PCR via a genomic reporter sequence. Mutant mixes at 0.625%, 1.25%, 2.5%, 5%, and 10% variant allele frequency were generated using 3030 total copies of human wild-type genomic DNA.

Each minor variant mix was run in quadruplicate. The assay was considered sensitive to the level tested if 75% of the replicates were positive (signal to noise ratio, SNR ≥5). LoD assessments were evaluated at Z-score 3. Specificity assessments were done with a minimum of 12 wild type samples and were also evaluated using z-score 3.

Assay Baseline

Assay baseline values were determined by processing 24 human wild-type DNA cell lines and FFPE samples using the HS Lung Panel v2 on the MassARRAY System as per the Baseline Creation Guide.[3]

Results

Assay sensitivity and specificity were determined using the Somatic Variant Report software, with a z-score of 3 and peak intensity of SNR ≥ 5. 78% of the assays have a limit of detection of 2.5% or lower variant allele frequency with an average specificity across all assays of 99.8%.

Table 2: HS Lung assay sensitivity and specificity

	Variant Allele Frequency (VAF)	Limit of Detection	Specificity
HS Lung	0.63%	9%	99.90%
	1.25%	45%	
	2.5%	78%	
	5%	97%	
	10%	100%	

The limit of detection and specificity for each assay in the HS Lung v2 panel at a z-score of 3 is detailed below.

Table 3: HS Lung Panel v2 limit of detection and specificity with gBlocks using a z-score of 3 and SNR of 5

Assay	Limit of Detection	Specificity
EGFR_c2155GtoATf_pG719SC_A	0.63	96%
EGFR_c2156GtoCAf_pG719AD_A	0.63	98%
EGFR_c2248GtoCr_Del19_C	0.63	100%
ERBB2_c2326_2327ins3f_pG776toVC_T	0.63	99%
KRAS_c34GtoACTf_pG12SRC_C	0.63	100%
KRAS_c34GtoACTf_pG12SRC_T	0.63	100%
KRAS_c38GtoAf_pG13D_A	0.63	100%
KRAS_c38GtoAr_pG13D_A	0.63	100%
BRAF_c1406GtoCTf_pG469AV_T	1.25	100%
BRAF_c1406GtoCTr_pG469AV_C	1.25	100%
BRAF_c1406GtoCTr_pG469AV_T	1.25	100%
BRAF_c1799TtoAf_pV600E_A	1.25	100%
EGFR_c2125GtoAr_pE709K_A	1.25	100%
EGFR_c2126AtoCGTr_pE709AGV_T	1.25	100%
EGFR_c2155GtoATf_pG719SC_T	1.25	100%
EGFR_c2156GtoCAf_pG719AD_C	1.25	100%
EGFR_c2156GtoCAr_pG719AD_A	1.25	100%
EGFR_c2156GtoCAr_pG719AD_C	1.25	100%
EGFR_c2235GtoAf_Del19_A	1.25	99%
EGFR_c2236GtoACTf_Del19_A	1.25	100%
EGFR_c2245GtoAr_Del19_A	1.25	100%
EGFR_c2247AtoCr_Del19_C	1.25	100%
EGFR_c2252CtoATr_Del19_A	1.25	100%
EGFR_c2252CtoATr_Del19_T	1.25	99%
EGFR_c2255CtoATr_Del19_T	1.25	98%
EGFR_c2256TtoAGr_Del19_A	1.25	100%
EGFR_c2256TtoAGr_Del19_G	1.25	100%
EGFR_c2303GtoACTf_pS768I_T	1.25	100%
EGFR_c2308GtoAf_Ins20_A	1.25	99%

Assay	Limit of Detection	Specificity
EGFR_c2309AtoCGf_Ins20_G	1.25	100%
EGFR_c2320GtoACf_Ins20_A	1.25	96%
EGFR_c2320GtoACf_Ins20_C	1.25	100%
EGFR_c2582TtoAGf_pL861QR_G	1.25	100%
ERBB2_c2326_2327ins3r_pG776toVC_T	1.25	100%
KRAS_c181CtoAGr_pQ61KE_A	1.25	100%
KRAS_c183AtoCTr_pQ61HH_C	1.25	100%
KRAS_c34GtoACTf_pG12SRC_A	1.25	100%
KRAS_c35GtoCATr_pG12ADV_A	1.25	100%
KRAS_c35GtoCATr_pG12ADV_C	1.25	100%
KRAS_c35GtoCATr_pG12ADV_T	1.25	100%
KRAS_c37GtoTf_pG13C_T	1.25	100%
BRAF_c1406GtoCTf_pG469AV_C	2.5	100%
BRAF_c1781AtoGf_pD594G_G	2.5	100%
BRAF_c1781AtoGr_pD594G_G	2.5	100%
EGFR_c2126AtoCGTf_pE709AGV_C	2.5	100%
EGFR_c2126AtoCGTf_pE709AGV_G	2.5	96%
EGFR_c2126AtoCGTf_pE709AGV_T	2.5	100%
EGFR_c2126AtoCGTr_pE709AGV_C	2.5	100%
EGFR_c2126AtoCGTr_pE709AGV_G	2.5	100%
EGFR_c2236GtoACTf_Del19_C	2.5	100%
EGFR_c2236GtoACTf_Del19_T	2.5	100%
EGFR_c2237AtoCTf_Del19_T	2.5	100%
EGFR_c2239TtoCGf_Del19_C	2.5	100%
EGFR_c2239TtoCGf_Del19_G	2.5	100%
EGFR_c2249CtoAr_Del19_A	2.5	100%
EGFR_c2255CtoATr_Del19_A	2.5	100%
EGFR_c2300CtoAr_Ins20_A	2.5	100%
EGFR_c2308GtoCTr_Ins20_T	2.5	100%
EGFR_c2309AtoCGf_Ins20_C	2.5	100%

Assay	Limit of Detection	Specificity
EGFR_c2310CtoTr_Ins20_T	2.5	100%
EGFR_c2369CtoTr_pT790M_T	2.5	100%
EGFR_c2573TtoGr_pL858R_G	2.5	100%
EGFR_c2582TtoAGf_pL861QR_A	2.5	100%
ERBB2_c2324_2325ins12r_pA775_G776insYVMA_G	2.5	100%
KRAS_c181CtoAGr_pQ61KE_G	2.5	100%
KRAS_c182AtoCGTf_pQ61PRL_C	2.5	100%
KRAS_c182AtoCGTf_pQ61PRL_T	2.5	100%
KRAS_c183AtoCTr_pQ61HH_T	2.5	100%
PIK3CA_c1624GtoAr_pE542K_A	2.5	100%
PIK3CA_c1633GtoAr_pE545K_A	2.5	100%
PIK3CA_c3140AtoGTr_pH1047RL_T	2.5	100%
EGFR_c2237AtoCTf_Del19_C	5	100%
EGFR_c2250AtoGr_Del19_G	5	100%
EGFR_c2296AtoGr_Ins20_G	5	100%
EGFR_c2303GtoACTf_pS768I_A	5	100%
EGFR_c2303GtoACTf_pS768I_C	5	100%
EGFR_c2308GtoCTr_Ins20_C	5	100%
EGFR_c2369CtoTf_pT790M_T	5	100%
EGFR_c2389TtoAf_pC797S_A	5	100%
EGFR_c2389TtoAr_pC797S_A	5	100%
EGFR_c2390GtoCr1_pC797S_C	5	100%
EGFR_c2573TtoGf_pL858R_G	5	100%
ERBB2_c2325_2326ins12r_pA775_G776insYVMA_T	5	100%
KRAS_c181CtoAGf_pQ61KE_A	5	100%
KRAS_c182AtoCGTf_pQ61PRL_G	5	100%
KRAS_c182AtoCGTr_pQ61PRL_C	5	100%
KRAS_c182AtoCGTr_pQ61PRL_T	5	100%
PIK3CA_c3140AtoGTr_pH1047RL_G	5	100%

Assay	Limit of Detection	Specificity
EGFR_c2240TtoCf_Del19_C	10	100%
KRAS_c181CtoAGf_pQ61KE_G	10	100%
KRAS_c182AtoCGTr_pQ61PRL_G	10	100%

The calibration curves derived from the LoD study display a linear response for most assays. These linear responses are used as correction coefficients to transform the HS Lung data into allele frequency. Additionally, the observed variance of each assay is accounted for with a confidence interval around the result. The allele frequency and confidence interval are reported in the results along with the z-score.

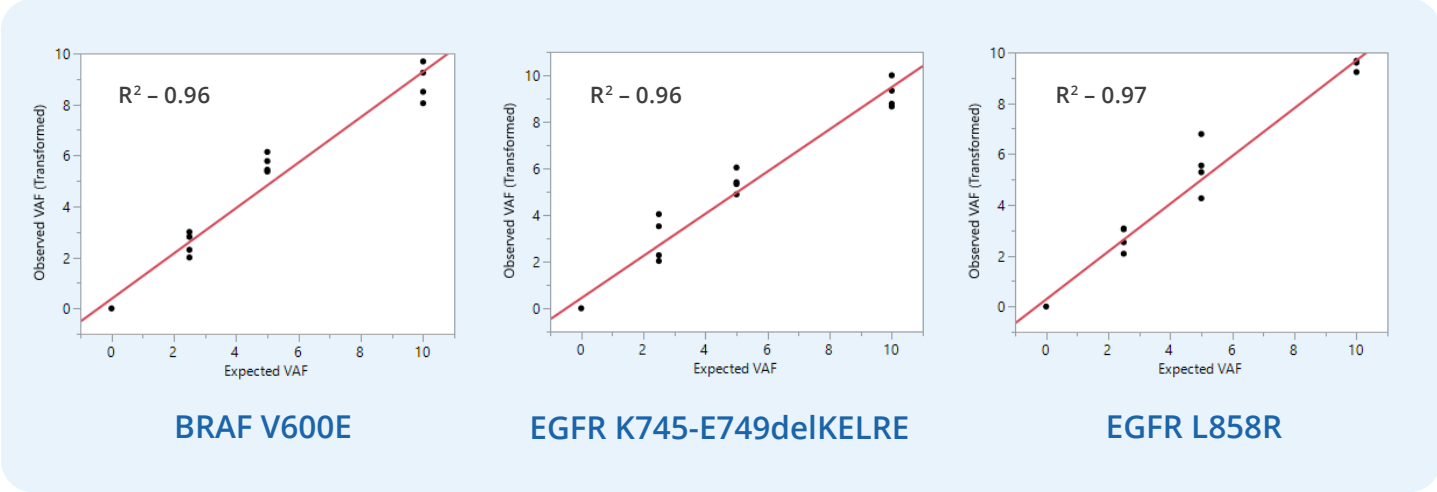


Figure 3. Example of expected vs. observed (transformed) allelic frequency

ACCURACY STUDY

Samples Tested - The SeraCare Seraseq® Tri-Level Tumor Mutation DNA Mix v2 HC (Catalog #: 0710-0097) and Horizon Tru-Q 0, 1, 2 and 3 (Catalog #: HD752, HD728, HD729, and HD730) series of reference standards from SeraCare and Horizon Discovery were used to verify assay performance. The SeraCare Seraseq Tri-Level mutation mix was also diluted by 25% and tested.

Results - The results from testing the reference standards were analyzed using the Somatic Variant Report software, with a z-score of 3 and an SNR cutoff of 5. All of the expected variants were successfully detected. Two false positive calls were encountered.

Table 4: iPLEX HS Lung Panel v2 assay performance with commercial reference standards

Sample	Expected Variant(s)	Detected Variants	FN	FP	Sensitivity	Specificity
HorizonTru-Q 0*	BRAF:p.V600E c.1799T>A, EGFR:p.G719S c.2155G>A, KRAS:p.G13D c.38G>A, PIK3CA:p.H1047R c.3140A>G	5	0	1	100%	99.2%
HorizonTru-Q 1	BRAF:p.V600E c.1799T>A, EGFR:p.G719S c.2155G>A, EGFR:p.T790M c.2369C>T, KRAS:p.G12A c.35G>C, KRAS:p.G12R c.34G>C, KRAS:p.G13D c.38G>A, PIK3CA:p.H1047R c.3140A>G	7	0	0	100%	100.0%
HorizonTru-Q 2	BRAF:p.V600E c.1799T>A, EGFR:p.G719S c.2155G>A, EGFR:p.L858R c.2573T>G, KRAS:p.G12V c.35G>T, KRAS:p.G13D c.38G>A, KRAS:p.Q61L c.182A>T, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	9	0	1	100%	99.1%
HorizonTru-Q 3	BRAF:p.V600E c.1799T>A, EGFR:p.E746_A750delELREA c.2235_2249del15, EGFR:p.G719S c.2155G>A, KRAS:p.G12S c.34G>A, KRAS:p.G13D c.38G>A, PIK3CA:p.E542K c.1624G>A, PIK3CA:p.H1047R c.3140A>G	7	0	0	100%	100.0%
SeraCare	BRAF:p.V600E c.1799T>A, EGFR:p.D770_N771insG c.2310_2311insGGT, EGFR:p.E746_A750delELREA c.2236_2250del15, EGFR:p.L858R c.2573T>G, EGFR:p.T790M c.2369C>T, ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC, KRAS:p.G12D c.35G>A, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	9	0	0	100%	100.0%
SeraCare-Dil	BRAF:p.V600E c.1799T>A, EGFR:p.D770_N771insG c.2310_2311insGGT, EGFR:p.E746_A750delELREA c.2236_2250del15, EGFR:p.L858R c.2573T>G, EGFR:p.T790M c.2369C>T, ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC, KRAS:p.G12D c.35G>A, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	9	0	0	100%	100.0%
WT	NA	0	0	0	100%	100.0%
WT_rep02	NA	0	0	0	100%	100.0%

FN = False Negative, FP = False Positive

*Horizon Tru-Q 0 is a wild type reference standard with background variants that are detectable by the HS Lung v2 Panel

REPRODUCIBILITY STUDY

The reproducibility of the HS Lung v2 panel was determined by testing gBlocks mixed with genomic DNA at 1.25% and 2.5% VAF for the KRAS G13D and EGFR L858R variants. These two variants have two redundant assays each in the panel. In addition, the SeraCare Complete Mutation Mix reference standard was tested at 5% (Catalog #0710-0528) and 2.5% (Catalog #0710-0529). The Seracare standard has nine variants detected in the HS Lung v2 via 12 assays. Each standard was evaluated in replicates of three

Table 5: Summary of reproducibility study with HS Lung panel v2

	Sensitivity			Specificity
	5% VAF	2.5% VAF	1.25% VAF	
Site 1 - gBlocks	N/A	100% PPA (12/12)	100% PPA (12/12)	99% NPA (818/819)
Site 1 – SeraCare	100% PPA* (36/36)	94% PPA* (34/36)	N/A	99% NPA (818/819)

**ERBB2_c2324_2325ins12r_pA775_G776insYVMA was not considered in the sensitivity calculation. gBlock assessment of this assay has LoD at 2.5%.*

EVALUATION WITH FFPE SAMPLES

Assay performance was also evaluated with FFPE samples. Variants were detected in 9 FFPE samples showing that the assay can successfully detect variants from FFPE specimens. FFPE specimens where not characterized prior to testing.

Table 6: Detection of variants in FFPE specimens

Sample	Variant Detected
FFPE1	BRAF:p.V600E c.1799T>A
	EGFR:p.D770G c.2309A>G
	EGFR:p.E709G c.2126A>G PartFw
FFPE18_1	EGFR:p.L858R c.2573T>G
FFPE19_1	EGFR:p.L858R c.2573T>G PartRev
	KRAS:p.G12V c.35G>T
	KRAS:p.Q61H c.183A>C
FFPE20_1	EGFR:p.V774M c.2320G>A
	ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC
FFPE20_5	EGFR:p.V774M c.2320G>A
	ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC
FFPE20_10	ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC
FFPE21_1	ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC
FFPE21_5	EGFR:p.V774M c.2320G>A
	ERBB2:p.A775_G776insYVMA c.2324_2325insATACGTGATGGC
FFPE4	KRAS:p.G13D c.38G>A
	PIK3CA:p.E545K c.1633G>A

SUMMARY

Results from the gBlocks and commercial reference standards studies demonstrate that the iPLEX HS Lung Panel v2 has excellent limit of detection with the majority of the assays being able to detect variants at ≤5% variant allele frequency with 99.8% average specificity and can be successfully used to detect variants from FFPE samples.

References

1. https://www.genomeweb.com/molecular-diagnostics/intermountains-edited-cancer-panel-reducing-rate-tests-rejected-due/
2. Frank Mularo, Jay E. Brock, Hema Liyanage, Scott Shell, Daniel Farkas. Evaluation of a screening tool to detect clinically actionable DNA variants in lung cancer patients. Poster session presented at Association of Molecular Pathology Annual Meeting; 2019
3. USG-CUS-135: Baseline Creation Guide - iPLEX® HS and UltraSEEK® Panels v2 and ClearSEEK™ Panels

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