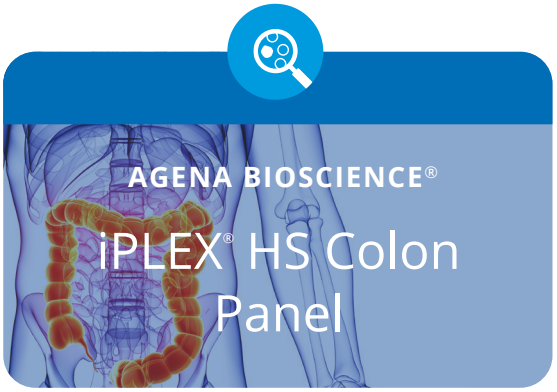
Reliable and Comprehensive Detection of Somatic Variants in Colorectal Cancer Specimens

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 (VAF), have a quick turnaround time with easy data analysis, and have a low cost. Next generation sequencing 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 and iPLEX® HS chemistry as an ideal solution for detecting somatic variants from colorectal cancer tissue specimens. Using as low as 20 ng of DNA input, the majority of the 100 variants 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.

IPLEX® HS COLON PANEL V2

The iPLEX HS Colon Panel v2 facilitates the detection of >100 variants implicated in colorectal 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]

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

Gene	Coverage
BRAF	Exon 11 - codon 469; Exon 15 - codons 594, 600
EGFR	Exon 12 - extracellular domain mutations
KRAS	Exon 2 – codons 12, 13; Exon 3 – codons 59, 61; Exon 4 – codons 117 and 146
NRAS	Exon 2 – codons 12, 13; Exon 3 – codons 59, 61; Exon 4 – codons 117 and 146
PIK3CA	Exon 9 – Codons 542, 545; Exon 20 – codon 1047

IPLEX HS CHEMISTRY

iPLEX HS chemistry consists of single global PCR amplification of a set of pre-defined loci harboring the variants of interest (Figure 1). 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 wild-type 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. The Somatic Variant Report software rapidly provides an automated, easy-to-interpret readout of variants detected within each sample.

With multiplexing of >100 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.

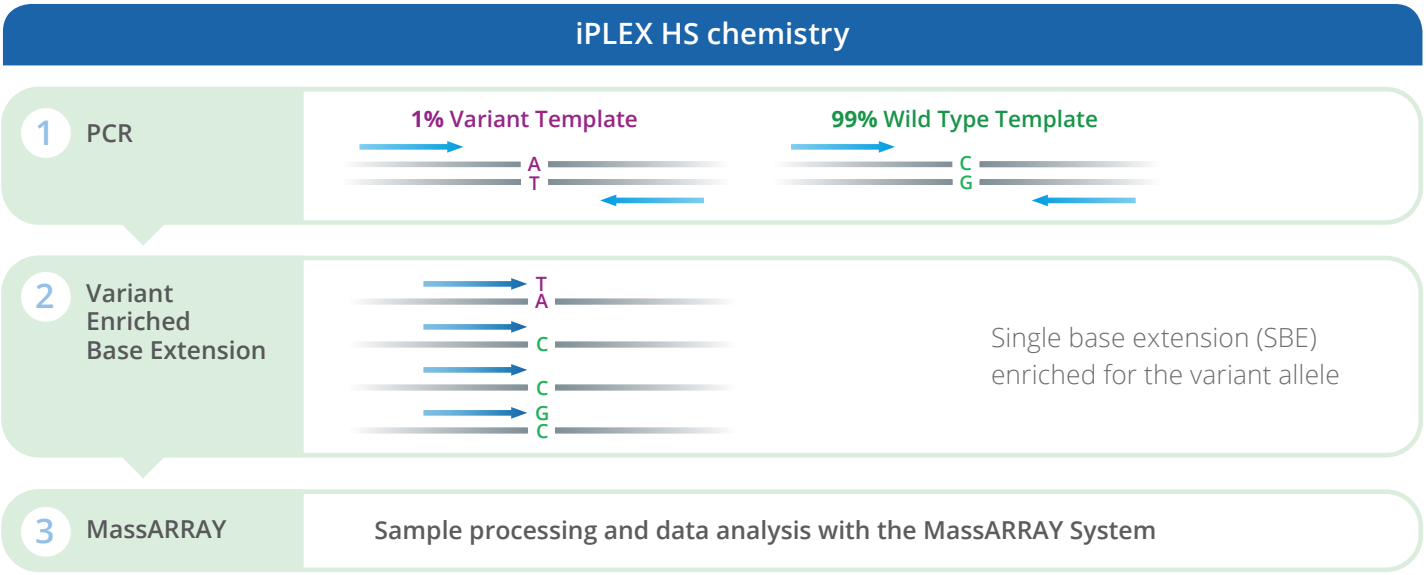

Figure 1: Somatic variant detection with iPLEX HSHS chemistry on the MassARRAY System

SOMATIC VARIANT REPORT

The Somatic Variant Report enables easy analysis of the results from the iPLEX HS Colon 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.

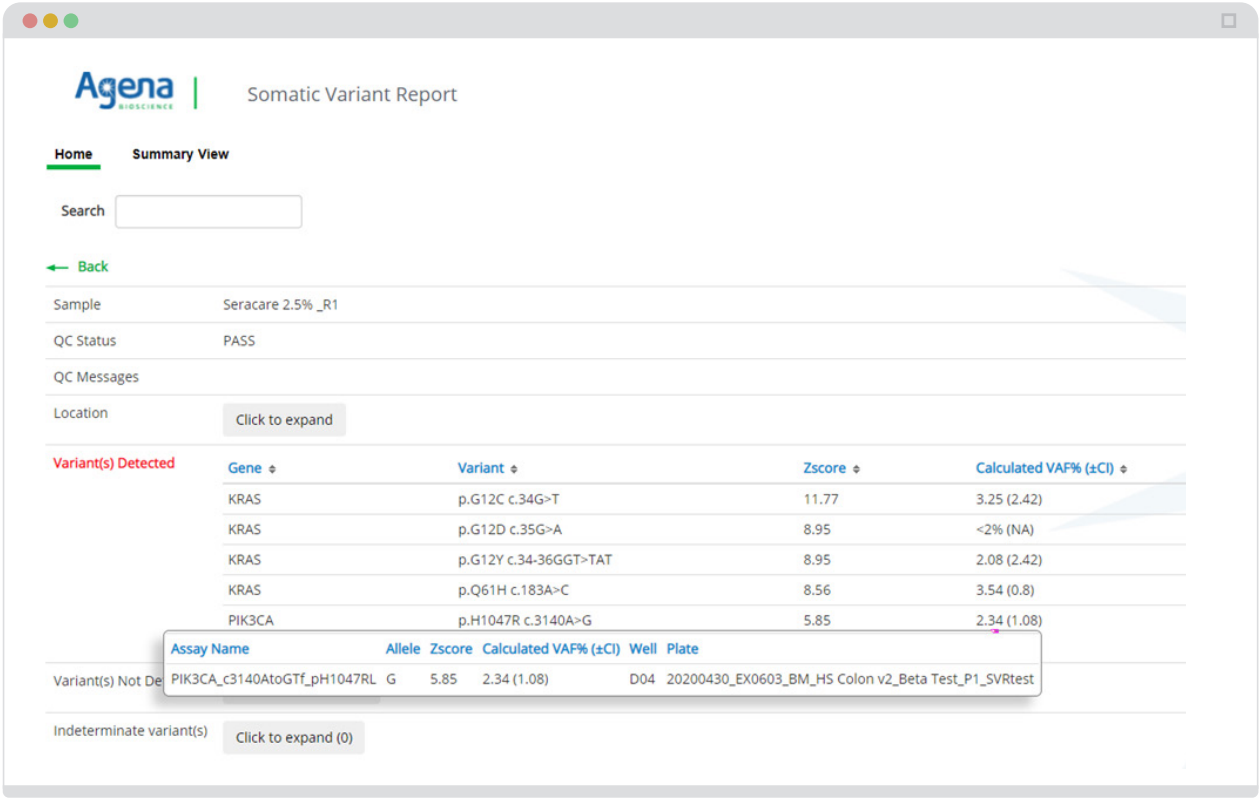

Figure 2: Example output from Somatic Variant Report showing variants detected, z-score, and variant allele frequency

LIMIT OF DETECTION (LOD) STUDY

Samples Tested

Limit of detection and specificity assessments were performed for every assay, using a contrived model system consisting of synthetic double-stranded constructs known as gBlocks™ from Integrated DNA Technologies (IDT). Each construct contains a single variant present in the iPLEX HS Colon 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. Variant mixes at 0.625%, 1.25%, 2.5%, 5%, and 10% VAF were generated using 3,030 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 a z-score of 3. Specificity assessments were done with a minimum of 12 wild-type samples and were also evaluated using a z-score of 3.

Assay Baseline

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

Results

Assay LoD and specificity were determined using the Somatic Variant Report software, with a z-score of 3 and peak intensity SNR of ≥ 5 cutoffs. 82% of the assays have a limit of detected of 2.5% or lower variant allele frequency with an average specificity of 99.9% across all assays.

Table 2: iPLEX HS Colon Panel v2 assay limit of detection and specificity

	Variant Allele Frequency (VAF)	Limit of Detection	Specificity
HS Colon	0.63%	16%	99.9%
	1.25%	56%	
	2.5%	82%	
	5%	97%	
	10%	100%	

The limit of detection and specificity for each assay in the HS Colon Panel v2 is shown in Table 3.

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

Assay	Specificity	Limit of Detection
KRAS_c34GtoCATf_pG12RSC_A	100%	0.63
KRAS_c34GtoCATr_pG12RSC_A	100%	0.63
KRAS_c34GtoCATr_pG12RSC_C	100%	0.63
KRAS_c34GtoCATr_pG12RSC_T	100%	0.63
KRAS_c35GtoCATf_pG12ADV_A	100%	0.63
KRAS_c35GtoCATf_pG12ADV_C	100%	0.63
KRAS_c35GtoCATf_pG12ADV_T	100%	0.63

Assay	Specificity	Limit of Detection
KRAS_c36TtoACGr_pG12Multi_C	100%	0.63
KRAS_c38GtoCATr_pG13ADV_A	100%	0.63
KRAS_c39CtoAGTr_pG13Multi_G	100%	0.63
NRAS_c182AtoCGTf_pQ61PRL_G	100%	0.63
NRAS_c36TtoACGr_pG12Multi_A	100%	0.63
NRAS_c37GtoCATf_pG13RSC_A	100%	0.63
PIK3CA_c1633GtoAr_pE545K_A	100%	0.63
KRAS_c175GtoATf_pA59TS_A	100%	1.25
KRAS_c182AtoCGTf_pQ61PRL_C	100%	1.25
KRAS_c182AtoCGTf_pQ61PRL_G	100%	1.25
KRAS_c182AtoCGTf_pQ61PRL_T	100%	1.25
KRAS_c34GtoCATf_pG12RSC_C	100%	1.25
KRAS_c34GtoCATf_pG12RSC_T	100%	1.25
KRAS_c35GtoCATr_pG12ADV_A	100%	1.25
KRAS_c35GtoCATr_pG12ADV_C	100%	1.25
KRAS_c35GtoCATr_pG12ADV_T	100%	1.25
KRAS_c36TtoACGr_pG12Multi_A	100%	1.25
KRAS_c36TtoACGr_pG12Multi_G	100%	1.25
KRAS_c37GtoCATf_pG13RSC_T	100%	1.25
KRAS_c38GtoCATr_pG13ADV_C	100%	1.25
KRAS_c38GtoCATr_pG13ADV_T	100%	1.25
KRAS_c39CtoAGTr_pG13Multi_A	100%	1.25
NRAS_c175GtoAf_pA59T_A	100%	1.25
NRAS_c182AtoCGTf_pQ61PRL_C	100%	1.25
NRAS_c349AtoGf_pK117E_G	100%	1.25
NRAS_c34GtoCATf_pG12RSC_A	100%	1.25
NRAS_c34GtoCATf_pG12RSC_C	100%	1.25
NRAS_c34GtoCATf_pG12RSC_T	100%	1.25

Assay	Specificity	Limit of Detection
NRAS_c351GtoCTf_pK117NN_C	100%	1.25
NRAS_c351GtoCTf_pK117NN_T	100%	1.25
NRAS_c35GtoCATf_pG12ADV_A	100%	1.25
NRAS_c35GtoCATf_pG12ADV_T	100%	1.25
NRAS_c35GtoCATr_pG12ADV_T	100%	1.25
NRAS_c37GtoCATf_pG13RSC_C	100%	1.25
NRAS_c37GtoCATf_pG13RSC_T	100%	1.25
NRAS_c38GtoCATr_pG13ADV_C	98%	1.25
NRAS_c38GtoCATr_pG13ADV_T	100%	1.25
NRAS_c436GtoCATr_pA146PTS_A	100%	1.25
NRAS_c436GtoCATr_pA146PTS_C	100%	1.25
NRAS_c436GtoCATr_pA146PTS_T	100%	1.25
NRAS_c437CtoGTr_pA146GV_T	100%	1.25
BRAF_c1406GtoAr_pG469E_A	100%	2.5
BRAF_c1781AtoGr_pD594G_G	100%	2.5
EGFR_c1476CtoAGr_pS492RR_A	100%	2.5
EGFR_c1476CtoAGr_pS492RR_G	100%	2.5
KRAS_c181CtoAGf_pQ61KE_G	100%	2.5
KRAS_c183AtoCTr_pQ61HH_C	100%	2.5
KRAS_c183AtoCTr_pQ61HH_T	100%	2.5
KRAS_c37GtoCATf_pG13RSC_C	100%	2.5
KRAS_c39CtoAGTr_pG13Multi_T	100%	2.5
KRAS_c437CtoGTf_pA146GV_G	100%	2.5
KRAS_c437CtoGTf_pA146GV_T	100%	2.5
NRAS_c176CtoGr_pA59G_G	100%	2.5
NRAS_c181CtoAGf_pQ61KE_A	100%	2.5
NRAS_c181CtoAGf_pQ61KE_G	100%	2.5
NRAS_c183AtoCTr_pQ61HH_C	96%	2.5

Assay	Specificity	Limit of Detection
NRAS_c183AtoCTr_pQ61HH_T	100%	2.5
NRAS_c35GtoCATr_pG12ADV_A	100%	2.5
NRAS_c35GtoCATr_pG12ADV_C	100%	2.5
NRAS_c36TtoACGr_pG12Multi_C	100%	2.5
NRAS_c38GtoCATr_pG13ADV_A	100%	2.5
NRAS_c437CtoGTr_pA146GV_G	100%	2.5
PIK3CA_c3140AtoGTf_pH1047RL_T	100%	2.5
EGFR_c1474AtoCf_pS492R_C	100%	5
KRAS_c175GtoATf_pA59TS_T	100%	5
KRAS_c176CtoAGr_pA59EG_A	100%	5
KRAS_c176CtoAGr_pA59EG_G	100%	5
KRAS_c181CtoAGf_pQ61KE_A	100%	5
KRAS_c37GtoCATf_pG13RSC_A	100%	5
KRAS_c436GtoACf_pA146TP_A	100%	5
NRAS_c182AtoCGTf_pQ61PRL_T	100%	5
NRAS_c350AtoGr_pK117R_G	100%	5
NRAS_c35GtoCATf_pG12ADV_C	100%	5
NRAS_c36TtoACGr_pG12Multi_G	100%	5
PIK3CA_c1624GtoAr_pE542K_A	100%	5
PIK3CA_c3140AtoGTf_pH1047RL_G	100%	5
KRAS_c351AtoCTr_pK117NN_C	100%	10
KRAS_c351AtoCTr_pK117NN_T	100%	10
KRAS_c436GtoACf_pA146TP_C	100%	10
NRAS_c436GtoCATr_pA146PTS_C	97%	0.63
NRAS_c436GtoCATr_pA146PTS_T	100%	1.25

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 iPLEX HS Colon 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.

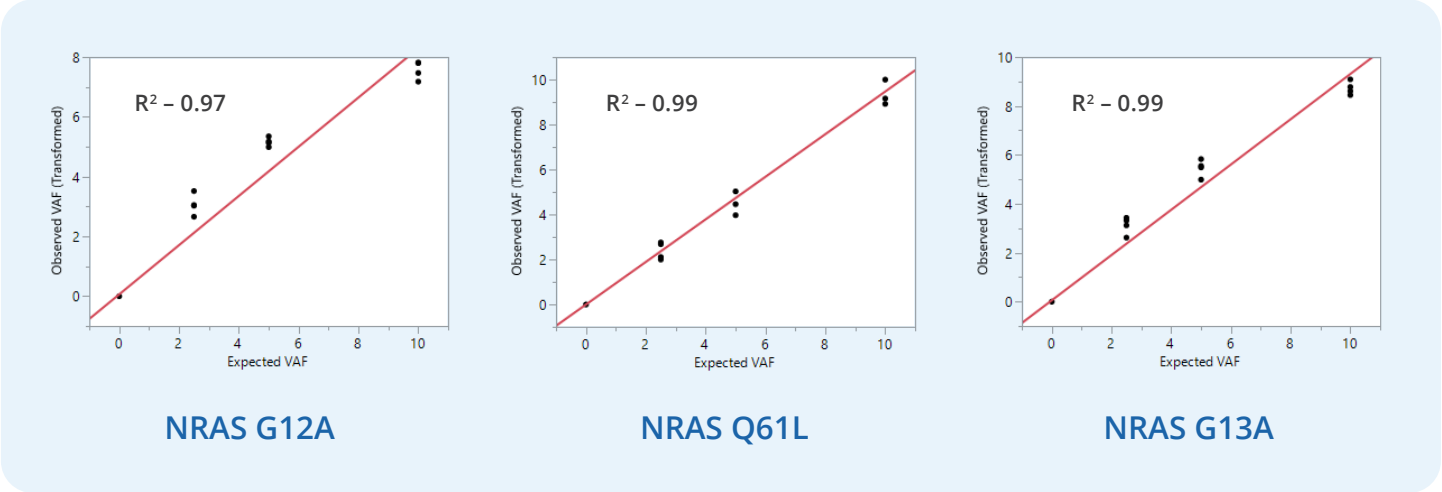

Figure 3: Example of expected vs. observed (transformed) allelic frequency

ACCURACY STUDY

Samples Tested

Assay performance was verified using SeraCare's Seraseq® Tri-Level Tumor Mutation DNA Mix v2 HC (Catalog #: 0710-0097) and Horizon Discovery's Tru-Q 0, 1, 2 and 3 (Catalog #: HD752, HD728, HD729, and HD730) reference standards. The SeraCare Seraseq Tri-Level mutation mix was also tested at a 25% dilution.

Results

Results from testing the reference standards were analyzed using the Somatic Variant Report software, with a z-score of 3 and an SNR cutoff ≥ 5. All the expected variants were successfully detected, and no false positive results were obtained.

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

Sample	Expected Variants(s)	Detected Variants	FN	FP	Sensitivity	Specificity
Horizon0*	BRAF:p.V600E c.1799T>A, KRAS:p.G13D c.38G>A, PIK3CA:p.H1047R c.3140A>G	3	0	0	100%	100%
Horizon1	BRAF:p.V600E c.1799T>A, KRAS:p.G12A c.35G>C, KRAS:p.G12R c.34G>C, KRAS:p.G13D c.38G>A, NRAS:p.Q61K c.181C>A, PIK3CA:p.H1047R c.3140A>G	6	0	0	100%	100%
Horizon2	BRAF:p.V600E c.1799T>A, KRAS:p.G12V c.35G>T, KRAS:p.G13D c.38G>A, KRAS:p.Q61L c.182A>T, NRAS:p.Q61L c.182A>T, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	7	0	0	100%	100%
Horizon3	BRAF:p.V600E c.1799T>A, KRAS:p.A146T c.436G>A, KRAS:p.G12S c.34G>A, KRAS:p.G13D c.38G>A, NRAS:p.Q61H c.183A>T, PIK3CA:p.E542K c.1624G>A, PIK3CA:p.H1047R c.3140A>G	7	0	0	100%	100%
NTC		0	0	0	100%	100%
SeraCare	BRAF:p.V600E c.1799T>A, KRAS:p.G12D c.35G>A, NRAS:p.Q61R c.182A>G, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	5	0	0	100%	100%
SeraCare Dilution	BRAF:p.V600E c.1799T>A, KRAS:p.G12D c.35G>A, NRAS:p.Q61R c.182A>G, PIK3CA:p.E545K c.1633G>A, PIK3CA:p.H1047R c.3140A>G	5	0	0	100%	100%
WT		0	0	0	100%	100%

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

FN = False Negative, FP = False Positive

REPRODUCIBILITY STUDY

The reproducibility of the HS Colon Panel v2 was determined by testing the Tru-Q (HD730) and the Complete Mutation mix (5%: 0710-0528; 2.5%: 0710-0529) reference standards from Horizon Discovery and SeraCare at two independent laboratories. Each standard has multiple variants interrogated in the panel and was replicated three times. Site one used both references, site two only used the Seracare reference.

Table 5: Summary of iPLEX HS Colon Panel v2 reproducibility study

Site/Panel	Sensitivity		Specificity
	5% VAF	2.5% VAF	
Site 1	100% PPA (48/48)	100% PPA (48/48)	99.3% NPA (858/864)
Site 2	100% PPA (24/24)	100% PPA (24/24)	99.2% NPA (756/762)

EVALUATION WITH FFPE SAMPLES

Assay performance was also evaluated with FFPE samples to verify performance in clinical specimens. Variants were successfully detected in FFPE specimens. The clinical "truth" of these samples was not known prior to testing.

Table 6

Sample	Variant(s) Detected
FFPE1	BRAF p.V600E c.1799T>A
FFPE2	None
FFPE3	KRAS p.G12C c.34G>T
FFPE4	KRAS p.G13D c.38G>A; PIK3CA p.E545K c.1633G>A
FFPE5	KRAS p.G12C c.34G>T
FFPE6	KRAS p.G13D c.38G>A, PIK3CA p.E545K c.1633G>A

SUMMARY

Results from the LoD and accuracy studies demonstrate that the iPLEX HS Colon Panel v2 has an excellent limit of detection, with the majority of the assays being able to detect variants at ≤5% with an average specificity of 99.99% 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. R.T. Birse, D. Irwin. Reliable Detection of Low Abundance Somatic Mutations of EGFR, KRAS, BRAF, NRAS and PIK3CA in Metastatic Colorectal Adenocarcinomas Using iPLEX HS, a New Highly Sensitive Assay for MassARRAY. Poster session presented at Association of Molecular Pathology Annual Meeting; 2016 Nov 10-12; Charlotte, NC.
3. Agena Bioscience. Baseline Creation Guide - iPLEX® HS and UltraSEEK® Panels v2 and ClearSEEK™ Panels. USG-CUS-135.

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