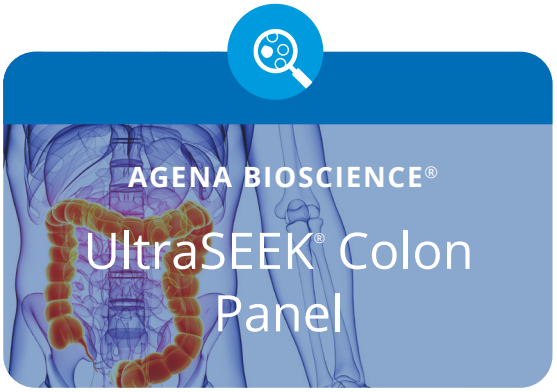
Ultra-sensitive and accurate detection of key variants in colorectal cancer liquid biopsy specimens

INTRODUCTION



Cancer is a complex disease with both inter- and intra- tumor heterogeneity, making it challenging to treat and manage effectively. Tissue biopsies provide only a snapshot of the disease as they are generally limited to a single site and are rarely repeated. In addition, rare acquired secondary and tertiary variants present at very low frequencies may easily be missed when analytical sensitivity of the detection assay is 5-10%. Liquid biopsies overcome these challenges by enabling the detection of circulating tumor DNA (ctDNA) present in blood plasma and derived from tumor sites across the body. While providing a more accurate picture of the disease, liquid biopsies have minimal risk and can easily be repeated to monitor disease and response to therapy.

The ideal technology for the detection of rare variants from cell-free DNA (cfDNA) would enable detection of multiple variants at as low as 0.1% variant allele frequency, have a quick turnaround time with simple analysis, and come at 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 the long turnaround times and extended costs associated with the increase in read depth to achieve the required limit of detection, they are not suited for rapid detection of known actionable targets and disease monitoring. Digital droplet (ddPCR) and real-time PCR (RT-PCR) assays are quick and not as expensive as NGS. However, they require a lot more 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 UltraSEEK® chemistry as an ideal solution for detecting rare variants from liquid biopsies in colorectal cancer (CRC). Using minimal DNA input of 20 ng, the majority of the variants can be detected at as low as 0.1% variant allele frequency. The MassARRAY uses a simple, PCR-based single-day workflow with easy analysis, making it ideally suited for disease monitoring and orthogonal validation studies.

Agena® BIOSCIENCE

ULTRASEEK® COLON PANEL V2

The UltraSEEK Colon Panel v2 was designed to enable study of disease progression and treatment monitoring from circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). The panel detects over 90 variants across five genes associated with CRC, using a low cfDNA input of 20 ng extracted from a single 10 mL blood draw.

Table 1: Variants detected by the UltraSEEK Colon v2 Panel

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

ULTRASEEK CHEMISTRY

UltraSEEK methodology uses multiplex PCR, followed by a variant-specific single base extension reaction (Figure 1). The extension reaction uses a variant-specific chain terminator labeled with a moiety for solid phase capture. After the capture, cleaning, and elution process, the extension products (analyte) are desalted, transferred to a SpectroCHIP® Array, and then loaded into the MassARRAY Analyzer and detected using time-of-flight measurements. Process and capture control assays are used to ensure the presence of DNA template in the reaction and success of the bead capture process. The Somatic Variant Report software rapidly provides an automated, easy to interpret readout of variants detected within each sample (Figure 2).

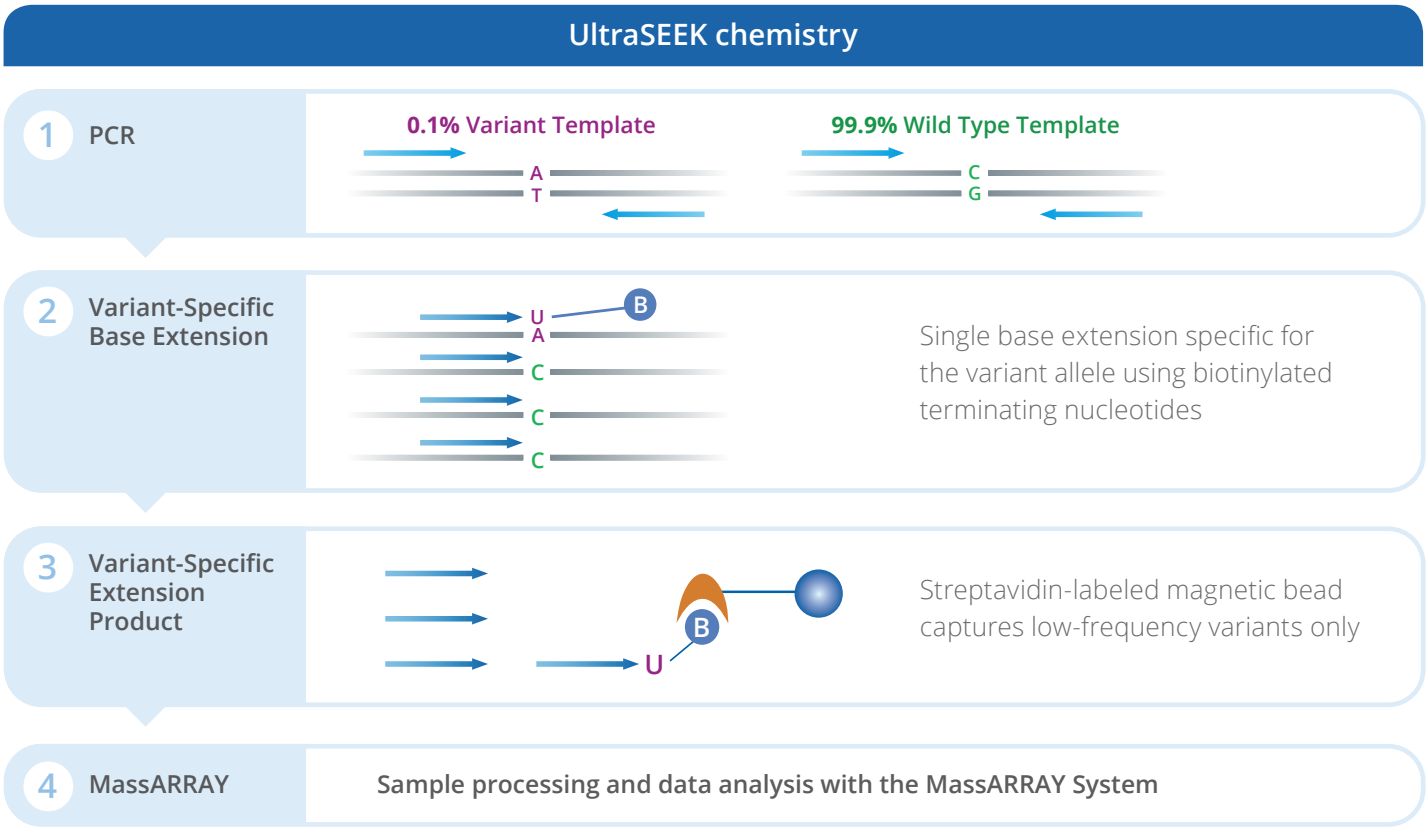


Figure 1: Rare variant detection with UltraSEEK chemistry on the MassARRAY system

SOMATIC VARIANT REPORT

The Somatic Variant Report enables easy analysis of the results from the UltraSEEK 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.

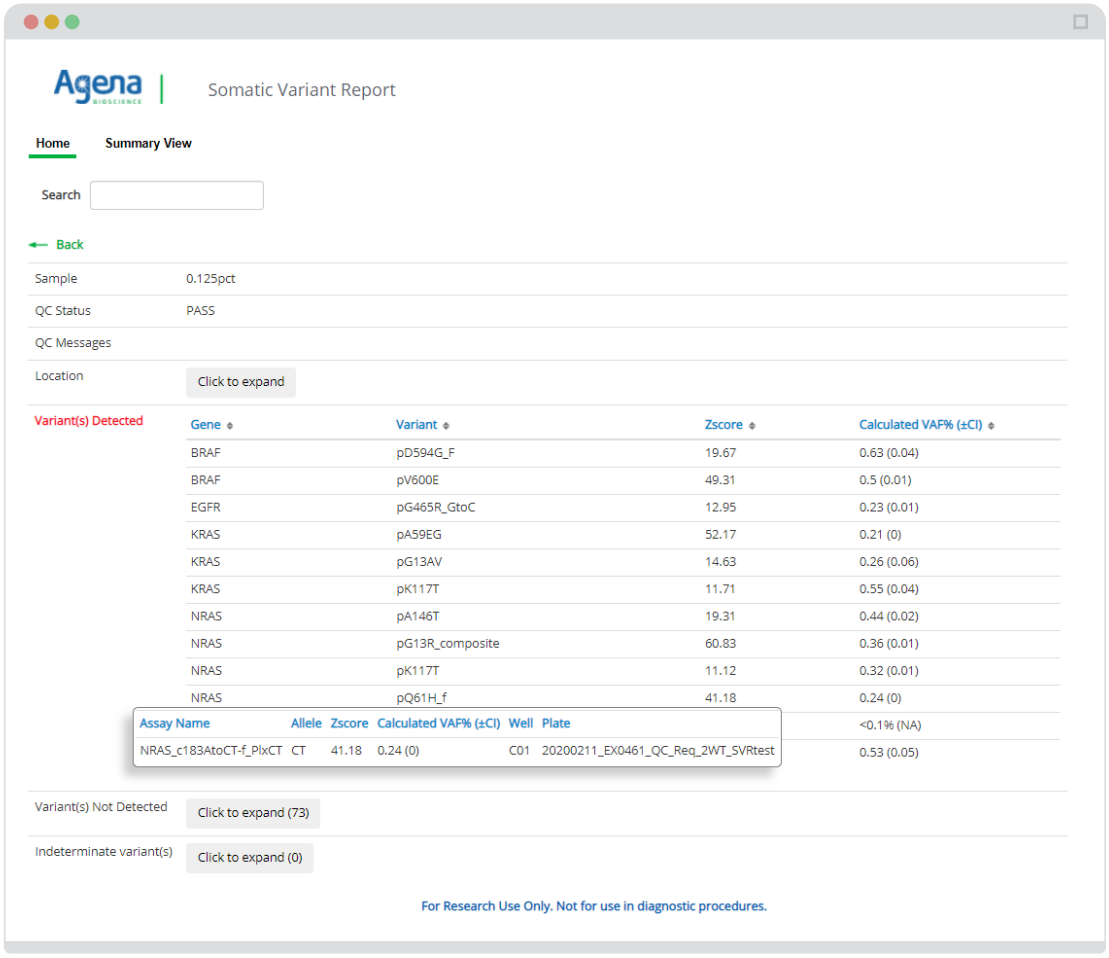


Figure 2: Example report output for Somatic Variant Report

LIMIT OF DETECTION (LOD) STUDY

Samples Tested

Limit of detection and specificity assessment were performed for every assay. A contrived model system was developed to assess the performance of each assay. The model consisted of synthetic double-stranded constructs known as gBlocks™ from Integrated DNA Technologies. Each construct contains a single variant present in the panel tested. 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 quantified for copy number by digital droplet PCR via a genomic reporter sequence. Allelic frequencies evaluated and corresponding copy numbers are listed in Table 2: Number of copies of mutant and wild type DNA used to generate mutant mixes.

Table 2: Number of copies of mutant and wild type DNA used to generate mutant mixes

% Mutant	2	1	0.5	0.25	0.125	0
Mutant Copy #/Rxn	160	80	40	20	10	0
WT Copy #/Rxn	8000	8000	8000	8000	8000	8000

Each minor variant mix was run in quadruplicate. The assay was considered sensitive to the level tested if three out of four (75%) of the replicates were positive (i.e., had a peak intensity [area] ≥5).

Assay Baseline

Assay baseline values were determined for each panel by processing 16 wildtype cfDNA samples on the MassARRAY System, as per the Baseline Creation Guide.[2]

Results

Assay limit of detection and specificity were determined using a z-score of 3 and 10 on the Somatic Variant Report software. The UltraSEEK Colon Panel v2 has high specificity ≥99.6% and low limit of detection ≤0.5% at both z-scores (Table 3).

Table 3: UltraSEEK Colon v2 Limit of Detection and Specificity Summary

Panel	Z-score = 3			Z-score = 10		
	Sensitivity		Specificity	Sensitivity		Specificity
	Variant Allele Frequency (VAF)	% of 96 assays detecting ≥75% of replicates		Variant Allele Frequency (VAF)	% of 96 assays detecting ≥75% of replicates	
UltraSEEK Colon v2	0.125%	72%	99.60%	0.125%	41%	100%
	0.25%	93%		0.25%	70%	
	0.5%	99%		0.5%	92%	
	1%	100%		1%	99%	
	2%	100%		2%	100%	

Sensitivity assessments were evaluated at z-scores 3-10 (Table 4), where sensitivity is defined as the number of assays detecting at least 3 out of 4 replicates at a given variant allele frequency. Specificity assessments were done with a minimum of 8 wild-type samples and were also evaluated using z-scores 3-10 (Table 5).

Table 4: Effect of z-score on sensitivity of UltraSEEK Lung v2 assays

Sensitivity	% of 96 assays detecting ≥75% of the replicates							
VAF	z3	z4	z5	z6	z7	z8	z9	z10
0.125%	72	66	61	54	51	48	43	41
0.25%	93	90	86	82	81	78	72	70
0.5%	99	99	99	97	96	95	92	92
1%	100	100	100	100	100	99	99	99
2%	100	100	100	100	100	100	100	100

Table 5: Effect of z-score on specificity of UltraSEEK Colon Panel v2 assays

	% of Total Assays at Each Given Specificity by zScore							
Specificity	**z3**	**z4**	**z5**	**z6**	**z7**	**z8**	**z9**	**z10**
100%	80	94	99	99	100	100	100	100
99%	8	5	1	1	0	0	0	0
98%	5	1	0	0	0	0	0	0
97%	3	0	0	0	0	0	0	0
96%	4	0	0	0	0	0	0	0

Table 6: UltraSEEK Colon Panel v2 Panel Sensitivity and Specificity with gBlocks using a z-score of 3 and area of 5

Assay	Variant	Limit of Detection	Specificity
BRAF_c1406GtoC-r_PlxG	BRAF_pG469A	0.125	98%
BRAF_c1799TtoA-r_PlxT	BRAF_pV600E	0.125	98%
EGFR_c1351CtoG-f_PlxG	EGFR_pR451G	0.125	99%
EGFR_c1393GtoC-r_PlxG	EGFR_pG465R	0.125	100%
EGFR_c1400AtoC-f_PlxC	EGFR_pK467T	0.125	100%
EGFR_c1474AtoC-f_PlxC	EGFR_pS492R	0.125	100%
EGFR_c1476CtoAG-r_PlxCT	EGFR_pS492R_G	0.125	100%
KRAS_c175GtoC-r_PlxG	KRAS_pA59P	0.125	100%
KRAS_c175GtoT-f_PlxT	KRAS_pA59S	0.125	100%
KRAS_c176CtoAG-r_PlxCT	KRAS_pA59E	0.125	100%
KRAS_c176CtoAG-r_PlxCT	KRAS_pA59G	0.125	100%
KRAS_c182AtoCT-f_PlxCT	KRAS_pQ61L	0.125	100%
KRAS_c182AtoCT-f_PlxCT	KRAS_pQ61P	0.125	100%
KRAS_c183AtoCT-f_PlxCT	KRAS_pQ61H_C	0.125	97%
KRAS_c183AtoCT-f_PlxCT	KRAS_pQ61H_T	0.125	97%
KRAS_c349AtoG-r_PlxC	KRAS_pK117E	0.125	100%
KRAS_c34GtoC-r_PlxG	KRAS_pG12R	0.125	100%
KRAS_c34GtoTC-f_PlxCT	KRAS_pG12C	0.125	100%

Assay	Variant	Limit of Detection	Specificity
KRAS_c34GtoTC-f_PlxCT	KRAS_pG12R	0.125	100%
KRAS_c350AtoC-r_PlxG	KRAS_pK117T	0.125	100%
KRAS_c350AtoG-r_PlxC	KRAS_pK117R	0.125	100%
KRAS_c351AtoCT-f_PlxCT	KRAS_pK117N_C	0.125	100%
KRAS_c35GtoA-r_PlxT	KRAS_pG12D	0.125	100%
KRAS_c35GtoCT-f_PlxCT	KRAS_pG12A	0.125	99%
KRAS_c35GtoCT-f_PlxCT	KRAS_pG12V	0.125	99%
KRAS_c36TtoAG-r_PlxCT	KRAS_pG12G_Multi_G	0.125	97%
KRAS_c36TtoAG-r_PlxCT	KRAS_pG12G_Multi_T	0.125	97%
KRAS_c37GtoA-r_PlxT	KRAS_pG13S	0.125	96%
KRAS_c37GtoCT-f_PlxCT	KRAS_pG13C	0.125	100%
KRAS_c37GtoCT-f_PlxCT	KRAS_pG13R	0.125	100%
KRAS_c38GtoA-r_PlxT	KRAS_pG13D	0.125	100%
KRAS_c38GtoC-r_PlxG	KRAS_pG13A	0.125	99%
KRAS_c38GtoCT-f_PlxCT	KRAS_pG13A	0.125	100%
KRAS_c38GtoCT-f_PlxCT	KRAS_pG13V	0.125	100%
KRAS_c436GtoC-r_PlxG	KRAS_pA146P	0.125	100%
KRAS_c437CtoG-f_PlxG	KRAS_pA146G	0.125	99%
NRAS_c175GtoA-r_PlxT	NRAS_pA59T	0.125	100%
NRAS_c176CtoG-r_PlxC	NRAS_pA59G	0.125	100%
NRAS_c181CtoAG-r_PlxCT	NRAS_pQ61E	0.125	100%
NRAS_c182AtoCT-f_PlxCT	NRAS_pQ61P	0.125	97%
NRAS_c182AtoG-r_PlxC	NRAS_pQ61R	0.125	100%
NRAS_c183AtoCT-f_PlxCT	NRAS_pQ61H_C	0.125	100%
NRAS_c183AtoCT-f_PlxCT	NRAS_pQ61H_T	0.125	100%
NRAS_c183AtoG-r_PlxG	NRAS_pQ61Q_Multi	0.125	96%
NRAS_c349AtoG-r_PlxC	NRAS_pK117E	0.125	100%
NRAS_c34GtoA-r_PlxT	NRAS_pG12S	0.125	99%
NRAS_c34GtoC-r_PlxG	NRAS_pG12R	0.125	98%
NRAS_c34GtoCT-f_PlxCT	NRAS_pG12R	0.125	100%

Assay	Variant	Limit of Detection	Specificity
NRAS_c350AtoC-r_PlxC	NRAS_pK117T	0.125	98%
NRAS_c350AtoG-r_PlxC	NRAS_pK117R	0.125	100%
NRAS_c351GtoCT-f_PlxCT	NRAS_pK117N_C	0.125	100%
NRAS_c35GtoA-r_PlxT	NRAS_pG12D	0.125	99%
NRAS_c36TtoAG-r_PlxCT	NRAS_pG12G_Multi_A	0.125	97%
NRAS_c36TtoAG-r_PlxCT	NRAS_pG12G_Multi_G	0.125	97%
NRAS_c37GtoA-r_PlxT	NRAS_pG13S	0.125	100%
NRAS_c37GtoC-r_PlxG	NRAS_pG13R	0.125	100%
NRAS_c37GtoCT-f_PlxCT	NRAS_pG13C	0.125	100%
NRAS_c37GtoCT-f_PlxCT	NRAS_pG13R	0.125	100%
NRAS_c38GtoA-r_PlxT	NRAS_pG13D	0.125	100%
NRAS_c38GtoC-r_PlxG	NRAS_pG13A	0.125	100%
NRAS_c38GtoCT-f_PlxCT	NRAS_pG13A	0.125	100%
NRAS_c436GtoCT-f_PlxCT	NRAS_pA146P	0.125	98%
NRAS_c436GtoCT-f_PlxCT	NRAS_pA146S	0.125	98%
NRAS_c437CtoG-f_PlxT	NRAS_pA146G	0.125	100%
NRAS_c437CtoT-f_PlxT	NRAS_pA146V	0.125	100%
PIK3CA_c1633GtoA-r_PlxT	PIK3CA_pE545K	0.125	96%
PIK3CA_c3140AtoG-r_PlxC	PIK3CA_pH1047R	0.125	100%
PIK3CA_c3140AtoT-f_PlxT	PIK3CA_pH1047L	0.125	98%
BRAF_c1406GtoA-r_PlxT	BRAF_pG469E	0.25	98%
BRAF_c1781AtoG-f_PlxG	BRAF_pD594G_F	0.25	100%
BRAF_c1781AtoT-f_PlxT	BRAF_pD594V_F	0.25	100%
EGFR_c1351CtoA-r_PlxT	EGFR_pR451S	0.25	100%
EGFR_c1391CtoT-f_PlxT	EGFR_pS464L	0.25	100%
EGFR_c1393GtoA-r_PlxT	EGFR_pG465R	0.25	100%
EGFR_c1473AtoG-r_PlxC	EGFR_pI491M	0.25	100%
KRAS_c175GtoA-r_PlxT	KRAS_pA59T	0.25	100%
KRAS_c181CtoAG-r_PlxCT	KRAS_pQ61E	0.25	100%
KRAS_c182AtoG-r_PlxC	KRAS_pQ61R	0.25	100%

Assay	Variant	Limit of Detection	Specificity
KRAS_c34GtoA-r_PlxT	KRAS_pG12S	0.25	100%
KRAS_c351AtoCT-f_PlxCT	KRAS_pK117N_T	0.25	100%
KRAS_c436GtoA-r_PlxT	KRAS_pA146T	0.25	100%
KRAS_c437CtoT-f_PlxT	KRAS_pA146V	0.25	100%
NRAS_c181CtoAG-r_PlxCT	NRAS_pQ61K	0.125	100%
NRAS_c182AtoCT-f_PlxCT	NRAS_pQ61L	0.25	97%
NRAS_c183AtoC-r_PlxC	NRAS_pQ61H	0.25	100%
NRAS_c34GtoCT-f_PlxCT	NRAS_pG12C	0.25	100%
NRAS_c351GtoCT-f_PlxCT	NRAS_pK117N_T	0.25	100%
NRAS_c35GtoCT-f_PlxCT	NRAS_pG12A	0.25	100%
NRAS_c436GtoA-r_PlxT	NRAS_pA146T	0.25	100%
PIK3CA_c1624GtoA-r_PlxT	PIK3CA_pE542K	0.25	99%
EGFR_c1351CtoT-f_PlxT	EGFR_pR451C	0.5	100%
EGFR_c1394GtoA-r_PlxT	EGFR_pG465E	1.0	100%
EGFR_c1476CtoAG-r_PlxCT	EGFR_pS492R_A	0.5	100%
KRAS_c181CtoAG-r_PlxCT	KRAS_pQ61K	0.5	100%
NRAS_c35GtoCT-f_PlxCT	NRAS_pG12V	0.5	100%
NRAS_c38GtoCT-f_PlxCT	NRAS_pG13V	0.5	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 normalized UltraSEEK 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 Somatic Variant Report, along with the z-score.

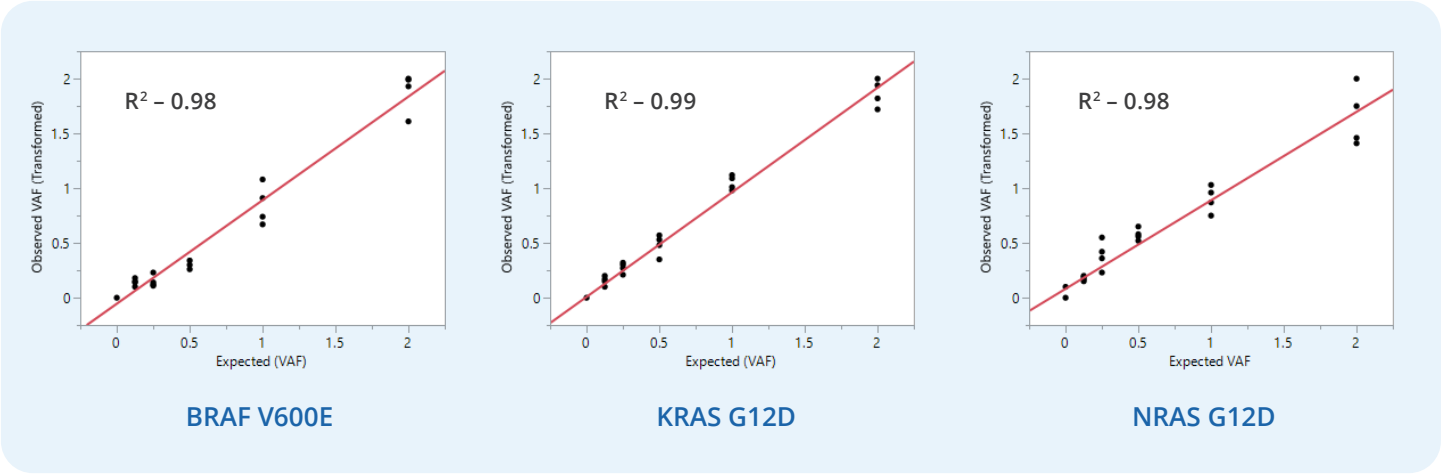


Figure 3: Example of expected vs. observed (transformed) allelic frequency

ACCURACY STUDY

Samples Tested

Assay performance in cfDNA samples was verified using the SeraCare Seraseq® ctDNA Complete™ Mutation Mix ctDNA reference standard, which contains 6 variants that can be detected using UltraSEEK Colon panel v2.

Allele frequencies of 1% (Catalog# 0710-0530), 0.5% (Catalog# 0710-0531), and 0.1% (Catalog# 0710-0532) were tested in triplicate, in addition to wild-type and non-template controls. The results for the reference standard were analyzed using a z-score of 3. 100% of the 6 variants assays characterized with this reference material were detected at ≥1% VAF (3/3 replicates).

Table 7: UltraSEEK Colon v2 Assay performance with reference standards

Variant	Assay	Allele Frequency	Results
BRAF_pV600E	BRAF_c1799TtoA-r_PlxT	1%	3/3
		0.5%	3/3
		0.1%	3/3
KRAS_pG12D	KRAS_c35GtoA-r_PlxGT	1%	3/3
		0.5%	3/3
		0.1%	3/3
KRAS_pG12CR	KRAS_c34GtoCT-f_PlxCT	1%	3/3
		0.5%	3/3
		0.1%	1/3
KRAS_pQ61H	KRAS_c183AtoCT-f_PlxCT	1%	3/3
		0.5%	3/3
		0.1%	3/3
NRAS_pQ61R	NRAS_c182AtoG-r_PlxC	1%	3/3
		0.5%	3/3
		0.1%	2/3
PIK3CA_pH1047R	PIK3CA_c3140AtoG-r_PlxC	1%	3/3
		0.5%	3/3
		0.1%	2/3

REPRODUCIBILITY STUDY

The reproducibility of the UltraSEEK Colon Panel v2 was determined by testing SeraCare Seraseq® ctDNA Complete Panel Mutation mix cfDNA reference standard at two independent laboratories at variant allele frequencies of 1% and 0.5%. Both sites were able to detect 100% of the variants at 1% VAF and ≥97% at 0.5% VAF demonstrating the robustness of the assay across different users and instruments (Table 8).

Table 8: Summary of reproducibility study

Variant	Reference Standard (Detected/Replicates)			
	Seracare 1%		Seracare 0.5%	
	Site #1	Site #2	Site #1	Site #2
PIK3CA pH1047R	(3/3)	(3/3)	(3/3)	(3/3)
NRAS pQ61R	(3/3)	(3/3)	(3/3)	(3/3)
KRAS pG12D	(3/3)	(3/3)	(3/3)	(3/3)
KRAS pG12R	(3/3)	(3/3)	(3/3)	(3/3)
KRAS pQ61H	(3/3)	(3/3)	(3/3)	(2/3)
BRAF pV600E	(3/3)	(3/3)	(3/3)	(3/3)
%PPA	**100% (36/36)**		**97% (35/36)**	

EVALUATION WITH cfDNA SAMPLES

The performance of the panels was evaluated with clinical cfDNA samples for demonstration of functionality of the panel in relevant sample types. A z-score of 3 was used for data analysis.

Table 9: UltraSEEK Colon Panel v2 results with cfDNA samples

Sample	Assay	Observed Variant	Calculated VAF (%)
LB15-0674	KRAS_c35GtoA-r_PlxT	KRAS_pG12D	>2
LB16-0023	KRAS_c37GtoCT-f_PlxCT	KRAS_pG13RC	>2
LB16-0066	KRAS_c183AtoCT-f_PlxCT	KRAS_pQ61H	>2
	BRAF_c1781AtoG-f_PlxG	BRAF_pD594G_F	>2
	KRAS_c34GtoA-r_PlxT	KRAS_pG12S	0.21
LB16-0514	WT	WT	NA
LB16-0681	KRAS_c183AtoCT-f_PlxCT	KRAS_pQ61H	0.07
LB17-0232	KRAS_c34GtoTC-f_PlxCT	KRAS_pG12CR	>2
	KRAS_c34GtoC-r_PlxG	KRAS_pG12R	0.94
LB17-1199	KRAS_c183AtoCT-f_PlxCT	KRAS_pQ61H	>2

SUMMARY

92% of the assays in the UltraSEEK Colon Panel v2 have a limit of detection ≤0.5%, and a specificity 100% (when z-score = 10). The UltraSEEK Colon panel is a reliable and ultrasensitive alternative for detecting clinically relevant variants in CRC and can be used to detect variants at a sensitivity appropriate for liquid biopsy samples. The range of somatic mutations in the panel allows the user to maximize coverage of clinically relevant variants while minimizing DNA input. All assays across the panel were characterized for limit of detection, and calibration curves were developed for each assay. These values are implemented in the Somatic Variant Report software, to enable reporting of variant allele frequencies for all assays. The linear response at liquid biopsy-relevant allelic frequencies makes the UltraSEEK Colon Panel v2 an ideal choice for monitoring the efficacy of treatment or residual disease in colorectal cancer.

References

1. DNA input and effect of copy number at different allele frequencies
https://blog.seracare.com/ngs/how-many-target-copies-are-present-in-your-plasma-dna-sample
2. Baseline Creation Guide - iPLEX® HS and UltraSEEK® Panels v2 and ClearSEEK™ Panels [USG-CUS-135]

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