Ultra-sensitive and accurate detection of key variants in melanoma 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 variants and disease monitoring. Digital droplet (ddPCR) and real-time PCR (RT-PCR) assays are quick and not as expensive as NGS. However, they require substantially 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 melanomas. Using minimal DNA input, more than 60 variants can be detected at as low as 0.1% variant allele frequency (VAF). The MassARRAY system 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® MELANOMA PANEL V2

The UltraSEEK Melanoma Panel was designed to enable study of disease progression and resistance from circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). The panel detects 61 variants across 76 assays using a low cell-free DNA (cfDNA) input of 20 ng extracted from a single 10 mL blood draw.

Table 1: Variants detected by the UltraSEEK Melanoma Panel v2

Gene	# of Variants
BRAF	*13*
CDKN2A	*1*
CTNNB1	*4*
IDH1	*2*
KIT	*7*
MAP2K1	*7*
NRAS	*19*
RAC1	*1*
RPS27	*1*
RQCD	*1*
SDHD	*3*
YAE1D1	*2*

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).

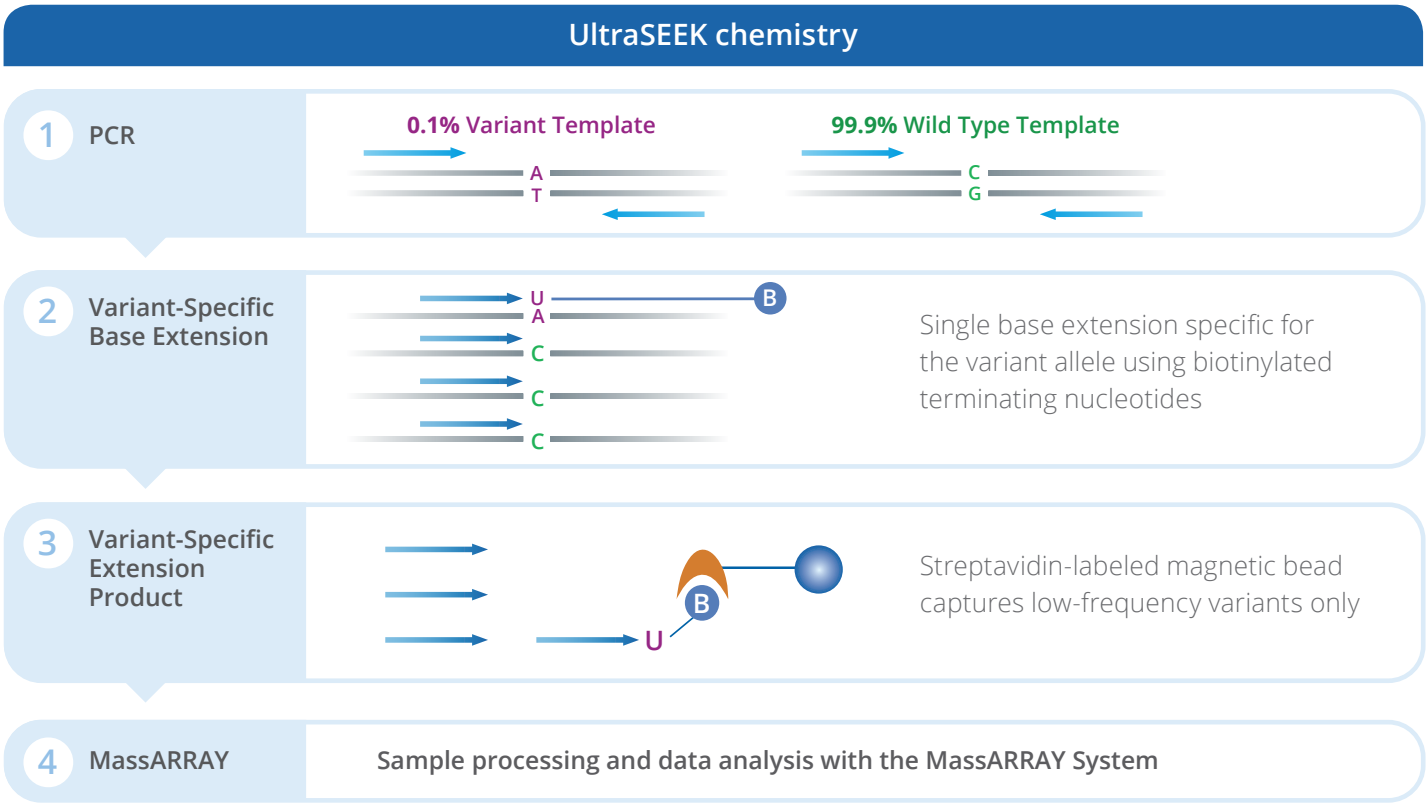

Figure 1: Rare variant detection with UltraSEEK chemistry on the MassARRAY System

SOMATIC VARIANT REPORT

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

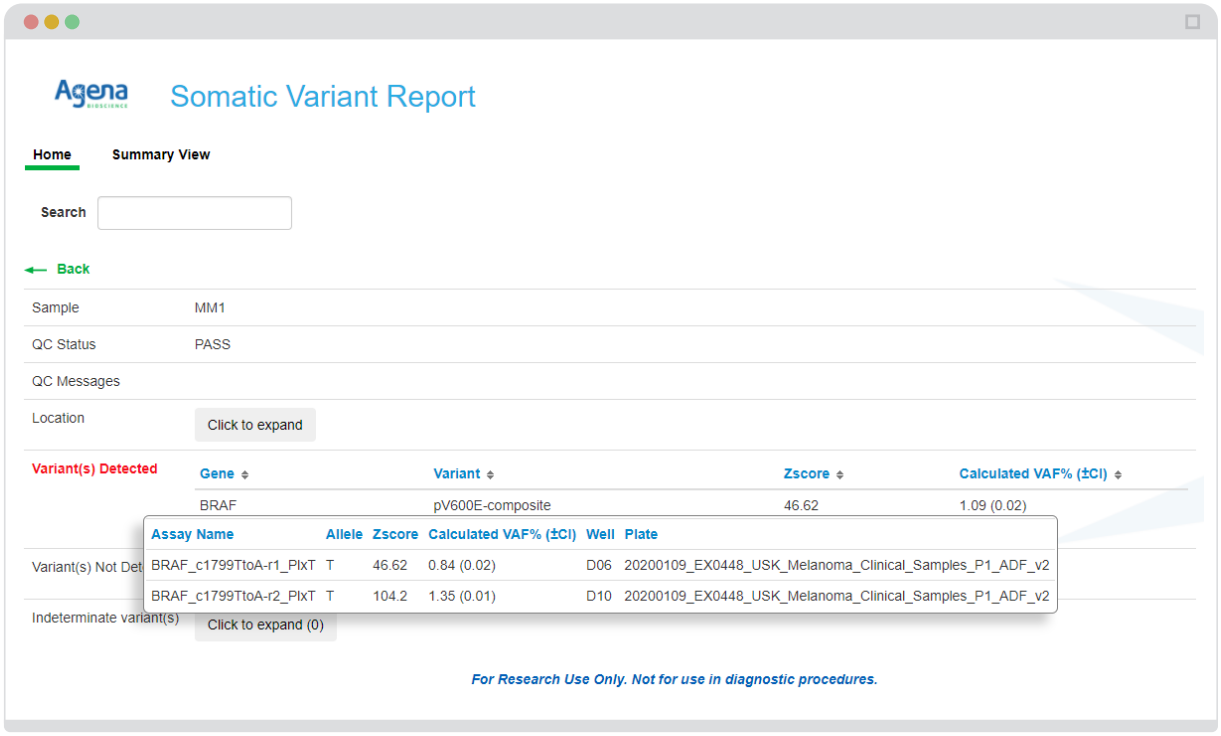

Figure 2: Example report output for Somatic Variant Report

LIMIT OF DETECTION

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 individually 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.

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

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. Both panels retain high specificity ≥98% and low limit of detection ≤0.5% at both z-scores (Table 3).

Table 3: UltraSEEK Melanoma v2 assay limit of detection and specificity

Panel	Z-score = 3			Z-score = 10		
	Sensitivity		Specificity	Sensitivity		Specificity
	Variant Allele Frequency (VAF)	% of 81 assays detecting ≥75% of replicates		Variant Allele Frequency (VAF)	% of 81 assays detecting ≥75% of replicates	
UltraSEEK Melanoma v2	0.125%	72%	99.60%	0.125%	40%	100%
	0.25%	91%		0.25%	67%	
	0.5%	99%		0.5%	89%	
	1%	100%		1%	96%	
	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 Melanoma v2 assays

	% of 81 assays detecting ≥75% of replicates							
Sensitivity	z3	z4	z5	z6	z7	z8	z9	z10
0.125	72	62	59	52	49	44	43	40
0.25	91	87	85	83	80	74	71	67
0.5	99	99	98	96	93	92	92	89
1	100	100	100	100	100	99	98	96
≥2	100	100	100	100	100	100	100	100

Z-score can also impact the specificity of the assays (the percentage of variants detected in wild-type samples). Note that the number of variants called is less than the total number of assays, due to assay redundancy.

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

	% of 81 Assays at Each Given Specificity							
Specificity	**z3**	**z4**	**z5**	**z6**	**z7**	**z8**	**z9**	**z10**
100%	91	96	98	98	98	98	99	99
99%	4	1	1	1	1	2	1	1
98%	2	1	0	0	0	0	0	0
97%	1	1	1	1	1	0	0	0
96%	1	0	0	0	0	0	0	0

The limit of detection and specificity for each assay in the UltraSEEK Melanoma Panel v2 (Table 6) at a z-score of 3, are shown below.

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

Assay	Variant	Limit of Detection (%VAF)	Specificity
BRAF_c1779TtoG-f1_PlxG	BRAF_D594N_TG>GA	0.125	100%
BRAF_c1780GtoA-r1_PlxT	BRAF_D594N	0.125	100%
BRAF_c1780GtoC-f1_PlxC	BRAF_D594H	0.125	100%
BRAF_c1782TtoA-r1_PlxT	BRAF_D594E	0.125	99%
BRAF_c1798GtoA-ASO2_PlxT	BRAF_V600K	0.125	100%
BRAF_c1798GtoA-r1_PlxT	BRAF_V600M	0.125	100%
BRAF_c1799TtoA-r1_PlxT	BRAF_V600E	0.125	100%
BRAF_c1799TtoA-r2_PlxT	BRAF_V600E	0.125	100%
BRAF_c1799TtoG-f1_PlxG	BRAF_V600G	0.125	100%
BRAF_c1799TtoG-f2_PlxG	BRAF_V600R_GT>AG	0.125	100%
BRAF_c1799TtoG-r1_PlxC	BRAF_V600G	0.125	100%
BRAF_c1799TtoG-r2_PlxC	BRAF_V600G	0.125	100%
BRAF_c1800GtoA-r1_PlxT	BRAF_V600E_TG>AA	0.125	100%
BRAF_c1800GtoT-f1_PlxT	BRAF_V600D_TG>AT	0.125	100%
BRAF_c1801AtoG-r1_PlxC	BRAF_K601E	0.125	100%
BRAF_c1801AtoG-r2_PlxC	BRAF_K601E	0.125	100%
CDKN2A_c238CtoT-f1_PlxT	CDKN2A_R80X	0.125	100%
CDKN2A_c238CtoT-f2_PlxT	CDKN2A_R80X	0.125	100%
CTNNB1_c110CtoA-r1_PlxT	CTNNB1_S37Y	0.125	100%
CTNNB1_c133TtoC-f1_PlxC	CTNNB1_S45P	0.125	99%
CTNNB1_c134CtoA-r1_PLxT	CTNNB1_S45Y	0.125	100%
CTNNB1_c134CtoT-f1_PlxT	CTNNB1_S45F	0.125	100%
IDH1_c394CtoT-f2_PlxT	IDH1_R132C	0.25	100%
IDH1_c395GtoA-r1_PlxT	IDH1_R132H	0.125	97%
IDH1_c395GtoA-r2_PlxT	IDH1_R132H	0.125	100%
KIT_c1669TtoA-r1_PlxT	KIT_W557R	0.125	98%

Assay	Variant	Limit of Detection (%VAF)	Specificity
KIT_c1669TtoA-r2_PlxT	KIT_W557R	0.125	100%
KIT_c1676TtoA-r1_PlxT	KIT_V559D	0.5	100%
KIT_c1676TtoC-f2_PlxC	KIT_V559A	0.125	100%
KIT_c1727TtoC-f2_PlxC	KIT_L576P	0.125	100%
KIT_c1924AtoG-r1_PlxC	KIT_K642E	0.125	100%
MAP2K1_c1144AtoC-f1_PlxC	MAP2K1_N382H	0.125	100%
MAP2K1_c157TtoC-f1_PlxC	MAP2K1_F53L	0.125	100%
MAP2K1_c157TtoC-f2_PlxC	MAP2K1_F53L	0.125	96%
MAP2K1_c332TtoG-f1_PlxG	MAP2K1_I111S	0.125	100%
MAP2K1_c370CtoT-f1_PlxT	MAP2K1_P124S	0.125	100%
MAP2K1_c370CtoT-f2_PlxT	MAP2K1_P124S	0.125	98%
NRAS_c181CtoG-f1_PlxG	NRAS_Q61E	0.125	100%
NRAS_c182AtoC-f1_PlxC	NRAS_Q61P	0.125	100%
NRAS_c183AtoC-r1_PlxG	NRAS_Q61H	0.125	100%
NRAS_c183AtoG-r1_PlxC	NRAS_Q61RL	0.125	100%
NRAS_c183AtoG-r2_PlxC	NRAS_Q61RL	0.125	100%
NRAS_c183AtoT-f1_PlxT	NRAS_Q61H	0.125	100%
NRAS_c183AtoT-f2_PlxT	NRAS_Q61H	0.125	100%
NRAS_c34GtoA-r1_PlxT	NRAS_G12S	0.125	100%
NRAS_c34GtoT-f1_PlxT	NRAS_G12C	0.125	100%
NRAS_c35GtoA-r1_PlxT	NRAS_G12D	0.125	100%
NRAS_c37GtoC-f1_PlxC	NRAS_G13R	0.125	100%
NRAS_c37GtoC-r1_PlxG	NRAS_G13R	0.125	100%
NRAS_c38GtoA-r1_PlxT	NRAS_G13D	0.125	100%
NRAS_c38GtoC-r1_PlxG	NRAS_G13A	0.125	100%
NRAS_c38GtoT-f1_PlxT	NRAS_G13V	0.125	100%
RPS27_c238CtoT-f1_PlxT	RPS27_UTR_MUT	0.25	100%
RQCD1_c392CtoT-f1_PlxT	RQCD1_P131L	0.125	100%
YAE1D1_c39605965GtoA-r1_PlxT	YAE1D1_MUT	0.125	100%
YAE1D1_c39605969GtoA-r1_PlxT	YAE1D1_MUT	0.125	100%

Assay	Variant	Limit of Detection (%VAF)	Specificity
KIT_c2447AtoT-f1_PlxT	KIT_D816V	0.125	100%
NRAS_c34GtoC-f1_PlxC	NRAS_G12R	0.125	100%
NRAS_c35GtoC-f1_PlxC	NRAS_G12A	0.125	100%
NRAS_c37GtoT-f1_PlxT	NRAS_G13C	0.125	100%
SDHD_c111957523-f1_PlxT	SDHD_MUT	0.125	100%
BRAF_c1781AtoT-f1_PlxT	BRAF_D594V	0.25	100%
IDH1_c394CtoT-f2_PlxT	IDH1_R132C	0.25	100%
KIT_c1676TtoC-f1_PlxC	KIT_V559A	0.25	100%
KIT_c2446GtoC-r1_PlxG	KIT_D816H	0.25	100%
MAP2K1_c332TtoG-r1_PlxC	MAP2K1_I111S	0.25	100%
MAP2K1_c362GtoC-f1_PlxC	MAP2K1_C121S	0.25	99%
MAP2K1_c362GtoC-f2_PlxC	MAP2K1_C121S	0.25	100%
MAP2K1_c607GtoA-r2_PlxT	MAP2K1_E203K	0.25	100%
MAP2K1_c790CtoT-f1_PlxT	MAP2K1_P264S	0.25	100%
NRAS_c181CtoA-r2_PlxT	NRAS_Q61K	0.25	100%
NRAS_c181CtoG-r1_PlxC	NRAS_Q61E	0.25	100%
NRAS_c182AtoG-r1_PlxC	NRAS_Q61R	0.25	100%
NRAS_c35GtoT-f1_PlxT	NRAS_G12V	0.25	100%
RAC1_c85CtoT-f1_PlxT	RAC1_P29S	0.25	100%
NRAS_c181CtoA-r1_PlxT	NRAS_Q61K	0.25	100%
SDHD_c111957541CtoT-f1_PlxT	SDHD_MUT	0.25	100%
KIT_c1727TtoC-f1_PlxC	KIT_L576P	0.5	100%
NRAS_c182AtoT-f1_PlxT	NRAS_Q61L	0.5	100%
SDHD_c111957544CtoT-f1_PlxT	SDHD_MUT	0.5	100%
MAP2K1_c607GtoA-r1_PlxT	MAP2K1_E203K	1	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.

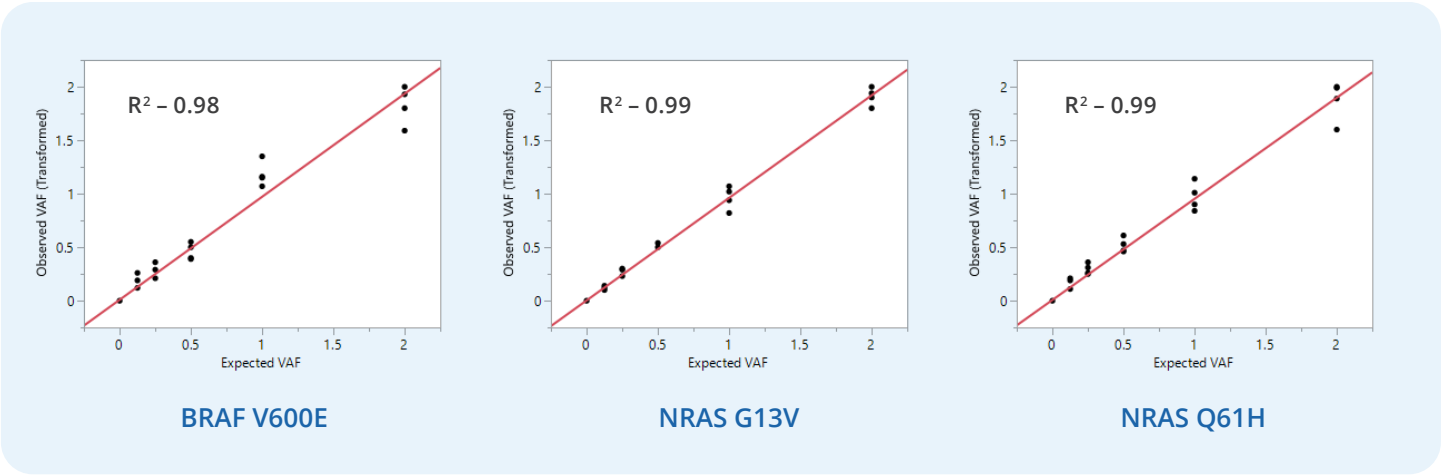

Figure 3: Example of expected vs. observed (transformed) allelic frequency

ACCURACY STUDY

Samples Tested

Assay performance in cfDNA samples was verified using the Seraseq® ctDNA Complete™ Mutation Mix ctDNA reference standard, which contains 4 variants that can be detected using 5 assays in the UltraSEEK Melanoma Panel v2.

Allele frequencies of 1% (Catalog# 0710-0530), 0.5% (Catalog# 0710-0531), 0.25% (), and 0.125% (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 variants were detected at VAFs of 1%, 0.5%, and 0.25%. At 0.125% VAF, the BRAF V600E and IDH1 132C variants were detected across all three replicates while the KIT D816V and NRAS Q61 variants were only detected in one and two of the replicates, respectively.

Table 7: UltraSEEK Melanoma v2 Assay performance with reference standards

Variant	Assay	Allele Frequency	Results
BRAF_V600E	BRAF_c1799TtoA-r1_PlxT	1.0%	3/3
		0.5%	2/2
		0.25%	3/3
		0.125%	3/3
BRAF_V600E	BRAF_c1799TtoA-r2_PlxT	1.0%	3/3
		0.5%	2/2
		0.25%	3/3
		0.125%	3/3
IDH1_R132C	IDH1_c394CtoT-f1_PlxT	1.000%	3/3
		0.5%	2/2
		0.25%	3/3
		0.125%	3/3
KIT_D816V	KIT_c2447AtoT-f1_PlxT	1.0%	3/3
		0.5%	2/2
		0.25%	3/3
		0.125%	1/3
NRAS_Q61R	NRAS_c182AtoG-r1_PlxC	1.0%	3/3
		0.5%	2/2
		0.25%	3/3
		0.125%	2/3
% PPA			**95% (52/55)**

EVALUATION WITH cfDNA SAMPLES

The performance of the panels was evaluated with clinical cfDNA samples. The UltraSEEK Melanoma Panel v2 successfully detected variants in cfDNA samples (Table 8). These samples were not previously characterized for the variants in the USK Melanoma panel. Each variant detected was positive across the redundant assays, providing greater confidence in the call.

Table 8

Sample	Observed Variant (zScore - 10)	Observed VAF
LB16-0031	IDH1_R132H*	0.25%
LB16-0299**	MAP2K1_pF53L*	0.15%
LB16-1039	BRAF_pV600E*	0.50%
MM1	BRAF_pV600E*	0.50%

** Agena USK Melanoma panel has redundant content confirming the result*

*** zScore - 3 used as threshold*

SUMMARY

89% of assays in the UltraSEEK Melanoma Panel have a limit of detection ≤0.5%, and a specificity 100% (when z-score = 10). This UltraSEEK panel is a reliable and ultrasensitive alternative for detecting clinically relevant variants in Melanoma and can be used to detect variants at a sensitivity appropriate for liquid biopsy samples. The range of somatic mutations allows the user to maximize coverage of clinically relevant variants while minimizing DNA input. All assays 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 Melanoma panel v2 an ideal choice for monitoring the efficacy of treatment or residual disease in melanomas.

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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