5-1-2019

Genomic analysis of circulating tumor DNA using a melanoma-specific UltraSEEK Oncogene Panel

Elin S. Gray
Edith Cowan University

Tom Witkowski

Michelle Pereira
Edith Cowan University

Leslie Calapre
Edith Cowan University

Karl Herron

See next page for additional authors

10.1016/j.jmoldx.2018.12.001
This is an Author's Accepted Manuscript of: Gray, E. S., Witkowski, T., Pereira, M., Calapre, L., Herron, K., Irwin, D., ... Wong, S. Q. (2019). Genomic analysis of circulating tumor DNA using a melanoma-specific UltraSEEK Oncogene Panel. *The Journal of Molecular Diagnostics, 21*(3), 418-426. Available here

TITLE:

Genomic analysis of circulating tumour DNA using a melanoma-specific UltraSEEK Oncogene panel.

FULL NAMES OF AUTHORS:

Elin S. Gray[1], Tom Witkowski[2], Michelle Pereira[1], Leslie Calapre[1], Karl Herron[3], Darryl Irwin[3], Brett Chapman[3], Muhammad A. Khattak[1,4,5], Jeanette Raleigh[6], , Athena Hatzimihalis[6], Jonathan Cebon[2], Shahneen Sandhu[6], Grant A. McArthur[6], Michael Millward[4,7], Melanie Ziman[1,8], Alexander Dobrovic[2,9.10], Stephen Q. Wong[6,11]

[1]School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, Australia

[2]Olivia Newton-John Cancer Wellness and Research Centre, Heidelberg, Victoria, Australia

[3]Agena Bioscience, Brisbane, Australia

[4]School of Medicine and Pharmacology, The University of Western Australia, Crawley, Western Australia, Australia

[5]Department of Medical Oncology, Fiona Stanley Hospital, Murdoch, Western Australia, Australia.

[6]Cancer Research, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

[7]Department of Medical Oncology, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia.

[8]School of Biomedical Sciences, The University of Western Australia, Crawley, Western Australia, Australia

[9]School of Cancer Medicine, La Trobe University, Victoria, Australia

[10]Department of Clinical Pathology, University of Melbourne, Victoria, Australia

[11]Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

SHORT RUNNING HEAD: MassArray for melanoma ctDNA

NUMBER OF TEXT PAGES: 10

NUMBER OF FIGURES/TABLES: 2 Tables, 3 Figure, 2 Supplementary Tables

GRANT NUMBERS AND SOURCES OF SUPPORT: EG is supported by a fellowship from the Cancer Research Trust. This work was supported by the Peter MacCallum Cancer Foundation and a Gundry Perpetual Endowment (1607) to SW. This study was partially funded by a Western Australia Cancer Council grant (1100249) to EG and a National Health and Medical Research (NHMRC) grant (1046711) to MZ.

DISCLOSURES: K. Herron, D. Irwin and B. Chapman are employees of Agena Bioscience. S.Q.Wong, E. Gray and T. Witkowski have received travel and accommodation funding from Bio-Rad Laboratories. No potential conflicts of interest were disclosed by the other authors.

CORRESPONDING AUTHOR: Elin Solomonovna Gray, Edith Cowan University, 270 Joondalup Drive, Joondalup, 6027, Western Australia, Phone#: 08 6304 5171, E-mail: e.gray@ecu.edu.au

ABSTRACT

The analysis of circulating tumour DNA (ctDNA) provides a minimally-invasive molecular interrogation that has the potential to guide treatment selection and disease monitoring. In this study, we evaluated a custom UltraSEEK melanoma panel for the MassARRAY® system, probing for 61 mutations over 13 genes. We compared the analytical sensitivity and clinical accuracy of the UltraSEEK melanoma panel to droplet digital PCR (ddPCR). The blinded analysis of 68 mutations detected in 48 plasma samples from stage IV melanoma patients revealed a concordance of 88% between the two platforms. Further comparison of both methods for the detection of *BRAF* V600E mutations in 77 plasma samples demonstrated a Cohen's κ of 0.826 (BCa 95% CI 0.669-0.946). Our results indicate that the UltraSEEK melanoma panel is as sensitive as ddPCR for the detection of ctDNA in this cohort of patients but highlight the need for detected variants to be confirmed orthogonally to mitigate any false positive results. The MassARRAY system enables rapid and sensitive genotyping for the detection of multiple melanoma-associated mutations in plasma.

KEYWORDS

UltraSEEK, melanoma, mutations, liquid biopsy, circulating tumor DNA

INTRODUCTION

Though comprising less than 2% of skin cancers, melanoma is responsible for the largest number of skin cancer–related deaths. Advances in targeted therapies and immune checkpoint inhibitors have revolutionised treatment in the metastatic setting [1-3]. Despite significant improvement in overall survival (OS), most patients on targeted therapies develop drug resistance within 12 months and immunotherapies are only effective in some patients [3]. Currently, radiological analysis and existing disease monitoring biomarkers (mainly LDH levels) are inadequate for guiding treatment selection, tracking response kinetics and the detection of emerging treatment resistance. Melanoma patients would benefit from a sensitive personalised test to monitor disease that can complement current therapies to melanoma.

Cell free DNA (cfDNA) are fragments of DNA shed into the bloodstream during cellular turnover, and in the case of tumour cells, the released DNA is referred to as circulating tumour DNA (ctDNA), which can be distinguished from normal cfDNA by the detection of tumour associated somatic mutations. Analysis of ctDNA offers the potential of a non-invasive method for identification of melanoma patients for molecularly based targeted therapies [4, 5]. In addition, ctDNA is emerging as a promising biomarker for early detection of disease status, particularly at times of treatment response or tumour regrowth [6-9]. CtDNA levels in plasma have been found to be strongly associated with tumour burden [8] and in particular with metabolic tumour burden [10], and low pre-treatment ctDNA levels are associated with better overall response rates and longer progression free survival (PFS) [4, 7, 11].

Droplet digital PCR (ddPCR) has emerged as one of the most cost effective and sensitive methods for the analysis of rare copies of mutant ctDNA. However, ddPCR at present only allows for the detection of one mutation at a time with some multiplex assays available targeting a limited number of specific hotspot mutations. While next generation sequencing (NGS) can detect mutations from a large breath of genes from plasma DNA, it is relatively costly, has a slow turnaround time, and requires high input material and complex bioinformatics platform analysis. Therefore, there is a requirement for a rapid, sensitive and cost effective assay that comprehensively screens for multiple commonly occurring mutations in melanoma.

Comprehensive genetic studies of melanomas have provided insights into the mutational landscape of melanoma, providing potentially important implications for prognosis and therapy [12-14]. In particular, the Cancer Genome Atlas (TCGA) annotation of melanomas define four genetic subclasses, *BRAF* mutant, *NRAS* mutant, *NF1* mutant and triple wild-type [14]. While most melanomas carry a mutation in *BRAF* codon V600 (~50%) or *NRAS* codons Q61 or G12/13 (~20%), a number of variants need to be

assessed for each position. *NF1* mutations are distributed across the whole gene with no defined hotspot mutations, making it difficult for targeted screening for somatic mutations [13]. Thus, other commonly mutated sites need to be targeted for ctDNA monitoring of *NF1* and triple-WT melanomas. For example, other melanoma associated mutations such as those in the *DPH3* promoter [15], *TERT* promoter [16], *RPS27* UTR [17] and *RAC1* [18, 19] amongst others provide alternatives for ctDNA monitoring in *BRAF/NRAS* wild type melanomas.

Here we evaluated 48 plasma samples from metastatic melanoma patients for mutations using a custom UltraSEEK melanoma panel on the MassARRAY system. This test allows analysis of 61 mutations over 13 genes within a single reaction. To determine the accuracy of the assay, results were compared to mutations identified in the same plasma samples by ddPCR.

MATERIALS AND METHODS

Plasma sample preparation and DNA extraction

Blood samples were collected from stage IV melanoma patients enrolled at the Sir Charles Gairdner Hospital and Fiona Stanley Hospital in Perth, Western Australia, the Olivia Newton-John Cancer Wellness & Research Centre (ONJCWRC) and the Peter MacCallum Cancer Centre in Melbourne, Victoria. Written informed consent was obtained from all patients under approved Human Research Ethics Committee protocols from Edith Cowan University (No. 11543), Sir Charles Gairdner Hospital (No. 2007-123), Peter MacCallum Cancer Centre (PMCC: 11/105) and Austin Hospital (HREC/14/Austin/425), with all methods performed in accordance with the relevant ethical guidelines and regulations of the Australian National Health and Medical Research Council. All tumour and plasma samples from the ONJCWRC were collected from patients as part of the Melbourne Melanoma Project. All tumour and plasma samples from the Peter MacCallum Cancer Centre were collected from patients as part of the Melanoma Biomarkers Study [8].

Blood was collected into EDTA vacutainer tubes or BCT tubes (Streck, La Vista, NE) and stored at 4°C until processing. Plasma was separated within 24 hours by centrifugation at 1600 g for 10 minutes, followed by a second centrifugation at 2000 g for 10 minutes, and then stored at -80°C until extraction. Cell free DNA (cfDNA) was isolated from between 2 to 5 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions and stored at -80°C until ctDNA quantification.

UltraSEEK Melanoma Panel

PCR was performed using 10 ng cfDNA in a single PCR reaction according to manufacturer's instructions (Agena Bioscience, San Diego, CA). Reactions were incubated initially at 94°C for 2 min. Forty-five cycles of PCR were performed at 94°C for 30 s, 56°C for 30 s, and 72°C for 1min. The PCR was completed with a final incubation of 5 minutes at 72°C. Thermocycling and incubation were performed in a Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA). Amplified products (70 µL) were treated with shrimp alkaline phosphatase for 40 minutes at 37°C, followed by denaturation for 5 minutes at 85°C. Single-base extension with biotinylated chain terminator nucleotides specific to the mutant allele was performed at 94°C for 30 s, followed by 40 cycles at 94°C for 5 s with five nested cycles of 52°C for 5 s, then 80°C for 5 s and incubation at 72°C for 3 min. Streptavidin-coated magnetic beads were used to capture the single base extended oligonucleotides. Beads with captured products were pelleted using a magnet and, suspended with 13mL of biotin competition solution, and incubated at 95°C for 5 minutes. Eluted products were conditioned with 2µL (2 mg) of anion exchange resin slurry. Finally, the analyte was dispensed onto a SpectroCHIP Array solid support using a MassARRAY RS1000 Nano-dispenser. Data were acquired via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the MassARRAY Analyzer. Data analysis were performed using Typer software version 4.0.26.74 (Agena Bioscience, San Diego, CA). Normalised intensity (NormInt) was calculated of the signal intensity of the mutant allele which has been normalised against the capture control peaks found in the spectrum. A value of one means the peak intensity of the observed mutant allele is equal to the peak intensity of the average of the 5 capture control peaks found in the spectrum. The capture control peaks are biotin labelled, non-reactive oligos, which are added to the extension reaction and used as an internal control for the streptavidin bead capture and elution of the mutant extension product steps.

Analytical Sensitivity and Specificity

The UltraSEEK Melanoma Panel assay validation used a model system developed to simulate samples harboring low frequency somatic mutations. Wild-type DNA (Coriell Cell Repositories, Camden, NJ) was spiked with different amounts of characterised cell lines (Horizon Diagnostics, Cambridge, UK) harbouring engineered mutations (Supplementary Table 1). The mixtures represented a 0.1%, 0.2%, 0.5% and a 1% mutant allele frequency, while keeping the total number of DNA molecules constant. Each dilution was analysed in four replicates. Each cell line harbouring a mutation for a specific assay was considered wild-type for all other assays in the multiplex.

Droplet digital PCR

PCR reactions were performed in a 20 µl reaction containing 1x droplet PCR supermix (No dUTP), and 250 nM of each probe, 900 nM primers and 8 µl of cfDNA. Commercially available and/or customised probes were used to analyse ctDNA. Previously described custom primer and probe sets were used for detection of mutation in *BRAF* [20, 21] and *DPH3* promoter (Calapre et al, submitted). Droplets were generated and analyzed using the QX200 system (Bio-Rad, Hercules, CA). DdPCR absolute quantification of mutant alleles and wild-type alleles was estimated by modeling as a Poisson distribution using the QuantaSoft analysis software v1.6.6 (Bio-Rad). Thresholds were defined based on the signal from empty droplets, wild-type DNA controls and mutant positive controls, as described in "Droplet Digital Application Guide" (Bio-Rad). The absolute number of mutant allele per mL of blood and mutant allele frequency were calculated from the QuantaSoft analysis software v1.6.6 outputs as follows:

Copies/mL plasma = (copies per mL of reaction as per QuantaSoft analysis software v1.6.6 [Bio-Rad]) X (volume of ddPCR reaction) X [(volume eluted/volume of DNA used in reaction)/4mL of plasma]

Mutant allele frequency= mutant copies/mL of plasma/ (mutant copies/mL of plasma + wild-type copies/mL of plasma)

Statistical Analysis

Cohen's kappa (κ) coefficient was used to assess agreement between ddPCR and UltraSEEK regarding the identification of *BRAF* V600E status (detected *vs*. undetected), using SPSS v24. Bias-corrected and accelerated (BCa) confidence intervals (95%CI) for Cohen's κ coefficient were constructed by bootstrapping, using 1,000 bootstrap replicates. According to Landis and Koch, the following κ values were used for interpretation: poor-to-fair (≤0.4), moderate (0.41-0.60), substantial (0.61-0.80) and almost perfect agreement (0.81-1.00) [22].

RESULTS

A custom UltraSEEK melanoma panel was devised containing 86 assays targeting 61 melanoma-associated mutations over 13 genes (Supplementary Table 1). We analysed the presence of somatic mutations in 48 plasma samples from stage IV melanoma patients recruited into liquid biopsy studies at four different hospitals across Australia. The selected plasma samples were previously screened for mutations by ddPCR or targeted sequencing. Characteristics of tumour and plasma samples are described in Supplementary Table 2. UltraSEEK analysis identified 80 mutations in these samples. Of those, 68 mutations could be compared to ddPCR results revealing concordant results for 60 (88%) of the mutations (Table 1). There was a significant correlation between the ddPCR mutational frequency

abundance and the UltraSEEK normalised intensity (Pearson's r=0.7056, p<0.0001) (Figure 1). Overall, UltraSEEK was able to detect mutations across a broad range of copies/mL of plasma (range 1.4-212,160) and fractional abundances (range 0.1-97.4) as defined by ddPCR analysis (Figure 2).

Three *BRAF* mutations previously identified by ddPCR in these samples were not detected by the UltraSEEK panel. This may suggest a limit of detection for some assays in the UltraSEEK panel. However, when plotted according to their frequency abundance the undetected mutations were neither at the lowest concentrations or frequency abundances (Figure 2). In one case, P9, a *BRAF* V600E mutation was identified by the UltraSEEK panel, while ddPCR indicated the presence of a *BRAF* V600K (Table 1, Figure 2). The tumour from this patient also contained a *BRAF* V600K mutation, confirming a false positive call by UltraSEEK.

Ten mutations detected by UltraSEEK but not confirmed by ddPCR were the *DPH3* promoter 8C>T (5), *CTNNB1* S45P (1) and *BRAF* V600E (4) (Bolded in Table 1). The latter may suggest low-level false positives or cross contamination with the UltraSEEK assays, as most were detected with minimal mutant signal intensity.

To further evaluate the specificity and sensitivity of the UltraSEEK melanoma panel, we next tested 49 plasma samples obtained from 20 healthy donors and 29 melanoma patients. Of the 29 melanoma patients, 22 were indicated to be negative for *BRAF* mutations in the archival pathology reports, and 7 were *BRAF* V600E or V600K positive in their tumour but found negative in the blood sample by ddPCR. None of the 7 plasmas from the plasma-negative *BRAF* mutant patients, were scored as positive by UltraSEEK, indicating the absence of detectable ctDNA in these samples by both UltraSEEK as well as ddPCR. Six of the 22 *BRAF* WT samples, were found to carry mutations using the UltraSEEK panel (Supplementary Table 3). One had a *BRAF* V600E mutation and one a *DPH3* promoter mutation that were not found by ddPCR. Two had *NRAS* Q61K/*DPH3* C>T and *NRAS* Q61K/*CDKN2A* R80X mutations that were confirmed by ddPCR. The *YAE1D1*_c39605965G>A mutation found in sample E40, but was not tested by ddPCR.

To perform unbiased assessment of concordance we compared both methods for the detection of *BRAF* V600E in 77 samples that were tested for this mutation in both platforms. We observed a substantial agreement with a Cohen's κ coefficient 0.826 (BCa 95% CI 0.669-0.946) (Table 2).

We next analysed longitudinally collected plasma from three melanoma patients treated with PD-1 inhibitors (pembrolizumab or nivolumab). Figure 3 shows that the normalised intensities for *BRAF*

V600E detected by UltraSEEK (right y-axis) correlated with copies by ddPCR (left axis), and with changes in disease status, declining with ongoing response to treatment and rising upon disease progression. These results demonstrate that the panel could potentially be utilised for non-invasive disease monitoring.

DISCUSSION

Advancements in ultrasensitive genotyping methods have created great interest in the application of somatic mutation detection from plasma DNA as a "liquid biopsy" for individualized patient management [23]. In the absence of a patient's tumour genotype, there is a need to accurately screen for multiple mutations from a blood sample at low mutant abundance and with small amounts of DNA input.

Here we assessed a comprehensive UltraSEEK panel specifically designed for the detection of melanoma-associated mutations. Samples analysed in the study were known to carry mutations in plasma by ddPCR but were blinded in the UltraSEEK analysis, including a set of 20 healthy control samples used for assessment of specificity.

The UltraSEEK Oncogene Panel assay uses a mass spectrometer for detection, and does not require the accessory equipment and support often needed with NGS-derived data. However, it is still able to interrogate multiple informative variants within a single reaction. The UltraSEEK chemistry is amenable to a manual workflow, but is also compatible with high-throughput processes using various automated liquid dispensing platforms. UltraSEEK differs from similar biochemistries in that it enriches the minor alleles by probing them specifically in a post-PCR primer extension step that omits the wild-type allele [24]. For this reason, UltraSEEK can only provide semi-quantitative measurement of the mutant allele. In comparison, methods like ddPCR provide absolute quantification of copies per volume of plasma of the mutant allele.

We made use of Cohen's kappa coefficient to compare both methodologies. The Cohen's kappa coefficient represents a considerable improvement over percent agreement calculations as the κ statistic provides a quantitative measure of agreement that has been adjusted for the degree of agreement expected solely on the basis of chance [25]. In addition, we use the κ coefficients with bias-corrected and accelerated 95%CIs, which automatically adjusts for bias and skewness in the bootstrap distribution [26]. We only analysed *BRAF* mutation for this comparison for two reasons: all samples were analysed for *BRAF* mutations in both assays and, secondly, many of the discordant results between the two platforms were observed in *BRAF* V600E mutations.

While no healthy donor samples were found to contain melanoma associated mutations, multiple melanoma samples were found to have mutations that were not confirmed by ddPCR. Given this potential for false positives in the current assay design, we would recommend any putative mutations detected by this assay to be confirmed using an orthogonal method, e.g. ddPCR or samples could be run in duplicate reactions. Moreover, we performed this study at the time when the panel was still being optimised by Agena Bioscience. A new version of the UltraSEEK panel is now available aiming to provide better specificity and sensitivity across all mutations.

In addition to the UltraSEEK panel performance, the observed discordant results may be attributed to the handling of the samples during shipment between laboratories and to differences between sample processing, as in some cases a separate plasma aliquot from the same blood collection time point was extracted to analyse by ddPCR. However, no specific event was identified to explain the discordant results (Supplementary Table 2), as all three samples where UltraSEEK failed to detect the *BRAF* V600E mutations were processed within 2 hours. Pre-analytical variables may have potentially confounded the concordance of the results in this study, e.g. freeze-thawing of the same sample tested leading to potential degradation. Our results therefore highlight that proper quality control assessment of a plasma DNA sample is warranted to ensure that the optimal amount of template and integrity of a sample is acceptable before testing.

While the screening of mutations in the *TERT* promoter were not possible in this current assay due to the GC richness of this loci, other highly recurrent promoter mutations were included in the panel including *DPH3* and *RPS27*, allowing serial mutation tracking in patients who are *BRAF/NRAS* wildtype. The panel can also be useful in the detection of resistance to mitogen-activated protein kinase inhibitors, e.g. *NRAS* mutations as highlighted in patient P16.

In conclusion, our results indicate that the UltraSEEK melanoma panel is as sensitive as droplet digital PCR for the detection of ctDNA. This highly multiplexed assay allows for rapid and sensitive screening for the detection of multiple melanoma-associated mutations in plasma.

REFERENCES

1. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP, Ribas A: Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 2013, 369:134-144.
2. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010, 363:711-723.
3. Homet B, Ribas A: New drug targets in metastatic melanoma. J Pathol 2014, 232:134-141.
4. Santiago-Walker A, Gagnon R, Mazumdar J, Casey M, Long GV, Schadendorf D, Flaherty K, Kefford R, Hauschild A, Hwu P, Haney P, O'Hagan A, Carver J, Goodman V, Legos J, Martin AM: Correlation of BRAF Mutation Status in Circulating-Free DNA and Tumor and Association with Clinical Outcome across Four BRAFi and MEKi Clinical Trials. Clinical cancer research : an official journal of the American Association for Cancer Research 2016, 22:567-574.
5. Ascierto PA, Minor D, Ribas A, Lebbe C, O'Hagan A, Arya N, Guckert M, Schadendorf D, Kefford RF, Grob JJ, Hamid O, Amaravadi R, Simeone E, Wilhelm T, Kim KB, Long GV, Martin AM, Mazumdar J, Goodman VL, Trefzer U: Phase II trial (BREAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. J Clin Oncol 2013, 31:3205-3211.
6. Lee JH, Long GV, Boyd S, Lo S, Menzies AM, Tembe V, Guminski A, Jakrot V, Scolyer RA, Mann GJ, Kefford RF, Carlino MS, Rizos H: Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. Ann Oncol 2017, 28:1130-1136.
7. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, Tembe V, Freeman J, Lee JH, Scolyer RA, Siew K, Lomma C, Cooper A, Khattak MA, Meniawy TM, Long GV, Carlino MS, Millward M, Ziman M: Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 2015, 6:42008-42018.

8. Wong SQ, Raleigh JM, Callahan J, Vergara IA, Ftouni S, Hatzimihalis A, Colebatch AJ, Li J, Semple T, Doig K, Mintoff C, Sinha D, Yeh P, Silva MJ, Alsop K, Thorne H, Bowtell DD, Gyorki DE, Arnau GM, Cullinane C, Kee D, Brady B, Kelleher F, Dawson MA, Papenfuss AT, Shackleton M, Hicks RJ, McArthur GA, Sandhu S, Dawson S-J: Circulating Tumor DNA Analysis and Functional Imaging Provide Complementary Approaches for Comprehensive Disease Monitoring in Metastatic Melanoma. JCO Precision Oncology 2017:1-14.

9. Tsao SC, Weiss J, Hudson C, Christophi C, Cebon J, Behren A, Dobrovic A: Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. Sci Rep 2015, 5:11198.

10. McEvoy AC, Warburton L, Al-Ogaili Z, Celliers L, Calapre L, Pereira MR, Khattak MA, Meniawy TM, Millward M, Ziman M, Gray ES: Correlation between circulating tumour DNA and metabolic tumour burden in metastatic melanoma patients. BMC Cancer 2018, 18:726.

11. Sanmamed MF, Fernandez-Landazuri S, Rodriguez C, Zarate R, Lozano MD, Zubiri L, Gracia JL, Martin-Algarra S, Gonzalez A: Quantitative Cell-Free Circulating BRAFV600E Mutation Analysis by Use of Droplet Digital PCR in the Follow-Up of Patients with Melanoma Being Treated with BRAF Inhibitors. Clin Chem 2014.

12. Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, Nickerson E, Auclair D, Li L, Place C, Dicara D, Ramos AH, Lawrence MS, Cibulskis K, Sivachenko A, Voet D, Saksena G, Stransky N, Onofrio RC, Winckler W, Ardlie K, Wagle N, Wargo J, Chong K, Morton DL, Stemke-Hale K, Chen G, Noble M, Meyerson M, Ladbury JE, Davies MA, Gershenwald JE, Wagner SN, Hoon DS, Schadendorf D, Lander ES, Gabriel SB, Getz G, Garraway LA, Chin L: A landscape of driver mutations in melanoma. Cell 2012, 150:251-263.

13. Krauthammer M, Kong Y, Ha BH, Evans P, Bacchiocchi A, McCusker JP, Cheng E, Davis MJ, Goh G, Choi M, Ariyan S, Narayan D, Dutton-Regester K, Capatana A, Holman EC, Bosenberg M, Sznol M, Kluger HM, Brash DE, Stern DF, Materin MA, Lo RS, Mane S, Ma S, Kidd KK, Hayward NK, Lifton RP, Schlessinger J, Boggon TJ, Halaban R: Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. Nat Genet 2012, 44:1006-1014.

14. Cancer Genome Atlas N: Genomic Classification of Cutaneous Melanoma. Cell 2015, 161:1681-1696.

15. Denisova E, Heidenreich B, Nagore E, Rachakonda PS, Hosen I, Akrap I, Traves V, Garcia-Casado Z, Lopez-Guerrero JA, Requena C, Sanmartin O, Serra-Guillen C, Llombart B, Guillen C, Ferrando J, Gimeno E, Nordheim A, Hemminki K, Kumar R: Frequent DPH3 promoter mutations in skin cancers. Oncotarget 2015, 6:35922-35930.

16. McEvoy AC, Calapre L, Pereira MR, Giardina T, Robinson C, Khattak MA, Meniawy TM, Pritchard AL, Hayward NK, Amanuel B, Millward M, Ziman M, Gray ES: Sensitive droplet digital PCR method for detection of TERT promoter mutations in cell free DNA from patients with metastatic melanoma. Oncotarget 2017, 8:78890-78900.

17. Dutton-Regester K, Gartner JJ, Emmanuel R, Qutob N, Davies MA, Gershenwald JE, Robinson W, Robinson S, Rosenberg SA, Scolyer RA, Mann GJ, Thompson JF, Hayward NK, Samuels Y: A highly recurrent RPS27 5'UTR mutation in melanoma. Oncotarget 2014, 5:2912-2917.

18. Watson IR, Li L, Cabeceiras PK, Mahdavi M, Gutschner T, Genovese G, Wang G, Fang Z, Tepper JM, Stemke-Hale K, Tsai KY, Davies MA, Mills GB, Chin L: The RAC1 P29S hotspot mutation in melanoma confers resistance to pharmacological inhibition of RAF. Cancer Res 2014, 74:4845-4852.

19. Mar VJ, Wong SQ, Logan A, Nguyen T, Cebon J, Kelly JW, Wolfe R, Dobrovic A, McLean C, McArthur GA: Clinical and pathological associations of the activating RAC1 P29S mutation in primary cutaneous melanoma. Pigment Cell Melanoma Res 2014, 27:1117-1125.

20. Reid AL, Freeman JB, Millward M, Ziman M, Gray ES: Detection of BRAF-V600E and V600K in melanoma circulating tumour cells by droplet digital PCR. Clin Biochem 2015, 48:999-1002.

21. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, Tembe V, Freeman J, Lee JH, Scolyer RA: Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. Oncotarget 2015, 6:42008–42018.

22. Landis JR, Koch GG: The measurement of observer agreement for categorical data. Biometrics 1977, 33:159-174.

23. Diaz LA, Jr., Bardelli A: Liquid biopsies: genotyping circulating tumor DNA. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2014, 32:579-586.

24. Mosko MJ, Nakorchevsky AA, Flores E, Metzler H, Ehrich M, van den Boom DJ, Sherwood JL, Nygren AO: Ultrasensitive Detection of Multiplexed Somatic Mutations Using MALDI-TOF Mass Spectrometry. The Journal of molecular diagnostics : JMD 2016, 18:23-31.

25. Viera AJ, Garrett JM: Understanding interobserver agreement: the kappa statistic. Fam Med 2005, 37:360-363.

26. Kang C, Qaqish B, Monaco J, Sheridan SL, Cai J: Kappa statistic for clustered dichotomous responses from physicians and patients. Stat Med 2013, 32:3700-3719.

Table 1. Comparison of mutations identified by UltraSEEK and droplet digital PCR analysis

Patient	Mutation	ddPCR		UltraSEEK	Patient	Mutation	ddPCR		UltraSEEK
		copies/ml	FA*	NormInt#			copies/ml	FA*	NormInt#
P1	*BRAF* V600K*	6518.8	73.8	1.21	**E11**	*BRAF* V600E2*	20.0	0.8	0.11
P3	*BRAF* V600K*	5024.0	45.9	1.09		***DPH3* c16306504C>T**	**0.0**	**0.0**	0.12
	IDH1 R132C	NT	NT	0.16		RAC1 P29S	6.8	0.2	0.16
P4	*BRAF* V600E*	63.0	2.4	0.33	**E12**	*BRAF* V600E*	6.8	0.6	0.10
P5	*BRAF* V600E*	268.0	3.4	1.13		IDH1 R132H	1.4	0.1	0.31
P7	*BRAF* V600R*	2506.0	75.0	0.26	**E13**	*BRAF* V600E*	56.0	8.3	1.86
	IDH1 R132C	NT	NT	0.27	**E14**	*BRAF* V600E*	18.0	0.7	0.31
	YAE1D1 c39605969G>A	NT	NT	1.28		***BRAF* V600E2 TG>AA**	**0.0**	**0.0**	1.46
P9	*BRAF* V600K*	95.5	1.6	**ND**	**E15**	*BRAF* V600E*	16.0	0.5	0.06
	***BRAF* V600E**	**0.0**	**0.0**	2.59	**E16**	*BRAF* V600R*	602.0	5.7	1.03
P10	*BRAF* V600E*	1527.0	16.3	2.59	**E17**	*BRAF* V600E*	971.1	23.1	2.95
	CDKN2A R80X	1020.0	18.8	2.48	**E18**	KIT L576P	342.0	12.4	3.28
P11	*BRAF* V600K*	206.0	11.5	0.39	**E19**	KIT V559A*	13.0	1.2	0.30
	CDKN2A R80X	176.0	14.3	2.29	**E21**	***DPH3* c16306504C>T**	**0.0**	**0.0**	0.14
	SDHD c111957523C>T	NT	NT	1.21	**E23**	*NRAS* Q61K*	382	5.4	0.98
P12	***CTNNB1* S45P**	**0.0**	**0.0**	0.44		*DPH3* c16306504C>T	36.0	2.0	1.11
P15	*NRAS* Q61K*	99.0	13.0	1.23	**E24**	*NRAS* Q61K*	9900	40	3.02
	DPH3 c16306504C>T	128.0	1.7	0.99		CDKN2A R80X	1396.0	14.0	3.82
	IDH1 R132C	NT	NT	0.22		***BRAF* V600E**	**0.0**	**0.0**	0.22
P16	*BRAF* V600E*	4265.3	28.9	3.39	**O2**	*BRAF* V600E*	26.0	1.3	**ND**
	NRAS Q61K		57.4	1.58		*DPH3* c16306504C>T	NT	NT	0.50
P33	*BRAF* K601E*	224.0	0.5	0.40	**O3**	CTNNB1 S45P	NT	NT	0.18
P35	*NRAS* G13R*	63.0	0.5	0.24		*DPH3* c16306504C>T	0.0	0.0	1.17
P37	*BRAF* V600K*	67.0	0.8	0.20		RAC1 P29S	1943.0	8.5	1.60
P38	*BRAF* V600K*	1018.0	5.3	0.30	**O4**	CTNNB1 S45F	NT	NT	0.54
P39	***BRAF* V600E***	**0.0**	**0.0**	0.29		*DPH3* c16306504C>T	1.3	0.1	0.30
	RQCD1 P131L	NT	NT	0.29		RPS27 c238CtoT	11.0	1.4	0.54
P40	*NRAS* Q61K*	127.0	3.1	0.69	**O6**	*NRAS* G12D	538.0	1.7	1.15
E1	*BRAF* V600E*	5.4	0.5	0.10	**O9**	*BRAF* V600E*	88	2.0	0.20
	BRAF K601E	NT	NT	0.47		*DPH3* c16306504C>T	25	1.3	0.20
E2	*BRAF* V600E*	126.0	3.1	0.83	**O10**	*BRAF* V600E*	38	1.8	0.20
E3	*BRAF* V600E*	100.0	2.1	0.62		***DPH3* c16306504C>T**	**0**	**0.0**	1.20
	BRAF K601E	NT	NT	0.51	**O11**	*BRAF* V600E*	276	8.4	0.90
E4	*BRAF* V600E*	4.8	0.3	0.14		*DPH3* c16306504C>T	5	2.9	1.50
E5	*BRAF* V600E*	24.0	0.4	0.26	**O12**	*BRAF* V600E*	391	15.2	2.80
E6	*NRAS* Q61K*	318.0	10.0	1.37		*DPH3* c16306504C>T	3	2.5	1.60
	DPH3 c16306504C>T	26.0	1.7	1.51	**O13**	*BRAF* V600E*	91	2.5	0.40
E7	*BRAF* V600E*	11.0	0.4	0.29		*DPH3* c16306504C>T	2	0.7	0.40
E8	*BRAF* V600E*	2380.0	31.5	3.38	**O14**	*BRAF* V600E*	33	1.3	**ND**
	DPH3 c16306504C>T	260.0	18.1	2.81		***DPH3* c16306504C>T**	**0**	**0.0**	0.40
E9	*BRAF* V600E*	202.0	3.1	1.00	**O15**	*BRAF* V600E*	26	1.3	**ND**
E10	*BRAF* V600R*	212160.0	97.4	0.45		***DPH3* c16306504C>T**	**0**	**0.0**	0.30
	CDKN2A R80X	NT	NT	2.76	**O16**	*BRAF* V600E*	219	5.6	0.60
	DPH3 c16306504C>T	1960.0	32.8	2.19		*DPH3* c16306504C>T	8	1.2	1.00
	MAP2K1 P124S*	31300.0	12.3	1.52					
	RAC1 P29S*	17420.0	62.2	2.62					

Red coloured cells indicate discordant results. FA: frequency abundance of mutant copies relative to wild-type DNA. NormInt: Normalised intensity. NT: Not tested. *Mutation identified in FFPE tumour tissue.

Table 2. *BRAF* mutation detection concordance between platforms.

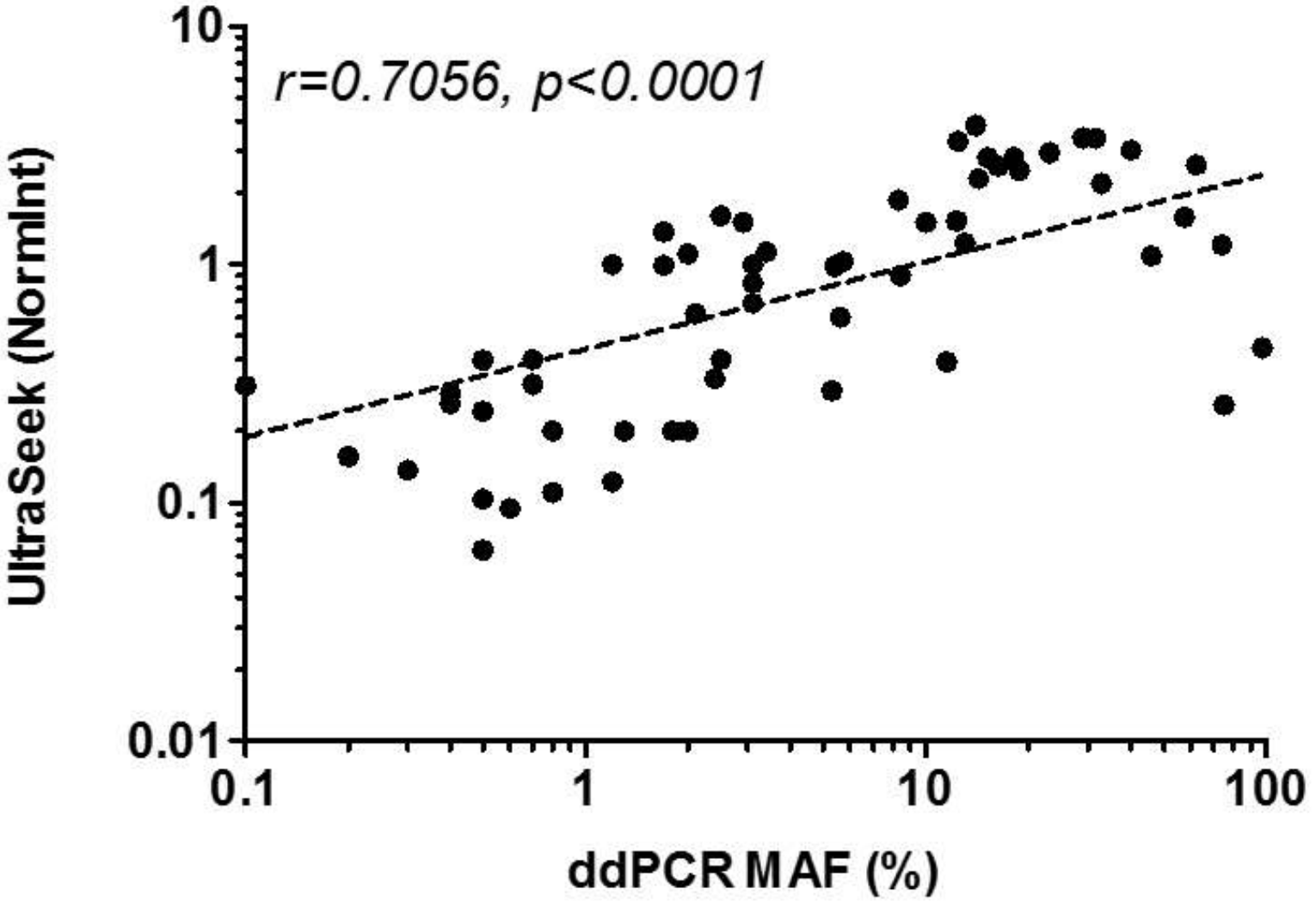
			ddPCR		Total
			Undetected	Detected	
UltraSEEK	Undetected	Count	48	3	51
		Expected Count	33.8	17.2	51.0
	Detected	Count	3	23	26
		Expected Count	17.2	8.8	26.0
Total		Count	51	26	77
		Expected Count	51.0	26.0	77.0

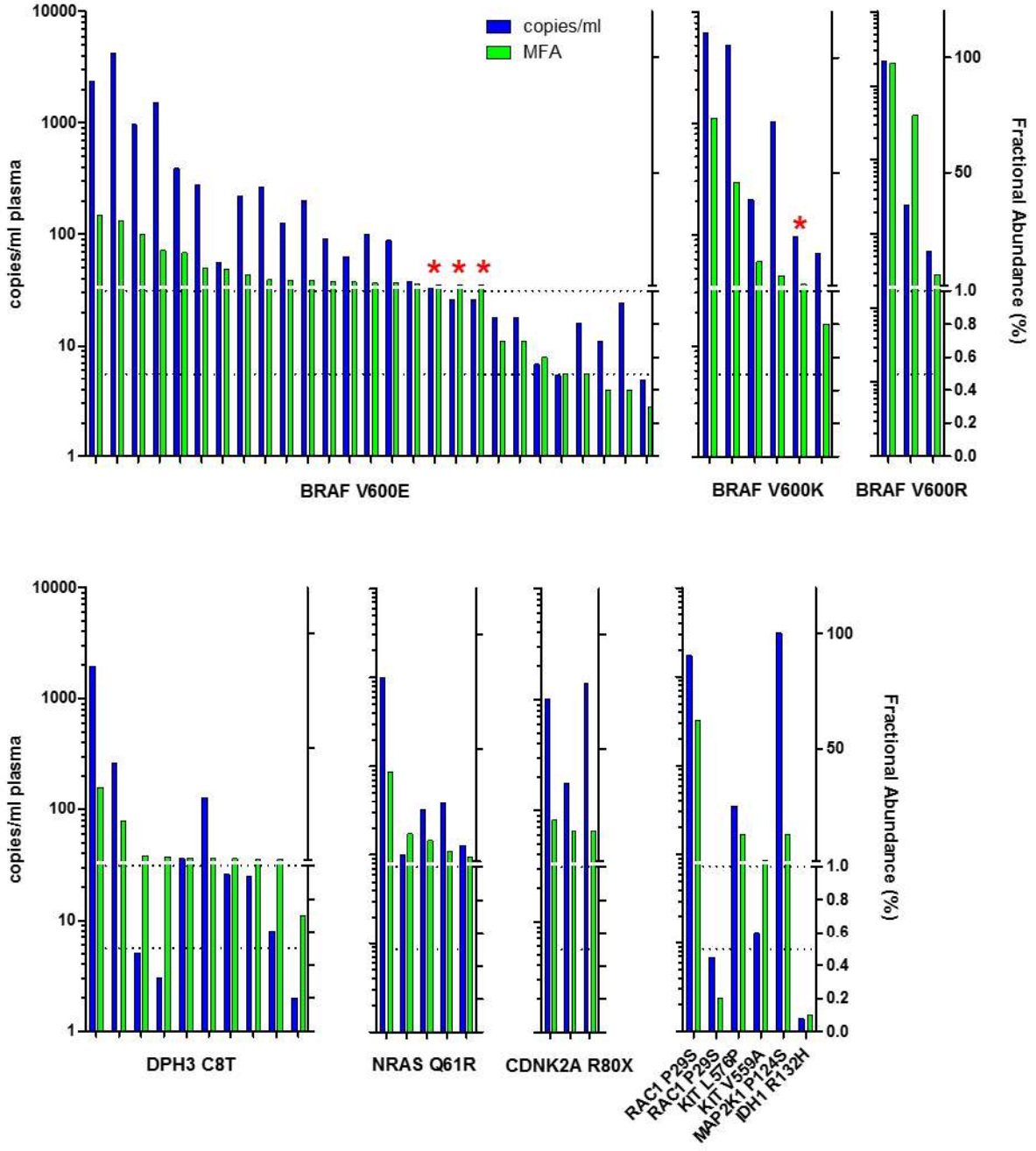
Figure Legends:

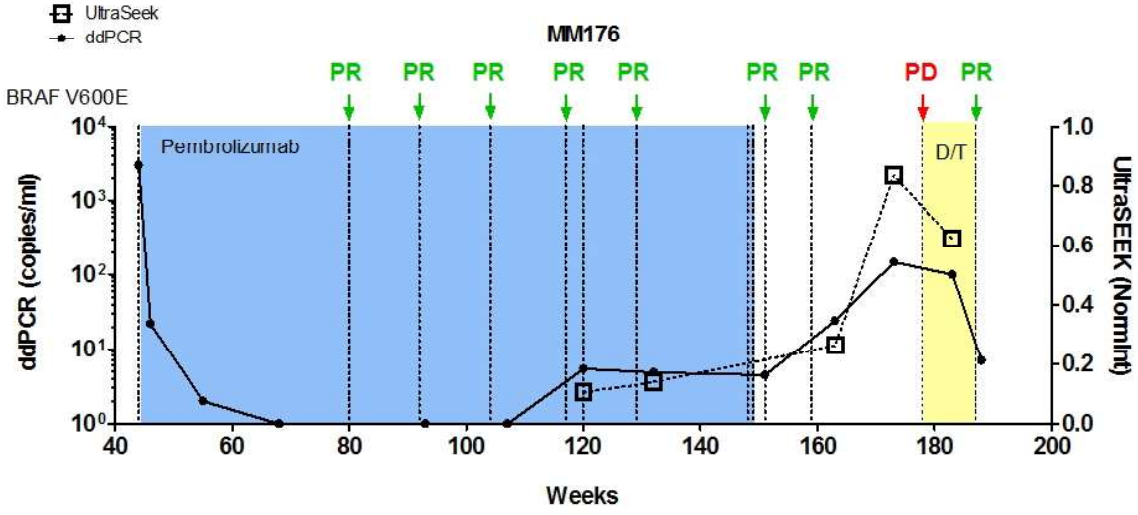
Figure 1: Correlation between ddPCR and UltraSEEK MassARRAY mutation detection in plasma cfDNA. Correlation between log transformed ddPCR mutational frequency abundance and UltraSEEK. Normalised Intensity of 59 mutations identified by both methods. Pearson r and correlation p-value are indicated. NormInt: Normalised intensity, ddPCR MAF: Droplet digital PCR mutant allele fraction

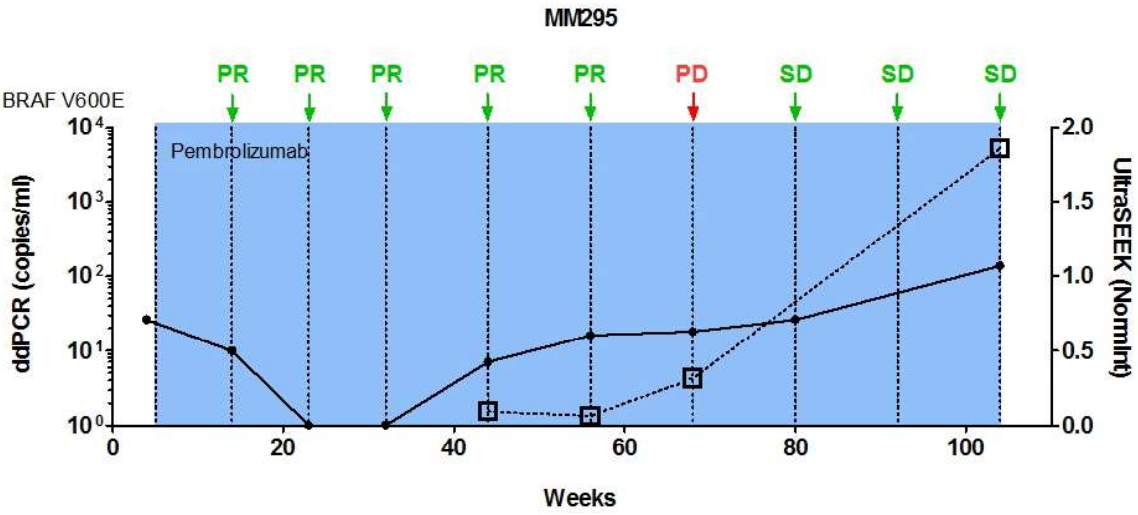
Figure 2: Range of copies/ml and frequency abundance by ddPCR of mutations in the plasmas analysed using MassArray UltraSEEK. Red asterisks indicate samples not detected by UltraSEEK.

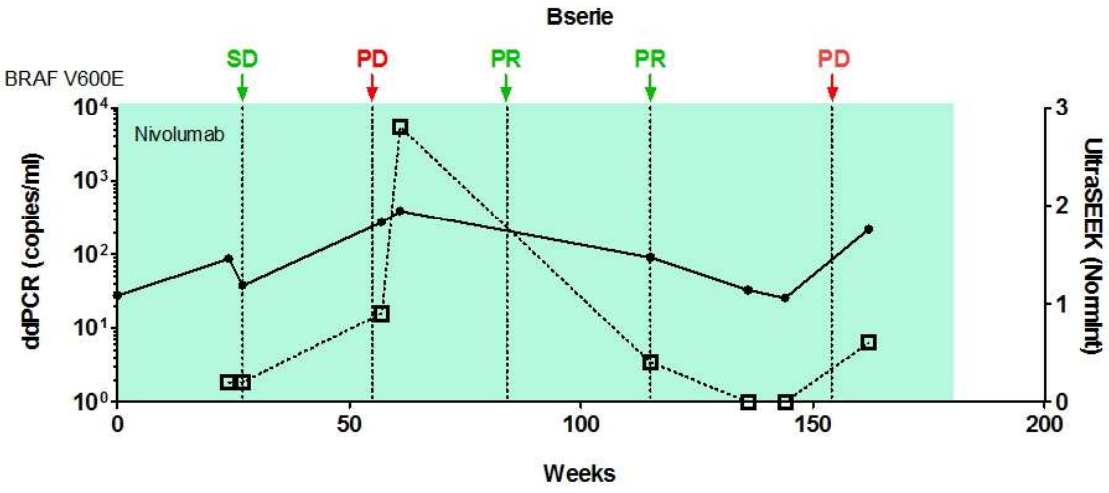
Figure 3: Longitudinal measurements of plasma ctDNA by ddPCR and UltraSEEK in 3 melanoma patients treated with PD-1/PDL-1 inhibitors. All three cases were tested for mutation *BRAF* V600E as a measurement of ctDNA quantity. Therapies are indicated by coloured boxes: blue – pembrolizumab, yellow- dabrafenib/trametinib (D+T), green- nivolumab. Disease statuses by radiological imaging are indicated by arrows and labelled as PR: partial response, SD: stable disease, MR: mixed response or PD: progressive disease.


r=0.7056, p<0.0001
UltraSeek (NormInt)
ddPCR MAF (%)
10
1
0.1
0.01
0.1
1
10
100


copies/ml
MFA
copies/ml plasma
Fractional Abundance (%)
10000
1000
100
10
1
100
50
1.0
0.8
0.6
0.4
0.2
0.0
BRAF V600E
BRAF V600K
BRAF V600R
DPH3 C8T
NRAS Q61R
CDNK2A R80X
RAC1 P29S
RAC1 P29S
KIT L576P
KIT V559A
MAP2K1 P124S
IDH1 R132H


UltraSeek
ddPCR
MM176
PR PR PR PR PR PR PR PD PR
BRAF V600E
Pembrolizumab
D/T
ddPCR (copies/ml)
UltraSEEK (Normlnt)
Weeks


MM295
PR PR PR PR PR PD SD SD SD
BRAF V600E
Pembrolizumab
ddPCR (copies/ml)
UltraSEEK (Normlnt)
Weeks


Bserie
SD PD PR PR PD
BRAF V600E
Nivolumab
ddPCR (copies/ml)
UltraSEEK (Normlnt)
Weeks

Supplementary Table 1: Mutations targeted on the Melanoma MassArray UltraSeek Panel

Assay_ID	Variant	Redundant Assay	LOD
BRAF_c1779TtoG-f1_PlxG	BRAF_D594N_TG>GA		
BRAF_c1780GtoA-r1_PlxT	BRAF_D594N		
BRAF_c1780GtoC-f1_PlxC	BRAF_D594H	Y	
BRAF_c1780GtoC-r1_PlxG	BRAF_D594H	Y	
BRAF_c1781AtoT-f1_PlxT	BRAF_D594V		
BRAF_c1782TtoA-r1_PlxT	BRAF_D594E		
BRAF_c1798GtoA-r1_PlxT	BRAF_V600M		
BRAF_c1798GtoA-r2_PlxT	BRAF_V600K		
BRAF_c1799TtoA-r1_PlxT	BRAF_V600E	Y	0.1%
BRAF_c1799TtoA-r2_PlxT	BRAF_V600E	Y	0.1%
BRAF_c1799TtoG-f1_PlxG	BRAF_V600G	Y	0.5%
BRAF_c1799TtoG-f2_PlxG	BRAF_V600R_GT>AG		0.5%
BRAF_c1799TtoG-r1_PlxC	BRAF_V600G	Y	1.0%
BRAF_c1799TtoG-r2_PlxC	BRAF_V600G	Y	0.1%
BRAF_c1800GtoA-r1_PlxT	BRAF_V600E_TG>AA		
BRAF_c1800GtoT-f1_PlxT	BRAF_V600D_TG>AT		
BRAF_c1801AtoG-r1_PlxC	BRAF_K601E	Y	
BRAF_c1801AtoG-r2_PlxC	BRAF_K601E	Y	
CDKN2A_c238CtoT-f1_PlxT	CDKN2A_R80X	Y	
CDKN2A_c238CtoT-f2_PlxT	CDKN2A_R80X	Y	
CTNNB1_c110CtoA-r1_PlxT	CTNNB1_S37Y		
CTNNB1_c133TtoC-f1_PlxC	CTNNB1_S45P		
CTNNB1_c134CtoA-r1_PLxT	CTNNB1_S45Y		
CTNNB1_c134CtoT-f1_PlxT	CTNNB1_S45F		
DPH3_c16306504CtoT-f1_PlxT	DPH3_MUT		
DPH3_c16306505CtoT-f1_PlxT	DPH3_MUT		
IDH1_c394CtoT-f1_PlxT	IDH1_R132C	Y	
IDH1_c394CtoT-f2_PlxT	IDH1_R132C	Y	
IDH1_c395GtoA-r1_PlxT	IDH1_R132H	Y	0.2%
IDH1_c395GtoA-r2_PlxT	IDH1_R132H	Y	0.1%
KIT_c1669TtoA-r1_PlxT	KIT_W557R	Y	
KIT_c1669TtoA-r2_PlxT	KIT_W557R	Y	
KIT_c1676TtoA-r1_PlxT	KIT_V559D		
KIT_c1676TtoC-f1_PlxC	KIT_V559A	Y	
KIT_c1676TtoC-f2_PlxC	KIT_V559A	Y	
KIT_c1676TtoC-r1_PlxG	KIT_V559A	Y	
KIT_c1727TtoC-f1_PlxC	KIT_L576P	Y	
KIT_c1727TtoC-f2_PlxC	KIT_L576P	Y	
KIT_c1924AtoG-r1_PlxC	KIT_K642E		
KIT_c2446GtoC-r1_PlxG	KIT_D816H		
KIT_c2447AtoT-f1_PlxT	KIT_D816V		
MAP2K1_c1144AtoC-f1_PlxC	MAP2K1_N382H	Y	

SNP_ID	EXT_CALL	Redundant Assay	LOD
MAP2K1_c1144AtoC-r1_PlxG	MAP2K1_N382H	Y	
MAP2K1_c157TtoC-f1_PlxC	MAP2K1_F53L	Y	
MAP2K1_c157TtoC-f2_PlxC	MAP2K1_F53L	Y	
MAP2K1_c332TtoG-f1_PlxG	MAP2K1_I111S	Y	
MAP2K1_c332TtoG-r1_PlxC	MAP2K1_I111S	Y	
MAP2K1_c362GtoC-f1_PlxC	MAP2K1_C121S	Y	
MAP2K1_c362GtoC-f2_PlxC	MAP2K1_C121S	Y	
MAP2K1_c370CtoT-f1_PlxT	MAP2K1_P124S	Y	
MAP2K1_c370CtoT-f2_PlxT	MAP2K1_P124S	Y	
MAP2K1_c607GtoA-r1_PlxT	MAP2K1_E203K	Y	
MAP2K1_c607GtoA-r2_PlxT	MAP2K1_E203K	Y	
MAP2K1_c790CtoT-f1_PlxT	MAP2K1_P264S		
NRAS_c181CtoA-r1_PlxT	NRAS_Q61K	Y	0.1%
NRAS_c181CtoA-r2_PlxT	NRAS_Q61K	Y	0.1%
NRAS_c181CtoG-f1_PlxG	NRAS_Q61E	Y	
NRAS_c181CtoG-r1_PlxC	NRAS_Q61E	Y	
NRAS_c182AtoC-f1_PlxC	NRAS_Q61P		
NRAS_c182AtoG-r1_PlxC	NRAS_Q61R		0.1%
NRAS_c182AtoT-f1_PlxT	NRAS_Q61L		0.1%
NRAS_c183AtoC-r1_PlxG	NRAS_Q61H		
NRAS_c183AtoG-r1_PlxC	NRAS_Q61RL	Y	
NRAS_c183AtoG-r2_PlxC	NRAS_Q61RL	Y	
NRAS_c183AtoT-f1_PlxT	NRAS_Q61H	Y	0.1%
NRAS_c183AtoT-f2_PlxT	NRAS_Q61H	Y	0.2%
NRAS_c34GtoA-r1_PlxT	NRAS_G12S		
NRAS_c34GtoC-f1_PlxC	NRAS_G12R		
NRAS_c34GtoT-f1_PlxT	NRAS_G12C		
NRAS_c35GtoA-r1_PlxT	NRAS_G12D		
NRAS_c35GtoC-f1_PlxC	NRAS_G12A		
NRAS_c35GtoT-f1_PlxT	NRAS_G12V		0.5%
NRAS_c37GtoC-f1_PlxC	NRAS_G13R	Y	
NRAS_c37GtoC-r1_PlxG	NRAS_G13R	Y	
NRAS_c37GtoT-f1_PlxT	NRAS_G13C		
NRAS_c38GtoA-r1_PlxT	NRAS_G13D		0.1%
NRAS_c38GtoC-r1_PlxG	NRAS_G13A		
NRAS_c38GtoT-f1_PlxT	NRAS_G13V		
RAC1_c85CtoT-f1_PlxT	RAC1_P29S		
RPS27_c238CtoT-f1_PlxT	RPS27_UTR_MUT		
RQCD1_c392CtoT-f1_PlxT	RQCD1_P131L		
SDHD_c111957523-f1_PlxT	SDHD_MUT		
SDHD_c111957541CtoT-f1_PlxT	SDHD_MUT		
SDHD_c111957544CtoT-f1_PlxT	SDHD_MUT		
YAE1D1_c39605965GtoA-r1_PlxT	YAE1D1_MUT		
YAE1D1_c39605969GtoA-r1_PlxT	YAE1D1_MUT		

Assay_ID: Assays used for detection of specific mutation. -f1,-f2: represents a forward directional assay (taking the direction the gene is transcribed into account) with the numbers representing redundancy in that direction for that assay. -r1, -r2, is the same but for reverse sequence.
Variant: Amino acid change identified by the probe. Cases where the change is mediated by two nucleotide changes are indicated.
LOD: Limit of detection calculated using Horizon Controls.

Supplementary Table 2: Characteristics of plasma and tumour samples used for study

Patient	Treatment	Plasma taken prior to treatment, during treatment or at progression	*BRAF* status	Method for tumour genotype	Other mutations	Method for tumour genotype of other mutations	Hours from blood collection	Volume of plasma used for extraction	Elution volume	Blood Tube Type
P1	Ipilimumab/Nivolumab	Prior to treatment	*BRAF* V600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P3	Dabrafenib/Trametinib	At progression	BRAFV600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P4	Dabrafenib/Trametinib	During treatment	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P5	Ipilimumab	During treatment	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P7	Ipilimumab	Prior to treatment	*BRAF* V600R	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P9	Dabrafenib	During treatment	*BRAF* V600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P10	Nivolumab	Prior to treatment	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P11	Vemurafenib	During treatment	*BRAF* V600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P12	Vemurafenib	During treatment	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P15	Ipilimumab	At progression	*BRAF* WT	HRM/Sanger Sequencing	*NRAS* Q61K	Pyrosequencing	<3hrs	2ml	50ul	EDTA
P16	Ipilimumab	At progression	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P33	Trametinib	Prior to treatment	*BRAF* K601E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P35	Pembrolizumab	At progression	*BRAF* WT	HRM/Sanger Sequencing	*NRAS* G13R	Pyrosequencing	<3hrs	2ml	50ul	EDTA
P37	Dabrafenib	During treatment	*BRAF* V600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P38	Vemurafenib	Prior to treatment	*BRAF* V600K	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P39	Ipilimumab	At progression	*BRAF* V600E	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
P40	Pembrolizumab	Prior to treatment	*BRAF* WT	HRM/Sanger Sequencing			<3hrs	2ml	50ul	EDTA
E1	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			6	5ml	40ul	EDTA
E2	Pembrolizumab	At progression	*BRAF* V600E	Cobas 4800/Sanger Sequencing			18	5ml	40ul	EDTA
E3	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			4	4.5ml	40ul	Streck
E4	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			6	5ml	40ul	EDTA
E5	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			24	5ml	40ul	EDTA
E6	Pembrolizumab	During treatment	*BRAF* WT	Cobas 4800/Sanger Sequencing	*NRAS* Q61K	NGS	24	5ml	40ul	EDTA
E7	Dabrafenib/Trametinib	At progression	*BRAF* V600E	Cobas 4800/Sanger Sequencing			6	5ml	40ul	EDTA
E8	-	Untreated	*BRAF* V600E	Cobas 4800/Sanger Sequencing			21	4ml	40ul	Streck
E9	Dabrafenib/Trametinib	At progression	*BRAF* V600E	Cobas 4800/Sanger Sequencing			19	5ml	40ul	EDTA
E10	Dabrafenib/Trametinib	At progression	*BRAF* V600R	Cobas 4800/Sanger Sequencing	*MAP2K1/ RAC*1P29S	NGS	20	4ml	40ul	Streck
E11	Dabrafenib/Trametinib	Prior to treatment	*BRAF* V600E2	Cobas 4800/Sanger Sequencing			20	3.3ml	30ul	Streck
E12	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			6	5ml	40ul	EDTA
E13	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			19	5ml	40ul	EDTA
E14	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			22	5ml	40ul	EDTA
E15	Pembrolizumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			22	5ml	40ul	EDTA
E16	Dabrafenib/Trametinib	Prior to treatment	*BRAF* V600R	Cobas 4800/Sanger Sequencing			25	5ml	40ul	EDTA
E17	Dabrafenib/Trametinib	Prior to treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			21	4.5ml	40ul	Streck
E18	Pembrolizumab	During treatment	*BRAF* WT	Cobas 4800/Sanger Sequencing			23	5ml	40ul	EDTA
E19	Ipilimumab	Prior to treatment	*BRAF* WT	Cobas 4800/Sanger Sequencing	*KIT* V559A	NGS	6	5ml	40ul	EDTA
E21	-	Untreated	*BRAF* WT	Cobas 4800/Sanger Sequencing			21	5ml	40ul	EDTA
E23	Ipilimumab/Nivolumab	Prior to treatment	*BRAF* WT	Cobas 4800/Sanger Sequencing	*NRAS* Q61K	ddPCR	23	5ml	40ul	EDTA
E24	Pembrolizumab	Prior to treatment	*BRAF* WT	Cobas 4800/Sanger Sequencing			23	5ml	40ul	EDTA
O1	-	Untreated	*NRAS* G13A	Cobas 4800/Sanger Sequencing	*NRAS* G13A	NGS	<2hrs	4ml	50uL	EDTA
O2	Nivolumab	During treatment	*BRAF* V600E	Cobas 4800/Sanger Sequencing			<2hrs	4ml	50uL	EDTA
O3	-	Untreated	*CTNNB1* S45P	Cobas 4800/Sanger Sequencing			<2hrs	4ml	50uL	EDTA
O4	-	Untreated	*CTNNB1* H45F	Cobas 4800/Sanger Sequencing			<2hrs	4ml	50uL	EDTA
O6	-	At progression	*NRAS* G12S	Cobas 4800/Sanger Sequencing			<2hrs	4ml	50uL	EDTA
O7	-	At progression	*NRAS* G12D	Cobas 4800/Sanger Sequencing			<2hrs	4ml	50uL	EDTA
O8	-	At progression	*CTNNB1* S45P	Cobas 4800/Sanger Sequencing	*NRAS* G12A	NGS	<2hrs	4ml	50uL	EDTA
O9	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O10	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O11	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O12	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O13	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O14	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O15	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA
O16	Nivolumab	During treatment	*BRAF* V600E	ddPCR			<2hrs	4ml	50uL	EDTA

HRM: High resolution Melt, EDTA: Ethylenediaminetetraacetic acid, NGS: Next generation sequencing

Supplementary Table 3: Extended comparison of UltraSEEK and ddPCR for specificity assessment

ID	HC or *BRAF* status	Mutation detected by UltraSEEK	NormInt	ddPCR	
				copies/ml	FA
E20	*BRAF* WT	ND			
E21	*BRAF* WT	ND			
E22	*BRAF* WT	ND			
E23	*BRAF* WT	*DPH3* c16306504C>T	0.14	0	0
E24	*BRAF* WT	ND			
E25	*BRAF* WT	ND			
E26	*BRAF* WT	ND			
E27	*BRAF* WT	ND			
E28	*BRAF* WT	ND			
E29	*BRAF* WT	ND			
E30	*BRAF* WT	ND			
E31	*BRAF* WT	*NRAS* Q61K	0.98	382	5.4
		DPH3 c16306504C>T	1.11	36.0	2.0
E32	*BRAF* WT				
E33	*BRAF* WT				
E34	*BRAF* WT	*NRAS* Q61K	3.02	9900	40
		CDKN2A R80X	3.82	1396.0	14.0
E35	*BRAF* WT	ND			
E36	*BRAF* WT	ND			
E37	*BRAF* WT	ND			
E38	*BRAF* WT	ND			
E39	*BRAF* WT	*BRAF* V600E	0.39	0	0
E40	*BRAF* WT	*YAE1D1*_c39605965G>A	0.34	NT	NT
E41	*BRAF* WT	ND			
E42	*BRAF* V600E	ND			
E43	*BRAF* V600E	ND			
E44	*BRAF* V600K	ND			
E45	*BRAF* V600E	ND			
E46	*BRAF* V600E	ND			
E47	*BRAF* V600E	ND			
E48	*BRAF* V600E	ND			

Supplementary Table 3: Extended comparison of UltraSEEK and ddPCR for specificity assessment (cont)

ID	HC or *BRAF* status	Mutation	Norm	ddPCR	
				copies/ml	FA
E49	HC	ND			
E50	HC	ND			
E51	HC	ND			
E52	HC	ND			
E53	HC	ND			
E54	HC	ND			
E55	HC	ND			
E56	HC	ND			
E57	HC	ND			
E58	HC	ND			
E59	HC	ND			
E60	HC	ND			
E61	HC	ND			
E62	HC	ND			
E63	HC	ND			
E64	HC	ND			
E65	HC	ND			
E66	HC	ND			
E67	HC	ND			
E68	HC	ND			

NormInt: Normalised intensity. FA: frequency abundance of mutant copies relative to wild-type DNA. ND: Not detected. HC: Healthy Control. NT: Not tested