Beyond V600E: Comprehensive genotyping of BRAF codon 600

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Introduction

BRAF mutations at codon 600 occur in 70 – 90% of metastatic melanomas and somewhat less frequently in colorectal, ovarian, thyroid and other tumors. The V600E (T1799A) activating mutation occurs with highest frequency across all tumor types, while lower frequency variants are of emerging importance. Pan-RAF and mutant-specific BRAF inhibitors have shown promising results in clinical trials. Lower frequency variants such as V600K (10 – 15%) have displayed differential responses to inhibitor therapies, demonstrating the importance of distinguishing between these variants. We have developed a robust semi-quantitative allele specific PCR assay that categorizes the codon 600 variants for identification of patients that may benefit from targeted therapies. To complement these AS-PCR assays, clinical samples with high mutant allele percentage (>10%) are reflexed to our validated Sanger sequencing assay. Together these assays allow complete characterization of high frequency V600E/K mutants and the rarer variants V600R, V600M, and V600D, as well as de-novo mutations that may arise.

Materials and methods

Genomic DNA was extracted from immortalized human melanoma cell lines and FFPE tumor tissues using internally validated protocols. Primers were designed and optimized to distinguish mutant variants. Sensitivity and specificity were evaluated using serial dilutions of mutant BRAF into wild type DNA. Precision and accuracy were determined using a blinded set of remnant clinical samples of known genotype. The AS-PCR assay utilizes 3 primer sets and a common TaqMan-MGB probe to generate an overlapping control amplicon and two allele-specific codon 600 amplicons that categorize E and K type variants (**Figure 1**). The Sanger sequencing assay provides bi-directional coverage spanning nucleotides 1742-1860 of exon 15 using standard PCR amplification and capillary electrophoresis.

BRAF allele-specific assay design distinguishes between V600E and V600K variants

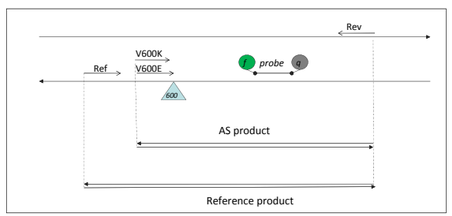


Figure 1. The AS-PCR assay utilizes 3 primer sets and a common TaqMan-MGB probe to discriminate between V600E and V600K variants. Rare variants D, M and R are also detected (see "Result Key," Table 2).

Results

Similar PCR efficiencies enable sensitive, semi-quantitative detection of codon 600 variants

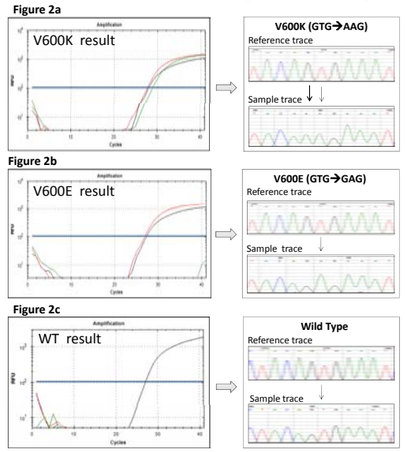


Figure 2a-c. Samples are run with the reference assay (black trace), V600E assay (red trace), and V600K assay (green trace). Amplification from all three assays indicates a V600K result (**Figure 2a**). Amplification from the Reference and V600E assays indicates a V600E result (**Figure 2b**). Amplification from the reference assay only indicates a wild type result (**Figure 2c**). ΔCts between the reference and mutation assays > 9 are classified as mutation negative. Positive results can be reflexed to Sanger sequencing.

Rare codon 600 mutations: amplification signatures can be used to categorize rare variants

The BRAF-AS PCR assays enable the identification of V600K while distinguishing E and D from M and R variants. The BRAF exon 15 Sanger sequencing assay can be used to further categorize rare mutants.

Mutations Captured

Mutation Name	% of BRAF mutations	Sequence at codon 600	Captured by
V600E	70-90%	GAG	V600E primer
V600K	10-30%	AAG	V600K primer
V600R	<5%	AGG	V600K primer
V600M	<5%	ATG	V600K primer
V600D	<5%	GAT	V600E primer

Table 1: Variants captured by BRAF AS-PCR assays and incidence among BRAF codon 600 mutations in melanoma patients[1,2].

Result key

Reference amplification	V600E primer amplification	V600K primer amplification	Genotype
✓			Wild Type
✓	✓		V600E, V600D
✓		✓	V600R, V600M
✓	✓	✓	V600K

Table 2: Amplification signatures of codon 600 variants.

Sensitivity: 0.2% mutant is detectable in a background of wild-type

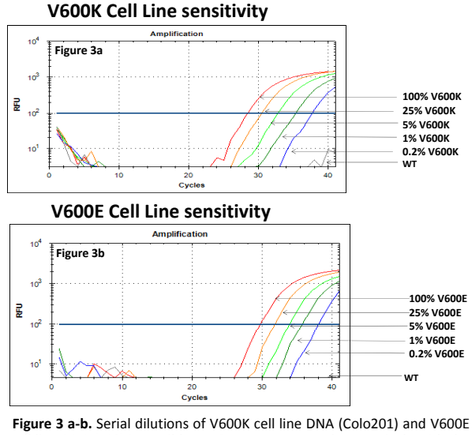
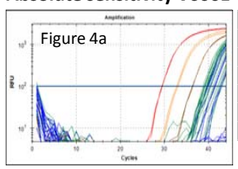


Figure 3 a-b. Serial dilutions of V600K cell line DNA (Colo201) and V600E cell line DNA (M6) into wild type DNA were assayed respectively with the V600K AS-PCR (**Figure 3a**) and the V600E AS-PCR (**Figure 3b**). Both assays demonstrate high specificity, with 0.2% mutant template (~10 copies) detectable against a background of wild type DNA.

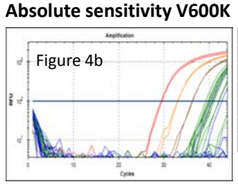
Absolute sensitivity: approaching single copy detection

Absolute sensitivity V600E



Copy #	Trace Color	% Mut	Mean Ct	Detected
3333.5	Red	50%	29.37	100%
333.4	Orange	5%	32.87	100%
33.3	Brown	0.50%	36.3	100%
3.3	Green	0.05%	40.3	100%
0.33	Blue	0.01%	41.18	35%
0	Black	0%	0	0%

Absolute sensitivity V600K



Copy #	Trace Color	% Mut	Mean Ct	Detected
3333.5	Red	50%	29.39	100%
333.4	Orange	5%	33.6	100%
33.3	Brown	0.50%	36.24	100%
3.3	Green	0.05%	41.13	75%
0.33	Blue	0.01%	41.65	10%
0	Black	0%	0	0%

Figure 4 a-b. Absolute sensitivity of the BRAF AS-PCR assays were examined by assaying V600E (**Figure 4a**) and V600K (**Figure 4b**) plasmids diluted to extinction. Copy numbers were calculated by mass from spectrophotometric measurement (nanodrop). Absolute sensitivity is ~1-3 copies per reaction.

Accuracy: 100% concordance of melanoma patient samples in blinded study

Sample	Reference Ct	V600E Ct	V600K Ct	V600E delta	V600K delta	Result	Known Genotype	Concordant
MMD001	30.49	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD002	32.1	32.65	Undet.	0.55	N/A	V600E	V600E	Yes
MMD003	35.28	35.82	Undet.	0.54	N/A	V600E	V600E	Yes
MMD004	28.93	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD005	28.17	28.49	29.37	0.32	1.2	V600K	V600K	Yes
MMD006	29.7	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD007	27.67	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD008	30.59	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD009	33.25	33.24	Undet.	-0.01	N/A	V600E	V600E	Yes
MMD010	30.67	Undet.	39.84	N/A	9.17	Negative	Negative	Yes
MMD011	34.05	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD012	31.3	31.56	31.85	0.26	0.55	V600K	V600K	Yes
MMD013	29.43	30.05	30.6	0.62	1.17	V600K	V600K	Yes
MMD014	32.03	32.18	Undet.	0.15	N/A	V600E	V600E	Yes
MMD015	28.3	30.35	Undet.	2.05	N/A	V600E	V600E	Yes
MMD016	28.53	30.21	Undet.	1.68	N/A	V600E	V600E	Yes
MMD017	34.69	35.24	35.02	0.55	0.33	V600K	V600K	Yes
MMD018	29.3	30.18	Undet.	0.88	N/A	V600E	V600E	Yes
MMD019	29.16	30.32	Undet.	1.16	N/A	V600E	V600E	Yes
MMD020	26.9	28.2	38.35	1.3	11.45	V600E	V600E	Yes
MMD021	34.05	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD022	30.5	29.95	30.46	0.55	0.04	V600K	V600K	Yes
MMD023	29.51	30.15	30.25	0.64	0.74	V600K	V600K	Yes
MMD024	30.46	30.88	31.19	0.42	0.73	V600K	V600K	Yes
MMD025	28.1	29.83	38.28	1.73	10.18	V600E	V600E	Yes
MMD026	36.65	34.25	34.19	2.4	2.46	V600K	V600K	Yes
MMD027	32.9	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes
MMD028	27.72	28.08	28.89	0.36	1.17	V600K	V600K	Yes
MMD029	28.22	28.67	29.45	0.45	1.23	V600K	V600K	Yes
MMD030	36.28	Undet.	Undet.	N/A	N/A	Negative	Negative	Yes

Table 3: Accuracy was determined using remnant clinical FFPE melanoma samples of known genotype. A ΔCt cut-off of 9 cycles was used to determine genotype (ΔCt of 9 is ~ 0.2% mutant). There was 100% concordance between the BRAF AS-PCR assay results and the known genotype.

Conclusions

The BRAF AS-PCR test has been validated to discriminate between V600E and V600K variants which may be significant in determining treatment. The assay design also captures rare codon 600 mutations, distinguishing E and D mutants from R and M. Determination of exact mutants can be further confirmed with allele frequencies within the sensitivity range of the Sanger sequencing assay (10 – 20 %). The AS-PCR test has been optimized for amplification of DNA isolated from FFPE specimens containing low levels of mutant target with a sensitivity to 0.2% (~10 copies). Combined, the AS-PCR test provides the flexibility of genotyping low level codon 600 mutations and the Sanger sequencing assay allows for complete surveillance of exon 15 to identify mutations that may influence therapeutic efficacy.

References

1. Jill C Rubinstein, Mario Sznol, Anna C Pavlick, Stephan Ariyan, Elaine Cheng, Antonella Bacchiocchi, Harriet M Kluger, Deepak Narayan, Ruth Halaban. 2010. Incidence of the V600K mutation among melanoma patients with BRAF mutations, and the potential therapeutic response to the specific BRAF inhibitor PLX4032. *Journal of Translational Medicine* 8:68.
2. Christine Lovly, Leora Horn, William Pao. My Cancer Genome. Vanderbilt-Ingram Cancer Center. http://www.mycancergenome.org/mutation.php?dz=melanoma&gene=BRAF&code=BV600

For further information

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