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 reclassified to our validated Sanger sequencing assay. Together these assays allow complete characterization of high frequency V600E/K mutants and the rarer variants V600R, V600D, and V600Q, 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 can be categorized into 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

Real-time PCR

Sanger Sequencing

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 V600K assays indicates a V600K result (Figure 2b). Amplification from the reference assay only indicates a wild type result (Figure 2c). AbsCts between the reference and mutation assays > 9 are classified as negative. Positive results can be referred 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 V600E 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

<table>
<thead>
<tr>
<th>Mutation Name</th>
<th>% of BRAF mutations</th>
<th>Sequence at codon 600</th>
<th>Captured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>V600E</td>
<td>70-95%</td>
<td>GAG</td>
<td>V600K primer</td>
</tr>
<tr>
<td>V600K</td>
<td>5%</td>
<td>AGG</td>
<td>V600K primer</td>
</tr>
<tr>
<td>V600M</td>
<td>5%</td>
<td>GAG</td>
<td>V600K primer</td>
</tr>
<tr>
<td>V600D</td>
<td>5%</td>
<td>GAT</td>
<td>V600K primer</td>
</tr>
</tbody>
</table>

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

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

Absoluteness: approaching single copy detection

Absolute sensitivity V600E

<table>
<thead>
<tr>
<th>Sample</th>
<th>Reference Ct</th>
<th>V600E Ct</th>
<th>V600K Ct</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLX1</td>
<td>100%</td>
<td>Undet.</td>
<td>Undet.</td>
<td>Yes</td>
</tr>
<tr>
<td>PLX2</td>
<td>100%</td>
<td>Undet.</td>
<td>Undet.</td>
<td>Yes</td>
</tr>
<tr>
<td>PLX3</td>
<td>100%</td>
<td>Undet.</td>
<td>Undet.</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 2: Amplification signatures of codon 600 variants

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

Conclusion
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 allelic 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 of 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

For further information
Please contact info@molecularmd.com or visit www.molecularmd.com.