High-sensitivity Target-enriching Sanger Sequencing Validation for the Human BRAF V600E Mutation Detection

Abstract

The low sensitivity of Sanger sequencing has prevented it from companion diagnostic applications. We have validated mutation-enriching Sanger sequencing for the identification of BRAF V600E mutation using our own Sanger sequencing reagents. The BRAF V600E mutation has been accurately and sensitively identified from both human genomic DNA variants and clinical samples of known BRAF V600 variants. The mutant-enriching Sanger sequencing has a great potential to be validated as a companion diagnostic tool in clinical labs.

Human BRAF is an important member in the Ras-Raf-MEK-ERK signal transduction pathway which controls cell proliferation. Mutations in BRAF have been found to be strongly associated with multiple cancers including melanoma, colorectal and non-small cell lung cancer (NSCLC). The most common mutation in BRAF is V600E at exon 15 (a T to A mutation at c.1799) and the mutant protein has higher and constitutive enzyme activity, leading to constant activation of downstream regulators. Other mutations such as V600K, V600D, and V600R are also found in these cancers at much lower frequencies.

The FDA approved multiple companion diagnostic tests for BRAF V600E targeted cancer therapies, including real-time PCR assay (qPCR), amplification-refractory mutation system (ARMS), and next generation sequencing (NGS). Other methods for BRAF V600E mutation testing include Sanger sequencing, mass spectrometry, high resolution melting (HRM), PNA or XNA-based qPCR, digital droplet PCR (ddPCR), and pyrosequencing. Most of these methods have a sensitivity of 1 to 10% of mutant/normal cell population, better than that of traditional Sanger sequencing (15 to 20%). However, HRM, qPCR, ARMS, and ddPCR assays fail to reveal the nature of the mutation and may also miss certain BRAF V600 mutations. The NGS assays, designed to be used for more complicated cancer detection and therapies, need to sequence multiple genes and take longer time. They are more costly for small sample sizes or detection of one or a few mutations.

Sanger sequencing is a routine technique used in molecular diagnostics labs. Due to its limited sensitivity, the technique has been more widely used for the detection of a single or a few gene mutations of genetic diseases and mutation confirmation for NGS in modern molecular diagnostics. However, if the sensitivity of Sanger sequencing is improved, it will have a potential to be used as a companion diagnostic assay for detection of a single or a few gene mutations to profile patients for target therapies. In this application note, we first use the BRAF V600E mutation enriching kit to selectively amplify the mutation and suppress amplification of the wild type BRAF sequence by PCR in the presence of BRAF V600E XNA. The mutation-enriching PCR product is then used as the template for Sanger sequencing to effectively and accurately identify BRAF V600E mutations. This method significantly improves the Sanger sequencing sensitivity for detection of low frequency mutations that are usually not confidently called. Therefore, there could be great interest to validate this method in CLIA labs for BRAF V600E mutation testing for companion diagnostics applications. Here we report the procedure used for validation of this novel mutation detection method on AB 3730XL using our own Sanger sequencing reagents.

MATERIALS AND METHODS

Samples for Sequencing

To demonstrate the mutant target-enriching Sanger sequencing, we used QClamp® BRAF V600E Mutation Enrichment Kit (referred as "the enrichment kit" below, DiaCarta Inc.) including the two positive controls with BRAF V600E mutation at 1.25% and 5% variant allele frequency (VAF), respectively. The genomic DNA isolated from Formalin Fixed Paraffin Embedded (FFPE) clinical samples used in the application note is also a gift from DiaCarta.

Enrichment of the BRAF V600E Mutation

Ten ng DNA from positive controls or clinical samples were used as the template. The enrichment PCR for the BRAF V600E mutation was conducted on the 9700 GeneAmp PCR instrument (Applied Biosystems) following the enrichment kit user manual. The PCR reactions were conducted in the presence or absence of the BRAF XNA from the kit. The PCR products were cleaned up by ADS Exo-Alp PCR Cleanup Mix (AdvancedSeq) according to the user manual. The purified product was diluted 8-fold and 2 µl was used for the downstream cycle sequencing reactions.

Sanger Sequencing

From cycle sequencing reactions to sample loading and running on capillary electrophoresis, all the Sanger sequencing reagents are from AdvancedSeq, including SupreDye v3.1 Cycle Sequencing Kit and SupreDye XT Purification Kit. The 10 µl sequencing reactions contains 0.4 µl SupreDye v3.1 premix and 0.5 µM BRAF V600E forward or reverse primer from the enrichment kit. The cycling conditions were: 95 °C for 30s, followed by 30 cycles of 95 °C for 10s, 50 °C for 5s, and 60 °C for 2 min. The sequencing reaction was then purified by the SupreDye™ XT Purification Kit according to the manual before loading onto AB 3730XL Genetic Analyzer with PwrPOP™-7 filled capillary array.

RESULTS AND DISCUSSION

The BRAF V600E Mutations in Positive Controls are Enriched and Identified by Sanger Sequencing

Due to its low sensitivity, Sanger sequencing has not been used for BRAF V600E detection in clinical labs for targeted cancer therapy. To improve the sensitivity of using Sanger sequencing for BRAF V600E mutation detection, we prepared the templates from both positive and negative controls using the enrichment kit in the presence or absence of BRAF XNA. The positive controls from the kit with 1.25% and 5% VAF mutation are sequenced to identify the BRAF V600E mutation comparing to the negative control (Figure 1). When no XNA is present, the BRAF V600E mutation in both positive controls is either undetectable or barely detectable (for 5% positive with forward primer). However, the BRAF V600E XNA strongly enriched the mutation, allowing only the mutation peaks to appear for both 1.25% and 5% VAF of templates. These results show the enrichment kit (with the XNA) significantly improve the sensitivity of the standard Sanger sequencing assay (when no XNA is present). These results indicate that the sensitivity of detection is at least 1.25% after mutation enrichment. In fact, the BRAF V600E XNA allows the Sanger sequencing sensitivity to reach 0.04% (unpublished data, DiaCarta).

Figure 1: Sequencing of the positive controls (BRAF V600E mutant with 1.25% and 5% VAF) and negative controls (0% mutant) for the BRAF V600E mutations. The negative control shows the wildtype sequence while the BRAF V600E mutation in positive controls are identified after enrichment in the presence of BRAF V600E XNA followed by Sanger sequencing using both forward and reverse primers. The arrows point to the base where the mutation can occur. For forward primer sequencing, the wildtype reads T and the mutant reads A; for reverse primer sequencing, the wildtype reads A and the mutant reads T.

Validation of BRAF V600E Mutations in Known Clinical Samples

We further validate the enrichment kit for known clinical samples. Again, all the five clinical samples (BRAF V600E mutant samples #82, #83, #86, and #90, and negative sample #87) were sequenced by Sanger sequencing after mutation enrichment. The sequencing results are 100% concordant with the known mutation status of the clinical samples. The sequencing results for samples #82, #86, and #87 are shown in Figure 2.

In the absence of BRAF V600E XNA, both the wild type and the mutant sequence peaks are shown in the two samples #82 and #86 with BRAF V600E mutations, but the BRAF V600E negative sample #87 only shows the wild type peaks for both forward primers and reverse primers. However, in the presence of BRAF XNA, the wild type peaks from both #82 and #86 mutant disappear and only the mutant peaks show up. On the other hand, for sample #87, only the wildtype peak shows up when using the reverse sequencing primer while the wildtype sequence could not be correctly amplified in the presence of the XNA using the forward sequencing primer. This is also observed when the forward primer was used to sequence the negative control wildtype BRAF in the presence of XNA (data not shown).

In the PCR without the BRAF XNA, which is the normal Sanger sequencing template amplification step, the BRAF V600E-positive clinical samples may or may not show the mutant peak in Sanger sequencing depending on the VAF of the mutant. If the VAF is low, the mutation may not appear or can be confidently called due to the limitation of the Sanger sequencing. However, the mutant can be clearly identified as a single mutant peak in the presence of BRAF V600E XNA, even the VAF is as low as 1.25%, as indicated when the genomic DNA was used (Figure 1). These results suggest that the mutant-enriching Sanger sequencing will work well for clinical samples.

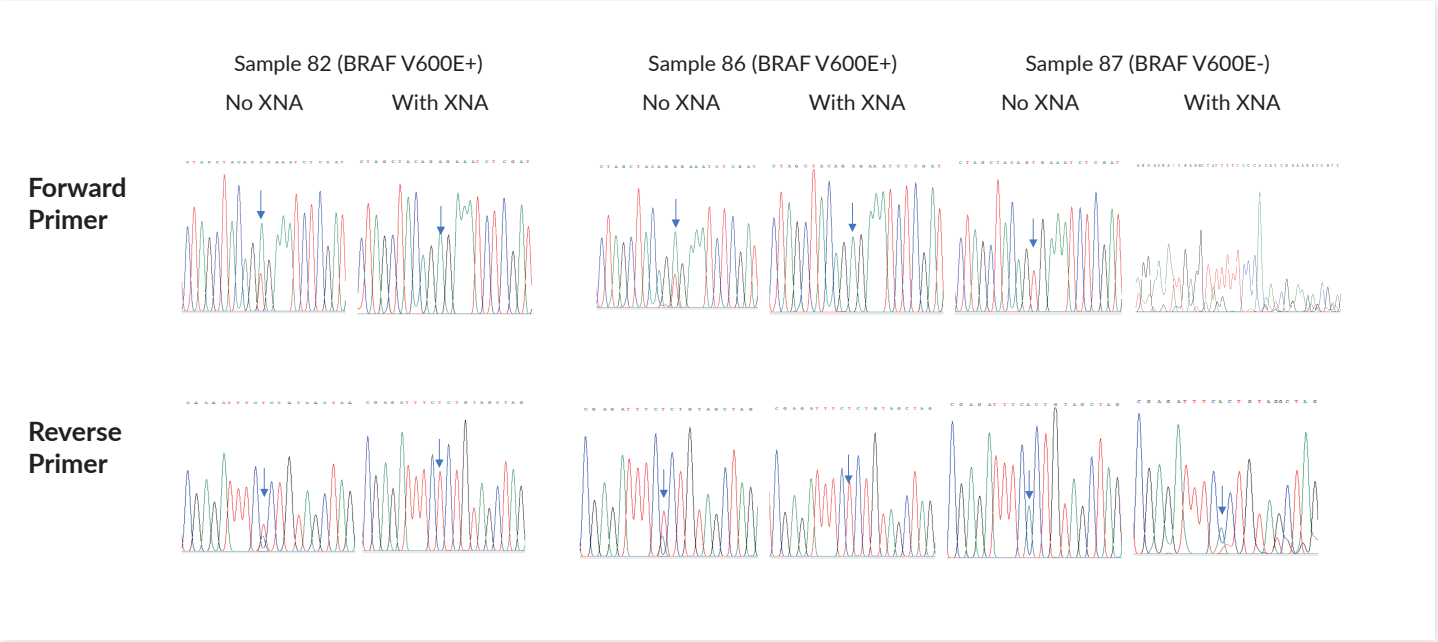

Figure 2. Clinical sample BRAF V600E testing using BRAF enrichment followed by Sanger sequencing. Genomic DNA from two BRAF V600E positive samples (#82 and #86) and one wildtype sample (#87) were enriched for BRAF V600E mutation in the presence or absence of BRAF XNA and the Sanger sequencing was performed using both forward and reverse primers. The arrows point to the base where the mutation can occur. For forward primer sequencing, the wildtype reads T and the mutant reads A; for reverse primer sequencing, the wildtype reads A and the mutant reads T.

CONCLUSION

In summary, we have demonstrated the application of QClamp® BRAF V600E Mutation Enrichment Kit and Sanger sequencing reagents from AdvancedSeq for the identification of BRAF V600E from both the genomic DNA mutants and clinical samples. The mutant target-enriching Sanger sequencing, without adding any additional steps, significantly improves the Sanger sequencing sensitivity of detecting BRAF mutations. The novel method can be validated and adopted in any CLIA labs for BRAF V600E mutation detection as a companion diagnostics assay.

We have validated the novel assay on AB 3730xL with our own v3.1 SupreDye Cycle Sequencing Kit and other sequencing reagents. It is evident that high-quality Sanger sequencing results can be achieved using our reagents, which can be used for validation and testing of this and other Sanger sequencing assays in CLIA labs.