



Yanggu Shi, Tyler Borcyk, Benjamin Legendre, Jr., Phil Eastlake, and Katherine Richardson
Transgenomic, Inc. 12325 Emmet St., Omaha, NE 68164, USA
Tel: 1-888-813-7253 Fax: 402-452-5401 Email: info@transgenomic.com

ABSTRACT

BRAF is a serine/threonine kinase which mediates the RAS/RAF/MAPK signal transduction pathway. Over 90% of the BRAF mutations observed is V600E (c.1796T>A), which constitutively activates mitogenic cascades leading to tumorigenesis. The V600E mutation is present in 70% melanoma, 100% hairy cell leukemia, 40% papillary thyroid cancer, and less frequently in other cancer types. Methods to determine the mutation status are valuable for clinical diagnosis, companion diagnostic tests, treatment guidance and outcome prediction. ICE COLD-PCR (Improved & Complete Enrichment CO-amplification at Lower Denaturation temperature PCR) is a novel mutant DNA enrichment technology that selectively amplifies the mutant DNA population by blocking normal DNA amplification with a modified reference strand (RS) oligo. After the enrichment with ICE COLD-PCR, the V600E mutation could be detected at a level as low as 0.05% with standard Sanger sequencing. For another clinically significant BRAF mutation G469A (c.1406G>C), the limit of detection reached 0.01%. We combined ICE COLD-PCR enrichment and dye terminator sequencing in one BLOCKer Sequencing (BLOcking Oligonucleotide Cycle Sequencing) reaction. In this application, a sequencing primer, a 5'-phosphorylated amplification primer, and a wild type blocking RS oligo were added to the cycle sequencing reaction, which contains additional thermal cycler steps to selectively denature the RS oligo from the mutant DNA sequence. This allows for amplification of the mutant sequence while simultaneously extending the sequencing primer. The interfering dye termination product from the amplification primer was then selectively degraded using lambda exonuclease, which digests the 5'-phosphorylated strand. The BLOCKer sequencing alone increased the V600E detection limit from 20% with standard Sanger sequencing to 1%, which compares favorably with other commercially available non-sequencing based mutation detection techniques. We expect even higher sensitivity by performing BLOCKer sequencing on samples pre-enriched with ICE COLD-PCR.

INTRODUCTION

Low level mutation detection has many important applications in biomarker discovery and genetic diagnostics. One way to achieve highly sensitive mutation detection is to selectively PCR amplify the mutant DNA in the sample. ICE COLD-PCR employs modified reference sequence oligos (RS) with the sequence identical to the wild type DNA to block the wild type extension at a critical temperature (T_c). Due to the thermodynamic difference between the perfectly matched (wild type) and mismatched (mutant) target DNA with the RS oligo, the PCR extension for the mutant DNA is not blocked (Fig.1).

We further combined ICE COLD-PCR with Sanger sequencing chemistry into a one tube reaction, producing sequencing traces with dramatically increased mutation detection sensitivity for mutant alleles (Fig. 2). Here we present the data for BRAF mutations V600E and G469A using the described technologies.

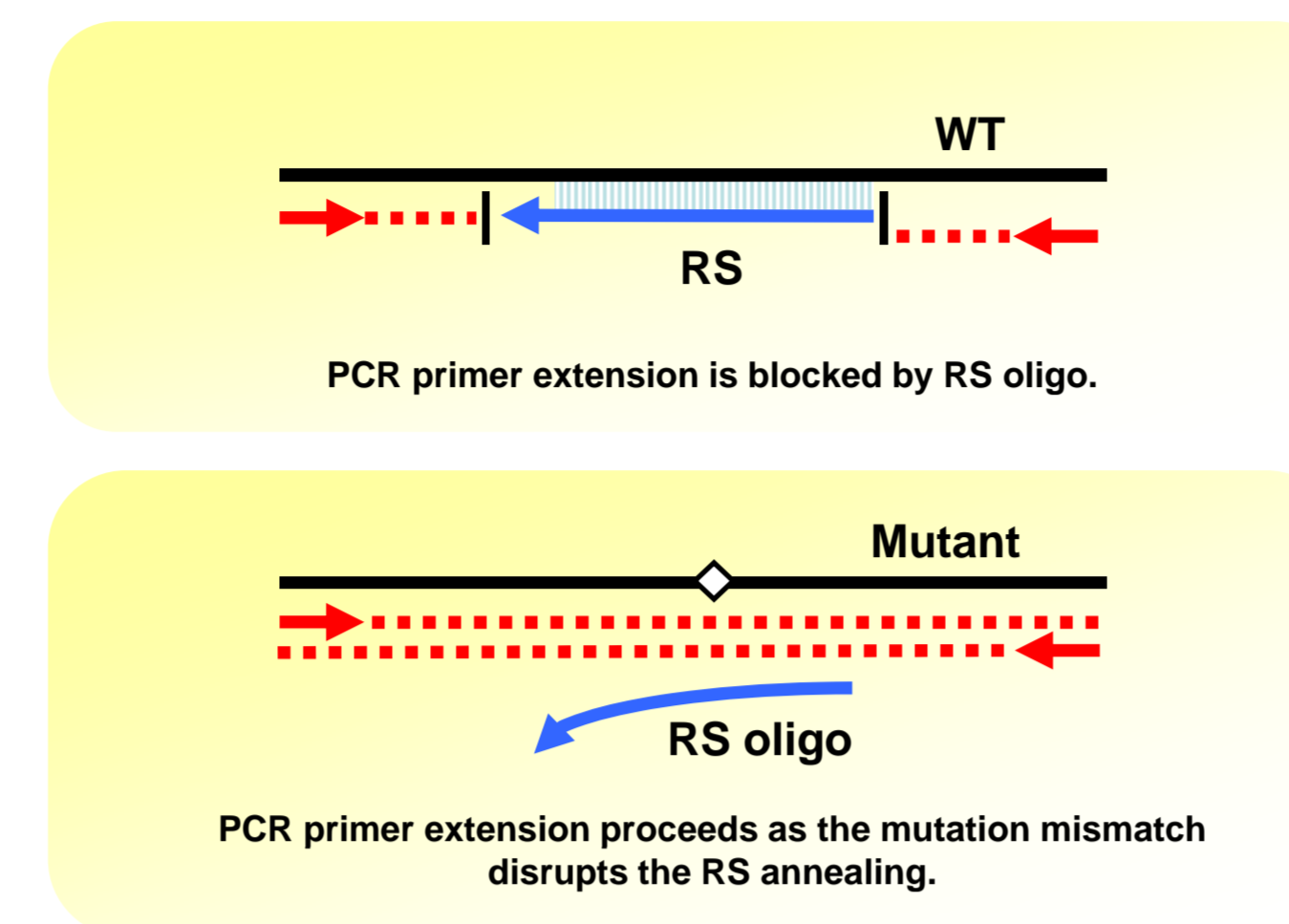


Fig.1. Mutant enrichment by ICE COLD-PCR at critical temperature T_c.

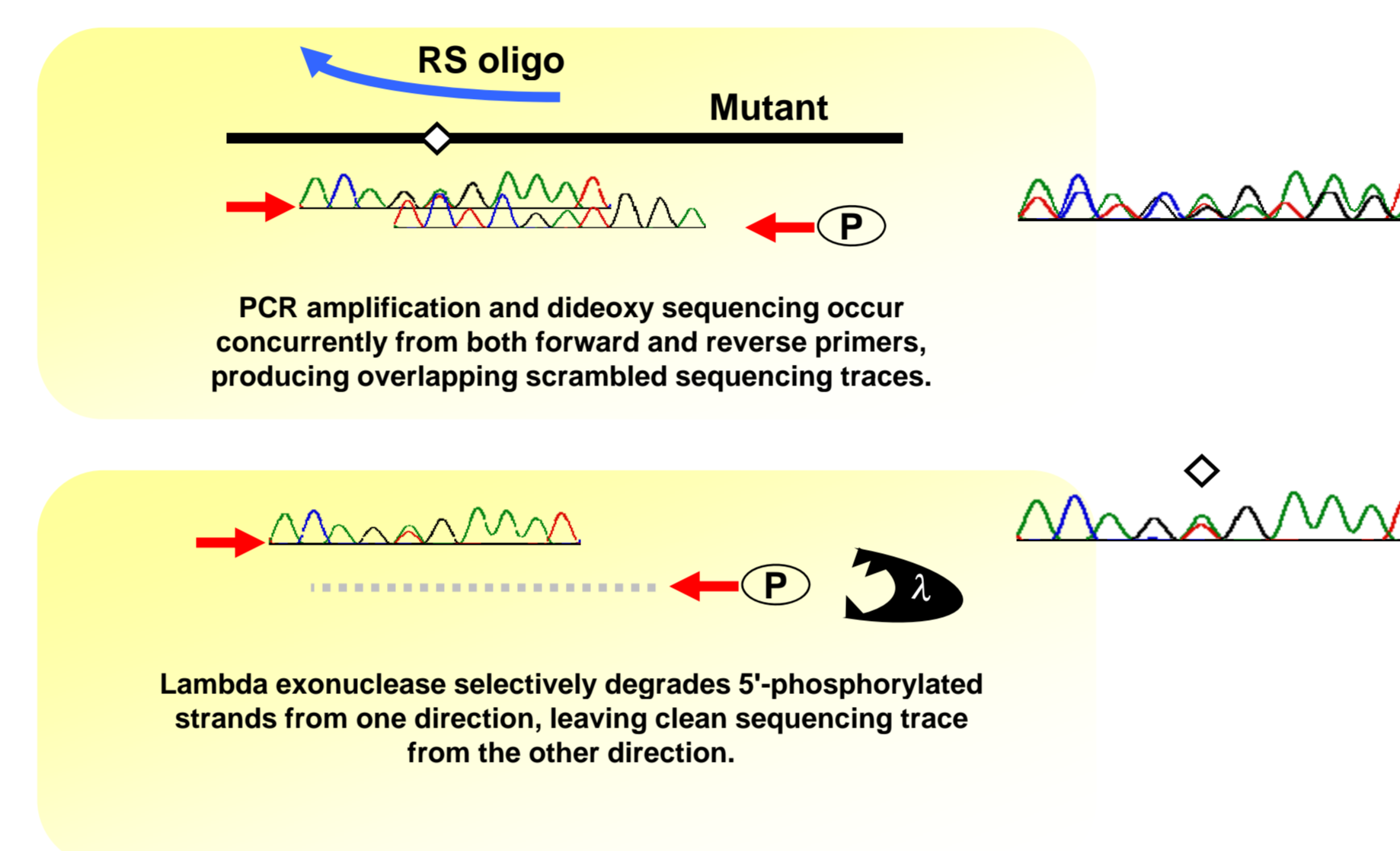


Fig.2. Combined ICE COLD-PCR to enrich mutant DNA and Sanger sequencing chemistry

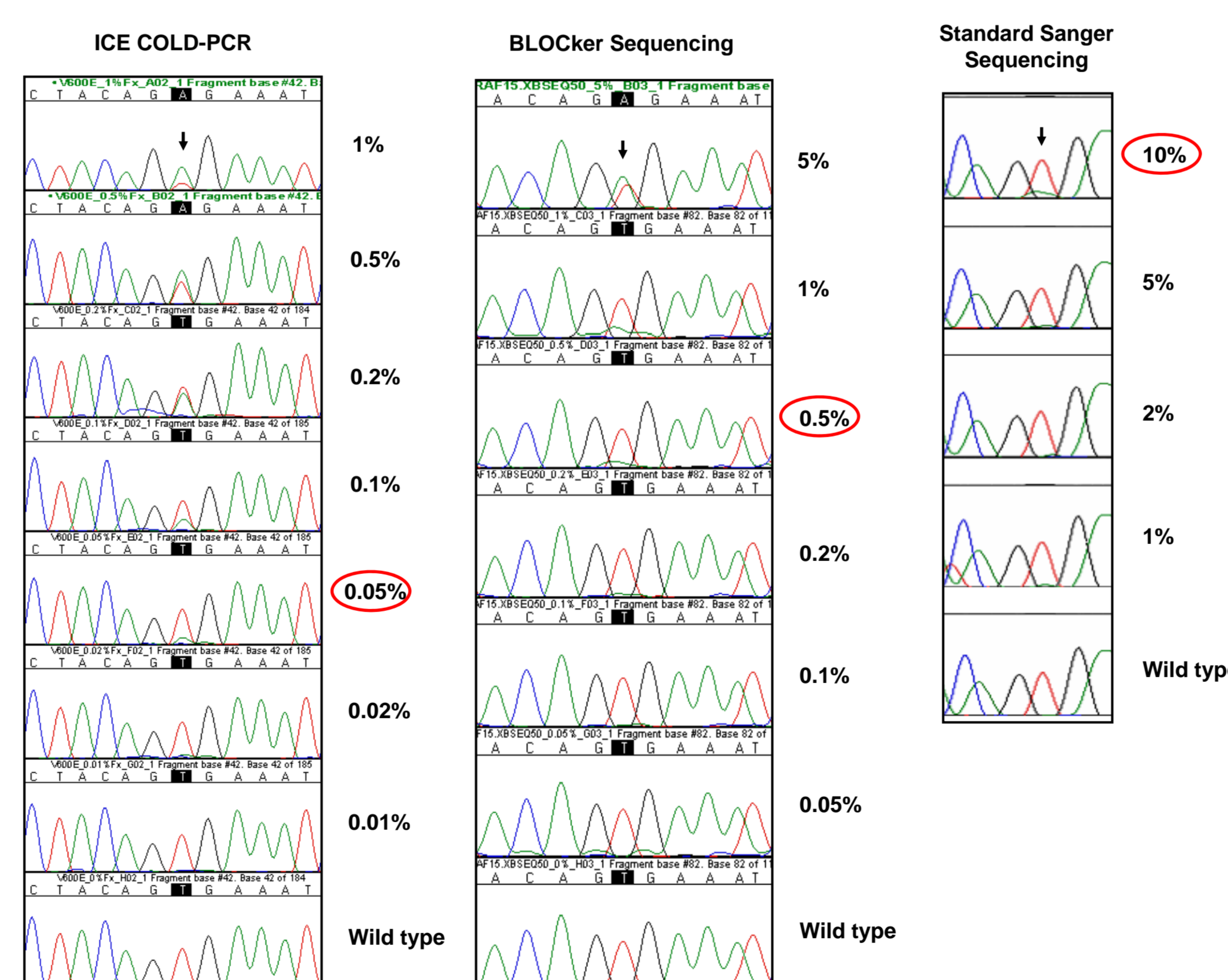


Fig.3. The LODs of V600E mutation in BRAF exon 15 were 0.05% by ICE COLD-PCR (left), 0.5% by BLOCKer Sequencing (middle), and 10% by Standard Sanger Sequencing (right). The arrows indicate the V600E mutation.

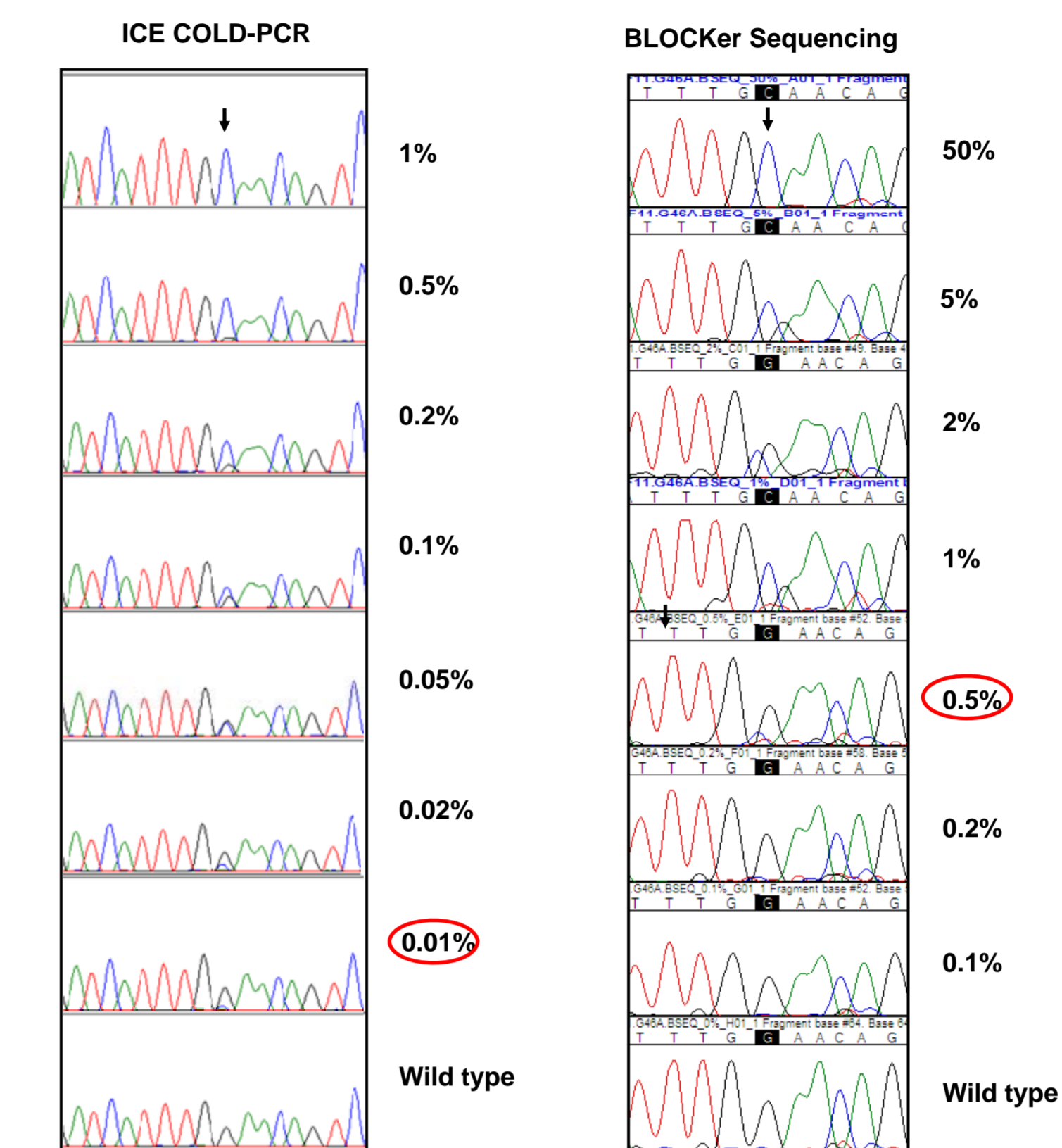


Fig.3. The LODs of G469A mutation in BRAF exon 11 were 0.01% by ICE COLD-PCR (left) and 0.5% by BLOCKer sequencing (right). The arrows indicate the G469A mutation.

MATERIALS AND METHODS

Locked Nucleic Acid (LNA) modified RS oligos were synthesized by Exiqon. Unmodified PCR primers were synthesized by Integrated DNA Technologies. The wild type genomic sequences of BRAF exon 15 and exon 11 were cloned into pNEB206A vector. The V600E and G469A mutations were introduced into the wild type plasmids by site directed mutagenesis. Serial dilutions of the mutant DNA in the wild type DNA at the same concentration were prepared for the LOD studies.

For ICE COLD-PCR, an initial pre-amplification PCR is performed with Phusion Polymerase (New England Biolabs). The ICE COLD-PCR reaction was optimized for primer and RS oligo concentration using T-TAQ Polymerase (Transgenomic, Inc.). The reactions were carried out in a thermal cycler with precise thermal control. Post ICE COLD-PCR products were subjected to standard sequencing.

For BLOCKer sequencing, one of the PCR primers was 5'-phosphorylated. ABI BigDye was used for the Sanger sequencing. Lambda exonuclease treatment was added before sequencing analysis.

All reactions were analyzed on an ABI 3730 XL DNA Sequencer.

CONCLUSIONS

1. Both ICE COLD-PCR and BLOCKer sequencing detected BRAF V600E and G469A with very high sensitivity.
2. Mutation detection by ICE COLD-PCR and BLOCKer sequencing offers familiar sequencing trace readouts as standard sequencing.
3. BLOCKer sequencing has the potential to be formulated for routine dye terminator/CE sequencing for high sensitivity mutation detection using standard sequencing instruments.
4. BLOCKer sequencing is well suited for mutation confirmation in conjunction with mutation scoring, such as dHPLC or Surveyor™ Nuclease mismatch cutting.