Sensitive Detection of *BRAF* Hotspot Mutation V600E using E-*ice*-COLD-PCR Combined with Pyrosequencing

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Abstract: It is important to sensitively detect *BRAF* V600E mutation, since it is an important biological marker in several types of cancers, such as melanomas and colorectal cancers. Here, we have combined enhanced improved and complete enrichment co-amplification at lower denaturation temperature-polymerase chain reaction (E-*ice*-COLD-PCR) with pyrosequencing for the detection of *BRAF* V600E mutation. A serial of mutation-containing dilutions was determined, and the detect limit of E-*ice*-COLD-PCR/pyrosequencing and conventional PCR/pyrosequencing assays were 0.1 and 5%, respectively. When *BRAF* V600E mutation in 10 melanoma patients were further determined, all samples with the V600E mutation detected by conventional PCR/pyrosequencing were also found to be detectable with E-*ice*-COLD-PCR/pyrosequencing. However, one sample was detected by using E-*ice*-COLD-PCR/pyrosequencing but not by conventional PCR/pyrosequencing. In summary, E-*ice*-COLD-PCR/pyrosequencing is sensitive, reliable and cost-effective for detecting *BRAF* V600E mutation in clinical samples.

1 INTRODUCTION

Mutations in *BRAF* have been found in a wide range of human cancers, including melanomas (59%), colorectal cancers (10%), thyroid cancers (30–70%), and early ovary cancers (30%) (Besaratinia 2008). Of those mutations, *BRAF* V600E mutation which replaces the valine by glutamate at codon 600 is the most frequent mutation, accounting for ~92% of all *BRAF* cancer mutations (Besaratinia 2008). Thus, it is clinically important to detect *BRAF* V600E mutation. Sanger sequencing is the gold standard for molecular diagnosis, and it is accurate and reliable (How-Kit 2013). However, it has several limitations in terms of costs effectiveness and sensitivity. For example, its limit of detection is only 10–30% of mutated alleles in a background of wild-type alleles (How-Kit 2013). Mostly, the DNA abundance or concentration of specimen samples obtained from tumor tissues and liquid biopsies, such as cell-free DNA (cfDNA), may be lower than 1%. In this case, the low sensitivity of Sanger sequencing will not be sufficient to detect the aforementioned low-abundance *BRAF* V600E mutation.

Pyrosequencing is a real-time DNA sequencing technique which measures the sensitivities of emitted light during DNA synthesis. It employs a set of enzymatic reactions to generate inorganic PPi and to convert it to visible light during the polymerization. This technology adds one nucleotide into the reaction at a time during DNA synthesis. Due to the previously known on the type of bases added, the sequence of the determined template can be interrogated sequentially (Tan 2008). Pyrosequencing offers a specific, sensitive, rapid and cost-effective alternative to dideoxy sequencing for the detection of *BRAF* V600E mutation (Tan 2008). Although pyrosequencing has a low detection limit of 5%, it still does not enable to detect the low percentage of mutated DNA.

To further decrease the limit of mutation detection, methods which are capable of enriching the unknown mutations in samples have been developed. For example, Co-amplification at lower denaturation temperature PCR (COLD-PCR) has been established

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to highly enrich low-abundance mutations from clinical samples, and the detection efficiency is independent of the mutation type and position (Li 2008). However, COLD-PCR is not able to effectively enrich and identify all forms of unknown mutations (Li 2008, Milbury 2011). Another drawback of COLD-PCR is that it needs to very precisely control a critical temperature (T_c) as a slight variation of 0.2°C may completely fail the mutation enrichment (Milbury 2011). Therefore, by the inclusive of a wild-type (WT) specific blocker probe, an improved and complete enrichment COLD-PCR (*ice*-COLD-PCR), in which a WT specific blocker probe was included, has been proposed to eliminate shortcoming of COLD-PCR that cannot enrich all mutation types (Milbury 2011). Further, by the incorporation of locked–nucleic acid (LNA) nucleotides in the WT blocker probe, a relatively novel technology, Enhanced-*ice*-COLD-PCR has also been developed. It maximizes the T_m differences between homo- and heteroduplexes for a single base mis-match and thus overcomes the above-mentioned issue that acquires a critical T_c in the COLD-PCR assays (How-Kit 2013). This technique provides a sensitive, reproducible and flexible technique for mutation enrichment.

In the present study, we demonstrate that E-*ice*-COLD-PCR is used to enrich low abundance *BRAF* V600E mutation and pyrosequencing is then applied as the downstream readout technology. The efficiency of E-*ice*-COLD-PCR/pyrosequencing is evaluated in detecting *BRAF* hotspot mutation V600E.

2 MATERIALS AND METHODS

2.1 Samples and DNA Extraction

Tumor specimens were obtained from 10 melanoma patients in this study. Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tissue was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's instructions. After extraction and purification, the DNA was quantified using a Qubit fluorometer (Thermo Fisher, Waltham, MA).

2.2 Plasmid Standards

Plasmid DNA templates harbored *BRAF* V600E mutation in exon 15 of *BRAF* gene were generated. A plasmid containing wild-type *BRAF* exon 15 was also constructed. The mutation type of plasmids was verified by using a PyroMark Q96 MD pyrosequencing instrument (Qiagen, Courtaboeuf, France). All the plasmids of the defined genotypes were used to generate amplicons for positive and negative controls.

2.3 Conventional PCR

Twenty-five nanogram of genomic DNA was used as template in a 25-μL PCR mix including 1× HotStar Taq DNA polymerase buffer, 1.6 mM of additional $MgCl_2$, 200 μM of dNTPs, 200 nM of the forward and reverse primers, and 2 U of HotStar Taq DNA polymerase. PCR cycling conditions contained an initial 95◦C denaturation for 3 min, followed by 40 cycles of 95◦C for 30 s, 56◦C for 30 s, 72◦C for 10 s, and 72°C for 5 min. Amplicons were verified by gel electrophoresis on a 2% agarose gel prior to pyrosequencing.

2.4 E-*ice*-COLD-PCR

E-*ice*-COLD-PCR reaction volume was 50 μL and the reaction mixtures contained 1× of Phusion DNA polymerase buffer, 25 ng of genomic DNA, 200 μM of dNTPs, 200 nM of each primer, 1.6 mM of additional $MgCl_2$, 40 nM of the blocker probe, and 5 U of Phusion™ DNA polymerase (New England Biolabs (NEB)). E-*ice*-COLD-PCR protocol contained an initial 95◦C denaturation for 10 min, followed by 6 cycles of 95◦C for 30 s, 60◦C for 20 s, 72◦C for 10 s, and then 44 cycles of 95◦C for 20 s, 70◦C for 30 s, 60◦C for 20 s, 72◦C for 10 s, and a final extension at 72°C for 5 min.

2.5 Pyrosequencing

Twenty μL of conventional PCR or E-*ice*-COLD-PCR products were purified and the purified products were applied to generate single-stranded for sequencing reaction by use of a PyroMark Q96 Vacuum Workstation (Qiagen, Courtaboeuf, France). Thereafter, the sequencing primer was annealed to the single-stranded target sequence after incubation at 80°C for 2 min. The order of nucleotide dispensation was C/A/G/A/T/G/A/T/C. After the run, the abundance of each genotype was determined by the AQ model of Pyromark Q96 MA software and shown upon the pyrogram.

3 RESULTS

3.1 The Determination of T_c

It is important to determinate the optimal critical T_c for E-*ice*-COLD-PCR reactions, at which mutant sequences were robustly amplified as well as

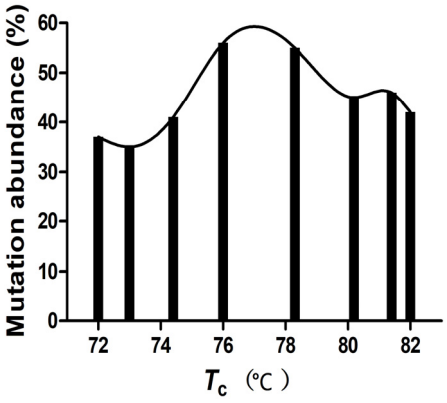

Figure 1: Determination of suitable critical denaturation temperatures (Tc) using a set of E-*ice*-COLD-PCR assays with gradient Tc (from 72◦C to 82◦C) on 5% mutation fraction in mixed plasmids and 40 nM of blockers.

at 5% mutant abundance with 40 nM of blocker probes. The PCR products were analyzed by pyrosequencing. As shown in Figure 1, with the increase of the Tc, the enrichment efficiency of mutant sequences increased. When Tc was 76°C, mutant sequences could be effectively enriched. However, when the Tc is higher than 78.3°C, the enrichment efficiency of mutant sequences gradually decreased. Thus, 76°C was chosen as the appropriate T_c for the E-*ice*-COLD-PCR in this study.

3.2 Comparison of Standard PCR/Pyrosequencing and E-*ice*-COLD-PCR/Pyrosequencing for V600E Mutation Detection

To compare conventional PCR/pyrosequencing and E-*ice*-COLD-PCR/pyrosequencing in detecting low-level *BRAF* V600E mutation, plasmid DNA with *BRAF* V600E mutation was diluted serially into wild-type DNA to generate the following fractions of mutations: 40, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0%. pyrosequencing (Figure 2). As shown, the mutation abundances obtained by both conventional PCR/pyrosequencing and E-*ice*-COLD-PCR/pyrosequencing decreased, as the ratios of the mutant to wild-type plasmids decreased. Since the reported detect limit of pyrosequencing was approximately 5% (Mauger 2017), mutation abundance lower than 5% was undetectable. Thus, E-*ice*-COLD-PCR/pyrosequencing was capable of detecting *BRAF* V600E mutation at a much lower ratio of mutant to wild-type alleles (0.1%) than conventional PCR/pyrosequencing (5%). That was to say, the detection limit of pyrosequencing was as low as 0.1% when combined with upstream enrichment E-*ice*-COLD-PCR.

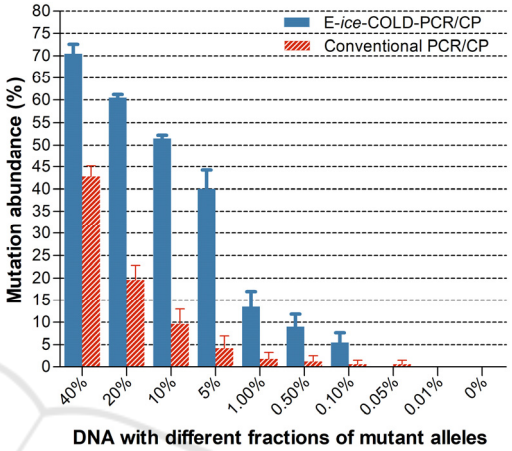

Figure 2: Comparison of conventional PCR/pyrosequencing and E-*ice*-COLD-PCR/pyrosequencing for *BRAF* V600E mutation detection.

3.3 E-*ice*-COLD-PCR/Pyrosequencing for BRAF V600E Detection in Clinical Samples

To further explore the potential clinical applications of E-*ice*-COLD-PCR/pyrosequencing for *BRAF* V600E detection in clinical samples, FFPE tissues from 10 melanoma patients were enriched by E-*ice*- COLD-PCR followed by pyrosequencing. The same samples were also analyzed by using conventional PCR/pyrosequencing for comparison. As shown in Table 1, the V600E mutations in all the five samples that were positive by conventional PCR/pyrosequencing were successfully detected by using E-*ice*-COLD-PCR/pyrosequencing. Furthermore, among the 5 undetectable samples, one of them with no V600E mutation detected by conventional PCR/pyrosequencing was successfully detected by E-*ice*-COLD-PCR/pyrosequencing. That was, E-*ice*-COLD-PCR/pyrosequencing increased the mutations detected in clinical melanoma specimens by 10% (1/10). Therefore, E-*ice*-COLD-PCR/pyrosequencing is more sensitive in detecting the V600E mutation when compare with conventional PCR/pyrosequencing, which might assist in cancer treatment and monitoring and in prenatal diagnosis.

Table 1: Comparison of conventional PCR/pyrosequencing and E-*ice*-COLD-PCR/pyrosequencing assays for *BRAF* V600E mutation detection in clinical samples.

No.	Mutation	Conventional PCR/pyrosequencing	E-*ice*-COLD-PCR/pyrosequencing
S1	c.1799T > A	WT[a]	WT
S2	c.1799T > A	WT	WT
S3	c.1799T > A	WT	MT
S4	c.1799T > A	MT[b]	MT
S5	c.1799T > A	MT	MT
S6	c.1799T > A	MT	MT
S7	c.1799T > A	WT	WT
S8	c.1799T > A	MT	MT
S9	c.1799T > A	WT	WT
S10	c.1799T > A	MT	MT

The grey highlighted sample was with no V600E mutation detected by conventional PCR/pyrosequencing but successfully detected by E-*ice*-COLD-PCR/pyrosequencing. a: Wild-type (WT). b: Mutant-type (MT).

4 DISCUSSIONS

With the introduction of precision medicine, the ability to detect low-abundance mutation or DNA has become more and more important in several clinical areas including diagnosis, treatment and prognosis of cancers, non-invasive prenatal diagnosis, forensic identification and so on. Here, we combined E-*ice*-COLD-PCR with pyrosequencing to detect *BRAF* V600E mutation in serial dilutions of mutant DNA and in clinical samples. The results demonstrated that E-*ice*-COLD-PCR/pyrosequencing showed higher levels of enrichment and sensitivity over conventional PCR/pyrosequencing. The detect limit of 0.1% in this work was consistent with the reported work for *KRAS* mutations detection (How-Kit 2013). However, it was slightly lower than that of the previously published work in which an E-*ice*-COLD-PCR/pyrosequencing assay has been used for detecting *BRAF* mutations with a detection limit of 0.01% (How-Kit 2014). The main reason might be that the different concentrations of blocker probes for different types of samples or different amounts of DNA input might show different detection quality and quantity (How-Kit 2014).

The widely used downstream read-out technologies includes HRM analysis, Sanger sequencing, and NGS. These methods are either not sensitive enough or too expensive when used in detecting low-abundance *BRAF* V600E mutation. Pyrosequencing is relatively sensitive (with a detection limit of 5%) and cost-effective for mutation detection. It enables to detect 0.1–0.01% of mutant DNA, when combined with E-*ice*-COLD-PCR to analyze mutations (How-Kit 2013, How-Kit 2014). In this study, the samples were analyzed in duplicates. A 5% mutation threshold for pyrosequencing was considered a simple as mutant type corresponding to the limit of detection of pyrosequencing. The samples were considered as mutated if their abundances showed a mutation level higher than 5%. In addition, when FFPE tissues were analyzed by E-*ice*-COLD-PCR/pyrosequencing, one sample with no mutation detected by conventional PCR/pyrosequencing was successfully detected by using E-*ice*-COLD-PCR/pyrosequencing. The results clearly demonstrated the power of E-*ice*-COLD-PCR/pyrosequencing in *BRAF* V600E mutation detection and identification in clinical samples.

5 CONCLUSIONS

All in all, we have combined E-*ice*-COLD-PCR with pyrosequencing to detect *BRAF* hotspot mutation V600E, and demonstrated that the use of E-*ice*-COLD-PCR/pyrosequencing increased the sensitivity for detecting *BRAF* V600E mutation with detection limit of 0.1%. When FFPE tissue specimens were detected, one sample was detected by using E-*ice*-COLD-PCR/pyrosequencing but not by conventional PCR/pyrosequencing. Thus, E-*ice*-COLD-PCR/pyrosequencing is high sensitivity, reproducible and flexible for identifying low-abundance *BRAF* V600E mutation in clinical specimens.

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REFERENCES

Besaratinia, A., Pfeifer, G.P. (2008) Sunlight ultraviolet irradiation and *BRAF* V600 mutagenesis in human melanoma. Hum. Mutat. 29, 983–991.

How-Kit, A., Mazaleyrat, N., Daunay, A., Nielsen, H.M., Terris, B., Tost, J. (2013) Sensitive detection of *KRAS* mutations using enhanced-*ice*-COLD-PCR mutation enrichment and direct sequence identification. Hum. Mutat. 34,1568–1580.

How-Kit, A., Lebbe, C., Bousard, A., Daunay, A., Mazaleyrat, N., Daviaud, C., Mourah, S., Tost, J. (2014) Ultrasensitive detection and identification of *BRAF* V600 mutations in fresh frozen, FFPE, and plasma samples of melanoma patients by E-*ice*-COLD-PCR. Anal. Bioanal. Chem., 406: 5513–5520.

Li, J., Wang, L., Mamon, H., Kulke, M.H., Berbeco, R., Makrigiorgos, G.M. (2008) Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat. Med. 14, 579–584.

Milbury, C.A., Li, J., Makrigiorgos, G.M. (2011) *Ice*-COLD-PCR enables rapid amplification and robust enrichment for low-abundance unknown DNA mutations. Nucleic Acids Res. 39, e2.

Mauger, F., How-Kit, A., Tost, J. (2017) COLD-PCR Technologies in the Area of Personalized Medicine: Methodology and Applications. Mol Diagn Ther. 21(3), 269-283.

Tan, Y.H., Liu, Y., Eu, K.W., Ang, P.W., Li, W.Q., Salto-Tellez, M., Iacopetta, B., Soong, R. (2008) Detection of *BRAF* V600E mutation by pyrosequencing. Pathology. 40, 295–298.