

The use of COLD-PCR and pyrosequencing for sensitive detection of *EGFR* T790M mutation

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Abstract. A sensitive and convenient method for the detection of epidermal growth factor receptor (*EGFR*) T790M mutation in non-small cell lung cancer (NSCLC) patients with acquired resistance to tyrosine kinase inhibitors (TKIs) would be desirable to guide treatment strategy. Consequently, studies have focused on sensitive characterization of *EGFR* T790M mutation. Herein, two methods of co-amplification at lower denaturation temperature PCR (COLD-PCR) and pyrosequencing were combined (COLD-PCR/pyrosequencing) for detecting *EGFR* T790M mutation. Evaluation of mutation-containing dilutions revealed that the sensitivities of COLD-PCR/pyrosequencing and conventional PCR/pyrosequencing assays for the detection of the T790M mutation were 0.1 and 5%, respectively, indicating a 50-fold increase in sensitivity. When the T790M mutation in 20 clinical NSCLC samples who had relapsed under first-generation *EGFR* TKI were further determined using COLD-PCR/pyrosequencing and conventional PCR/pyrosequencing, the detection rates were 35% (7/20) and 25% (5/20), respectively. All patients who were positive for the T790M mutation with conventional PCR/pyrosequencing were also found to be positive with COLD-PCR/pyrosequencing. The discordant cases were 2 samples with no T790M mutation detected with conventional PCR/pyrosequencing, but which were positive with COLD-PCR/pyrosequencing. COLD-PCR/pyrosequencing is a sensitive and cost-effective tool for detecting the T790M mutation which will permit an improvement of therapeutic management.

1 Introduction

In recent years, the use of epidermal growth factor receptor tyrosine kinase inhibitors (*EGFR*-TKIs) has significantly improved the survival of patients with non-small cell lung cancer (NSCLC) harboring activating *EGFR* mutations ([1]). However, most patients eventually acquire resistance to the drug, resulting in disease progression ([2]). Half of all resistance to *EGFR*-TKIs is caused by a threonine-methionine amino acid substitution at position 790 (T790M) in exon 20 of *EGFR* gene ([3]). Thus, it is clinically crucial to detect T790M mutation in exon 20 of the *EGFR* gene. However, the ability to detect *EGFR* T790M mutation can be difficult since the specimen samples retrieved from tumor tissues and liquid biopsies such as cell-free DNA (cfDNA) may be low in tumor abundance or DNA concentration. As a result, accurate and sensitive molecular techniques and methods for *EGFR* T790M mutation analysis are acquired. The current methods for detecting of T790M mutation in clinical practice include real-time PCR assays ([4]), allele-specific (AS) PCR ([5]), amplification refractory mutation system (ARMS) ([1]), droplet digital PCR (ddPCR) ([1, 5]), high-resolution melting (HRM) ([6]), Sanger sequencing ([6]) and next-generation sequencing (NGS) ([7]). Among those PCR-based methods, some do not allow to detect

low-abundance *EGFR* T790M mutation, while others are highly sensitive. But there is a potential for false-positive results. These false-positive results might result from the amplifications of small amounts of DNA ([8, 9]). In addition, HRM is a simple, rapid and sensitive method for detecting *EGFR* T790M mutation, however it is hampered by the inability to identify the specific nucleotide change ([10]). Sanger sequencing enables to interrogate the specific nucleotide change, whereas it has a low analytical sensitivity of 20% of the mutated allele in a WT background ([11]). NGS is another sequencing technique enabled to mutation detection, but it is high cost, time-consuming and requiring a large amount of sequencing to detect rare mutations, rendering it not suitable for clinical routine use in a lot of laboratories ([12]). Therefore, finding a sensitive and convenient detection assay is important.

Pyrosequencing also has the potential for mutation detection. It is a sequencing-by-synthesis (SBS) based method, in which one of the four nucleotides (A, G, C, and T) is sequentially incorporated at a time during a cyclic primer extension step. Each allelic variant will give a unique peak compared to the two other variants, which allows to specifically discriminate different alleles by use of pyrosequencing ([13]). Compared to Sanger sequencing, pyrosequencing is more sensitive with the limit of detection as low as 5% of the mutant allele in

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wild-type DNA ([11]). Although having higher sensitivity in variant detection, pyrosequencing is unable to selectively enrich the variant alleles, which would be requested in detecting low percentage of mutated DNA.

Co-amplification at lower denaturation temperature PCR (COLD-PCR) is a modified PCR method which allows to selectively amplify low-abundance alleles from a mixture of wild-type and mutant sequences, irrespective of the mutation type and position ([14, 15]). It reduces the limitations in low-abundance mutation detection by use of a critical denaturation temperature (T_c) during PCR amplification. Low-abundance mutation enrichment by use of COLD-PCR has been demonstrated in combination with several downstream approaches such as TaqMan-based real-time PCR ([16]), HRM ([17]), Sanger sequencing ([6]) and pyrosequencing ([14]). When combined with pyrosequencing, COLD-PCR can improve mutation detection and identification by approximately 5-fold to 35-fold ([14]).

Here, we present the combination of COLD-PCR with pyrosequencing for the detection of *EGFR* T790M mutation. We demonstrate that upstream COLD-PCR assay allows for the enrichment of *EGFR* T790M mutation in a sensitive and inexpensive manner and downstream pyrosequencing of COLD-PCR products allows for the discrete identification of low-abundance *EGFR* T790M mutation.

2 Materials and methods

2.1 Samples and DNA extraction

Tumor specimens were obtained from 20 NSCLC patients from Southwest Hospital who had relapsed under first-generation *EGFR* TKI. For all the specimens, the T790M mutation in *EGFR* gene was assessed by PCR/pyrosequencing and 5 results were positive. Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tissue was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen) following the manufacturer's instructions. After extraction and purification, DNA was quantified by a Qubit fluorometer (Thermo Fisher, Waltham, MA).

2.2 Plasmid construction

Plasmid standards were generated containing *EGFR* exon 20 with the T790M mutation. A plasmid containing wild-type *EGFR* exon 20 was also constructed. The sequences of the plasmids were confirmed by bidirectional Sanger sequencing. The constructed plasmids were then used to generate amplicons for positive and negative controls.

2.3 Conventional PCR

The templates harboring mutational sites were amplified using the following primers to obtain a 85-bp amplicon: Forward, 5'- CTGGGCATCTGCCTCACCT -3'; Reverse:

5'-biotin-TGTGTTCCCGGACATAGTCCA -3'. Primers were synthesized by Shenzhen Huada Gene Technology Co. LTD. (Shenzhen, China). Reactions for conventional PCR were performed using 25 ng of genomic DNA in a total volume of 25- μ L containing a final concentration of 1 \times HotStar Taq DNA polymerase Buffer, 1.6 mM of additional $MgCl_2$, 200 μ M of dNTPs, 200 nM of each primer and 2 U of HotStar TaqDNA polymerase. Amplification by conventional PCR was performed with an initial denaturing at 98 °C for 30 s, 40 cycles of 98 °C for 10 s, 50.3 °C for 20 s, and 72 °C for 10 s, and final extension at 72 °C for 10 min. The reactions are carried out on a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). After PCR amplification, electrophoresis on 2% agarose gel was performed to confirm successful amplification of the 85-bp PCR product before pyrosequencing.

2.4 Determination of critical denaturation temperature, T_c

The most important thing in designing a COLD-PCR assay is to determine the best T_c for the reaction. To define optimal T_c for the given amplicon with *EGFR* T790M mutation, several series of COLD-PCR assays with gradient T_c were performed to check different temperatures decreasing from 83.8 to 80.5°C using standard mixed plasmids at 3% mutation fraction. Direct sequencing of PCR product was used to determine the degree to which the mutant allele was enriched. The denaturation temperature that reproducibly produces good quality PCR amplicons with strong enrichment should be selected as the T_c .

2.5 COLD-PCR

COLD-PCR can be performed in two formats, full-COLD-PCR and fast-COLD-PCR ([14]). Here, full-COLD-PCR was performed to enrich *EGFR* T790M mutation, since full-COLD-PCR enabled to identify all possible mutations. COLD-PCR was performed following the introduction in the literature ([14]), with minor modification. The COLD-PCR reaction mixtures consisted of 25 ng of genomic DNA, 1 \times of Phusion DNA polymerase Buffer, 1.6 mM of additional $MgCl_2$, 200 μ M of dNTPs, 200 nM of each primer and 5 U of Phusion™ DNA polymerase (New England Biolabs (NEB)). The COLD-PCR protocol started with 5 cycles of the conventional PCR amplification for an initial buildup of all amplicons, followed by 39 COLD-PCR cycles to selectively enrich for mutant sequences. The full COLD-PCR cycling conditions included an initial denaturation for 30 s at 98°C, followed by 5 cycles of standard PCR (10 s denaturation at 98°C, 20 s annealing at 50.3°C, and 10 s elongation at 72°C) and 39 cycles of COLD-PCR (10 s denaturation at 98°C, 30 annealing at 70°C, 10 s at T_c , 20 s primer annealing at 50.3°C and 10 s elongation at 72°C), followed by a final extension for 5 min at 72°C. Amplicons were analyzed by gel electrophoresis on a agarose gel prior to pyrosequencing.

2.6 Pyrosequencing

According to our previous work, 20 μ L of PCR or COLD-PCR products were purified and rendered single-stranded on a PyroMark Q96 Vacuum Workstation (Qiagen, Courtaboeuf, France) ([18]). Mutation detection was performed on a PyroMark Q96 MD (Qiagen, Hilden, Germany) following the manufacturer's instructions using the pyrosequencing primer, 5'-CCGTGCAGCTCATCA-3'. Each sample was sequenced with nucleotide dispensation order: 5'-GCTGCTAGC-3'. Quantitative mutation detection was analyzed with the Pyromark MS software (Qiagen) in the allele quantification (AQ) mode. After the run, Pyromark Q96 MA software was able to automatically analyze pyrogram (A series of peaks were called a pyrogram) outputs and the percentage of mutated amplicons was automatically shown upon the pyrogram. Each sample used for *EGFR* T790M analysis was also reanalyzed in triplicate to confirm the reproducibility.

2.7 Determination of the sensitivity of COLD-PCR/pyrosequencing assays

A plasmid DNA sample containing *EGFR* T790M mutation was used as the source of the mutant allele. This mutation-containing DNA sample was serially diluted into wild-type DNA to obtain the following percentages: 10, 5, 1, 0.5, 0.1, 0.05, and 0%. Additionally, several replicates of wild-type DNA (0% mutant) were included in each experiment and evaluated in parallel. All the DNA mixtures were simultaneously subjected to conventional PCR and COLD-PCR and then interrogated by pyrosequencing. All samples were run in duplicate.

3 Results

3.1 Determination of optimal T_c for *EGFR* T790M detection

When COLD-PCR is performed to enrich mutant sequence, the use of a very precise T_c is crucial, because a slight variation of as little as 0.2-0.3 $^{\circ}$ C from the T_c could make it difficult to obtain satisfactory mutation enrichment ([19]). Furthermore, for each DNA sequence, T_c is strongly dependent on DNA sequence, and it is determined practically for each specific sequence. To precisely determine the optimal T_c for *EGFR* T790M detection, COLD-PCR assay was evaluated using templates with 5% mutant-to-wild-type ratios and a range of lower denaturation temperatures (80.5, 81.4, 81.9, 82.7, 83.3, and 83.8 $^{\circ}$ C). Subsequently, amplification products were tested by pyrosequencing (Fig. 1). As shown, after COLD-PCR, 13, 14, 14, 15, 16, and 8% mutant-to-wild-type ratios were obtained at different T_c . When lower denaturation temperature was

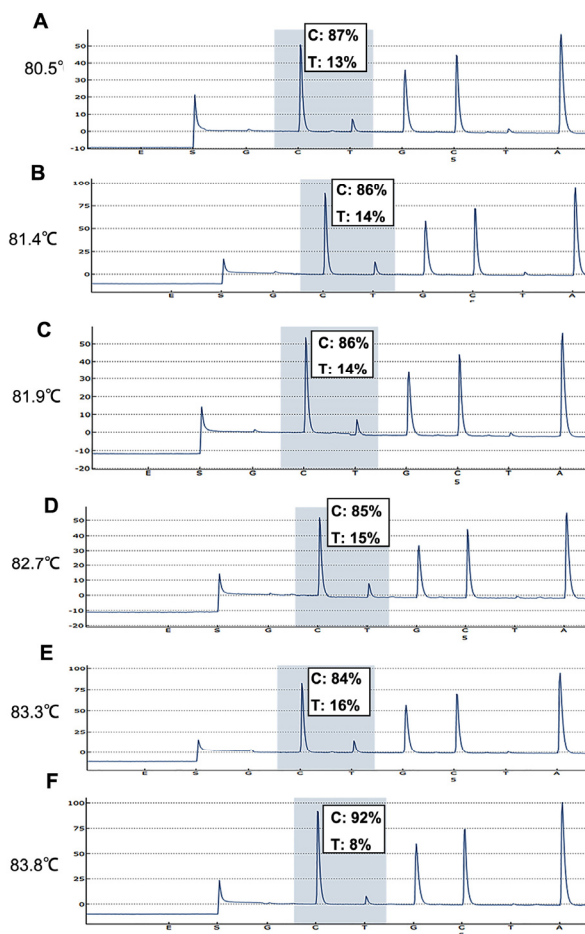


Fig. 1. The pyrograms from pyrosequencing after COLD-PCR. (A)-(F) lower denaturation temperatures applied was 80.5, 81.4, 81.9, 82.7, 83.3, and 83.8 $^{\circ}$ C. As shown, 83.3 $^{\circ}$ C was designed as the optimal T_c .

set to 83.3 $^{\circ}$ C, the best mutant-sequence enrichment provided, with efficient amplification. Thus, the optimal T_c for *EGFR* T790M detection was 83.3 $^{\circ}$ C.

3.2 Sensitivity of COLD-PCR/pyrosequencing for *EGFR* T790M detection

To evaluate the theoretical sensitivity of COLD-PCR/pyrosequencing for detecting *EGFR* T790M mutation, a mutation-containing DNA sample was serially diluted with a wild-type DNA sample to obtain 10, 5, 1, 0.5, 0.1, 0.05 and 0% mutant-to-wild-type ratios. These reconstituted samples were simultaneously submitted to conventional PCR and COLD-PCR followed by pyrosequencing (Fig. 2). As shown, pyrosequencing analysis of COLD-PCR amplicons demonstrated more efficient enrichment of the T790M mutation. However, for conventional PCR, there is nearly no inherent potential for preferential enrichment. For example, in the pyrograms 10% initial *EGFR* T790M appeared to be enriched to 28% and 11% after COLD-PCR and conventional PCR, respectively. The pyrograms of amplicons obtained with conventional PCR demonstrated that the minimum detectable percentage was approximately 5%, which was consistent

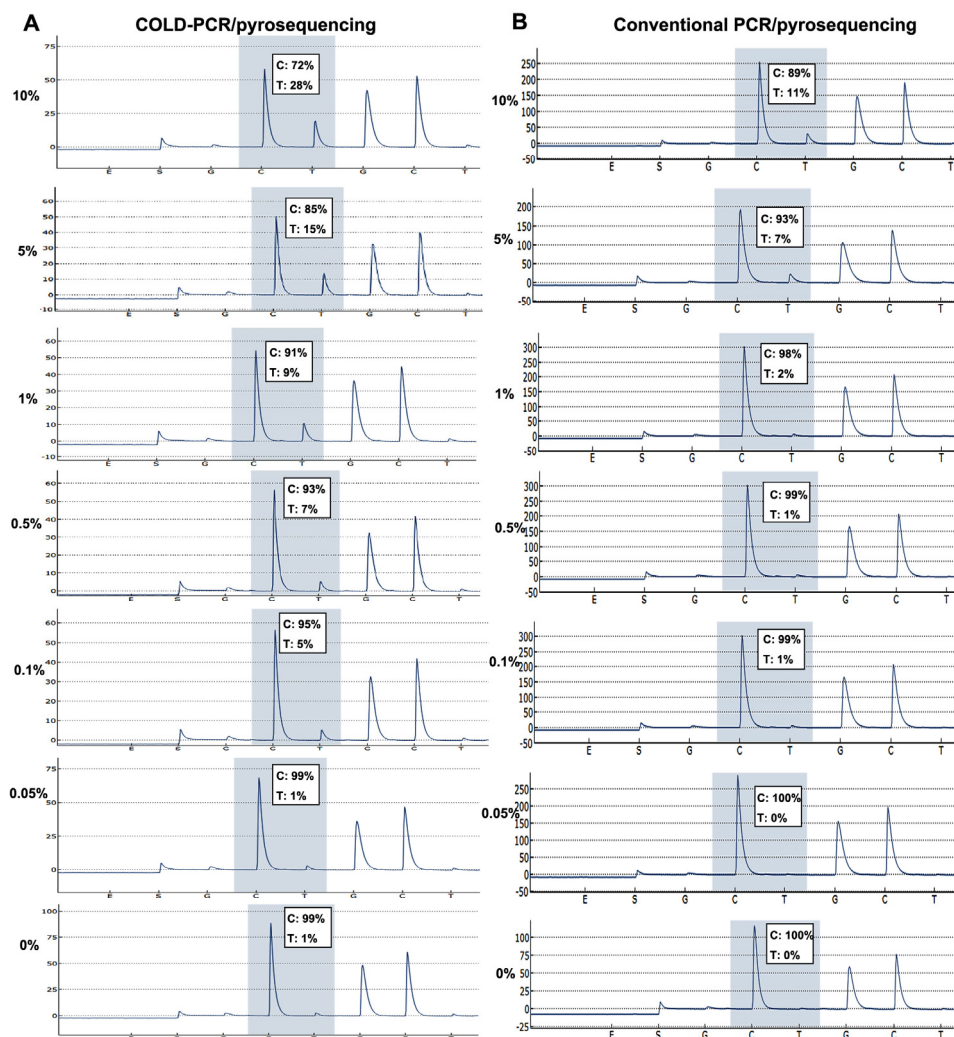


Fig. 2. Pyrosequencing analysis of the 85-bp amplicon after COLD-PCR and conventional PCR, respectively. Pyrograms were presented for (A) COLD-PCR and (B) Conventional PCR. Serial dilutions (10, 5, 1, 0.5, 0.1, 0.05 and 0%) of *EGFR* T790M mutation-containing DNA into wild-type DNA were presented.

with previous research results ([11]). Conversely, after COLD-PCR/pyrosequencing 0.1% *EGFR* T790M were easily detectable, providing a 50-fold increase in the detection of this mutant.

We further evaluated the enrichment potential of COLD-PCR/pyrosequencing for *EGFR* T790M mutation detection. As shown in Fig. 3, when the initial abundance of *EGFR* T790M was larger than 0.5%, lower abundance of initial mutation abundance exhibited a greater enrichment in COLD-PCR platforms, compared to the relatively higher mutant fractions. That was, within a certain abundance range (>0.5% for *EGFR* T790M mutation), the mutation enrichment of COLD-PCR/pyrosequencing was highly related to the initial starting abundance of the *EGFR* T790M mutation.

3.3 COLD-PCR/pyrosequencing for *EGFR* T790M detection in clinical samples

We further examined the potential clinical applications of COLD-PCR/pyrosequencing over conventional PCR/pyrosequencing for detecting *EGFR* T790M mutation in clinical samples. 20 NSCLC patients who

were first tested using conventional PCR/pyrosequencing to assess whether the T790M mutation was positive, and 5 results were positive. All the 20 NSCLC patients were further enriched by COLD-PCR followed by pyrosequencing. As shown in Table 1, COLD-PCR/pyrosequencing successfully detected the T790M mutation in all the 5 samples that were positive by conventional PCR/pyrosequencing. Furthermore, among the 15 undetectable samples, 2 of them with no T790M mutation detected by conventional PCR/pyrosequencing were successfully detected by using COLD-PCR/pyrosequencing with the mutant-to-wild-type ratio of 10% and 8%, respectively. That was, COLD-PCR/pyrosequencing increased the mutations detected in clinical NSCLC tumor specimens by 10% (2/20). Additionally, COLD-PCR/pyrosequencing enhanced the ratios of the mutant-to-wild-type approximately 1.4-2.6-fold. Thus, this protocol allowed for the detection of much lower abundant T790M mutations in comparison with conventional PCR/pyrosequencing, which permitted earlier detection and an improvement of therapeutic management.

4 Discussions

Low-abundance DNA mutations (e.g., *EGFR* T790M) in

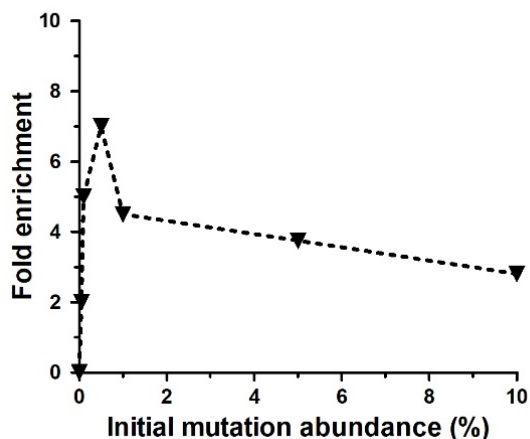


Fig. 3. Enrichment potential for COLD-PCR/pyrosequencing.

heterogeneous specimens may cause drug resistance and can be clinically significant biomarkers of disease progression. Therefore, it is crucial to design accurate and sensitive detection methods for such low-abundance mutations. Here, we have described COLD-PCR/pyrosequencing, a novel methodology that combined COLD-PCR with downstream pyrosequencing for detecting the *EGFR*-encoded T790M mutant in serial dilutions of mutant DNA and in biological samples. As a result, the combination of COLD-PCR with pyrosequencing quantitatively detected as little as 0.1% mutant alleles with a 50-fold mutation enrichment, compared to conventional PCR/pyrosequencing. That was, COLD-PCR/pyrosequencing exhibited higher levels of enrichment and sensitivity over conventional PCR/pyrosequencing. Our results were in agreement with a previous study ([20]). Furthermore, the detect limit of 0.1% in our work was slightly lower than that of the previously published work in which a COLD-PCR/pyrosequencing assay has also been used for detect-

Table 1. Comparison of mutant-to-wild-type-ratio by COLD-PCR/pyrosequencing and conventional PCR/pyrosequencing assays for the detection of *EGFR* T790M mutations. Samples with no T790M mutation identified by both conventional PCR/pyrosequencing and COLD-PCR/pyrosequencing were not listed.

No.	Mutation (aa)	Ratio by PCR ^a (%)	Ratio by COLD-PCR ^b (%)	Enhancement efficiency (fold)
S1	p.Thr790Met	15	39	2.6
S2	p.Thr790Met	NF ^c	10	-
S6	p.Thr790Met	38	57	1.5
S9	p.Thr790Met	NF	8	-
S12	p.Thr790Met	16	30	1.9
S15	p.Thr790Met	57	68	1.2
S19	p.Thr790Met	25	35	1.4

a: Samples were identified by conventional PCR/pyrosequencing. b: Samples were identified by COLD-PCR/pyrosequencing, c :not found.

ing *EGFR* T790M, reducing the limit of detection to 0.125% and yielding a 40-fold improved selectivity compared with standard PCR/pyrosequencing ([20]). COLD-PCR has also been applied to combine with HRM and TaqMan-based real-time PCR for the detection and identification of *EGFR* T790M mutation with a limit of detection of 0.01 and 0.8% respectively. ([16, 17]). Several studies have demonstrated that COLD-PCR exhibited high ability to identify much lower mutant fractions, improving the ability to detect minor allele variants in the early stages of disease progression ([14, 21, 22]). Furthermore, COLD-PCR is cost effectiveness in comparison to other methods enabled to detect such small clones (eg, NGS) since it can be performed with conventional PCR reagents and laboratory equipment. These features allow it to become available to the normal routine processing without causing any additional costs.

Pyrosequencing is a relatively sensitive downstream technology, and the combination of pyrosequencing with COLD-PCR enables to detect as low as 0.1% of mutant-DNA, making it widely be used as the downstream sequencing technology for mutation analysis ([23, 24]). In Fig. 2, pyrogram from 0% mutation-containing DNA sample also detected 1% mutated alleles, it might result from the background signal. In addition, enrichments were represented as fold changes of the average mutation-to-wild-type ratios of the same dilution between COLD-PCR and conventional PCR amplifications. Here, the mutation-to-wild-type ratio was automatically shown upon the pyrogram by Pyromark Q96 MA software.

As for low-abundance mutation detection, a number of different factors might affect the sensitivity and the specificity, such as a low concentration of tumor cell, the presence of DNA fragment, poor quality of DNA, and non-sensitive downstream technologies. The combination of COLD-PCR with pyrosequencing enables to enrich the mutant content and to improve the detect limit of downstream technologies, providing a powerful tool for determinating low-abundance *EGFR* T790M mutations in clinical samples.

5 Conclusion

In conclusion, we have combined COLD-PCR with pyrosequencing for detecting *EGFR* T790M, and demonstrated that the use of COLD-PCR/pyrosequencing increased the sensitivity for detecting *EGFR* T790M mutation by approximately 50-fold with the limit of detection of 0.1% and increased the T790M mutation detected in clinical NSCLC tumor specimens by 10% (2/20). All in all, COLD-PCR/pyrosequencing is high sensitivity, cost-effective and ease of use for identifying low-abundance *EGFR* T790M mutation in clinical specimens.

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