A multiple detection method for distinguishing gene mutations based on melting curves of extended quenching probes

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Keywords:
- EGFR gene
- Gene mutations
- Melting curve
- Multiple DNA target Detection
- Non-small cell lung cancer

Abstract

Conventional PCR methods can detect only a few targets simultaneously and do not fulfill most clinical requirements, especially those for detecting plasma circulating DNA. By designing characteristic universal fluorescent probes, combining multiplex PCR with the invasive reaction, and analyzing the resulting differences in the melting curves formed by extension with double-stranded probes, we developed a new method to distinguish between three mutations in the same fluorescent channel and nine mutations in three fluorescent channels in a single tube. After optimization, this method was used to distinguish between 27 mutations using only three reactions, and mutations representing as low as 0.2%–0.5% of DNA could be detected, even when up to nine mutations were present at the same time. Testing of nine clinical samples, including three L858R-positive, four 19 del-positive, and two L861Q-positive samples, showed consistent results with digital PCR tests. Compared with the conventional PCR method, our method expands the capabilities of fluorescence detection by achieving multiplex detection in a single-tube, thereby providing a simple, low-cost tool for clinical applications.
good biomarker for drug targeting (Heng et al., 2019; Pratama et al., 2019). In contrast, the T790M mutation does not respond to drug targeting, but together with the C797S mutation, it shows cis-resistance and trans-sensitivity to third-generation targeting drugs (Madic et al., 2018; Arulananda et al., 2019). Therefore, before EGFR-TKIs are taken by patients with NSCLC, it is necessary to determine whether the tumor tissue harbors gene mutations and identify the type of mutation in the EGFR gene (Joy et al., 2020). Thus, the growing number of roles discovered for different mutations increases the clinical value of discriminating between types of mutations.

When simultaneously discriminating between mutations, the traditional PCR method requires multiple tubes for parallel detection (Yang et al., 2021). Next-generation sequencing can handle a large amount of data but also has the disadvantages of being complex, expensive, and time consuming (Toledo et al., 2018; Yokouchi et al., 2020). Therefore, it is necessary to develop a low-cost and easy-to-use method to detect multiple mutation sites simultaneously. In this paper, we introduce a method that combines multiplex PCR and the invasive reaction. The invasive reaction is highly specific because the signal corresponding to the recognized sequence is produced only when the “invasive structure” is formed by the upstream and downstream probes with the target template (Liu et al., 2018; Xiang et al., 2018). The invasive reaction is widely used for detection of various nucleic acids, in particular gene mutations (Usami et al., 2008; Yamamoto et al., 2009). Our method uses the different melting peaks formed by the fluorescent signal molecules produced during the invasive reaction, which extend along the quenching probes (QPs) to further expand the fluorescence detection capacity, i.e., to detect three mutation sites in a single fluorescence channel. Using FAM, ROX, and CY5 fluorescence channels, nine mutation sites could be detected in the EGFR gene, not only providing more accurate results but also tackling the issues of sample scarcity and cost.

1. Materials and methods

1.1. Equipment and reagents

SLAN-96 Fluorescence PCR (Shanghai Hongshi Medical Technology Co., Ltd., catalogue number: SLAN-96S, Shanghai, China), the Naica™ Crystal digital PCR system (Stillia Technologies Inc., France), a Nanodrop ultraviolet spectrophotometer (Thermo Fisher Scientific, catalogue number: ND-ONE-W, USA), and a two-person biosafety cabinet and chemical hood (Shandong Biobase Co., China) were used. Taq DNA (Shanghai Promega Co., Ltd., catalogue number: M1661S, Shanghai, China) and a Nucleic acid extraction kit and Afu endonuclease (Guangzhou Biotron Technology Co., Ltd., China) were used. All other chemicals and solvents (analytical reagent grade) were purchased from Sigma (St. Louis, MO, USA). The target sequences of the EGFR gene were obtained from the NCBI (https://www.ncbi.nlm.nih.gov/) and COMSIC databases (https://cancer.sanger.ac.uk/cosmic). Twenty-seven mutations were located on exons 18–21 (Table 1). Primers and probes compatible with different gene sequences were designed using IDT DNA online software (https://sg.idtdna.com/pages/tools/) and Oligo 7 design software (http://www.oligo.net/), and the sequences are shown in Supplementary Table S1. Primers and probes were obtained by Sangon Biotech, Co., Ltd., (Shanghai, China) and plasmid was obtained from Bioligo Co., Ltd., (Shanghai, China).

1.2. DNA extraction from clinical samples

DNA was obtained from clinical residual plasma samples using the nucleic acid extraction kit. Nucleic acid concentration and purity were detected using the UV spectrophotometer. 1 × TE buffer (10 mmol/L Tris-
HCl, 1 mmol/L EDTA (pH 8.0)) was used to dilute samples to 10–30 ng according to the concentration obtained from the UV spectrophotometer.

1.3. Detection of multiple mutation sites

A 40-μL reaction system was prepared that included the following: 1× PCR buffer, 50–200 nmol/L primers (P-Fs, P-Rs), 100–500 nmol/L upstream probe (UP), 200–600 nmol/L fluorescent probe (DP), 500 nmol/L QP, 2.5 U Taq DNA polymerase and 80 ng Afu endonuclease. The working concentrations of all primers and probes are listed in detail in Supplementary Table S2. The 1× PCR buffer contained 15 mmol/L Tris-HCl (pH 8.5), 30 mmol/L NaCl, 6 mmol/L MgCl₂, 0.05% Tween-20, and 0.05% IGEPAL® CA-630. The reaction procedure was as follows: 45 cycles of 2 min at 95°C, 10 s at 95°C, 40 s at 69°C; 15 min at 60°C; 15 s at 42°C; 15 s at 45°C; and melting curve analysis at 60–95°C. Different amounts of each sample were mixed with wild-type DNA and different proportions of corresponding plasmids. Clinical samples were analyzed by assessing characteristic melting curves and results were further validated using digital PCR.

2. Results and discussion

2.1. Detection principle

In order to detect different mutations in a single tube, multiplex PCR was combined with the nucleic acid invasion reaction, and the characteristic melting curves of the multiple fluorescent probes were used to identify three mutation sites in the corresponding fluorescence channels.

Figure 1. Schematic of the assay procedure for detecting EGFR mutations. 1A shows a schematic of the process for distinguishing between EGFR mutations. 1B shows the dynamic fluorescence intensity from 60°C to 95°C. Three melting peaks can be observed on a single fluorescence channel.

Figure 2. Results of different amounts of QPs and DPs in the FAM, ROX, and CY5 channels. 2A–2C represent the FAM, ROX, and CY5 channels, respectively. The three peaks correspond to Tm1, Tm2, and Tm3 for each fluorescence channel.
The detection principle of this method is shown in Figure 1. EGFR mutation sites were used as an example (Figure 1A). Primers (P–F, P-R) were designed to amplify different targets. UPs and DPs were designed, with the UP sequence at the 3' terminal end of the mismatched template and invading the first position of the complementary sequence between the DP and the template. The 5' end of the DP sequence was unrelated to the target nucleic acid and was modified with a fluorescent group. The middle sequence of the DP was modified with a quenching group. The fluorescence of the DP was quenched when the probe was intact, but fluorescence was produced when the probe was cleaved. The target nucleic acid hybridized with the UP and DP to form a unique "invasive structure", which was recognized and cleaved by Afu endonuclease to generate a signal molecule (S) with a fluorescent group that gradually accumulated as reaction time increased. The 3' end of the QP was

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Figure 3. Simultaneous detection of different numbers of EGFR mutations. 3A shows the detection of two EGFR mutants. Figure 3B shows the detection of three EGFR mutants. Figure 3C shows the detection of six EGFR mutants. Figure 3D shows the detection of nine EGFR mutants. The three melting peaks (Tm1, Tm2, and Tm3) in the ROX, FAM, and CY5 channels correspond to H773_V774insH, D770_N771insG, L858R, T790M, L747_P753>Q, S768I, C797S, V769_D770insASV, and L861Q, respectively.

Figure 4. The LOD of the method for single-plex EGFR mutation. 4A–4F represent T790M (FAM), S768I (FAM), C797S (CY5), V769_D770insASV (CY5), H773_V774insH (ROX), and L858R (ROX), respectively. Samples with concentrations of 20%, 5%, 2%, 0.5%, 0.2%, and 0% were detected using this method.
completely complementary to S, and each QP had a corresponding S. At low temperatures in the presence of DNA polymerase, the S hybridized with the QP and extended into a double-stranded structure with a specific melting peak (Tm). Different QPs have different Tm values, and when a target is present, its corresponding Tm value can be obtained by melting temperature analysis for the resulting S (Figure 1B) and used to determine whether there is a corresponding mutation.

### 2.2. Optimization of DPs and QPs

Because DPs and QPs have a significant impact on Tm, we optimized the amount of DPs and QPs in each of the three fluorescence channels. The final concentrations of QP in the experiment were 0, 0.02, 0.04, 0.1, 0.2, 0.5, 1, and 2 μmol/L, and equal concentrations of the corresponding DP were used (Figure 2). The results of Figures 2A-2C showed that as the amount of QP increased, Tm became more significant. However, the melting peak of each fluorescence channel was significantly different when the amount of QP exceeded 0.5 μmol/L, the case in point are the melting peaks of the ROX and FAM fluorescence channel, which are indicated in Figures 2B and 2C, respectively. Consequently, 0.5 μmol/L of QP and a corresponding amount of DP were used in the reaction. The Tm values for each channel are shown in Table 1. Tm1, Tm2, and Tm3 were 69.5, 76.5, and 86.5 °C in the CY5 fluorescence channel (Figure 2A), 71.2, 78.5, and 87.5 °C in the ROX fluorescence channel (Figure 2B), and 69.5, 74.5, and 85.0 °C in the FAM fluorescence channel (Figure 2C), respectively.

### 2.3. Multiple detection performance

Clinical samples may contain multiple mutations. Therefore, we tested the multiple detection capability of this method. Using tube 3 as an example, different numbers of mutation targets (2-plex, 3-plex, 6-plex, and 9-plex targets) were detected, and the results are shown in Figures 3A–3D. The results confirm that the method we established can simultaneously distinguish between mutation targets, whether the number of targets is two, three, six, or nine. Even up to a 9-plex reaction, each mutation could still be detected without errors, indicating that the method can be used to simultaneously detect multiple mutations.

### 2.4. Limit of detection (LOD)

To evaluate the LOD for the mutations, we detected single or multiple mutations using gradient dilution in tube 3. Two mutations were selected for gradient dilution in each fluorescence channel. Samples with concentrations of 20%, 5%, 2%, 0.5%, 0.2%, and 0% were used, and the results are shown in Figure 4. The results in Figures 4A–4F show that the minimum prevalence of combined mutations that could be detected by this method was >0.5%, but some mutations with lower prevalences could be detected, such as L858R, C797S, and H773_V774insH, for which 0.2% of the samples were distinguishable from the wild-type sample (0%). Therefore, the LOD for this method is >0.5%, which is better than most methods for detecting mutations based on qPCR (Chulakasian et al., 2010).

Next, six mutations from tube 3 (T790M, L747_P753 > Q, S768I, H773_V774insH, D770_N771insG, and L858R) in the FAM and ROX channels were selected, and the positive template was diluted to concentrations of 20%, 3%, 0.5%, 0.1%, and 0%. The results obtained using this method are shown in Figure 5. Figures 5A, 5B, and 5C show that positive results were detected at concentrations of 20%, 3%, and 0.5% (solid lines in Figures 5A–5C) for the sample containing six mutation sites when compared with wild-type samples (0%; dotted lines in Figures 5A–5C). Figure 5D shows that negative results were detected at 0.1% (solid line in Figure 5D) for the sample containing six mutation sites compared with wild-type samples (0%; dotted line in Figure 5D). These results reveal that this method can be used to detect samples with concentrations as low as 0.5% when six mutations are present at the same time. Thus, this method has a low LOD and suitable performance for multiple mutation detection. Existing gene mutation detection methods based on qPCR rarely achieve an LOD as low as 0.2%, and hardly any existing method based on qPCR can distinguish between multiple mutations (Ho et al., 2014; Feng et al., 2017).

To verify the precision of this method, samples with concentrations of 0.2% (L858R), 0.5% (D770_N771insG) and 0% were chosen for each of
two mutation sites from tube 3, and eight replicates were performed for each sample. As shown in Supplementary Figure S1, the mean intensities ± standard deviations of the melting peaks were 33.3 ± 2.64 for 0.2% L858R and 32.4 ± 1.49 for 0.5% D770_N771insG and were significantly different from those of the negative sample (16.2 ± 1.32 and 18.2 ± 1.52). These results showed that our method has high precision for sample detection.

2.5. Analysis of clinical samples

Nine clinical samples from NSCLC patients were analyzed to verify the feasibility of this method for biological sample detection, as shown in Figure 6. Figures 6A–6I represent the results of samples 1–9. Of the nine clinical samples, three cases were L858R-positive, four cases were 19 del-positive (two cases contained L747_P753 > S and two cases contained E746_A750del (2)), and two cases were L861Q-positive.

Figure 6. Results of nine clinical samples detected using this method. 6A–6I represent the results of samples 1–9. Of the nine clinical samples, three cases were L858R-positive, four cases were 19 del-positive (two cases contained L747_P753 > S and two cases contained E746_A750del (2)), and two cases were L861Q-positive.

Figure 7. Results of digital PCR detection of samples 2 and 5. 7A: Sample 2 was L858R-positive. 7B: Sample 5 was E746_A750del (2)-positive.

2.5. Analysis of clinical samples

Nine clinical samples from NSCLC patients were analyzed to verify the feasibility of this method for biological sample detection, as shown in Figure 6. Figures 6A–6I show the detection results of samples 1–9 (Supplementary Table S3), of which three were L858R-positive (2573T>G), four were 19 del-positive (two samples were L747_P753 > S-positive (2240_2257del18) and two samples were E746_A750del (2)-positive (2236_2250del15)), and two were L861Q-positive (2582T>A). The results for these nine samples detected using our method were consistent with those of ARMS-PCR (Supplementary Figure S2), which is widely used for clinical diagnosis (Zhang et al., 2019; Okada et al., 2022). To further confirm the reliability of our results, samples 2 and 5 were verified by digital PCR, and the results were showed in Figures 7A and 7B, respectively. The results of samples 2 and 5 were consistent with those of our method (Sample 2: L858R-positive, Sample 5: E746_A750del (2)-positive), indicating that this method can be used for clinical mutation detection.

3. Conclusion

Molecular diagnosis-assisted targeted therapy for NSCLC is among the most successful applications of cancer therapies (Yung et al., 2009; Hu et al., 2012). As personalized targeted drugs and the therapeutic effects of
different mutation types are developed and understood, clinical genotyping will become more and more important (Watanabe et al., 2015; Akher et al., 2020). However, existing methods cannot fully meet the clinical needs (Diehl et al., 2008). In this article, we developed a new method based on multiple fluorescence and characteristic melting peaks that performs well in the detection of multiple gene mutations. The method can be used to identify 27 known mutation sites in the EGFR gene using only three tubes, which is a great improvement over existing methods. Our results demonstrate that the LOD can reach 0.2%–0.5% and confirm the feasibility of detecting multiple mutations. The advances include the minimal requirements for clinical sample DNA (such as plasma DNA) and the low-cost of clinical genotyping. This study provides a new method with practical value and contributes to the development of clinical molecular testing.

**Declarations**

**Author contribution statement**

Wang Jianping, Liu Zipeng, Pan Tengfei; Zhang Song: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Data availability statement**

Data included in article/supp. material/referenced in article.

**Declaration of interests statement**

The authors declare no conflict of interest.

**Additional information**

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