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Research Article

Method for Solving Non-specific Amplification Interference of Fluorescence Quantitative PCR in Gene Detection

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Abstract: ***Objective:*** To explore a method to solve the issue of interference in fluorescence quantitative PCR non-specific amplification for gene detection. ***Method:*** A three-step method was used for amplification, and the quantitative fluorescence signal collection process was set in the extension stage. ***Results:*** Three-step amplification has the advantages of wide application range; improved accuracy; and reduced primer design requirements. ***Conclusion:*** The interference of non-specific amplification signals was effectively avoided, the melting curve plotting process was omitted, the reaction time was shortened, and the detection accuracy was improved.

Keywords: Fluorescence quantitative PCR; Specific amplification; Gene detection

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Fluorescence quantitative PCR technology is a kind of quantitative PCR detection technology. It uses fluorescent dyes or fluorescently-labeled specific probes to label and track PCR amplification products, monitor the reaction process online in real-time, and then analyze the products in combination with corresponding software to calculate the initial concentration of the template. There are different labeling methods for fluorescence quantitative PCR technology. The commonly used methods are fluorescent dye method and hydrolysis probe method[1]. Currently, PCR amplification is usually carried out by one-step or two-step methods in the process of gene detection. It is necessary to collect fluorescent signals throughout the process, and it is inevitable that non-specific amplification signals such as dimers will inevitably interfere with the detection. The result is based on the reaction time and fluorescence signal changes to plot the melting curve, where the process is cumbersome, the reaction time is long, and the detection accuracy is low. Nevertheless, improving the current technical problems and developing a method that is convenient to operate, with shortened reaction time and improved detection accuracy hold important practical significance.

1 Materials and Methods

1.1 General Information

The fluorescent dye method is to add excessive SYBR Green and other fluorescent dyes in the PCR reaction system. SYBR Green fluorescent dyes can be non-specifically incorporated into the DNA double-strand to emit fluorescent signals, while SYBR Green dye molecules not incorporated in the strands will not emit any fluorescent signals. This ensures that the increase of the fluorescent signal is completely synchronized with the increase of the PCR product to monitor the progress of the PCR reaction[2]. Fluorescent dye method real-time quantitative PCR technology has strong applicability, low cost and is easy to use. However, as dyes such as SYBR Green are non-specifically incorporated into all amplified double-stranded DNA fragments, it will inevitably lead to fluorescent signals that interfere with the

detection due to the incorporation of dyes to non-specific fragments[3].

Currently, the process of gene detection often uses one-step or two-step PCR amplification. The fluorescence signal needs to be collected throughout the process, and it is inevitable that non-specific amplification signals such as dimers will interfere with the detection. The results are based on the reaction time and fluorescence signal changes to plot the melting curve, where the process is cumbersome, the reaction time is long, and the detection accuracy is low[2].

1.2 Methods

Three-step amplification was adopted, and the fluorescence quantitative signal collection process was set in the extension stage, which not only effectively avoided the interference of non-specific amplification signals, but also eliminated the melting curve plotting process, shortened the reaction time and improved the detection accuracy[4].

PCR amplification reaction conditions are: pre-denaturation at 90-95°C for 1-15min; denaturation at 90-95°C for 10-30s; annealing at 50-65°C for 30s-90s; extension at 70-85°C for 10s-30s; 35-50 cycles; The signal collection was carried out during the extension stage; the 20μL PCR reaction system included the following components: the final concentration of 2×SYBR Green PCR Master Mix was 1; the final concentration of primers were both 200nM; the final concentration of the DNA template was 10-100ng; dH_2O was 20 μL[5].

The primer selection includes any one of the following 7 pairs of specific primers:

Primer pair 1:
TGAGGTTATTGTGTTTGGAA;
GTAGGTCACAGGCTCTAC;
Primer pair 2:
CACTGTTTGAGGTTATTGTG;
GTAGGTCACAGGCTCTAC;
Primer pair 3:
ACCACTGTTTGAGGTTATTG;
GTAGGTCACAGGCTCTAC;
Primer pair 4:
AGACCACTGTTTGAGGTTA;
GTAGGTCACAGGCTCTAC;
Primer pair 5:
AGTGCTTAGAGACCACTG;
GTAGGTCACAGGCTCTAC;
Primer pair 6:
GAGCATAGGAAAGTGCTTA;
GTAGGTCACAGGCTCTAC;
Primer pair 7:
GAGCATAGGAAAGTGCTTA;
CACAGGCTCTACGTGTAG;

1.3 Implementation

The implementation of the PCR amplification method will now be described in detail.

(1) Experimental Group (after improvement)

First, fresh samples of normal human peripheral blood were used, and DNA was extracted from the blood sample for the following experimental applications, and then the human K-Ras gene was detected by the SYBR Green fluorescent PCR method, and different primer pairs were tested separately. Fluorescence quantitative PCR amplification system (20μL) was prepared, as shown in table (1.3-1):

Table 1.3-1. Fluorescence Quantitative PCR Amplification System (20 μL)

Reagents	Dosage
1× SYBR Green. PCR• Master mix	10 μL
Upstream Primer	0.4 μL (200 nM)
Downstream Primer	0.4 μL (200 nM)
DNA Template	10-100 ng
dH_2O fill up to 20	

TFinally, the PCR amplification reaction conditions were obtained: 95°C pre-denaturation for 10 minutes; denaturation at 95°C for 10 seconds, annealing at 60°C for 50 seconds, extension at 80°C for 10 seconds, 40 cycles, and fluorescence signal collection at 80°C; melting curve process at 95°C for 1 minute, 65°C for 30s, 95°C for 30s, the fluorescence signal was collected during the whole process.

(2) Control Group (before improvement)

The differences from the experimental group (improved) is that the PCR amplification reaction conditions were: pre-denaturation at 95°C for 10 minutes; denaturation at 95°C for 10 seconds, annealing at 60°C for 50 seconds, 40 cycles, and

fluorescence signals collected at 60°C; melting curve process: The fluorescence signal was collected during the whole process at 95°C for 1min, 65°C for 30s, 95°C for 30s.

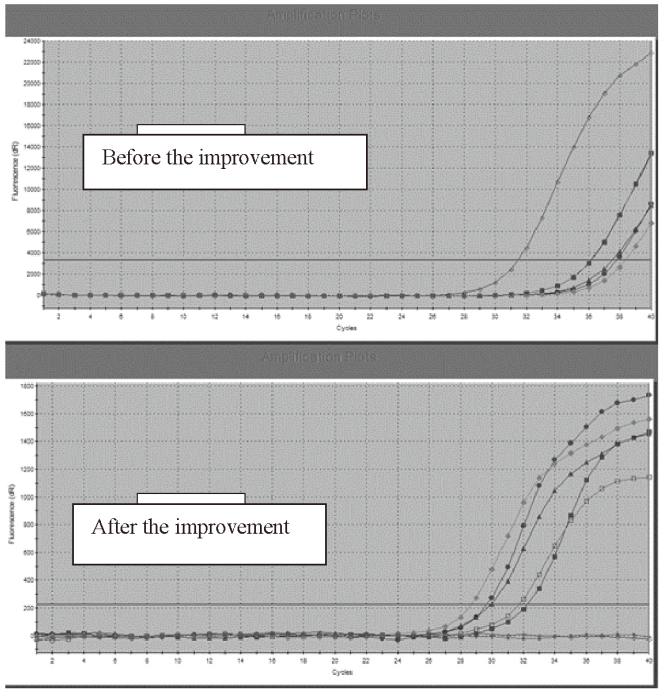

Fig. 1.3-2

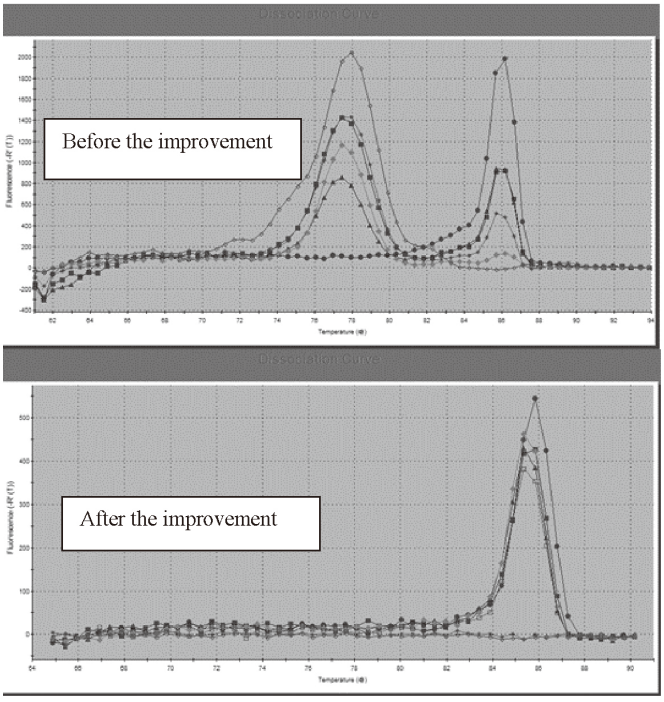

Fig. 1.3-3

2 Results

Figure (1.3-2) shows the comparison of the amplification curves of the experimental group (after improvement) and the control group (before improvement) in the detection of K-Ras gene by the two fluorescence quantitative PCR methods, and Figure (1.3-3) shows the comparison of the melting curves of the experimental group (after improvement) and the control group (before improvement) in the detection of K-Ras gene by two fluorescence quantitative PCR methods. It can be seen from the results that the normal SYBR Green fluorescence PCR amplification curve of the control group (before the improvement) has a larger Ct value and amplification curve, but the melting curve is messy and there are multiple peaks at different positions, indicating the presence of a primer dimer; for the improved method, the Ct value of the amplification curve is smaller, the amplification efficiency is improved, the melting curve shows a single peak at consistent position, eliminating the amplification interference of the primer dimer[1].

The melting curve reaction process in the experimental group (after improvement) was only used to compare the two fluorescence PCR amplifications procedures with the control group (before improvement), which can be omitted in the actual test. It can be seen from the results that it has the following advantages: (1) Wide application range: the process of the fluorescence quantitative PCR reaction is improved without additional reagents or changing the ratio of the reaction system, which can be applied to most dye-based fluorescence quantification PCR reaction. (2) Improved accuracy: The interference of non-specific amplification is eliminated, the increase in target gene fragments is more positively correlated with the increase in fluorescence signal intensity, and the analysis and assessment of experimental results are more accurate. (3) Lower primer design requirements: The signal of the target gene fragment can also be detected normally in the presence of structural interference such as primer dimers.

3 Conclusion

In the process of quantitative PCR gene detection based on the fluorescent dye method, the added fluorescent dyes such as SYBR Green are not selective for the amplified DNA double-strands, so some non-specific amplified fragments will incorporate the dye to produce fluorescence signal interference in detection. The presence of amplification primers may

also lead to the formation of primer dimers, which will also affect the detection of fluorescent signals. In addition, two-step fluorescence quantitative PCR detection is generally used currently, and this detection method requires signal collection during the entire process, and after the fluorescence signal is collected, the melting curve reaction process needs to be carried out, which leads to a long reaction time and various interferences resulting in low detection accuracy. A change is applied to the conventional two-step fluorescence quantitative PCR detection by adopting a three-step method, and the fluorescence signal collection is designed to be carried out during the extension stage (at 80 ℃ high temperature). Since non-specific amplification products are often short fragments, they will undergo denaturation and uncoiling in the range of 70-85 ℃ and cannot bind to fluorescent dyes to emit fluorescence, so all the fluorescent signals collected at this time come mainly from the amplified fragments of the target gene, thereby effectively eliminate the interference caused by non-specific amplification to the fluorescent dye quantitative PCR reaction[1]. The method disclosed in this experiment to solve the non-specific amplification interference of fluorescent quantitative PCR in gene detection is suitable for most fluorescent dye-based quantitative PCR reactions. It can reduce the occurrence of fluorescent signal interference caused by various situations, improve the detection accuracy, omit the melting curve reaction process and shorten the reaction time, which is of great significance for improving the detection efficiency of fluorescent dye-based quantitative PCR in the scientific research process[2].

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