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ORIGINAL ARTICLE

The Impact of Sharing Primer, the Quantity of the Internal Control Gene and the Primer Dimer on Reaction System in Duplex PCR

Wencan Jiang [1], Shang He [1], Jianan Wang [1, 2], Chen Chen [1], Yingjiao Sha [1], Huizhen Sun [1], Xiaozhou Yuan [1], Xiaoting Liu [1], Chengbin Wang [1]

[1] *Department of Clinical Laboratory Medicine, Chinese People's Liberation Army General Hospital & Postgraduate Medical School, Beijing, People's Republic of China*
[2] *College of Laboratory Medicine and Life Science, Wenzhou Medical University, Wenzhou, People's Republic of China*

SUMMARY

Background: **In duplex real time quantitative PCR (qPCR), there are several factors affecting the sensitivity of duplex qPCR, such as sharing primer, quantity of the internal control (IC) gene, and the primer dimer (PD). The aim of the study is to find out the relationship of interference among templates with different primer pairs, the internal control gene, and the PD in duplex PCR.**
Methods: **We designed and synthesized plasmids with partial same sequence and different types of primers include central-homo primer pair, ordinary primer pair, and complementary primer pair. Then we compared the amplification efficiencies of different kinds of primer pairs. Besides, we adjusted the amount of IC plasmid and IC primer to find out the key factor that influences the sensitivity of the target template.**
Results: **The concentration ratios of two plasmids showed interference in sharing the universal primer pair, sharing one forward primer, and sharing no primer were 50:1, 200:1 and 500:1, respectively. The amplification efficiency of the ordinary primer pair was higher than that of the universal primer pair for the plasmid. Sensitivity of the duplex qPCR remained unchanged when the amount of PDs increased, but it declined rapidly when the quantity of the IC increased.**
Conclusions: **IC is the major factor influencing the sensitivity of the duplex qPCR and it would be better to use one universal primer for IC and target template to achieve minimum interference.**
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Correspondence:
Chengbin Wang
Fuxing Road 28
Haidian District, Beijing
China
Phone: +86 15901056535
Fax: +86 10-66937771
Email: wangcbin301@163.com

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INTRODUCTION

There are two types of real time quantitative PCR (qPCR), one type uses nonspecific DNA binding dyes and the other type uses labeled probes [1,2]. DNA binding dyes, e.g., SYBR Green, can bind to double-stranded DNA and luminesce to record the amplification of the target gene [3]. However, it may result in false positive outcomes when primer dimer (PD) or other nonspecific amplification products are present [4,5]. As for

the other type of qPCR, the labeled probe can only bind to a specific target sequence. It can bypass the false positive results caused by the PD, but interference due to PD is still unavoidable [6]. Excessive PD can inhibit the amplification of the target template and result in false negative outcomes [7]. This problem could be partly solved by adding an internal control (IC) in the reaction system to monitor the PCR process [8-10], but at the same time, it will also increase the complexity of the reaction system substantially and causes mutual interferences between the IC gene and the target template in a duplex PCR system [11].

So far in the literature, research focusing on the interference relationships between different types of IC and target template in the duplex PCR system are very limited and possible solutions to this problem have not been addressed. In our work, we examined interference relationships among templates using customized plasmids with partial same sequence and different types of primers and used homo β-globin and hepatitis B virus (HBV) genes to find key factors affecting the detection sensitivity of the target template in duplex PCR. In addition, the primer concentration and Ct value of the IC template were also adjusted to compare the detection sensitivity of the target template under different conditions.

MATERIALS AND METHODS

Reagents and instruments

For the PCR reagents used in this research, β-globin and HBV plasmid were provided by Beijing TagArray Molecular Co., Ltd. Plasmid "pU1" and "pU2" were synthesized at Shanghai Sangon Biotechnology Co., Ltd. The PCR reaction mix was bought from Beijing ComWin Biotech Co., Ltd. DNA amplification and product detection were performed on TL 988, bought from Xi 'an TianLong Co., Ltd.

Cloning protocol of different sequence plasmids and primer probes

Sequences of different viruses were searched from the GeneBank. Then the plasmid pU1 and pU2 were designed and synthesized according to the sequence of human herpes virus. The sequence of pU1 is:

5'-
CGGGGTGCAGCACGGTCTTCTGCCCTGCCTGCA
CGTGGCCGCCACCGTGACGACCATCGGCCGCG
AGATGCTCCTCGCGACGCGCGCGTACGTGCAC
GCGCGCTGGGCGGAGTTCGATCAGCTGCTGGC
CGACTTTCCGGAGGCGGCCGGCATGCGCGCCC
CCGGTCCGTACTCCATGCGCATCATCTACGGGG
ACACGGACTCCATTTTCGTTTTGTGCCGCGGCC
TCACGGCCGCGGGCCTGGTGGCCATGGGCGAC
AAGATGGCGAGCCACATCTCGCGCGCGCTGTTC
CTCCCCCCGATCAAGCTCGAGTGCGAAAAAAC
GTTCACCAAGCTGCTGCTCATCGCCAAGAAAA
AGTACATCGGCGTCATCTGCGGGGGCAAGATG
CTCATCAAGGGCGTGGATCTGGTGCGCAAAA-
3'.

the sequence of pU2 is:

5'-
CGGAGGAGAGCAGCACGTTACCGGAGGGGCTC
GAAACATCATCAGGGGGCTTGGACGAACGGCG
GGTGGAGGCGCGGGTCATCTACGGGGACACGG
ACAGCGTGTTTGTCCGCTTTCGTGGCCTGACGC
CGCAGGCTCTGGTGGCGCGTGGGCCCAGCCTG
GCGCACTACGTGACGGCCTGTCTTTTTGTGGAG
CCCGTCAAGCTGGAGTTTGAAAAGGTCTTCGTC
TCTCTTATGATGATCTGCAAGAAACGTTACATC
GGCAAAGTGGAGGGCGCCTCGGGTCTGAGCAT
GAAGGGCGTGGATCTGGTGCGCAAGACGGCCT
GCGAGTTCGTCATGGCAGTCAACGGTTAGCGTG
ACGTCCTCT-3',

The same bases of the two sequences were underlined. The structure of the two plasmids were shown in Figure 1, the underlined bases indicate the sequences that are the same in both plasmids.

Designation of primers and probes

DNAMAN V6.0 and Primer Premier 5.0 were used to design different primers. The primer pair comF/comR was universal for the two plasmids, whereas the primer pair PF1/PR1 was specific for pU1 and the primer pair PF2/PR2 was specific for pU2. The probe P1 and P2 were specific probes for pU1 and pU2, respectively (Table 1). In addition, sequences of HBV DNA were obtained from the GeneBank (sequence number: KT-991435.1), and the highly conserved sequence of its core region C was selected as target template to design central-homo primer pair and probe. The sequence of the forward primer HBVF1 was

5'- aaa tgc ccc tat ctt ctc aac-3',

the reverse primer HBVR1 was

5'- aga ttg aga tct tct gcg ac-3',

and probe HBVP was

5'- FAM-cgt ctg cga ggc gag gga gtt ctt ctt cgc ag-BHQ1-3'.

The underlined parts in the primer F and R were the central-homo sequences. To verify the practicability of the central-homo primer pair, two additional central-homo primer pairs HBVF2/HBVR2, HBVF3/HBVR3 and ordinary primer pairs HBVF4/HBVR4, HBVF5/HBVR5 were designed. The sequences were as follows,

HBVF2:

5'- agg tcc cct aga aga aga ac-3';

HBVR2:

5'- aca ggt aca gta gaa gaa taa ag-3';

HBVF3:

5'- gga aga gaa act gtt ctt gag t-3';

HBVR3:

5'- gcg agg gag ttc ttc ttc-3';

HBVF4:

5'- aac tgt tct tga gca ttt ggt g-3';

HBVR4:

5'- aac agt agt ttc cgg aag tg-3';

HBVF5:

5'- tcc tcc tgc tta cag acc a-3';
HBVR5:
5'- tcc cac ctt atg agt cca a-3'.
The underlined bases of the primer pairs HBVF2/HBVR2 and HBVF3/HBVR3 are the central-homo sequences. The primers, probes and plasmids were all synthesized at Shanghai Sangon Biotechnology Co., Ltd.

Probe real time fluorescence quantitative PCR reaction
The PCR mixture for TaqMan real-time PCR was 25 μL including 0.5 μL of 10 μmol F/R primer, 12.5 μL of PCR mix, 0.5 μL of 5 μmol/L probe, 5 μL of template and ddH_2O. The reaction surface was sealed by 30 μL mineral oil. The cycle program was set as 10 minutes at 95°C, and followed by 40 cycles of 30 seconds at 95°C and 60 seconds at 58°C. The fluorescence values were read at 58°C.

Interference tests of plasmid pU1 and pU2 with different primers
The concentration ratios of plasmid pU1 and pU2 were set as 1,000:1, 500:1, 200:1, 50:1, 1:50, 1:200, 1:500, and 1:1,000, then three sets of interference tests were carried out. In the first set, the universal primer pair comF/comR was used to amplify the two plasmids; in the second set, the two plasmids shared the same forward primer, namely, pU1 used the primer pair comF/PR1, pU2 used the primer pair comF/PR2; and for the third set, pU1 used the primer pair PF1/PR1 and pU2 used PF2/PR2. The interference was evaluated by the Ct values of the two plasmids.

Comparisons of the amplification efficiency between the universal primer pair and the optimized ordinary primer pair
In order to compare the amplification efficiency of the universal primer pair and the ordinary primer pair, the plasmid pU2 was diluted 10-fold and detected by the primer pair comF/comR and PF2/PR2. Then the Ct values of the same dilution were compared.

PD tests of different HBV primer pairs by template-free qPCR (dye method)
To verify the performance of central-homo primer pairs, levels of PD formed by central-homo primer pairs HBVF1/HBVR1, HBVF2/HBVR2, HBVF3/HBVR3 and ordinary primer pairs HBVF4/HBVR4, HBVF5/HBVR5 were tested in a template-free qPCR system using SYBR Green I dye. The volume of the template-free PCR mixture was 25 μL and included 0.5 μL of 10 μmol/L F/R primers, 12.5 μL of 2 x Premix, 1.0 μL of 25 x SYBR Green I, and 10.5 μL of ddH_2O. The reaction surface was sealed by 30 μL mineral oil. The cycle program was: 10 minutes at 95°C, and followed by 40 cycles of 30 seconds at 95°C and 60 seconds at 58°C. The fluorescence values were read at 58°C. The results were analyzed and the Ct values exceeding 37 were recognized as negative.

Ascertaining the concentration of IC primer and plasmids
To explore the key factor influencing the amplification of the target gene, the homo β-globin plasmid was chosen as the internal control. The concentrations of the IC primer were set at 10 μmol/L, 8 μmol/L, 5 μmol/L, 2 μmol/L, and 1 μmol/L, then serial 10-fold diluted β-globin plasmids were tested using these primers. Under different concentration of the IC primer, based on our prior experiments, the concentration of plasmid with a Ct value around 30 was determined as the concentration of IC template concentration subsequently in the system.

PD tests and the sensitivities of different HBV detection systems containing serial concentrations of IC primers
The HBV plasmid was chosen as the target template and treated with a series of 10-fold dilutions. The above mentioned 25 μL detection systems containing IC were used to detect these serially diluted templates. The Ct value of the IC was selected around 30, and based on this, the concentrations of the IC primers were set at 10 μmol/L, 8 μmol/L, 5 μmol/L, 2 μmol/L, and 1 μmol/L and the single PCR without IC system was set as the comparison. The Ct value of PDs in each system was detected by the dye method.

The sensitivity of duplex qPCR with different concentrations of IC plasmid
The concentration of the IC plasmids, with corresponding Ct values around 15, 20, 25, and 30, were selected according to the previous determinate concentration of IC primers and plasmids. Then serially diluted HBV plasmids were tested by PCR systems with different concentrations of IC plasmids. All the IC primer concentrations used were 5 μmol/L and the original concentration of the HBV plasmid was 5×10^9 IU/mL. The sensitivity of duplex qPCR with different IC was compared.

The effects of IC template on the sensitivity of the duplex qPCR HBV detection system
In order to prove the influence of the IC gene, two reaction systems were designed. System A: the IC primer pair (5 μmol/L) with the IC plasmid with a Ct value around 15. System B: the IC primer pair (5 μmol/L) without the IC plasmid. The results of the two systems were compared to verify if there was a significant difference in sensitivity between the two systems. Reaction procedure and results analysis were the same as mentioned above.

Table 1. Primer and Probe sequences for pU1 and pU2.

Primer	Sequence (5'-3')
comF	5'- cat cta cgg gga cac gga c-3'
comR	5'- cac cag atc cac gcc ctt-3'
PF1	5'- ggg ctc gaa aca tca tca g-3'
PR1	5'- taa cgt ttc ttg cag atc atc-3'
PF2	5'- agc tgc tgg ccg act ttc-3'
PR2	5'- gag cag cac ctt gtt gaa c-3'
P1	5'- FAM-ttt gtc cgc ttt cgt ggc ctg acg cgg a-TAMRA-3'
P2	5 - Texas Red-cga caa gat ggc gag cca cac ctc cg-BHQ2-3'

Note: FAM and Texas Red were different fluorophore labels, TAMRA and BHQ2 were different quenchers.

Table 2. PD amount of different groups and interference relationship between pU1 and pU2.

Group	1	2	3
Primer for pU1	comF/comR	comF/PR1	PF1/PR1
Primer for pU2	comF/comR	comF/PR2	PF2/PR2
Concentration fold	50	200 - 500	500 - 1,000
Ct value of PD	34.7	33.67	29.78

Table 3. The Ct value of different primer pairs for plasmid pU2.

Dilution ratio	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Blank
comF/PR2	15.03	18.57	21.30	24.14	27.79	31.31	-
comF/comR	17.14	19.87	23.26	26.89	30.58	34.55	-

Table 4. Ct values of PDs of central-homo primer pairs and ordinary primer pairs.

Primer pair	HBVF1/HBVR1	HBVF2/HBVR2	HBVF3/HBVR3	HBVF4/HBVR4	HBVF5/HBVR5
The Ct value of PDs	36.07	35.71	-	32.17	31.72

Table 5. Plasmid concentrations corresponding to different primer concentrations (Ct value about 30).

Concentration of IC primer (μmol/L)	10	8	5	2	1
Concentration of IC plasmid (IU/mL)	5×10^4	5×10^4	5×10^4	5×10^5	5×10^7

Table 6. Sensitivity of qPCR HBV detection system containing different IC primer and PD test.

Concentration of IC primer (μmol/L)	HBV target template concentration (IU/mL)							ddH_2O	Ct Value of PD
	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2		
10	14.93	19.49	23.17	26.38	29.34	33.10	-	-	27.82
8	15.84	19.77	22.53	26.27	29.64	32.81	-	-	28.76
5	14.57	18.24	22.30	26.35	29.71	32.91	-	-	29.89
2	15.61	19.98	24.19	27.20	30.16	33.48	-	-	32.99
1	17.04	20.12	23.71	27.17	31.24	33.25	-	-	34.21
Single PCR	14.66	19.03	23.57	26.84	30.40	33.04	35.28	-	36.07

Table 7. Comparison of sensitivities of duplex qPCR with different concentrations of IC.

Ct value of IC	Concentration of target HBV template (IU/mL)							dH_2O
	5×10^8	5×10^7	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	
15	14.80	19.87	23.51	27.08	31.17	-	-	-
20	15.67	19.58	22.88	27.23	31.17	34.57	-	-
25	15.72	19.70	23.99	27.46	31.07	34.12	-	-
30	14.57	18.24	22.30	26.35	29.71	32.91	-	-
Single PCR	14.66	19.03	23.57	26.84	30.40	33.04	35.28	-

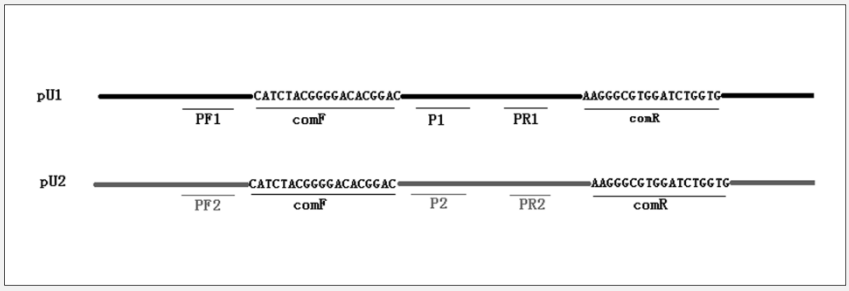

Figure 1. Sequence structures of pU1 and pU2.

RESULTS

Interference tests of plasmid pU1 and pU2 with different primer pairs

The results are shown in Table 2. In the first group, pU1 and pU2 sharing the universal primer pair, the amplification of the plasmid with the lower concentration was significantly inhibited when the concentration difference between the two plasmids was over 50-fold. In the second group, pU1 and pU2 sharing the same forward primer, interference only appeared when there were 200-fold difference between the two plasmids, whereas in the third group, pU1 and pU2 using different primer pairs, significant interference was observed when the concentration difference was 500-fold. Among these three groups, the Ct value of PD in the third group was significantly smaller than the other two groups, which means much more PD was produced in the third group than in the others.

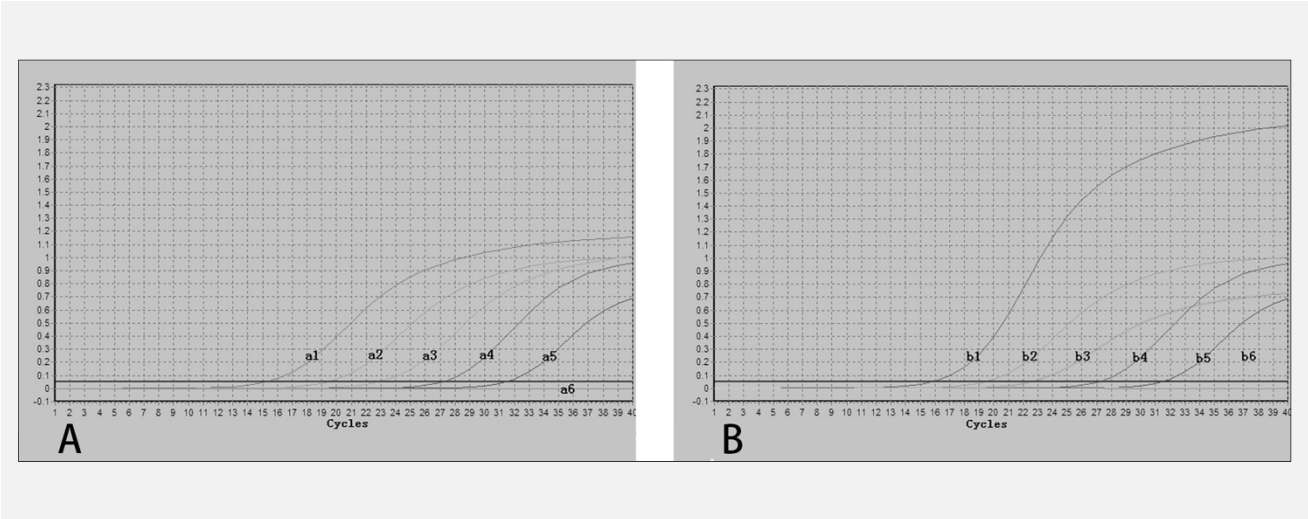

Figure 2. Sensitivity of duplex RT-PCR with and without IC template.

Note: a1 - a6 are the results of serial 10-fold diluted target template detected by the duplex qPCR containing IC template with a Ct value about 15, and the limit of detection was the fifth dilution with a corresponding concentration of 5 x 10^4 IU/mL. b1 - b6 are the results of serial 10-fold diluted target template detected by the duplex RT-PCR without IC template, and the limit of detection is the sixth dilution with a corresponding concentration of 5 x 10^3 IU/mL.

Comparison of the amplification efficiency between the universal primer pair and the ordinary primer pair

As shown in Table 3, the Ct value of primer pair comF/comR was higher than comF/PR2 in all dilutions of pU2, and the amplification efficiency of the universal primer pair of pU2 decreased as the dilution ratio went down.

PD tests of different HBV primer pairs by template-free qPCR (dye method)

The results in Table 4 showed that Ct values of PDs of central-homo primer pairs HBVF1/HBVR1, HBVF2/HBVR3, and HBVF3/HBVR3 were above 36, whereas the Ct values of PDs of ordinary primer pairs HBVF4/HBVR4 and HBVF5/HBVR5 were around 30 - 34. This indicated that amount of PDs formed by ordinary primer pairs were more than that of central-homo primer pairs.

Ascertaining the concentration of IC primer and plasmids

As shown in Table 5, controlling the Ct value at 28 - 30, the higher concentration of the IC primer was used, the lower concentration of the IC plasmid that was added. The amount of the different plasmids were selected for IC primer pairs with different concentrations.

The sensitivity of different HBV detection systems containing IC primers with serial concentration and PD quantity test

The results are shown in Table 6. The sensitivities of the target template in all the duplex qPCR systems with IC were 5 x 10^3 IU/mL, while the sensitivity of the single PCR without IC was 5 x 10^2 IU/mL. There was a negative correlation between the amount of PDs and the concentration of the IC primer pairs.

The sensitivity of duplex qPCR with different IC gene Ct values

As shown in Table 7, raising the concentration of IC (smaller Ct value) in duplex qPCR containing IC led to the increase of detection sensitivity of the target template or the bigger Ct value of target template with a low concentration.

The influence of IC template on the sensitivity of duplex qPCR HBV detection system

The minimum detectable concentration of system A was 5 x 10^4 IU/mL containing the IC template with a Ct value around 15, whereas for the duplex PCR without IC template the minimum detectable concentration was 5 x 10^3 IU/mL. Results were shown in Figure 2.

DISCUSSION

PD is one of the main factors influencing the detection sensitivity of single qPCR. A high concentration of PD would compete with target template for primers and

thus inhibit the amplification of target template, which may lead to false negative results. Satterfield's study showed that the amplification of target template would be totally inhibited when the concentration of PD was 10 times more than that of the target template [3]. The internal control system and central-homo primer pair are generally adopted in qPCR to deal with the PD interference, which can reduce the PD amount and thus decrease the suppression effect of PD on the target template [4,7]. Therefore, it is vitally important to determine the type of the IC gene.

Results in Table 2 show that when two different templates in duplex qPCR share the same primer pair, the amplification of the template with lower concentration would be inhibited if the concentration of the two templates has a 50-fold difference. But when the two templates share the same forward primer, the interference appeared only after the concentration difference of two templates reached 200-fold, and this was similar to the third group in which the two templates use their own primers. As for the amount of PD, the second group sharing the same forward primer was similar with that of the first group sharing the universal primer pair. In addition, as shown in Table 3, the amplification efficiency of the universal primer pair was not as high as that of the optimized primer pair. It was difficult to guarantee the amplification efficiency of every template when using one pair of the universal primer. Therefore, under the situation that there are primer pairs which can amplify both IC template and target template in duplex qPCR system, it would be better to choose one universal primer. In this way, using as few primers as possible in duplex qPCR can not only control the system complexity, but also reduce the interference between the two templates. When fixing the primer at one end, we can adjust the position of the primer at the other end, thereby increasing the freedom of primer design and ensuring the highest amplification efficiency of the designed primer.

From Table 6, we can see that for duplex qPCR, when the Ct value of the IC was kept at 28 - 30, the amount of IC primer had no significant influence on the sensitivity of the detection system. Different concentrations of the IC primer pair only leads to changes of the PD amount, but it had no effect on the sensitivity of duplex qPCR systems. The minimum detectable concentration was 5×10^3 IU/mL, whereas the minimum detectable concentration is 5×10^2 IU/mL for the single PCR using central-homo primer pair, indicating that the simplex PCR was more sensitive than duplex PCR. As shown in Table 7, when the concentration of the IC primer was kept in a certain range, the more IC template used, the more serious the influence of the IC amplicon would have on the target template. These results suggested that the concentration variation of IC template had more significant influence on the detection sensitivity of target template than the amount of PDs. The possible reason was that in duplex qPCR, PDs from IC template and other primers, except for PDs from target template primers, had no competition with target template for target primer, but competed for Taq polymerase and some other components. Therefore, when the Ct value of IC template was controlled at 28 - 30, and the Ct value of PDs was controlled above 30 (namely the amount of PD was lower than that of the IC amplicon), the IC template would play a dominant role in the non-competitive inhibition.

Above all, PD is the main factor affecting the sensitivity of single PCR, whereas in duplex PCR, PDs as well as the IC gene have non-competitive inhibition on target template amplification. For a PCR system using ordinary primers without central-homo sequences, adding the IC system could increase the detection sensitivity, because by the dispersal effect among primers, the IC system could decrease the amount of PD, which competes with the target template. Despite central-homo primer pairs in simplex PCR being able to control the amount of PD at a low level, the effect of central-homo primer pairs would be limited because of the interference between IC and other PDs. There was seldom a difference between the ordinary primer pair PCR and central-homo primer pair after adding the IC system. Choosing a simplex PCR system with central-homo primer pairs in the TaqMan probe method could avoid the interference of the IC gene and decrease the influence of PD at the same time. However, to assure the specificity of the detection results, the IC system is indispensable. Further study should focus on how to increase the detection sensitivity of the duplex qPCR containing IC system.

CONCLUSION

Under the situation that one primer pair can amplify both IC template and target template in the duplex qPCR system, it would be better to choose one universal primer. The internal control gene is the major influence factor on the sensitivity of the duplex PCR.

Declaration of Interest:
The authors declare that they have no competing interests.

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