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Preliminary Study on Identification of Hypertension Drug Related Genes by Taqman Probe and Locked Nucleic Acid Multiple Primers

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Abstract: The ADRB1 and CYP2D6 gene sequences were designed according to Ensembl website. Self-designed primers and probes for ADRB1 and CYP2D6. Single qPCR systems for ADRB1 and CYP2D6 gene detection were established respectively, and then PCR reaction conditions were optimized to establish multiple qPCR systems. The multiplex qPCR system can simultaneously genotype ADRB1 and CYP2D6, so as to judge whether the target gene is mutated, which can provide guidance for accurate medication of hypertension patients. The experimental results show that the detection method is simple, rapid, specific and reproducible, which can improve the flux and reduce the cost. Therefore, the multiplex qPCR system model for ADRB1 and CYP2D6 gene detection was successfully constructed.

Keywords: Hypertension; ADRB1; CYP2D6; Genotyping; Multiple qPCR.

1. Introduction

Hypertension is a common chronic disease of cardiovascular disease. The global more than 1 billion hypertension patients need about getting the drugs. At present the treatment of hypertension rely mainly on oral medications, so individual genes guide treatment more and more important in the treatment of hypertension.

Gene polymorphisms have different effects on drug efficacy by influencing drug metabolic enzymes, transporters and drug targets. The study on the polymorphism of drug-related genes in hypertension can provide basis for individualized treatment [1]. YANG Mei et al. [2] found that in the study of gene polymorphisms associated with antihypertensive drugβThe gene sites associated with receptor blockers are CYP2D6 * 10 and ADRB1 (1165G>C). The genotyping of CYP2D6 and ADRB1 can provide guidance for precise medication of hypertension patients.

Multiple real-time fluorescence quantitative PCR is the simultaneous detection of multiple pathogenic genes by adding multiple pairs of primers and multiple probes to the same reaction, which has greatly saved human and material resources [3]. In the experiment, we should try to avoid the possible inhibition of culture medium and nucleic acid extraction reagent on PCR [4-7], avoid the mutual interference between various primers, probes and luminous groups, and avoid non-specific amplification between various target fragments. At the same time, ensure that each primer and probe has a similar annealing temperature, so that each target fragment has a similar amplification efficiency. Through the screening of primers and probes, the optimization of multiple PCR reaction system, the specificity detection and the confirmation of detection limit in genes, a multiple fluorescence quantitative PCR reaction system with strong specificity and high discrimination was established [3].

At present, there are many studies on the polymorphism of hypertension drug-related genes, but the research method is relatively simple, and it is time-consuming and laborious to detect individual genes separately. In this study, a multiplex qPCR rapid genotyping method was established, which can simultaneously detect CYP2D6 and ADRB1 genes. Six primers and two probes were added to the same reaction system to realize the simultaneous detection of multiple pathogenic genes. Although it increases the difficulty of experimental design, it can improve the flux, reduce the cost, and be more simple and fast.

2. Materials and Equipment

2.1. Plasmid Samples

The samples of ADRB1 and CYP2D6 wild plasmids and mutant plasmids were designed by our laboratory. Confirm the target gene sequence on Ensemble website and entrust Biotechnology (Shanghai, China) Co., Ltd. to synthesize ADRB1 homozygous wild-type plasmid, ADRB1 homozygous mutant plasmid, CYP2D6 homozygous wild-type plasmid and CYP2D6 homozygous mutant plasmid. The sample concentration was measured with the Implen ultramicro spectrophotometer for nucleic acid quantitative determination. The gene sequence is shown in Table 1.

2.2. Design and Synthesis of Primers

Based on the ADRB1 gene sequence and CYP2D6 gene sequence as templates, using Primer Express 3.0 software (Thermo Fisher Scientific), the ADRB1 wild-type forward primer, ADRB1 mutant forward primer, ADRB1 reverse primer, CYP2D6 wild-type forward primer, CYP2D6 mutant forward primer, and CYP2D6 reverse primer were designed. The wild type and mutant of ADRB1 gene share a reverse primer, and the wild type and mutant of CYP2D6 gene share a reverse primer. In addition, the 3 'end of the wild-type forward primer and the mutant forward primer were modified with locked-nucleotide respectively to ensure that the primer and template DNA can be specifically paired. Six primers were commissioned to be synthesized by Bioengineering (Shanghai, China) Co., Ltd. The primer sequence is shown in Table 2.

Table 1. ADRB1 and CYP2D6 wild and mutant sequences

Name	Sequence
Homozygous wild sequence of ADRB1	AAGGCCTTCCACCGCGAGCTGGTGCCCGACCGCCTCTTCGTCTTCTTCAACTGG CTGGGCTACGCCAACTCGGCCTTCAACCCCATCATCTACTGCCGCAGCCCCGAC TTCCGCAAGGCCTTCCAG**G**GACTGCTCTGCTGCGCGCAGGGCTGCCCGCCGGC GCCACGCGACCCACGGAGACCGGCCGCGCGCCTCGGGCTGTCTGGCCCGGCCC GGACCCCCGCCATCGCCCGGGGCCGCCTCGGACGACGAC
Homozygous mutation sequence of ADRB1	AAGGCCTTCCACCGCGAGCTGGTGCCCGACCGCCTCTTCGTCTTCTTCAACTGG CTGGGCTACGCCAACTCGGCCTTCAACCCCATCATCTACTGCCGCAGCCCCGAC TTCCGCAAGGCCTTCCAG**C**GACTGCTCTGCTGCGCGCGCAGGGCTGCCCGCCG GCGCCACGCGACCCACGGAGACCGGCCGCGCGCCTCGGGCTGTCTGGCCCGGC CCGGACCCCCGCCATCGCCCGGGGCCGCCTCGGACGACGAC
Homozygous wild sequence of CYP2D6	GCCTGGTCCTCTGTGCCTGGTGGGGTGGGGGTGCCAGGTGTGTCCAGAGGAGC CCATTTGGTAGTGAGGCAGGTATGGGGCTAGAAGCACTGGTGCCCCTGGCCGTG ATAGTGGCCATCTTCCTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGCTGG GCTGCACGCTAC**C**CACCAGGCCCCCTGCCACTGCCCGGGCTGGGCAACCTGCT GCATGTGGACTTCCAGAACACACCATACTGCTTCGACCAG
Homozygous mutation sequence of CYP2D6	GCCTGGTCCTCTGTGCCTGGTGGGGTGGGGGTGCCAGGTGTGTCCAGAGGAGC CCATTTGGTAGTGAGGCAGGTATGGGGCTAGAAGCACTGGTGCCCCTGGCCGTG ATAGTGGCCATCTTCCTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGCTGG GCTGCACGCTAC**T**CACCAGGCCCCCTGCCACTGCCCGGGCTGGGCAACCTGCT GCATGTGGACTTCCAGAACACACCATACTGCTTCGACCAG

Table 2. ADRB1 and CYP2D6 primer sequences

Name	Sequence
ADRB1 forward wild primer	CAAGGCCTTCCAGG-LNA
ADRB1 forward mutation primer	CAAGGCCTTCCAGC-LNA
ADRB1 reverse primer	TGGGTCGCGTGGCG
CYP2D6 forward wild primer	GCTGCACGCTACC-LNA
CYP2D6 forward mutation primer	GGCTGCACGCTACT-LNA
CYP2D6 reverse primer	CCTCCCTCACCTGGTCGAA

2.3. Probe Design and Synthesis

Based on the ADRB1 gene sequence and CYP2D6 gene sequence as templates, the ADRB1 Taqman probe and CYP2D6 Taqman probe are designed using Primer Express 3.0 software (Thermo Fisher Scientific). The wild type and mutant type of the ADRB1 gene Taqman probe need not be designed separately. They use the same Taqman probe. The same is true for CYP2D6. The fluorescent reporting group is designed at the 5 'end of TaqMan probe, and the quenching group is designed at the 3' end of TaqMan probe. The fluorescence reporting group of ADRB1 Taqman probe is CY3, and the quenching group is BHQ2. The fluorescence reporting group of CYP2D6 Taqman probe is FAM, and the quenching group is BHQ1. Two probes were commissioned to be synthesized by Yingwei Jieji (Shanghai, China) Trading Co., Ltd. The Taqman probe sequence is shown in Table 3.

Table 3. ADRB1 and CYP2D6 Taqman probe sequences

Name	Sequence
ADRB1 Taqman probe	CY3-ACTGCTCTGCTGCGCGCGC-BHQ2
CYP2D6 Taqman probe	FAM-ACCAGGCCCCCTGCCACTGC-BHQ1

2.4. Instruments and Equipment

ABI 7500 Fast fluorescent quantitative PCR instrument, fluorescent quantitative PCR reaction tube, pcr instrument, Implen ultramicro spectrophotometer, agarose, UV gel imager.

3. Experimental Method

3.1. Construction and Optimization of Single qPCR Detection System

Take ADRB1 or CYP2D6 wild-type and mutant plasmid DNA as templates, add corresponding specific primers and Taqman probes, and conduct real-time fluorescence quantitative PCR reaction. The reaction system is: 2x Luna Universal Prob qPCR mix, reverse primer, forward wild-type (mutant) primer, Taqman probe, template DNA, adding water to supplement 20ul. Add the above reaction components into the qPCR reaction tube and mix them, and conduct amplification reaction in ABI 7500PCRFast fluorescence quantitative PCR instrument. Reaction procedure: 95 ℃, 60S, 40X (95 ℃, 15S, 60 ℃, 30S). After the reaction is completed, click the Analyze button in the instrument program to view the experimental results.

3.2. Construction and Optimization of Multiple qPCR Detection System

Based on the single qPCR system, a multiple qPCR detection system was constructed. The concentration of primers, probes and templates in the system was optimized to determine the best reaction system. The experiment was as follows: the multiple qPCR reaction solution for ADRB1 and CYP2D6 gene detection was prepared with 20 ul. Reaction system: 2xLuna Universa Prob qPCR mix, ADRB1 forward mutation primer, ADRB1 reverse primer, CYP2D6 forward wild-type primer, CYP2D6 reverse primer, ADRB1Taqman probe, CYP2D6 Taqman probe, ADRB1 template, CYP2D6 template, add water to supplement 20 ul. Add the above reaction components into the qPCR reaction tube and mix them, and conduct amplification reaction in ABI 7500 PCR Fast fluorescence quantitative PCR instrument. Reaction procedure: 95 ℃, 60S, 40X (95 ℃, 15S, 60 ℃, 30S). After the reaction is completed, click the Analyze button in the instrument program to view the experimental results.

3.3. Real-time Fluorescence Quantitative PCR Specificity Experiment

Different templates were tested in the multiple qPCR reaction system and procedure to test the specificity of the established ADRB1 and CYP2D6 gene detection multiple qPCR method. The experiment was carried out in three groups. One group added homozygous wild-type plasmid DNA template; In the second group, homozygous mutant plasmid DNA template was added; The three groups added homozygous wild-type plasmid DNA and homozygous mutant plasmid DNA into the system at a ratio of 1:1 to simulate the heterozygous mutant plasmid.

3.4. Real-time Fluorescence Quantitative PCR Repeatability Test

The homozygous wild plasmids, homozygous mutant plasmids and heterozygous mutant plasmids of ADRB1 and CYP2D6 were tested for repeatability at the same concentration (0.5ng/20ul), and the mean and SD of CT values were calculated, and the statistical results were analyzed to check the stability of the data.

4. Experimental Results

4.1. Plasmid Concentration

Bioengineering (Shanghai, China) Co., Ltd. was entrusted to synthesize homozygous wild type and homozygous mutant plasmids of ADRB1 and CYP2D6. The concentrations of four plasmids were determined by IMPLEN ultramicro spectrophotometer: CYP2D6 homozygous wild type was 45ng/ul; The homozygous mutant of CYP2D6 was 47ng/ul; The homozygous wild type of ADRB1 was 50 ng/ul; The homozygous mutant of ADRB1 was 48 ng/ul. The purity of the four plasmids was tested, and the A260/280 was around 1.8. It showed that the four plasmids were relatively pure, and were not contaminated during the experimental preparation process.

4.2. Evaluation of Primer Specificity

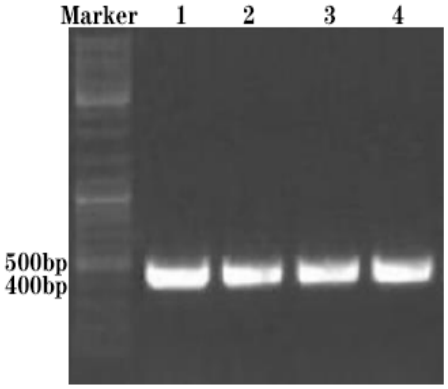


Figure 1. gel electrophoresis of primer sequence

Note: Marker: GeneRuler DNA Ladder Mix (SM0331); 1) CYP2D6 wild-type forward primer; 2) CYP2D6 mutant positive primer; 3) ADRB1 wild-type forward primer; 4) ADRB1 mutant forward primer

Take ADRB1 or CYP2D6 plasmid as the template, add the corresponding specific primer designed by ourselves, and amplify in the PCR instrument. After the reaction is completed, take an appropriate amount of reaction solution for 2.5% agarose gel electrophoresis, and then detect the amplification results on the UV gel imaging instrument. After repeated experiments, suitable specific primers were selected. The specific fragments corresponding to the four genes have obvious bands and good specificity. The agarose gel electrophoresis results showed that the bands were bright, without primer dimer, band tail, etc. The results are shown in Figure 1.

4.3. Single qPCR Detection System

After repeated experiments, the optimal conditions were obtained by optimizing the qPCR system. When the final concentration of template DNA is 0.5ng/20ul, the fluorescence signal of the reaction is the best. The optimal single-weight qPCR reaction system is as follows: 2x Luna Universal Prob qPCR mix 10ul, reverse primer (10uM) 0.8ul, forward primer (10uM) 0.8ul, Taqman probe (10uM) 0.4ul, template 0.5ng, and water supplement 20ul. Reaction procedure: 95 ℃, 60S, 40X (95 ℃, 15S, 60 ℃, 30S).

4.4. Multiple qPCR Detection System

After repeated experiments, on the basis of single qPCR system, multiple qPCR system optimization experiments obtained the best results. The optimal multiple qPCR reaction system is as follows: 2xLuna Universal Prob qPCR mix 10ul, ADRB1 forward mutation primer (10uM) 10ul, ADRB1 reverse primer (10uM) 10ul, CYP2D6 forward wild primer (10uM) 0.8ul, CYP2D6 reverse primer (10uM) 0.8ul, ADRB1Taqman probe (10uM) 0.4ul, CYP2D6 probe (10uM) 0.4ul, ADRB1 template 0.5ng, CYP2D6 template 0.5ng, and water supplement 20ul. Reaction procedure: 95 ℃, 60S, 40X (95 ℃, 15S, 60 ℃, 30S).

4.5. Multiple qPCR Specificity Test Results

According to 4.4, a multiple qPCR system was established to detect the specificity of the ADRB1 and CYP2D6 genotyping methods. The results showed that the CT value was clearly differentiated, indicating that this method has good specificity. The established multiple qPCR system can distinguish the homozygous wild type, homozygous mutant and heterozygous mutant of ADRB1 and CYP2D6 genes through the CT value, so it can be used to detect the genotype of ADRB1 and CYP2D6. The results are shown in Table 4.

Table 4. Test results of specific experiment

Group name	Template gene name	Detection channel	CT
Group 1	Homozygous wild plasmid of CYP2D6 Homozygous wild plasmid of ADRB1	FAM	16.67
		CY3	32.75
Group 2	Homozygous mutant plasmid of CYP2D6 Homozygous mutant plasmid of ADRB1	FAM	26.23
		CY3	17.08
Group 3	Heterozygous mutant plasmid of CYP2D6 Heterozygous mutant plasmid of ADRB1	FAM	18.90
		CY3	21.54

4.6. Multiple qPCR Repeatability Test Results

The homozygous wild-type and homozygous mutant gene

plasmids of ADRB1 and CYP2D6 were used as templates to detect the repeatability. The samples were repeatedly tested for 10 times according to the same concentration. The experimental results were relatively average, and the coefficient of variation was less than 5%, indicating good repeatability. The repeatability results are shown in Table 2 and Table 5, and the coefficient of variation is shown in Table 6.

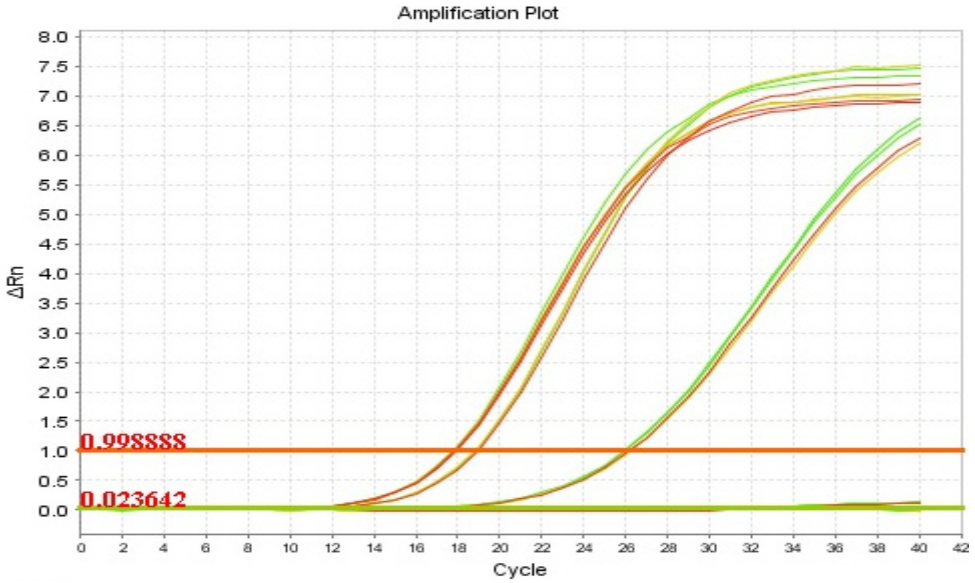


Figure 2. Detection diagram of qPCR repeatability verification

Table 5. Results of qPCR repeatability verification

Template gene name	Homozygous wild plasmid		Homozygous mutant plasmid		Heterozygous mutant plasmid	
	FAM Channel	CY3 Channel	FAM Channel	CY3 Channel	FAM Channel	CY3 Channel
CT1	16.07	32.78	25.83	18.04	19.44	22.54
CT2	15.57	31.58	25.98	17.15	19.58	21.08
CT3	17.55	33.01	26.54	16.84	19.11	22.47
CT4	16.55	31.87	26.78	18.15	20.78	22.55
CT5	16.78	32.55	26.48	16.87	18.28	21.98
CT6	15.47	32.59	26.48	17.10	19.48	23.95
CT7	16.64	31.98	26.44	17.45	21.11	23.02
CT8	16.38	33.21	26.25	17.48	18.57	21.54
CT9	16.25	32.11	25.78	17.28	20.15	21.34
CT10	17.88	32.01	26.21	16.87	19.05	22.47
Mean CT± SD	16.51±0.77	32.37±0.54	26.27±0.33	17.32±0.46	19.56±0.90	22.49±0.85

Table 6. Statistical table of coefficient of variation

Template gene name	Detection channel	CV(SD/Mean)
Homozygous wild plasmid	FAM	4.6%
Homozygous mutant plasmid	FAM	1.6%
	CY3	1.2%
Heterozygous mutant plasmid	FAM	2.6%
	CY3	4.6%

5. Discussion

In this paper, a rapid genotyping method of multiple qPCR, which can simultaneously detect the ADRB1 and CYP2D6 genes, was studied. Six primers and two probes were added to the same reaction system to realize the simultaneous detection of multiple pathogenic genes. The differential fluorescence labeling of the probe with the markers of different detection channels can realize the simultaneous and rapid detection of two or more diseases. The ADRB1 and CYP2D6 genes were simultaneously typed by fluorescence quantitative PCR. The method of judging the experimental results is to judge the genotyping results according to CT values. The results of CYP2D6 genotyping were detected by FAM channel. When the CT value was 16 (±1), it showed that CYP2D6 was a homozygous wild-type gene; When the CT value is 26 (±1), it shows the CYP2D6 homozygous mutant gene; When the CT value is 19 (±1), it shows the CYP2D6 heterozygous mutant gene; CY3 channel was used to detect the results of ADRB1 genotyping. When the CT value was 32 (±1), it showed that it was a homozygous wild-type gene of ADRB1; When the CT value is 17 (±1), it shows CYP2D6 homozygous mutant gene; When the CT value is 22 (±1), it shows the CYP2D6 heterozygous mutant gene.

In multiple PCR of human DNA single nucleotide polymorphism target, LNA primer has higher specificity. The specific primers studied in this paper were modified with LNA in the multiple detection system. LNA is a nucleic acid derivative with 2 '- O, 4' - C-methylene [8]. The resulting bicyclic structure locks the ribose into the C3 '- endo conformation, which can enhance the hybrid Tm [9]and hybridization specificity [10]. The improvement of specificity may be due to the formation of fewer template connections by specific primers with 3 'LNA terminal, which affects the utilization of DNA polymerase substrate [11]. At present, the clinical method for genotyping of antihypertensive drugs is based on PCR technology. A pair of primers, a wild-type

probe and a mutant probe are designed to genotype a target through the probe. The method studied in this paper improves the deficiency that each detection system can only carry out single PCR reaction, and each reaction system can only detect polymorphism of one gene site. This method designs a probe, a wild (mutant) type forward primer and a reverse primer according to each gene, which reduces the use of the probe and reduces the cost of the experiment. In addition, the 3 'terminal base of the specific forward primer was modified with locked-nucleotide to enable it to bind specifically with the template DNA efficiently, and the Taqman-BHQ fluorescent probe labeled with different fluorescent dye groups was used for real-time detection and analysis of the two genes at the same time. This shows that the method of this study - multiple fluorescence quantitative PCR method can overcome the shortcomings of poor specificity, improve specificity and reduce costs; It can also reduce experimental operation and experimental pollution. It is a good method for genotyping.

Because the target reaction may be stronger than the internal control reaction when amplifying in real-time fluorescent PCR, the internal reference substance is omitted in this study. The repeatability of the constructed multiplex PCR system was verified, and the coefficient of variation was less than 5%. The results were stable. Therefore, the method studied in this paper can genotyping ADRB1 and CYP2D6 at the same time, so as to determine whether the target gene is mutated, which can provide guidance for precise drug use in patients with hypertension. In this paper, the detection model of multiple qPCR system was initially constructed by plasmid, and the feasibility of the system was further tested by using clinical samples in the next step.

6. Conclusion

In this study, a multiple qPCR system was constructed with Taqman probe and locked-nucleic acid primer to genotype the hypertension drug-related genes ADRB1 and CYP2D6. It can accurately and clearly distinguish the wild type and mutant type of ADRB1 and CYP2D6. The detection method is simple and fast, improves the flux, reduces the cost, has strong specificity and good repeatability, can reduce the experimental operation, reduce the experimental pollution and error. Therefore, the multiplex qPCR system model for ADRB1 and CYP2D6 gene detection was successfully constructed.

References

[1] Jun YANG, Jing WANG, Shuang WANG,et al.Research Progress of Drug-related Gene Polymorphisms in Hypertension [J]. Studies of Trace Flements and Health, 2019, 36 (5):63-65.

[2] YANG Mei, XIAO Zhilin,CHEN Xiaobin,et al.Distribution of polymorphism of antihypertensive drug related genes in Changsha[J].Journal of Clinial Cardiology (China), 2019, 35 (6):559-563.

[3] WANG Xiao. Simultaneous determination of 4 kinds of foodborne pathogens in frozen vegetables by multiplex quantitative real-time PCR[J].Journal of Food Safety and Quality ,2018, 9(22):5917-5921.

[4] Seheu PM, Berghof K, Stahl U, et al. Detection of pathogenic and spoilage miero-organisms in food with the polymerase chain reaction [J]. Food Microb, 1998, 15(l): 13-31.

[5] Rodriguez-LaZaro D, D'Agostino M, Pla M, et al. Construction strategy for an internal amplification control for real-time diagnostic assays using nucleic Acid sequence-based amplification: development and clinical application [J]. J Clin Mierobiol, 2004, 42(12): 5832-5836.

[6] Hoorfar J, Cook N, Malorny B, et al. Making internal amplification control mandatory for diagnostic PCR [J]. J Clin Microbiol, 2003, 41(12):5835-5841.

[7] JI Dong,XIN Shao−jie. Development and Data Analysis of Real−Time Fluorescent Quantitative PCR[J]. LETTERS IN BIOTECHNOLOGY,2009,20(4): 598-600.

[8] KOSHKIN A A,SINGH S K,NIELSEN P,et al.LNA (Locked Nucleic Acids) : Synthesis of the adenine, cytosine , guanine ,5-methyleytosine, thymine and uracil bicyclonucleoside monomers , oligomerisation, and unprecedented nucleic acid recognition [J].Tetrahedron, 1998 , 54 (14) :3607-3630.

[9] NIELSEN C B,SINGH S K, WENGEL J,et al.The solution structure of a locked nucleic acid (LNA) hybridized to DNA[J]. Journal of Biomolecular Structure and Dynamics, 1999, 17(2):175-191.

[10] BRAASCH D A, JENSEN S, LIU Y H, et al. RNA interference in mammalian cells by chemically. -modified RNA [J]. Biochemistry , 2003, 42 (26) :7967-7975.

[11] LATORRA D,HOPKINS D ,CAMPBELL K , et al.Multiplex allele -specific PCR with optimized locked nucleic acid primers [J]. BioTechniques ,2003 ,34(6) :1150-1152.