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A primer design strategy for PCR amplification of GC-rich DNA sequences

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ABSTRACT

Objectives: To establish a primer design method for amplification of GC-rich DNA sequences.

Design and methods: A group of 15 pairs of primers with higher T_m (>79.7 °C) and lower level ΔT_m (<1 °C) were designed to amplify GC-rich sequences (66.0%–84.0%). The statistical analysis of primer parameters and GC content of PCR products was performed and compared with literatures. Other control experiments were conducted using shortened primers for GC-rich PCR amplifications in this study, and the statistical analysis of shortened primer parameters and GC content of PCR products was performed compared with primers not shortened. A group of 26 pairs of primers were designed to test the applicability of this primer designing strategy in amplifications of non-GC-rich sequences (35.2%–53.5%).

Results: All the DNA sequences in this study were successfully amplified. Statistical analyses show that the T_m and ΔT_m were the main factors influencing amplifications.

Conclusions: This primer designing strategy offered a perfect tool for amplification of GC-rich sequences. It proves that the secondary structures cannot be formed at higher annealing temperature conditions (>65 °C), and we can overcome this difficulty easily by designing primers and using higher annealing temperature.

Introduction

Polymerase chain reaction (PCR), widely applied in many research fields, is one of the routine molecular biology techniques. However, PCR amplifications of GC-rich sequences involve much greater difficulties than those of non-GC-rich ones [1,2]. Although only about 3% of the human DNA sequences are GC-rich, the majority of the important regulatory domains including promoters, enhancers, and control elements consist of GC-rich sequences [3], and most housekeeping genes, tumor suppressor genes, and approximately 40% of tissue-specific genes contain GC-rich sequences in their promoter region [4]. Obviously, ineffective PCR amplifications of these GC-rich DAN sequences hamper the progress of the study into these gene sequences.

The conventional practice for cracking this hard nut of ineffective PCR amplification is adding certain organic additives such as betaine, dimethylsulfoxide, formamide, polyethylene glycerol, non-ionic detergents, 7-deaza-dGTP, dUTP and their combinations [5–16] to the reaction mixture, or jointly using such highly effective DNA polymerase as AmpliTaq™, Taq Gold™, and KOD Hot-Start polymerase [4], Optimase DNA polymerase, Platinum® Taq DNA Polymerase High-Fidelity, etc. [17], during the course of PCR amplification. In addition, techniques such as template denaturation with NaOH, hot start PCR, stepdown PCR, and slowdown PCR can also improve the PCR amplification of GC-rich sequences [18–21]; other factors including adjusting magnesium concentration, buffer pH, denaturing and annealing time and/or temperature, and PCR cycling numbers are sometimes also to be taken into account.

It is widely believed that the major cause of ineffective amplifications of GC-rich templates is the formation of secondary structures such as loops or hairpins brought about by GC-rich DNA templates and/or primers' self-complementary or other conformational features [1].

Primer is a crucial factor for successful PCR amplifications. Precise primer designing and analyzing, especially that of their secondary structures such as self-dimers, hairpins, and cross-dimers [22], before PCR amplifications, are necessary. In this study, an in-depth study was made into fifteen pairs of primers recorded in the related literature for amplifications of GC-rich templates. It was found that

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all the primers studied shared some defects such as over-low melting temperature (T_m), over-high level of T_m difference of primer sets (ΔT_m), or mismatching between the primer and multiple loci of the templates. All these defects with the primer will lead to either annealing failure or annealing of multiple loci of the DNA templates, which would further give rise to the ineffective amplifications or non-specific ones. Given that these primer defects are common reasons for invalid PCR amplifications of GC-rich sequences, it just shows that the solution to this problem lies in nothing but the effective primer designing, especially for amplifications of GC-rich DNA templates. In this study, a specific primer designing strategy featuring high T_m and low ΔT_m was established. According to this strategy, fifteen pairs of primers with specific parameters for amplifications of GC-rich DNA sequences and twenty-six more pairs were designed for testing the applicability of this strategy in amplifications of non-GC-rich sequences.

Materials and methods

DNA templates

A total of fifteen GC-rich and twenty-six non-GC-rich DNA target sequences were employed in this study. The fifteen GC-rich DNA sequences are from eight genes: HBA2 (NC_000016.9), FMR1 (NC_000023.10), APOE (NC_000019.9), HRES1 (NC_000001.9), CSTB (NC_000021.8), INSR (NC_000019.9), AR (NC_000023.10), and GATA4 (NC_000008.10), with the GC content of these primers ranging from 66.0% to 84.0%; the twenty-six non-GC-rich DNA target sequences are from twenty-six exons of F8 gene located in Xq28, whose GC content range from 35.2% to 53.5%. (All sequences of these genes were obtained from http://www.ncbi.nlm.nih.gov/nucleotide/).

Genomic DNA is isolated from human white blood cells using the conventional phenol–chloroform method.

Primer designing and optimizing

The fifteen pairs of primers for amplifications of GC-rich sequences were designed manually. In order for the primers to have the highest possible T_m (>79.7 °C) and the smallest possible ΔT_m (1.0 °C), several nucleotides were added to or removed from the 5′ and/or 3′ ends of each primer based on DNA targets, under the precondition that the primers were complementary to the DNA sequence templates. The GC contents of PCR products and the primer parameters including T_m, ΔT_m, the maximum ΔG free energy of self-dimer, cross-dimer, and hairpin were worked out by means of the software Oligo 6.64 (Molecular Biology Insights, Inc., CO).

The fifteen pairs of primers in this study were shortened by reducing two bases from primer 5′ ends as control experiments.

In addition, twenty-six other pairs of primers with higher T_m and lower ΔT_m of primer sets were designed according to the strategy mentioned above, for testing the applicability of the strategy in the amplifications of non-GC-rich sequences. All the primers in this study were synthesized by Invitrogen (Guangzhou, PR China). The parameters of the two groups of primers designed for this study and the fifteen pairs recorded in the related reference literature were detailed in Tables 1-3, respectively.

PCR system and amplification conditions

All PCRs were carried out under the following conditions: 50–200 ng of human genomic DNA, 0.2 μM of each primer, 50 μM of each dNTPs, 2.0 U of Taq DNA polymerase enzyme, and 5 μL of 10× buffer supplemented with 100 mM Tris–HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$ (Takara, Dalian, PR China). The final volume was adjusted to 50 μL with water.

The reactions were performed in a Gene Amp PCR system 9700 (PE Applied Biosystems). The amplifications of the GC-rich DNA

Table 1
Parameters of fifteen pairs of primers for GC-rich DNA amplifications in this study.

Gene	Primer				Max ΔG free energy (kcal/mol) of primer			PCR products	
	Sequence (5′–3′)	Length (mer)	T_m	ΔT_m	Self-dimer	Cross-dimer	Hairpin	GC %	Size (bp)
INSR	F: CGCGGCCCCCAGCGCCTCTT	20	86.5	0.1	10.3	13.4	2.6	82.8	180
	R: GCCCCGCGGCGCCCAGTAGCA	20	86.6		16.5		1.0		
HRES1 (a)	F: GGCCCGGGCTGGCGGGGCG	19	91.3	1.0	22.2	22.7	5.5	84.0	206
	R: CCCCCGCGCCCCGCGCAC	18	90.3		10.3		5.1		
HRES1 (b)	F: GGAAACACATCCCCAGCTGAGGGC	24	81.0	0.5	10.1	6.2	2.1	68.9	1226
	R: GCAAGAGGAAACTTGAAAAGGCGGATCAC	29	80.5		5.4		1.7		
HRES1 (c)	F: GGAAACACATCCCCAGCTGAGGGCCGGGAG	30	90.1	0.1	10.1	6.7	2.9	68.9	1226
	R: GCAAGAGGAAACTTGAAAAGGCGGATCACGCCAATGC	37	90.0		9.8		5.4		
FMR1	F: GCCCCGCACTTCCACCACCAGCTC	24	85.4	0.4	6.3	6.7	0	80.9	283
	R: GGCGCTCAGCTCCGTTTCGGTTTCACTTC	29	85.8		9.8		1.5		
HBA2 (a)	F: CCCCACAGACTCAGAGAGAACCCACCA	27	81.4	0.6	3.2	5.0	2.0	66.3	885
	R: CAGGTAAACACCTCCATTGTTGGCACATTCC	31	82.0		6.0		1.5		
HBA2 (b)	F: CCCCACAGACTCAGAGAGAACCCACCA	27	81.4	0.0	3.2	5.0	2.0	68.0	764
	R: GGGGAGGCCCAAGGGGCAAG	20	81.4		9.3		4.1		
APOE (a)	F: CCCGGTGGCGGAGGAGACG	19	81.2	0.5	9.8	9.4	2.2	75.5	322
	R: GTCGCGGCCCTGTTCCACCAG	21	81.7		10.3		0.8		
APOE (b)	F: GCCTGGGGCAGGGGGAGAACAGC	23	84.6	0.2	6.6	9.3	2.1	67.4	282
	R: GCCCGACCCCGAGTAGCTCTCCTGAGAC	28	84.4		6.3		2.0		
HBA2 (c)	F: CGCGGGTTGCGGGAGGTGTAG	21	81.2	0.7	10.3	9.4	0	66.2	343
	R: CCGGGATAGAGAGAACCCAGGCACAC	26	80.5		9.8		0		
CSTB (a)	F: CGGCGCCCGGAAAGACGATAC	21	80.0	0.1	16.0	9.8	1.5	76.7	473
	R: CGGGGCCAAAGCGGCTTCTT	20	79.9		9.3		1.8		
CSTB (b)	F: GCCCCCACCCAGCCTGGAG	19	80.2	0.3	6.6	12.4	1.8	76.0	786
	R: CGGGGCCAAAGCGGCTTCTT	20	79.9		9.3		0.9		
GATA4 (a)	F: GCGGGTGCCCTCCTCCGTG	19	81.3	0.5	6.7	6.7	2.4	78.8	510
	R: CCCTCGCGCTCCTACTCACCGAGA	24	81.8		10.3		0		
GATA4 (b)	F: GAGCCTAGAGCCCTTTGCTCAATGCTG	27	79.7	0.7	6.3	9.4	1.9	66.5	500
	R: GGGGTGTAAGCGGCTCCGTCG	21	80.4		6.7		0		
AR	F: TCGGCCGCCGTCCAAGACCTAC	22	81.6	0.2	16.5	19.6	2.3	66.0	380
	R: CGGCGGCTCCAGGCTCTGG	19	81.4		6.6		1.7		

Table 2
Parameters of twenty-six pairs of primers for non-GC-rich DNA amplifications in this study.

Exons of F8 gene	ID no.	Primer			PCR products	
		Sequence (5′–3′)	T_m	ΔT_m	GC %	Size (bp)
Exon 1	1	F: CCAGTGGGTAAAGTTCCTTAAAATGCTC	74.0	0.3	44.4	365
		R: CCTTCCTCCAAGCAGACTTACATCC	73.7			
Exon 2	2	F: CCAGCTGCTTTTTGAAGTGTCCAC	74.7	0.1	42.0	381
		R: GCAGTTGGCATAGACCAAGCAGAG	74.8			
Exon 3	3	F: GGGCATGCTTCTCCACTGTGAC	74.9	0.3	45.0	302
		R: CGCCACCATTACAAAGCACACA	74.6			
Exon 4	4	F: GAGGCGGACGTTGCTGTGA	74.5	0.2	41.6	721
		R: GGCAGAGGACAGTAGAAATCACTACTGATAAAG	74.3			
Exon 5	5	F: AGCCAAGTTATCAAGAGACAGAAGACTACATT	73.0	1.1	35.2	324
		R: CCCATCTCCTTCATTCCTGAACAGT	74.1			
Exon 6	6	F: CGGTCATTCATGAGACACATGCTTAG	74.3	0.5	47.4	304
		R: GAGGCCAGAGATCTTCAAGTATTCAGTG	73.8			
Exon 7	7	F: GGTGATTGGTGACCTTGGCAAG	74.4	0.9	39.9	549
		R: CGGGATCTTGGCTGAGGTCTAATAC	73.5			
Exon 8 plus 9	8	F: CAGCCTAATATAGCAAGACACTCTGACATTGT	74.5	1.2	40.1	805
		R: CTTCTTACCTGACCTTAAATCTTTTCTTCAACTTAC	73.3			
Exon 10	9	F: GTCCTGGAACCAATCCTCCATG	73.4	1.0	35.6	393
		R: GGCCAACAGCTGGAGAAAGGA	74.4			
Exon 11	10	F: GTGCGACTTTAGCTTCCACTTGCT	73.5	0.9	40.8	380
		R: CCAGCACTTGGAAAGGCAAGA	73.2			
Exon 12	11	F: GCCATCGCTTTCATCATAGACTGC	74.5	0.4	44.5	272
		R: CACCACCCACTGGACTTAAGTGCT	74.1			
Exon 13	12	F: CCTAATTGTATCATGACAATCACAATCCAA	73.8	0.6	39.2	362
		R: GGGCAAAAGAGCATACGAATGG	73.2			
Exon 14	13	1F: GGGAATGGGAGAGAACCTCTAACAGA	74.2	0.3	41.9	810
		1R: GGGGAGATGACTTTTTGCCAAATAGA	74.5			
	14	2F: GGACCCCCAAGTATGCCAGTTC	74.3	0.1	36.6	925
		2R: GAGGCAAAACTACATTCTCTTGGATTAATGTT	74.2			
	15	3F: GGGTGAATTTACAAAGGACGTAGGACTC	74.1	0.2	38.9	923
		3R: GGGAGCCAACCTCTCTTTGATCA	74.3			
	16	4F: GGAGATGACTGGTGATCAAAGAGAGGT	74.4	0.4	43.8	880
		4R: GGTGTCATCATCTGGTAAAGTCAAATGTC	74.0			
Exon 15	17	F: GGCAAGAGTATTTCAAGGAAGATGAAGTG	74.3	0.1	38.5	374
		R: CCAAAAGTGGGAATACATTATAGTCAGCAAG	74.4			
Exon 16 plus 17	18	F: GGGATGTAAACCCTAAGGACCTTAAGATC	73.2	0.7	39.7	880
		R: CCCTGGATCAAGTCTCATTTGTCAA	73.9			
Exon 18	19	F: GGGCTCCCTGCAATATCCAGAT	74.0	0.1	36.7	754
		R: GGTGCCCTATGGGATTTGAGATG	74.1			
Exon 19 plus 20	20	F: GGGGTCCAAAAGTAGTGGAACTACCA	74.5	0.1	37.4	960
		R: CAGCCCAGGTTCTTGGAGCA	74.4			
Exon 21	21	F: GGACAACAAGGATAAGCAATATCCTTAGATAGTAATT	73.8	0.0	36.5	594
		R: GGGGATTGGAGTACTTAAGGACACGT	73.8			
Exon 22	22	F: GGCTGGGAACTCAATAAGCATCTTTC	74.4	0.6	39.3	468
		R: GGAAGCTAAGAGTGTTTGTCCAATATCTGA	73.8			
Exon 23	23	F: TTGGATGCTGTTGAGAAACGCA	74.5	0.4	40.8	341
		R: CCCCCAGTCTCAGGATAACTAGAACAGT	74.1			
Exon 24	24	F: CCCTAGAATATCAGTGGAAGCTGCTCA	74.9	0.8	44.1	426
		R: CCAGGAGGTTCAATTAGAGATCATTTCC	74.1			
Exon 25	25	F: TTCTGGGAGTAAATGGTGACCAAGAG	73.9	0.3	39.9	479
		R: GGGAATCAAGGTGCTGATGAGG	73.6			
Exon 26	26	F: GTCCTGTCAGACAACCAATAAATGCTATC	73.2	1.5	53.5	226
		R: GCAGTGGCAGGTGCTGCA	74.7			

templates were under the following conditions: an initial denaturation step at 96 °C for 5 min; denaturation at 96 °C for 45 s in all 35 PCR cycles; the other conditions including annealing temperature (T_a) and extension time at 72 °C as listed in Table 4; a final extension at 72 °C for 10 min. At the same time, the amplifications using fifteen pairs from literatures were performed under the same PCR conditions.

The control experiments' PCRs were under the following conditions: an initial denaturation at 95 °C for 5 min followed by 35 PCR cycles of denaturating at 96 °C for 45 s in all; annealing at 56, 58, 60, 63, and 65 °C for 30 s; extension time at 72 °C as listed in Table 4; a final extension at 72 °C for 10 min.

The conditions for amplifications of the non-GC-rich DNA templates were as follows: an initial denaturation at 95 °C for 5 min followed by 30 PCR cycles of denaturating at 94 °C for 30 s in all; annealing at 59 °C for 30 s; extension at 72 °C for 1 min; a final extension at 72 °C for 10 min.

An electrophoresis of the amplification products was conducted on 1.5% agarose gels, in which 5 μL reaction products were loaded with 1 μL loading buffer stained with ethidium bromide, with the gels run at 80 V for 50 min.

Statistical analysis

An independent-sample *t*-test, by means of SPSS 13.0 software program (SPSS Inc., Chicago, USA), was conducted of the parameters of the 15 pairs of primers for amplification of GC-rich templates in this study, the 15 pairs obtained from the literature, and the GC content of the PCR amplification products.

Table 3
Parameters of primers for GC-rich DNA amplifications in references.

References	Primer			Max ΔG free energy (kcal/mol) of primer			PCR products
	Sequence (5′–3′)	T_m	ΔT_m	Self-dimer	Cross-dimer	Hairpin	GC %
[9]	FP: CCCGCACTGAGCTCCTACAC	71.8	9.1	9.5	4.7	0	79.1
	RP: GGACGTCGCCTTCGCCATCG	80.9		9.4		0	
[9]	FP: TGCCTGGGGAGTCCCAAGG	76.4	5.6	8.1	6.2	3.6	66.8
	RP: AAGCGTCCGTGCGCCTGGCT	82.0		9.8		2.4	
[9]	FP: TGCTTCACCGTCTCTCCTTCCGT	77.0	19.1	3.6	3.6	0	72.9
	RP: TACCCGCTCGCCCACTCGCCCGCC	91.1		3.6		0	
[4]	FP: CCAAGGCGTCGAAGTCTG	69.1	2.6	6.8	8.7	0	78.9
	RP: TCATCTTCTTCGTCCTCCAG	66.4		3.6		0	
[5]	FP: GCTCAGCTCCGTTTCGGTTTCACTTCCGGT	85.7	5.6	9.8	6.7	2.6	80.4
	RP: AGCCCCGCACTTCCACCACCAGCTCCTCCA	91.3		6.3		2.0	
[7]	FP: AAACACTGCTGTGGTGGA	64.5	2.0	3.2	6.6	1.3	52.4
	RP: TAGCTCAACAGAATCCAGGC	66.5		6.3		0	
[19]	FP: ATGGGTAGCAACAAGAGCAAGCCCAA	79.3	8.0	8.1	10.9	3.4	62.8
	RP: CTAGAGGTTCTCCCCGGGCTGGTACTGGGGCT	87.3		16.0		4.8	
[19]	FP: ATGGGCACCGGGGGCCGGCGGGGGGCGGCG	103.8	21.0	16.0	6.7	5.7	55.6
	RP: TTAGGAAGGATTGGACCGAGGCAAGGTCAG	82.8		6.0		1.9	
[20]	FP: GAGCGTTGGCGTCGTGC	73.9	0.2	3.6	6.2	0	83.8
	RP: GAGGAGGAGGGCCGAGGA	73.7		9.6		0	
[20]	FP: TCCGGGACGAGCTCCGGGAA[a]	81.9	0.7	9.8	12.9	4.1	80.9
	RP: GGGGACACCCCGGTGAATGAGC[a]	81.2		9.8		5.7	
[20]	FP: CCTCCTTCCCCGGGAACAGGC[a]	80.4	1.6	16.0	9.8	1.4	78.7
	RP: CCGAGGGTGCGGGAGCAGTAG[a]	78.8		6.7		2.4	
[20]	FP: CTCGGTTATAAGATGGCGGCGCTGA[a]	80.5	1.4	9.8	6.7	0	74.6
	RP: AGTCGGGAGGGCGGCAGGGT[a]	81.9		3.6		0	
[20]	FP: CCTGGAAGAGGTCCCAGCGTGAGT[a]	79.2	4.0	6.6	6.6	2.5	61.1
	RP: GACAAGACCAGAAGGAGCTGAGGG[a]	75.2		6.3		0.8	
[20]	FP: GGGATGGTTGCCCAGGCTGGA[a]	81.1	3.1	6.6	8.1	2.1	59.6
	RP: TGATGGGTGCCGTGGTCTCCT[a]	77.9		3.6		0	
[20]	FP: CTCCCACGAGAGCATCATCTG	71.7	1.0	3.6	6.5	0	60.4
	RP: GCAGTTGAAGTCGTCGTAGCC	70.7		3.6		1.3	

[a] Primer has too many mismatch loci.

Results

PCR amplifications of fifteen GC-rich sequences

With no enhancers or techniques such as touch-down PCR employed, all the fifteen GC-rich DNA sequences in this study were successfully amplified by just using the common Taq polymerase enzyme (Fig. 1). None of the amplifications with fifteen pairs from literatures was successfully amplified (Fig. 2), and almost all of the amplifications with shortened primers have failed (Fig. 3).

Statistical analysis of primers' parameters and GC content of amplification products

It was displayed by the independent-sample *t*-test that significant differences existed in the T_m and ΔT_m between the two groups of primers ($P=0.012<0.05$, $P=0.007<0.05$, respectively), and the differences in the ΔG free energy of self-dimers, cross-dimers, hairpins, and in the GC contents of PCR products between the two groups were not statistically significant ($P=0.770>0.05$, $P=0.073>0.05$, $P=0.403>0.05$, and $P=0.360>0.05$, respectively) (Table 5). Significant differences existed in the T_m and ΔT_m between the shortened (T_m: 78.2800 ± 4.3743, ΔT_m: 1.5867 ± 1.5222) and not shortened primers ($P=0.000<0.05$, $P=0.006<0.05$, respectively), and the differences in the ΔG free energy of self-dimers, cross-dimers, hairpins, and in the GC contents of PCR products between the two groups were not statistically significant ($P=0.175>0.05$, $P=0.070>0.05$, $P=0.213>0.05$, and $P=0.624>0.05$, respectively; Table 5).

Table 4
Annealing and extension conditions of PCR amplifications of GC-rich sequences in this study.

Gene	Annealing conditions	Extension time at 72 °C
INSR	68 °C, 45 s	30 s
HRES1 (a)	65 °C, 45 s	30 s
HRES1 (b)	65 °C, 45 s	1 min 10 s
HRES1 (c)	65 °C, 45 s	1 min 10 s
FMR1	65 °C, 45 s	30 s
HBA2 (a)	65 °C, 45 s	50 s
HBA2 (b)	65 °C, 45 s	50 s
Apo E (a)	68 °C, 45 s	30 s
Apo E (b)	65 °C, 45 s	30 s
HBA2 (c)	65 °C, 45 s	30 s
CSTB (a)	66 °C, 45 s	40 s
CSTB (b)	68 °C, 45 s	50 s
GATA4 (a)	68 °C, 45 s	40 s
GATA4 (b)	67 °C, 45 s	40 s
AR	67 °C, 45 s	40 s

Testing of this primer design strategy applied in amplifications of non-GC-rich sequences

It was demonstrated by the result of the electrophoresis that all of the twenty-six target sequences derived from exons of F8 gene were successfully amplified, as was witnessed by the specificity of the bands of the PCR products (Fig. 4).

Discussion

It is generally held that GC-rich primers and DNA templates can hamper the denaturation, annealing, and extension in the course of PCR amplifications, which constitutes the major reason for ineffective amplifications of GC-rich templates. Based on this point of view, many approaches aimed at reducing the stability of the double-helix of DNA template have been devised to crack this hard technical nut, such as adding certain organic additives and jointly using highly effective DNA polymerase to reaction mixture during PCR amplifications. However, the performance of these organic chemical compounds is quite unpredictable [19] and, moreover, even in the presence of such additives, the amplifications of GC-rich templates are often ineffective.

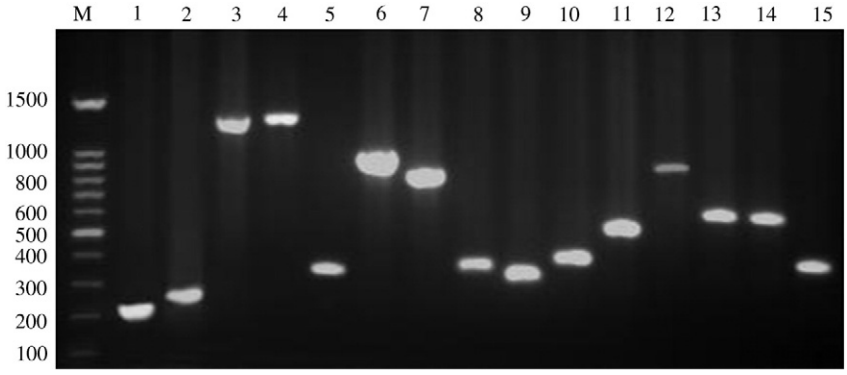

Fig. 1. Electrophoresis analysis of PCR products amplified with primers for GC-rich DNA sequences in this study with 2% agarose gel. Lane M: 100 bp DNA ladder marker (D015-2; Dingguo, Beijing, PR China); lanes 1–15: INSR, HRES1 (a), HRES1 (b), HRES1 (c), FMR1, HBA2 (a), HBA2 (b), APOE (a), APOE (b), HBA2 (c), CSTB (a), CSTB (b), GATA4 (a), GATA4 (b), and AR, respectively.

Other techniques employed for reducing the stability of the complex secondary structures include hot start PCR, stepdown PCR, slowdown PCR, and methods such as template pre-denaturating with NaOH, adjusting magnesium concentration, buffer pH, denaturing and annealing time and/or temperature, and cycle numbers, all of which are used more or less. However, all the optimizing methods mentioned above are, besides being time and labor consuming and involving high costs, only effective for PCR amplifications of some, but not all, GC-rich DNA sequences [20,23].

Typically, a PCR amplification cycle comprises three steps: DNA templates melting, primer annealing, and extension. With the primary cause leading to ineffective PCR amplifications being usually the problem of primer annealing, primer properties are of vital importance for PCR amplifications, especially for those of GC-rich DNA templates. The predominant part of the related literature holds that formation of secondary structures of primers should be avoided in primer designing but pays scarce attention on how to adjust the other parameters of the primers.

Theoretically, the primers for amplifications of GC-rich sequences should have a high T_m and a high T_a, and it is impossible for primers with a low T_m to anneal under high T_a conditions. In this study, we made attempts at making PCR amplifications at a low T_a with some primers which, according to the literature, have a high T_m and a high ΔT_m used, but all failed. The failure may be attributed to either that the two denatured GC-rich DNA strands with the high T_m has renaturated before primer annealing, or that, even if the primers has annealed successfully, the extension is cramped due to the possible renaturation of the two denatured GC-rich DNA strands with the high T_m, either of which can result in annealing/extension failure and thus ineffective amplifications. Moreover, the amplification attempts made under the high T_a condition also ended in failure. What underlies this might be that 1) the primers with the lower T_m could successfully anneal, but those with the higher T_m would anneal with multiple loci in the GC-rich DNA templates, which consumes the primers with higher T_m in large amounts; or 2) the primer with the lower T_m could not anneal under the conditions where the primer with the higher T_m could. Either of these circumstances will lead to asynchronous primer annealing and thus non-specific amplifications and/or low yield products. We have tested the literatured primers' PCR amplifications under the experiment conditions of this study, but all of them failed (Fig. 2). Otherwise, all aforementioned has been proven by our control experiments by using shortened primers with lower T_m and higher ΔT_m (Fig. 3). Based on the above analysis, we try to find the rules to regular T_m and ΔT_m of primers for amplifications of GC-rich DNA templates.

Proceeding from the above considerations, we designed fifteen primer sets and successfully amplified fifteen GC-rich templates (66.0%–84.0%) without using any enhancers or highly effective DNA polymerase. All of the 15 pairs of primers had a higher T_m (83.1167 ± 3.5254 °C) and lower level ΔT_m (0.3933 ± 0.2840 °C). It was demonstrated by the statistical analysis that no significant difference in the GC content existed between the 15 DNA templates employed in this study and the 15 ones recorded in the literature ($P = 0.360 > 0.05$), a result giving testimony to the fact that, at a higher T_a or under the 72 °C extension condition, the secondary structure of GC-rich sequences could not be formed. Therefore, it could be claimed that the primers are the major factors leading to invalid amplifications compared with the secondary structures of GC-rich templates. Moreover, the statistical analysis of the primer parameters showed that the predominant factors influencing amplifications of GC-rich sequences should be the T_m and ΔT_m of primers.

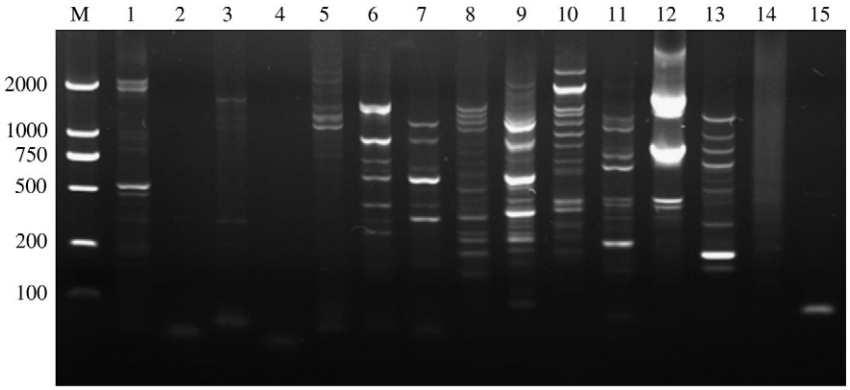

Fig. 2. Electrophoresis analysis of PCR products amplified with primers from literatures with 2% agarose gel. Lane M: 100 bp DNA ladder marker (D015-2; Dingguo, Beijing, PR China); lanes 1–15: orders as same as the primer pairs listed in Table 3.

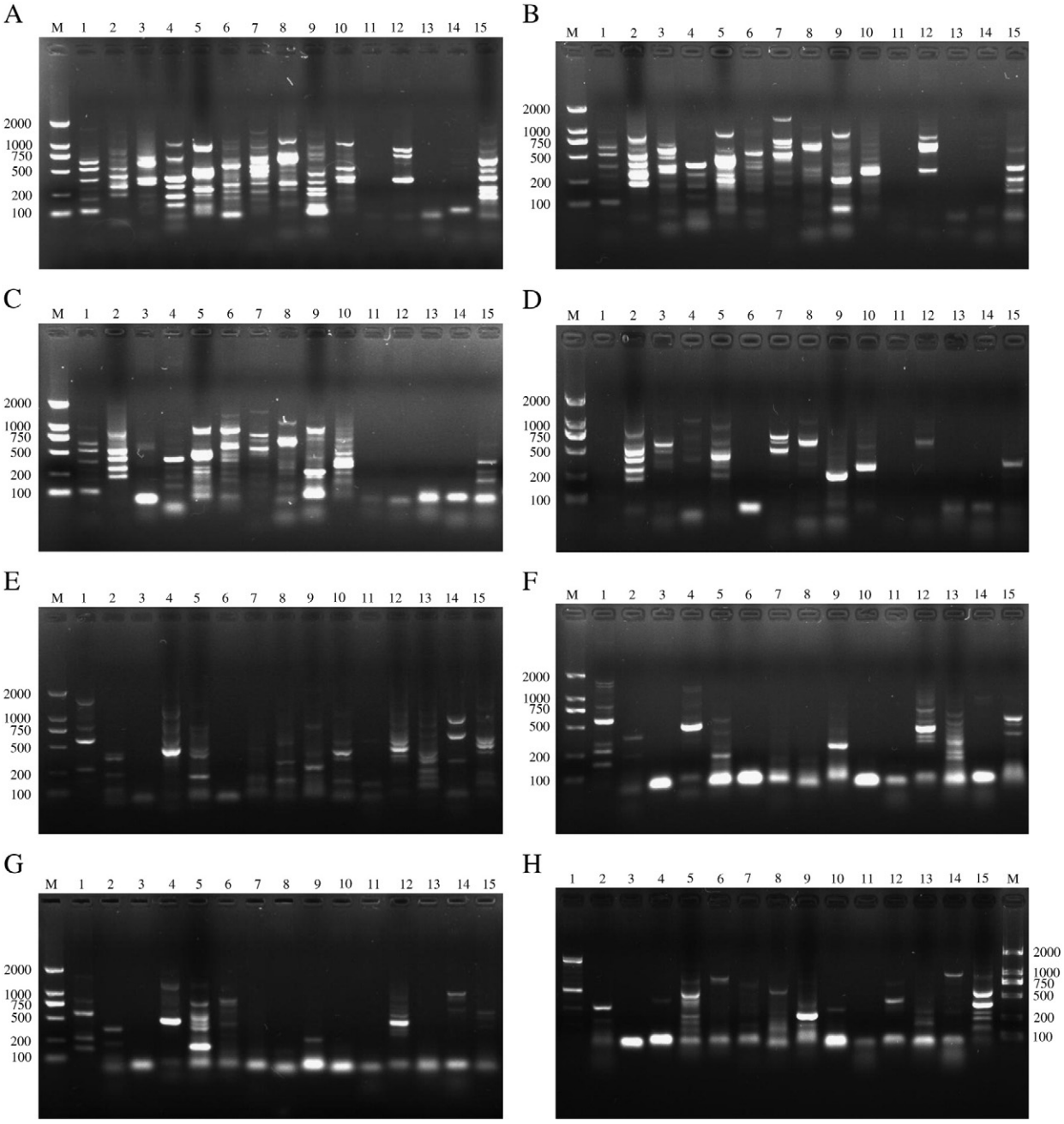

Fig. 3. Electrophoresis analysis of PCR products amplified with shortened primers with 2% agarose gel. Lane M: 100 bp DNA ladder marker (D015-2; Dingguo, Beijing, PR China); lanes 1–15: orders as same as the primer pairs listed in Table 1. The annealing temperatures of A–G was 58, 59, 60, 61, 62, 63, 64, and 65 °C, respectively.

In order to ascertain the applicability of this primer designing strategy in the amplifications of non-GC-rich DNA templates, we, according to the exons of F8 gene, designed twenty-six pairs of primers, the GC contents of whose PCR products ranged from 35.2% to 53.5%. To ensure that the primers achieve the amplification under the same conditions, we designed the primers in such a way as to enable them to be with a

Table 5
Independent-samples *t* test analysis of primer parameters and GC content of PCR products.

Parameters	Primers					PCR products
	T_m (n = 30)	ΔT_m ($n = 15$)	Self-dimer ($n = 30$)	Cross-dimer ($n = 15$)	Hairpin ($n = 30$)	GC % ($n = 15$)
This study	83.1167 ± 3.5254	0.3933 ± 0.2840	9.3300 ± 4.0901	10.1133 ± 5.1360	1.9600 ± 1.4848	72.8600 ± 6.6230
References	78.8000 ± 8.2224	5.6667 ± 6.4148	7.5100 ± 3.7390	7.3933 ± 2.3696	1.6000 ± 1.8107	69.8667 ± 10.4988
Control experiments	78.2800 ± 4.3743	1.5867 ± 1.5222	8.7695 ± 5.0124	9.5462 ± 5.0894	1.8769 ± 1.4237	72.8501 ± 6.2011
P-value	0.012[a]/0.000[b]	0.007[a]/0.006[b]	0.770[a]/0.175[b]	0.073[a]/0.070[b]	0.403[a]/0.213[b]	0.360[a]/0.624[b]

All primer parameters data expressed as mean ± SD.
[a] Independent-samples *t*-test between this study and references.
[b] Independent-samples *t*-test between this study and control experiments.

Fig. 4. Electrophoresis analysis of PCR products of twenty-six exons of F8 gene with 1.5% agarose gel. Lane M: 100 bp DNA ladder marker (D015-2; Dingguo, Beijing, PR China); lanes 1–7: exons 1–7; lane 8: exon 8 plus 9; lanes 9–12: exon 10–13; lanes 13–16: four fragments of exon 14; lane 17: exon 15; lane 18: exon 16 plus 17; lane 19: exon 18; lane 20: exon 19 plus 20; lane 21–26: exon 21–26, respectively.

close T_m (74.0788 ± 0.4791 °C) and a low ΔT_m (0.5038 ± 0.3995 °C). Against the GC content of their PCR products, the T_a of the 26 pairs of primers was on the high end. As was demonstrated by the electrophoresis result, this primer designing strategy was also competent for non-GC-rich sequence PCR amplification, a result bearing testimony to the importance of the primer for PCR amplifications.

Usually, the requirements for primer design include the primer length should be between 18–30 bases; the ΔT_m value of primer sets should be lower than 5 °C; the mismatched bases between the primer 3′ end and template should not be more than 3; the ΔG of the secondary structures of the primer should be less than 5 kcal/mol. The originality of the primer design strategy formulated in this study is that it is revealed that the major factors influencing amplifications of GC-rich DNA sequences are the T_m and ΔT_m of primer sets while other factors do not exert significant influence. Furthermore, we have proposed that, in amplifying GC-rich DNA sequences, the T_m value should be greater than 79.7 °C and, the ΔT_m, less than 1 °C.

To put it in a nutshell, primer parameters of T_m and ΔT_m, besides the factor of specificity, are the most important factors having a significant bearing on amplifications of GC-rich sequences. Primers with a higher T_m and lower ΔT_m can easily overcome the technical difficulties in the amplification of GC-rich DNA templates under routine PCR conditions and can thus make success of the amplifications.

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