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PCR reagents

Successful amplification of AT-rich DNA sequences with Phusion Plus DNA Polymerase

Introduction

PCR is one of the most widely used techniques in molecular biology for *in vitro* amplification of DNA fragments, and its accuracy and sensitivity make it highly reliable. Although many DNA sequences can be easily analyzed using PCR-based methods, successful amplification of problematic sequences like AT-rich regulatory elements and genes in AT-rich genomes often requires time-consuming PCR optimization. Here we describe PCR conditions that allow amplification of extremely AT-rich (up to 90%) DNA targets. We also provide guidelines for PCR optimization with Thermo Scientific™ Phusion™ Plus DNA Polymerase. Phusion Plus DNA Polymerase is a hot-start proofreading enzyme with over 100 times the fidelity of *Taq* polymerase, and it can efficiently amplify DNA with AT content as high as 90% with minimal optimization. The innovative reaction buffer enables universal annealing of most primers at 60°C, and it works effectively with long and GC-rich targets.

Materials and methods

AT-rich regions in the genome of *Staphylococcus aureus* subsp. *aureus* strain Seattle 1945 (ATCC Cat. No. 25923D5) served as template DNA. Primers were designed to amplify 200 bp regions with AT content of 65%, 70%, 75%, 80%, and 85%. We also designed primers to amplify 100 bp fragments with 90% AT content, since there were no 200 bp intervals with AT content that high in the genome of this particular strain of *S. aureus*. The sequences of the forward and reverse PCR primers are shown in Table 1.

Table 1. PCR primer sequences.

AT content	Sequence	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon size
65%	1	ATATGATGACATCTAAGACAACCGTAG	TAATTCTGTTACAAGTTCTGCTGTTG	200 bp
	2	TGAATATAAACAAGCATATTTCAAAGG	GGTCGATATCTAACATTACACCTTG	
	3	GTATATGACGAAAAGGGCTCC	CCTAGTGATAGTGCTTTTTGGAC	
	4	CTTGATGATTCCTGATTTGAGCAG	CGTAATCGTGGCGATCTTTGC	
70%	1	TTACTCAACTTTCCTAAAAGATACTGAGC	TATTACTATAATTTGCTAATTCTTCAGTAGT	200 bp
	2	CAAATGAATTTATTAAATCAATTCGTGATAACG	CTTTGGCGGTCGATCACTCG	
	3	GGCTAATTGTTGATATTACGCCACC	TGTAATTGGTTTTCCTAATAATTGTGAATATGC	
	4	TAGAGAGCTTACAGATTTCTCATTACC	TATATAACAGATTACATTTCCCAAAGTTTCC	
75%	1	CAAGTTGAATATAAAAGTTAAACGCCG	CTACAACTTCTTGAACCAAAATAATCACTC	200 bp
	2	TAAAATAGAGTAGACGTAAGTGTGAATGCAAG	TTAAGCACAAGATAGATTATAGCACAGC	
	3	TTTGTAGCGAATGATGAAACACT	CTCTCTTCAACATTATGCAATTCTAAACC	
	4	TATATTGAAGATGTAGATAAAGCTCATTATGATG	AAACATAGGTGATAAGTTACTACAATCTG	
80%	1	AACAGTTGAATGGGATAGTTAATGA	CTACTCTTTAAGGTTTACTAAAATAAAAGTTG	200 bp
	2	ATATGTCAATAATTCCTATAATACATTATTAAAACATC	ATGTGCTGAATGATACCAACCAC	
	3	AATTATTTTCACTACTCGTTTTTATTTTGA	ACTAATTTGTTTAATCGTTTCATCATC	
	4	ACATATTGACATTAAATAAATTGACAAAATAAG	TTGTTACAATTAAAGAACCTACTGTTGC	
85%	1	ATATTAATTTTAAGGGCAAATAATACTATTTCT	TAAATAATACAATTGTTTTAAATACAAAAATGTATATC	200 bp
	2	AGATTCTAAGATGATATTAAATAATTCTTGTAATA	TTTTATTTTTTATAAACGTAATTTCTTAACAAATC	
	3	ATATCTTTTTAAGTTCACTTATCATTTTATTACT	CATGAGATTTATTTAATATCTAAAAATGTAAAGA	
	4	AGTAAATAAGCTACTAAATTATTGCATAAACTG	GTAATACTTAAAGAAAATATTAAACTTAAAGC	
90%	1	ACCTAAAATATAGAAAATACATAAAAGTAAGTATAGTTAT	AATTAATTATAATTCTACATTTTTACAATTAATTAATAAA	100 bp
	2	ACCTAAAATATAGAAAATACATAAAAG	AATTAATTATAATTCTACATTTTTAC	
	3	TTTGGTTATGAATATGTAAATACCATTAAT	ATAATATTTAAGAATAATAATAATATTAAATATTTATT	
	4	TTTTATATTATAAAATTAACTTTAATATATTTTTTACAAT	AAATATATATTATTTTTAAATAAAATCTCTAGTATAATAA	

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The target sequences were amplified under standard reaction conditions on the Applied Biosystems™ ProFlex™ 3 x 32-Well PCR System (Cat. No. 4484073) using Phusion Plus DNA Polymerase. The composition of the final PCR mix and the thermal cycling protocol are shown in Table 2 and Table 3, respectively.

Table 2. Composition of final PCR mixture (standard protocol).

Component	Final composition
5X Phusion Plus buffer*	1X
Forward primer	0.5 µM
Reverse primer	0.5 µM
10 mM dNTPs	200 µM each
S. aureus gDNA	10 ng
Phusion Plus DNA Polymerase	0.2 µL
Nuclease-free water	Add to 20 µL final

* Provides 1.7 mM $MgCl_2$ at 1X.

Table 3. Standard thermal cycling protocol.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Annealing	60°C	10 sec	
Extension	72°C	30 sec/kb	
Final extension	72°C	5 min	1
Hold	4°C	–	–

PCR reagents

- Phusion Plus DNA Polymerase (Cat. No. F630S)
- Thermo Scientific™ 10 mM dNTP Mix (Cat. No. R0191)
- Thermo Scientific™ 25 mM $MgCl_2$ (Cat. No. AB0359)
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)

Analysis of PCR products

PCR products were diluted 2.5-fold in nuclease-free water to a final volume of 15 µL and analyzed on precast 2% Invitrogen™ E-Gel™ *48* Agarose Gels with SYBR™ Safe DNA Gel Stain (Cat. No. G820802). An equal volume of Invitrogen™ E-Gel™ 1 Kb Plus DNA Ladder (Cat. No. 10488090) or Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091) was run on each gel for sizing and approximate quantitation. Electrophoresis was run for 20 minutes as recommended in the standard protocol for 2% E-Gel *48* agarose gels on the Invitrogen™ E-Gel™ Power Snap Plus Electrophoresis System (Cat. No. G9101).

Results

Amplification of AT-rich regions in the *S. aureus* genome under standard PCR conditions

The complete genome of *S. aureus* subsp. *aureus* strain Seattle 1945 is a 2,778,850 bp circular chromosome with an AT content of 67.14%, which makes it ideal for evaluating amplification of sequences with various AT content levels. Electrophoresis of the PCR products revealed successful amplification, under standard conditions, of sequences with up to 75% AT content (Figure 1).

At an annealing temperature of 60°C, the specificity of all primer sets was high and amplicons were obtained in high yields. The results indicated that Phusion Plus DNA Polymerase enabled efficient amplification, without optimization, of sequences with AT content up to 75%. The amplicon yield began to decrease once the AT content reached 80%, and no PCR products were detected for sequences with 90% AT content. Optimization would thus be required to efficiently amplify targets with ≥80% AT content.

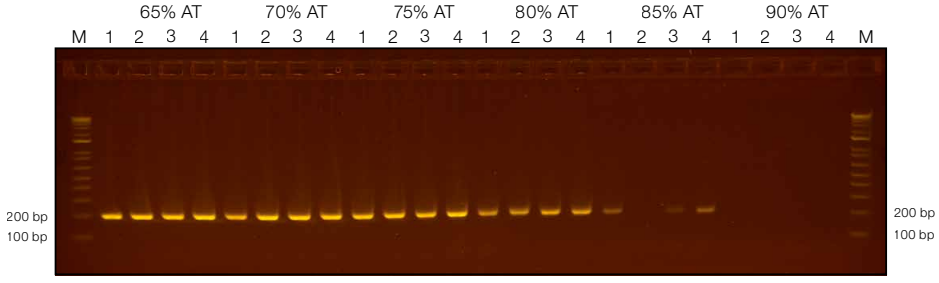

Figure 1. Amplification of AT-rich DNA targets under standard (non-optimized) PCR conditions. Genomic DNA (gDNA) targets in *S. aureus* subsp. *aureus* Seattle 1945 with varying AT content were amplified on the ProFlex PCR system using Phusion Plus DNA Polymerase with annealing at 60°C. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA. The PCR products were run on a 2% E-Gel *48* agarose gel containing SYBR Safe stain. Lane M: E-Gel 1 Kb Plus DNA Ladder.

Optimizing amplification of AT-rich sequences using Phusion Plus DNA Polymerase

Successful PCR requires an optimal combination of chemistry, primer design, and cycling conditions. When beginning PCR optimization, it is critical to consider the thermal cycling parameters. Since the A–T pair is held together by just two hydrogen bonds, the optimal annealing temperatures for primers with exceptionally high AT content are likely to be below 60°C. To find optimal annealing temperatures for targets with 80–90% AT content, 10 annealing temperatures ranging from 44°C to 62°C were tested by gradient PCR after preparing reactions as shown in Table 2. As expected, the optimal annealing temperatures for most of the AT-rich targets were below 60°C. The optimal annealing temperatures for targets with 90% AT content ranged from 44°C to 54°C (Table 4).

Table 4. Optimal annealing temperatures for targets with 80–90% AT content.

AT content	Amplicon	Annealing temperature (°C)	Amplicon size
80%	1	60	200 bp
	2	60	
	3	58	
	4	60	
85%	1	60	200 bp
	2	58	
	3	58	
	4	58	
90%	1	54	100 bp
	2	44	
	3	54	
	4	52	

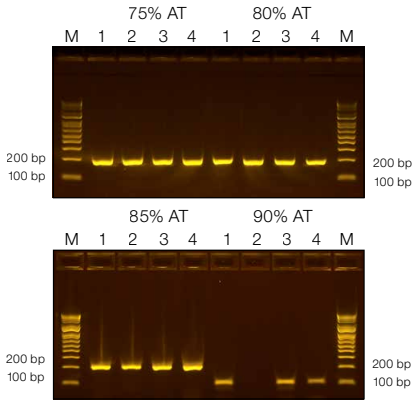

Figure 2. Amplification of sequences with 75–90% AT content under optimized thermal cycling conditions. *S. aureus* gDNA target sequences with varying AT content were amplified on the ProFlex PCR system using Phusion Plus DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA. Thermal cycling conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 10 sec at optimal annealing temperature (Table 4), 1 min/kb at 64°C; 5 min at 64°C. The PCR products were run on a 2% E-Gel *48* agarose gel containing SYBR Safe stain. Lane M: E-Gel 1 Kb Plus DNA Ladder.

PCR was then performed with annealing at the optimized temperatures. Extension was tested at 60°C, 64°C, 68°C, and 72°C, and the extension time was increased from 30 sec/kb to 60 sec/kb. Amplification was most efficient with extension at 64°C. Even the 90% AT sequences were efficiently amplified, with the exception of amplicon 2 (Figure 2), although the bands were not as bright as those of the 200 bp amplicons.

The bands containing 90% AT amplicons were less bright than the bands containing 75–85% AT amplicons, so we varied the $MgCl_2$ concentration to see whether we could improve PCR efficiency. Mg^{2+} is an essential cofactor that influences the activity and fidelity of DNA polymerase as well as annealing. To determine the optimal $MgCl_2$ concentration for amplifying AT-rich sequences, separate PCR reactions were set up with final $MgCl_2$ concentrations of up to 4.2 mM. (Note: The Phusion Plus buffer contributes 1.7 mM $MgCl_2$.) PCR efficiency was highest with an additional 1–2.5 mM $MgCl_2$ (Figure 3), and no significant differences in efficiency were observed in this range.

Additional $MgCl_2$ enabled Phusion Plus DNA Polymerase to amplify all but one of the sequences with 90% AT content (Figure 3). However, this required extremely low annealing temperatures of 44°C to 54°C. The primers for amplicon 2 were shorter, but they targeted the same sequence in the *S. aureus* genome as primers for the first 90% AT amplicon (Table 1). This clearly showed that primer design played a crucial role in amplification of AT-rich sequences. Optimal primers for AT-rich sequences should be 21–34 nt in length, and the melting temperature (T_m) should be ~60°C. You can use the **T_m calculator** on the Thermo Fisher Scientific website to determine the best melting temperatures for your primers.

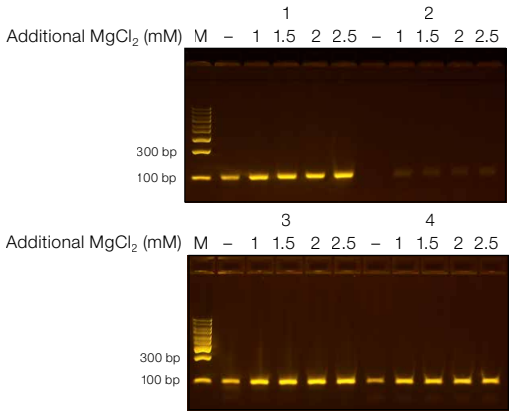

Figure 3. Effect of increasing $MgCl_2$ concentration on amplification of 90% AT targets. AT-rich *S. aureus* gDNA target sequences were amplified on the ProFlex PCR system using Phusion Plus DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA and an additional 1 mM, 1.5 mM, 2 mM, or 2.5 mM $MgCl_2$. Thermal cycling conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 10 sec at optimal annealing temperature (Table 4), 1 min/kb at 64°C; 5 min at 64°C. The PCR products were run on a 2% E-Gel *48* agarose gel containing SYBR Safe stain. Lane M: E-Gel 1 Kb Plus Express DNA Ladder.

If you have a primer with a very low T_m, you can try increasing the length of the primer by a few bases. The primers used in this study were up to 40 nt long. You could also redesign the primer to target a sequence with higher GC content. If possible, a primer designed to amplify an AT-rich sequence should terminate with a G or C at the 3′ end. However, the primer should have no more than two G or C nucleotides at the 3′ end. Do not use primers with significant sequence homology or self-complementarity. The primer should be specific for the target, so verify its specificity by performing a search with the BLAST™ tool at ncbi.nlm.nih.gov/blast.

To directly compare amplification efficiency with targets that had different AT content, we amplified sequences with 75–90% AT content under our optimized PCR conditions with extension at 64°C and a final $MgCl_2$ concentration of 2.7 mM. We also adjusted the annealing temperature for each amplicon. With the exception of one faint band, the bands for all amplicons were clearly visible (Figure 4). The overall intensity and uniformity of the bands indicated the PCR conditions were well optimized. Targets with 90% AT content were amplified as efficiently as targets with 75% AT content as long as appropriate primers were selected. The forward and reverse primers for amplicon 2 (90% AT) were only 27 nt and 22 nt long, respectively, and they annealed at 44°C. Phusion Plus DNA Polymerase amplified the target sequence, although amplification efficiency was notably lower (Figure 4).

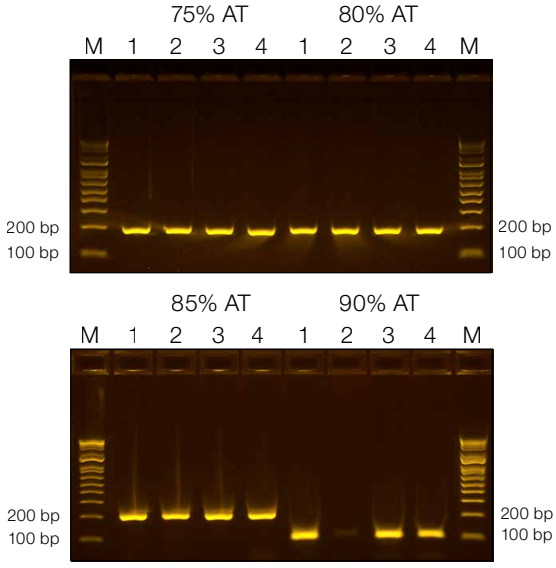

Figure 4. Amplification of AT-rich sequences under optimized conditions with Phusion Plus DNA Polymerase. Targets in the *S. aureus* genome with 75–90% AT content were amplified on the ProFlex PCR system using Phusion Plus DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA and 2.7 mM $MgCl_2$. Thermal cycling conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 10 sec at optimal annealing temperature (Table 4), 1 min/kb at 64°C; 5 min at 64°C. The PCR products were run on a 2% E-Gel *48* agarose gel containing SYBR Safe stain. Lane M: E-Gel 1 Kb Plus DNA Ladder.

Recommendations for AT-rich PCR

Based on this study, we have established some guidelines for using Phusion Plus DNA Polymerase to amplify AT-rich sequences.

1. Pay special attention to primer design. Ideally, primers will have the following properties:
   - Length: 21–34 nt
   - Melting temperature (T_m): ~60°C
   - No homo- or hetero-complementarity
   - G or C at the 3′ ends
   - High target specificity

   Use the **T_m calculator** to determine the melting temperatures of your primers. If any primer you are using has an extremely low T_m, try lengthening it by a few bases. The primers used to amplify AT-rich sequences in this study were up to 40 nt in length. We also recommend performing a BLAST search of your template at ncbi.nlm.nih.gov/blast to check the specificity of your primers.

2. Prepare PCR reactions as shown in Table 5 and use the thermal cycling protocol in Table 6 to amplify AT-rich sequences with Phusion Plus DNA Polymerase.

Table 5. Reaction composition for PCR amplification of AT-rich sequences. Changes from the original protocol are in bold.

Component	Final composition
5X Phusion Plus buffer*	1X
Forward primer**	0.5 µM
Reverse primer**	0.5 µM
Additional $MgCl_2$†	**1–2.5 mM**
10 mM dNTPs	200 µM each
Template DNA	0.01–10 ng (plasmid DNA) 5–100 ng (gDNA)
Phusion Plus DNA Polymerase	0.2 µL per 20 µL reaction
Nuclease-free water	Add to 20 µL final volume

* Provides 1.7 mM $MgCl_2$ at 1X concentration.

** Reduce the concentration of each primer to 0.2 µM when amplifying >5 kb gDNA targets.

† Recommended only for targets with >85% AT content. Start by adding 1 mM $MgCl_2$ and work up from there.

Table 6. Thermal cycling protocol for amplification of AT-rich sequences. Changes to the original protocol are in bold.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	**5–10 sec**	**25–35**
Annealing*	**44–60°C**	10 sec	
Extension**	**72°C or 64°C**	**15–60 sec/kb†**	
Final extension**	**72°C or 64°C**	5 min	1
Hold	4°C	–	–

* Determine annealing temperatures experimentally on a temperature gradient to amplify ≥75% AT sequences.

** Set the extension temperature at 64°C when amplifying ≥75% AT sequences.

† For extension at 64°C, increase the extension time to 30–60 sec/kb.

Summary

PCR reaction conditions can be easily adjusted to amplify sequences with AT content of up to 90% using Phusion Plus DNA Polymerase. Here we highlight the importance of optimizing thermal cycling conditions—specifically, reducing the annealing and extension temperatures. To amplify extremely AT-rich sequences with Phusion Plus DNA Polymerase, we recommend setting the extension temperature at 64°C and performing gradient PCR to optimize the annealing temperature. We also recommend adding $MgCl_2$ to a final concentration of 2.7 mM if the AT content of the target sequence is particularly high (>85%).

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