PCR reagents

Successful amplification of AT-rich DNA sequences with Platinum SuperFi II 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, amplification of challenging targets like the genes of organisms with AT-rich genomes often requires fine-tuning. Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase is a hot-start proofreading enzyme with an ultralow sequence error rate. It has over 300 times the fidelity of *Taq* DNA polymerase, and it can efficiently amplify DNA with AT content of up to 90% with minimal optimization. Platinum SuperFi II DNA Polymerase enables robust long-range and GC-rich PCR, and the innovative reaction buffer allows annealing of most primers at 60°C. Here we demonstrate the capability of Platinum SuperFi II DNA Polymerase to amplify targets with AT content of up to 90% and outline a PCR optimization strategy.

Important notes

- Platinum SuperFi II DNA Polymerase can be used to amplify DNA targets with high AT content. For successful amplification of AT-rich sequences, the optimal annealing temperature should be determined via gradient PCR. We also recommend performing extension at 60°C for 30 sec/kb.
- It may be necessary to adjust the final $MgCl_2$ concentration in the PCR mix up to 4.25 mM, particularly if the AT content of the target sequence is extremely high (≥85%).
- After PCR is complete, reaction mixtures should be diluted 2- to 20-fold for optimal separation on Invitrogen™ E-Gel™ agarose gels.

Materials and methods

AT-rich regions in the genome of *Staphylococcus aureus* subsp. *aureus* strain Seattle 1945 (ATCC Cat. No. 25923) 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 this 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.

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

PCR reagents

- Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase (Cat. No. 12361010)
- Invitrogen™ 10 mM dNTP Mix (Cat. No. 18427013)
- Invitrogen™ 1 M $MgCl_2$ (Cat. No. AM9530G)
- Invitrogen™ Water, nuclease-free (Cat. No. AM9938)
- Forward and reverse PCR primers for amplification of AT-rich sequences (Table 1)

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 Invitrogen™ SYBR™ Safe 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).

Table 1. PCR primer sequences.

AT content	No.	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	ATAATATTTAAGAATAATAATAATATATTAAATATTTATT	
	4	TTTTATATTATAAAATTAACTTTAATATATTTTTTACAAT	AAATATATATTATTTTTAAATAAAATCTCTAGTATAATAA	

Results

Amplifying 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%. The genome of this strain was thus ideal for evaluating amplification of sequences with various AT content levels. Electrophoresis of the PCR products showed that under standard PCR conditions, sequences containing up to 80% AT were successfully amplified by Platinum SuperFi II DNA Polymerase (Figure 1).

Table 2. Standard reaction conditions for PCR with Platinum SuperFi II DNA Polymerase.

Component	Final composition
5X SuperFi II buffer*	1X
Forward primer	0.5 µM
Reverse primer	0.5 µM
10 mM dNTPs	200 µM each
S. aureus gDNA	10 ng
Platinum SuperFi II DNA Polymerase	0.4 µL
Nuclease-free water	Add to 20 µL final volume

* Provides 1.75 mM $MgCl_2$ at 1X concentration.

Table 3. Standard thermal cycling protocol for PCR with Platinum SuperFi II DNA Polymerase.

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	Hold	Hold

Targets with 75% AT content were amplified in good yields with high specificity, indicating that an annealing temperature of 60°C was suitable for amplifying 75% AT targets without optimization. All of the 80% AT sequences were amplified under standard conditions, but yields were lower. Amplification of the 85% AT targets was poor, and no 90% AT amplicons were detected on the E-Gel agarose gel. Therefore, optimization would be necessary to efficiently amplify the ≥80% AT targets.

Optimizing amplification of AT-rich sequences using Platinum SuperFi II DNA Polymerase

Efficient PCR requires an optimal combination of primer design, chemistry, and cycling parameters. When beginning PCR optimization, it is critical to consider the thermal cycling parameters. Since the A–T pair is held together by only two hydrogen bonds, the optimal annealing temperatures for primers with very high AT content may be below 60°C. To determine the optimal annealing temperatures for 80–90% AT targets, 10 annealing temperatures ranging from 44°C to 62°C were tested in gradient PCR using the reaction mix composition shown in Table 2. As expected, optimal annealing occurred at temperatures below 60°C in most cases. Amplification of the 90% AT sequences required the lowest annealing temperatures (Table 4).

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 to 60 sec/kb. Amplification was most efficient with extension at 60°C. Even the 90% AT sequences were efficiently amplified with the exception of amplicon 2 (Figure 2). To amplify AT-rich sequences with Platinum SuperFi II DNA Polymerase, we recommend lowering the extension temperature to 60°C and performing gradient PCR to determine the optimal annealing temperature.

Table 4. Optimized annealing temperatures for targets containing 80–90% AT.

AT content	Amplicon	Annealing temperature (°C)	Amplicon size
80%	1	58	200 bp
	2	60	
	3	56	
	4	58	
85%	1	58	200 bp
	2	58	
	3	58	
	4	58	
90%	1	50	100 bp
	2	44	
	3	46	
	4	44	

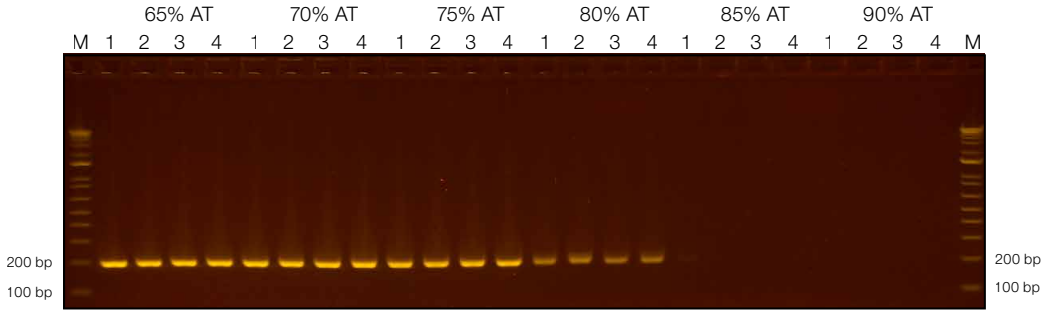

Figure 1. Amplification of AT-rich targets under standard (non-optimized) PCR conditions. Targets with different AT content were amplified from *S. aureus* subsp. *aureus* Seattle 1945 genomic DNA (gDNA) on the ProFlex PCR system using Platinum SuperFi II DNA Polymerase. Each 20 µL reaction contained 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 60°C, 30 sec/kb at 72°C; 5 min at 72°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.

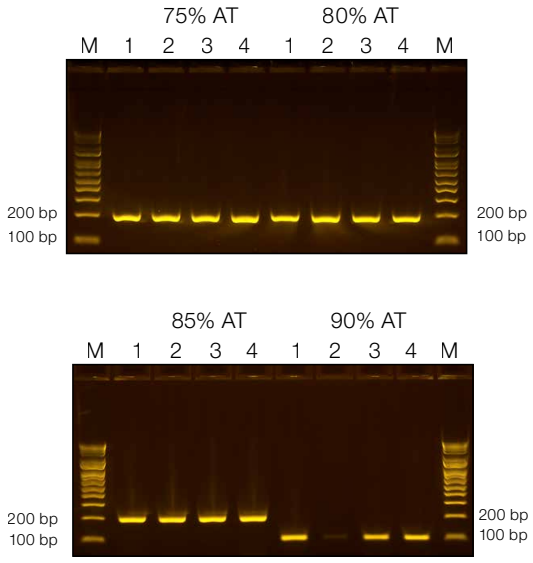

Figure 2. Amplification of 75–90% AT targets under optimized thermal cycling conditions. Target sequences with varying AT content were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum SuperFi II DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA. Thermal cycling conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 10 sec at optimized annealing temperature (Table 4), 1 min/kb at 60°C; 5 min at 60°C. The PCR products were run on 2% E-Gel *48* agarose gels containing SYBR™ Safe DNA Gel Stain. Lane M: E-Gel 1 Kb Plus DNA Ladder.

Since the bands containing 90% AT amplicons were less bright than the bands containing 75–85% AT amplicons after adjusting the thermal cycling parameters (Figure 2), we investigated whether increasing the final $MgCl_2$ concentration would improve reaction efficiency. Mg^{2+} is a crucial PCR cofactor that affects primer annealing and the activity and fidelity of DNA polymerase. To determine the optimal $MgCl_2$ concentration for amplifying AT-rich (≥85% AT) sequences, separate PCR reactions were set up with final $MgCl_2$ concentrations of up to 4.25 mM. (Note: The SuperFi II buffer contributes 1.75 mM $MgCl_2$.) PCR efficiency was enhanced by adding $MgCl_2$ to final concentrations of between 2.75 mM and 4.25 mM. Bands containing amplicon 2 of the 90% AT target were notably brighter with additional $MgCl_2$ (Figure 3). To amplify extremely AT-rich sequences, we recommend adding $MgCl_2$ to a final concentration of 2.75 mM. Higher $MgCl_2$ concentrations did not improve amplification efficiency further.

The additional 1 mM of $MgCl_2$ enabled Platinum SuperFi II DNA Polymerase to effectively amplify a 90% AT target that had previously been amplified in extremely low yield. Although the primers used to obtain amplicon 2 were shorter, they targeted the same sequence in the *S. aureus* genome as the primers used to obtain the first 90% AT amplicon (Table 1). The results clearly demonstrate that successful AT-rich PCR depends heavily on the use of appropriately designed primers. An optimal primer for AT-rich PCR should be 21–34 nt in length and have a melting temperature (T_m) of ~60°C. Use the **T_m calculator** and instructions on the Thermo Fisher Scientific website to determine the melting temperatures of your primers.

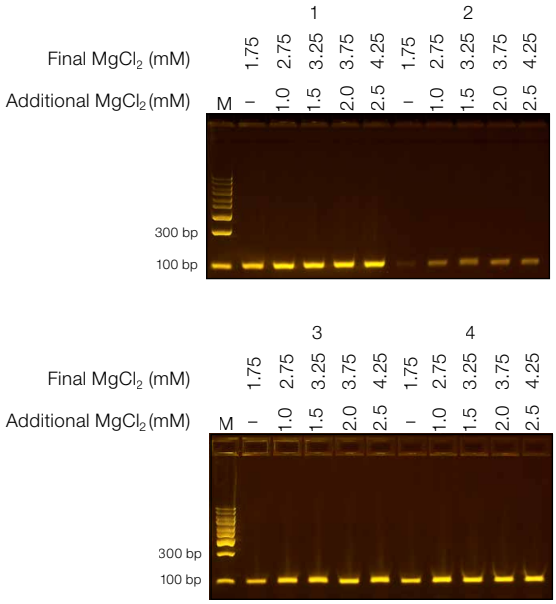

Figure 3. Effects of increasing final $MgCl_2$ concentration on amplification of 90% AT targets with extension at 60°C. AT-rich target sequences were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum SuperFi II DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* 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 optimized annealing temperature (Table 4), 1 min/kb at 60°C; 5 min at 60°C. The PCR products were run on 2% E-Gel *48* agarose gels containing SYBR Safe stain. Lane M: E-Gel 1 Kb Plus Express DNA Ladder.

If the T_m of a primer is very low, lengthen the primer by a few bases or select a portion of the target sequence with higher GC content to design a new primer. The primers used in this study were up to 40 nt long. If possible, the primer 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 60°C and an additional 1 mM $MgCl_2$. We also adjusted the annealing temperature for each target. All amplicons were detected, confirming that Platinum SuperFi II DNA Polymerase could efficiently amplify AT-rich sequences. The consistently high intensity of the bands across samples indicated that optimal PCR reaction conditions were selected. Targets with 90% AT content were amplified as effectively as targets with 75% AT content when well-designed primers were used (Figure 4). Even when suboptimal primer pairs were used to amplify a 90% AT target, amplification efficiency with Platinum SuperFi II DNA Polymerase was only slightly lower.

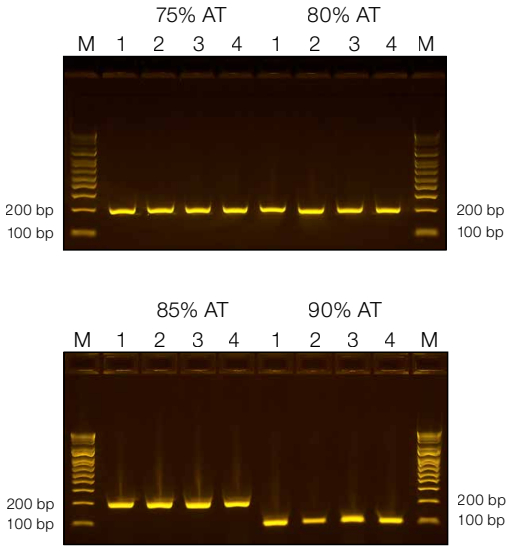

Figure 4. Amplification of AT-rich sequences under optimized conditions with Platinum SuperFi II DNA Polymerase. Targets with 75–90% AT content were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum SuperFi II DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA and an additional 1 mM $MgCl_2$. Thermal cycling conditions: 30 sec at 98°C; 35 cycles of 10 sec at 98°C, 10 sec at optimized annealing temperature (Table 4), 1 min/kb at 60°C; 5 min at 60°C. The PCR products were run on 2% E-Gel *48* agarose gels 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 amplification of AT-rich targets with Platinum SuperFi II DNA Polymerase.

1. Pay special attention to primer design. Ideally, primers should have the following properties:
   - Length: 21–34 nt
   - Melting temperature (T_m): ~60°C. Use the **T_m calculator** to determine the melting temperatures of your primers. When the T_m of a primer is extremely low, lengthen the primer by a few bases. The primers used to amplify AT-rich sequences in this study were up to 40 nt long.
   - No homo- or hetero-complementarity
   - Terminate with a G or C at the 3´ end (if possible)
   - High target specificity. Perform a BLAST search of your template at **ncbi.nlm.nih.gov/blast**
2. Use the protocols outlined in Tables 5 and 6 to amplify AT-rich sequences with Platinum SuperFi II DNA Polymerase.

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

Component	Final concentration
5X SuperFi II 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)
Platinum SuperFi II DNA Polymerase	0.4 µL per 20 µL reaction
Nuclease-free water	Add to 20 µL final volume

* Provides 1.75 mM $MgCl_2$ at 1X concentration.

** Reduce the primer concentration 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 from the original protocol are shown 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 60°C****	**15–60 sec/kb†**	
Final extension	**72°C or 60°C****	5 min	1
Hold	4°C	Hold	Hold

* Determine annealing temperatures experimentally via temperature gradient PCR to amplify ≥75% AT sequences.

** Extend at 60°C to amplify ≥75% AT sequences.

† Extend for 30–60 sec/kb at an extension temperature of 60°C.

Summary

PCR reaction conditions can be easily adjusted to amplify targets with extremely high (90%) AT content with Platinum SuperFi II DNA Polymerase. In this study, we developed a PCR protocol that enabled amplification of targets with up to 90% AT content in high yields. We highlight the importance of using appropriately designed primers, carefully adjusting the annealing and extension temperatures, and increasing the final $MgCl_2$ concentration for successful AT-rich PCR. These recommendations can be useful for amplification of AT-rich DNA sequences for use in a variety of downstream applications.

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