PCR reagents

Platinum II *Taq* Hot-Start DNA Polymerase enables amplification of AT-rich DNA sequences

Introduction

PCR is one of the most widely used techniques in molecular biology for *in vitro* amplification of DNA fragments. Although many DNA sequences can be easily analyzed using PCR-based methods, the amplification of challenging targets like sequences with high AT content (e.g., genes of organisms with AT-rich genomes) often requires fine-tuning. Invitrogen™ Platinum™ II *Taq* Hot-Start DNA Polymerase is designed for fast and easy PCR, with its unique combination of high-performance *Taq* DNA polymerase and hot-start technology. Platinum II *Taq* Hot-Start DNA Polymerase is engineered to be tolerant of inhibitors and enables successful amplification with samples of suboptimal purity and high GC content. Here we demonstrate that Platinum II *Taq* Hot-Start DNA Polymerase can effectively amplify targets with up to 90% AT content with only minor optimization of reaction conditions.

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 for amplification of 100 bp fragments with 90% AT content, since there were no 200 bp intervals with an AT content of 90% 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 II *Taq* Hot-Start 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™ II *Taq* Hot-Start DNA Polymerase (Cat. No. 14966001)
- Invitrogen™ 10 mM dNTP Mix (Cat. No. 18427013)
- Invitrogen™ 1 M $MgCl_2$ (Cat. No. AM9530G)
- Invitrogen™ 2 M KCl, RNase-free (Cat. No. AM9640G)
- Thermo Scientific™ tetramethylammonium chloride, TMAC (Cat. No. 138331000)
- Invitrogen™ Water, nuclease-free (Cat. No. AM9938)
- Forward and reverse PCR primers for amplification of AT-rich sequences (Table 1)

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	

Primer design

The primers 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 at thermofisher.com/tmcalculator to determine the melting temperatures of your primers. If the T_m of the primer is very low, try choosing a portion of the sequence with high GC content or lengthening the primer by a few bases. If possible, the primer should terminate with a G or C at the 3′ end. However, do not use a primer with 3 or more G or C nucleotides in a row at the 3′ end or a primer that is likely to form homo- or heterodimers. Primers must be target-specific, so double-check specificity by performing a search with the BLAST™ tool at ncbi.nlm.nih.gov/blast.

Standard PCR reaction conditions using Platinum II *Taq* Hot-Start DNA Polymerase

Table 2. Reaction conditions using Platinum II *Taq* Hot-Start DNA Polymerase (standard protocol).

Component	Final composition
5X Platinum II PCR buffer*	1X
Forward primer	0.2 µM
Reverse primer	0.2 µM
10 mM dNTPs	200 µM each
S. aureus gDNA	10 ng
Platinum II *Taq* Hot-Start DNA Polymerase	0.16 µL
Nuclease-free water	Add to a final volume of 20 µL

* Provides 1.5 mM $MgCl_2$ at 1X concentration.

Table 3. Cycling protocol using Platinum II *Taq* Hot-Start DNA Polymerase (standard protocol).

Step	Temperature	Time	Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	15 sec	35
Annealing	60°C	15 sec	
Extension	68°C	15 sec/kb	
Hold	4°C	Hold	Hold

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). Prepared DNA samples and an equal volume of E-Gel 1 Kb Plus DNA Ladder (Cat. No. 10488090) or E-Gel 1 Kb Plus Express DNA Ladder (Cat. No. 10488091) were loaded into the wells of the gel. 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 using 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 content were successfully amplified by Platinum II *Taq* Hot-Start DNA Polymerase (Figure 1). PCR products with 75% AT content were amplified with high yield and specificity using a universal primer annealing temperature of 60°C without need for optimization. All 80% AT sequences were amplified under standard conditions, but in lower yields. However, amplification of 85% AT targets was poor, and no amplicons for 90% AT sequences were visible on the E-Gel agarose gel. Therefore, optimization was necessary to efficiently amplify ≥80% AT targets.

Optimizing amplification of AT-rich sequences using Platinum II *Taq* Hot-Start DNA Polymerase

Efficient PCR requires an optimal combination of primer design, chemistry, and cycling parameters. The thermal conditions are the most important parameters to test at the beginning of PCR optimization. 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 best annealing temperatures for 80–90% AT targets, 10 different temperatures ranging from 44°C to 62°C were tested in a gradient PCR run using the reaction composition described in Table 2. As expected, the optimal annealing temperatures were below 60°C for all 80–90% AT targets (Table 4).

PCR was then performed using the experimentally determined annealing temperatures with extension at 60°C, 64°C, and 68°C. The extension time was increased to 30 sec/kb. Extension at 60°C resulted in the most effective amplification. Even the 90% AT sequences were successfully amplified under these conditions (Figure 2). We recommend lowering the extension temperature to 60°C and using gradient PCR to determine the annealing temperature when amplifying sequences with high AT content using Platinum II *Taq* Hot-Start DNA Polymerase.

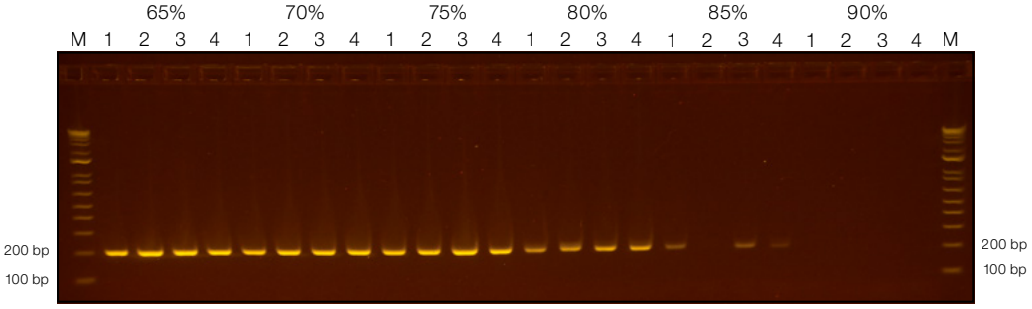

Figure 1. Amplification of AT-rich targets using standard (non-optimized) PCR conditions. Target sequences with different AT content were amplified from *S. aureus* subsp. *aureus* Seattle 1945 genomic DNA (gDNA) on the ProFlex PCR system using Platinum II *Taq* Hot-Start DNA Polymerase and a universal annealing temperature of 60°C. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA. Thermal cycling conditions: 2 min at 94°C; 35 cycles of 15 sec at 94°C, 15 sec at 60°C, 15 sec/kb at 68°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.

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

AT content	Amplicon	Annealing temperature (°C)	Amplicon size
80%	1	56	200 bp
	2	54	
	3	54	
	4	54	
85%	1	54	200 bp
	2	52	
	3	50	
	4	52	
90%	1	46	100 bp
	2	50	
	3	44	
	4	50	

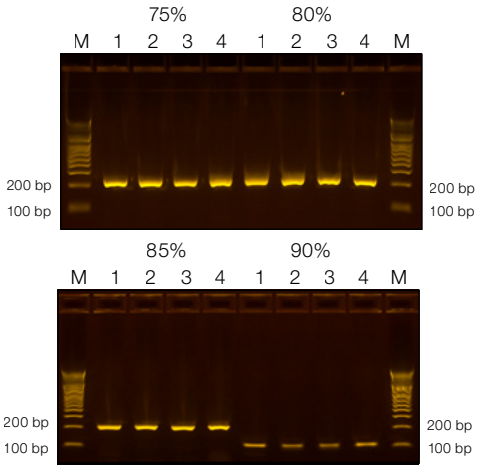

Figure 2. Amplification of 75–90% AT targets using optimized thermal cycling conditions. Target sequences with different AT content were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum II *Taq* Hot-Start DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA. Thermal cycling conditions: 2 min at 94°C; 35 cycles of 15 sec at 94°C, 15 sec at optimal annealing temperature (Table 4), 30 sec/kb at 60°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.

Since the 90% AT amplicon bands were less bright than the 75–85% AT amplicon bands after thermal cycling adjustment (Figure 2), we continued optimization by examining the effects of adding $MgCl_2$, KCl, and tetramethylammonium chloride (TMAC) to further improve reaction efficiency. Mg^{2+} is a crucial cofactor in PCR that has an impact on primer annealing as well as the activity and fidelity of DNA polymerase. To determine the optimal concentration of $MgCl_2$ for amplification of extremely AT-rich sequences, separate PCR reactions were prepared with final $MgCl_2$ concentrations of up to 4.0 mM. (Note: The Platinum II PCR buffer contributes 1.5 mM $MgCl_2$.) PCR efficiency in this study was enhanced by additional $MgCl_2$ in final concentrations of 2.5 mM and 4.0 mM (Figure 3A). When amplifying extremely AT-rich (≥85% AT) sequences, we recommend adding $MgCl_2$ to a final concentration of 2.5 mM (additional 1 mM $MgCl_2$). We have observed no significant differences in the product yields when more than 1–2.5 mM of additional $MgCl_2$ is used.

We then investigated the effects of adding KCl. KCl in PCR buffer acts by neutralizing the charge on the backbone of DNA. It reduces the repulsion between the negatively charged primers and template, thereby stabilizing AT-rich primer and template binding. The impact of KCl on AT-rich PCR was assessed by titrating the KCl concentration from 30 mM to 90 mM in increments of 20 mM. The addition of 30–90 mM KCl increased the yield of AT-rich amplicons regardless of concentration (Figure 3B). To enhance AT-rich PCR, we suggest supplementing the reaction mixture with 30 mM KCl.

Finally, we examined the impact of TMAC on AT-rich PCR. TMAC increases the melting temperature of DNA by increasing AT base pair stability such that it is closer to that of a GC base pair. The melting temperatures of AT-rich primers may thus be higher in the presence of TMAC. To explore the effect of TMAC, a series of PCR assays was performed in the presence and absence of TMAC for targets that contained 90% AT. In the presence of 50–110 mM TMAC, Platinum II *Taq* Hot-Start DNA Polymerase increased the yields of 90% AT-rich amplicons to a similar extent (Figure 3C). We thus recommend adding 50 mM TMAC to PCR reactions prepared with Platinum II *Taq* Hot-Start DNA Polymerase to improve the efficiency of amplification of AT-rich sequences.

Overall, $MgCl_2$, KCl, and TMAC were found to have positive effects on AT-rich PCR (Figure 3). To boost AT-rich PCR efficiency, we recommend adding either 30 mM KCl, 50 mM TMAC, or an additional 1 mM $MgCl_2$.

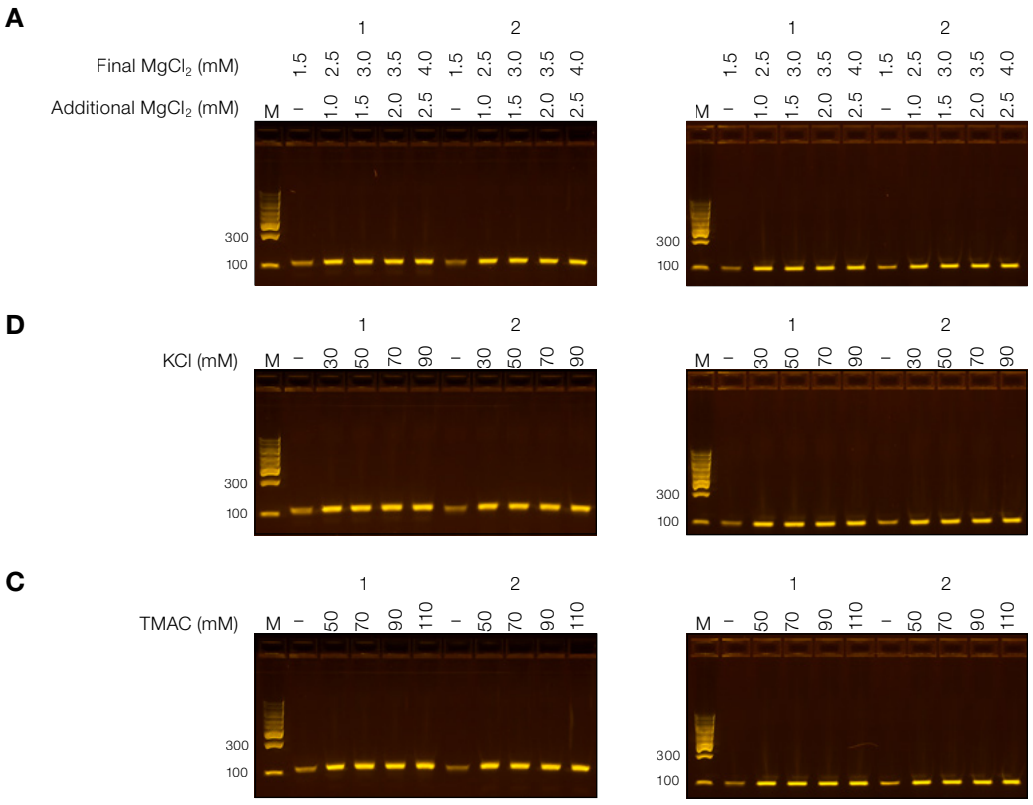

Figure 3. Effects of $MgCl_2$, KCl, and TMAC on the amplification of 90% AT targets at an extension temperature of 60°C. AT-rich target sequences were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum II *Taq* Hot-Start DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA and an additional **(A)** 1, 1.5, 2, or 2.5 mM $MgCl_2$, **(B)** 30, 50, 70, or 90 mM KCl, or **(C)** 50, 70, 90, or 110 mM TMAC. Thermal cycling conditions: 2 min at 94°C; 35 cycles of 15 sec at 94°C, 15 sec at optimal annealing temperature (Table 4), 30 sec/kb at 60°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.

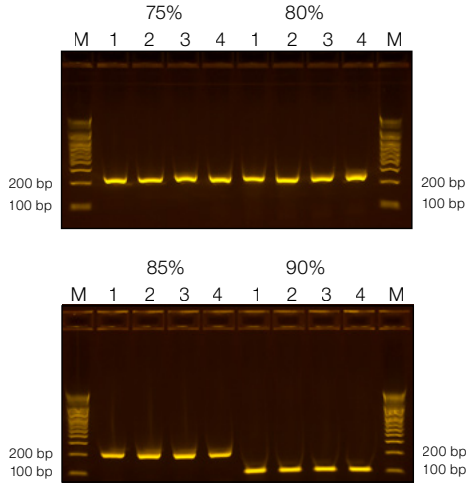

Figure 4. Amplification of AT-rich sequences using optimized conditions and Platinum II *Taq* Hot-Start DNA Polymerase. Target sequences with 75–90% AT content were amplified from *S. aureus* gDNA on the ProFlex PCR system using Platinum II *Taq* Hot-Start DNA Polymerase. Each 20 µL reaction contained 10 ng of *S. aureus* gDNA and an additional 1 mM of $MgCl_2$. Thermal cycling conditions: 2 min at 94°C; 35 cycles of 15 sec at 94°C, 15 sec at optimal annealing temperature (Table 4), 30 sec/kb at 60°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.

To directly compare PCR efficiency with targets that had different AT content, 75–90% AT sequences were amplified under the following conditions: extension temperature of 60°C, adjusted annealing temperature for each target, and an additional 1 mM $MgCl_2$. All amplicons were detected, which indicated successful AT-rich PCR. The results showed that Platinum II *Taq* Hot-Start DNA Polymerase enabled efficient AT-rich amplification. The strong and consistent intensity of the bands across samples indicated that optimal PCR reaction conditions were selected. The 90% AT targets were amplified as effectively as targets with 75% AT content (Figure 4).

Recommendations for AT-rich PCR

Based on this study, we have established some guidelines for amplifying AT-rich targets with Platinum II *Taq* Hot-Start 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 our **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 in length
   - No homo- or hetero-complementarity
   - G or C at the 3´ end (if possible)
   - High target specificity. Perform a BLAST search for your template at **ncbi.nlm.nih.gov/blast**
2. Use the protocols described in Tables 5 and 6 for amplification of AT-rich sequences with Platinum II *Taq* Hot-Start 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 Platinum II PCR buffer*	1X
Forward primer	0.2 µM
Reverse primer	0.2 µM
Additional $MgCl_2$/KCl/TMAC**	**1–2.5 mM $MgCl_2$, 30–90 mM KCl, or 50–110 mM TMAC**
10 mM dNTPs	200 µM each
Template DNA	0.01–10 ng (plasmid DNA) 5–100 ng (gDNA)
Platinum II *Taq* Hot-Start DNA Polymerase	0.16 µL per 20 µL reaction
Nuclease-free water	Add to 20 µL final volume

* Provides 1.5 mM $MgCl_2$ at 1X concentration.

** Recommended only for targets with ≥85% AT content. If additional reagents are used, we recommend the following ranges: 1–2.5 mM $MgCl_2$ (2.5–4.0 mM final concentration), 30–90 mM KCl, or 50–110 mM TMAC. We observed no differences in PCR efficiency when the additional reagents were tested within these ranges.

Table 6. Thermal cycle program for amplification of AT-rich sequences. Changes from the original protocol are in bold.

Cycle step	Temperature	Time	Cycles
Initial denaturation	94°C	2 min	1
Denaturation	94°C	15 sec	**25–35**
Annealing	**44–60°C***	15 sec	
Extension	**68 or 60°C****	**15–30 sec/kb†**	
Hold	4°C	Hold	Hold

* Determine annealing temperatures experimentally via temperature gradient when amplifying ≥75% AT sequences.

** Use extension temperature of 60°C when amplifying ≥75% AT sequences.

† Increase extension time to 30 sec/kb when using an extension temperature of 60°C.

Summary

This study demonstrates that Platinum II *Taq* Hot-Start DNA Polymerase can be used to amplify AT-rich fragments. We showed that PCR conditions can be easily adjusted for amplification of extremely AT-rich (90%) sequences. Possible modifications include lowering the annealing and extension temperatures and adjusting the concentration of $MgCl_2$, KCl, or TMAC. These recommendations can be useful for enhancing the amplification of AT-rich DNA sequences for a variety of downstream applications.

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