Code No. 6140

For research

TaKaRa

*In vitro*Transcription T7 Kit

(for siRNA Synthesis)

manual

v202106Da

Content

This product is transcribed in vitro using T7 RNA Polymerase (*in vitro*This kit uses T7 RNA Polymerase to transcribe a linear double-stranded DNA containing a T7 promoter sequence, efficiently synthesizing

single-stranded RNA. The kit also includes a control template (linear plasmid DNA). ,
Using 20 ng of the control template as a template, a 20 μl transcription reaction can produce approximately

10 μg of 2 kb single-stranded RNA. This kit also contains RNase T1, which specifically cleaves the 3'-

terminal phosphate of guanosine nucleotides in single-stranded RNA. siRNA can be synthesized after in vitro

transcription.

(Note) This kit is suitable for synthesizing large amounts of RNA and is not recommended for preparing RNA probes with high specific activity.

● Product content (50 times, 20 μl reaction system)

10×Transcription Buffer T7 RNA	**100 μl**
Polymerase (50 U/μl) RNase	**100 μl**
Inhibitor (40 U/μl) ATP Solution (50	**25 μl**
mM)	**100 μl**
GTP Solution (50 mM)	**100 μl**
CTP Solution (50 mM)	**100 μl**
UTP Solution (50 mM)	**100 μl**
RNase free DNase I (5 U/μl)	**200 μl**
Control Template (50 ng/μl) RNase	**20 μl**
T1 (100 U/μl)	**25 μl**
RNase T1 Dilution Buffer	**700 μl**
RNase free dH2	**1 ml**
10×Annealing Buffer*	**500 μl**

*** Used for annealing of two oligo DNA strands. Buffer**

composition: 100 mM Tris-HCl (pH 8.0) , 500 mM CH_3COOK, 10 mM EDTA.

- **Save:-20℃**

- **Experimental operation precautions**

1. Strictly avoid mixing RNase into the reaction system.
2. Experimental equipment (such as gun tips, microtubes, etc.) must strictly use RNase-free supplies.
3. Please wear disposable gloves during experimental operations to prevent RNase contamination.

● Preparation of template DNA

Contains T7 promoter, linearized plasmid or PCR

amplification product can be used as template. T7 promoter

sequence

The bold +1 G initiates RNA transcription.

1. Plasmid as template

Insert the target DNA into the plasmid vector containing T7 promoter, and then treat it with restriction enzyme. Linearize the plasmid using a restriction enzyme that has no recognition site in the inserted DNA fragment.Please use a 5'

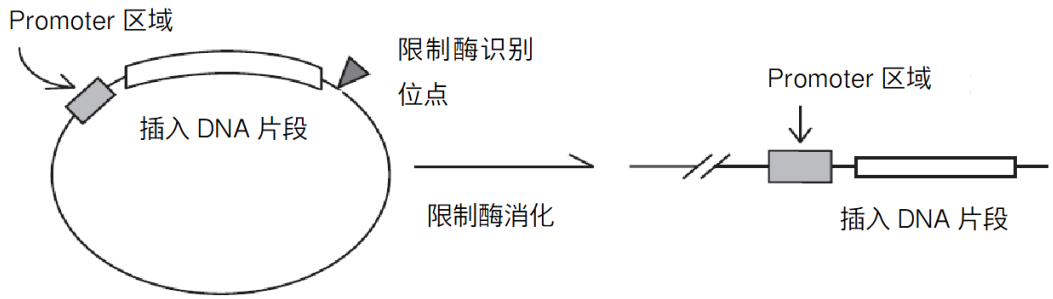
After the reaction, perform a Proteinase K treatment and a purification reaction.

1) Add Proteinase K to a final concentration of 100 μg/ml and SDS to a final concentration of

0.5% to the restriction enzyme reaction solution. 2) Incubate at 37°C for 30 minutes.

3) TE-saturated phenol/chloroform treatment.
4) Add 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol for ethanol precipitation.
5) 5) Wash the recovered DNA precipitate with 80% ethanol.

6) Dissolve RNase-free TE Buffer.



2. PCR amplification product as template

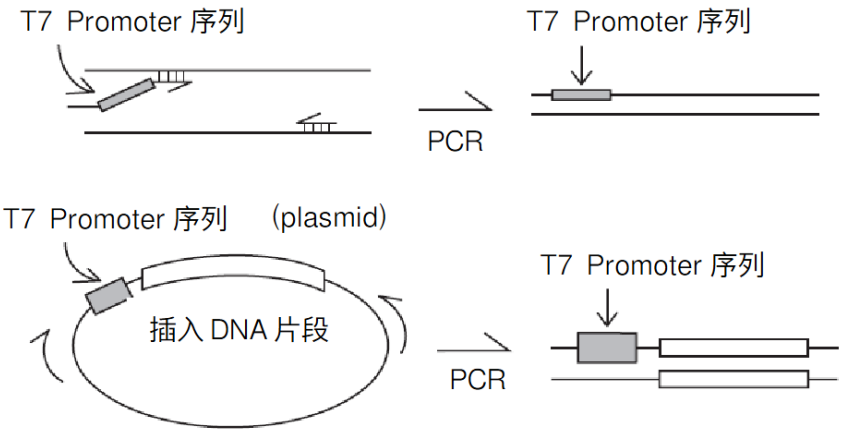
The T7 promoter (TAATACGACTCACTATAGGG) is added to the 5' end of the sense primer. (In this case, adding 6 to 10 nucleotides upstream of the T7

promoter sequence can more effectively promote the binding of T7 RNA polymerase and the synthesis of RNA.) , or insert the target slice

PCR amplification was performed using a sense primer and an anti-sense primer upstream of the T7

promoter in the plasmid DNA fragment. The amplified product can be purified using NucleoSpin Gel

and PCR Clean-up (Cat. No. 740609 .10/.50/.250).



T● ***in vitro*transcriptional response**

(1) Prepare the following reaction mixture.

Reagents	Usage
10×Transcription Buffer	**2 μl**
ATP Solution	**2 μl**
GTP Solution	**2 μl**
CTP Solution	**2 μl**

UTP Solution	**2 μl**
RNase Inhibitor	**0.5 μl**
T7 RNA Polymerase	**2 μl**
RNase-free dH2O	**X μl**
linear template DNA*	**20 ng～1 μg**
Total	**20 μl**

*10× Transcription Buffer contains spermidine. Spermidine forms complexes with nucleic acids, which can easily cause precipitation. Template DNA must be added last.

The above reaction system is 20 μl, and the reaction system can be increased or decreased appropriately.

(2) Mix the above solutions thoroughly and centrifuge gently. Collect the transcription reaction mixture at the bottom of the reaction tube and incubate at 42°C for 1-2 hours. Note: A white precipitate may form after the reaction. This is the free pyrophosphate that reacts with the magnesium ions in the reaction mixture to form magnesium pyrophosphate. This does not affect subsequent experiments. To remove it, add EDTA. If adding EDTA affects subsequent experiments, centrifugation and recovery of the supernatant is acceptable.

● DNase treatment

If you want to remove DNA, perform DNase treatment as follows after the transcription reaction.

(1) After the transcription reaction, add 10-20 U/20 μl of RNase-free DNase I, mix well, and incubate at 37°C

(2) for 30 minutes.

● Transcribed RNA purification

A. Perform phenol/chloroform extraction and isopropanol precipitation as follows. When the reaction volume is less than 100 μl, add RNase-free dH2Make up to 100 μl with 0.5% O. (1)

An equal volume of phenol (pH 4.5)/chloroform/isoamyl alcohol (25:24:1) was added, stirred with a vortex, and centrifuged at 12,000 rpm at room temperature for 2 minutes.

(2) Transfer the upper layer (aqueous layer) to a new tube and add an equal volume of chloroform/isoamyl alcohol (24:1). , vortex stirring, 12,000 rpm Centrifuge at room temperature for 2 minutes.

(3) Transfer the upper (aqueous) layer to a new tube, add 1/10 volume of 3 M sodium acetate and an equal volume of isopropanol, and mix thoroughly. After standing at room temperature for 5 minutes, centrifuge at 15,000 rpm for 5

(4) minutes.

(5) The supernatant was removed and the precipitate was washed with 80% ethanol.

(6) After drying, add RNase-free dH2Dissolve the precipitate in 1% O or TE

(7) Buffer. If necessary, aliquot and store at -20℃ to -70℃.

B. The following commercial products can also be used (when transcript RNA size

>200 bases): NucleoSpin RNA Clean-up

(740948.10/740948.50/740948.250) NucleoSpin RNA Clean-up XS

(740903.10/740903.50/740903.250)

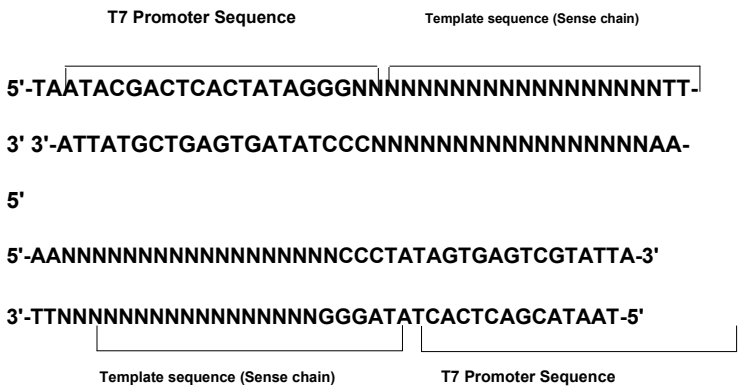
● Production of siRNA

This kit can be used to produce siRNA using the following

method.

<Step 1>

Two sets of double-stranded DNA oligonucleotides were prepared to attach the siRNA sequence to the T7 promoter sequence.



<Step 2>

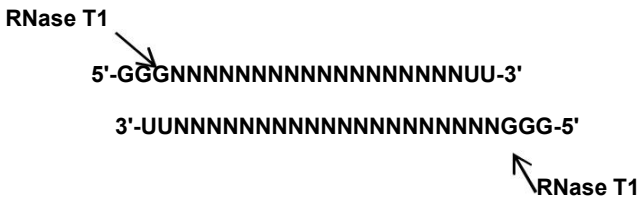
Two sets of double-stranded DNA oligonucleotides were prepared as templates in a tube.*in vitro*The transcription reaction occurs. Sense and anti-sense RNAs are synthesized simultaneously. The synthesized RNA can form double strands.

5'-GGGNNNNNNNNNNNNNNNNNUU-3'

3'-UUNNNNNNNNNNNNNNNNNNNGGG-5'

<Step 3>

DNase I digests the template DNA oligonucleotide, and RNase T1 degrades the GGG portion of one RNA strand.



<Step 4>

siRNA with a 2-base overhang at the 3' end was purified and recovered.

5'-NNNNNNNNNNNNNNNNNNUU-3' 3'-

UUNNNNNNNNNNNNNNNNNN-

5' DNA Oligonucleotide Design

Regarding the design of synthetic oligonucleotides for preparing siRNA, First, the target siRNA sequence needs to be determined. Then the sense of siRNA, A set of anti-sense primers were designed separately, and a total of four oligonucleotides were synthesized in the appropriate direction of the T7 promoter. Adding about six arbitrary bases upstream of the T7 promoter can improve transcription efficiency.

● Experimental example

Synthesize RNA interference against GFP and use siRNA for RNA interference experiments.Confirm that the siRNA synthesized by in vitro transcription using this kit

Effects in RNA interference experiments.

1. Synthesize the following single-stranded oligo DNA

Synthesize 4 single-stranded oligo DNAs for normal experiments (see Figure 1) and 4 single-stranded oligo DNAs for negative control

experiments (see Figure 2) , DNA sequences of the bold black parts of the four single-stranded oligo DNAs used in the Negative Control experiment,

It is a DNA sequence obtained by shuffling the same portion of the four single-stranded oligo DNAs used in normal experiments, and will not cause RNA interference with GFP.

Note: To ensure efficient reaction with T7 RNA Polymerase, add six bases before the T7 Promoter sequence (toward the 5' end) when designing synthetic oligo DNA. In our experience, adding GATCAC is optimal.

T7 Promoter Sequence

5'-GATCACTAATACGACTCACTATAGGG**GGAGTTGTCCCAATTCTTG**TT**-3' Oligo-1**

3'-CTAGTGATTATGCTGAGTGATATCCC**CCTCAACAGGGTTAAGAAC**AA**-5' Oligo-2**

5'-AAGGAGTTGTCCCAATTCTTGCCCTATAGTGAGTCGTATTA**GTGATC-3' Oligo-3 3'-**

TT CCTCAACAGGGTTAAGAACGGGATATCA**CTCAGCATAAT**CACTAG-5' Oligo-4**

T7 Promoter Sequence

Figure 1. Four single-stranded oligo DNA sequences used in normal experiments

T7 Promoter Sequence

5'-GATCACTAATACGACTCACTATAGGG**GGGATGT**CTCACATCTTGTTT**-3' n-Oligo-1 3'-**

CTAGTGATTATGCTGAGTGATATCCCCCCTACAGAGTGTAGAACA**AA**-5' n-Oligo-2 5'-**

AAGGGATGTCTCACATCTTGTCCCTATAGTGAGTCGTATTAGTGATC**-3' n-Oligo-3 3'-**

TTCCCTACAGAGTGTAGAACAGGGATATCACTCAGCATAATCACTAG**-5' n-Oligo-4**

T7 Promoter Sequence

Figure 2. Four single-stranded oligo DNA sequences used in negative control experiments

2. Preparation of Double-stranded Oligo DNA

① Dissolve the synthesized single-stranded oligo DNA in sterile distilled water to prepare a 100 pmol/μl DNA solution. Prepare the oligo DNA annealing reaction solution according to the following ingredients.

Reagents	Usage
10×Annealing Buffer	**2 μl**
100 pmol/μl Oligo A*	**2 μl**
100 pmol/μl Oligo B*	**2 μl**
sterile water	**14 μl**

*** A and B must be paired. The specific pairings are as follows: Oligo-1 and Oligo-2; Oligo-3 and Oligo-4; n-Oligo-1 and n-Oligo-2; n-Oligo-3 and n-Oligo-4.**

② Place the above oligo DNA annealing reaction solution on a PCR amplifier, treat it at 95℃ for 2 minutes.This is followed by the annealing phase, which consists of a gradual cooling ramp to 25°C over 45 minutes, and a subsequent 10-minute hold. At this time, a pair of single-stranded oligo DNAs are annealed to form double-stranded oligo DNA. The concentration is 10 pmol/μl. This solution can be used as template DNA. Store at -20°C when not in use.

3. In vitro transcription reaction

① Prepare RNA in vitro transcription reaction solution according to the following components.

Reagents	Usage
10×Transcription Buffer	**2 μl**
ATP Solution	**2 μl**
GTP Solution	**2 μl**
CTP Solution	**2 μl**
UTP Solution	**2 μl**
RNase Inhibitor	**0.5 μl**
T7 RNA Polymerase	**2 μl**
RNase-free dH_2O	**X μl**
10 pmol/μl double-stranded oligo DNA solution (Oligo-1/-2)*1	**1 μl**
10 pmol/μl double-stranded oligo DNA solution (Oligo-3/-4)*1	**1 μl**
Total	**20 μl*2**

***1. 10× Transcription Buffer contains spermidine. Spermidine forms complexes with nucleic acids, which can easily cause precipitation. Template DNA must be added last.**

*** 2. The reaction system can be scaled up**

② Evenly mix the above solutions and centrifuge gently. Collect the transcription reaction solution at the bottom of the reaction tube and react at 42°C for 2 hours. Take a portion of

the reaction solution for gel electrophoresis to confirm the RNA product after in vitro transcription.

4. Nuclease Treatment

① Add the following solutions to the reaction tube after in vitro transcription reaction.

Reagents	Usage
RNase free DNase I (5 U/μl)	**2 μl**
RNase T1 (4 U/μl)*	**1 μl**

***: 4 U/μl RNase T1: Dilute the RNase T1 in the kit to 4 U/μl using RNase T1 Dilution Buffer.**

(The diluted RNase T1 should be used as soon as possible and should not be stored.)

② Incubate at 37°C for 2 hours.

5. Purification of siRNA

① Add an equal volume of water-saturated acidic phenol/chloroform/isoamyl alcohol (25:24:1) to the above reaction solution. , after mixing thoroughly

Centrifuge at 10,000 rpm for 5 minutes at room temperature.

② Transfer the upper (aqueous) layer to a new centrifuge tube and add an equal volume of 5 M ammonium acetate (pH 5.6) and four times the volume of 99.5% ethanol. Let stand at room temperature for 5 minutes, then centrifuge at 15,000 rpm for 10 minutes. Remove the supernatant, add 100 μl of 80% ethanol, and centrifuge at 15,000 rpm for 5 minutes.

③ Remove the supernatant, dry slightly, and add 20-50 μl of RNase Free dH2Note: If the above purification method cannot completely remove NTP, or the reaction system is scaled up, it is recommended to use CHROMA SPIN™ +TE-30 columns (Code No. 636069).

④ Measure the OD of the above solution260The purity of RNA was confirmed by gel electrophoresis.

6. RNA Interference Experiment (siRNA Effect Assay)

① Simultaneously introduce GFP expression plasmid and siRNA solution into 293T cells to verify the RNA interference effect.

use*Trans*IT®-293 (Code No. MIR2700) and*Trans*IT-TKO®(Cat. No. MIR2150) The GFP expression plasmid and the above-prepared siRNA solution were simultaneously introduced into 293T cells. On the second day of cell culture, the GFP fluorescence intensity (mean value) in the cells was measured using FCM (Flow Cytometry). .

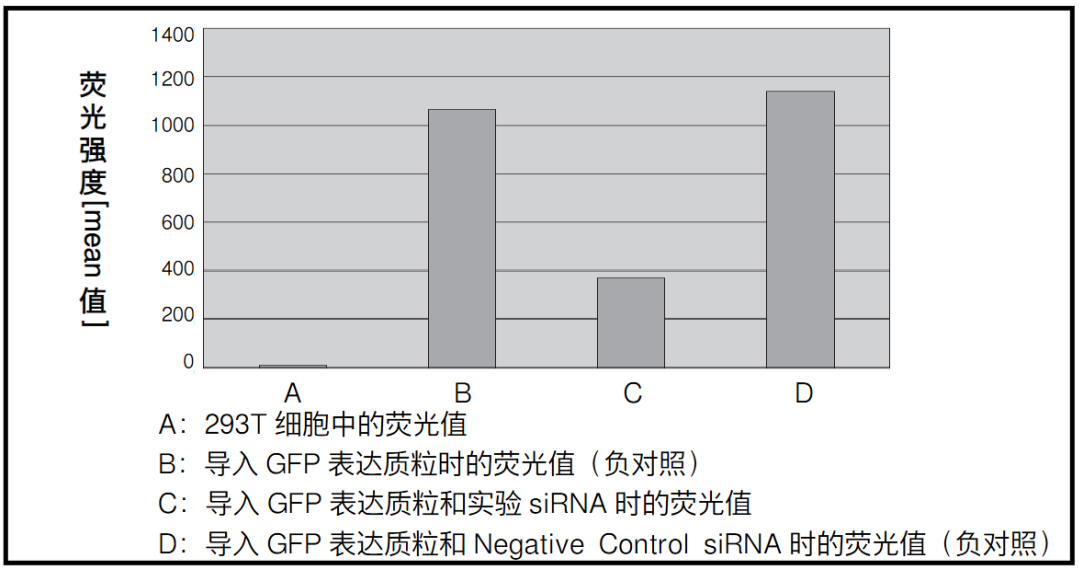


图 3. RNA 干扰实验结果图（荧光检测结果）

A: Fluorescence value in 293T cells

B: Fluorescence value when GFP expression plasmid was introduced (negative control) C: Fluorescence value when GFP expression plasmid and experimental siRNA were introduced

D: Fluorescence value when GFP expression plasmid and Negative Control siRNA were introduced (negative control)

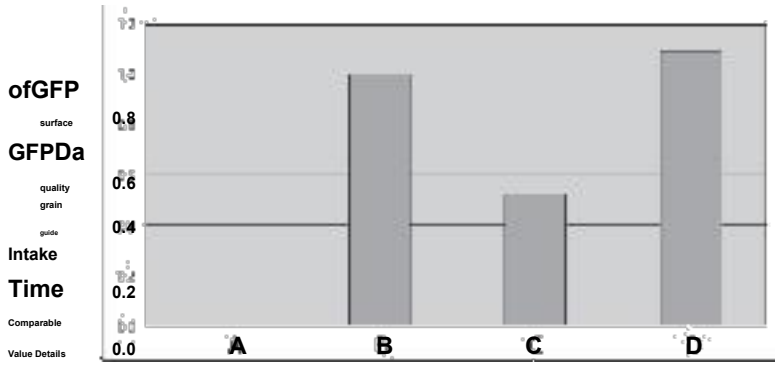
Figure 3. RNA interference experiment results (fluorescence detection results)

② Total RNA was extracted from the various experimental cells in ① above, and Real Time RT-PCR reaction was performed to detect the GFP mRNA expression level in the cells under various conditions.

Using the Smart Cycler II System (Cepheid), chimeric assay, 50 ng Total RNA as template, four Housekeeping genes correct for RNA expression.

The results show (see Figure 4), GFP mRNA expression decreased, and the siRNA synthesized by in vitro transcription in this experiment showed good RNA Interference effect.

Cells and middle 1.2 1.0



A: GFP mRNA ratio in 293T cells

B: GFP mRNA ratio when GFP expression plasmid was introduced (negative control) C: GFP mRNA ratio when GFP expression plasmid and experimental siRNA were introduced

D: GFP mRNA ratio when GFP expression plasmid and Negative Control siRNA were introduced (negative control)

Figure 4. RNA interference experiment results (GFP mRNA level detection results)

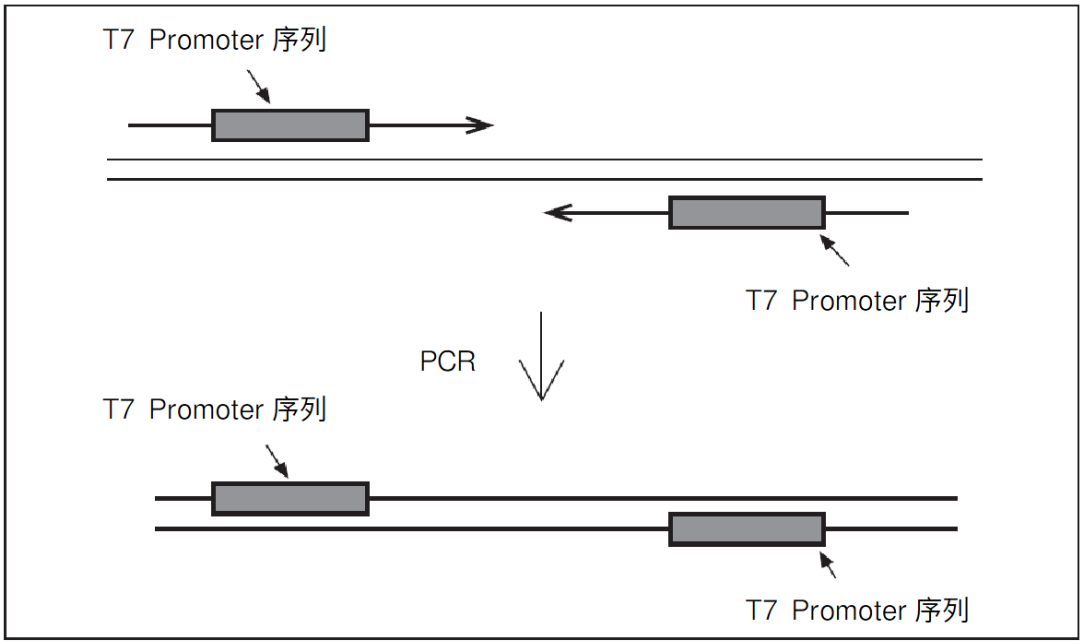
（refer to）

Using this kit, double-stranded RNA synthesized according to the following method can serve as a substrate for Dicer and RNase III.

(A) PCR amplification was performed using a pair of primers with T7 promoter sequences added to the 5' end. Made into 5'Double-stranded DNA containing a T7 promoter (See Template Example 1) . The double-stranded DNA was used as a template for*in vitro*transcription reactionIf. the synthesized RNA is 800 base

(B) The following can be annealed to form double-stranded RNA, but the yield is lower than (B). PCR amplification was performed using a primer with a T7 promoter sequence added to the 5' end and a conventional primer to produce the amplified product of Template Example 2. The same amount of PCR amplified product was used as a template and the same amount of PCR amplified product was used simultaneously in the same tube.*in vitro*transcription reaction. Alternatively, use the PCR amplification product as a template for*in vitro*After the transcription reaction, double-stranded RNA is annealed. The yield is higher than (A).

(C) Clone the desired RNA fragments in the forward and reverse directions into a vector containing a T7 promoter sequence, and then linearize them separately (see Template Example 3). Two equal amounts of linearized plasmids were used as templates and the reaction was performed simultaneously in one tube.*in vitro*transcription reaction. Or separately*in vitro*After the transcription reaction, double-stranded RNA is annealed.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T7 Promoter 序列
PCR
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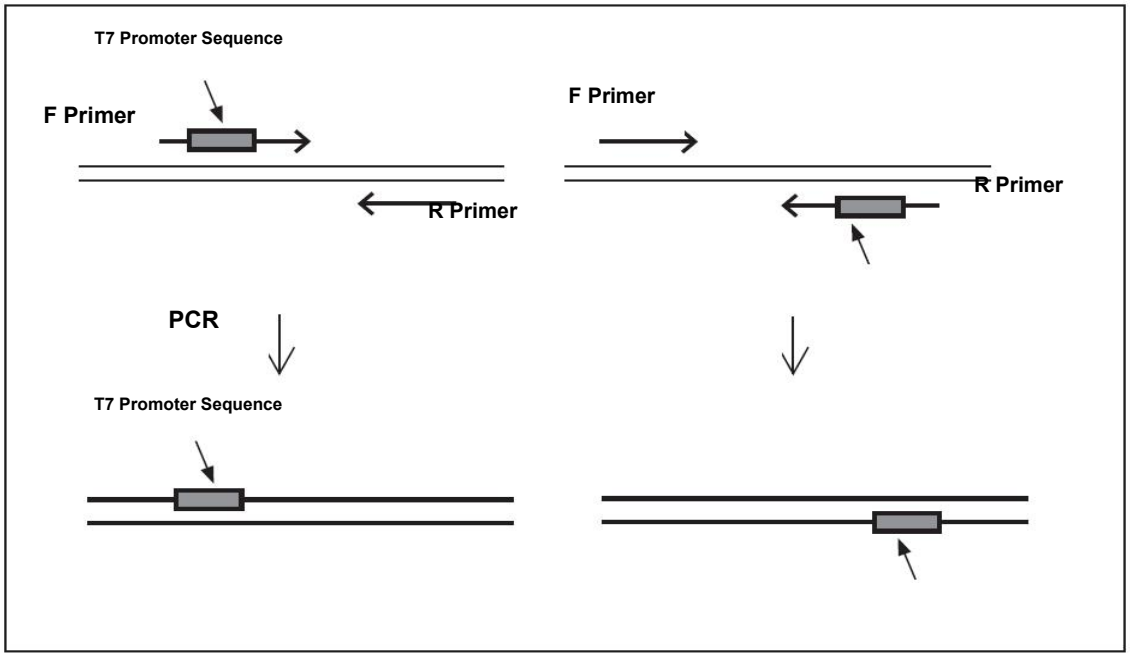
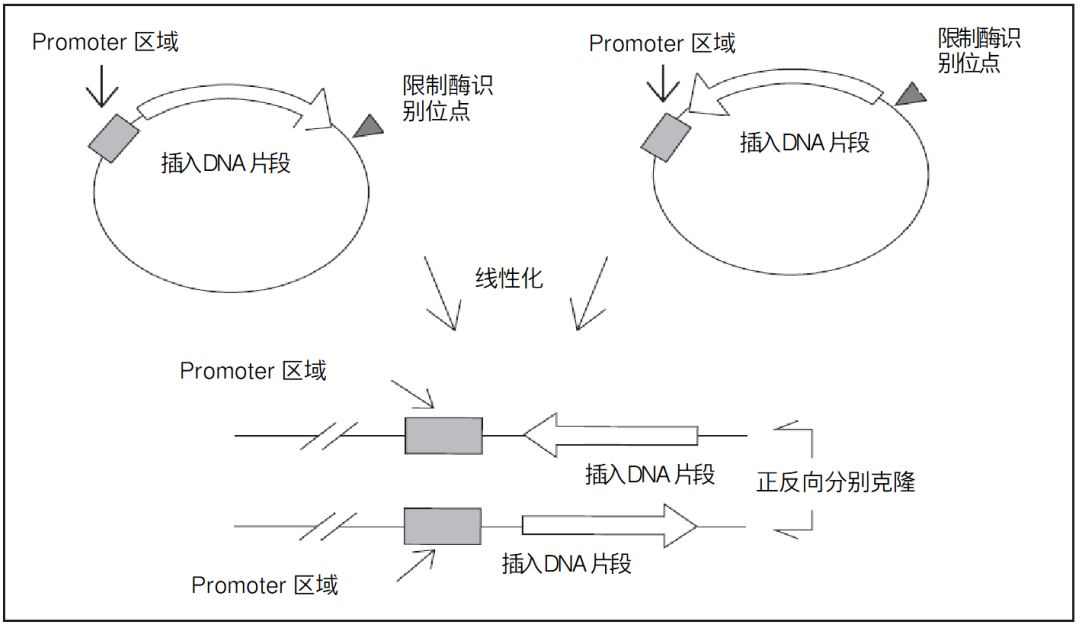
T7 Promoter 序列

模板例 1



Promoter Area



模板例 3

● Troubleshooting

1. Low RNA synthesis

(1) Template DNA is mixed with RNase or EDTA, etc.

→ Template DNA is treated with Proteinase K.

Proteinase K at a final concentration of 100 μg/ml and SDS at a final concentration of 0.5% were added to the template solution. After incubation at 37°C for 30 minutes, the solution was treated with phenol/chloroform, ethanol precipitated, and then washed with 80% ethanol.

(2) The template DNA contains NaCl.

→ High concentrations of NaCl (>30 mM) reduce RNA polymerase activity. Remove the NaCl by desalting (using a spin column or performing another ethanol precipitation and washing twice with 80% ethanol).

(3) Small amount of template DNA.

→ The prepared template DNA needs to measure not only the absorbance (OD260) , electrophoresis confirmation is also required to ensure the accuracy of the template DNA amount.

(4) Short reaction time.

→ Ensure the reaction time is 1-2 hours. Depending on the template DNA, the reaction time may need to be extended. Try extending the reaction time to approximately 4 hours.

2. Multiple reaction products or no desired product may be observed.

(1) A restriction enzyme that forms a 3' overhang was used.

→ When plasmid DNA is used as a template, if 3' overhangs are formed during linearization, very long transcription RNA will be synthesized (see reference 3) Do not use restriction enzymes that form 3' overhangs. If other restriction enzymes cannot be used, perform smoothing. (2) Incomplete enzyme digestion during plasmid linearization.

→ Confirm the linearization by electrophoresis. If the enzyme digestion is incomplete, perform the enzyme digestion again.

(3) RNA forms secondary structures during electrophoresis.

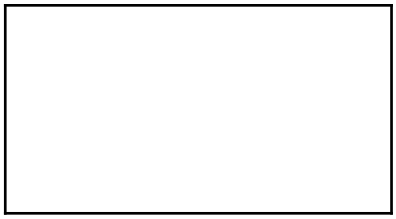
→*in vitro*RNA synthesized in the transcription reaction is typically electrophoresed using nondenaturing gel electrophoresis. If secondary structures are formed, larger bands or double bands may appear. In this case, use denaturing gel electrophoresis. Alternatively, mix the synthesized RNA with denaturing buffer (64% formamide, 26 mM MOPS, pH 7.2, 6.45 mM sodium acetate, 0.6 mM EDTA) and perform electrophoresis at 65°C for 15 minutes to eliminate bands associated with RNA secondary structures.

● References

(1) Davanloo, P., Rosenberg, AH., Dunn, JJ., Studier, FW. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. :*Proc Natl Acad Sci USA*. (1984)81: 2035-2039.
(2) Browning, K S. Transcription and translation of mRNA from polymerase chain reaction-generated DNA. *Amplifications*. (1989)3: 14-15.
(3) Schenborn, E T. and Mierendorf Jr, R C. A novel transcription property of SP6 and T7 RNA polymerase : dependence on template structure.*Nuc Acids Res.*(1985)13: 6223-6236.
(4) Heinonen, J E., Smith, CI., and Nore, B F. Silencing of Bruton's tyrosine kinase (Btk) using short interfering RNA duplexes (siRNA).*FEBS Letter*. (2002)527: 274-278.

● Related products

T7 RNA Polymerase (Code No. 2540A/B)
NucleoSpin Gel and PCR Clean-up (Code No. 740609.10/.50/.250)
CHROMA SPIN™+TE-30 Columns (Code No. 636069) NucleoSpin RNA Clean-up (740948.10/.50/.250)
NucleoSpin RNA Clean-up XS (740903.10/.50/.250)



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