Cat. # RR014A

For Research Use

TaKaRa

PrimeScript™ RT-PCR Kit

Product Manual

v202108Da

Table of Contents

I. Description

PCR (Polymerase Chain Reaction) is a process that amplifies specific DNA fragments by using two different primers on either end of the target DNA region. PCR is a simple and powerful tool, but it cannot be used to directly amplify RNA. However with RT-PCR, cDNA is synthesized from RNA using reverse transcriptase and a target region is amplified by PCR using the cDNA as a template, thereby enabling the PCR process to be applied for RNA analysis. The RT-PCR method can be used in various applications including structural analysis of RNA, efficient cDNA cloning, and analysis of gene expression at the RNA level.
This kit for 2-step RT-PCR, is designed to provide excellent extensibility and efficient amplification. PrimeScript reverse transcriptase was developed by Takara Bio Inc. based on RTase from M-MLV. PrimeScript RTase shows excellent extensibility for the RNA template. For PCR, *TaKaRa Ex Taq*® HS polymerase is used. This is hot start PCR enzyme enables amplification with high efficiency and high specificity. Combining our RT-PCR technology with this enzyme allows this kit to efficiently generate RT-PCR products at standard reverse transcription temperature (42℃), even when RNA templates contain higher-order structures. It is not necessary to perform reactions at a high temperature when there is a risk of RNA decomposition.

This kit includes all reagents necessary for the reverse transcription of RNA to cDNA and cDNA amplification using PCR.

II. Components (50 reactions)*1

1.	PrimeScript RTase (for 2 step)	25 μl
2.	5X PrimeScript Buffer	200 μl
3.	RNase Inhibitor (40 U/μl)	25 μl
4.	dNTP Mixture (10 mM each)	150 μl
5.	Oligo dT Primer (2.5 μM)	50 μl
6.	Random 6 mers (20 μM)	50 μl
7.	*TaKaRa Ex Taq* HS (5 U/μl)	25 μl
8.	10X PCR Buffer II	250 μl
9.	Control F-1 Primer*2 (20 μM)	10 μl
10.	Control R-1 Primer*3 (20 μM)	10 μl
11.	Positive Control RNA (2×10^5 copies/μl)	20 μl
12.	RNase Free dH_2O	1 ml

＊1 50 reactions [Reverse transcription 20 μl → PCR 50 μl]
＊2 Upstream sense primer for Positive Control RNA
＊3 Downstream anti-sense primer for Positive Control RNA

【Primer sequence】

Primer	Sequence
Random 6 mers	pd $(N)_6$
Oligo dT Primer	dT sequence: original design by Takara Bio*4
Control F-1 Primer	5'-CTGCTCGCTTCGCTACTTGGA-3'
Control R-1 Primer	5'-CGGCACCTGTCCTACGAGTTG-3'

＊4 This sequence is different from TaKaRa RNA PCR™ Kit (AMV) Ver. 3.0 (Cat. #RR019A/B), Oligo dT Adaptor Primer, and it does not contain M13 Primer M4 complementary section.

【Positive Control RNA】
The supplied Positive Control RNA is an RNA prepared by *in vitro* transcription using SP6 RNA polymerase from plasmid pSPTet3 containing a DNA fragment (approximately 1.4 kb) from the tetracycline resistance gene from pBR322 downstream of a SP6 promoter.

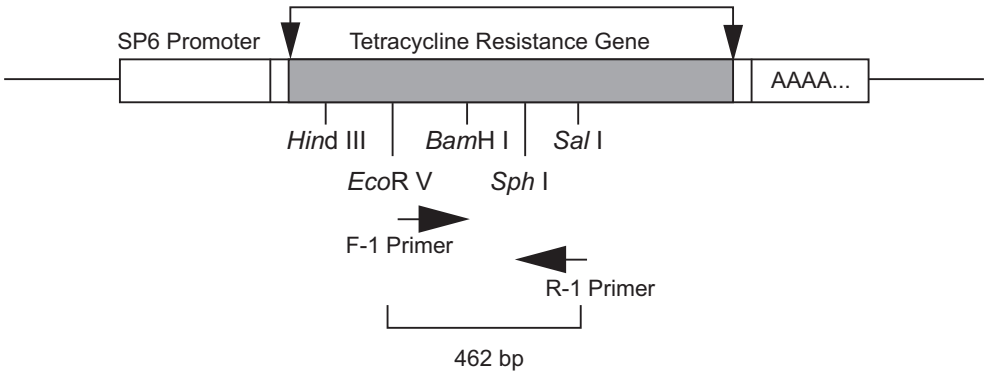


Figure 1. Control RNA: Amplified DNA fragment using Control Primers

Materials Required but not Provided

1. DNA Amplification System (authorized instruments)
   TaKaRa PCR Thermal Cycler Dice™ *Touch* (Cat. #TP350)*
   TaKaRa PCR Thermal Cycler Dice Gradient (Cat. #TP600)*, etc.
2. Agarose gel
   Agarose L03 [TAKARA] (Cat. #5003/5003B)
   PrimeGel™ Agarose PCR-Sieve (Cat. #5810A), etc.
3. Electrophoresis Apparatus
4. Microcentrifuge
5. Micropipettes and pipette tips (autoclaved)

＊ Not available in all geographic locations. Check for availability in your area.

III. Storage

-20℃

IV. Principle

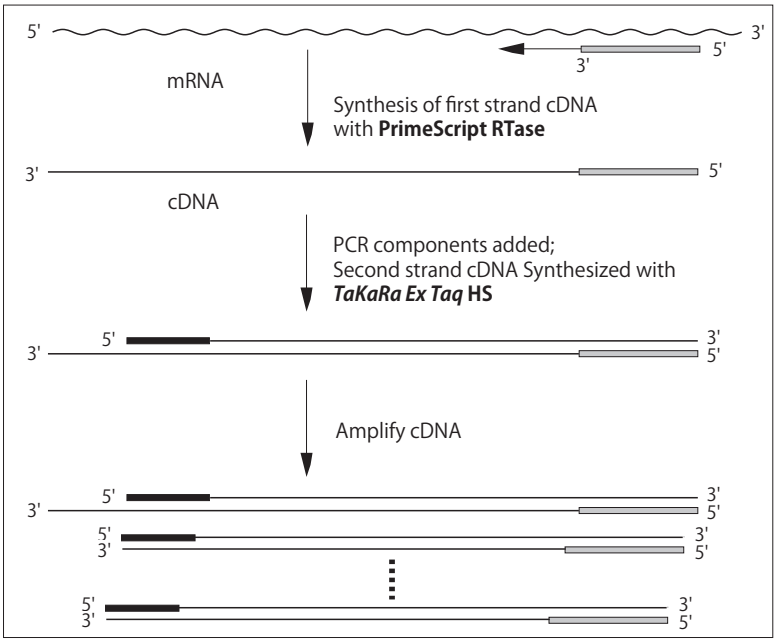


Figure 2. Principle of PrimeScript RT-PCR Kit

Reverse Transcription
Mix reverse transcription primer, dNTP mixture, and template RNA.
↓
65℃ 5 min
4℃
↓
Add buffer, enzyme, and RNase inhibitor.
↓
30℃ 10 min*
42 (- 50)℃ 15 - 30 min
95℃ 5min
4℃

* Necessary when Random 6 mers are used.

↓

Use a portion of the reverse transcription mixture as a template for PCR using *TaKaRa Ex Taq* HS.

Example
94℃ 30 sec
55 - 65℃ 30 sec
72℃ 1 min/kb
30 cycles

Figure 3. Flowchart of PrimeScript RT-PCR Kit

This kit is designed to perform reverse transcription of RNA to cDNA by using PrimeScript RTase and subsequent PCR amplification using a portion of the synthesized 1st strand cDNA as a template and *TaKaRa Ex Taq* HS.
Random 6 mers, Oligo dT Primer or the specific downstream primer can be used for cDNA synthesis of the RNA.

V. Features

RNA template	General
Amplified size	~ 12 kb
Reverse Transcriptase	PrimeScript RTase (preferred temperature, 42℃)
DNA Polymerase	*TaKaRa Ex Taq* HS
RNase Inhibitor	Supplied in the Kit
Primer for 1st strand cDNA synthesis	Random 6 mers, Oligo dT Primer, or specific downstream primer.

VI. Notes

Read carefully before using the kit.

1. For both reverse transcription and PCR amplification, prepare a master mix of reagents for all samples and aliquot to individual tubes. A master mix allows accurate reagent dispensing, minimizing pipetting errors and avoiding repeated dispensing of each reagent. This will also help to minimize variation of the data from experiment to experiment.
2. PrimeScript RTase, RNase Inhibitor, and *TaKaRa Ex Taq* HS should be mixed gently. Avoid generating bubbles. Gently spin down the solution prior to pipetting. Pipet the enzymes slowly as the enzyme contains 50% glycerol and is very viscous.
3. Keep the enzymes at -20℃ until just before use and return to the freezer promptly after use.
4. Avoid repeated freeze-thawing of the Positive Control RNA. Aliquot and store the Positive Control RNA at -70 to -80℃.
5. Use new disposable pipette tips to avoid contamination between samples for transferring reagent.

[Primer Selection]

The primer for reverse transcription should be selected from any of the following 3 types: Random 6 mers, Oligo dT Primer, or specific downstream primer.

Oligo dT Primer:

Use only for reverse transcription of mRNA with polyA tails.

(Note: RNA of prokaryotes, rRNA and tRNA of eukaryotes, and mRNA of some species of eukaryotes do not have polyA tails.)

Random 6 mers:

Best used for reverse transcription of long RNAs, and RNA with hairpin structures. Also, it can be used to reverse transcribe all types of RNA (rRNA, mRNA, tRNA, etc.).

Specific downstream primer (anti-sense primer for PCR):

Requires target sequence to synthesize oligonucleotide that has complementary sequences with the template.

VII. Protocol

<A. Denaturation of template RNA and reverse transcription>

A-1. Prepare the following reaction mixture

Reagent	Volume
dNTP Mixture (10 mM each)	1 μl
Oligo dT Primer (2.5 μM) or Random 6 mers (20 μM) or Specific Primer (2 μM)*1	1 μl
Template RNA*2 (or Positive Control RNA	2 μl)
RNase Free dH_2O	up to 10 μl

＊1 The primer for 1st strand cDNA synthesis should be either Oligo dT Primer, Random 6 mers, or a specific downstream primer (for Control RNA, use R-1 Primer). (See Notes from previous section, "[Primer Selection]" for selecting the appropriate primers to use.)

＊2 Template RNA: use up to 8 μl. When total RNA is used, up to 5 μg can be added (recommended amount: 100 pg - 1 μg).

A-2. Place the tubes in the Thermal Cycler and set the parameters according to the following conditions for denaturation and annealing.

65℃ 5 min
4℃

NOTE: This denaturation/annealing process facilitates reverse transcription by ensuring efficient denaturation of template RNA and efficient annealing of reverse transcription primer to template RNA.

A-3. Prepare the reagent mixture by combining the reagents in the proportions shown as below.

Reagent	Volume
Reaction mixture from A-2	10 μl
5X PrimeScript Buffer	4 μl
RNase Inhibitor (40 U/μl)	0.5 μl
PrimeScript RTase (for 2 step)	0.5 μl
RNase Free dH_2O	5 μl
Total	20 μl per sample

A-4. Place the tubes in a Thermal Cycler and perform reverse transcription using the following program.

(30℃	10 min)*3
42 (- 50)℃	15 - 30 min
95℃	5 min*4
4℃	

*3 When use Random 6 mers at reverse transcription, react the mixture at 30℃ for 10 minutes before 42℃ incubation. The efficiency of reverse transcription can be increase by elongation of Random 6 mers during 30℃ incubation.

*4 For amplification of longer targets, inactivation at 70℃ for 15 min is recommended, so there will be no damage to the 1st strand cDNA (i.e., nicking).

NOTE: In general, the reaction temperature should be 42℃ for PrimeScript RTase. However, when using specific downstream primers like the reverse primer for PCR, there may be some unusual amplification due to mispriming. This can be avoided by changing the reaction temperature to 50℃.

<B. Reaction of PCR>

B-1. Prepare the reaction mixture by combining the following reagents.

Reagent	Volume	Final conc.
10X PCR Buffer II	5 μl	1X
dNTP Mixture (10 mM each)	2 μl	400 μM
Upstream Primer (20 μM)*5 (Sense)	0.5 μl	0.2 μM
Downstream Primer (20 μM)*6 (Anti-sense)	0.5 μl	0.2 μM
TaKaRa Ex Taq HS (5 U/μl)	0.5 μl	
Reverse transcription mixture	≦ 5 μl	
Sterile water	up to 50 μl	

＊5 Use F-1 Primer, for Positive Control RNA.
＊6 Use R-1 Primer, for Positive Control RNA.

B-2. After mixing reagents, put all tubes in the thermal cycler, and start PCR using the following conditions.

General Condition

(A) 3 step PCR

94℃	30 sec	┐
55 - 65℃	30 sec	├ 30 cycles
72℃	1 min/kb	┘

(B) 2 step PCR

98℃	10 sec	┐
68℃	1 min/kb	┘ 30 cycles

Positive Control RNA*7

94℃	30 sec	┐
60℃	30 sec	├ 30 cycles
72℃	1 min	┘

＊7 A 462 bp amplification product is obtained by PCR using Control Primer F-1 and R-1.

VIII. PCR Conditions

- Annealing Temperature
  60℃ is optimal for amplification of the Control RNA; however, is necessary to change the annealing temperature (55 - 65℃) depending on the specific target. It may be necessary to determine the optimal annealing temperature experimentally in the range of 45 - 65℃.

- Extension time
  The extension time depends on the target length. Usually, *TaKaRa Ex Taq* HS extends DNA at 1 kb per minute at 72℃.

- Number of cycles
  40 - 50 cycles are recommended if the cDNA amount is small.

- Most of the PCR products amplified using this kit have a 3' A overhang. Therefore, it is possible to clone the PCR product directly into a T-Vector. In addition, it is possible to clone into a blunt end vector by blunting the ends and phosphorylation. This can be done using Mighty Cloning Reagent Set (Blunt End)(Cat. #6027) for blunt end vector cloning.

IX. Applications

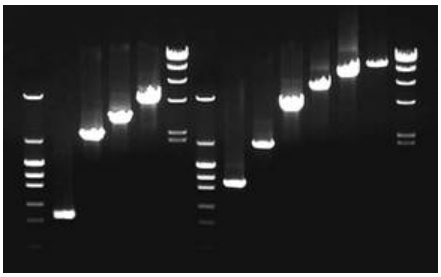
1. Using human heart total RNA or HL60 cell total RNA as a template, targeted genes of various lengths were reverse transcribed and subsequently amplified.

   Reverse transcription; 1 μg total RNA/20 μl RT reaction
   ↓
   PCR; 2 μl reverse transcription reaction/50 μl PCR reaction
   (total RNA equivalent of 100 ng/50 μl PCR reaction)

Target gene	total RNA used
Dystrophin Transferrin receptor (TFR)	Human Heart HL60 cell

PCR Condition:

0.5 - 6 kb			8 - 12 kb		
94℃	1 min		94℃	1 min	
↓			↓		
94℃	30 sec	⎫	98℃	10 sec	⎫
55℃	30 sec	⎬ 30 cycles	68℃	8 or 15 min	⎬ 30 cycles
72℃	1 min/kb	⎭			⎭

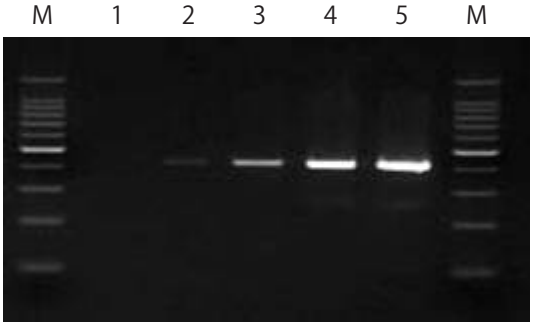


M1: pHY Marker
1: TFR 0.5 kb
2: TFR 2.2 kb
3: TFR 3 kb
4: TFR 4.4 kb
5: Dystrophin 1 kb
6: Dystrophin 2 kb
7: Dystrophin 4 kb
8: Dystrophin 6 kb
9: Dystrophin 8 kb
10: Dystrophin 12 kb
M2: λ-*Hin*d III digest

Excellent extension and amplification were confirmed at 0.5 - 12 kb.

2. HL60 total RNA was used as a the template. GAPDH gene expression was measured by RT-PCR using Oligo dT Primers for reverse transcription. The kit protocol was followed for RT and PCR.

Target: GAPDH 428 bp
PCR Condition: 94℃ 1 min
↓
94℃ 30 sec
55℃ 30 sec
72℃ 1 min
] 40 cycles



RNA template (total RNA)
1: 0.1 pg
2: 1 pg
3: 10 pg
4: 100 pg
5: 1 ng
M: 100 bp DNA Ladder

The detection was possible even when using 1 pg of total RNA.

X. Preparation of RNA Samples

This kit is designed to perform reverse transcription of RNA to cDNA and subsequent amplification. It is important to use a high purity RNA sample for greater yields of cDNA. Therefore, it is essential to inhibit the activity of RNase in the cells and to prevent contamination of RNase derived from equipment and solutions used. Additional precautions should be taken during the sample preparation, such as using clean disposable gloves, dedicating a workspace exclusively for RNA preparation, and avoiding unnecessary talking during operation to prevent contamination of RNase from sweat or saliva.

【 Equipment 】

Disposable plastic equipment should be used. For glass tools, treat with the following procedure prior to use.

(1) Treat the glass tools with 0.1% DEPC at 37℃ for 12 hours.
(2) Autoclave at 120℃ for 30 min to remove DEPC.

It is recommended that RNase-OFF® (Cat. #9037) be used for RNase removal from table, instruments, tubes, and others. It is also recommended that all equipment be used exclusively for RNA preparation.

【 Preparation of RNA sample 】

Simple purification methods can yield enough RNA for reverse transcription and subsequent PCR; however, it is recommended to use highly purified RNA obtained by the GTC (Guanidine thiocyanate) method, etc.

XI. Related Products

PrimeScript™ Reverse Transcriptase (Cat. #2680A/B/C)
PrimeScript™ II Reverse Transcriptase (Cat. #2690A/B/C)
PrimeScript™ One Step RT-PCR Kit Ver.2 (Cat. #RR055A/B)
PrimeScript™ One Step RT-PCR Kit Ver.2 (Dye Plus) (Cat. #RR057A)
PrimeScript™ High Fidelity RT-PCR Kit (Cat. #R022A/B)*
PrimeScript™ 1st strand cDNA Synthesis Kit (Cat. #6110A/B)
PrimeScript™ II 1st strand cDNA Synthesis Kit (Cat. #6210A/B)*
TaKaRa PCR Thermal Cycler Dice™ *Touch* (Cat. #TP350)*
TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600)*
Agarose L03 [TAKARA] (Cat. #5003/5003B)
PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)
RNase-OFF® (Cat. #9037)
Mighty Cloning Reagent Set (Blunt End) (Cat. #6027)

* Not available in all geographic locations. Check for availability in your area.