Cat. # RR019A

For Research Use

TaKaRa

TaKaRa RNA PCR™ Kit (AMV) Ver.3.0

Product Manual

v202006Da

Table of Contents

I. Description

PCR (Polymerase Chain Reaction) is a simple and powerful method which allows *in vitro* amplification of DNA fragments through a succession of three incubation steps at different temperatures. In principle, PCR is a method to amplify DNA segments, and not directly amplify RNA. However, synthesis of cDNA from RNA using reverse transcriptase enables to apply PCR to the RNA analysis. Many reports of various fields have been made by applying this method, such as of structual analysis of RNA, efficient cDNA cloning, analysis of gene expression at the RNA level, etc.
TaKaRa RNA PCR Kit (AMV) Ver.3.0 is designed to perform the reverse transcription of RNA to cDNA using AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and subsequent PCR amplification using *TaKaRa Ex Taq* HS® in a single tube. By including all reagents necessary for the reverse transcription and for subsequent cDNA amplification, this kit allows simple and efficient analysis of RNA.
The Oligo dT-Adaptor Primer is improved to elevate efficiency of cDNA synthesis from 3'-termini of poly(A)$^+$ RNA. This enables amplificatoin of unknown 3'-termini utilizing 3'-RACE System. As this kit uses the enzyme for Hot Start PCR, *TaKaRa Ex Taq* HS, non-specific amplification deriving from mispriming or primer-dimers can be avoided.

II. Components (For 100 reactions*1)

1.	AMV Reverse Transcriptase XL (originated from Avian Myeloblastosis Virus)	(5 U/μl)	50 μl
2.	RNase Inhibitor	(40 U/μl)	25 μl
3.	Random 9 mers*2	(50 pmol/μl)	50 μl
4.	Oligo dT-Adaptor Primer*2	(2.5 pmol/μl)	50 μl
5.	RNase Free dH_2O		1 ml
6.	*TaKaRa Ex Taq* HS	(5 U/μl)	25 μl
7.	M13 Primer M4*2	(20 pmol/μl)	50 μl
8.	10X RT Buffer 100 mM Tris-HCl (pH 8.3) 500 mM KCl		1 ml
9.	5X PCR Buffer		1 ml
10.	dNTP Mixture	(10 mM each)	150 μl
11.	$MgCl_2$	(25 mM)	1 ml
12.	Control R-1 Primer*2 (downstream primer for Positive Control RNA)	(20 pmol/μl)	25 μl
13.	Control F-1 Primer*2 (upstream primer for Positive Control RNA)	(20 pmol/μl)	25 μl
14.	Positive Control RNA*3 (transcribed poly(A)$^+$ RNA of pSPTet3 plasmid)	(2 x 10^5 copies/μl)	25 μl

*1 One reaction means 10 μl RT followed by 50 μl PCR.
*2 Primers Sequence
- Random 9 mers : dp (5'-NNNNNNNNN-3')
- Oligo dT-Adaptor Primer: This original primer includes dT and the region to M13 Primer M4.
- Control F-1 Primer : 5'-CTGCTCGCTTCGCTACTTGGA-3'
- Control R-1 Primer : 5'-CGGCACCTGTCCTACGAGTTG-3'
- M13 Primer M4 : 5'-GTTTTCCCAGTCACGAC-3'

*3 Positive Control RNA
Supplied control RNA is *in vitro* transcribed RNA using SP6 RNA polymerase from plasmid pSPTet3 inserted with DNA fragment (approximately 1.4 kb) having tetracycline resistant gene, originated from pBR322, in the downstream of SP6 promoter.
This control RNA is a poly(A)$^+$ RNA containing 30 bases of poly(A) at the tail. When full-length double-stranded cDNA is synthesized from this control RNA, tetracycline resistant plasmid is obtained by inserting this cDNA.

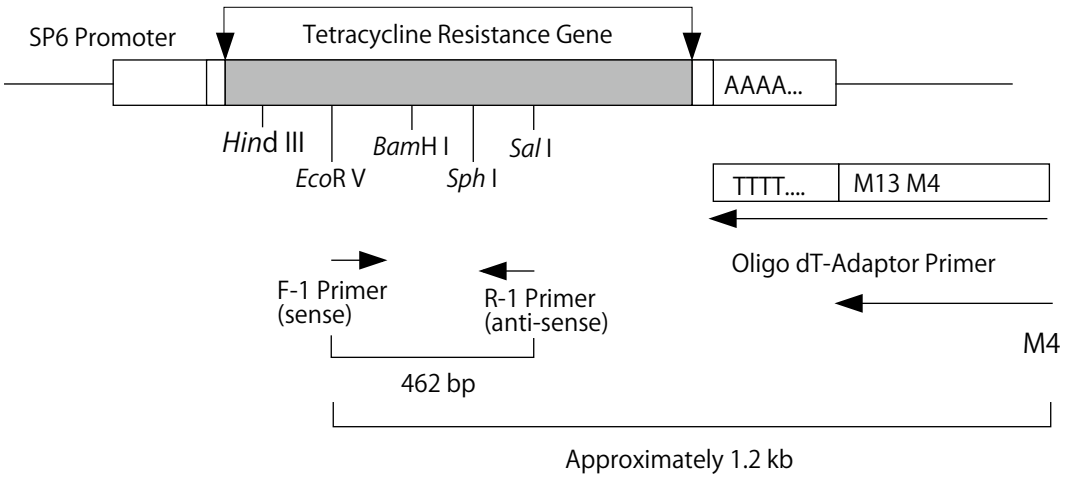

Figure 1. Amplified DNA fragments using Positive Control RNA and several primers

III. Materials Required but not Provided

1. Reagents
PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)
Agarose L03「TAKARA」(Cat. #5003/5003B)
PrimeGel Agarose LE 1-20K GAT (Cat. #5801A)

2. Materials
- Authorized instruments for PCR
  ex. TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600)
  TaKaRa PCR Thermal Cycler Dice *Touch* (Cat. #TP350)*
- Agarose gel electrophoresis apparatus
  ex. Mupid-2plus (Cat. #M-2P)
  Mupid-exU (Cat. #EXU-1)
  Mupid-One (Cat. # O1-01)
- Microcentrifuge
- Micropipets and pipette tips (autoclaved)

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

IV. Storage -20℃

V. Principles

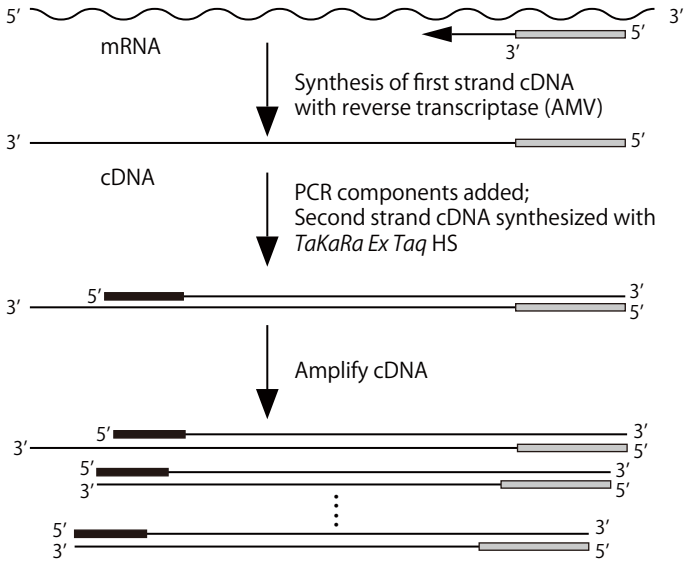

Figure 2. Schematic diagram of RNA PCR

mRNA

mRNA

Prepare reaction mixture by combining AMV RTase and other reagents necessary for 1st strand cDNA synthesis.

Condition of thermal cycling:

(30℃	10 min)*	1 cycle
42 - 60℃	15 - 30 min	
95℃	5 min	
5℃	5 min	

* Random 9 mers should be reverse transcribed at 30℃, 10 min, prior to cDNA synthesis to have enough length to be used for annealing at 42 - 60℃.

Into the same tube, add *TaKaRa Ex Taq* HS and other reagents necessary for PCR.

Condition of thermal cycling:

94℃	30 sec	25 - 30 cycles
55 - 65℃	30 sec	
72℃	1 min/kb	

cDNA

Perform agarose gel electrophoresis for the analysis of the amplified products.

This kit allows reverse transcription from RNA to cDNA using AMV RTase and subsequent amplification in the same tube utilizing *TaKaRa Ex Taq* HS DNA Polymerase. Radom 9 mers, Oligo dT-Adaptor Primer, or a specific downstream primer which acts as an anti-sense primer in PCR can be used for cDNA synthesis. Oligo dT-Adaptor Primer is used for 3'-RACE System.

VI. Features

Template RNA	General
RNA to be transcribed and amplified	≦ 5.0 kb
Reverse Transcriptase	AMV Reverse Transcriptase XL
DNA Polymerase	*TaKaRa Ex Taq* HS
RNase Inhibitor	Supplied in the kit
Primer for 1st strand cDNA synthesis	Random 9 mers, or Oligo dT-Adaptor Primer or Specific downstream PCR primer
3'-RACE System	This kit is available for 3'-RACE System by using Oligo dT-Adaptor Primer in RT, and by using M13 Primer M4 in PCR
Protocol	Single tube reaction (RTase is heat inactivated prior to PCR)

VII. Preparation of RNA Sample

This kit is designed to perform the reverse transcription of RNA to cDNA and subsequent amplification.
The purity of RNA sample will affect the yield of cDNA synthesis. So it is essential to inhibit the activity of RNase in the cells and also to prevent the contamination of RNase derived from equipments and solutions used. Extra precautions should be taken during the sample preparation; put on clean disposable gloves, dedicate a table to exclusive use for RNA preparation, and avoid unnecessary talks during the operations to prevent the contamination of RNase from operators' sweat or saliva.

A. Equipment

Disposable plastic equipments shall be used. In case using glass tools, treat the glass tools with DEPC (diethylpyrocarbonate) prior to use.

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

It is recommended to prepare all the equipments as the exclusive use for RNA preparation.

B. Reagent

Reagents for RNA preparation, including purified water, shall be prepared with heat sterilized glass tools (180℃, 60 min), or if possible those treated with 0.1% DEPC solution and autoclaved. Reagents and purified water should be exclusively used for RNA preparation.

C. Preparation method

It is necessary to prepare highly-pure RNA. Impurities such as polysaccharides and protein may inhibit the cDNA synthesis reaction. In addition, prevent genomic DNA contamination. Preparation of RNA from tissues and cells should be performed as quickly as possible after sample collection. If this is not possible, store samples at -80℃ or in liquid nitrogen.The guanidium thiocyanate phenol chloroform method (AGPC method), or a commercial RNA reagent or kit for the isolation and purification of RNA may be used.

Examples: RNAiso Plus (Cat. #9108/9109)
NucleoSpin RNA (Cat. #740955.10/.50/.250)

D. RNA Sample Amount

Approximately 500 ng of total RNA is appropriate per one reaction.

VIII. Notes

1) For both reverse transcription and PCR amplification, master mix of reagents (containing RNase Free dH2O, buffers, dNTP Mixtures, $MgCl_2$, etc) for all samples can be prepared first, then aliquoted to tubes. Using such mixtures will allow accurate reagents dispense: minimize reagents pipetting losses, and avoid repeat dispensing of the each reagent. This helps to minimize variation of the data among the experiments.

2) Enzymes such as RTase, *TaKaRa Ex Taq* HS, and RNase Inhibitor shall be mixed gently by pipetting. Avoid generating bubbles. Gently spin down the solution prior to mixing. Pipette enzymes carefully and slowly as the viscosity of the buffer can lead to pipetting errors.

3) Keep enzymes at -20℃ until just before use and return into the freezer promptly after use.

4) Use new disposable pipette tips to avoid contamination between samples.

5) PCR condition
Optimum PCR condition varies depending on the thermal cycler used for PCR. It is recommended to perform a control experiment to determine the condition prior to using a sample.

6) Primer selection
Depend on many factors, the primer for reverse transcription should be selected from either of Random 9 mers, Oligo dT-Adaptor Primer, or specific downstream PCR primer. For short mRNAs with no hairpin structure, any one of the above three primers can be used.

[General guideline of the primer selection]

Random 9 mers :
Use for the transcription of long RNAs or of RNA with hairpin structure. Also can be used to reverse transcribe all RNA (rRNA, mRNA, and tRNA). Any pairs of PCR primers work equally well in PCR of cDNA synthesized with Random 9 mers.

Specific downstream primer (anti-sense primer in PCR) :
Use for the target RNA which sequence is already determined.

Oligo dT-Adaptor Primer :
Use only for mRNAs with poly(A) tails (Note: Prokaryotic RNA, eukaryotic rRNA and tRNA, and some eukaryotic mRNA do not have poly(A) tails). This primer was designed originally by Takara Bio for efficient cDNA synthesis. It will allow 3'-RACE method utilizing M13 Primer M4 which is complementary to Adaptor region after reverse transcription.

IX. Protocol

Standard RT-PCR

A. Reverse Transcription

1. Prepare the reaction mixture in a tube as shown below. The primer for a cDNA synthesis should be chosen from either of Random 9 mers, Oligo dT-Adaptor Primer, or specific downstream primer. (Control R-1 Primer for the control experiment). See "VIII Notes 6) Primer selection".

Reagent	Amount	Final conc.
$MgCl_2$	2 μl	5 mM
10X RT Buffer	1 μl	1X
RNase Free H_2O	3.75 μl	
dNTP Mixture	1 μl	1 mM
RNase Inhibitor	0.25 μl	1 U/μl
AMV Reverse Transcriptase XL[*1]	0.5 μl	0.25 U/μl
Random 9 mers	0.5 μl	2.5 μM
or Oligo dT-Adaptor Primer		or 0.125 μM
or Specific downstream PCR primer (R-1 Primer)		or 1.0 μM
Positive Control RNA	1 μl	[2 x 10^5 copies]
or Experimental sample		or [≦ 500 ng total RNA]
Total	10 μl per sample	

2. Place the tube in a Thermal Cycler and set the parameters by the following condition.

(30℃	10 min)[*2]	
42 - 60℃[*1]	15 - 30 min	1 cycle
95℃	5 min[*3]	
5℃	5 min	

*1 AMV Reverse Transcriptase can work at 60℃. However, when using long RNA (> 2 kb), it is advisable to perform reverse transcription at 42℃. When Positive Control RNA is used as template, reverse transcription at 50℃ is recommended.

*2 When using Random 9 mers, perform reverse transcription in advance at 30℃ for 10 minutes to obtain enough length to anneal with primer at 42 - 60℃.

*3 AMV RTase binds to cDNA and inhibits PCR amplification. Heat treatment of 95℃, 5 minutes inactivates the reverse transcriptase and removes the inhibitory effect on PCR. If the concentration of AMV RTase increases, inactivation of AMV RTase becomes difficult. Therefore, for long RNA, it is advisable to increase the incubation time during AMV RTase rather than increase the amount of AMV RTase added.

B. PCR

1. Prepare reaction mixture.

(per 50 μl mixture)

Reagent	Amount	Final conc. in PCR reaction
5X PCR Buffer	10 μl	1X
Sterile purified water	28.75 μl	
TaKaRa Ex Taq HS	0.25 μl	1.25 U/50 μl
Upstream PCR primer (F-1 Primer for Control RNA)	0.5 μl	0.2 μM (20 pmol)
Downstream PCR primer*1	0.5 μl	0.2 μM (20 pmol)
[For Control RNA, R-1 Primer or M13 Primer M4 (when Oligo dT Adaptor primer is used in reverse transcription)]		
Total	40 μl per sample	

*1 When downstream PCR primer is used in reverse transcription, add 0.5 μl of sterile purified water instead of downstream primer.

2. Add 40 μl of the mixture into a tube containing the cDNA obtained at Step A-2.
3. Spin for approximately 10 sec with a microcentrifuge.
4. Place the tubes in a Thermal Cycler and perform PCR amplification under the optimal condition.*2

Standard Condition

94℃	30 sec	25 - 30 cycles
55 - 65℃	30 sec	
72℃	1 min/kb	

Positive Control RNA

94℃	30 sec	30 cycles
60℃	30 sec	
72℃	1 min	

*2 PCR condition

- Annealing temperature
  60℃ is optimal for amplification of the Control RNA; however, it is necessary to change the annealing temperature (55 - 65℃) depending on the targets. If amplification does not perform well, 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 cycle
  40 - 50 cycles are recommended if 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 or phosphorylation. The Mighty Cloning Reagent Set (Blunt End) (Cat. #6027) is available for blunt end vector cloning.

5. After the amplification is completed, apply 5 - 10 μl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments.*3

*3 The PCR amplified product can be stored frozen until subsequent analysis.

In a reaction with Positive Control RNA

Primer for reverse transcription	PCR Primer	Amplified fragment
Oligo dT-Adaptor Primer	F1 and M13 Primer M4	Approximately 1.2 kb
	or F-1 and R-1	462 bp
Random 9 mers	F-1 and R-1	462 bp
Control R-1 Primer	F-1 and R-1	462 bp

3'-RACE System

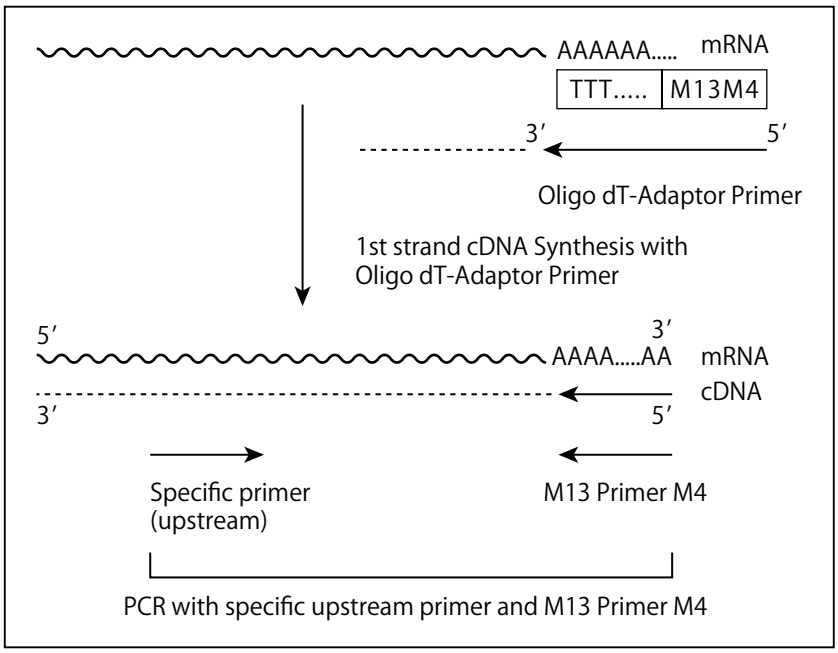

Figure 3. Schematic diagram RT-PCR with 3'-RACE System

A. Reverse Transcription

1. Prepare the reaction mixture in a tube.

Reagent	Amount	Final conc.
$MgCl_2$	2 μl	5 mM
10X RT Buffer	1 μl	1X
RNase Free H_2O	3.75 μl	
dNTP Mixture	1 μl	1 mM
RNase Inhibitor	0.25 μl	1 U/μl
Reverse Transcriptase	0.5 μl	0.25 U/μl
Oligo dT-Adaptor Primer	0.5 μl	0.125 μM
total RNA (500 ng/μl)	1 μl	500 ng/10 μl
Total	10 μl	

2. Place all tubes in a Thermal Cycler and set the parameters by the following condition.

42 - 60℃ 30 min
95℃ 5 min
5℃ 5 min
] 1 cycle

B. PCR

1. Prepare reaction mixture.

(per 50 μl mixture)

Reagent	Amount	Final conc. in PCR reaction
5X PCR Buffer	10 μl	1X
Sterile purified water	28.75 μl	
TaKaRa Ex Taq HS	0.25 μl	1.25 U/50 μl
M13 Primer M4 (20 μM)	0.5 μl	0.2 μM
Specific upstream primer (20 μM)	0.5 μl	0.2 μM
Total	40 μl	per sample

2. Add 40 μl of the mixture into a tube containing the cDNA obtained at Step A-2.

3. Place the tubes in a Thermal Cycler and perform amplification by the following condition.

94℃ 30 sec
55℃ 30 sec
72℃ 0.5 - 5 min
] 30 cycles

4. After the amplification is completed, apply 5 μl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments. Target cDNA can be verified by the amplified fragment.

X. References

1) Kawasaki E S and Wang A M. *PCR Technology* (Erlich, H. A. ed.), *Stockton Press*. (1989) 89-97.
2) Lynas C, Cook S D, Laycock K A, Bradfield J W B, and Maitland N J. *J Pathology*. (1989) **157**: 285-289.
3) Frohman M A, Dush M K, and Martin G R. *Proc Natl Acad Sci USA.* (1988) **85**: 8998-9002.

XI. Related Products

Reverse Transcriptase XL (AMV) for RT-PCR (Cat. #2630A)
Recombinant RNase Inhibitor (Cat. #2313A/B)
Ribonuclease Inhibitor (Porcine liver) (Cat. #2311A/B)
TaKaRa Ex Taq® Hot Start Version (Cat. #RR006A/B)
Random Primer (pd(N)$_9$) (Cat. #3802)
Mighty Cloning Reagent Set (Blunt End) (Cat. #6027)
PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)
Agarose L03「TAKARA」(Cat. #5003/5003B)
PrimeGel Agarose LE 1-20K GAT (Cat. #5801A)
TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600)
TaKaRa PCR Thermal Cycler Dice™ *Touch* (Cat. #TP350)*
Mupid-2plus (Cat. #M-2P)
Mupid-exU (Cat. #EXU-1)
Mupid-One (Cat. # O1-01)

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