

For Research Use

TaKaRa

**TaKaRa RNA LA PCR™ Kit
(AMV) Ver.1.1**

Product Manual

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I. Description

TaKaRa RNA LA PCR Kit (AMV) Ver.1.1 is designed to achieve longer and more accurate PCR with *TaKaRa LA Taq*® following reverse transcription. *TaKaRa LA Taq* is an enzyme developed by Takara Bio as the key component of LA (long and accurate) PCR. Based on the advantage of LA Technology, this kit performs reverse transcription of RNA to cDNA using AMV (Avian Myeloblastosis Virus) Reverse Transcriptase and subsequent amplification of long cDNA using *TaKaRa LA Taq* all in a single tube.

The supplied Oligo dT-Adaptor Primer is designed to allow more efficient cDNA synthesis from 3'-termini of poly A⁺ RNA. This enables amplification of unknown 3'-termini utilizing 3'-RACE System.

II. Components (for 100 reactions*1)

1.	AMV Reverse Transcriptase XL *2 (originated from Avian Myeloblastosis Virus)	(5 U/μl)	50 μl
2.	RNase Inhibitor	(40 U/μl)	25 μl
3.	Random 9 mers *3	(50 pmol/μl)	50 μl
4.	Oligo dT-Adaptor Primer *3	(2.5 pmol/μl)	50 μl
5.	RNase Free dH ₂ O		1 ml
6.	<i>TaKaRa LA Taq</i>	(5 U/μl)	25 μl
7.	M13 primer M4 *3	(20 pmol/μl)	50 μl
8.	10X RNA PCR Buffer 100 mM Tris - HCl (pH 8.3) 500 mM KCl		120 μl
9.	10X LA PCR Buffer II (Mg ²⁺ free)		500 μl
10.	dNTP Mixture	(each 10 mM)	150 μl
11.	MgCl ₂	(25 mM)	1 ml
12.	Control R-1 primer *3 (downstream primer for Positive control RNA)	(20 pmol/μl)	25 μl
13.	Control F-1 primer *3 (upstream primer for Positive control RNA)	(20 pmol/μl)	25 μl
14.	Positive control RNA *4 (transcribed poly A ⁺ RNA of pSPTet3 plasmid)	(2 x 10 ⁵ copies/μl)	25 μl

* 1 This kit is designed for 100 reactions. (the total reaction amount ; RT reaction 10 μl, PCR 50 μl)

* 2 Manufactured by Life Sciences Advanced Technologies, Inc.

* 3 Primers Sequence

• Random 9 mers :

5' - NNNNNNNNNN - 3'

• Oligo dT-Adaptor Primer :

The original primer includes dT and complementary region to M13 primer M4.

• Control F-1 primer :

5' - CTGCTCGCTTCGCTACTTGGA - 3'

• Control R-1 primer :

5' - CGGCACCTGTCTACGAGTTG - 3'

• M13 primer M4 :

5' - GTTTTCCCAGTCACGAC - 3'

* 4 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 in an appropriate plasmid.

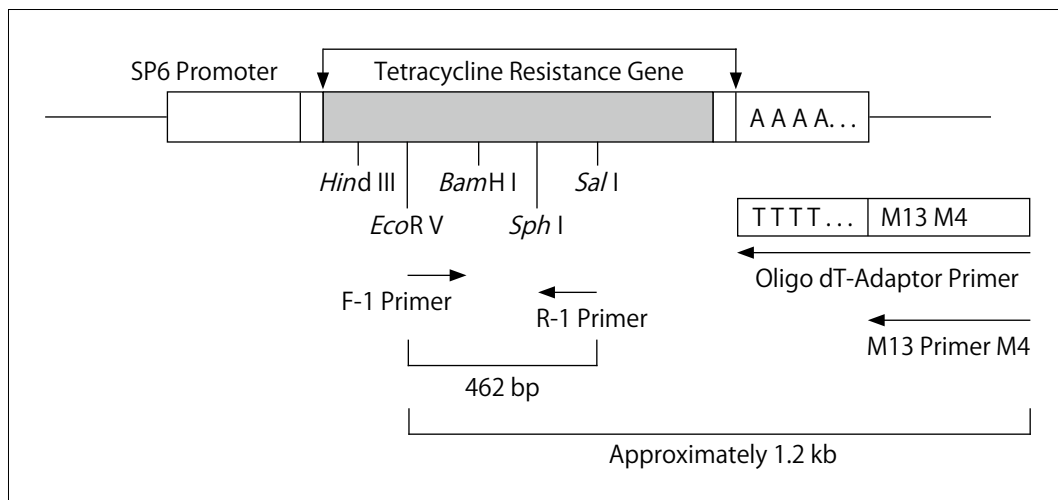


Figure 1. Amplified DNA fragments using control RNA and several primers

III. Materials Required but not Provided

1. Reagents

- Agarose
 - ex. 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)

IV. Storage -20°C

V. Principles

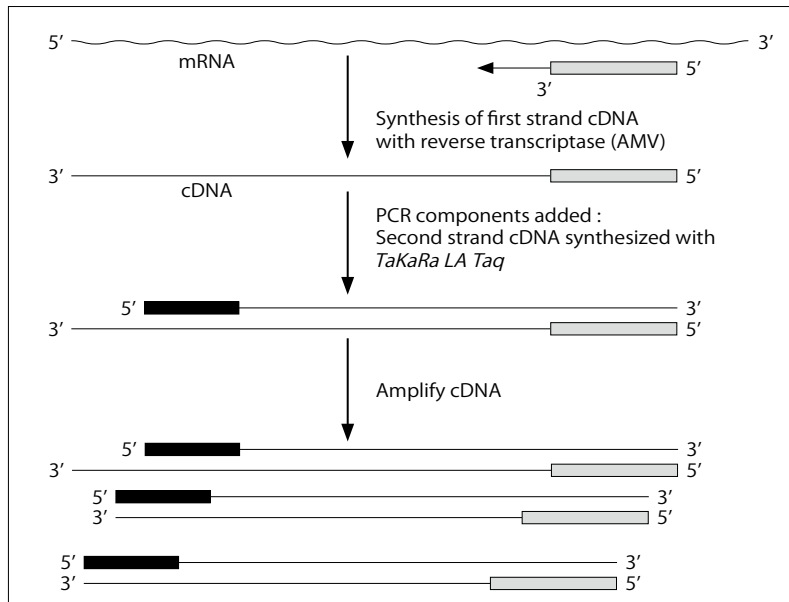


Figure 2. Schematic diagram of RNA LA PCR

mRNA												
<p>Prepare reaction mixture by combining AMV RTase and other reagents for reverse transcription.</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding-right: 20px;">RT-reaction :</td> <td style="padding-right: 20px;">(30°C 10 min)*</td> <td rowspan="4" style="font-size: 3em; padding: 0 10px;">}</td> <td rowspan="4" style="vertical-align: middle;">1 cycle</td> </tr> <tr> <td></td> <td>42 - 60°C 15 - 30 min</td> </tr> <tr> <td></td> <td>99°C 5 min</td> </tr> <tr> <td></td> <td>5°C 5 min</td> </tr> </table> <p>* Random 9 mers should be reverse transcribed at 30°C, 10 min, prior to cDNA synthesis to have enough length to be used for annealing at 42 - 60°C.</p>	RT-reaction :	(30°C 10 min)*	}	1 cycle		42 - 60°C 15 - 30 min		99°C 5 min		5°C 5 min		
RT-reaction :	(30°C 10 min)*	}			1 cycle							
	42 - 60°C 15 - 30 min											
	99°C 5 min											
	5°C 5 min											
<p>Add <i>TaKaRa LA Taq</i> into the same tube and other reagents necessary for PCR reaction.</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td style="padding-right: 20px;">PCR reaction :</td> <td style="padding-right: 20px;">94°C 2 min</td> <td rowspan="2" style="font-size: 3em; padding: 0 10px;">}</td> <td rowspan="2" style="vertical-align: middle;">1 cycle</td> </tr> <tr> <td></td> <td>94°C 0.5 min</td> </tr> <tr> <td></td> <td>60°C 0.5 min</td> <td rowspan="2" style="font-size: 3em; padding: 0 10px;">}</td> <td rowspan="2" style="vertical-align: middle;">28 cycles</td> </tr> <tr> <td></td> <td>72°C 1.5 min</td> </tr> </table>	PCR reaction :	94°C 2 min	}	1 cycle		94°C 0.5 min		60°C 0.5 min	}	28 cycles		72°C 1.5 min
PCR reaction :	94°C 2 min	}			1 cycle							
	94°C 0.5 min											
	60°C 0.5 min	}	28 cycles									
	72°C 1.5 min											
PCR product												
<p>Perform agarose gel electrophoresis to verify the amplified products.</p>												

This kit allows reverse transcription from RNA to cDNA using AMV RTase and subsequent amplification in the same tube utilizing *TaKaRa LA Taq*.

Random 9 mers, Oligo dT-Adaptor Primer or a specific downstream primer which act as an antisense primer in PCR can be used for cDNA synthesis. Oligo dT-Adaptor Primer is used for 3'-RACE System.

VI. Features

Template RNA	General
Amplified size	up to 12 kb
Reverse Transcriptase	AMV Reverse Transcriptase (in the range of 42 - 60°C)
PCR Polymerase	<i>TaKaRa LA Taq</i>
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

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 sample 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

Commercially available sterile disposable plastic equipment can be considered RNase-free and can be used in experiments as is. Equipment such as microtubes, and tips for micropipettes should be autoclaved before use. Glassware and spatula are done dry heat sterilization at 160°C for at least 2 hours. Items that cannot be sterilized by dry-heating should be treated with 0.1% diethylpyrocarbonate (DEPC) solution for 12 hours at 37°C and then autoclaved (to prevent carboxymethylation of RNA by the DEPC) before use.

Designate equipment for RNA experiments and separate from other equipment. Additionally, because the most common source of RNase contamination is barehands, wear plastic gloves and a face mask when performing RNA experiments.

B. Reagent

Whenever possible, treat reagents with 0.1% DEPC and autoclave before use. For reagents that cannot be autoclaved, prepare using equipment and water that have been sterilized and then filter sterilize after preparation. Solutions and sterile purified water that are used to prepare reagents should be designated for use in RNA experiments only.

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°C 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, MgCl₂ etc) for all samples can be prepared first, then aliquoted to individual tubes. Using such mixtures will allow accurate reagents dispense : minimize reagents pipetting losses, and avoid repeat dispensing and mixing of the each reagent. This helps to minimize variation of the data among the experiments.
- 2) Reagents such as RTase, *TaKaRa LA Taq* and RNase Inhibitor shall be mixed gently by pipetting. Avoid generating bubbles. Spin down quickly the solution prior to mixing. Pipette the reagents carefully and slowly due to these viscosity.
- 3) Keep enzymes at -20°C until just before use and return at -20°C 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. It is recommended to perform preliminary experiment to determine the condition prior to using a sample.
- 6) Primer Selection
The primer for reverse transcription should be selected from either Random 9 mers, Oligo dT-Adaptor Primer, or specific downstream PCR primer. For short mRNAs formed no hairpin structure, any one of the above three primers can be used.

[Guideline of the primer selection]

- Random 9 mers
Use for transcription of long RNA or of RNA having hairpin structure. Also use 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. This primer will allow 3'-RACE method utilizing M13 primer M4 which is complementary to Adaptor region after reverse transcription.

IX. Protocol

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 Random 9 mers, Oligo dT-Adaptor Primer, or specific downstream primer. For the control experiment, use Control R-1 primer. (Refer to "VIII . 6) Primer Selection".)

Reagent	Amount	Final conc.
MgCl ₂	2 μl	5 mM
10X RNA PCR Buffer	1 μl	1X
RNase Free dH ₂ O	4.25 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 or Oligo dT-Adaptor Primer or Specific downstream PCR primer	0.5 μl	2.5 μM or 0.125 μM or 1.0 μM
Positive control RNA or Total RNA Sample	0.5 μl	[1 x 10 ⁵ copies] or [≤ 500 ng total RNA]
Total	10 μl per reaction	

2. Place tubes in a Thermal Cycler and set the parameters below.

(30°C 42 - 60°C *3 99°C 5°C	10 min) *2 15 - 30 min 5 min *4 5 min	}	1 cycle
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- * 1 AMV RTase binds to cDNA and inhibits PCR amplification. Heat treatment at 99°C for 5 min inactivates the RTase and removes the inhibitory effect on PCR. For long RNA, it is advisable to increase the incubation time during AMV RTase rather than increase the amount of AMV RTase.
- * 2 When using Random 9 mers, perform reverse transcription in advance at 30°C for 10 min to obtain enough length to anneal with primer at 42 - 60°C.
- * 3 AMV RTase can work at 60°C. However, when the reactivity is low at 60°C, it is advisable to perform reverse transcription at around 42°C. When Positive control RNA is used as template, reverse transcription at 50°C is recommended.
- * 4 For amplification of longer targets, the inactivation of AMV RTase at 70°C for 15 min is recommended, so there will be no damage to the 1st strand cDNA.

B. PCR

1. Prepare reaction mixture as shown below.

Reagent	Amount	(per 50 μl mixture) Final conc.
MgCl ₂	3 μl	2.5 mM
10X LA PCR Buffer II (Mg ²⁺ free)	4 μl	1X
TaKaRa LA Taq	0.25 μl	1.25 U/50 μl
Upstream PCR Primer (Control F-1 Primer for Control RNA)	0.5 μl	0.2 μM
Downstream PCR Primer* ¹ (For Control RNA, Control R-1 Primer or M13 primer M4 when Oligo dT-Adaptor Primer is used in RT)	0.5 μl	0.2 μM
Sterile purified water	31.75 μl	
Total	40 μl per reaction	

* 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 the tube at Step A-2.
3. Spin for approximately 10 sec with a microcentrifuge.
4. Set the tubes in a Thermal Cycler and perform PCR under the optimal condition.

94°C	2 min	} 28 cycles	1 cycle
94°C	30 sec		
60°C	30 sec		
72°C	1 - 2 min/kb		

5. After the reaction is completed, apply 5 - 10 μl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments.*²

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

X. PCR Conditions

- **Annealing Temperature**
Optimize annealing temperature of PCR within the range of 37 - 65°C as necessary. The temperature is 60°C at reaction of Control RNA.
- **Extension time**
Usually, TaKaRa LA Taq extends 1 kb per 1 - 2 min at 72°C.
- **Number of cycles**
When small amount of cDNA is used, the repetition of 40 - 50 cycles are required for PCR amplification.
- **As PCR products amplified with this kit have one A added at 3'-termini, the PCR product can be directly cloned into T-vector by TA cloning. Also it is possible to clone the PCR product in blunt-end vectors after blunting and phosphorylation at the end of the PCR products.**

XI. Experimental Examples

(1) RT-PCR of long mRNA (approx. 12 kb)

Template RNA :	human heart poly A ⁺ RNA
Target gene :	human dystrophin
Amplified DNA fragment size :	12 kb

A. Reverse Transcription

1. Prepare the reaction mixture as shown below.

Reagent	Amount	Final conc.
MgCl ₂	2 μl	5 mM
10X RNA PCR Buffer	1 μl	1X
RNase Free dH ₂ O	4.5 μl	
dNTP Mixture	0.25 μl	1 mM
RNase Inhibitor	0.5 μl	1 U/μl
Reverse Transcriptase	0.5 μl	0.25 U/μl
Random 9 mers	0.25 μl	2.5 μM
human heart poly A ⁺ RNA	0.25 μl	0.25 μg/10 μl RT
Total	10 μl per reaction	

2. Place the tubes in a Thermal Cycler and perform the reaction by the following condition.

30°C	10 min	} 1 cycle
42°C	50 min	
70°C	15 min*1	

* 1 For amplification of longer targets, the inactivation at 70°C for 15 min is performed.

B. PCR

1. Prepare reaction mixture for PCR as shown below.

Reagent	Amount	(per 50 μl mixture) Final conc.
MgCl ₂	3 μl	2.5 mM
10X LA PCR Buffer II (Mg ²⁺ free)	4 μl	1X
Sterile purified water	31.75 μl	
TaKaRa LA Taq	0.25 μl	1.25 U/50 μl
Primers	each 0.5 μl	each 0.2 μM
Total	40 μl per reaction	

2. Add 40 μl of the mixture into the tube at Step A-2.
3. Place the tubes in a Thermal Cycler and perform PCR under the following condition.

94°C	30 sec	} 30 cycle
65°C	15 min	

4. After the reaction is completed, apply 5 μl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments. The target cDNA is verified with the amplified fragment of 12 kb.

(2) 3'-RACE System

Template RNA : human HL60 total RNA
 Target gene : Transferrin receptor (TFR)
 Amplified DNA fragment size : 1,105 bp

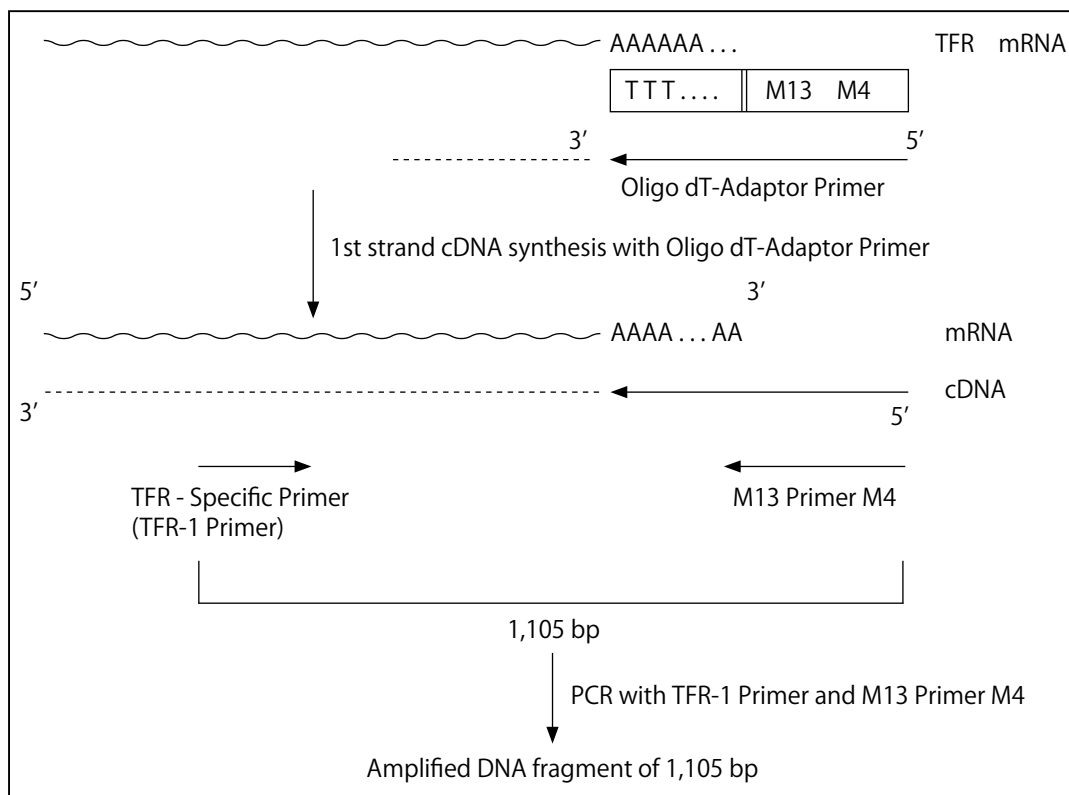


Figure 3. Schematic diagram of RT - PCR of HL60 total RNA with 3'-RACE System

A. Reverse Transcription

1. Prepare the reaction mixture as shown below.

Reagent	Amount	Final conc.
MgCl ₂	2 μl	5 mM
10X RNA PCR Buffer	1 μl	1X
RNase Free dH ₂ O	4.25 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
HL60 total RNA (1 μg/μl)	0.5 μl	0.5 μg/10 μl RT
Total	10 μl	per reaction

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

30°C	10 min	} 1 cycle
50°C	30 min	
99°C	5 min	
5°C	5 min	

B. PCR

1. Prepare reaction mixture for PCR as shown below.

Reagent	Amount	(per 50 μ l mixture) Final conc.
MgCl ₂	3 μ l	2.5 mM
10X LA PCR Buffer II (Mg ²⁺ free)	4 μ l	1X
Sterile purified water	31.75 μ l	
<i>TaKaRa LA Taq</i>	0.25 μ l	1.25 U/50 μ l
M13 Primer M4	0.5 μ l	0.2 μ M
TFR-1 Primer	0.5 μ l	0.2 μ M
Total	40 μl per reaction	

2. Add 40 μ l of the mixture into the tube at Step A-2.
3. Place the tubes in a Thermal Cycler and perform PCR under the following condition.

94°C	30 sec	}	30 cycles
55°C	30 sec		
72°C	5 min		
4. After the reaction is completed, apply 5 μ l of the reactant for agarose gel electrophoresis to verify the amplified DNA fragments. The target cDNA is verified with the amplified fragment of 1,105 bp.

XII. References

- 1) Kawasaki E S and Wang A M. (1989) PCR Technology (Erich, H. A. ed.), Stockton Press, 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 Sc. USA*. (1988) **85**: 8998 - 9002.

XIII. Related Products

AMV Reverse Transcriptase XL for RT-PCR (Cat. #2630A)
Recombinant RNase Inhibitor (Cat. #2313A/B)
Ribonuclease Inhibitor (Porcine liver) (Cat. #2311A/B)
Takara LA Taq® (Cat. #RR002A/B)
Random Primer (nonadeoxyribonucleotide mixture; pd(N)₉) (Cat. #3802)
TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600)
Mupid-2plus (Cat. #M-2P)
Mupid-exU (Cat. #EXU-1)
Mupid-One (Cat. #O1-01)
PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)
PrimeGel™ Agarose LE 1-20K GAT (Cat. #5801A)
NucleoSpin RNA (Cat. #740955.10/.50/.250)

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