

For Research Use

TaKaRa

**PrimeScript™ RT reagent Kit
(Perfect Real Time)**

Product Manual

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I. Description

PrimeScript RT reagent Kit is designed to perform the reverse transcription optimized for real-time RT-PCR. It uses PrimeScript Reverse Transcriptase, which features excellent extendibility. The kit makes fast, efficient cDNA template synthesis for real-time PCR possible. The step of experimental procedure in this kit is simple and suitable for high throughput analysis. This kit can be used in combination with real-time PCR reagent, such as SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820S/A/B), SYBR Fast qPCR Mix (Cat. #RR430S/A/B), SYBR *Premix Ex Taq* (Tli RNaseH Pluse) (Cat. #RR420S/A/B), or Probe qPCR Mix (Cat. #RR391A/B)*, for 2 step real-time RT-PCR. The optimized protocol for assay can be selected in each assay condition using either SYBR Green or a probe.

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

II. Components (200 reactions, 10 µl per reactions)

1.	5X PrimeScript Buffer (for Real Time)* ¹	400 µl
2.	PrimeScript RT Enzyme Mix I* ²	100 µl
3.	Oligo dT Primer	50 µM 100 µl
4.	Random 6 mers	100 µM 400 µl
5.	RNase Free dH ₂ O	1 ml
6.	EASY Dilution (for Real Time PCR)* ³	1 ml

*¹ Contains dNTP Mixture and Mg²⁺.

*² Contains RNase Inhibitor.

*³ To be used when producing serial dilutions of total RNA and cDNA. EASY Dilution (for Real Time PCR) makes it possible to obtain a precise dilution down to very low concentrations. Moreover, this solution will not affect reverse-transcription or PCR reactivity. You can use the diluted template solution directly in reverse-transcription and PCR reactions.

EASY Dilution (for Real Time PCR) may also be purchased separately (Cat. #9160).

Note: Please use EASY Dilution (for Real Time PCR) in combination with Takara Bio's Real-Time PCR Reagents. Compatibility with products from other manufacturers has not yet been verified.

Materials Required but not Provided

- Thermal Cycler (or 37°C, 42°C water bath, 85°C heat block)
- 0.2 ml and 1.5 ml microtube (for reverse transcription)
- Micropipettes and pipette tips (autoclaved)

III. Storage -20°C

IV. Features

1. Makes fast, efficient synthesis of cDNA templates for real-time PCR possible. This kit is best suited for 2 step real-time RT-PCR.
2. The kit includes Random 6 mers and Oligo dT Primer for use as reverse transcription primers. The reaction can be performed using mixture these two primers, or the primer can be selected based on the purpose of the experiment. Furthermore, specific primers of a gene can be used for detection of a specific gene.
3. Protocols are prepared for use with SYBR Green qPCR assay and probe qPCR assay. Please select the protocol based on the assay method to be used for real-time PCR.

There are the following differences between the protocols for SYBR Green qPCR assay and probe qPCR assay.

- Amount of RT Primer Mix used in reverse-transcription reaction
- Amount of total RNA capable of using in reverse-transcription reaction

4. A standard curve must be generated for the quantitation of real-time RT-PCR. It is important that accurate dilution of total RNA or cDNA after reverse transcription is performed by lower concentrations for a proper standard curve. However, dilution with water or TE can narrow the range of the curve due to be unstable at data obtained from low concentrations. Using EASY Dilution (for Real Time PCR) for dilution causes the results to be accurate at lower concentrations and facilitates creation of a wide-range standard curve.

V. Precautions

Following is the protocol for this kit. Please read it carefully before you use.

1. It is convenient to prepare a master mix of reagents containing RNase Free dH₂O, buffer, enzymes, etc. Using such a mixture allows accurate dispensing of reagents, minimizes pipetting losses, and avoids repeated mixing of each reagent. This helps to minimize experimental variability.
2. Gently spin down the PrimeScript RT Enzyme Mix I prior to pipetting. Pipet enzymes slowly and carefully because of the viscosity of the 50% glycerol in this solution.
3. Use new disposable pipette tips to avoid contamination between samples when transferring reagents.

VI. Protocol: Reverse Transcription

(Refer to VIII. B. Preparation of RNA sample.)

【 For SYBR Green qPCR assay 】

1. Prepare the following reaction mixture on ice.
Prepare a slightly larger amount of master mix than required to compensate for pipetting losses. After dispensing aliquots of this mixture into the microtubes, add the RNA sample.

<Per reaction>

Reagent	Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 μ l	1X
PrimeScript RT Enzyme Mix I	0.5 μ l	
Oligo dT Primer (50 μ M)* ¹	0.5 μ l	25 pmol
Random 6 mers (100 μ M)* ¹	0.5 μ l	50 pmol
total RNA		
RNase Free dH ₂ O		
Total	10 μ l* ²	

- *¹ Using both Oligo dT Primer and Random 6 mers, efficient synthesis of cDNA from total RNA can be accomplished. The required amount of primer for exclusive use of each primer or a gene specific primer is as follows.

Primer	Amount	Total Amount (pmol)
Oligo dT Primer (50 μ M)	0.5 μ l	25 pmol
Random 6 mers (100 μ M)	0.5 μ l	50 pmol
Gene specific primer (2 μ M)	0.5 μ l	1 pmol

- *² It is possible to scale up the RT reaction as needed. Up to 500 ng of total RNA can be reverse transcribed in 10 μ l of the reaction mixture.

2. Incubate the reaction mixture under the following condition.

37°C 15 min*³ (Reverse transcription)
 85°C 5 sec (Inactivation of reverse transcriptase with heat treatment)
 4°C

- *³ When using a gene specific primer:
 Perform the reverse transcription at 42°C for 15 minutes. If non-specific amplification products are observed at the PCR step, resetting this temperature to 50°C may improve the results.

Note:

- When the reaction mixture obtained in step 2 is used for real-time PCR, the volume of the mixture should be less than 10% of the total PCR reaction volume for real-time PCR.
- The protocol of reverse transcription for probe qPCR assay (p.6) is not recommended, because background of SYBR Green might increase in real-time PCR reaction.

【 For probe qPCR assay 】

1. Prepare the following reaction mixture on ice.
Prepare a slightly larger amount of master mix than required to compensate for pipetting losses. After dispensing aliquots of this mixture into the microtubes, add the RNA sample.

<Per reaction>

Reagent	Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 μ l	1X
PrimeScript RT Enzyme Mix I	0.5 μ l	
Oligo dT Primer (50 μ M)* ¹	0.5 μ l	25 pmol
Random 6 mers (100 μ M)* ¹	2 μ l	200 pmol
total RNA		
RNase Free dH ₂ O		
Total	10 μ l* ²	

- *¹ Using both Oligo dT Primer and Random 6 mers, efficient synthesis of cDNA from total RNA can be accomplished. The required amount of primer for exclusive use of each primer or a gene specific primer is as follows.

Primer	Amount	Total Amount (pmol)
Oligo dT Primer (50 μ M)	0.5 μ l	25 pmol
Random 6 mers (100 μ M)	2 μ l	200 pmol
Gene specific primer (2 μ M)	0.5 μ l	1 pmol

- *² It is possible to scale up the RT reaction as needed. Up to 1 μ g of total RNA can be reverse transcribed in 10 μ l of the reaction mixture.

2. Incubate the reaction mixture under the following condition.

37°C 15 min*³ (Reverse transcription)
 85°C 5 sec (Inactivation of reverse transcriptase with heat treatment)
 4°C

- *³ When using a gene specific primer:
 Perform the reverse transcription at 42°C for 15 minutes. If non-specific amplification products are observed at the PCR step, resetting this temperature to 50°C may improve the results.

Note:

- When the reaction mixture obtained in step 2 is used for real-time PCR, the volume of the mixture should be less than 10% of the total PCR reaction volume for real-time PCR.
- It is possible to use the protocol for SYBR Green qPCR assay (p.5). In the case using the protocol, however, up to 500 ng of total RNA can be reverse transcribed in 10 μ l of the reaction mixture.

VII. Protocol: Real-time PCR

The following protocol is for real-time PCR using SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A) with templates generated with this kit.

[Protocol when using Thermal Cycler Dice™ Real Time System //]

1. Prepare the PCR mixture shown below.

<Per reaction>

Reagent	Amount	Final conc.
SYBR <i>Premix Ex Taq</i> II (2X)	12.5 μ l	1X
PCR Forward Primer (10 μ M)	1 μ l	0.4 μ M *1
PCR Reverse Primer (10 μ M)	1 μ l	0.4 μ M *1
RT reaction solution (cDNA solution)*2	2 μ l	
Sterile purified water	8.5 μ l	
Total	25 μ l *3	

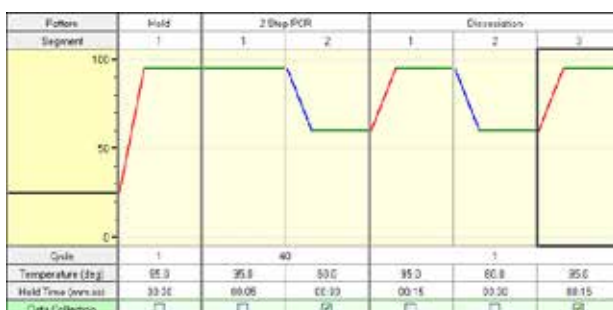
*1 A final primer concentration of 0.4 μ M is most likely to yield a good result. Nevertheless, if there is an issue with reactivity, try to find an optimal concentration between 0.2 and 1.0 μ M.

*2 It is recommended to apply cDNA template corresponding 10 pg - 100 ng total RNA per 25 μ l of reaction mixture. In addition, the volume of RT reaction solution (cDNA) should be in less than 10% volume of PCR reaction mixture.

*3 The recommended reaction volume is 25 μ l.

2. Initiate the reaction.

The shuttle PCR standard protocol is recommended for PCR. Try this protocol first and optimize PCR conditions as necessary. Perform a 3 step PCR when using a primer with low T_m value or when a shuttle PCR is not feasible.

**Shuttle PCR standard protocol**

Hold (initial denaturation)

Cycle: 1

95°C 30 sec

2 Step PCR

Cycles: 40

95°C 5 sec

60°C 30 sec

Dissociation

Note:

TakaRa Ex Taq® HS used in this product is a hot-start PCR enzyme utilizing an anti-*Taq* antibody that suppresses polymerase activity. The initial denaturation step prior to PCR should be at 95°C for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if an assay will be performed.
When using Thermal Cycler Dice Real Time System //, please refer to its instruction manual to read analytical methods.

[Protocol when using Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System and StepOnePlus Real-Time PCR System]

* Follow the instrument manual recommended conditions.

1. Prepare the PCR reaction described below.

<Per reaction>

Reagent	Amount	Amount	Final conc.
SYBR Premix Ex Taq II (2X)	10 μ l	25 μ l	1X
PCR Forward Primer (10 μ M)* ¹	0.8 μ l	2 μ l	0.4 μ M* ¹
PCR Reverse Primer (10 μ M)* ¹	0.8 μ l	2 μ l	0.4 μ M* ¹
ROX Reference Dye or Dye II (50X)* ²	0.4 μ l	1 μ l	1X
RT reaction solution (cDNA solution)* ³	2 μ l	4 μ l	
Sterile purified water	6 μ l	16 μ l	
Total	20 μ l* ⁴	50 μ l* ⁴	

*¹ A final primer concentration of 0.4 μ M is most likely to yield a good result. Nevertheless, if there is an issue with reactivity, try to find an optimal concentration between 0.2 and 1.0 μ M.

*² The concentration for ROX Reference Dye II (50X) is lower than that for ROX Reference Dye (50X).

- Use ROX Reference Dye II (50X) when performing analyses with Applied Biosystems 7500/7500 Fast Real-Time PCR System.
- Use ROX Reference Dye (50X) when using Applied Biosystems 7300 Real-Time PCR System and StepOnePlus Real-Time PCR System.

*³ It is recommended to apply cDNA template corresponding 10 pg - 100 ng total RNA per 20 μ l of reaction mixture. In addition, the volume of RT reaction solution (cDNA) should be in less than 10% volume of PCR reaction mixture.

*⁴ Prepare in accordance with the recommended volume for each instrument.

2. Initiate the reaction.

The recommended protocol for PCR is the shuttle PCR standard protocol described below. Try this protocol first and optimize PCR conditions as necessary. Perform a 3 step PCR when using a primer with low T_m value or when a shuttle PCR is not feasible.

< Applied Biosystems 7300/7500 Real-Time PCR System and StepOnePlus >



Shuttle PCR standard protocol

Stage 1: Initial denaturation

Reps: 1
95°C 30 sec

Stage 2: PCR reaction

Reps: 40
95°C 5 sec
60°C 30 - 34 sec*

Dissociation Stage

* With StepOnePlus, set to 30 sec;
with 7300 set to 31 sec;
with 7500 set to 34 sec

< Applied Biosystems 7500 Fast Real-Time PCR System >

Shuttle PCR standard protocol

Holding Stage

Number of cycle: 1
95°C 30 sec

Cycling Stage

Number of Cycles: 40
95°C 3 sec
60°C 30 sec

Melt Curve Stage

Note:

Takara Ex Taq HS used in this product is a hot-start PCR enzyme utilizing an anti-*Taq* antibody that suppresses polymerase activity. The initial denaturation step prior to PCR should be at 95°C for 30 sec. Longer heat treatment may decrease enzyme activity and affect amplification efficiency and quantification.

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if an assay will be performed.
Please refer to the instruction manual for your real time PCR instrument to read about analytical methods.

VIII. Appendix

A. Experimental example:

Reverse Transcription Reaction Time and Amount of cDNA Synthesis

[Process]

Reverse Transcription

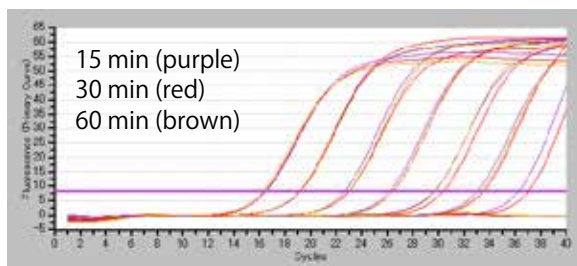
Reagent: PrimeScript RT reagent Kit (Perfect Real Time)
 Template: Mouse liver total RNA (2 pg - 2 μg and sterile purified water)
 Reaction mixture: 20 μl
 Primer: Random 6 mers
 Reaction condition: 37°C 15, 30, 60 min → 85°C, 5 sec → 4°C

Real-time PCR

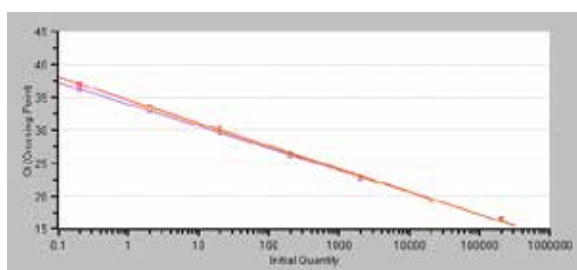
Reagent: SYBR Premix Ex Taq (Perfect Real Time)
 Template: 2 μl of reverse transcription reaction mixtures from above
 20 μl reactions
 Reaction mixture: 25 μl
 Target gene: *Actb*
 Reaction condition: Thermal Cycler Dice Real Time System Standard protocol

[Result]

Amplification curve



Standard curve



	Time	RSq	Eff (%)	Standard Curve
purple	15 min	0.999	92.3	$Y = -3.522 * \text{LOG}(X) + 33.94$
red	30 min	0.999	93.3	$Y = -3.495 * \text{LOG}(X) + 34.61$
brown	60 min	0.999	95.2	$Y = -3.441 * \text{LOG}(X) + 34.28$

The reverse transcription reaction time was set at 15, 30, and 60 min and a comparison was performed. In this experiment, all three reaction times showed equally efficient reverse transcription over a wide range of the template concentration.

B. Preparation of RNA sample

It is important to use a highly pure RNA samples for better cDNA yield. It is essential to inhibit RNase activity in the cells and also to prevent RNase derived from equipment and solutions used. Extra precautions should be taken during the sample preparation, including use of clean disposable gloves, dedication of a exclusively table for exclusive use for RNA preparation, and avoiding unnecessary speaking during assembly to prevent RNase contamination from operator sweat or saliva.

[Equipment]

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

- (1) Hot-air sterilization (180°C, 60 min), or
- (2) Treatment with 0.1% diethylpyrocarbonate (DEPC) at 37°C for 12 hours, followed by autoclaving at 120°C for 30 min to remove DEPC.

It is recommended that all the equipment be used exclusively for RNA preparation.

[Reagent]

All reagents to be used in this experiment must be prepared using tools which were treated as described in previous section (Hot-air sterilization (180°C, 60 min) or DEPC treatment), and all purified water must be treated with 0.1% DEPC and autoclaved.

All reagents and purified water should be used exclusively for RNA experiments.

[Preparation of RNA sample]

Use of highly purified RNA obtained by GTC (Guanidine thiocyanate) method, etc. is recommended. The RNA isolation kits such as RNAiso Plus (Cat. #9108/9109) also can be used for isolating high purity of total RNA. The purified RNA sample should be dissolved in sterile purified water or sterilized TE buffer at process of RNA isolation lastly.

[Contamination with Genomic DNA and its countermeasure]

Some cases, a total RNA sample may contain a small amount of genomic DNA, which could potentially become a PCR template. This could result in inaccurate results.

To avoid this to situation, the following countermeasure is recommended:

- (1) design primers which will not amplify genomic DNA, or
- (2) remove genomic DNA by DNase I treatment

- (1) Designing a primer which will not amplify genomic DNA:

It is possible to design a primer that will not amplify genomic origin of DNA based on the exon and intron structure of genomic DNA. First, confirm the target genomic structure, and select a large intron region. Then design primers on the upstream and downstream sides of the intron region. If the intron is large enough, genomic DNA amplification will not occur. Even if size of the intron is not large, products resulting from amplification of genomic templates would be larger than products originating from cDNA, allowing identification by dissociation curve analysis. However, this approach cannot be applied for single-exon genes or genes that contain pseudogene. Moreover, species without introns or new species without well-identified genomic structures could demonstrate similar problems. In this case, we recommend performing the DNase I treatment described in (2).

- (2) After extract total RNA remove genomic DNA by Recombinant DNase I (RNase-free) (Cat. #2270A) treatment. After the reaction, DNase I should be inactivated by either heat treatment or phenol/chloroform extraction.

Procedure

1. Prepare the following reagents:

total RNA	20 - 50 μ g
10X DNase I Buffer	5 μ l
RNase Inhibitor	20 U
DNase I (RNase-free)	2 μ l (10 U)
DEPC treated water	to 50 μ l

2. Incubate at 37°C for 20 min.
3. Perform one of the following procedures to inactivate DNase I.
- A. Heat treatment
- (1) Add 2.5 μ l of 0.5 M EDTA, and incubate at 80°C for 2 min.
 - (2) Increase reaction volume to 100 μ l with DEPC treated water.
- B. Phenol/Chloroform extraction
- (1) Mix 50 μ l of DEPC treated water and 100 μ l of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) together.
 - (2) Centrifuge at 15,000 rpm for 5 min, at room temperature transfer the upper layer to a new tube.
 - (3) Add equal amount of Chloroform/isoamyl alcohol (24 : 1) and mix.
 - (4) Centrifuge at 15,000 rpm for 5 min, at room temperature transfer upper layer to new tube.
4. Add 10 μ l of 3 M sodium acetate and 250 μ l of cold ethanol, then incubate on ice for 10 min.
5. Centrifuge at 15,000 rpm for 15 min at 4°C and remove the top clear liquid.
6. Wash the precipitate, with 70% ethanol and then, centrifuge at 15,000 rpm for 5 min at 4°C and remove the supernatant.
7. Dry the precipitate.
8. Dissolve the precipitate in the proper amount of DEPC treated water.

[Confirmation of Genomic DNA Contamination]

The presence of genomic DNA can be verified by real-time PCR without reverse transcription. It is convenient to use a primer that can do PCR amplification from both genomic DNA and mRNA for this experiment, and one tube should include an exogenous genomic DNA aliquot. In addition, a primer which was designed not to amplify genomic DNA could amplify derived from pseudogene. In this case, this procedure can also be use to verify DNA.

IX. Related Products

PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A)
 PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Cat. #RR047A)
 SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A)
 SYBR® Fast qPCR Mix (Cat. #RR430A)
 SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A)
 SYBR® Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A)*
 Probe qPCR Mix (Cat. #RR391A)*
 EASY Dilution (for Real Time PCR) (Cat. #9160)
 RNAiso Plus (Cat. #9108/9109)

Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
 Thermal Cycler Dice™ Real Time System II (Cat. #TP900/TP960)*
 Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)*

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

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SYBR is a registered trademark of Molecular Probes, Inc.

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