Table of Contents

I. Components 2
II. Storage 2
III. Features 3
IV. Precautions 3
V. Protocol : Reverse Transcription 4
VI. Protocol : Real-time PCR 6
VII. Appendix 9
VIII. Related Products 12

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), SYBR® *Premix Ex Taq* (Tli RNaseH Pluse) or *Premix Ex Taq* (Probe qPCR), for 2 step real-time RT-PCR. The optimized protocol for assay can be selected in each assay condition using either SYBR® Green or TaqMan® probe.

I. Components

1.	5X PrimeScript Buffer (for Real Time) ＊1		40 μl
2.	PrimeScript RT Enzyme Mix I ＊2		10 μl
3.	Oligo dT Primer	50 μM	10 μl
4.	Random 6 mers	100 μM	40 μl
5.	RNase Free dH_2O		100 μl
6.	EASY Dilution (for Real Time PCR) ＊3		100 μl

＊1 : Contains dNTP Mixture and Mg^{2+}.
＊2 : Contains RNase Inhibitor.
＊3 : Used as dilute solution for making of diluents of total RNA or cDNA.
In contrast to dilution with water or TE, EASY Dilution (for Real Time PCR) facilitates more accurate dilution to low concentrations, and thus allows a standard curve with more dynamic range. Diluted template reagent can be applied as the template for reverse transcription or PCR reactions, because Easy Dilution Solution does not inhibit either reverse transcription or PCR enzyme activity. EASY Dilution Solution is also available separately. EASY Dilution (for Real Time PCR) (Cat. #9160)

Note : EASY Dilution (for Real Time PCR) has been tested with TaKaRa's real-time PCR reagents. Compatibility with products from other manufacturers has not yet been verified.

Reagents and instruments not supplied in this kit

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

II. Storage

-20℃

III. 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) Two protocols for both SYBR® Green assay and TaqMan® probe assay are present in this manual. Select a proper protocol adjusting a method of real-time PCR assay.

Note: There are the following differences between the reverse transcription protocols for SYBR® Green assay and TaqMan® probe assay.
- Maximum amount of Random 6 mers used for reverse transcription
- Maximum amount of total RNA used for reverse transcription

(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.

IV. 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 dH_2O, 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 transfering reagents.

V. Protocol : Reverse Transcription

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

[For SYBR® Green 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.

Reagent	Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 μl	1X
PrimeScript RT Enzyme Mix I	0.5 μl	
Oligo dT Primer (50 μM) ＊1	0.5 μl	25 pmol
Random 6 mers (100 μM) ＊1	0.5 μl	50 pmol
total RNA		
RNase Free dH_2O		
Total	10 μl＊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 μM)	0.5 μl	25 pmol
Random 6 mers (100 μM)	0.5 μl	50 pmol
Gene specific primer (2 μM)	0.5 μl	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 μl of the reaction mixture.

2. Incubate the reaction mixture under the following condition.

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

＊3 : When using a gene specific primer :
Perform the reverse transcription at 42℃ for 15 minutes. If non-specific amplification products are observed at the PCR step, resetting this temperature to 50℃ 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 TaqMan® probe assay (p.5) is not recommended, because background of SYBR® Green might increase in real-time PCR reaction.

[For TaqMan® Probe 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.

Reagent	Amount	Final conc.
5X PrimeScript Buffer (for Real Time)	2 μl	1X
PrimeScript RT Enzyme Mix I	0.5 μl	
Oligo dT Primer (50 μM) ＊1	0.5 μl	25 pmol
Random 6 mers (100 μM) ＊1	2 μl	200 pmol
total RNA		
RNase Free dH_2O		
Total	10 μl＊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 μM)	0.5 μl	25 pmol
Random 6 mers (100 μM)	2 μl	200 pmol
Gene specific primer (2 μM)	0.5 μl	1 pmol

＊2 : It is possible to scale up the RT reaction as needed. Up to 1 μg of total RNA can be reverse transcribed in 10 μl of the reaction mixture.

2. Incubate the reaction mixture under the following condition.

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

＊3 : When using a gene specific primer :
Perform the reverse transcription at 42℃ for 15 minutes. If non-specific amplification products are observed at the PCR step, resetting this temperature to 50℃ 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 Assay (p.4). In the case using the protocol, however, up to 500 ng of total RNA can be reverse transcribed in 10 μl of the reaction mixture.

VI. 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. If performing real-time PCR with TaqMan® probe detection, TaKaRa recommends use of *Premix Ex Taq* (Probe qPCR) (Cat. #RR390A)

< Protocol when using Thermal Cycler Dice Real Time System *II* >

1. Prepare the PCR mixture shown below.

<Per reaction>

Reagent	Amount	Final conc.
SYBR® *Premix Ex Taq* II (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM ＊1
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM ＊1
RT reaction solution (cDNA solution)＊2	2.0 μl	
dH_2O (sterile distilled water)	8.5 μl	
total	25 μl ＊3	

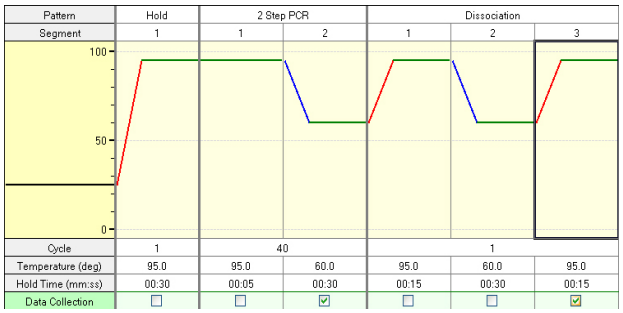
＊1 : A final primer concentration of 0.4 μM is most likely to yield a good result. Nevertheless, if there is an issue with reactivity, try to find an optimal concentration between 0.2 and 1.0 μM.

＊2 : It is recommended to apply DNA template corresponding 10 pg - 100 ng total RNA per 25 μl 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 μl.

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℃ 30 sec

2 Step PCR
Cycle: 40
95℃ 5 sec
60℃ 30 sec

Dissociation

Note : This product combines the high performance of *TaKaRa Ex Taq* HS, which is an enzyme for hot start PCR utilizing *Taq* antibody. Initial denaturation step prior to PCR should be at 95℃ for 30 sec. No need to heat at 95℃ for (5-)15 min. as the initial denaturation, required for chemically modified Taq polymerase. If longer heat treatment is provided, the enzyme activity decreases and the amplification efficiency and the accuracy in quantification can also be affected.

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 *II*, 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 (Life Technologies).

1. Prepare the PCR reaction described below.

<Per reaction>

Reagent	Amount	Amount	Final conc.
SYBR® *Premix Ex Taq* II (2X)	10 μl	25 μl	1X
PCR Forward Primer (10 μM) ＊1	0.8 μl	2.0 μl	0.4 μM ＊1
PCR Reverse Primer (10 μM) ＊1	0.8 μl	2.0 μl	0.4 μM ＊1
ROX Reference Dye or Dye II (50X) ＊2	0.4 μl	1.0 μl	1X
RT reaction solution (cDNA solution) ＊3	2.0 μl	4.0 μl	
dH_2O (sterilized distilled water)	6.0 μl	16 μl	
Total	20 μl ＊4	50 μl ＊4	

＊ 1 : A final primer concentration of 0.4 μM 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 μM.

＊ 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 DNA template in 10 pg - 100 ng per 20 μl of reaction mixture. In addition, the volume of RT reaction solution (cDNA) should be in less than 10% volume of PCR reaction mixture.

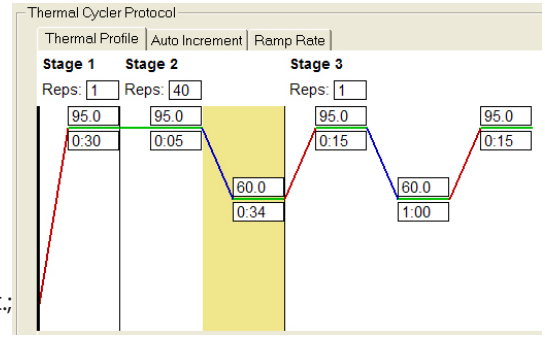
＊ 4 : Prepare in accordance with the recommended volume for each instrument.

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 Tm value or when a shuttle PCR is not feasible.

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

[Shuttle PCR standard protocol]

Stage 1 : Initial denaturation
Reps : 1
95℃ 30 sec.
Stage 2 : PCR reaction
Reps : 40
95℃ 5 sec.
60℃ 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
Reps：1
95℃ 30 sec.
Cycling Stage
Number of Cycles：40
95℃ 3 sec.
60℃ 30 sec.
Melt Curve Stage

Note : This product combines the high performance of *TaKaRa Ex Taq* HS, which is an enzyme for hot start PCR utilizing *Taq* antibody. Initial denaturation step prior to PCR should be at 95℃ for 30 sec. No need to heat at 95℃ for (5-)15 min. as the initial denaturation, required for chemically modified Taq polymerase. If longer heat treatment is provided, the enzyme activity decreases and the amplification efficiency and the accuracy in quantification can also be affected.

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.

VII. Appendix

A. Experimental example :

Reverse Transcription Reaction Time and Amount of cDNA Synthesis

[Process]

Reverse Transcription

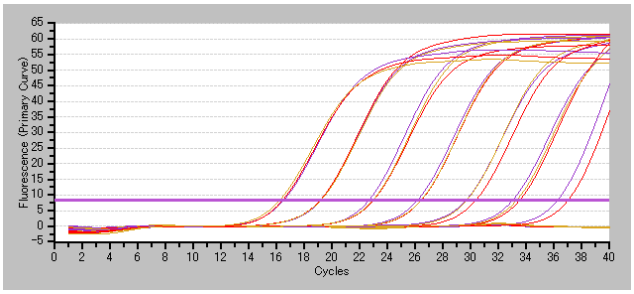
Reagent : PrimeScript RT reagent Kit (Perfect Real Time)
Template : Mouse liver total RNA (2 pg - 2 μg and sterilized water)
Reaction mixture : 20 μl
Primer : Random 6 mers
Reaction Condition : 37℃ 15, 30, 60 min. → 85℃, 5 sec. → 4℃

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 : Mouse Actb
Reaction Condition : Thermal Cycler Dice Real Time System Standard protocol

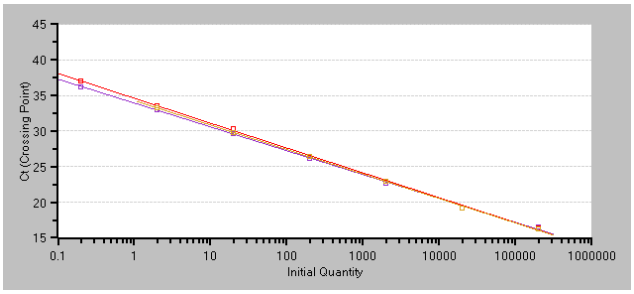
[Result]

Amplification curve



15 min. (purple)
30 min. (red)
60 min. (brown)

Standard curve



15 min. (purple)	Rsq : 0.999	Eff = 98.7%	Y= − 3.522 ∗ LOG (X) + 33.94
30 min. (red)	Rsq : 0.999	Eff = 93.3%	Y= − 3.495 ∗ LOG (X) + 34.61
60 min. (brown)	Rsq : 0.999	Eff = 95.2%	Y= − 3.441 ∗ 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 operater 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℃, 60min), or
(2) Treatment with 0.1% diethylpyrocarbonate (DEPC) at 37℃ for 12 hours, followed by autoclaving at 120℃ 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℃, 60 min.) or DEPC treatment), and all distilled water must be treated with 0.1%DEPC and autoclaved.

All reagents and distilled 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 sterilized distilled 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 μg
10X DNase I Buffer	5 μl
RNase Inhibitor	20 U
DNase I (RNase-free)	2 μl (10 U)
DEPC treated water	to 50 μl

2. Incubate at 37℃ for 20 min.
3. Perform one of the following procedures to inactivate DNase I
   A. Heat treatment
      (1) Add 2.5 μl of 0.5 M EDTA, incubate at 80℃ for 2 min.
      (2) Increase reaction volume to 100 μl with DEPC treated water
   B. Phenol/Chloroform extraction
      (1) Mix 50 μl of DEPC treated water and 100 μl 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 at15,000 rpm for 5 min. , at room temperature transfer upper layer to new tube.
4. Add 10 μl of 3 M sodium acetate and 250 μl of cold ethanol, then incubate on ice for 10 min.
5. Centrifuge at 15,000 rpm for 15 min. at 4℃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℃ 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.

VIII. Related Products

PrimeScript™ RT reagent Kit (Perfect Real Time) (Cat. #RR037A)
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/B)
SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
SYBR® Premix DimerEraser ™ (Perfect Real Time) (Cat. #RR091A)
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A/B)
EASY Dilution (for Real Time PCR) (Cat. #9160)
RNAiso Plus (Cat. #9108/9109)

- SYBR® is a registered trademark of Molecular Probes Inc.
- TaqMan® is a registered trademark of Roche Molecular Probes Inc.