Cat. # RR036Q

For Research Use

TaKaRa

PrimeScript™ RT Master Mix (Perfect Real Time) Sample

Product Manual

v201204Da_2

Table of Contents

I. Description

This product is a reverse transcription reagent kit designed to perform reverse transcription optimized for 2 step real-time RT-PCR (RT-qPCR). It contains a 5X pre-mixed reagent containing all of the components needed for quantitative RT-PCR reverse transcription (PrimeScript RTase, RNase Inhibitor, Random 6mers, Oligo dT Primer, dNTP Mixture, and reaction buffer), and a reaction can be started simply by adding template RNA and water. Because it uses PrimeScript RTase, which features excellent extendibility, this product, like PrimeScript RT reagent Kit (Perfect Real Time) (Cat. #RR037A/B), makes it possible to synthesize template cDNA for real-time PCR efficiently in a short time.

The cDNA obtained with this product can be used in both the SYBR® Green Assay and TaqMan® Probe Assay. Please use this product in combination with quantitative PCR reagents, such as SYBR® *Premix Ex Taq* II (Tli RNaseH Plus) and *Premix Ex Taq* (Probe qPCR), depending upon your objectives.

Note: TAKARA BIO is under a license agreement with Molecular Probes Inc. for the use of SYBR® Green I as a reagent for research purposes. SYBR® is a registered trademark of Molecular Probes Inc.

II. Components (for 20 x 10 μl reactions)

1.	5X PrimeScript RT Master Mix (Perfect Real Time)＊1	40 μl
2.	RNase Free dH_2O	1 ml
3.	EASY Dilution (for Real Time PCR)＊2	1 ml

＊1: Contains PrimeScript RTase, RNase Inhibitor, Oligo dT Primer, Random 6mers, dNTP Mixture, and reaction buffer (containing Mg^{2+}).

＊2: To be used when preparing serial dilutions of total RNA and cDNA. EASY Dilution makes it possible to obtain a precise dilution down to very low concentrations. Moreover, this solution will not affect reverse-transcription and PCR reactivity. You can use the diluted 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 in combination with Takara Bio's Real-Time PCR Reagents. Compatibility with products from other manufacturers has not yet been verified.

III. Materials Required but not Provided

- Thermal cycler
  (or 37℃ water bath and 85℃ heat block)
- 0.2 ml and 1.5 ml microtubes (for reverse-transcription reaction)
- Micropipettes and tips (autoclaved)

IV. Storage

-20℃

V. Features

(1) The reaction can be started simply by adding template RNA and water.

(2) Template cDNA for real-time PCR can be efficiently synthesized in a short time. This kit is best suited for 2 step real-time RT-PCR.

(3) Optimal template cDNA for real-time PCR can be synthesized with 2 kinds of reverse-transcription primers: Oligo dT Primer and Random 6 mer.

(4) A standard curve must be generated for the quantification of real-time RT-PCR. To generate a precise standard curve, it is important to dilute the total RNA and reverse-transcribed cDNA precisely to low concentrations. However, dilution of templates with water or TE buffer may narrow the range of the curve due to template instability at low concentrations. Using EASY Dilution (for Real Time PCR) makes for more accurate results at low concentrations, facilitating the creation of a wide-range standard curve.

VI. Precautions for Use

Read these precautions before use and follow them when using this product.

(1) Briefly centrifuge the 5X PrimeScript RT Master Mix before use and allow the reagent to settle to the bottom of the tube. Since the 5X PrimeScript RT Master Mix has high viscosity, you will need to perform pipetting slowly and carefully, and also mix the reaction solution well.

(2) When dividing up the reagent, please be sure to use new disposable tips to avoid contamination among samples.

(3) This product is a completely pre-mixed reagent with reverse-transcription primers already added (Oligo dT Primer and Random 6 mers).

Note: This product cannot be used for reverse-transcription reactions using Gene-Specific Primer.

VII. Protocol

Reverse transcription reaction

For RNA preparation method, please see Section X.

1. Prepare the reverse-transcription reaction solution on ice.

<Per reaction>

Reagent	Amount	Final conc.
5X PrimeScript RT Master Mix (Perfect Real Time)	2 μl	1X
total RNA	*	
RNase Free dH_2O	up to 10 μl	

＊: The scale of the reverse-transcription reaction can be increased as necessary. Reverse transcription of as much as 500 ng of total RNA is possible with 10 μl of reaction solution.

2. Perform the reverse-transcription reaction after gently mixing the reaction solution.

37℃ 15 min. (reverse-transcription)
85℃ 5 sec. (for heat inactivation of reverse transcriptase)
4℃

Note: When the reverse-transcription solution obtained in Step B is to be introduced to a real-time PCR reaction, the volume of the solution should be 10% or less of the reaction volume for real-time PCR.

VIII. Real-Time PCR

The protocol for performing real-time PCR using SYBR® *Premix Ex Taq* II (Tli RNaseH Plus) (Cat. #RR820A) after performing a reverse-transcription reaction with this kit is shown below.

If real-time PCR is to be performed with TaqMan® probe detection, please use *Premix Ex Taq* (Probe qPCR) (Cat. #RR390A).

< Protocol when using Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System and StepOnePlus™ Real-Time PCR System >

＊ Please follow the procedures provided in the manual of the respective instrument.

1. Prepare the PCR mixture shown below.

<Per reaction>

Reagent	Amount	Amount	Final conc.
SYBR® *Premix Ex Taq* II (2X)	10 μl	25 μl	1X
PCR Forward Primer (Tli RNaseH Plus) (10 μM)	0.8 μl	2 μl	0.4 μM ＊1
PCR Reverse Primer (10 μM)	0.8 μl	2 μl	0.4 μM ＊1
ROX Reference Dye (50X) or Dye II (50X) ＊2	0.4 μl	1 μl	1X
RT reaction solution (cDNA solution) ＊3	2 μl	4 μl	
dH_2O (sterile distilled water)	6 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 Real-Time PCR System or 7500 Fast Real-Time PCR System.
- Use ROX Reference Dye (50X) when using StepOnePlus™ or 7300 Real-Time PCR System.

＊ 3: In a 20 μl reaction volume, it is preferable to use a quantity of cDNA corresponding to 10 pg - 100 ng of total RNA template. In addition, the reverse-transcription reaction solution volume should correspond to less than 10% of the PCR reaction solution volume.

＊ 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.

   1) Applied Biosystems 7300/7500 Real-Time PCR System, StepOnePlus™

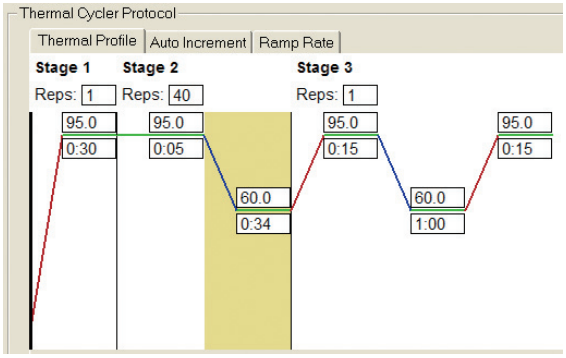


Figure 1. Shuttle PCR standard protocol

Stage 1: Initial denaturation
Reps: 1
95℃ 30 sec.

Stage 2: PCR
Reps:40
95℃ 5 sec.
60℃ 30 - 34 sec. ＊

Dissociation stage

＊ : With StepOnePlus™, set to 30 sec.; with 7300, set to 31 sec.; and with 7500, set to 34 sec.

   2) 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:

- *TaKaRa Ex Taq* HS in this kit is a hot start PCR enzyme with an anti-Taq antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be at 95℃ for 30 sec. Enzyme activity decreases with longer heat treatment and the amplification efficiency and quantification accuracy can be affected.
- Even for the initial denaturation of template before PCR, 95℃ for 30 sec is generally sufficient.

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.

< Method Using Thermal Cycler Dice Real Time System *II* >

1. Prepare the PCR reaction mixture.

<Per reaction>

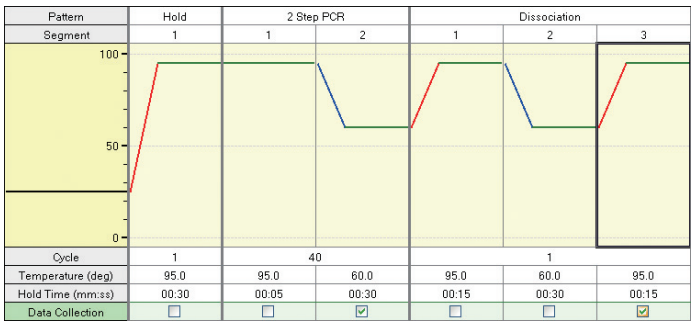
Reagent	Amount	Final conc.
SYBR® *Premix Ex Taq* II (Tli RNaseH Plus) (2X)	12.5 μl	1X
PCR Forward Primer (10 μM)	1 μl	0.4 μM ＊1
PCR Reverse Primer (10 μM)	1 μl	0.4 μM ＊1
RT reaction solution (cDNA solution)	2 μl ＊2	
dH_2O (sterilized distilled water)	8.5 μl	
Total	25 μl	

＊1: Good results are mostly obtained with a final primer concentration of 0.4 μM, but when there is a problem with reactivity, it is best to consider an optimal concentration in the range of 0.2 - 1.0 μM.

＊2: It is preferable to use a quantity of cDNA corresponding to 10 pg - 100 ng of total RNA template. In addition, the reverse-transcription solution introduced should correspond to 10% or less of the PCR reaction solution volume.

2. Start the reaction.

Shuttle PCR standard protocol (below) is recommended. Try this protocol first, and optimize the reaction condition if needed. When the shuttle protocol is difficult due to a primer with low Tm value, etc., try a 3 step PCR protocol.



Hold
(initial denaturation)
Cycle：1
95℃ 30 sec.
2 Step PCR
Cycle：40
95℃ 5 sec.
60℃ 30 - 60 sec.
Dissociation

Figure 2. Shuttle PCR standard protocol

Note:

- *TaKaRa Ex Taq* HS is a hot start PCR enzyme with an anti-Taq antibody that inhibits polymerase activity. The initial denaturation step prior to PCR should be at 95℃ for 30 sec. Enzyme activity decreases with longer heat treatment and the amplification efficiency and quantification accuracy can be affected.
- Even for the initial template denaturation before PCR, 95℃ for 30 sec. is generally sufficient.

3. After the reaction is complete, check the amplification and melting curves and plot a standard curve if a quantitative assay will be performed.

When using Thermal Cycler Dice Real Time System *II*, please refer to its instruction manual to read analytical methods.

IX. Experimental Example

Reverse-transcription reaction time and amount of cDNA synthesized.

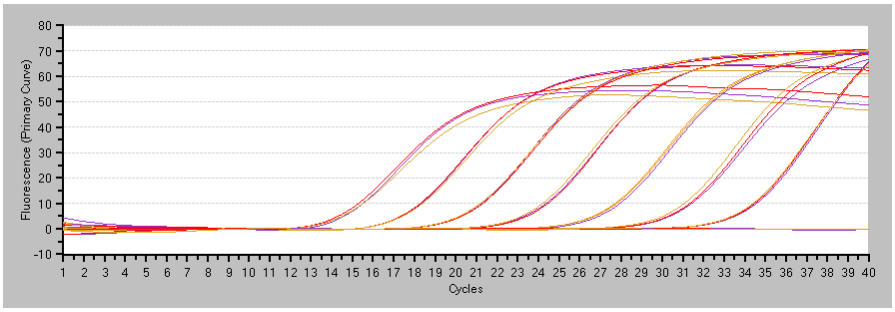
[Method]

1. Reverse-Transcription Reaction
   Reagent: PrimeScript RT Master Mix (Perfect Real Time)
   Template: Human Placenta total RNA 2 pg - 2 μg and sterilized water
   Reaction volume: 20 μl
   Reaction conditions: 37℃ for 15, 30, or 60 min. → 85℃ for 5 sec. → 4℃

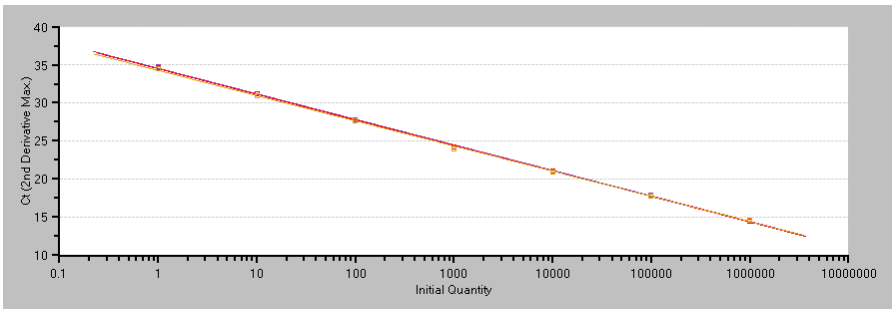
2. Real Time PCR
   Reagent: SYBR® *Premix Ex Taq* II (Perfect Real Time)
   Template: 2 μl of each of the aforementioned reverse-transcription reactions
   Reaction volume: 25 μl
   Target gene: Human ACTB
   Reaction conditions:
   Standard protocol for use with Thermal Cycler Dice Real Time System

[Results]

Amplification curve



Standard curve



	Time	RSq	Eff	Standard Curve
Violet	15 min.	0.999	99.0	Y = -3.346 * LOG(X)+34.52
Red	30 min.	1.000	98.9	Y = -3.349 * LOG(X)+34.46
Yellow	60 min.	0.999	100.9	Y = -3.301 * LOG(X)+34.24

Reactivity was compared for cases where the reaction time was set at 15, 30, or 60 min. This experiment showed that reactions could proceed with equivalent efficiency over a wide range of template concentrations regardless of the reaction time.

X. Appendix

Preparation of RNA Samples

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.

A. 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℃, 60 min.)

(2) Sterilize glassware in 0.1% diethyl pyrocarbonate (DEPC) solution at 37℃ for 12 hours. Autoclave (120℃, 30 min.) to remove any residual DEPC.

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

B. Reagents

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.

C. Method of Preparation for RNA Samples

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

D. Contamination with Genomic DNA and its countermeasure

In 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, either design primers which will not amplify genomic DNA or 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 extraction of 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	up 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 then 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 then transfer the 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.

E. 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.

XI. Related Products

SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A)
EASY Dilution (for Real Time PCR) (Cat. #9160)
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)＊1
PrimeScript™ RT Reagent Kit (Perfect Real Time) (Cat. #RR037A)
PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Cat. #RR047A/B)
RNAiso Plus (Cat. #9108/9109)＊2

＊1: Not available for sale in the U.S. or Europe.
＊2: Not available for sale in Europe.