Cat. # RR064A

For Research Use

TaKaRa

One Step PrimeScript™ RT-PCR Kit (Perfect Real Time)

Product Manual

v202202Da

Table of Contents

I. Description

The One Step PrimeScript RT-PCR Kit (Perfect Real Time) is designed for one step, real-time reverse transcription PCR (RT-PCR) using probe detection. With this kit, all RT-PCR steps can be performed in a single tube. Therefore, it is not necessary to add additional reagents during the reaction, minimizing the risk of contamination and simplifying the workflow. Amplified products are monitored in real time without need for gel electrophoresis after PCR. This kit is suitable for detection of small amounts of RNA.

This kit uses PrimeScript Reverse Transcriptase, which allows efficient and rapid cDNA synthesis, and *TaKaRa Ex Taq*® HS, which enables highly efficient hot start PCR.

Compatible instrument systems include:

- Applied Biosystems 7300/7500 Real-Time PCR System (Thermo Fisher Scientific)
- LightCycler (Roche Diagnostics)
- Smart Cycler System/Smart Cycler II System (Cepheid)
- Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
- Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued)
- Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)

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

II. Principle

The One Step PrimeScript RT-PCR Kit (Perfect Real Time) allows cDNA synthesis from RNA using PrimeScript Reverse Transcriptase followed by PCR amplification by *TaKaRa Ex Taq* HS in one uninterrupted procedure. PCR amplification products are detected and monitored in real time with probes.

1. PCR

PCR amplifies targeted DNA sequences from small amounts of template DNA. Each cycle of PCR includes heat denaturation of DNA, primer annealing, and primer extension by DNA polymerase. Iterative PCR cycles allow exponential amplification of the target DNA in a short time period. Use of a hot start PCR enzyme, *TaKaRa Ex Taq* HS, prevents mispriming and formation of primer dimers during reaction mixture preparation or other pre-cycling steps, thereby allowing highly specific amplification.

2. RT-PCR

During RT-PCR, cDNA synthesized from RNA template is amplified by PCR. This kit uses a one step RT-PCR protocol as shown in Figure 1. A gene-specific reverse primer is used for reverse transcription. Using the synthesized cDNA as a template, specific forward and reverse primers are used for PCR amplification. Random primers and oligo dT primers cannot be used for reverse transcription with this kit.

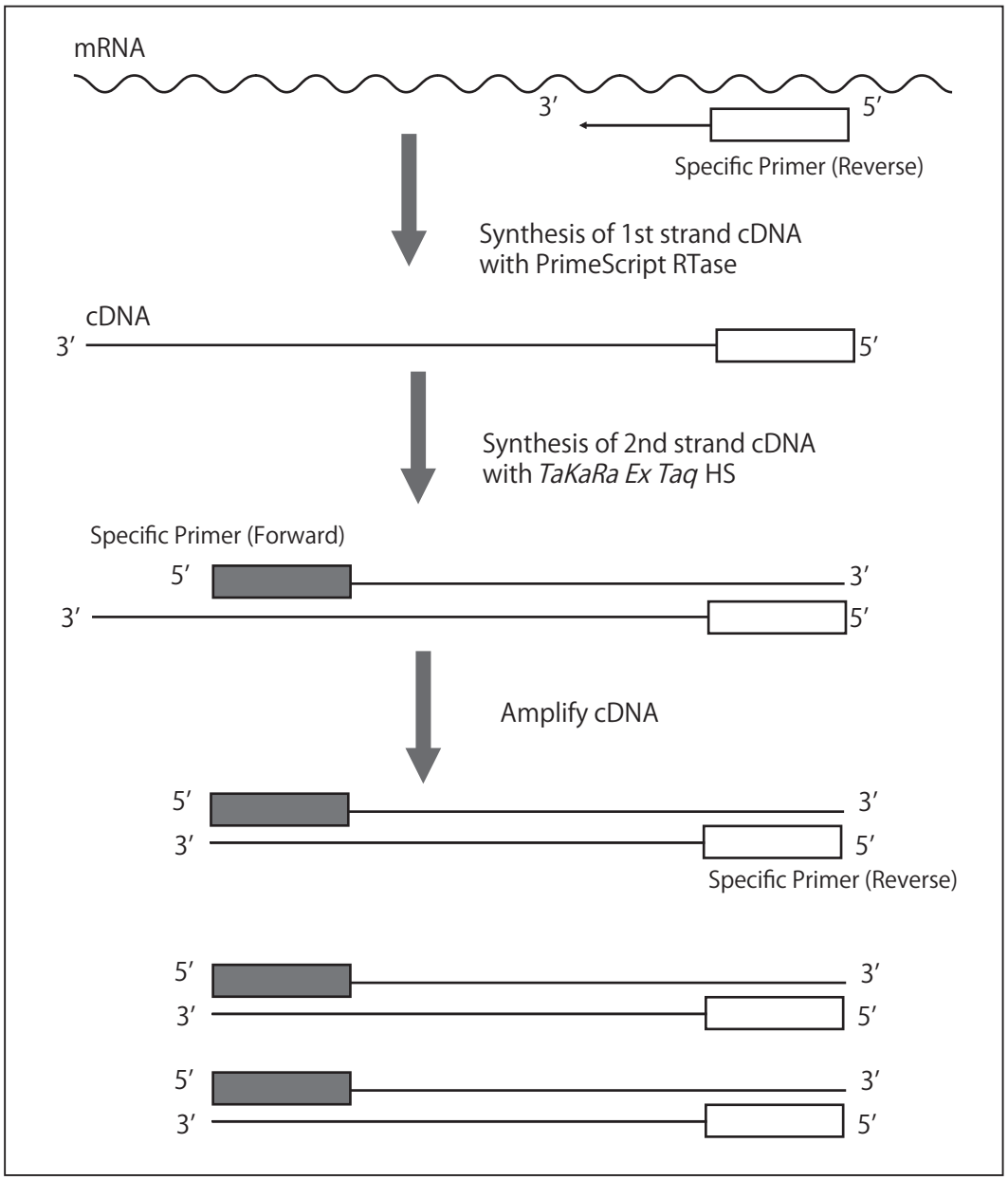
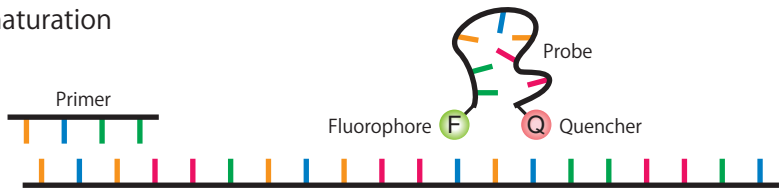


Figure 1. Principle of One Step RT-PCR

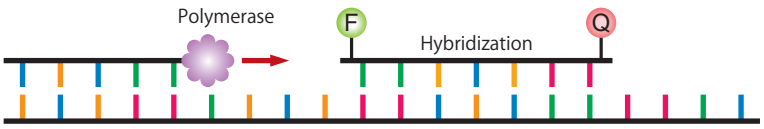
3. Fluorescence Detection

Oligonucleotides modified with a fluorophore (e.g., FAM) at the 5′ end and quencher (e.g., TAMRA) at the 3′ end are included in the reaction.
During the annealing step, the probe specifically hybridizes to the template DNA and fluorescence of the fluorophore is suppressed by the quencher. During the extension step, the 5′→3′ exonuclease activity of *Taq* DNA polymerase degrades the probe hybridized to the template. This prevents quenching, allowing fluorescence emission. The amount of amplified product can be monitored by measuring the fluorescence intensity.

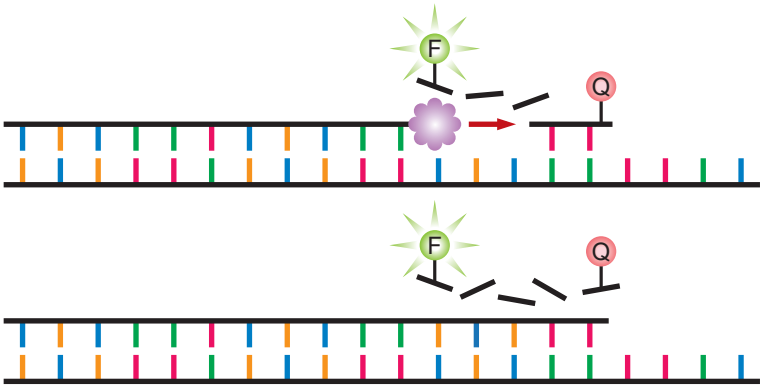
1) Heat denaturation



2) Primer annealing/probe hybridization



3) Extension



III. Components (for 100 reactions; 50 μl reaction system)

1. 2X One Step RT-PCR Buffer III*1	840 μl x 3
2. *TaKaRa Ex Taq* HS (5 U/μl)	100 μl
3. PrimeScript RT enzyme Mix II*2	100 μl
4. RNase Free dH_2O	1.25 ml x 2
5. ROX Reference Dye (50X conc.)*3	100 μl
6. ROX Reference Dye II (50X conc.)*3	100 μl

*1 Includes dNTP Mixture, Mg^{2+}.

*2 Includes RNase Inhibitor.

*3 ROX Reference Dye/Dye II is used for normalization by background subtraction. For the Applied Biosystems 7300 Real-Time PCR System, the use of ROX Reference Dye (50X) is recommended. For Applied Biosystems 7500 Real-Time PCR System, the use of ROX Reference Dye II is recommended. ROX is not required for use with the LightCycler, Smart Cycler or Thermal Cycler Dice Real Time System real time instruments.

Materials required but not provided.

- Real-Time PCR system (authorized instruments)
- Reaction tube or plate designed specifically for Real-Time PCR system
- PCR primers
- Probe for detection (TaKaRa qPCR Probe , etc.)
- Micropipettes and tips (sterile, with filter)

IV. Storage

-20℃

V. Features

(1) One Step RT-PCR allows accurate, rapid analysis of small amounts of RNA such as viral RNA.
(2) *TaKaRa Ex Taq* HS enables highly efficient, specific amplification and detection.
(3) One Step RT-PCR Buffer III is a 2X premix, which allows simple preparation of the reaction with minimal risk of contamination.

VI. Precautions before Use

This section describes precautions for using this product. Read before use.

(1) When mixing reagents for PCR, mix enough for all reactions in a master mix, plus extra to allow for pipetting error. Using master mixes allows accurate reagent dispensing, minimizes reagent pipetting errors, and reduces repeated dispensing of the each reagent. This helps to minimize variability from experiment to experiment and well to well.
(2) Mix PrimeScript RT Enzyme Mix II and *TaKaRa Ex Taq* HS gently. Avoid generating bubbles. Gently spin down the solution prior to pipetting. Pipet the enzymes slowly as the enzyme is highly viscous (contains 50% glycerol). Keep the enzyme at -20℃ until just before use and return to the freezer promptly after use.
(3) If a precipitate appears when 2X One Step RT-PCR Buffer III is thawed, vortex to dissolve the precipitate completely before using the reagent.
(4) Use new disposable pipette tips to avoid cross-contamination between samples when transferring reagents.
(5) This kit requires gene-specific primers for reverse transcription. Do not use random primers or oligo-dT primers.

VII. Protocol

【 Protocol using LightCycler 】

Note: Follow the instruction manual for the LightCycler for operation.

1. Prepare the following reagents on ice.

< Per reaction >

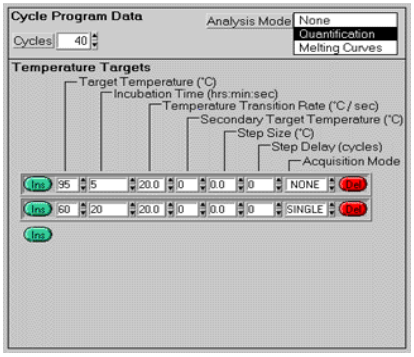
Reagent	Volume	Final Conc.
2X One Step RT-PCR Buffer III	10 μl	1X
TaKaRa Ex Taq HS (5U/μl)	0.4 μl	
PrimeScript RT enzyme Mix II	0.4 μl	
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM*1
Probe	0.8 μl*2	
total RNA	2 μl*3	
RNase Free dH_2O	5.6 μl	
Total	20 μl	

*1 Use primers at a final concentration of 0.2 μM for most reactions. If further optimization is needed, determine the optimal concentration within the range of 0.1 - 1.0 μM.

*2 Probe concentration differs depending on the real-time PCR instrument and type of fluorescent label. Refer to the instrument operation manual and product documents supplied with the probe.

*3 Use 10 pg - 100 ng of total RNA as template.

2. Briefly centrifuge the PCR capillaries and set them in the LightCycler to initiate the reaction. Use the standard protocol described below for the reaction. If needed, the PCR conditions may be modified further.
(For a detailed explanation, please refer to page 12 “PCR Reaction Conditions”.)



Standard protocol

Stage 1: Reverse transcription
42℃ 5 min 20℃/sec
95℃ 10 sec 20℃/sec
1 Cycle
Stage 2: PCR reaction
95℃ 5 sec 20℃/sec
60℃ 20 sec 20℃/sec
40 Cycles

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy for quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 10 sec.

3. After the reaction is complete, verify the amplification curve. Prepare a standard curve when quantification is desired. Please refer to the instruction manual for your real-time PCR instrument to determine the appropriate analysis methods.

【 Protocol using Applied Biosystems 7300/7500 Real-Time PCR System 】

1. Prepare the following reagents on ice.

< Per reaction >

Reagent	Volume	Volume	Final Conc.
2X One Step RT-PCR Buffer III	10 μl	25 μl	1X
TaKaRa Ex Taq HS (5 U/ μl)	0.4 μl	1 μl	
PrimeScript RT enzyme Mix II	0.4 μl	1 μl	
PCR Forward Primer (10 μM)	0.4 μl	1 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.4 μl	1 μl	0.2 μM*1
Probe	0.8 μl	2 μl*2	
ROX Reference Dye or Dye II (50X)*3	0.4 μl	1 μl	
total RNA	2 μl	4 μl*4	
RNase Free dH_2O	5.2 μl	14 μl	
Total	20 μl*5	50 μl*5	

*1 Use primers at final concentration of 0.2 μM for most reactions. If further optimization is needed, determine the optimal concentrations within the range of 0.1 - 1.0 μM.

*2 The optimal probe concentration differs depending on the real-time PCR instrument and the type of fluorescent label. Refer to the instrument operation manual and product documents supplied with the probe.

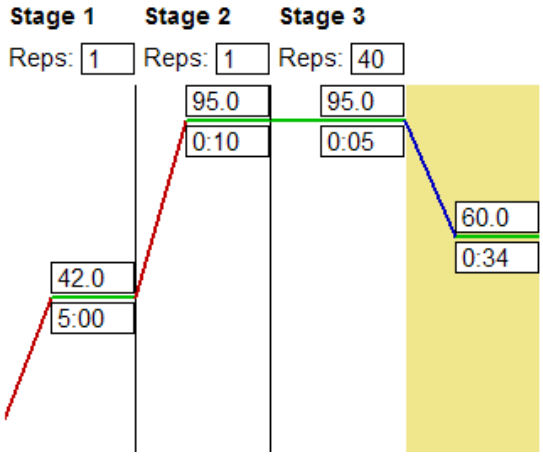
*3 ROX Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities among wells.

For Applied Biosystems 7300 Real-Time PCR System, the use of ROX Reference Dye (50X) is recommended. For Applied Biosystems 7500 Real-Time PCR System, the use of ROX Reference Dye II is recommended.

*4 Use 20 pg - 200 ng of total RNA as template in each 50 μl reaction.

*5 Prepare reactions according to the recommended volume for each instrument.

2. Start reaction
   Use the standard protocol described below for the reaction. If needed, the PCR conditions may be modified further. (For a detailed explanation, please refer to page 12.)



Standard protocol

Stage 1, 2: Reverse transcription
Reps: 1
42℃ 5 min
95℃ 10 sec
Stage 3: PCR reaction
Reps: 40
95℃ 5 sec
60℃ 31 or 34 sec*6

*6 Set at 31 sec for 7300, and 34 sec for 7500.

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy for quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 10 sec.

3. After the reaction is complete, verify the amplification curve. Prepare a standard curve when quantification is desired. Please refer to the instruction manual for your real-time PCR instrument to determine the appropriate analysis methods.

【 Protocol using Smart Cycler II System 】

1. Prepare the following reagents on ice.

< Per reaction >

Reagent	Volume	Final Conc.
2X One Step RT-PCR Buffer III	12.5 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.5 μl	
PrimeScript RT enzyme Mix II	0.5 μl	
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
Probe	1 μl*2	
total RNA	2 μl*3	
RNase Free dH_2O	7.5 μl	
Total	25 μl	

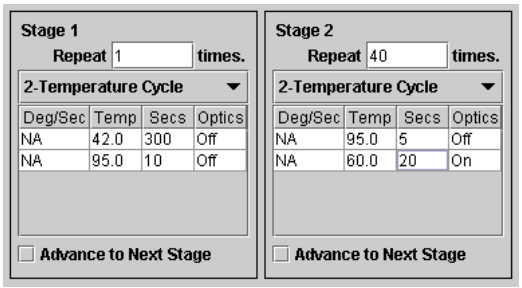
*1 Use primers at a final concentration of 0.2 μM for most reactions. If further optimization is needed, determine the optimal concentration within the range of 0.1 - 1.0 μM.

*2 Optimal probe concentration differs depending on the real-time PCR instrument and type of fluorescence labeling material. Refer to the instrument operation manual and product documents supplied with the probe. Generally, when performing detection using the Smart Cycler System, the final probe concentration should be 0.1 - 0.5 μM.

*3 Use 10 pg - 100 ng of total RNA as template.

2. Gently spin down the reaction tubes with the Smart Cycler-specific centrifuge, then start the reaction.

   Use the standard protocol described below for the reaction. If needed, the PCR conditions may be modified further. (For a detailed explanation, please refer to page 12.)



Standard protocol

Stage 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Stage 2: PCR reaction
Repeats: 40 times
95℃ 5 sec
60℃ 20 sec

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy for quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 10 sec.

3. After the reaction is complete, verify the amplification curve. Prepare a standard curve when quantification is desired. For analysis methods when using the Smart Cycler System, please refer to the instruction manual provided with this system.

【 Protocol using Thermal Cycler Dice Real Time System III, *II,* and *Lite* 】

1. Prepare the following reagents on ice.

< Per reaction >

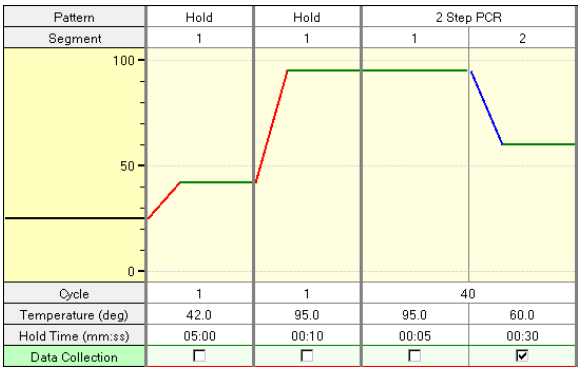
Reagent	Volume	Final Conc.
2X One Step RT-PCR Buffer III	12.5 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.5 μl	
PrimeScript RT enzyme Mix II	0.5 μl	
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
Probe	1 μl*2	
total RNA	2 μl*3	
RNase Free dH_2O	7.5 μl	
Total	25 μl	

*1 Use primers at final concentration of 0.2 μM for most reactions. If further optimization is needed, determine the optimal concentration within the range of 0.1 - 1.0 μM.

*2 Probe concentration differs depending on the real-time PCR instrument and type of fluorescent label. Refer to the instrument operation manual and product documents supplied with the probe. Generally, when performing detection using the Thermal Cycler Dice Real Time System, the optimal final probe concentration should be 0.1 - 0.5 μM.

*3 Use 10 pg - 100 ng of total RNA as template.

2. Start the reaction.
Use the standard protocol described below for the reaction. If needed, the PCR conditions may be modified further. (For detailed explanation, please refer to page 12.)



Standard protocol

Pattern 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Pattern 2: PCR reaction
Cycles: 40
95℃ 5 sec
60℃ 30 sec

Note:
This product contains *TaKaRa Ex Taq* HS, which includes a *Taq* antibody. There is no need to heat at 95℃ for 5 - 15 min for initial denaturation (as is required for chemically modified *Taq* polymerases). If longer heat treatment is used with this kit, the enzyme activity decreases and amplification efficiency and the accuracy for quantification may also be affected.
Perform heat inactivation of RTase prior to PCR at 95℃ for 10 sec.

3. After the reaction is complete, verify the amplification curve. Prepare a standard curve when quantification is desired. For the analysis methods using Thermal Cycler Dice Real Time System, please refer to the instruction manual.

< PCR Reaction Conditions >

Shuttle PCR

Step	Temp.	Time	Detection	Notes
Denature	95℃	3 - 5 sec	Off	Since real-time PCR targets are generally shorter than 300 bp, denaturation at 95℃ for 3 - 5 sec is sufficient.
Annealing/ Extension	56 - 64℃	20 - 30 sec (31, 34 sec)*	On	When optimizing reaction conditions, evaluate results using an annealing/extension temperature in the range of 56℃ to 64℃. If further optimization is needed, increasing incubation time for this step may improve results.

* Some systems do not allow a setting of 30 sec or less for the detection step. For the Applied Biosystems 7300 system, set at 31 seconds or longer; for the Applied Biosystems 7500 system, set at 34 seconds or longer.

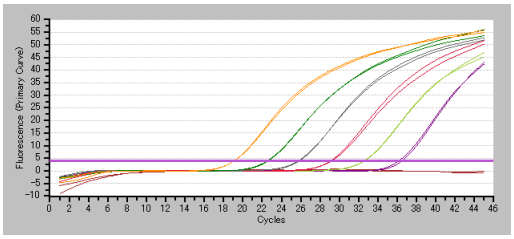
Recommended number of cycles: 30 - 45

VIII. Experimental Example

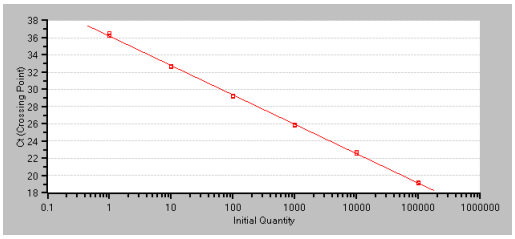
1. Detection of Mouse Actb
   Real-time one step RT- PCR was performed using 1 pg - 100 ng of mouse liver total RNA or sterilized water (negative control) as template. The Thermal Cycler Dice Real Time System was used to quantify PCR products detected with primers and probe of TaqMan Gene Expression Assays (Thermo Fisher Scientific).

2. Results

< Crossing Point method >

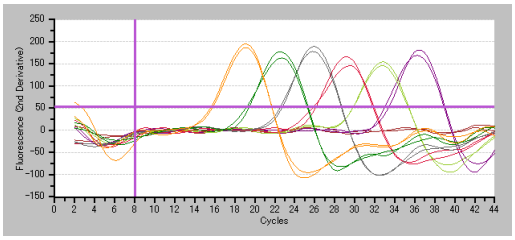


Amplification Curve

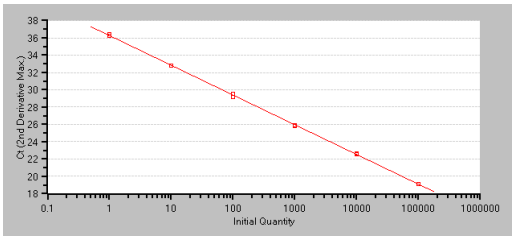


Standard Curve

< 2nd Derivative Maximum method >



Amplification Curve



Standard Curve

3. Discussion
   Actb was detected using a range of 1 pg - 100 ng of total RNA as a template.
   Linearity of the standard curve was obtained over a wide range of template DNA.

IX. Appendix

Preparation of RNA

This kit is designed to perform reverse transcription of RNA to cDNA and subsequent amplification by PCR. It is important to use high purity RNA samples for optimal cDNA yield. Therefore, it is essential to inhibit cellular RNase activity and also to prevent RNase contamination. Extra precaution should be taken during the sample preparation, including the use of clean disposable gloves, dedication of an area exclusively for RNA preparation, and avoidance of RNase contamination from sweat or saliva.

[Equipment]

Use disposable plastic equipment. Treat glassware with either protocol (1) or protocol (2) below prior to use.

(1) Heat sterilization (180℃ for 60 min).

(2) Treatment with 0.1% DEPC at 37℃ for 12 hours followed by autoclaving at 120℃ for 30 min to remove DEPC.

Note: Reserve dedicated equipment to be used exclusively for RNA preparation.

[Reagents]

All reagents used in this procedure must be prepared using tools which were treated as described in previous section (heat sterilization at 180℃ for 60 min or DEPC treatment). Treat distilled water with 0.1% DEPC followed by autoclaving. All reagents and distilled water should be used exclusively for RNA experiments.

[Preparation of RNA sample]

Use highly purified RNA obtained by the GTC (Guanidine thiocyanate) method, etc.

X. Related Products

Probe qPCR Mix (Cat. #RR391A/B)
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A/B)
One-Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*

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