Cat. # RR096A

For Research Use

TaKaRa

One Step TB Green®
PrimeScript™ PLUS RT-PCR Kit
(Perfect Real Time)

Product Manual

v202202Da

Table of Contents

I. Description

The One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) is designed for intercalator-based one-step, real-time RT-PCR using TB Green for detection. RT-PCR can be performed in a single tube, minimizing the risk of contamination. Also, amplified products are monitored in real time, so there is no need to verify them by electrophoresis following PCR. This kit is also suitable for detecting tiny amounts of RNA such as RNA viruses.
This kit uses PrimeScript PLUS RTase, which has exceptionally strong strand displacement activity and can efficiently synthesize cDNA in a short time period, and *TaKaRa Ex Taq*® HS, a high-efficiency hot-start PCR enzyme, which are optimized for one-step RT-PCR. The combination of *TaKaRa Ex Taq* HS, a hot-start PCR enzyme that includes an anti-*Taq* antibody, and a buffer optimized for real-time PCR, results in higher reaction specificity. Combining TaKaRa Bio RT-PCR technology with these enzymes allows this kit to provide more efficient amplification of RT-PCR products.

Compatible real-time PCR instruments:

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

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

II. Principle

The One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) synthesizes cDNA from RNA using PrimeScript PLUS Reverse Transcriptase, and amplifies this cDNA using *TaKaRa Ex Taq* HS, within a single tube. PCR amplification products are monitored in real time using TB Green as an intercalator.

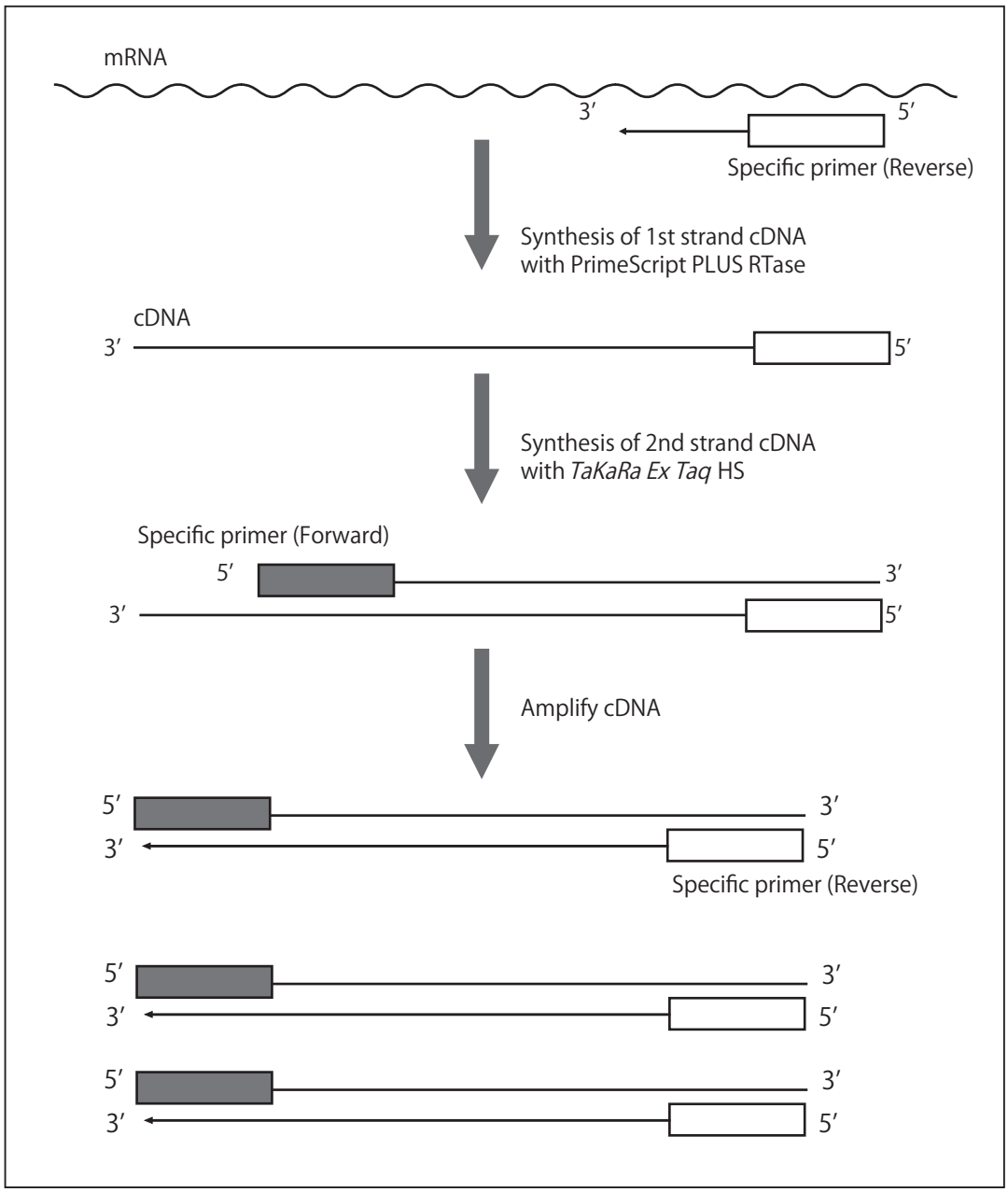
1. PCR

PCR is a technique used to amplify specific target sequences from min amounts of DNA. By repeating three cycles of heat denaturation, primer annealing, and primer extension, the target fragment is amplified up to a million times by DNA polymerase within a short time.

This product uses *TaKaRa Ex Taq* HS, a hot-start PCR enzyme that prevents nonspecific amplification resulting from mispriming or primer dimer formation during reaction mixture preparation or other pre-cycling steps, thereby allowing high-sensitivity detection.

2. RT-PCR

Although RNA cannot be used directly as a template for PCR, PCR can be used for highly sensitive RNA detection and analysis after synthesizing cDNA from RNA using reverse transcriptase (RT-PCR). This kit uses one-step RT-PCR, as shown below. With the One Step TB Green PrimeScript PLUS RT-PCR Kit, cDNA is synthesized from RNA using PrimeScript PLUS RTase with a specific reverse primer, and then PCR amplification is carried out in the same reaction tube using *TaKaRa Ex Taq* HS with specific forward and reverse primers. (Random primers and Oligo dT primers cannot be used for reverse transcription.)



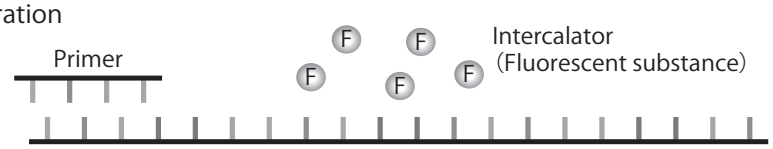
Principle of One-Step RT-PCR

3. Fluorescent spectrophotometer method

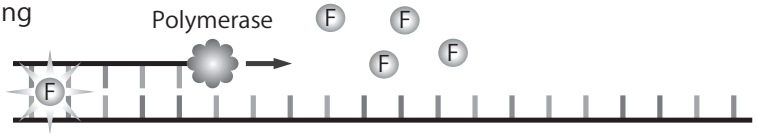
This method uses a DNA intercalator (e.g., TB Green) that emits fluorescence when bound to double-strand DNA. Monitoring fluorescence allows for quantification of amplification products.

Measuring the fluorescence intensity also provides the melting temperature of amplified DNA.

1) Heat denaturation



2) Primer annealing



3) Extension

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

1.	2X One Step TB Green RT-PCR Buffer 4＊1	840 μl x 3
2.	PrimeScript PLUS RTase Mix＊2	100 μl
3.	*TaKaRa Ex Taq* HS Mix＊3	300 μl
4.	RNase Free dH_2O	1.25 ml x 2
5.	ROX Reference Dye (50X conc.)＊4	100 μl
6.	ROX Reference Dye II (50X conc.)＊4	100 μl

＊1 Contains dNTP Mixture, Mg^{2+} and TB Green.
＊2 Contains reverse transcriptase and RNase inhibitor.
＊3 Contains *TaKaRa Ex Taq* HS and the accessory protein.
＊4 ROX Reference Dye and ROX Reference Dye II are intended to be used with instruments that correct for between-well fluorescent signal, such as the real-time PCR devices by Thermo Fisher Scientific.

◆ Use the ROX Reference Dye
- Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific)
- StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Use the ROX Reference Dye II
- Applied Biosystems 7500/7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ Not required
- Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*5
- Thermal Cycler Dice Real Time System *III*/*Lite* (Cat. #TP900/TP960/TP700/TP760: discontinued)
- LightCycler (Roche Diagnostics)
- Smart Cycler System (Cepheid)

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

Materials Required but not Provided

1. Real-time PCR instrument
2. Reaction tube or plate suitable for the real-time PCR instrument
3. PCR Primer＊6
4. Micropipettes and pipette tips (autoclaved)

＊6 Please refer to IX-B. Guidelines for Primer Design.

IV. Storage

-20℃
Store 2X One Step TB Green RT-PCR Buffer 4 protected from light.

V. Features

(1) One Step RT-PCR makes accurate and rapid analysis of small amounts of RNA possible.

(2) This kit offers highly efficient amplification and sensitive detection by combining a reverse transcriptase, PrimeScript PLUS RTase and *TaKaRa Ex Taq* HS, a *Taq* DNA polymerase containing a hot start antibody that strongly suppresses nonspecific amplification.

(3) One Step TB Green RT-PCR Buffer 4 is a 2X premix containing TB Green, simplifying reaction preparation.

VI. Precautions before Use

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

(1) For the reaction solution, prepare master mix for the required number of reactions plus a few extra. Preparation of a master mix minimizes losses and error due to pipetting, allowing the reagents to be dispensed more accurately. This reduces experimental variability.

(2) PrimeScript PLUS RTase Mix and *TaKaRa Ex Taq* HS Mix should be mixed gently. Avoid generating bubbles. Gently spin down the solution prior to pipetting. Pipet the enzymes slowly as the enzyme contains 50% glycerol and is very viscous. Keep the enzyme at -20℃ until just before use and return to the freezer promptly after use.

(3) If precipitate appears during thawing of 2X One Step TB Green RT-PCR Buffer 4, dissolve precipitate completely before use by vortexing.

(4) Always use fresh disposable tips to avoid any potential cross-contamination between samples when preparing or dispensing reaction mixtures.

(5) Use a gene-specific reverse primer for reverse transcription reactions performed with this kit. Do not use random primers or Oligo dT primer.

VII. Protocol

【For the LightCycler】

Note: Please follow the procedures provided in the LightCycler manual (Roche Diagnostics).

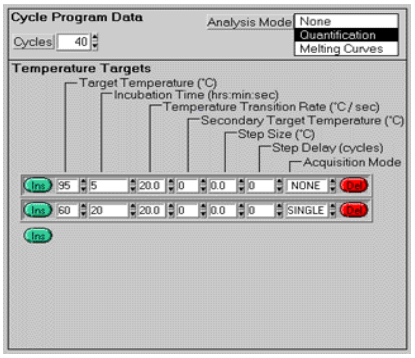
1. Prepare the PCR mixture shown below.

< Per reaction >

Reagent	Amount	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	10 μl	1X
TaKaRa Ex Taq HS Mix	1.2 μl	
PrimeScript PLUS RTase Mix	0.4 μl	
PCR Forward Primer (10 μM)	0.8 μl	0.4 μM*1
PCR Reverse Primer (10 μM)	0.8 μl	0.4 μM*1
total RNA	2 μl*2	
RNase Free dH_2O	4.8 μl	
Total	20 μl	

*1 A final primer concentration of 0.4 μM likely will provide good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.
*2 It is recommended to use 10 pg - 100 ng total RNA as template.

2. Gently spin down PCR capillaries, then start the reaction after placing them in the LightCycler.
It is recommend to use the standard protocol described below. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. (For guidelines on optimizing PCR conditions, refer to Page 12.)



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
Stage 3: melting curve analysis
95℃ 0 sec 20℃/sec
65℃ 15 sec 20℃/sec
95℃ 0 sec 0.1℃/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 completed, verify amplification curve and melting curve. Establish the standard curve when quantitative analysis is necessary.
Please refer to the instruction manual for your real time PCR instrument to read about analytical methods.

【 For the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System and StepOnePlus Real-Time PCR System 】

Note: Please follow the procedures provided in the manual of the respective instrument.

1. Prepare the PCR mixture shown below on ice.

< Per reaction >

Reagent	Amount	Amount	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	10 μl	25 μl	1X
TaKaRa Ex Taq HS Mix	1.2 μl	3 μl	
PrimeScript PLUS RTase Mix	0.4 μl	1 μl	
PCR Forward Primer (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 or Dye II (50 X)*3	0.4 μl	1 μl	
total RNA	2 μl	4 μl*2	
RNase Free dH_2O	4.4 μl	12 μl	
Total	20 μl*4	50 μl*4	

*1 A final primer concentration of 0.4 μM likely will provide good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.

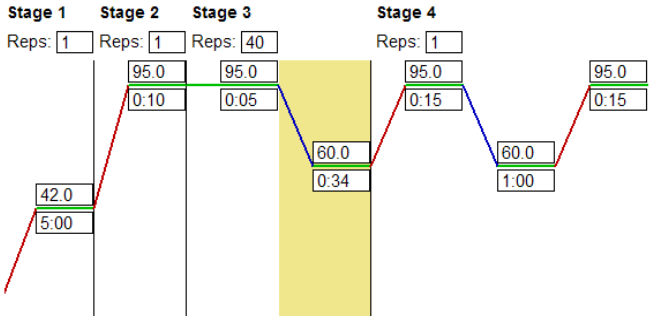
*2 It is recommended to use 20 pg - 200 ng total RNA as template in a 50 μl reaction volume.

*3 The ROX Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities among wells when used with real time PCR instruments that have option.
Use ROX Reference Dye (50X) when using Applied Biosystems 7300 Real-Time PCR System and StepOnePlus Real-Time PCR System.
Use ROX Reference Dye II (50X) when using Applied Biosystems 7500/7500 Fast Real-Time PCR System.

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

2. Start the reaction.
It is recommended to use the standard protocol described below. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when using primers with low Tm values or when shuttle PCR is not feasible. (For guidelines on optimizing PCR conditions, refer to Page 12.)

< 7300/ 7500 Real-Time PCR System, StepOnePlus Real-Time PCR System >



Stage 1, 2: Reverse transcription
Reps: 1
42℃ 5 min
95℃ 10 sec

Stage 3: PCR reaction
Reps: 40
95℃ 5 sec
60℃ 30 - 34 sec＊5

Stage 4: Dissociation Protocol

＊5 With StepOnePlus, set to 30 sec, with 7300, set to 31 sec, and with 7500, set to 34 sec.

< 7500 Fast Real-Time PCR System >

Holding Stage
42℃ 5 min
95℃ 10 sec

Cycling Stage
Number of Cycles: 40
95℃ 3 sec
60℃ 30 sec

Melt Curve Stage

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, check the amplification and melting curves and plot a standard curve if absolute quantification will be performed.
Please refer to the instruction manual for your real time PCR instrument for information on analytical methods.

【 For the Smart Cycler II System 】

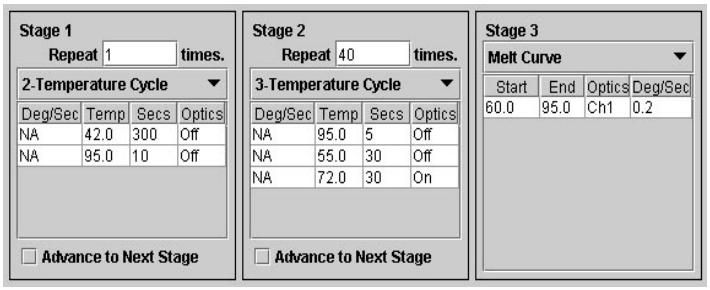
1. Prepare the PCR reaction mixture shown below on ice.

< Per reaction >

Reagent	Amount	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	12.5 μl	1X
TaKaRa Ex Taq HS Mix	1.5 μl	
PrimeScript PLUS RTase Mix	0.5 μl	
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM＊1
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM＊1
total RNA	2.0 μl＊2	
RNase Free dH_2O	6.5 μl	
Total	25 μl	

＊1 A final primer concentration of 0.4 μM likely will provide good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.
＊2 It is recommended to use 10 pg - 100 ng total RNA as template.

2. Briefly centrifuge reaction tubes with the Smart Cycler centrifuge and then place them in the Smart Cycler instrument to initiate the reaction.
It is recommended to use the standard protocol described below. Try this protocol first and optimize PCR conditions as necessary.
(For guidelines on optimizing PCR conditions, refer to Page 12.)



Stage 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Stage 2: PCR reaction
Repeats: 40 times
95℃ 5 sec
55℃ 30 sec
72℃ 30 sec
Stage 3: Melt Curve

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, check the amplification and melting curves and plot a standard curve if absolute quantification will be performed.
For information on analytical methods when using the Smart Cycler System, please refer to the instruction manual for the Smart Cycler System.

【For the Thermal Cycler Dice Real Time System series】

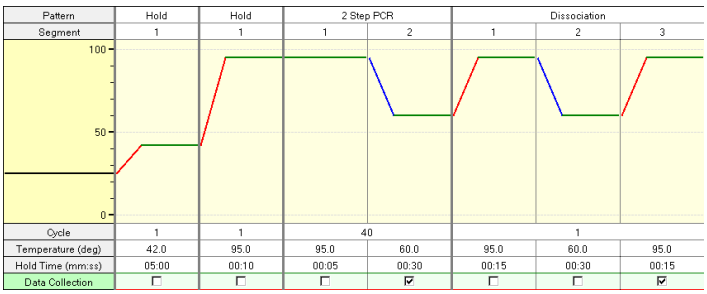
1. Prepare the PCR reaction mixture shown below on ice.

< Per reaction >

Reagent	Amount	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	12.5 μl	1X
TaKaRa Ex Taq HS Mix	1.5 μl	
PrimeScript PLUS RTase Mix	0.5 μl	
PCR Forward Primer (10 μM)	1.0 μl	0.4 μM*1
PCR Reverse Primer (10 μM)	1.0 μl	0.4 μM*1
total RNA	2.0 μl*2	
RNase Free dH_2O	6.5 μl	
Total	25 μl	

*1 A final primer concentration of 0.4 μM likely will provide good results. However, if there is an issue with reactivity, use a primer concentration between 0.1 and 1.0 μM.
*2 It is recommended to use 10 pg - 100 ng total RNA as template.

2. Gently spin down the reaction tubes or plate with a centrifuge, then start the reaction after setting them in the Thermal Cycler Dice Real Time System.
First try the standard protocol described as follows. Optimize PCR reaction conditions as needed. Use 3-step PCR if shuttle PCR is difficult, for example when using primers with low Tm values. (For guidelines on optimizing PCR conditions, refer to Page 12.)



Stage 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Stage 2: PCR reaction
Repeats: 40 times
95℃ 5 sec
60℃ 30 sec
Stage 3: Dissociation

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 completed, verify the amplification curve and melting curve. Establish the standard curve when quantification is done.
Refer to the operation manual of Thermal Cycler Dice Real Time System and the following Experiment Examples for the analysis method with Thermal Cycler Dice Real Time System.

PCR Reaction Conditions

If the recommended standard protocol do not provide sufficient reactivity, follow the procedures below to optimize primer concentration and PCR conditions.

Select PCR conditions based on comprehensive analysis of both reaction specificity and amplification efficiency. Using conditions that balance these two aspects will allow accurate measurement over a wide range of concentrations.

○ System with a high reaction specificity

- With a no template control, nonspecific amplification (e.g., primer-dimers) does not occur.
- Nonspecific amplification products, those other than the target product, are not generated.

○ System with a high amplification efficiency

- Amplification product is detected at earlier cycles (small Ct value).
- PCR amplification efficiency is high (near the theoretical value of 100%).

1. Evaluation of primer concentration

The relationship between primer concentration and reaction specificity and amplification efficiency is illustrated below. Reducing the primer concentration raises reaction specificity. In contrast, increasing the primer concentration raises amplification efficiency.

(Primer concentration)	Low (0.1 μM)		High (1.0 μM)
Specificity	high	←	low
Efficiency	low	→	high

2. Evaluation of PCR conditions

○ To improve reaction specificity

Raising the annealing temperature may improve reaction specificity. Perform optimization while checking amplification efficiency.

[Shuttle PCR] Standard protocol		Raise the annealing temperature	
95℃	5 sec	95℃	5 sec
60℃	30 sec	up to 64℃	30 sec

○ To raise amplification efficiency

Increasing the elongation time or switching to 3-step PCR may improve amplification efficiency.

[Shuttle PCR] Standard protocol		Prolong the extension time		[3-step PCR]		Prolong the elongation time	
95℃	5 sec	95℃	5 sec	95℃	5 sec	95℃	5 sec
60℃	30 sec	60℃	1 min -	55℃	30 sec	55℃	30 sec
				72℃	30 sec	72℃	1 min -

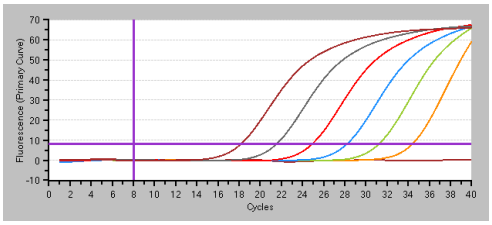
VIII. Experimental Example

Detection of Mouse Ppia (peptidylproly I isomerase A)
(Thermal Cycler Dice Real Time System is used)

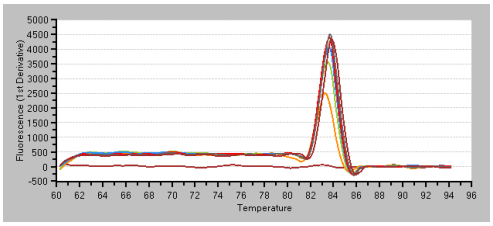
1. Procedure

   Real-time, one-step RT- PCR was performed using 2 pg - 200 ng of total RNA that was prepared from mouse liver and sterile purified water (negative control) as the template.

2. Result

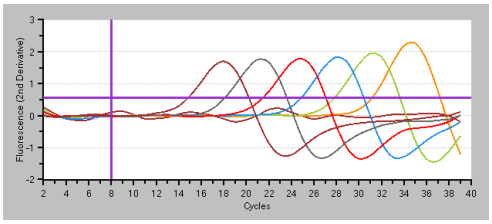


Amplification Curve

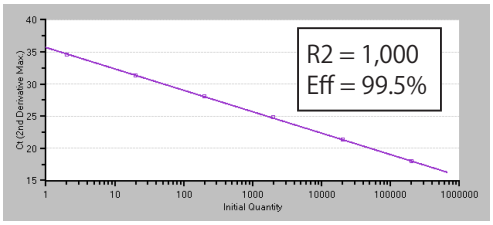


Melting Curve

After performing the reaction, the Ct value was obtained from the 2nd derivative of the amplification curve, and then used to create the standard curve.



2nd derivative



Standard Curve

3. Discussion

   It was possible to detect the target gene using 2 pg - 200 ng total RNA. The melting curve shows that the same amplification products were obtained, even when different amounts of template were used. The standard curve remained linear within the concentration range of the template.

IX. Appendix

A. Preparation of RNA Samples

The kit is used for synthesis of cDNA from RNA and PCR amplification of a target gene. For successful cDNA synthesis, it is essential to obtain highly pure RNA. Great care must be taken to inhibit RNase from both endogenous and external sources. To prevent RNase contamination (e.g., from sweat or saliva introduced while handling and preparing the RNA), take measures such as avoiding unnecessary talking, wearing clean disposable gloves, and using a dedicated laboratory bench for preparing RNA.

Note: It is recommended that all the equipment be used as the exclusive use for RNA preparation.

[Equipment]

Disposable plastic equipment shall be used. Glass tools should be treated with one of the following protocols prior to use.

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

[Reagent]

All reagents to be used in this experiment must be prepared using glasswares which were treated as described in previous section (Hot-air sterilization or DEPC treatment). 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 Samples]

Since RT-PCR usually requires only small amounts of RNA, common purification methods are usually sufficient. However, we recommend that the guanidine thiocyanate (GTC) method be used if possible. In general, RNA should be of the highest purity possible.

When preparing high purity total RNA from cell cultures or tissue samples, NucleoSpin RNA (Cat. #740955.10/.50/.250) or the AGPC method simplified reagent RNAiso Plus (Cat. #9108/9109) can be used. For blood samples, NucleoSpin RNA Blood (Cat. #740200.10/.50) or RNAiso Blood (Cat. #9112/9113) can be used.

B. Guidelines for Primer Design

Designing primers which allow good reactivity is critical to efficient real-time PCR. Please follow the guidelines below to design primers that yield high amplification efficiency without nonspecific amplification.

■ Amplification product

Amplified size	The optimal size is 80 - 150 bp (amplification up to 300 bp is possible).

■ Primer

Length	17 - 25 mer
GC content	40 - 60% (45 - 55% is recommended.)
Tm	Make sure that the Tm values for the forward primer and the reverse primer do not differ greatly. Use primer design software to determine Tm values. OLIGO＊1: 63 - 68℃ Primer 3 : 60 - 65℃
Sequence	Make sure that there are no overall base sequence biases. Avoid having any GC-rich or AT-rich regions in the sequence (particularly at the 3′ end). Avoid having consecutive T/C pairings (polypyrimidine). Avoid having consecutive A/G pairings (polypurine).
Sequence of 3' end	Avoid having any GC-rich or AT-rich sequence at the 3′ end. It is preferable to have a G or C as the 3′ end-base. Avoid primers with T as the 3′ end-base.
Complementarity	Avoid having any complementary sequences of 3 bases or more within a primer and between primers. Avoid having any complementary sequences of 2 bases or more at the primer's 3′ ends.
Specificity	Verify primer specificity by BLAST search＊2.

＊1 OLIGO Primer Analysis Software (Molecular Biology Insights, Inc.)
＊2 https://blast.ncbi.nlm.nih.gov/Blast.cgi

X. Related Products

One Step TB Green® PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B)*
One Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
TB Green® Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B)*
One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR064A/B)
Probe qPCR Mix (Cat. #RR391A/B)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
NucleoSpin RNA (Cat. #740955.10/.50/.250)*
NucleoSpin RNA Blood (Cat. #740200.10/.50)*

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