Cat. # RR066A

For Research Use

TaKaRa

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

Product Manual

v202202Da

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I. Description

The One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) is designed for intercalator-based one step, real-time RT-PCR using TB Green for detection. All reactions can be performed in a single tube, therefore operation is simple, and the risk of contamination is minimized.
Amplified products are monitoring in real time, so there is no need for electrophoresis after PCR. This kit is suitable for detection of tiny amounts of RNA such as RNA virus.

This kit uses PrimeScript RTase, which has excellent extension capability and can efficiently synthesize cDNA in short time, and *TaKaRa Ex Taq*® HS, a high efficiency hot start PCR enzyme. Both enzymes are optimized for one step RT-PCR, allowing efficient generation of RT-PCR products.

Applicable real time PCR instruments:

- 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)
- Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System, StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
- Smart Cycler System/Smart Cycler II System (Cepheid)
- LightCycler (Roche Diagnostics), and other

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

II. Principle

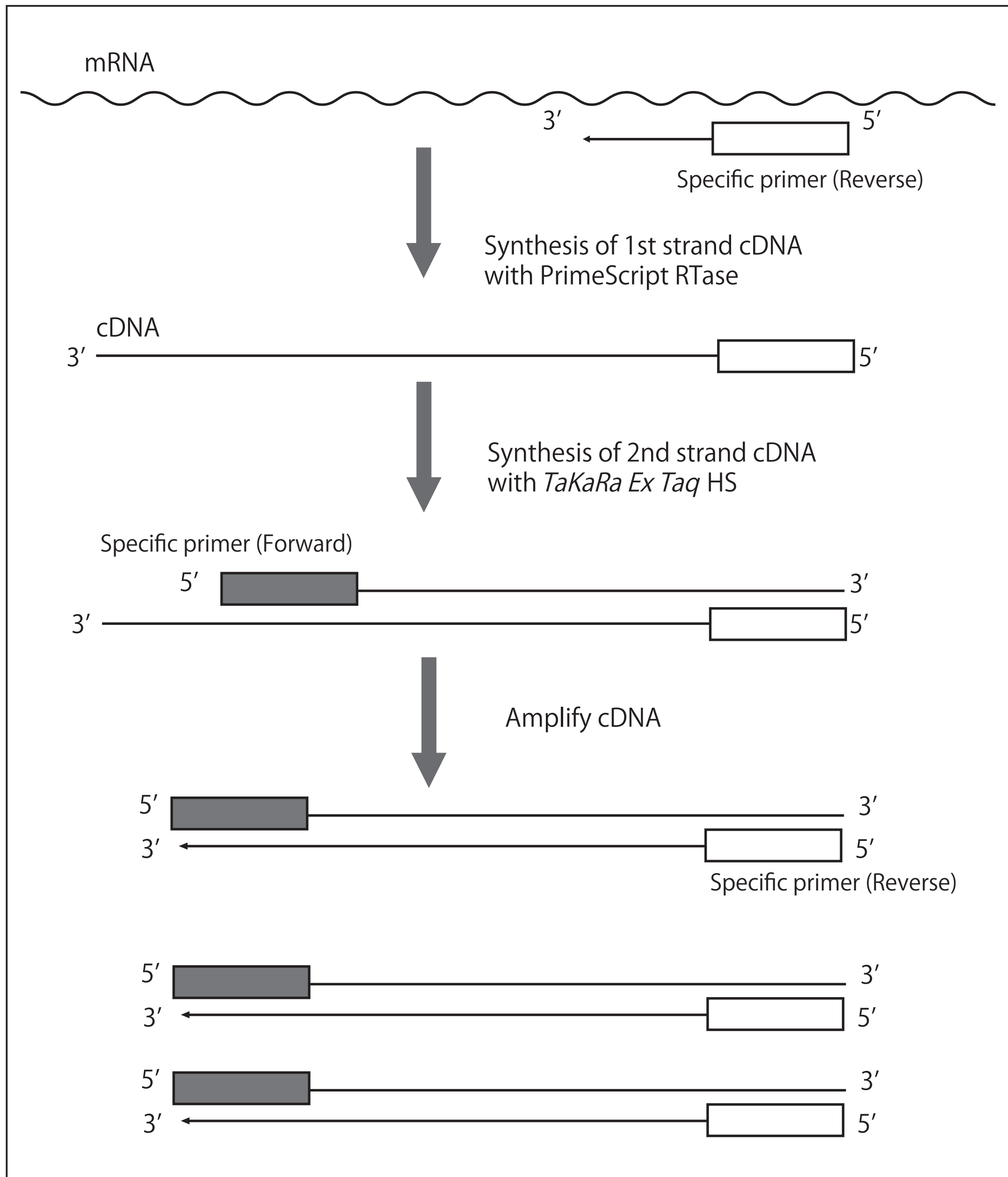
The One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) enables cDNA synthesis from RNA using reverse transcriptase, PrimeScript RTase, and PCR amplification using *TaKaRa Ex Taq* HS in one tube. PCR amplification products are monitored in real time with TB Green as an itercalator

1. PCR

PCR is a technique that amplifies a targeted DNA region from a small amount of DNA. One cycle of PCR includes heat denaturation of DNA, primer annealing, and primer extension by DNA polymerase. By repeating this process, PCR allows amplification of a targeted gene segment to one million times in a short time period. The use of a hot start PCR enzyme, *TaKaRa Ex Taq* HS, for amplification prevents miss-priming during preparation of reagents, and non-specific amplification caused by primer dimer.

2. **RT-PCR**

Although RNA cannot be used as a direct template of PCR, PCR can be applied for RNA analysis when cDNA is first synthesized from RNA with reverse transcriptase (RT-PCR). The principle of one step RT-PCR is shown below; a specific primer (reverse) is used for reverse transcription. Then using the synthesized cDNA as a template, PCR amplification is performed with specific primers (forward, reverse). (Random Primer and Oligo dT Primer cannot be used for reverse transcription.)



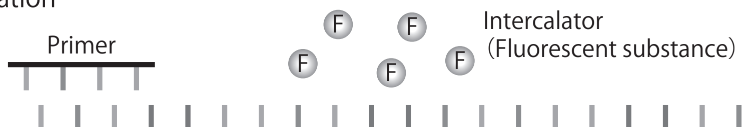
Principle of One Step RT-PCR

3. Fluorescence detection

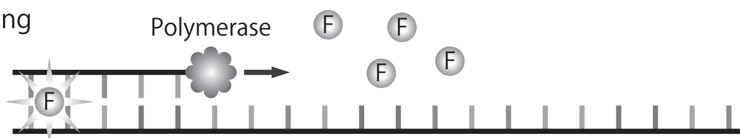
[Intercalator method]

This method detects fluorescence produced during amplification by adding an Intercalator (e.g., TB Green) that fluoresces when bound to double strand of DNA. By detecting this fluorescence, the amount of amplified DNA can be quantified and the melting point can be measured.

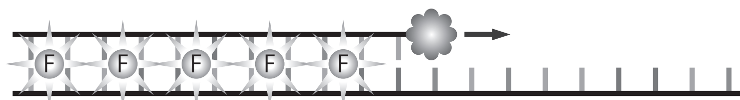
1) Heat denaturation



2) Primer annealing



3) Extension



III. Components (100 reactions, 50 μl volume)

1.	2X One Step TB Green 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+}, and TB Green. Store protected from light.

*2 Includes PrimeScript RTase and RNase Inhibitor.

*3 ROX Reference Dye/Dye II is used for normalization of the fluorescent signal by background subtraction.

◆ 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)*4
- Thermal Cycler Dice Real Time System *II*/*Lite* (Cat. #TP900/TP960/TP700/TP760: discontinued)
- Smart Cycler System/Smart Cycler II System (Cepheid)
- LightCycler (Roche Diagnostics)

*4 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 plates suitable for the real-time PCR instrument
3. PCR primers*5
4. Micropipettes and pipette tips (autoclaved)

*5 Refer to XI. Guidelines for Primer Design.

IV. Storage

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

V. Features

(1) One Step RT-PCR is intended for accurate and rapid analysis of RNA viruses or small amounts of RNA.

(2) For PCR, *TaKaRa Ex Taq* HS, a high efficiency hot start PCR enzyme, is used. The buffer system is optimized for real-time PCR, thus high quality detection is possible. Moreover, One Step TB Green RT-PCR Buffer III is a 2X concentration of premix with TB Green, so preparation of reaction mixtures is simple.

VI. Precautions

Please read it carefully before use.

(1) When mixing reagents for PCR, mix enough for 10 reactions (master mix). Using a master mix allows accurate reagent dispensing, minimizes reagent pipetting errors, and avoids repeated dispensing of each reagent. This helps to minimize variation between experiments.

(2) PrimeScript RT enzyme Mix II and *TaKaRa Ex Taq* HS 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 into the freezer promptly after use.

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

(4) Use new disposable pipette tips to avoid contamination between samples for transferring reagent.

(5) Use the gene-specific reverse primer for reverse transcription. Random Primer or Oligo-dT Primer should not be used.

VII. Protocol

【For the Thermal Cycler Dice Real Time System III (*II* and *Lite*: disdontinued)】

1. Prepare the following reagents on ice.

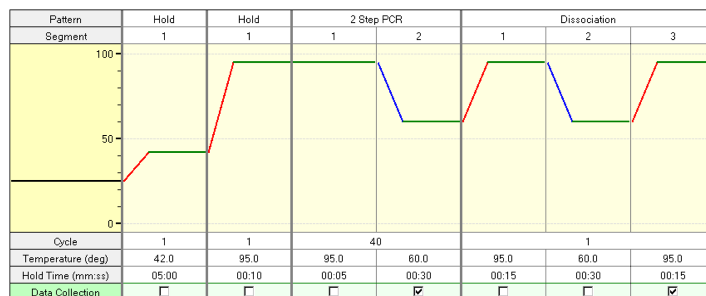
< Per reaction >

Reagent	Volume	Final conc.
2X One Step TB Green 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
total RNA*2	2 μl	
RNase Free dH_2O	8.5 μl	
Total	25 μl	

*1 The final concentration of primers can be 0.2 μM in most reactions. However, if there is insufficient 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 a template.

2. Gently spin down the reaction tubes or plate, then start the reaction after setting them in the Thermal Cycler Dice Real Time System.
Use the standard protocol described as follows. Try this protocol first and optimize PCR condition as necessary. Perform 3-step PCR when shuttle PCR is difficult, for example for primer with low Tm value.
To optimize PCR conditions further, refer to section VIII. Optimization.



Pattern 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Pattern 2: PCR
Cycles: 40
95℃ 5 sec
60℃ 30 sec
Pattern 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 complete, verify the amplification curve and melting curve. Plot a standard curve if absolute quantification is desired.
Refer to the instrument manual of Thermal Cycler Dice Real Time System and the following application examples for the analysis method with Thermal Cycler Dice Real Time System.

【 StepOnePlus Real-Time PCR System 】

Note: Follow manuals of each instrument to operate them.

1. Prepare the following reagents on ice.

< Per reaction >

Reagent	Volume	Volume	Final Conc.
2X One Step TB Green 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
ROX Reference Dye or Dye II (50 X)*3	0.4 μl	1 μl	
total RNA*2	2 μl	4 μl	
RNase Free dH_2O	6 μl	16 μl	
Total	20 μl*4	50 μl*4	

*1 The final concentration of primers can be 0.2 μM in most reactions. However, if there is insufficient 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 a template in a 50 μl reaction.

*3 The ROX Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities between wells when using the real-time PCR instruments below. For Applied Biosystems 7300 Real-Time PCR System and StepOnePlus Real-Time PCR System, use ROX Reference Dye (50X). For Applied Biosystems 7500/7500 Fast Real-Time PCR System, use ROX Reference Dye II (50X).

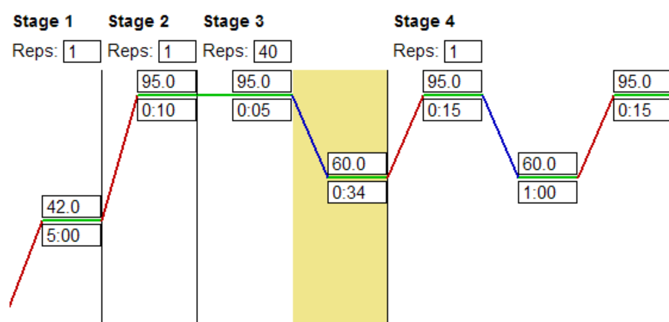
*4 Prepare in accordance with the recommended volume for each instrument.

2. Start reaction

Use the standard protocol described below. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when shuttle PCR is difficult, for example for primer with low Tm value.

To optimize PCR conditions further, refer to section VIII. Optimization.

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



Stage 1, 2: Reverse transcription
Reps: 1
42℃ 5 min
95℃ 10 sec
Stage 3: PCR
Reps: 40
95℃ 5 sec
60℃ 30 - 34 sec*5
Stage4: Dissociation Protocol

*5 Set up at 30 sec for StepOnePlus, 31 sec for 7300, and 34 sec for 7500.

< Applied Biosystems 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, verify the amplification curve and dissociation curve. Plot the standard curve if quantification is done.
   Refer to the operation manual of the real-time PCR instrument for additional analysis methods.

【 For the LightCycler 】

Note: Follow the manual of LightCycler (Roche Diagnostics).

1. Prepare the following reagents on ice.

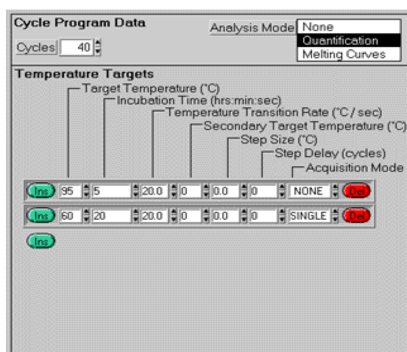
< Per reaction >

Reagent	Volume	Final Conc.
2X One Step TB Green RT-PCR Buffer III	10 μl	1X
TaKaRa Ex Taq HS (5 U/ 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
total RNA*2	2 μl	
RNase Free dH_2O	6.4 μl	
Total	20 μl	

*1 The final concentration of primers can be 0.2 μM in most reactions. However, if there is insufficient 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 a template.

2. Gently spin down the PCR capillaries, then start the reaction after setting them onto LightCycler.
Use standard protocol described below. Try this protocol first and optimize PCR conditions as necessary. Perform 3-step PCR when shuttle PCR is difficult, for example for primers with low Tm values.
To optimize PCR conditions further, refer to section VIII. Optimization.



Stage 1: Reverse transcription
42℃ 5 min 20℃/sec
95℃ 10 sec 20℃/sec
1 cycle

Stage 2: PCR
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 complete, verify amplification curve and melting curve. Plot the standard curve if absolute quantification is desired.
Refer to the operation manual of the LightCycler for additional analysis methods.

【 For the Smart Cycler II System 】

1. Prepare the following reagents on ice.

< Per reaction >

Reagent	Volume	Final Conc.
2X One Step TB Green RT-PCR Buffer III	12.5 μl	1X
TaKaRa Ex Taq HS (5U/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
total RNA*2	2 μl	
RNase Free dH_2O	8.5 μl	
Total	25 μl	

*1 The final concentration of primers can be 0.2 μM in most reactions. However, if there is insufficient reactivity, use a primer concentration between 0.1 and 1.0 μM.
*2 It is recomended to use 10 pg - 100 ng total RNA as a template.

2. Gently spin down the reaction tubes with a Smart Cycler specific centrifuge, then start the reaction after setting them onto Smart Cycler.
   Use standard protocol described below. First, try this protocol and optimize PCR condition as necessary. Perform 3-step PCR when shuttle PCR is difficult to process, for example for primer with low Tm value.
   To optimize PCR conditions further, refer VIII. Optimization.

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

Stage 1 — Repeat 1 times. — 2-Temperature Cycle

Deg/Sec	Temp	Secs	Optics
NA	42.0	300	Off
NA	95.0	10	Off

Advance to Next Stage

Stage 2 — Repeat 40 times. — 2-Temperature Cycle

Deg/Sec	Temp	Secs	Optics
NA	95.0	5	Off
NA	60.0	20	On

Advance to Next Stage

Stage 3 — Melt Curve

Start	End	Optics	Deg/Sec
60.0	95.0	Ch1	0.2

Stage 1: Reverse Transcription
Hold
42℃ 5 min
95℃ 10 sec
Stage 2: PCR
Repeats: 40 times
95℃ 5 sec
60℃ 20 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, verify the amplification curve and melting curve. Plot the standard curve if absolute quantification is desired.
   Refer to the operation manual of Smart Cycler for additional analysis methods.

VIII. Optimization

If the recommended conditions (shuttle PCR standard protocol) provide unsatisfactory reactivity, follow the procedures below to evaluate what primer concentration and PCR conditions to use.

Select PCR conditions based on comprehensive analysis, taking into consideration both reaction specificity and amplification efficiency. A PCR system balanced between these two aspects allows accurate assay over a wide range of concentrations.

○ System with a high reaction specificity

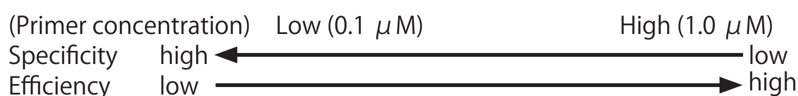
- With no template control, non-specific amplification such as primer-dimer formation do not take place.
- Non-specific amplification products, those other than the target product, are not generated.

○ System with a high amplification efficiency

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

1. Evaluation of primer concentration

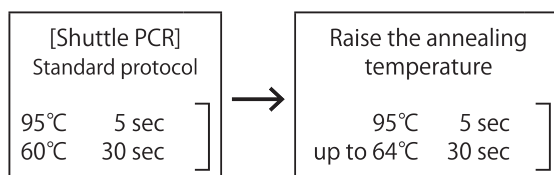
The relationship between primer concentration and reaction specificity and amplification efficiency is illustrated below. Reducing the primer concentration raises reaction specificity. Increasing the primer concentration, in contrast raises amplification efficiency.



2. Evaluation of PCR condition

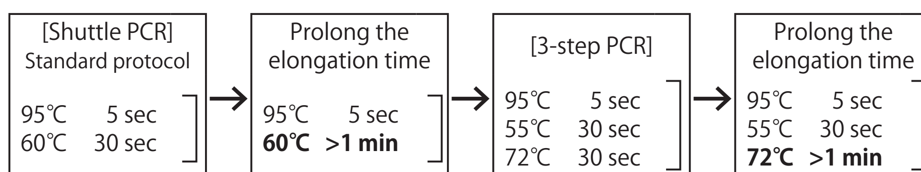
○ To raise reaction specificity

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



○ To raise amplification efficiency

Prolonging the elongation time or switching to a 3-step PCR may improve amplification efficiency. Perform optimization using the steps below.

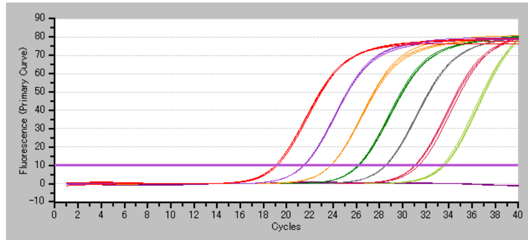


IX. Experimental Example

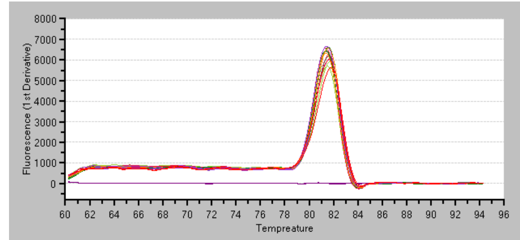
Detection of Rat Rplp2 (ribosomal protein, large P2)
(Thermal Cycler Dice Real Time System is used here.)

1. Procedure
   Using total RNA 6.4 pg - 100 ng that was prepared from rat liver or sterile purified water (negative control) as a template, real-time one step RT- PCR was performed.

2. Result

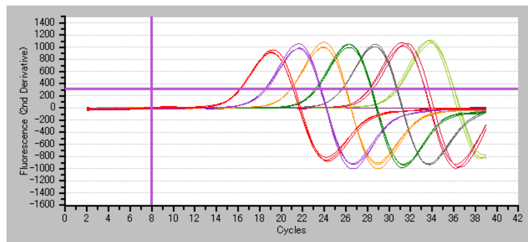


Amplification Curve

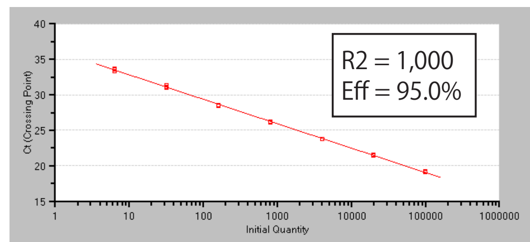


Melting Curve

After reaction, obtain Ct value from 2nd derivative of amplification curve and plot the standard curve.



2nd derivative



Standard Curve

Target DNA was detected. Using 6.4 pg - 100 ng of total RNA, the melting curve shows that the same amplification products were obtained even different amounts of template. The linearity of standard curve was obtained over the wide range of template concentrations.

X. Appendix

Preparation of RNA sample

This kit is designed to perform the reverse transcription of RNA to cDNA and subsequent PCR amplification. It is important to use high purity of RNA sample for better yields of cDNA synthesis. So, it is essential to inhibit cellular RNase activity and also to prevent contamination with RNases derived from equipment and solutions used. Extra precaution should be taken during sample preparation, including use of clean disposable gloves, dedication of a work surface exclusively for RNA preparation, and avoiding unnecessary taking during assembly, to prevent the RNase contamination from operates' sweat or saliva.

[Equipment]

Disposable plastic equipment should be used. Glass tools should be treated with the following protocol 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.

Note: It is recommended that all equipment are used exclusively for RNA preparation.

[Reagent]

All reagents used in this experiment must be prepared using tools treated as described above (hot-air sterilization(180℃, 60 min) or DEPC treatment), and 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]

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.

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

XI. Guidelines for Primer Design

It is essential to design primers that allow good reactivity for successful real-time PCR reactions. Follow the guidelines below to design primers which offer high amplification efficiency and minimize non-specific reaction.

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 overall there are no 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

XII. Related Products

One Step TB Green® PrimeScript™ PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B)*
One Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)
One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR064A/B)
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420/A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820/A/B)
TB Green® Premix DimerEraser™ (Perfect Real Time) (Cat. #RR091A/B)*
Probe qPCR Mix (Cat. #RR391A/B)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
RNAiso Plus (Cat. #9108/9109)
RNAiso Blood (Cat. #9112/9113)

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

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