Cat. # RR086A

For Research Use

TaKaRa

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

Product Manual

We have begun the process of changing the names for Takara Bio's intercalator-based real-time PCR (qPCR) products to the "TB Green series".
These products can be used the same way as before, as only the names are changing.
Catalog number and product performance are unaffected by this transition.

v202008Da

Table of Contents

I. Description

One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) is designed for intercalator-based one-step, real-time RT-PCR using TB Green for detection. All steps of RT-PCR can be performed in a single tube allowing a simple procedure and minimizing the risk of contamination. Also, amplified products are monitored in real time, so there is no need to verify by 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 extendibility and can efficiently synthesize cDNA quickly, and *TaKaRa Ex Taq*® HS, a high efficiency hot start PCR enzyme, optimized for one step RT-PCR. In comparison to One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A), this kit has an improved buffer system, which results in improved reaction specificity. In addition, this kit achieves simple and convenient procedure with premixed components.

Applicable real time PCR instruments: ＊1

- Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System, StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
- LightCycler (Roche Diagnostics)
- Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990) ＊2
- Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued)

＊1 Use One Step TB Green PrimeScript RT-PCR Kit (Perfect Real Time) (Cat. #RR066A) ＊2 or One Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real Time) (Cat. #RR096A/B) ＊2 for analysis with Smart Cycler System/ Smart Cycler II System (Cepheid).

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

II. Principle

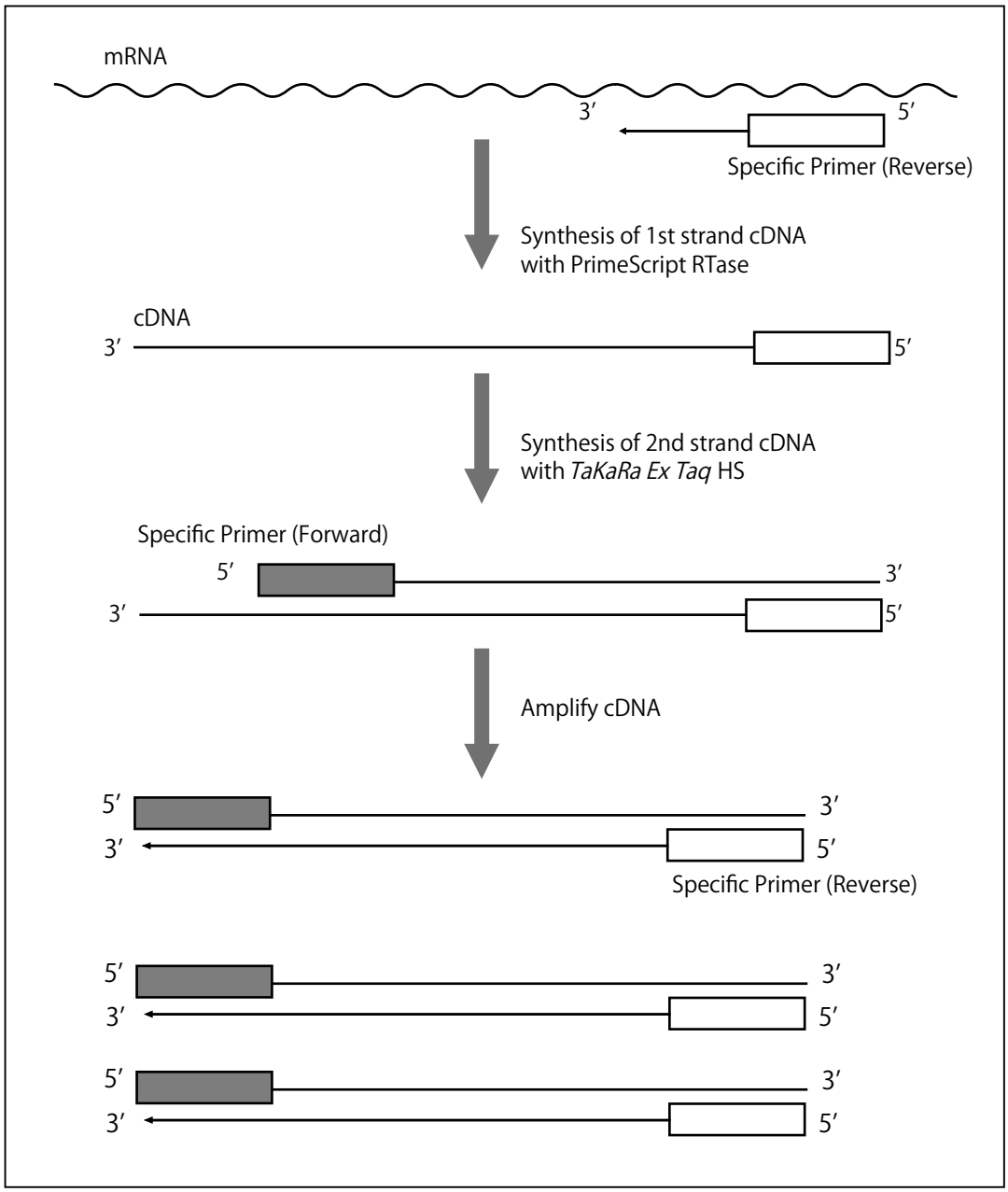
One Step TB Green PrimeScript RT-PCR Kit II (Perfect Real Time) performs cDNA synthesis from RNA using PrimeScript Reverse Transcriptase and PCR amplification with *TaKaRa Ex Taq* HS DNA Polymerase within one tube continuously. PCR amplification products are monitored in real time using TB Green as an intercalator.

1. PCR

PCR is a technique that amplifies a targeted region of a gene from a small amount of DNA. One cycle of PCR includes heat denaturation of DNA, primer annealing, and extension with DNA polymerase. By repeating this process, PCR allows exponential amplification of the targeted gene fragment in a short time period. Use of a hot start PCR enzyme, such as *TaKaRa Ex Taq* HS, for amplification avoids mispriming prior to the reaction and non-specific amplification caused by primer dimer formation allowing high quality detection.

2. RT-PCR

Although RNA is not a direct template of PCR, PCR can be applied for RNA analysis when cDNA is first synthesized from RNA with reverse transcriptase (RT-PCR). This kit uses One Step RT-PCR. The principle of this is shown below. For One Step RT-PCR, use Specific Primer (Reverse) for reverse transcription. Then, PCR amplification is performed using Specific primers (Forward, Reverse) with the synthesized cDNA as a template. (Random Primer and Oligo dT Primer cannot be used for reverse transcription.)

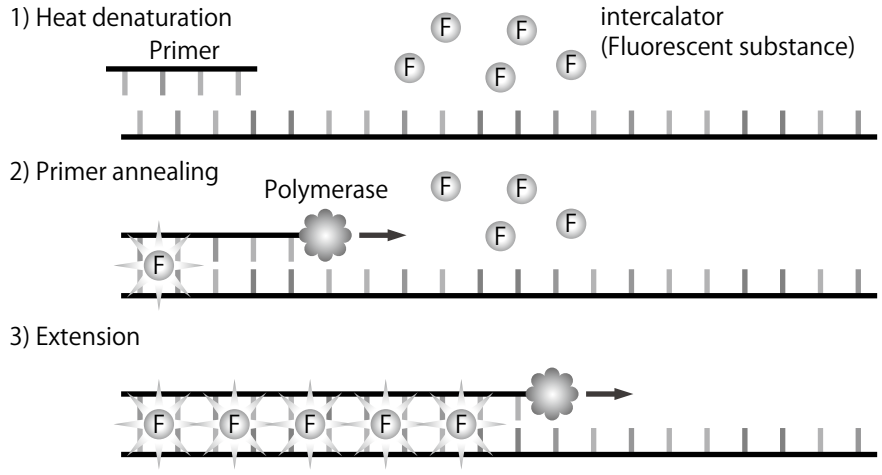


Principle of One Step RT-PCR

3. Fluorescent spectrophotometer method

[Intercalator method]

This method detects fluorescence which produced during the amplification process by adding DNA intercalators (for example: TB Green) that fluoresce by bonding to double strand DNA. It fluoresces when bound to double stranded DNA that is synthesized by polymerase reaction. From detecting this fluorescence, not only the quantity of amplified DNA, but also, the melting point can be measured.



III. Components (100 reactions, 50 μl reaction system)

1.	2X One Step TB Green RT-PCR Buffer 4＊1	840 μl x 3
2.	PrimeScript 1 step Enzyme Mix 2＊2	200 μl
3.	RNase Free dH_2O	1.25 ml x 2
4.	ROX Reference Dye (50X conc.)＊3	100 μl
5.	ROX Reference Dye II (50X conc.)＊3	100 μl

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

＊2 Includes PrimeScript RTase, RNase Inhibitor, and *TaKaRa Ex Taq* HS.

＊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 series (Cat. #TP950/TP900: discontinued etc.)
- LightCycler (Roche Diagnostics)

Reagents or equipment not included in the kit.

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

＊ Refer to section X. Guidelines for Primer Design.

IV. Storage

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

V. Features

(1) One Step RT-PCR 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 amplification is effective and high quality detection is possible. Moreover, One Step TB Green RT-PCR Buffer 4 is a 2X concentration of premix with TB Green, so reaction preparation is simple.

VI. Note

This section describes precautions for using this kit. Read them before use.

(1) When mixing reagents for PCR, mix enough for 10 reactions for the master mix. Using master mixes allows accurate reagent dispensing, minimized reagent pipetting errors, and no repeat dispensing of the each reagent. This helps to minimize variation of the data from experiment to experiment.

(2) PrimeScript 1 step Enzyme Mix 2 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 completely before using by vortex.

(4) Use new disposable pipette tips to avoid contamination between samples when transferring reagents.

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

VII. Protocol

【 Protocol using LightCycler 】

＊Follow manual of LightCycler (by Roche Diagnostics) for operation.

1. Prepare the following reagents on ice.

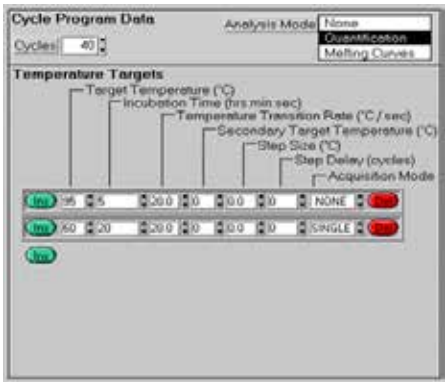
< Per reaction >

Reagent	Volume	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	10 μl	1X
PrimeScript 1 step Enzyme Mix 2	0.8 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	5.6 μl	
Total	20 μl	

＊1 The final concentration of primers can be 0.4 μM for most reactions. If this does not work, determine the optimal concentration within the range of 0.2 - 1.0 μM.

＊2 It is recommended to use 10 pg - 100 ng total RNA as templates.

2. Gently spin down PCR capillaries, then start the reaction after setting them in the LightCycler. 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. (Refer PCR reaction condition on page 11, for detail explanation.)



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.
Refer to the operation manual of LightCycler.

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

＊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 4	10 μl	25 μl	1X
PrimeScript 1 step Enzyme Mix 2	0.8 μl	2 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 (50X)＊3	0.4 μl	1 μl	
total RNA	2 μl	4 μl	＊2
RNase Free dH_2O	5.2 μl	14 μl	
Total	20 μl＊4	50 μl＊4	

＊1 The final concentration of primers can be 0.4 μM for most reactions. If this does not work, determine the optimal concentration within the range of 0.2 - 1.0 μM.

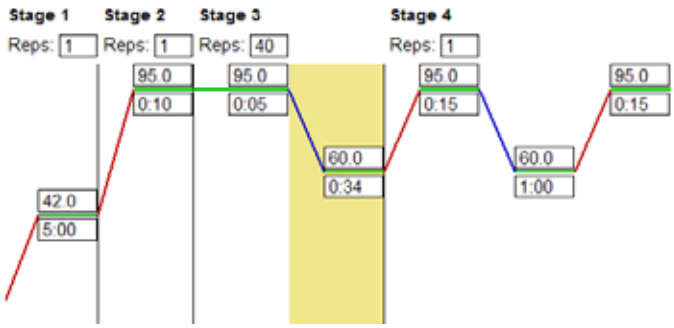
＊2 Use 20 pg - 200 ng total RNA as the template in 50 μl reaction.

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

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

2. 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. (Refer PCR reaction conditions on page 11, for a detailed explanation.)

< 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 reaction
Reps: 40
95℃ 5 sec
60℃ 30 - 34 sec *
Stage 4: Dissociation Protocol

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

< 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 completed, verify the amplification curve and dissociation curve. Establish the standard curve when quantification is done.
Refer to the operation manual of the real-time PCR instrument.

【 Protocol using Thermal Cycler Dice Real Time System series 】

1. Prepare the following reagents on ice.

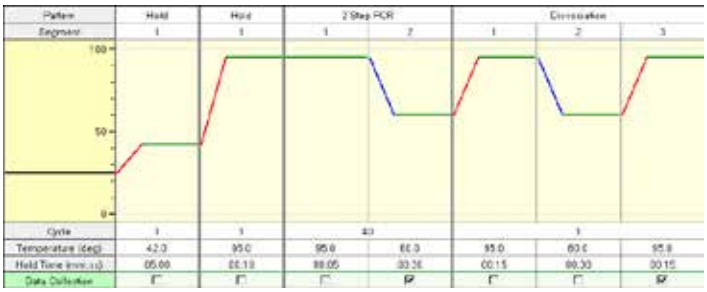
< Per reaction >

Reagent	Volume	Final Conc.
2X One Step TB Green RT-PCR Buffer 4	12.5 μl	1X
PrimeScript 1 step Enzyme Mix 2	1.0 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	7.5 μl	
Total	25 μl	

＊1 The final concentration of primers can be 0.4 μM in most reactions. If this does not work, determine the optimal concentrations within the range of 0.2 - 1.0 μM.

＊2 Use 10 pg - 100 ng total RNA as templates.

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. (Refer PCR reaction condition on page 11, for detail explanation.)



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 Condition

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.

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

2. Evaluation of PCR condition

○ To raise 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 60℃ 30 sec		95℃ 5 sec up to 64℃ 30 sec

○ 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.

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

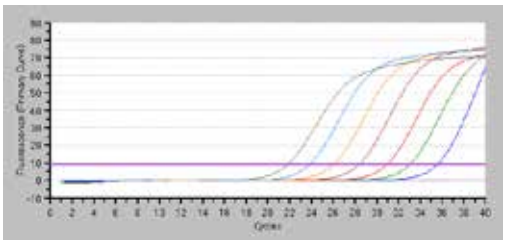
VIII. Experimental Example

Detection of Human HPRT1 (hypoxanthine phosphoribosyl transferase1)
(Thermal Cycler Dice Real Time System)

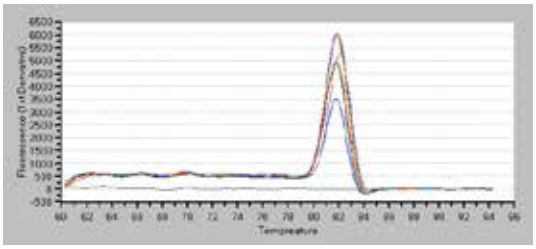
1. Procedure

Using total RNA 6.4 pg - 100 ng that was prepared from Human Liver and sterile purified water (negative control) as an template, Real-Time One Step RT- PCR was performed.

2. Result

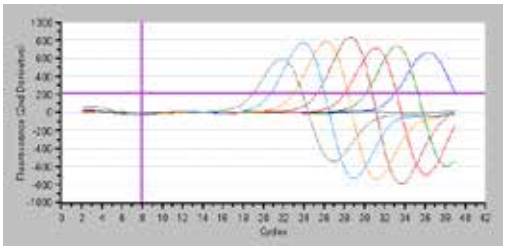


Amplification Curve

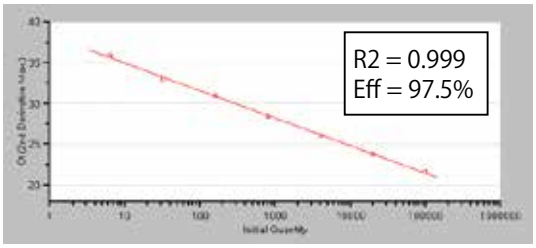


Melting Curve

After reaction completion, Ct values from the 2nd derivative of amplification curve was obtained, and the Standard curve was created.



2nd derivative



Standard Curve

3. Discussion

Target DNA were detected using total RNA 6.4 pg - 100 ng as templates. The melting curve shows that the same amplification products were obtained even when different amounts of template were used. Good linearity of standard curve was obtained within the wide range concentration of template.

IX. Appendix

Preparation of RNA sample

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

[Equipment]

Use disposable plastic equipment. Glass tools should be treated with either 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.

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

[Reagents]

All reagents to be used in this experiment must be prepared using tools which were treated as described in the previous section (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.

X. Guidlines for Primer Design

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

Amplification product

Amplified size	80 - 150 bp is recommended. (Possible to amplify a target up of 300 bp.)

Primer

Length	17 - 25 mer
GC content	40 - 60% (45 - 55% is recommended.)
Tm	Tm values of Forward primer and Reverse primer must not largely differ. Tm value is calculated with the software for calculation of Tm value. e.g. OLIGO＊1: 63 - 68℃ Primer 3 : 60 - 65℃
Sequence	The sequence should not be partially rich in any base in the whole region. Avoid high GC or AT content (especially at the 3' end). Do not include polypyrimidine (serial T/C sequence). Do not include polypurine (serial A/G sequence).
Sequence of 3' end	The 3' terminal portion should not have high content of GC or AT. G or C at the 3' end is recommended. T at the 3' end is not recommended.
Complementarity	Complementary sequences of more than 3 bases should not exist within a primer or between primer pairs. Primer pairs should not have a complementary sequences of more than 2 bases at the 3' end each.
Specificity	Specificity of primers should be confirmed through BLAST search.＊2

＊1 OLIGO Primer Analysis Software (Molecular Biology Insights, Inc.)
＊2 http://www.ncbi.nlm.nih.gov/BLAST/

XI. 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 (Perfect Real Time) (Cat. #RR066A/B)＊
One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR064A/B)＊
TB Green® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
Probe qPCR Mix (Cat. #RR391A/B)＊

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