Cat. # RR067A

For Research Use

TaKaRa

One Step SYBR® *Ex Taq*™ qRT-PCR Kit (Perfect Real Time)

Product Manual

v201202Da

Table of Content

I. Description

One Step SYBR® *Ex Taq* qRT-PCR Kit (Perfect Real Time) is designed for Real-Time One Step RT-PCR using SYBR® Green Detection.*1, 2 The RT-PCR is performed in a single tube for simple operation and minimal risk of contamination. In addition, amplified products are monitored in real time and do not require electrophoresis after PCR. This kit is suitable for detection of small amounts of RNA (i.e. RNA virus).
This kit contains a new RTase, which has excellent extendibility and can efficiently synthesize cDNA quickly, and *TaKaRa Ex Taq* HS, for high efficiency and specificity PCR, which have been optimized for one step RT-PCR. The combination of TaKaRa Bio's RT-PCR technology with these enzymes makes this kit an excellent choice for any qRT-PCR experiment.

Applicable real time PCR instruments ;

- Thermal Cycler Dice Real Time System *II* (Cat. #TP900)*3
- ABI PRISM® 7000, Applied Biosystems® 7500/7500 Fast Real-Time PCR System (Life Technologies™)
- LightCycler® (Roche Diagnostics)
- Smart Cycler® II System*4 (Cepheid)

*1 : SYBR® Green I is licensed by Molecular Probes Inc. for research reagents. SYBR® is a registered trademark of Molecular Probes Inc. (U.S. and Europe).
*2 : Use One Step *Ex Taq* qRT-PCR Kit for Real-Time One Step RT-PCR using TaqMan® probe. TaqMan® is a registered trademark of Roche Molecular Systems. The 5' nuclease process is covered by patents owned by Roche Molecular Systems, Inc. and F. Hofmann-La Roche Ltd. Purchase of the product does not provide a license to use this patented technology.
*3 : This instrument is not available in the U.S. and Europe.
*4 : Smart Cycler® is a registered trademark of Cepheid.

II. Principle

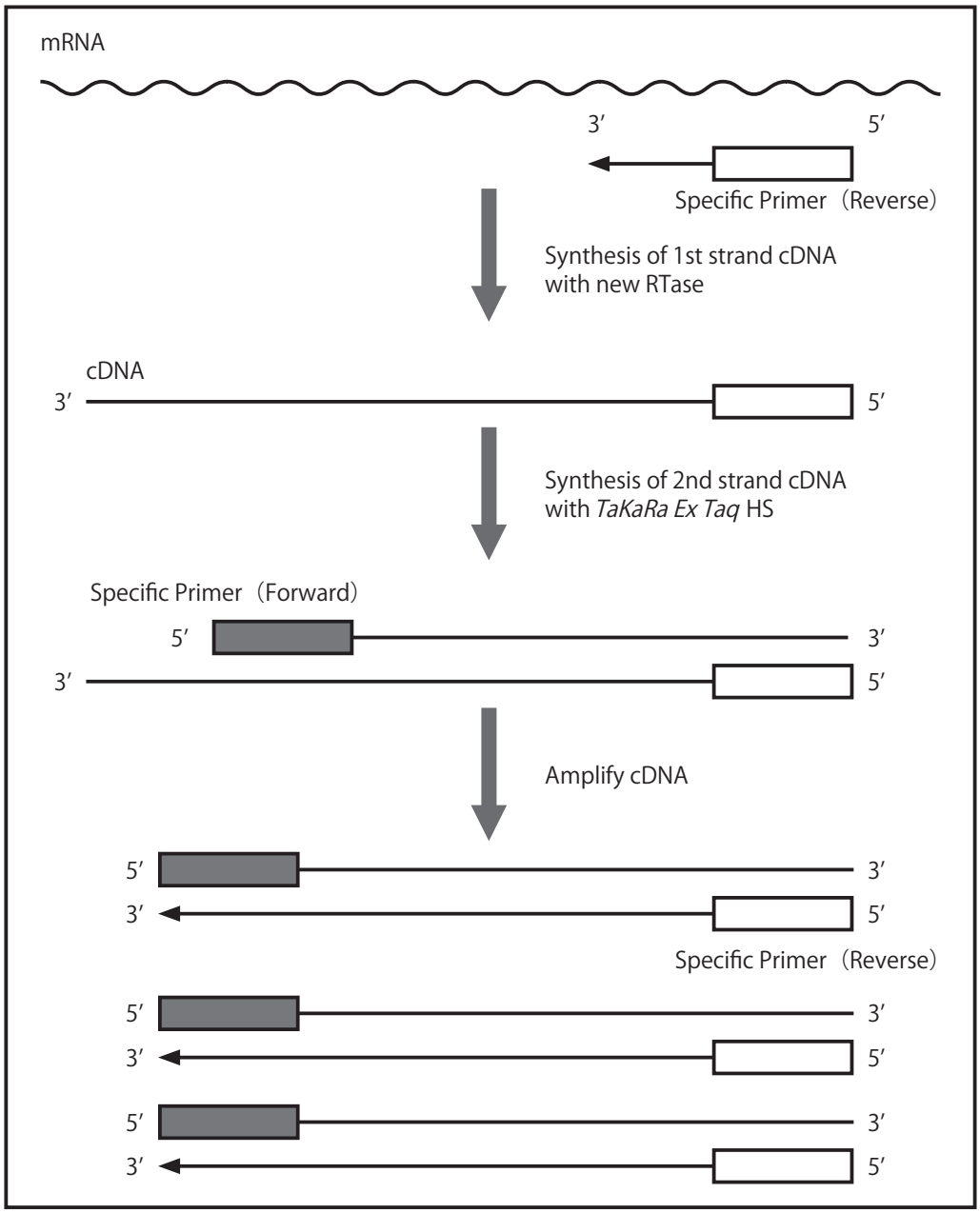
The One Step SYBR® *Ex Taq* qRT-PCR Kit is used to perform cDNA synthesis from RNA using a reverse transcriptase, new RTase, and PCR amplification using *TaKaRa Ex Taq* HS all in one tube. PCR amplification products are detected using the SYBR® Green I method in real time monitoring.

1. PCR

PCR is a technique that amplifies only a targeted section of a gene from small quantities of DNA. One cycle of PCR includes denaturation of DNA, annealing of the primers, and extension of the DNA using a DNA polymerase. By repeating this process, PCR allows exponential amplification of a targeted gene segment in a short period of time. Using *TaKaRa Ex Taq* HS for amplification helps to avoid mis-priming and non-specific amplification caused by primer dimer.

2. RT-PCR

RNA cannot be a direct template for PCR, however, PCR can be used for RNA analysis when cDNA is synthesized from RNA using reverse transcriptase. This is called RT-PCR and is a high quality technique used for detection of RNA. This kit uses One Step RT-PCR. The principle of this is shown in figure below. For One Step RT-PCR, a Specific Primer (Reverse) is used for reverse transcription. Next the synthesized cDNA is used as a template, for the next PCR amplification using the specific primers (Forward, Reverse). (Random Primer and Oligo dT Primer cannot be used for reverse transcription.)



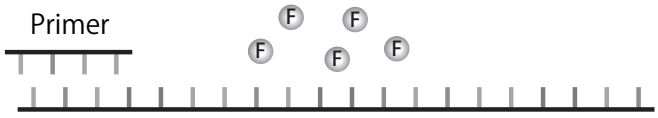
Principle of One Step RT-PCR

3. Fluorescent spectrophotometer method

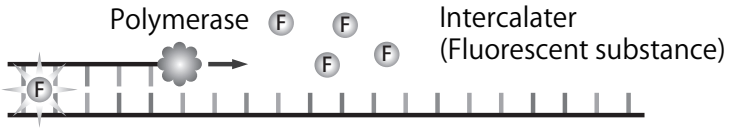
[Intercalator method]

The Intercalator method detects fluorescence produced during the amplification process when the dye (i.e. SYBR® Green I) binds to double strand DNA. Fluorescence occurs when the intercalator is bound with double stranded DNA that has been synthesized by the polymerase reaction. Detecting fluorescence for quantification of amplified DNA and measuring the melting point can be performed using this method.

1) Heat denaturation



2) Primer annealing



3) Extension

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

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

＊1 : Includes dNTP Mixture, Mg^{2+} and SYBR® Green I.

＊2 : ROX™ Reference Dye/Dye II is used for normalization of intensity by background subtraction. For ABI PRISM®7000, the use of ROX™ Reference Dye is recommended. For Applied Biosystems® 7500/7500 Fast Real Time PCR System, the use of ROX™ Reference Dye II is recommended. The use of ROX™ Reference Dye or Dye II is optional. It is not required for use with Smart Cycler® or LightCycler® real time instruments.

Reagents or equipment not included in the kit.

1. Gene amplification system for Real-Time PCR (authorized instruments)
2. Reaction tube or plate exclusive for the real time PCR
3. PCR Primer＊
4. Micropipettes and pipette tips (autoclaved)

＊ Please refer to X. Guideline for designing of primer.

IV. Storage

-20℃
Store 2X One Step SYBR® RT-PCR Buffer should be protected from light after its receipt.

V. Features

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

(2) *TaKaRa Ex Taq* HS allows for high efficiency and specificity PCR. The *TaKaRa Ex Taq* HS buffer system is optimized for Real-Time PCR for high quality detection.

(3) The One Step RT-PCR Buffer is a 2X premix for simple preparation of the reaction with minimal risk of contamination.

VI. Note

Please read note and protocol carefully before you use the kit.

(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 each reagent. This helps to minimize variation of the data from experiment to experiment or well to well.

(2) The RTase Enzyme Mix 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.

(3) Keep the enzyme at -20℃until just before use and return to the freezer promptly after use.

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

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

VII. Protocol

< Protocol using Smart Cycler® II System >

1. Prepare the following reagents on ice.

< Per reaction >

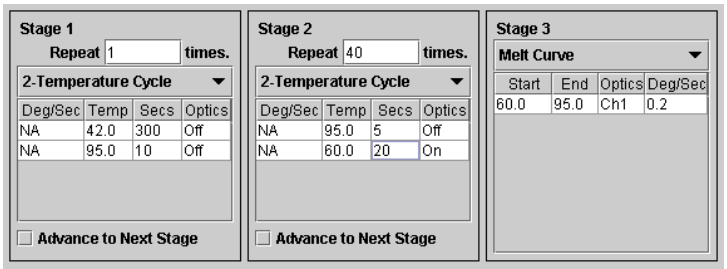
Reagents	Volume	Final Conc.
2X One Step SYBR® RT-PCR Buffer	12.5 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.5 μl	
RTase Enzyme Mix	0.5 μl	
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
total RNA	2 μl *2	
RNase Free dH_2O	8.5 μl	
Total	25 μl	

*1 : The final concentration of primers can be 0.2 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.1 - 1.0 μM.

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

2. Start the reaction

Gently spin down the reaction tubes with Smart Cycler® specific centrifuge, then start the reaction after setting them onto Smart Cycler®. Use the standard protocol recommendations as described for each reaction. First, try this protocol; modify PCR reaction condition as needed. Use 3-step PCR when shuttle PCR is difficult to process (i.e. Primer with low T_m value). (Refer to PCR reaction condition on page 10, for detailed explanation.)



Stage 1：Reverse Transcription
Hold
42℃ 5 min.
95℃ 10 sec.
Stage 2：PCR reaction
Repeat：40 times
95℃ 5 sec.
60℃ 20 sec.
Stage 3：Melt Curve

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start enzyme utilizing a *Taq* antibody. The heat inactivation step prior to PCR should be at 95℃ for 10 sec. There is no need to heat at 95℃ for (5-) 15 min. as the initial denaturation, required for chemically modified *Taq* polymerase. If a longer heat treatment is used, the enzyme activity will decrease and the amplification efficiency and accuracy in quantification may be affected.

3. After the reaction is completed, verify the amplification curve and melting curve. Establish the standard curve when quantification is done.
For the analytical method when using Smart Cycler® System, please refer to the instruction manual for Smart Cycler® System.

< Protocol using ABI PRISM® 7000 and Applied Biosystems® 7500/7500 Fast Real-Time PCR System >

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

1. Prepare the following reagents on ice.

< Per reaction >

Reagents	Volume	Volume	Final Conc.
2X One Step RT-PCR Buffer	10 μl	25 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.4 μl	1 μl	
RTase Enzyme Mix	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 (50X)＊3	0.4 μl	1 μl	
total RNA	2 μl	4 μl ＊2	
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. When it does not work, determine the optimal concentrations within the range of 0.1 - 1.0 μM.

＊2 : It is recommended to use 10 pg - 100 ng total RNA as templates for 20 μl reaction.

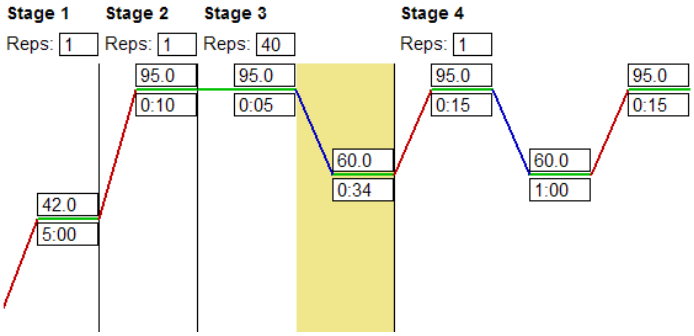
＊3 : The ROX™ Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities among wells when used with real time PCR instruments that have option. For ABI PRISM® 7000, the use of ROX™ Reference Dye is recommended. For Applied Biosystems® 7500/7500 Fast Real-Time PCR System, the use ROX™ Reference Dye II is recommended.

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

2. Start the reaction

Use the standard protocol described as follows for the reaction. First, try this protocol; modify PCR reaction condition as needed. Use 3-step PCR when shuttle PCR is difficult, for example Primer with low T_m value. (Refer PCR reaction condition on page 10, for detail explanation.)

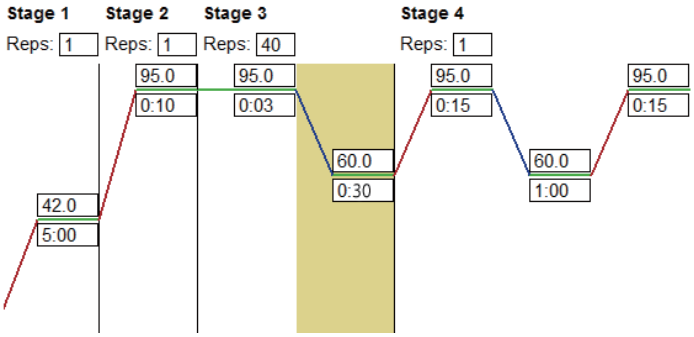
< ABI PRISM® 7000 and Applied Biosystems® 7500 Real-Time PCR System >



Stage 1, 2：Reverse transcription
Reps：1
42℃ 5 min.
95℃ 10 sec.
Stage 3：PCR reaction
Reps：40
95℃ 5 sec.
60℃ 31 or 34 sec. ＊
Stage 4：Dissociation Protocol

＊：With 7000, set to 31 sec.; and with 7500, set to 34 sec.

< Applied Biosystems® 7500 Fast Real-Time PCR System >



Stage 1, 2：Reverse transcription
Reps：1
42℃ 5 min.
95℃ 10 sec.
Stage 3：PCR reaction
Reps：40
95℃ 3 sec.
60℃ 30 sec.
Stage 4：Dissociation Protocol

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start enzyme utilizing a *Taq* antibody. The heat inactivation step prior to PCR should be at 95℃ for 10 sec. There is no need to heat at 95℃ for (5-) 15 min. as the initial denaturation, required for chemically modified *Taq* polymerase. If a longer heat treatment is used, the enzyme activity will decrease and the amplification efficiency and accuracy in quantification may be affected.

3. After the reaction is complete, verify the amplification curve and dissociation curve. Establish a standard curve when quantification is done.

＊Refer to the Instruction manual of the real time PCR instrument used.

< Protocol using LightCycler® >

＊ Follow instruction manual for the LightCycler® (by Roche Diagnostics) for operation.

1. Prepare the following reagents on ice.

< Per reaction >

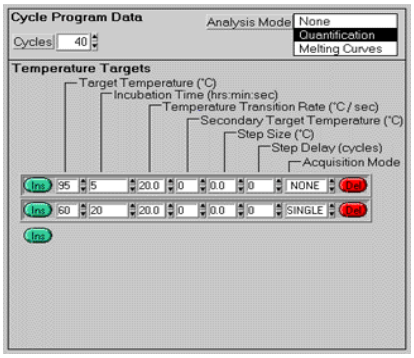
Reagents	Volume	Final Conc.
2X One Step SYBR® RT-PCR Buffer	10 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.4 μl	
RTase Enzyme Mix	0.4 μl	
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM＊1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM＊1
total RNA	2 μl ＊2	
RNase Free dH_2O	6.4 μl	
Total	20 μl	

＊1 : The final concentration of primers can be 0.2 μM in most reactions. When it does not work, determine the optimal concentrations within the range of 0.1 - 1.0 μM.

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

2. Start the reaction

Gently spin down PCR capillaries, then start the reaction after putting them into the LightCycler®. Use the standard protocol described as follows for the reaction. First, try this protocol; modify PCR reaction condition as needed. Use 3-step PCR when shuttle PCR is difficult, for example Primer with low T_m value. (Refer PCR reaction condition on page 10, 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 Cycle
Stage 3：melting curve analysis
95℃ 0 sec. 20℃/sec.
65℃ 15 sec. 20℃/sec.
95℃ 0 sec. 0.1℃/sec.

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start enzyme utilizing a *Taq* antibody. The heat inactivation step prior to PCR should be at 95℃ for 10 sec. There is no need to heat at 95℃ for (5-) 15 min. as the initial denaturation, required for chemically modified *Taq* polymerase. If a longer heat treatment is used, the enzyme activity will decrease and the amplification efficiency and accuracy in quantification may be affected.

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.

PCR Reaction Condition

Shuttle PCR

Step	Temp.	Time	Detection	Remark
Denature	95℃	3 - 5 sec.	Off	Since the target size amplified for real time PCR is generally shorter than 300 bp, the denaturation at 95℃ for 3 - 5 seconds is a sufficient for denaturation.
Annealing/ Extension ＊	60 - 66℃	20 - 30 sec.	On	Please try each standard protocol at first. The temperature should be optimized within the range of 60 - 66℃ if optimization is required. When reaction does not proceed efficiently, extend the time or change the reaction to 3 step PCR.

3 step PCR

Step	Temp.	Time	Detection	Remark
Denature	95℃	3 - 5 sec.	Off	Since the target size amplified for real time PCR is generally shorter than 300 bp, the denaturation at 95℃ for 3 - 5 seconds is a sufficient for denaturation.
Annealing	55 - 60℃	10 - 20 sec.	Off	Please try 55℃ for 10 seconds first. When non-specific amplified products are generated or when the amplification efficiency is low, optimization of the annealing temperature may be necessary. Longer annealing time may improve the amplification efficiency.
Extension ＊	72℃	6 - 15 sec.	On	When the amplified size is less than 300 bp, the time should be determined within the range of 6 - 15 seconds. Longer extension time can cause non-specific amplification.

Cycle : 30 - 45cycles

＊: The detection step must be set at more than 30 seconds for the Applied Biosystems® instrument. 7000 should be set at 31 seconds, and 7500 should be set at 34 seconds.

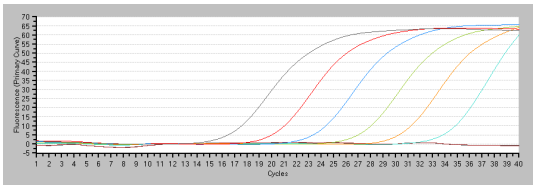
VIII. Experiment Example

Detection of Mouse Gapdh (glyceraldehydes-3-phosphate dehydrogenase)
(Thermal Cycler Dice Real Time System is used here)

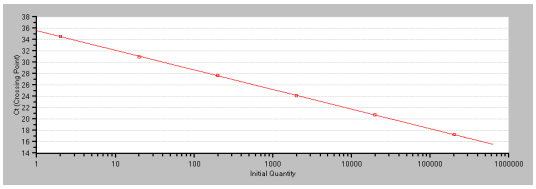
1. Procedure
   Using total RNA (2 pg - 200 ng) prepared from Mouse Liver and a negative control of sterilized water as templates, Real-Time One Step RT- PCR was performed.

2. Result

< Crossing Point method >



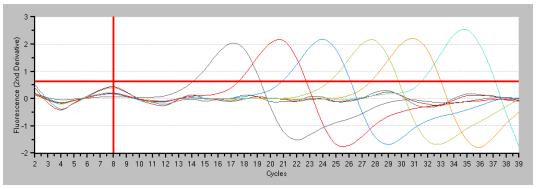
Amplification Curve



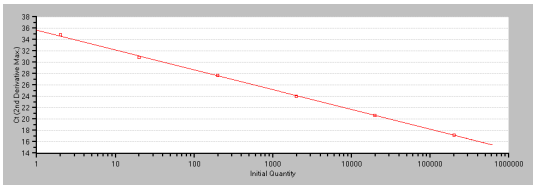
Melting Curve

After reaction, obtain Ct value form 2nd derivative of amplification curve and create a standard curve.

< 2nd Derivative Maximum method >



2nd derivative



Analytical Curve

3. Discussion
   Detection of target DNA was possible using total RNA of 2 pg-200 ng. The melting curve shows that the same product was amplified even though different amounts of template were used. The linearity of the standard curve was obtained within the wide range of templates.

IX. Appendix

A. Preparation of RNA sample

This kit is designed to perform reverse transcription of RNA to cDNA and subsequent amplification. It is important to use high purity RNA samples for better yields of the cDNA synthesis. In addition, it is essential to inhibit cellular RNase activity and 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 researcher's sweat or saliva.

[Equipment]

Disposable plastic equipment should be used. If 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.

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

[Reagent]

All reagents used in this experiment must be prepared using tools treated as described in the previous section (Hot-air sterilization [180℃, 60min] or DEPC treatment), and distilled water must be treated with 0.1% DEPC and autoclaved. All reagents and distilled water should be used exclusively for RNA experiments.

[Preparation of RNA sample]

It is recommended to use highly purified RNA obtained by GTC (Guanidine thiocyanate) method, etc.

X. Guideline for designing of primer

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

Amplification product

Amplified size	80 - 150 bp is highly recommended. (It is possible to amplify a target up to 300 bp.)

Primer

Length	17 - 25 mer
GC content	40 - 60% (45 - 55% is recommended.)
T_m	T_m values of Forward primer and Reverse primer must not differ each other. T_m values are calculated with calculation software. e.g. OLIGO™＊1 : 63 - 68℃ Primer 3＊2 : 60 - 65℃
Sequence	The primer sequence should not contain high numbers of either base in the entire sequence. Avoid including parts which have high GC or AT content, (especially 3' -end). Do not include polypyrimidine (serial T/C sequence). Do not include polypurine (serial A/G sequence).
Sequence of 3' end	The termini part of the 3' end should not contain a high content of GC or AT. It is recommended to have a G or C at the 3' end. It is not recommended to have a T at the 3' end.
Complementarity	Do not have a complementary sequence of more than 3 bases within a primer or between primer pairs. Primer pairs should not have a complementary sequence of more than 2 bases at the 3' end.
Specificity	Specificity of primers should be confirmed through a BLAST search.＊3

＊1 : OLIGO™ Primer Analysis Software (Moleculor Biology Insights, Inc.)
＊2 : Primer3 (http://www-genome.wi.mit.edu/ftp/distribution/software/)
＊3 : http://www.ncbi.nlm.nih.gov/BLAST/

XI. Related Products

One Step *Ex Taq*™ qRT-PCR Kit (RR068A/B)
One Step SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR066A/B)
One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Cat. #RR086A/B)
SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (Cat. #RR064A/B)
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A/B)

NOTICE TO PURCHASER: LIMITED LICENSE

[P5] PCR Notice
Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,994,056 and 6,171,785. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

[L01] One Step RNA PCR / One Step RT-PCR
Use of this product is licensed from bioMerieux, is covered by US Patent 5,817,465 and equivalents, and is for Research Use Only.

[L11] SYBR® Green I
This product is covered by the claims of U.S. Patent No. 5,436,134 and 5,658,751 and their foreign counterpart patent claims.
Takara PCR products containing SYBR® Green I are sold under license from Molecular Probes Inc. only for the usage in Real-time PCR for internal research purpose. These products are not to be used for the purpose such as; providing medical, diagnostic, or any other testing, analysis or screening services or providing clinical information or clinical analysis in return for compensations.

[L15] Hot Start PCR
Licensed under U.S. Patent No. 5,338,671 and 5,587,287 and corresponding patents in other countries.

[L46] SYBR/Melting Curve Analysis
The purchase of this product includes a limited, non-transferable license for all fields other than human or veterinary in vitro diagnostics under specific claims of U.S. Patent Nos. 6,174,670, 6,569,627 and 5,871,908, owned by the University of Utah Research Foundation or Evotec Biosystems GmbH and licensed to Idaho Technology, Inc. and Roche Diagnostics GmbH, to use only the enclosed amount of product according to the specified protocols. No right is conveyed, expressly, by implication, or by estoppel, to use any instrument or system under any claim of U.S. Patent Nos. 6,174,670, 6,569,627 and 5,871,908, other than for the amount of product contained herein.

[L52] Rox Reference Dye (Research Field)
Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,928,907. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser' s own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

[M57] LA Technology
This product is covered by the claims 6-16 of U.S. Patent No. 5,436,149 and its foreign counterpart patent claims.

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

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