Table of Content

I. Description

One Step Ex Taq qRT-PCR Kit (Perfect Real Time) is designed for Real-Time One Step RT-PCR using the TaqMan® probe.*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 real time RT-PCR experiment.

Various real time PCR instruments have been tested. Among them are:

- Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System (Life Technologies)
- LightCycler® (Roche Diagnostics)
- Smart Cycler® II System*3 (Cepheid)

*1 : 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.

*2 : Use of the One Step SYBR® Ex Taq qRT-PCR Kit (Perfect Real Time) for Real-Time One Step RT-PCR using SYBR® Green I is possible. 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).

*3 : Smart Cycler® is a registered trademark of Cepheid.

II. Principle

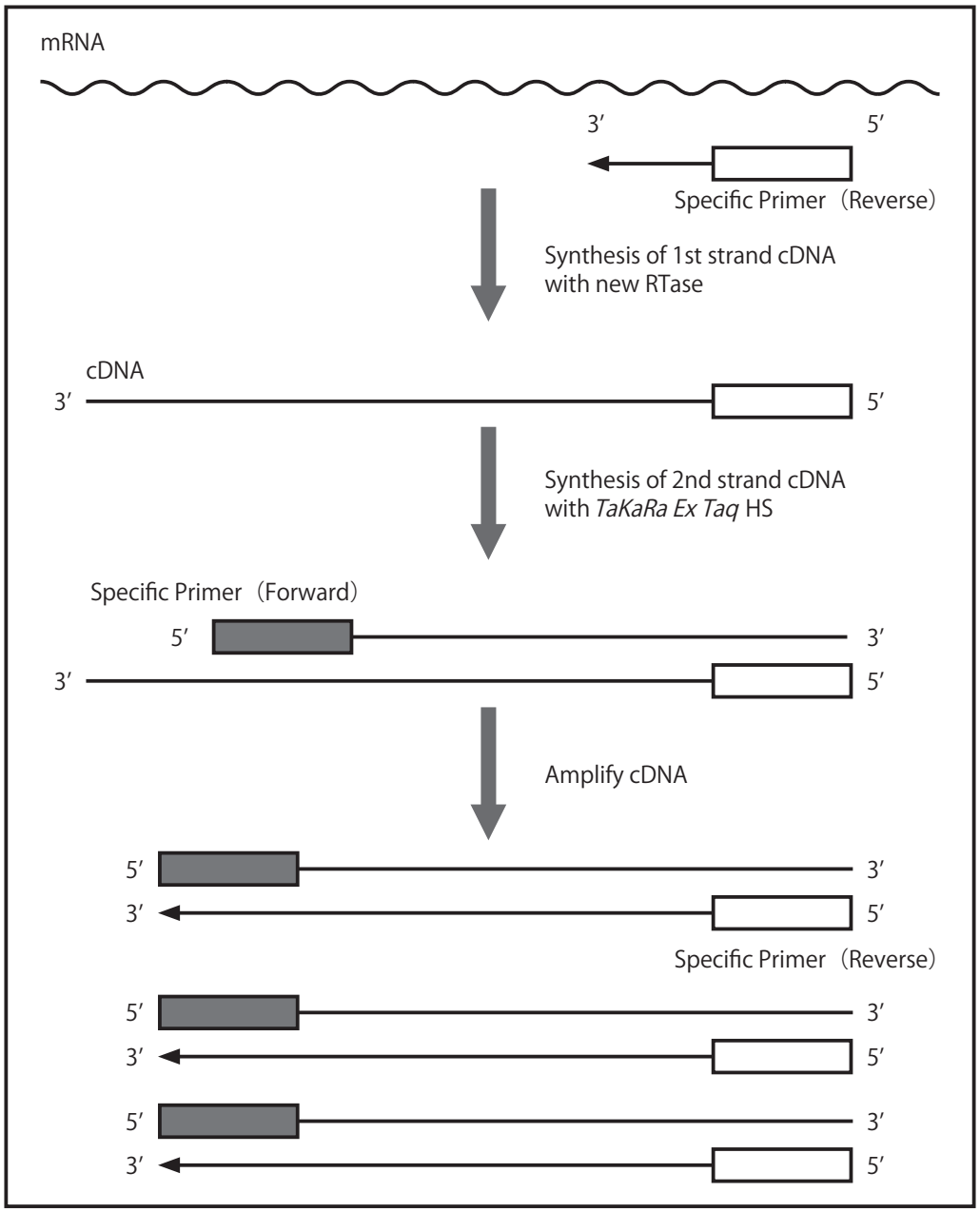
This kit is used to perform cDNA synthesis from RNA using a reverse transcriptase, new RTase, and PCR amplification using *TaKaRa Ex Taq* HS all in one tube. PCR amplification products are detected using the TaqMan® probe method in real time monitoring.

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 mispriming 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.)



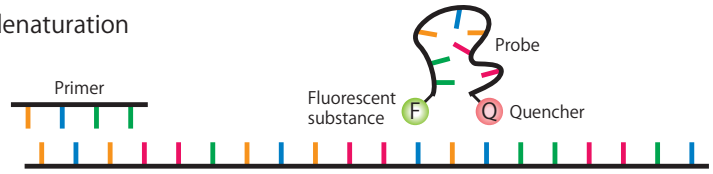
Principle of One Step RT-PCR

3. Fluorescence detection

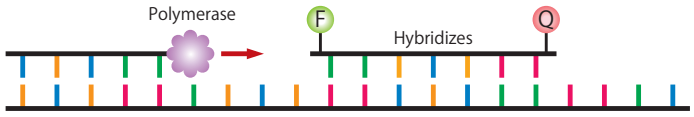
TaqMan® Probe method :

The TaqMan® method is based on a combination of TaqMan Technology and a real time PCR instrument. An oligonucleotide is modified with a fluorescent substance (e.g.FAM) at the 5'-end and with a quencher (e.g.TAMRA) at the 3'-end and is added to a reaction system. During the annealing step, the TaqMan® probe specifically binds to the template DNA, and fluorescence is suppressed by the quencher. During the extension step, the 5' → 3' exonuclease activity of the Taq DNA polymerase degrades the TaqMan® probe hybridized to a template and allows the emission of fluorescence. By measuring the fluorescence intensity the amount of amplified product is monitored.

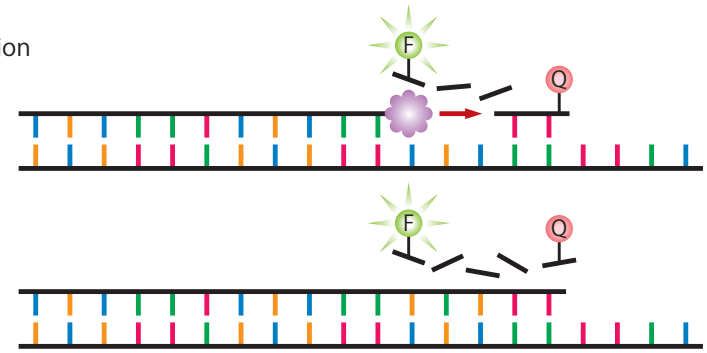
1) Heat denaturation



2) Primer annealing/Probe hybridization



3) Extension



III. Kit Contents (for 10 reactions; 50 μl reaction system) :

1.	2X One Step RT-PCR Buffer*1	250 μl
2.	*TaKaRa Ex Taq* HS (5 U/μl)	10 μl
3.	RTase Enzyme Mix	10 μl
4.	RNase Free dH_2O	1.25 ml
5.	ROX Reference Dye (50X conc.)*2	10 μl
6.	ROX Reference Dye II (50X conc.)*2	10 μl

*1 : Contains dNTP Mixture and Mg^{2+}.

*2 : ROX Reference Dye/Dye II is used for normalization of intensity by background subtraction. Use ROX Reference Dye (50X) for Applied Biosystems 7300 Real Time PCR System, and use ROX Reference Dye II (50X) for Applied Biosystems 7500/7500 Fast Real Time PCR System.
This component is not required with Smart Cycler® or LightCycler®.

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. TaqMan® Probe (licensed probe)
5. Micropipetts and pipette tips (autoclaved)

IV. Storage -20℃

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 when using Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System >

1. Prepare the PCR mixture shown below on ice.

< Per reaction >

Reagents	Amount	Amount	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
TaqMan® Probe	0.8 μl	2 μl	＊2
ROX Reference Dye or Dye II (50X)＊3	0.4 μl	1 μl	
total RNA	2 μl	4 μl	＊4
RNase Free dH_2O	5.2 μl	14 μl	
Total	20 μl ＊5	50 μl ＊5	

＊1 : 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 : Probe concentration differs depending on kind of real time PCR instrument, and kind of fluorescence labeling material. The adding amount should be determined by referring to the operation manual of instrument and a product insert supplied with probe.

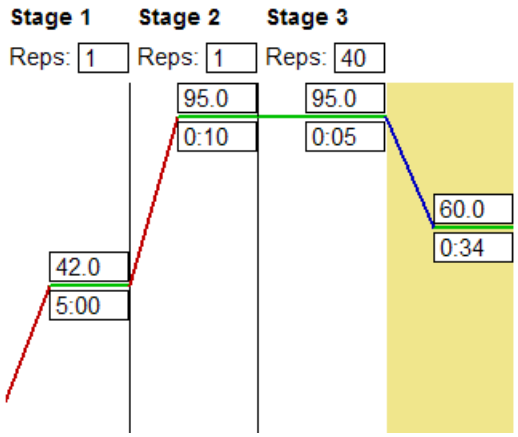
＊3 : The ROX Reference Dye/Dye II is supplied for performing normalization of fluorescent signal intensities among wells when used with real time PCR instruments that have option.
Use ROX Reference Dye (50X) for Applied Biosystems 7300 Real-Time PCR System.
Use ROX Reference Dye II (50X) for Applied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

＊4 : It is recommended to use 10 pg - 100 ng total RNA as templates per 20 μl of reaction mixture.

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

2. Start reaction
Use the standard protocol as recommended for reaction. Try this protocol first and optimize PCR conditions as necessary.
(For detailed explanation, please refer to page 10 on PCR reaction condition.)

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



Standard protocol

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

＊: With 7300, set to 31 sec;
with 7500, set to 34 sec.

< Applied Biosystems 7500 Fast Real-Time PCR System >

Standard protocol
Holding Stage
Reps: 1
42℃ 5 min.
95℃ 10 sec.
Cycling Stage
Number of Cycles: 40
95℃ 3 sec.
60℃ 30 sec.

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start PCR 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. Establish the standard curve when quantification is done. Please refer to the instruction manual for your real time PCR instrument to read about analytical methods.

< Protocol when using Smart Cycler® II System >

1. Prepare the PCR mixture shown below on ice.
   < Per reaction >

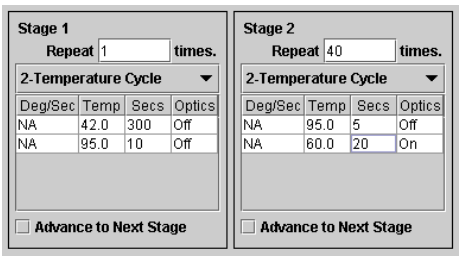
Reagents	Amount	Final Conc.
2X One Step RT-PCR Buffer	12.5 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.5 μl	
RTase Enzyme Mix	0.5 μl	
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM＊1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM＊1
TaqMan® Probe	1 μl	＊2
total RNA	2 μl	＊3
RNase Free dH_2O	7.5 μl	
Total	25 μl	

＊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 : Probe concentration differs depending on type of real time PCR instrument, and type of fluorescence labeling material. The amount added should be determined by referring to the operation manual of the instrument and the product insert supplied with the probe. Generally, when performing detection using the Smart Cycler® System, probe concentration should be determined using 0.1 - 0.5 μM as the range for the final concentration.

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

2. Briefly centrifuge reaction tubes with Smart Cycler® centrifuge and then set them in Smart Cycler® to initiate the reaction.
   Use the standard protocol recommendations as described for each reaction. Try this protocol first and optimize PCR conditions as necessary. (For detailed explanation, please refer to page 10 on PCR reaction condition.)



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

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start PCR 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. 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 when using LightCycler® >

＊ Please follow the procedures provided in the LightCycler® manual (Roche Diagnostics).

1. Prepare the PCR mixture shown below on ice.
   < Per reaction >

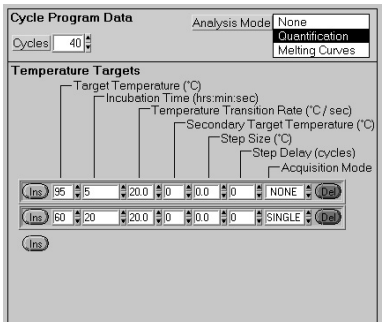
Reagents	Amount	Final Conc.
2X One Step RT-PCR Buffer	10 μl	1X
TaKaRa Ex Taq HS (5 U/μl)	0.4 μl	
RTase Enzyme Mix	0.4 μl	
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM＊1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM＊1
TaqMan® Probe	0.8 μl	＊2
total RNA	2 μl	＊3
RNase Free dH_2O	5.6 μl	
Total	20 μl	

＊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 : Probe concentration differs depending on kind of real time PCR instrument, and kind of fluorescence labeling material. The adding amount should be determined by referring to the operation manual of instrument and a product insert supplied with probe.

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

2. Briefly centrifuge PCR capillaries and set them in LightCycler® to initiate the reaction. Use the standard protocol as recommended for reaction. Try this protocol first and optimize PCR conditions as necessary. (For detailed explanation, please refer to page 10 on PCR reaction condition.)



Standard protocol

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

Note : This product uses high performance *TaKaRa Ex Taq* HS which is a hot start PCR 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, check the amplification curve and plot a standard curve if an assay will be performed.
   Please refer to the instruction manual for your real time PCR instrument to read about analytical methods.

PCR Reaction Condition

Shuttle PCR Cycle : 30 - 45cycles

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 sufficient for denaturation.
Annealing/ Extension	56 - 64℃	20 - 30 sec. (31 or 34 sec.)＊	On	Please try each standard protocol initially. The temperature should be optimized within the range of 56 - 64℃ if optimization is required. When reaction does not proceed efficiently, extend the time or change the reaction into 3 step PCR.

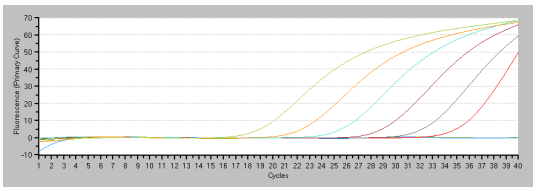
＊: Detection step must be set at longer than 30 seconds for device of Applied Biosystems. With 7300, set to 31 sec; with 7500, set to 34 sec.

VIII. Experiment Example

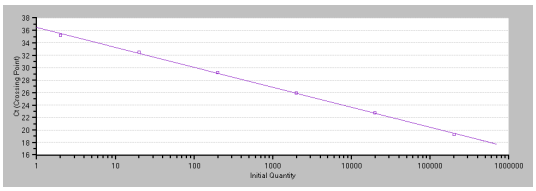
1. Detection of Mouse Actb
   Using total RNA (2 pg - 200 ng) prepared from Mouse Liver as a template and sterilized water as a negative control, Real-Time One Step RT- PCR was performed using a Thermal Cycler Dice Real Time System. PCR products were detected with the Primer/Probe Mix of TaqMan® Gene Expression Assays and TaqMan® Probe (Life Technologies).

2. Result

< Crossing Point method >

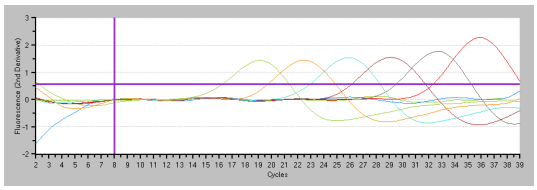


Amplification Curve

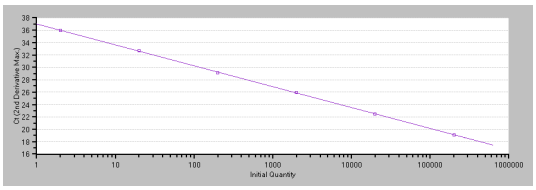


Standard Curve

< 2nd Derivative Maximum method >



Amplification Curve



Standard Curve

3. Discussion
   Target DNA was able to be detected using total RNA from 2 pg - 200 ng.
   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 the 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 strongly recommended that all equipment be used exclusivly for RNA preparation.

[Reagent]

All reagents to be used in this experiment must be prepared using tools which were treated as described in previous section (Hot-air sterilization [(180℃, 60 min) 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 the GTC (Guanidine thiocyanate) method, etc.

X. Related Products

One Step Ex Taq™ qRT-PCR Kit (Perfect Real Time) (Cat. #RR068A)
One Step SYBR® Ex Taq™ qRT-PCR Kit (Perfect Real Time) (Cat. #RR067A)
SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (Cat. #RR820A/B)
SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Cat. #RR420A/B)
Premix Ex Taq™ (Probe qPCR) (Cat. #RR390A/B)

NOTICE TO PURCHASER: LIMITED LICENSE

[L1] 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.

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

[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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