COSMO BIO CO., LTD.
Inspiration for Life Science

TOYOBO

Realtime PCR Master Mix

Code No. QPK-101, 101T

For research purposes

Handling instructions

Distributor

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Inspiration for Life Science

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[Warning]

This product is a reagent for use in research. Never use for diagnosis or other clinical purposes. When using this product, follow general laboratory precautions and pay attention to safety.

This product is sold under license agreements with F. Hoffmann-La Roche Ltd., Roche Molecular Systems Inc. and Applied Biosystems.
"Purchase of this product is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) process for The Research Field in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems or as purchased, i.e., an authorized thermal cycler."

[1] Introduction

1. General Outline

This product is a 2 x master mix, containing all necessary elements other than primers and sample DNA. Its use in combination with probes and primers allows realtime QPCR using TaqMan® assay, hybridization probe assay and other methods.

2. Features

- High Versatility

This product contains BSA so that it can be applied to systems using glass capillaries (e.g., LightCycler™ of Roche Diagnostics Inc.). Passive reference compatible with ABI PRISM® 7700 (Applied Biosystems Inc.), etc., is also included with this kit. It has been verified that this passive reference does not affect use even in systems (LightCycler™, etc.) that are not originally designed to use it.

- Rapid Hot Start

To suppress nonspecific reactions, hot start PCR is adopted in this product, with anti-Taq monoclonal antibody used for Taq DNA polymerase. This adoption allows highly fast reactivation of Taq DNA Polymerase. The 1st denaturation step for reactivation of polymerase can be finished within 1 minute, whereas more than 10 minutes are required in conventional methods. Each cycle of denaturation step is settable according to the time necessary to denature nucleic acids. High-speed Thermal Cycler like LightCycler™ proves the merit on time reduction.

[2] Precautions

1. Precautions for Use

- This product is a reagent for use in research. Never use it for diagnosis or other clinical purposes. The full range of hazards that may be caused by this reagent have not been investigated entirely. Take adequate care when handling this product, e.g., use protective equipment and clothing.

- Immediately after the product is thawed, it needs to be placed upside down and agitated gently to become fully uniform.

2. Storage

- As a rule, this product should be stored frozen at -20°C or lower, shielded from light. After thawing, it can be stored at 4°C for a maximum of 2 months. Shielding from light is also necessary in this case too. Use a light-shielding box or the aluminum bag provided with the kit for this purpose (QPK-101 has another aluminum bag. Use the two bags separately for storage at different temperatures (-20°C and 4°C).
- It is recommended that only one pack is thawed at a time and that it is used within 2 months of thawing. In case a thawed tube has to be stored for a long period, it should be frozen again at -20°C. In our internal test, any reduction in performance was not observed when a thawed solution was stored at 4 ▪ for about 80 days. Even a solution after thawed and freezed more than 20 times could also be used for realtime PCR without showing any reduction in performance.

[3] Composition

[Realtime PCR Master Mix]

This is a double-concentrated solution containing all of the elements needed for PCR (excluding primers). It contains dNTP, $MgCl_2$, Taq DNA polymerase, anti-Taq monoclonal antibody, etc.

[4] What is needed to use this product?

1. Reagents and devices

[Primers and probes]

Use of this kit requires the preparation of primer pairs and probes corresponding to the gene sequence to be detected or quantified. Primers and probes should be set in accordance with the precautions specific to each assay system. Usually, the length of the area to be amplified is also important. Nonspecific reactions tend to occur more

frequently and amplification efficiency decreases as the region to be amplified becomes longer. Thus, the length of the region to be amplified affects sensitivity and quantitation. It is desirable that the region to be amplified is 200 bp or less in length (preferably 150 bp or less).

[Distilled water]

Use water of a purity applicable in ordinary PCR and the like.

[Realtime PCR device]

Devices for realtime PCR such as ABI PRISM® 7700 (Applied Biosystems Inc.), LightCycler™ (Roche Diagnostics Inc.) and LineGene (Bio Flux Corporation) should be used. Use devices in conformance with their handling instructions.

2. Sample DNA

[cDNA]

- Use samples that have been purified by phenol treatment, ethanol sedimentation, etc., after 1st strand cDNA synthetic reaction. Either Random Primer or oligo-dT primer may be used as the primer for 1st strand cDNA synthesis, but care is needed with regard to the amount of primer carried forward to PCR.
- The reaction solution ReverTra Ace -α-® (Code: FSK-101) contained in our company's 1st strand cDNA synthesis kit can be used simply by diluting 10-fold or more. Amplification is possible even when the solution is used undiluted, but primer or reverse transcriptase contamination can affect QPCR adversely (even when heat-treated) during cDNA synthesis. We therefore recommend use of the solution after dilution.

[Genomic DNA, etc.]

DNA samples with purity levels high enough for use in PCR should be used. In cases of human genomic DNA, etc., about 1-10 ng is appropriate.

[5] Protocol

1. TaqMan® assay using ABI PRISM® 7700

(1) Preparation of reaction solution

[Example of PCR reaction solution] (primer 0.4 μM, probe 0.2 μM, scale 50 μl)

Distilled water	14 μl
Realtime PCR Master Mix	25 μl
Primer-1 (10 μM)	2 μl
Primer-2 (10 μM)	2 μl
TaqMan® probe (5 μM)	2 μl
Sample solution	5 μl
Total volume	50 μl

- So long as the volume of Realtime PCR Master Mix remains 1/2 of the total volume, the volume of the other elements may be changed as needed to achieve optimal composition. If the total volume (reaction scale) is modified, maintain the optimal ratio of elements.
- Primers should be used in the range between 0.2 and 1 μM. The probe should be used at a final concentration between 0.05 and 0.3 μM. In the case that amplification efficiency is low, adding primer might contribute to improvement.
- When a commercially available primer probe mix is used, use at the concentration designated by the manufacturer. SNPs typing reagents can also be used.
- After preparing a master mix of all components but template for the number of tubes needed plus some tubes extra as specified above, dispense appropriate volumes into PCR tubes. Add template DNA to the individual PCR tubes containing the master mix last.

(2) Performing PCR

[PCR cycle (example 1)] (two-step, annealing/elongation 60°C, 60 seconds)

95°C, 60 seconds

↓

95°C, 15 seconds ⎫ × 40 cycles

60°C, 60 seconds (data collection)

- The temperature for annealing/elongation should be adjusted for the primer and probe used. When a commercially available primer probe mix is used, perform realtime PCR in the cycle condition designated by the manufacturer.
- 60 seconds at the 1st denaturation step and 15 seconds at each cycle of denaturation step are enough for PCR, because reactivation of polymerase can be completed at very short times in this product. Heating a solution longer time than necessary may cause weakening of the polymerase activity. Avoid more than 5 minutes of denaturation step particularly.
- Elongation time does not require adjustment, as realtime PCR usually does not involve amplification of long chains. If it needs to be changed, the data collection step should be 30 seconds or more.
- Refer to the PCR device's handling instructions for details of PCR conditions.

2. Hybridization probe assay using Roche LightCycler™

(1) Preparation of reaction solution

[Example of PCR reaction solution] (primer 0.3 μM, probe 0.2/0.4 μM, scale 20 μl)

Distilled water	5.6 μl
Realtime PCR Master Mix	10 μl
Primer-1 (10 μM)	0.6 μl
Primer-2 (10 μM)	0.6 μl
HybriProbe 1 (5'-acceptor) (10 μM)	0.8 μl
HybriProbe 2 (3'-acceptor) (10 μM)	0.4 μl
Sample solution	2 μl
Total volume	20 μl

- Hybridization probe assays use fluorescent energy transfer (FRET) between two probes for the purpose of detection. The 5'-terminal of probe 1 is bound to acceptor fluorescent dye (LC-Red 640, etc.), and the 3'-terminal of probe 2 is bound to donor fluorescent dye (FITC, etc.).

- So long as Realtime PCR Master Mix remains 1/2 of the total volume, the volume of the other elements may be changed as needed to achieve optimal composition. However, the volume of probe 1 should usually be larger than that of probe 2. If the total volume (reaction scale) is modified, maintain the optimal ratio of elements.
- In the case that amplification efficiency is low, adding primer might contribute to improvement.
- After preparing a master mix of all components but template for the number of tubes needed plus some tubes extra as specified above, dispense appropriate volumes into PCR capillaries. Add template DNA to the individual PCR capillaries containing the master mix last.

(2) Performing PCR

[PCR cycle (example)] (annealing 50°C, 15 seconds/elongation 75°C, 30 seconds)

95°C, 30 seconds

↓

95°C, 5 seconds
50°C, 15 seconds (data collection)] × 40 cycles
75°C, 30 seconds

- Set the temperature for the annealing step lower than the Tm value for the probe. Probe binding occurs in this step and data are collected.
- Set the temperature for elongation higher than the Tm value for the probe. The probe is freed, and elongation advances. Set temperatures at optimal levels for the primer and probe used.
- 30 seconds at the 1st denaturation step and 5 seconds at each cycle of denaturation step are enough for PCR, because reactivation of polymerase can be completed at very short times in this product. Heating a solution longer time than necessary may cause weakening of the polymerase activity. Avoid more than 5 minutes of denaturation step particularly.
- Refer to the PCR device's handling instructions for details of PCR conditions.

3. One-step RT-PCR with the addition of reverse transcriptase

(1) Overview

- This reagent is for use in PCR targeting DNA samples (cDNA, genomic DNA, etc.) but if reverse transcriptase is added, it can also be used in one-step RT-PCR with RNA samples.
- We have confirmed the detection capability of this product when used with TaqMan® assay. However, it must be borne in mind that one-step RT-PCR is more likely to undergo nonspecific responses than ordinary PCR, leading to reduced sensitivity so please investigate as necessary prior to use. Validation has not been carried out for other assay systems.
- The protocol shown here pertains to cases using our ReverTra Ace® reverse transcriptase (Code: TRT-101, 102). Optimal conditions may change when using other manufacturers' reverse transcriptase.

(2) Preparation of the reaction solution

- Ordinary reaction solution for PCR is combined with RNase Inhibitor (Code: SIN-101, 102) at a final concentration of 0.5 U/μl and ReverTra Ace® at a final concentration of 0.01 - 1 U/μl. No other modifications to composition are made.
- ReverTra Ace® is difficult to use non-diluted. We recommend you dilute it before use. RNase Inhibitor is diluted in distilled water to a concentration of 5U/μl, to create a diluent. This diluent is used to dilute ReverTra Ace®. Dilute RT solution should be prepared immediately before use, and the unused portion of the diluted RT solution should be discarded.
- We recommend incorporating an RT(-) control sample (reaction solution free of reverse transcriptase) to check for amplification due to DNA contamination (e.g., contamination by genomic DNA).

[Dilute RT solution (example)] (ReverTra Ace® 3U/μl (final concentration 0.3 U/μl))

Diluent for RT (RNase Inhibitor 5 U/μl)	32 μl
ReverTra Ace® (100 U/μl)	1 μl

Total volume	33 µl

[PCR reaction solution] (example: TaqMan® assay)	
Distilled water	9 µl
Realtime PCR Master Mix	25 µl
Primer-1 (10 µM)	2 µl
Primer-2 (10 µM)	2 µl
TaqMan® probe (5 µM)	2 µl
Dilute RT solution (ReverTra Ace® 3 U/µl)	5 µl
Sample solution	5 µl
Total volume	50 µl

- So long as the volume of Realtime PCR Master Mix remains 1/2 of the total volume, the volume of the other elements may be changed as needed for optimal composition. If the total volume (reaction scale) is modified, maintain the optimal ratio of elements.
- With regard to the concentration of primer and probe, refer to Protocol 1 or 2. In the case that amplification efficiency is low, adding primer might contribute to improvement.
- When a commercially available primer probe mix is used, use at the concentration designated by the manufacturer.
- After preparing a master mix of all components but template for the number of tubes needed plus some tubes extra as specified above, dispense appropriate volumes into PCR tubes. Add template DNA to the individual PCR tubes containing the master mix last.

(3) Performing PCR

- As a rule, it is necessary to incorporate a reverse transcription step (at 42°C for about 20 minutes) before the first step of the ordinary PCR cycle (see Protocols 1 and 2), to extend the duration of the denaturation step to about 5 minutes (to completely inactivate reverse transcriptase and prevent inhibition of PCR). After that, PCR should be performed by normal cycle condition referring to Protocol 1, 2 or 3.
- The temperature for annealing should be adjusted for the Tm value of the primer and probe used.

- When a commercially available primer probe mix is used, perform realtime PCR in the cycle condition designated by the manufacturer.

[ABI PRISM® 7700 (example)]

42°C, 20 minutes

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95°C, 5 minutes

↓

95°C, 15 seconds
60°C, 60 seconds (data collection)] × 40 cycles

[6] Trouble shooting

1. Absence or disturbance of amplification curves

(1) Problems related to device settings (refer to handling instructions for each device)

Cause	Countermeasure
Detection settings, etc., are not suitable for the probe used	Correct detection settings and repeat analysis.
Inappropriate data collection settings	Correct settings and repeat assay.
Erroneous positioning of the sample	Re-position the sample and repeat analysis or assay.
Other malfunction or failure	Check the device with reference to its handling instructions.

(2) Problems related to reagents

Cause	Countermeasure
Inappropriate PCR Cycle condition, concentration or arrangement of the primer and/or probe	Consider changing the concentration or the cycle condition. Reviewing methods vary according to an assay method. Refer to each Protocol. If the problem persists, redesigning is recommended.
Low purity of the sample	Repeat assay after purification by phenol extraction or ethanol sedimentation. Residual reverse transcriptase can affect cDNA assays.

2. Variation in quantitation

(1) Problems related to device settings (refer to handling instructions for each device)

Cause	Countermeasure

Device malfunction or failure	Temperature and detectability can vary due to trouble with devices. Check the device with reference to its handling instructions.

(2) Problems related to reagents

Cause	Countermeasure
Inappropriate PCR Cycle condition, concentration or arrangement of the primer and/or probe	If amplifying system efficiency is low, variations in measurement tend to be greater. Consider changing the concentration or the cycle condition. Reviewing methods vary according to an assay method. Refer to each Protocol. If large variations remain, redesigning is recommended.
Low purity of the sample	Inappropriate samples can cause variations in measurement. Homogeneous samples should be used. Residual reverse transcriptase can affect cDNA assays. In such cases, repeat assay after purification by phenol extraction or ethanol sedimentation.
Variation in readings	Repeat assay after elevating reaction scale.

[7] Related products

Product Names	Package	Code No.
ReverTra Ace -α-®	100 rxns	FSK-101
ReverTra Ace®	10,000 U × 1 50,000 U × 1	TRT-101 TRT-102
RNase Inhibitor	2,500 U × 1 2,500 U × 5	SIN-101 SIN-102
SYBR® Green Realtime PCR Master Mix	1 ml × 1 1 ml × 5	QPK-201T QPK-201

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