RT-PCR Quick Master Mix

PCR-311F 50 reactions

Store at -20°C

Contents

CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precaution and utilize safety while using this kit.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO (SHANGHAI) BIOTECH, CO., LTD.
Tel(86)-21-58794900

[1] Introduction

Description

The RT-PCR Quick Master Mix provides a 2 x Master Mix for RT-PCR using a thermostable DNA polymerase derived from *Thermus thermophilus* (Tth) HB8[1)]. Tth DNA polymerase exhibits reverse transcriptase activity in addition to DNA polymerase activity in the presence of Mn^{2+} ions. Therefore, this system enables "one-step RT-PCR" including reverse transcription and PCR steps. This kit is suitable for high-throughput RT-PCR, and decreases contamination risks.

Features

-This kit enables one-step RT-PCR including reverse transcription and PCR steps. This kit is suitable for high-throughput RT-PCR and decreases contamination risks.

-Compared with normal reverse transcriptases from retroviruses (e.g., M-MLV reverse transcriptase), Tth DNA polymerase can work at a higher temperature. Therefore, this kit has the advantage of amplifying targets with complex conformations and GC-rich content.

-Hot-start technology using anti-Tth DNA polymerase antibodies enables highly efficient and specific amplification.

Notes

The PCR fidelity of this kit is not good because PCR is performed with Mn^{2+} instead of Mg^{2+}. Do not use this kit for DNA cloning.

[2] Components

This kit includes the following reagents for application with 50 reactions. All reagents should be stored at - 20 °C.

2 x RT-PCR Quick Master Mix	625 µl x 2
50 mM $Mn(OAc)_2$	200 µl
Nuclease-free water	1100 µl

2 x RT-PCR Quick Master Mix

-This solution contains the reaction buffer, dNTPs, rTth DNA polymerase, and anti-DNA polymerase antibodies as a 2-times concentrated solution.

-Do not freeze and thaw more than 10 times. This reagent can be stored at 4 °C within 2 months.

50 mM $Mn(OAc)_2$

This reagent is used with the 2 x RT-PCR Quick Master Mix. Tth DNA polymerase can work as a DNA polymerase and reverse transcriptase in the presence of Mn^{2+}.

Nuclease-free water

This nuclease-free water is prepared without DEPC treatment.

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[3] Protocol

1. Template RNA for RT-PCR

The following RNAs are appropriate for highly efficient reverse transcription.

(1) Total RNA

Total RNA usually contains 1-2% mRNA. Total RNA can be used as template directly with this kit. RNA prepared by AGPC (Acid Guanidium-Phenol-Chloroform) or the column method contains genomic DNA, so total RNA should be treated with DNase I before transcription.

(2) Poly(A)$^+$ RNA (mRNA)

Poly (A)$^+$ RNA is useful to detect low-level expressing mRNA. However, poly (A)$^+$ RNA should be treated carefully because Poly(A)+ RNA is more sensitive to RNase than total RNA.

2. Primers for reverse transcription and PCR

Only gene-specific primers can be used for reverse transcription in this kit. In general, reverse primers for PCR can work as reverse transcription primers. PCR primers can be designed according to the usual rule.

3. Protocol for RT-PCR

(1) Mix the following reagents.

Component	Amount	Final concentration
Nuclease-free water*1	X µl	
2 x RT-PCR Quick Master Mix	25 µl	
50 mM $Mn(OAc)_2$	2.5 µl	2.5 mM *2
Forward Primer	10-30 pmol	0.2-0.6 µM *3
Reverse Primer	10-30 pmol	0.2-0.6 µM *3
RNA sample Total RNA Poly(A)$^+$ RNA	 < 2.5 µg < 500 ng	
Total volume	50 µl	

Notes

*1 This kit contains nuclease-free water for 50 reactions but not for the dilution of the RNA samples. Nuclease-free water prepared without DEPC treatment is recommended for RNA sample dilution.

*2 Using a higher concentration of $Mn(OAc)_2$ can increase the detection sensitivity and amplification efficiency. However, non-specific amplification is also increased with increasing Mn^{2+} concentration.

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*3 Using higher concentrations of primers can increase the detection sensitivity and amplification efficiency. However, non-specific amplification is also increased with higher primer concentrations.

(2) Perform the following heat cycle.

Denaturation :	90°C , 30 sec.*1	
RT*2 :	60° C, 30 min.	
Pre-denaturation :	94 °C, 1 min.	
Denaturation :	94 °C, 30 sec.	↰ 25-40 cycles*3
Annealing :	50-70 °C*4, 30 sec.	
Extension :	72 °C, 1 min.*5	
Extension :	72 °C, 7 min.	

Notes

*1 The PCR Master Mix contains the anti-Tth DNA polymerase antibodies for hot-start PCR. The first denaturation step (90 °C, 30 sec.) is enough to inactivate the antibodies. Do not prolong this denaturation step.

*2 The temperature of reverse transcription (RT) should be set below Tm of primers. The temperature can be optimized between 50 and 60 °C.

*3 The number of cycles can be adjusted depending on the expression level of the target mRNA.

*4 The optimal annealing temperature is around Tm -5 °C. Using a lower annealing temperature can increase the detection sensitivity and amplification efficiency. However, non-specific amplification is also increased at lower annealing temperatures.

*5 This condition is applicable to the amplification of targets up to 1kb. For longer targets, a longer extension step is necessary.

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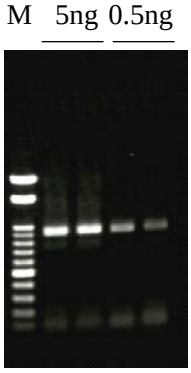
[4] Example

Example 1. Amplification of human cdc2 gene from poly $(A)^+$ RNA.



Template: human mRNA (from HeLa cell)
5ng or 0.5ng/ 50 µl (reaction)
Target: cdc2 mRNA ORF 900bp
M: 100bp Ladder Markers

PCR cycling condition:

90℃ 30 sec.
60℃ 30 min.
94℃ 1 min.
94℃ 30 sec.
60℃ 30 sec.] 40 cycles
72℃ 1 min.
72℃ 7 min.

Cdc2-F : 5'-CCATACCATTGACTAACTATGGAAGAT-3' (27mer)
Cdc2-R : 5'-GTCAGAAAGCTACATCTTCTTAATCTG -3' (27mer)

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[5] Related Protocol

1. DNase I treatment of total RNA

Total RNA prepared by general methods contains genomic DNA. Genomic DNA can be eliminated by the following method.

(1) Mix the following reagents.

Nuclease-free water	X µl
Total RNA (<10 µg)	Y µl
10 x DNase I Buffer [*e.g.* 100 mM Tris-Cl, 20 mM $MgCl_2$(pH 7.5)]	1 µl
RNase-free DNase I (10 U/µl)	0.5 µl
Total volume	10 µl

(2) Incubate on ice for 10-30 min.

(3) Purify the treated RNA according to the following step.

DNase I-treated RNA
↓ ← Add nuclease-free water (adjust volume to 100 µl)
↓ ← Add 100 µl TE-saturated phenol

Vortex
Keep on ice for 5 min
↓ Centrifuge at 12,000 rpm for 5 min

Supernatant
↓ ← Add 100 µl chloroform: isoamyl alcohol (24:1), Vortex
↓ Centrifuge at 12,000 rpm for 5 min

Supernatant
↓ ←Add 100 µl 5 M ammonium acetate + 200 µl isopropanol
+ [5 µl 20 mg/ml glycogen* (for coprecipitation) : optional]
Vortex
Incubate at - 20 °C for 30 min
↓ Centrifuge at 12,000 rpm for 5 min

Discard supernatant
Precipitate
↓ ←Add 70% ethanol
↓ Centrifuge at 12,000 rpm for 5 min

Discard supernatant
Precipitate
↓ ←Dissolve in appropriate volume of nuclease-free water

RNA solution

*Molecular biology grade

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[6] Trouble shooting

Symptom	Cause	Solution
No amplification	Low purity of the RNA template	Repurify the RNA sample.
	Degradation of the RNA template	Prepare the RNA sample again.
	Insufficient amount of the target RNA	Increase the template RNA.
	Inappropriate annealing temperature	The optimal annealing temperature is around Tm -5 °C. Using a lower annealing temperature can increase the detection sensitivity and amplification efficiency. However, non-specific amplification is also increased at a lower annealing temperature.
	Low concentrations of primers	The optimal final concentration of primers is 0.2-0.6 μM. Using higher concentrations of primers can increase the detection sensitivity and amplification efficiency. However, non-specific amplification is also increased with increasing primer concentrations. The efficiency can be improved by using a higher concentration (up to 1μM) of the reverse primer.
Non-specific amplification	High concentrations of primers	Using higher concentrations of primers can increase the detection sensitivity and amplification efficiency. Decrease the primer concentration to 0.2 μM or less.
	Low specificity of primers	-Redesign the primers according to the following optimal conditions. A GC content of 40-60% -No homology around the 3' terminal end of each primer. -No complementary area within the primer.
Amplification on blank samples	Contamination or carry-over	Use fresh nuclease-free water and primers.

[7] References

1) Myers T. W. and Gelfand D. H. , *Biochemistry*, 30: 7661-6 (1991)