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Inspiration for Life Science

Instruction manual *RNA-direct*<sup>™</sup> SYBR<sup>®</sup> Green Realtime PCR Master Mix 1705

F0930K

# *RNA-direct*<sup>™</sup> SYBR<sup>®</sup> Green Realtime PCR Master Mix

QRT-201 0.5mLx5

Store at -20°C, protected from light

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## 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.

-LightCycler<sup>™</sup> is a trademark of Idaho Technology, Inc. and Roche Molecular Systems, Inc.

-SYBR<sup>®</sup> is a registered trademark of Molecular Probes, Inc.

-RNA-direct<sup>™</sup> is a trademark of Toyobo Co., Ltd. in Japan.



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## [1] Introduction

### Description

This product is a 2 x Master Mix for “1-step real-time PCR” using a thermostable DNA polymerase derived from *Thermus thermophilus* (Tth) HB8<sup>1)</sup>. Tth DNA polymerase exhibits reverse transcriptase activity in the presence of Mn<sup>2+</sup> ions. This system allows for “1-step real-time PCR”, including reverse transcription and PCR steps. This reagent can be applied to an intercalation assay with SYBR<sup>®</sup> Green I.

### Features

- This reagent is suitable for high-throughput real-time PCR and increases reliability of product, due to lowered risk of contamination.
- This reagent can be used in systems using glass capillaries (e.g., LightCycler, Roche Molecular Systems, Inc.).
- This reagent can be used in systems using passive reference (e.g., 7700, Applied Biosystems, Inc.). The passive reference dye does not affect any other systems.
- Hot Start technology, using anti-Tth DNA polymerase antibodies, allows for high specificity and reproducible amplification.

## [2] Components

This reagent includes the following components for 100 reactions, with a total of 50 µl per reaction. All reagents should be stored at -20 °C.

<QRT-201>

|  |         |
|--|---------|
| <i>RNA-direct</i> <sup>™</sup> SYBR <sup>®</sup> Green Realtime PCR Master Mix | 0.5mlx5 |
| 50mM Mn(OAc) <sub>2</sub>  | 0.5mlx1 |

### Notes:

This reagent can be stored at 4°C for up to 2 months, protected from light. For longer storage, this reagent should be kept at -20°C and protected from light.

## [3] Primer

### 1. Primer conditions

Primers should be designed according to the following guidelines:

- Primer length: 20-30 mer
- GC content of primer: 40-60%
- Target length: ≤200 bp (optimally, 50-150 bp)

Notes:

Longer targets (>200 bp) reduce efficiency and specificity of amplification. The ideal optimal target length range is 50-150 bp.

## [4] Detection

This reagent can be used with the following devices:

### 1. Normal devices, such as:

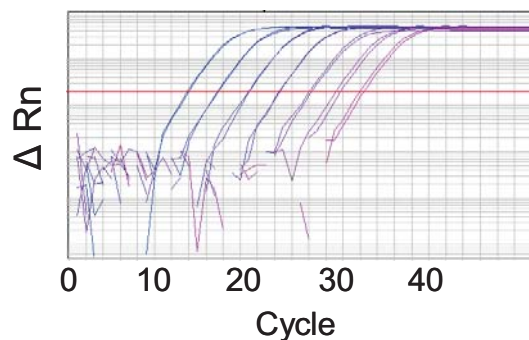
-LineGene, Bioer etc.

### 2. Devices using a glass capillary or a passive reference, such as:

-LightCycler, Roche Molecular Systems

-7000, 7700, and 7900, Applied Biosystems

**Notes:** The passive reference mode of detectors should be set at "ROX".



**Fig. 1** Detection of  $\beta$ -actin expression with SYBR<sup>®</sup> Green I

Device: Applied Biosystems 7900HT

Target:  $\beta$ -actin

Assay method: SYBR Green<sup>®</sup> I

Template: cDNA from total RNA (HeLa cells)

## [5] Specimen

The following RNAs are appropriate for highly efficient 1-step real-time PCR.

### 1. Total RNA

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

### 2. Poly(A)<sup>+</sup> RNA (mRNA)

Poly(A)<sup>+</sup> RNA can be used to detect low-level expressing mRNA. However, poly(A)<sup>+</sup> RNA should be treated carefully, because Poly(A)<sup>+</sup> RNA is more sensitive to RNase than total RNA.

## [6] Protocol

### 1. Intercalation assay protocol using Applied Biosystem 7900HT

The following is an intercalation assay protocol using 7900HT. If using other detection devices, this protocol should be slightly altered, according to each instruction manual.

#### (1) Preparation of reaction solution

| Component   | Volume      | Final Concentration       |
|---|-------------|---------------------------|
| Nuclease-free water   | X $\mu$ l   |                           |
| <i>RNA-direct</i> <sup>TM</sup> SYBR <sup>®</sup> Green Realtime PCR Master Mix | 25 $\mu$ l  | 1x                        |
| 50mM Mn(OAc) <sub>2</sub>   | 2.5 $\mu$ l | 2.5 mM                    |
| 10 pmol / $\mu$ l (10 $\mu$ M) Primer #1  | 1.0 $\mu$ l | 0.2 $\mu$ M               |
| 10 pmol / $\mu$ l (10 $\mu$ M) Primer #2  | 1.0 $\mu$ l | 0.2 $\mu$ M               |
| Template RNA  | Y $\mu$ l   |                           |
| Total RNA   |             | <2.5 $\mu$ g / 50 $\mu$ l |
| Poly (A) <sup>+</sup> RNA   |             | <500 ng / 50 $\mu$ l      |
| Total volume  | 50 $\mu$ l  |                           |

#### Notes

- Primer concentrations can be further optimized, if needed. The optimal range of primers is 0.2-0.6  $\mu$ M. In the case of commercially available primers, those recommended condition should be used.
- The final concentration of Mn(OAc)<sub>2</sub> should be adjusted to 2-3.5 mM. Lower Mn concentrations result in decreased non-specific amplification; higher Mn concentrations result in increased amplification efficiency.
- Nuclease-free water prepared without DEPC-treatment is recommended.

#### (2) Cycling condition

The following is a cycling condition the “standard mode” of 7900HT.

| <3-step cycle>         |                                  |             |
|------------------------|----------------------------------|-------------|
| Denaturation :         | 90 °C, 30 sec.                   |             |
| RT* :                  | 61 °C, 20 min.                   |             |
| Pre-denaturation :     | 95 °C, 1 min.                    |             |
| Denaturation :         | 95 °C, 15 sec.                   | } 45 cycles |
| Annealing :            | 55-65 °C, 15 sec.                |             |
| Extension :            | 74 °C, 45 sec. (data collection) |             |
| Melting curve analysis |                                  |             |

\*The temperature of reverse transcription (RT) should be set below T<sub>m</sub> of primers. The temperature can be optimized between 50 and 61 °C.

#### Notes

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

-The annealing temperature should to  $T_m - 5$  °C. The optimal annealing temperature range is 55-65°C.

## 2. Intercalation assay protocol using Roche LightCycler™

The following is an intercalation assay protocol using Roche LightCycler™. In the case of other detection devices, this protocol should be slightly altered, according to each instruction manual.

### (1) Preparation of reaction solution

| Component   | Volume      | Final Concentration     |
|---|-------------|-------------------------|
| Nuclease-free water                                     | X $\mu$ l   |                         |
| <i>RNA-direct</i> ™ SYBR® Green Realtime PCR Master Mix | 10 $\mu$ l  | 1x                      |
| 50 mM Mn(OAc) <sub>2</sub>                              | 1 $\mu$ l   | 2.5 mM                  |
| 10 pmol / $\mu$ l (10 $\mu$ M) Primer #1                | 0.4 $\mu$ l | 0.2 $\mu$ M             |
| 10 pmol / $\mu$ l (10 $\mu$ M) Primer #2                | 0.4 $\mu$ l | 0.2 $\mu$ M             |
| Template RNA  | Y $\mu$ l   |                         |
| Total RNA   |             | <1 $\mu$ g / 20 $\mu$ l |
| Poly(A) <sup>+</sup> RNA                                |             | <200 ng / 20 $\mu$ l    |
| Total volume  | 20 $\mu$ l  |                         |

### Notes

- The primer concentration can be further optimized, if needed. The optimal range of primers is 0.2-0.6  $\mu$ M. In the case of commercially available primers, those recommended conditions should be used.
- The final concentration of Mn(OAc)<sub>2</sub> should be adjust to 2-3.5 mM. Lower Mn concentrations result in decreased non-specific amplification; higher Mn concentrations result in increased the amplification efficiency.
- Nuclease-free water prepared without DEPC-treatment is recommended.

### (2) Cycling condition

The following condition is recommended.

| <3-step cycle>     |                                  |             |
|--------------------|----------------------------------|-------------|
| Denaturation :     | 90 °C, 30 sec.                   | } 45 cycles |
| RT* :              | 61 °C, 20 min.                   |             |
| Pre-denaturation : | 95 °C, 30 sec.                   |             |
| Denaturation :     | 95 °C, 5 sec.                    |             |
| Annealing :        | 55-65 °C, 10 sec.                |             |
| Extension :        | 74 °C, 15 sec. (data collection) |             |

\*The temperature of reverse transcription (RT) should be set below  $T_m$  of primers. The temperature can be optimized between 50 and 61 °C.

### Notes

- The PCR Master Mix contains anti-Tth DNA polymerase antibodies for Hot Start PCR. The first denaturation step (90°C, 30 sec.) is sufficient to inactivate the antibodies. Do not prolong this denaturation step.
- The temperature transition rate should be set to 20 °C/sec. Poor amplification may be improved by adjusting the temperature transition rate to 2 °C/sec.
- If the target length is ≤ 200 bp, the extension time should be adjusted to 15 sec. Data collection steps should be at least 15 sec.

## [7] 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<br>[e.g. 100 mM Tris-Cl, 20 mM MgCl <sub>2</sub> (pH 7.5)] | 1 μl         |
| RNase-free DNase I (10 U/μl)   | 0.5 μl       |
| <b>Total volume</b>  | <b>10 μl</b> |

#### (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 at 4 °C

#### Supernatant

- ↓ ← Add 100 μl 5 M ammonium acetate + 200 μl isopropanol  
+ [5 μl 2 mg/ml glycogen\* (for coprecipitation) : optional]

#### Vortex

#### Incubate at - 20 °C for 30 min

- ↓ Centrifuge at 12,000 rpm for 10-15 min at 4 °C

#### Discard supernatant



**Precipitate**

- ↓ ←Add 1 ml 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

## [ 8 ] Troubleshooting

| Symptom                    | Cause  | Solution  |
|----------------------------|--|---|
| No amplification           | Incorrect setting of detector mode for the fluorescence dye. | Confirm the detector setting.   |
|                            | Incorrect setting for data collection.                       | Confirm the data collection setting.  |
|                            | Incorrect setting for sample position.                       | Reposition the sample tubes.  |
|                            | Inappropriate concentration of primers or probes.            | Optimize primer or probe concentration according to instructions (see [6]).                     |
|                            | Inappropriate design of primers or probes.                   | Confirm specificity and T <sub>m</sub> of primers and probes.                                   |
|                            | Inappropriate cycle conditions.                              | Confirm T <sub>m</sub> of the primers and probes.   |
|                            | Low purity or quality of samples                             | Check the purity or quality of sample DNA.  |
|                            | Low concentration of Mn(OAc) <sub>2</sub> .                  | Increase the Mn concentration to 3.5 mM.  |
| Variation in detection     | Failure or malfunction of device                             | Check the device  |
|                            | Low quality of sample DNA.                                   | Repurify DNA sample by phenol/chloroform extraction and ethanol precipitation, or other method. |
|                            | Inappropriate concentration of primers or probes.            | Optimize primer or probe concentration according to the instructions (see [6]).                 |
|                            | Inappropriate design of primers or probes.                   | Confirm specificity and T <sub>m</sub> of primers and probes.                                   |
|                            | Inappropriate cycle conditions.                              | Confirm T <sub>m</sub> of the primers and probes.   |
|                            | Variation of dispensed volume                                | Increase the reaction volume  |
| Signals in blank reactions | Contamination of amplicons or sample DNAs.                   | Use fresh PCR grade water. Re-make primer solution, probe solution, and master mix.             |
|                            | Detection of a non-specific amplification.                   | Optimize the primer and cycle conditions.   |

## [ 9 ] Related products

| Product name  | Package           | Code No.            |
|---|-------------------|---------------------|
| High efficient cDNA synthesis kit for real-time PCR<br><b>ReverTra Ace<sup>®</sup> qPCR RT Kit</b>  | 200 rxns          | FSQ-101             |
| High efficient cDNA synthesis master mix for real-time PCR<br><b>ReverTra Ace<sup>®</sup> qPCR RT Master Mix</b>  | 200 rxns          | FSQ-201             |
| High efficient cDNA synthesis master mix for real-time PCR with genomic DNA remover<br><b>ReverTra Ace<sup>®</sup> qPCR RT Master Mix<br/>with gDNA remover</b> | 200 rxns          | FSQ-301             |
| High efficient revers transcriptase<br><b>ReverTra Ace<sup>®</sup></b>  | 10,000U           | TRT-101             |
| <b>RNase inhibitor (Recombinant type)</b>   | 2,500U            | SIN-201             |
| Realtime PCR master mix for probe assay<br><b>THUNDERBIRD<sup>®</sup> Probe qPCR Mix</b>  | 1mLx1<br>1.67mLx3 | QPS-101T<br>QPS-101 |
| Realtime PCR master mix for SYBR <sup>®</sup> Green assay<br><b>THUNDERBIRD<sup>®</sup> SYBR qPCR Mix</b>   | 1mLx1<br>1.67mLx3 | QPS-201T<br>QPS-201 |
| Realtime PCR master mix for probe assay<br><b>Realtime PCR Master Mix</b>   | 1mLx5             | QPK-101             |
| Realtime PCR master mix for SYBR <sup>®</sup> Green assay<br><b>SYBR<sup>®</sup> Green Realtime PCR Master Mix</b>  | 1mLx5             | QPK-201             |
| One-step realtime PCR master mix for probe assay<br><b>RNA-direct<sup>™</sup> Realtime PCR Master Mix</b>   | 0.5mLx5           | QRT-101             |

## [ 10 ] References

- Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. Myers T. W. and Gelfand D. H. , *Biochemistry*, 30: 7661-6 (1991)





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