

# TaqMan<sup>®</sup> Small RNA Assays

TaqMan<sup>®</sup> MicroRNA Assays, Custom TaqMan<sup>®</sup> Small RNA Assays, and TaqMan<sup>®</sup> siRNA Assays

Pub. No. 4412551 Rev. D

**Note:** For safety and biohazard guidelines, see the “Safety” appendix in the *TaqMan<sup>®</sup> Small RNA Assay User Guide* (Pub. No. 4364031). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This Quick Reference is intended as a benchtop reference for experienced users of TaqMan<sup>®</sup> Small RNA Assays. For detailed instructions, supplemental procedures, and troubleshooting, see the *TaqMan<sup>®</sup> Small RNA Assay User Guide* (Pub. No. 4364031).

## Perform reverse transcription

### Guidelines for isolation of high-quality RNA

- Prepare samples using a method that preserves small RNAs.
- To prevent the loss of the longer control transcripts (such as snoRNAs), size fractionation is *not* recommended.

### Guidelines for RNA input

- Use 1–10 ng of total RNA per 15- $\mu$ L RT reaction.
- For optimal reverse transcription, input RNA should be:
  - Free of inhibitors of reverse transcription (RT) and PCR.
  - Dissolved in PCR-compatible buffer.
  - Free of RNase activity.
  - Nondenatured total RNA (not applicable for double-stranded templates).

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**IMPORTANT!** Do not denature the total RNA.

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- Use 10 to 10,000 cells per sample for the Cells-to-C<sub>t</sub> kit.
- When working with double-stranded template such as siRNAs and miRNA mimics, denature the siRNA template with sequence-specific RT Primer before performing the reverse transcription.
- Prepare the RT reactions in an area free of artificial templates, amplified material, and siRNA transfections. High-copy-number templates can easily contaminate the reactions.
- When working with siRNA use total RNA from non-transfected cells as a control in *in-vitro* and *in-vivo* studies. The amount of input total RNA for optimal detection depends on the transfection protocol used.

**Note:** When testing for more than 10 targets, it might be more efficient to create primer pools. For details see *Protocol for Creating Custom RT and Preamplification Pools Using TaqMan<sup>®</sup> MicroRNA Assays* (Pub. No. 4465407).

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**IMPORTANT!** TaqMan<sup>®</sup> MicroRNA Assays and TaqMan<sup>®</sup> Small RNA Assays are optimized for the MuLV Reverse Transcriptase contained in the TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit. Assay performance with other reverse transcriptase enzymes cannot be guaranteed.

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## Before you begin

- Thaw components of the reverse transcription kit on ice.
- Thaw the RT Primers on ice, vortex briefly, then centrifuge briefly to collect the contents at the bottom of the tube.
- (*Large assays only*) Dilute the 20 $\times$  or 60 $\times$  RT Primer to a 5 $\times$  working solution using 0.1 $\times$  TE Buffer. Store for up to one year at -25°C to -15°C.

## Prepare the RT Reaction Mix

1. In an appropriately-sized microcentrifuge tube, prepare RT Reaction Mix according to the following table.

| Component                                   | Volume (1 reaction) | Volume (10 reactions) <sup>[1]</sup> |
|---|---------------------|--------------------------------------|
| 100mM dNTPs (with dTTP)                     | 0.15 µL             | 1.65 µL                              |
| MultiScribe™ Reverse Transcriptase, 50 U/µL | 1.00 µL             | 11.00 µL                             |
| 10× Reverse Transcription Buffer            | 1.50 µL             | 16.50 µL                             |
| RNase Inhibitor, 20 U/µL                    | 0.19 µL             | 2.09 µL                              |
| Nuclease-free Water                         | 4.16 µL             | 45.76 µL                             |
| <b>Total RT Reaction Mix volume</b>         | <b>7.00 µL</b>      | <b>77.00 µL</b>                      |

<sup>[1]</sup> Includes 10% overage.

2. Invert to mix, then centrifuge briefly to collect the contents at the bottom of the tube.

Place the RT Reaction Mix on ice. Proceed immediately to “Prepare the RT reaction” on page 2.

### Prepare the RT reaction

Use one of the following procedures.

Prepare RT reaction with double-stranded small RNA

Silencer Select miRNAs are double-stranded miRNA-mimicking molecules.

1. Combine 3 µL of 5× RT Primer and 5 µL of double-stranded template in a reaction tube or in each well of a reaction plate.
2. Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube or wells.
3. Incubate at 85°C for 5 minutes.
4. Incubate at 60°C for 5 minutes, then place on ice.
5. Add 7 µL of RT Reaction Mix to each reaction tube or well.  
RT Reaction Mix was prepared in “Prepare the PCR Reaction Mix” on page 3.
6. Seal the tube or reaction plate.
7. Centrifuge briefly to collect the contents at the bottom of the tubes or wells.

Place on ice and proceed immediately to “Perform reverse transcription” on page 3.

Prepare RT reaction with single-stranded small RNA

1. Combine 7 µL of RT Reaction Mix and 5 µL of total RNA in a reaction tube or in each well of a reaction plate.  
RT Reaction Mix was prepared in “Prepare the RT Reaction Mix” on page 1.
2. Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tubes or wells.
3. Add 3 µL of 5× RT Primer to each reaction tube or each well of a reaction plate.
4. Seal the tubes or reaction plate.
5. Centrifuge briefly to collect the contents at the bottom of the tubes or wells.

Place on ice and proceed immediately to “Perform reverse transcription” on page 3.

## Perform reverse transcription

Place the reaction plate or tubes into a thermal cycler, then incubate using standard cycling, a reaction volume of 15.0  $\mu\text{L}$ , and the following settings.

| Step                  | Temperature | Time       |
|-----------------------|-------------|------------|
| Reverse transcription | 16°C        | 30 minutes |
|                       | 42°C        | 30 minutes |
| Stop reaction         | 85°C        | 5 minutes  |
| Hold                  | 4°C         | Hold       |

The RT reaction product can be stored at  $-25$  to  $-15^\circ\text{C}$  for up to one week.

## Perform PCR amplification

### Procedural guidelines for performing real-time PCR

- Follow best practices when preparing or performing PCR.
- Prepare the PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Protect the assays from light and store in a freezer. Excessive exposure to light might affect the fluorescent probes.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.
- Perform three replicates of each reaction.
- Include the following reactions:
  - A small RNA assay for each cDNA sample.
  - Endogenous control assays for each cDNA sample.
  - No-template controls (NTCs) for each assay on the plate.

**Note:** Use of NTC reactions to evaluate background signal is strongly recommended.

### Before you begin

- (For large assays) Dilute 60 $\times$  assays to 20 $\times$  working solutions before use.
- Thaw assays (20 $\times$ ) and cDNA templates on ice, vortex gently, then centrifuge briefly to bring the contents to the bottom of the tube.

### Prepare the PCR Reaction Mix

1. Mix the PCR Master Mix reagent by gently swirling the bottle.
2. Prepare the PCR Reaction Mix in appropriately-sized microcentrifuge tubes, according to the following table. Add 10% overage to account for pipetting loss.

| Component                            | Volume per reaction                    |                                       |
|--------------------------------------|--|---------------------------------------|
|                                      | 384-well or 96-well fast (0.1mL) plate | 96-well standard (0.2 mL) plate       |
| TaqMan® Small RNA Assay (20X)        | 0.50 $\mu\text{L}$                     | 1.00 $\mu\text{L}$                    |
| PCR Master Mix                       | 5.00 $\mu\text{L}$                     | 10.00 $\mu\text{L}$                   |
| Nuclease-free water <sup>[1]</sup>   | 3.50 $\mu\text{L}$                     | 7.00 $\mu\text{L}$                    |
| <b>Total PCR Reaction Mix volume</b> | <b>9.00 <math>\mu\text{L}</math></b>   | <b>18.00 <math>\mu\text{L}</math></b> |

<sup>[1]</sup> Adjust quantity of water to account for cDNA template.

3. Vortex to mix the PCR Reaction Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
2. Add cDNA template (1 pg to 100 ng in nuclease-free water), or nuclease-free water for NTC, to each well.
  - 384-well or 96-well fast (0.1 mL) plate: 1  $\mu$ L
  - 96-well standard (0.2 mL) plate: 2  $\mu$ L

**Note:** This is the maximum amount of cDNA template that can be added to a reaction, because the RT Primer must be diluted a minimum of 1:15 in the final PCR reaction.

**Note:** Adjust the volume of nuclease-free water in the PCR Reaction Mix for a larger volume of cDNA.

3. Seal the plate with optical adhesive film, then centrifuge the plate briefly to bring the Reaction Mix to the bottoms of the wells.
4. Apply a compression pad to the plate, if required by your real-time PCR system.

## Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

**Note:** The instrument must be configured with the block appropriate for the plate type.

1. Select the cycling mode appropriate for the Master Mix.

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**IMPORTANT!** The cycling mode depends on the Master Mix that is used in the reaction. The cycling mode does not depend on a Standard or a Fast plate format.

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2. Set up the thermal protocol for your instrument.

**Table 1** TaqMan® Fast Advanced Master Mix (StepOne™, StepOnePlus™, ViiA™ 7, and QuantStudio™ systems with fast cycling mode)

| Step                      | Temperature | Time       | Cycles |
|---------------------------|-------------|------------|--------|
| (Optional) UNG activation | 50°C        | 2 minutes  | 1      |
| Enzyme activation         | 95°C        | 20 seconds | 1      |
| Denature                  | 95°C        | 1 second   | 40     |
| Anneal / Extend           | 60°C        | 20 seconds |        |

**Table 2** TaqMan® Fast Advanced Master Mix (7500 and 7500 Fast systems with fast cycling mode)

| Step                      | Temperature | Time       | Cycles |
|---------------------------|-------------|------------|--------|
| (Optional) UNG activation | 50°C        | 2 minutes  | 1      |
| Enzyme activation         | 95°C        | 20 seconds | 1      |
| Denature                  | 95°C        | 3 seconds  | 40     |
| Anneal / Extend           | 60°C        | 30 seconds |        |

**Table 3** TaqMan® Universal Master Mix II, no UNG, TaqMan® Universal Master Mix II, with UNG, TaqMan® Universal PCR Master Mix, no AmpErase™ UNG, or TaqMan® Universal PCR Master Mix (any compatible instrument with standard cycling mode)

| Step                      | Temperature | Time       | Cycles |
|---------------------------|-------------|------------|--------|
| (Optional) UNG activation | 50°C        | 2 minutes  | 1      |
| Enzyme activation         | 95°C        | 10 minutes | 1      |
| Denature                  | 95°C        | 15 seconds | 40     |
| Anneal / Extend           | 60°C        | 60 seconds |        |

3. Set the appropriate reaction volume.
4. Load the plate into the real-time PCR instrument.
5. Start the run.

## Analyze the results

For detailed information about data analysis, see the appropriate documentation for your instrument. Use the absolute or relative quantification ( $\Delta\Delta C_t$ ) methods to analyze results.

The general guidelines for analysis include:

- View the amplification plot; then, if needed:
  - Adjust the baseline and threshold values.
  - Remove outliers from the analysis.
- In the well table or results table, view the  $C_t$  values for each well and for each replicate group.

For more information about real-time PCR, see *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239) or go to [thermofisher.com/qpcreducation](http://thermofisher.com/qpcreducation).

## Secondary analysis software

DataAssist™ Software is a simple, powerful data analysis tool for sample comparison when using the comparative  $C_t$  ( $\Delta\Delta C_t$ ) method for calculating relative quantitation of gene expression. The software uses a filtering procedure for outlier removal and various normalization methods based on lists of single or multiple genes. It provides relative quantification analysis of gene expression through a combination of statistical analysis and interactive visualization.

DataAssist™ Software is available for download from [thermofisher.com/dataassist](http://thermofisher.com/dataassist).

## Algorithms for data analysis

**Table 4** Algorithm recommendations for single-tube assays

| Algorithm                       | Recommendation  |
|---------------------------------|---|
| Threshold ( $C_t$ )             | Recommended.  |
| Relative threshold ( $C_{rt}$ ) | <i>(Optional)</i> Use for troubleshooting abnormal or unexpected results. |

The relative threshold algorithm is available in the Relative Quantification application on the Thermo Fisher Cloud ([thermofisher.com/cloud](http://thermofisher.com/cloud)).

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**Revision history:** Pub. No. 4412551

| Revision | Date             | Description   |
|----------|------------------|---|
| D        | 12 February 2019 | <ul style="list-style-type: none"><li>• Removed the optional UNG activation step from PCR protocols.</li><li>• Added new instruments, Master Mixes, and other applicable products.</li><li>• Added thermal cycling protocols for all compatible Master Mixes.</li><li>• Updated options for secondary analysis software.</li><li>• Updated for general style, formatting, and branding.</li></ul> |
| C        | January 2011     | Baseline for this revision history.   |

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