

# TaqMan™ Advanced miRNA Assays

## Single-tube assays

Catalog Number A25576

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**Note:** For safety and biohazard guidelines, see the “Safety” appendix in the following product documentation: *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897). 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™ Advanced miRNA Assays and the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Cat. No. [A28007](#); sold separately). For detailed instructions, supplemental procedures, and troubleshooting, see the *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897).

## Prepare cDNA templates

### Procedural guidelines

#### Guidelines for preparing cDNA templates

- Keep the TaqMan™ Advanced miRNA Assays in storage until ready for use.
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 15% overage.

#### Guidelines for RNA input

- Prepare samples using a total RNA isolation method that preserves small RNAs.
- For tissue samples, use 2 ng of total RNA per reaction.
- For blood, serum, or plasma samples, use 2 µL of sample eluent (from the sample isolation procedure) per reaction. If RNA can be quantified, use 2 ng of total RNA per 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

## Perform the poly(A) tailing reaction

1. Thaw samples and cDNA synthesis reagents on ice, gently vortex, then centrifuge briefly.

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**IMPORTANT!** The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (see “Perform the adaptor ligation reaction” on page 3).

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2. In a 1.5 mL microcentrifuge tube, prepare sufficient Poly(A) Reaction Mix for the required number of reactions according to the following table.

| Component                                | Number of reactions |                  |                  |
|------------------------------------------|---------------------|------------------|------------------|
|                                          | 1                   | 4 <sup>[1]</sup> | 8 <sup>[1]</sup> |
| 10X Poly(A) Buffer                       | 0.5 µL              | 2.3 µL           | 4.6 µL           |
| ATP                                      | 0.5 µL              | 2.3 µL           | 4.6 µL           |
| Poly(A) Enzyme                           | 0.3 µL              | 1.4 µL           | 2.8 µL           |
| RNase-free water                         | 1.7 µL              | 7.8 µL           | 15.6 µL          |
| <b>Total Poly(A) Reaction Mix volume</b> | <b>3.0 µL</b>       | <b>13.8 µL</b>   | <b>27.6 µL</b>   |

<sup>[1]</sup> Volumes include 15% overage.

3. Vortex the Poly(A) Reaction Mix, then centrifuge briefly.
4. Add 2 µL of sample to each well of a reaction plate or each reaction tube.  
**Note:** (*Optional*) Before adding the sample to the reaction plate or tube, add RNase Inhibitor to each sample to minimize the effects of RNase contamination.
5. Add 3 µL of Poly(A) Reaction Mix to each well or tube.  
 The total volume should be 5 µL per well or tube.  
**Note:** Decrease RNase-free water as required to compensate for RNase Inhibitor.
6. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
7. Centrifuge the reaction plate or tubes briefly to collect the contents at the bottom and eliminate air bubbles.
8. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling.

| Step            | Temperature | Time       |
|-----------------|-------------|------------|
| Polyadenylation | 37°C        | 45 minutes |
| Stop reaction   | 65°C        | 10 minutes |
| Hold            | 4°C         | Hold       |

Proceed immediately to “Perform the adaptor ligation reaction” on page 3.

## Perform the adaptor ligation reaction

1. In a 1.5 mL microcentrifuge tube, prepare sufficient Ligation Reaction Mix for the required number of reactions according to the following table.

| Component                                 | Number of reactions |                  |                  |
|-------------------------------------------|---------------------|------------------|------------------|
|                                           | 1                   | 4 <sup>[1]</sup> | 8 <sup>[1]</sup> |
| 5X DNA Ligase Buffer                      | 3 µL                | 13.8 µL          | 27.6 µL          |
| 50% PEG 8000 <sup>[2]</sup>               | 4.5 µL              | 20.7 µL          | 41.04 µL         |
| 25X Ligation Adaptor                      | 0.6 µL              | 2.8 µL           | 5.5 µL           |
| RNA Ligase                                | 1.5 µL              | 6.9 µL           | 13.8 µL          |
| RNase-free water                          | 0.4 µL              | 1.8 µL           | 3.7 µL           |
| <b>Total Ligation Reaction Mix volume</b> | <b>10 µL</b>        | <b>46 µL</b>     | <b>92 µL</b>     |

<sup>[1]</sup> Volumes include 15% overage.

<sup>[2]</sup> 50% PEG 8000 is very viscous. Follow the instructions in the Important section for accurate pipetting.

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**IMPORTANT!** For accurate pipetting of 50% PEG 8000:

- Use 50% PEG 8000 at room temperature.
  - Aspirate and dispense the solution slowly.
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2. Vortex the Ligation Reaction Mix, then centrifuge briefly.
3. Transfer 10 µL of the Ligation Reaction Mix to each well of the reaction plate or each reaction tube containing the poly(A) tailing reaction product.  
The total volume should be 15 µL per well or tube.
4. Seal the reaction plate or tubes, then vortex briefly or shake (1,900 rpm for 1 minute with an Eppendorf™ MixMate™ to thoroughly mix the contents.

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**IMPORTANT!** Correct mixing is necessary for efficient ligation. For correct mixing when vortexing, you need to observe a swirling motion of the adaptor ligation reaction.

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5. Centrifuge the reaction plate or tubes briefly to collect the contents at the bottom.
6. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

| Step     | Temperature | Time       |
|----------|-------------|------------|
| Ligation | 16°C        | 60 minutes |
| Hold     | 4°C         | Hold       |

Proceed immediately to “Perform the reverse transcription (RT) reaction” on page 4.

## Perform the reverse transcription (RT) reaction

1. In a 1.5 mL microcentrifuge tube, prepare sufficient RT Reaction Mix for the required number of reactions according to the following table.

| Component                           | Number of reactions |                  |                  |
|-------------------------------------|---------------------|------------------|------------------|
|                                     | 1                   | 4 <sup>[1]</sup> | 8 <sup>[1]</sup> |
| 5X RT Buffer                        | 6 µL                | 27.6 µL          | 55.2 µL          |
| dNTP Mix (25 mM each)               | 1.2 µL              | 5.5 µL           | 11.0 µL          |
| 20X Universal RT Primer             | 1.5 µL              | 6.9 µL           | 13.8 µL          |
| 10X RT Enzyme Mix                   | 3 µL                | 13.8 µL          | 27.6 µL          |
| RNase-free water                    | 3.3 µL              | 15.2 µL          | 30.4 µL          |
| <b>Total RT Reaction Mix volume</b> | <b>15 µL</b>        | <b>69 µL</b>     | <b>138 µL</b>    |

<sup>[1]</sup> Volumes include 15% overage.

2. Vortex the RT Reaction Mix, then centrifuge briefly.
3. Transfer 15 µL of the RT Reaction Mix to each well of the reaction plate or each reaction tube containing the adaptor ligation reaction product.  
The total volume should be 30 µL per well or tube.
4. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
5. Centrifuge the reaction plate or tubes briefly to collect the contents at the bottom.
6. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

| Step                  | Temperature | Time       |
|-----------------------|-------------|------------|
| Reverse transcription | 42°C        | 15 minutes |
| Stop reaction         | 85°C        | 5 minutes  |
| Hold                  | 4°C         | Hold       |

Proceed to “Perform the miR-Amp reaction” on page 4, or store the RT reaction product at –20°C for up to 2 months.

## Perform the miR-Amp reaction

1. In a 1.5 mL microcentrifuge tube, prepare sufficient miR-Amp Reaction Mix for the required number of reactions according to the following table.

| Component                                | Number of reactions |                  |                  |
|------------------------------------------|---------------------|------------------|------------------|
|                                          | 1                   | 4 <sup>[1]</sup> | 8 <sup>[1]</sup> |
| 2X miR-Amp Master Mix                    | 25 µL               | 115 µL           | 230 µL           |
| 20X miR-Amp Primer Mix                   | 2.5 µL              | 11.5 µL          | 23 µL            |
| RNase-free water                         | 17.5 µL             | 80.5 µL          | 161 µL           |
| <b>Total miR-Amp Reaction Mix volume</b> | <b>45 µL</b>        | <b>207 µL</b>    | <b>414 µL</b>    |

<sup>[1]</sup> Volumes include 15% overage.

2. Vortex the miR-Amp Reaction Mix, then centrifuge briefly.
3. Transfer 45 µL of the miR-Amp Reaction Mix to each well of a reaction plate or reaction tubes.

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**IMPORTANT!** Use a *new* reaction plate or *new* reaction tubes.

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4. Add 5 µL of the RT reaction product to each well or tube.  
The total volume should be 50 µL per well or tube.

- Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
- Centrifuge the reaction plate or tubes briefly to collect the contents at the bottom.
- Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings, maximum ramp speed, and standard cycling:

| Step              | Temperature | Time       | Cycles |
|-------------------|-------------|------------|--------|
| Enzyme activation | 95°C        | 5 minutes  | 1      |
| Denature          | 95°C        | 3 seconds  | 14     |
| Anneal/Extend     | 60°C        | 30 seconds |        |
| Stop reaction     | 99°C        | 10 minutes | 1      |
| Hold              | 4°C         | Hold       | Hold   |

**Note:** If the  $C_t$  value is high, the number of cycles can be increased, up to 18 cycles.

Proceed to “Perform real-time PCR” on page 5. Alternatively, store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

## Perform real-time PCR

### Procedural guidelines for performing real-time PCR

- Keep the assays protected from light and stored as indicated until ready for use.
- We recommend four replicates of each reaction.
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 15% overage.
- For reaction volumes that are different from those detailed, scale all components proportionally. Reaction volumes <10  $\mu$ L are not recommended.

### Prepare the PCR reaction plate

- Thaw the assays on ice, gently vortex, then centrifuge briefly.
- Prepare 1:10 dilution of cDNA template.
- Gently shake the bottle of master mix. Do not invert the bottle.
- In a 1.5-mL microcentrifuge tube, prepare sufficient PCR Reaction Mix for the required number of reactions according to the following table.

| Component                             | Number of reactions         |                             |                              |
|---------------------------------------|-----------------------------|-----------------------------|------------------------------|
|                                       | 1                           | 4 <sup>[1]</sup>            | 8 <sup>[1]</sup>             |
| TaqMan™ Fast Advanced Master Mix (2X) | 10 $\mu$ L                  | 46.0 $\mu$ L                | 92.0 $\mu$ L                 |
| TaqMan™ Advanced miRNA Assay (20X)    | 1 $\mu$ L                   | 4.6 $\mu$ L                 | 9.2 $\mu$ L                  |
| RNase-free water                      | 4 $\mu$ L                   | 18.4 $\mu$ L                | 36.8 $\mu$ L                 |
| <b>Total PCR Reaction Mix volume</b>  | <b>15 <math>\mu</math>L</b> | <b>69 <math>\mu</math>L</b> | <b>138 <math>\mu</math>L</b> |

<sup>[1]</sup> Volumes include 15% overage.

- Vortex the PCR Reaction Mix, then centrifuge briefly.
- Transfer 15  $\mu$ L of the PCR Reaction Mix to each well of a PCR reaction plate.
- Add 5  $\mu$ L of the diluted cDNA template to each reaction well of the plate.  
The total volume should be 20  $\mu$ L per reaction well.

8. Seal the reaction plate with an adhesive cover, then vortex briefly to thoroughly mix the contents.
9. Centrifuge the reaction plate briefly to collect the contents at the bottoms of the wells.

## 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.

1. Set the appropriate experiment settings and PCR thermal cycling conditions for your instrument. Select the fast cycling mode if it is an option on your instrument.

**IMPORTANT!** Fast cycling mode is selected for TaqMan™ Fast Advanced Master Mix. The cycling mode does not depend on a Standard or a Fast plate format.

**Table 1 StepOnePlus™ Real-Time PCR Systems, ViiA™ 7 Real-Time PCR Systems, and QuantStudio™ Real-Time PCR Systems**

| Step              | Temperature | Time       | Cycles |
|-------------------|-------------|------------|--------|
| Enzyme activation | 95°C        | 20 seconds | 1      |
| Denature          | 95°C        | 1 second   | 40     |
| Anneal / Extend   | 60°C        | 20 seconds |        |

**Table 2 7500/7500 Fast Real-Time PCR Systems**

| Step              | Temperature | Time       | Cycles |
|-------------------|-------------|------------|--------|
| Enzyme activation | 95°C        | 20 seconds | 1      |
| Denature          | 95°C        | 3 seconds  | 40     |
| Anneal / Extend   | 60°C        | 30 seconds |        |

2. Set the reaction volume appropriate for the reaction plate.
3. Load the reaction plate in the real-time PCR instrument.
4. Start the run.

## Analyze the results

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

The general guidelines for analysis include:

- View the amplification plot; then, if needed:
  - Adjust the baseline and threshold values.
 

**Note:** A threshold value of 0.1 is recommended.
  - 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, go to: [thermofisher.com/qpcducation](http://thermofisher.com/qpcducation).

## Algorithms for data analysis

**Table 3 Algorithm recommendations for single-tube assays**

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

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



**Revision history:** Pub. No. 100027898 F

| Revision | Date             | Description                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|----------|------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| F        | 25 April 2025    | The overage was updated to 15% when preparing reactions.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| E        | 21 August 2023   | <ul style="list-style-type: none"><li>The guidelines for RNA input were updated to indicate an amount of RNA from blood, serum, and plasma samples if the RNA can be quantified ("Guidelines for RNA input" on page 1).</li><li>The volumes provided for multiple reactions were updated to 8 reactions from 10 reactions. Eight is a more common factor for setting up reactions.</li><li>The instructions for the miR-Amp reaction was updated to indicate that the number of cycles can be increased to up to 18 if the C<sub>t</sub> value is low ("Perform the miR-Amp reaction" on page 4).</li><li>A note was added to indicate that fast cycling mode is selected for TaqMan™ Fast Advanced Master Mix. The cycling mode does not depend on a Standard or a Fast plate format ("Set up and run the real-time PCR instrument" on page 6).</li><li>Information was added about algorithms for data analysis ("Algorithms for data analysis" on page 6).</li></ul> |
| D        | 2 September 2016 | Update general formatting to streamline content.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
| C        | 13 July 2015     | Correct typos in poly(A) tailing reaction preparation table and in miR-Amp reaction thermal cycling conditions table.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| B        | 19 June 2015     | Correct typo in PCR reaction preparation table.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| A        | 7 April 2015     | New document.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |

The information in this guide is subject to change without notice.

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