

TaqMan[®] Advanced miRNA Assays

USER GUIDE

Single-tube assays

for use with:

TaqMan[®] Advanced miRNA cDNA Synthesis Kit

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Revision C

Manufacturer's address: Life Technologies Corporation | 6055 Sunol Blvd | Pleasanton, CA 94566

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| Revision | Date | Description |
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| C | 09 September 2016 | <ul style="list-style-type: none">• Update Assay ID for hsa-miR-320a to 478594_mir• Update graphics for cDNA and qPCR workflows• Remove Assay ID 478289_mir from exogenous control list• Update general formatting to streamline content |
| B | June 2015 | <ul style="list-style-type: none">• Correct typo• Update software name• Add information about compression pad |
| A | April 2015 | New document. |

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Product information

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Product description

TaqMan[®] Advanced miRNA Assays are pre-formulated primer and probe sets that are designed for analysis of microRNA (miRNA) expression levels using Applied Biosystems™ real-time PCR instruments. The assays can detect and quantify the mature form of the miRNA from 1–10 ng of total RNA from tissue, or 2 µL of total RNA from serum or plasma. For more information about PCR detection with TaqMan[®] Advanced miRNA Assays, see page “TaqMan[®] Advanced miRNA Assays chemistry overview” on page 22.

The TaqMan[®] Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007; sold separately) is required for preparing the cDNA template that is used with the TaqMan[®] Advanced miRNA Assays. The kit enables the analysis of:

- Multiple miRNAs from a single amplified sample.
- Samples that are limited in quantity, including serum, plasma, or other biological fluids.

This document describes procedures to prepare cDNA templates from miRNA followed by PCR amplification of the cDNA template and subsequent data analysis. In the first stage of the workflow, mature miRNAs from total RNA are modified by 1) extending the 3' end of the mature transcript through poly(A) addition, then 2) lengthening the 5' end by adaptor ligation. The modified miRNAs then undergo universal reverse transcription followed by amplification to increase uniformly the amount of cDNA for all miRNAs (miR-Amp reaction). For more information about cDNA synthesis of templates for TaqMan[®] Advanced miRNA Assays, see page “cDNA template preparation” on page 21.

TaqMan[®] Advanced miRNA Assays of interest are then used for quantification of miRNA expression levels by qPCR analysis. Predesigned TaqMan[®] Advanced miRNA Assays are available for most human miRNAs in miRBase (the miRNA sequence repository). For a current list of assays, go to thermofisher.com/advancedmirna.

Note: TaqMan[®] Advanced miRNA Assays are for analysis of mature miRNA only. For analysis of siRNA, or other small RNAs that are fewer than 200 bases in length go to thermofisher.com/taqmanmirna.

The procedures in this document are for use with TaqMan[®] Advanced miRNA Assays supplied in the single-tube format.

Contents and storage

Pre-designed and inventoried TaqMan[®] Advanced miRNA Assays are manufactured and stocked in advance.

Table 1 TaqMan[®] Advanced miRNA Assays (single-tube format)

| Item | Cat. No. | Amount | Storage |
|--|----------|------------------------|---------|
| TaqMan [®] Advanced miRNA Assay | A25576 | 1 tube 250 µL (20X) | -20°C |

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**.
MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 2 TaqMan[®] Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007): 50 rxns

| Component | Part No. | Storage |
|-------------------------|-----------|------------|
| 10X Poly(A) Buffer | 100032529 | -20°C |
| ATP, 10 mM | 100032528 | |
| Poly(A) Enzyme, 5 U/µL | 100029178 | |
| 5X DNA Ligase Buffer | 100029179 | |
| RNA Ligase, 10 U/µL | 100029180 | |
| 50% PEG 8000 | 100029181 | |
| 25X Ligation Adaptor | 100029182 | |
| 10X RT Enzyme Mix | 100029183 | |
| 5X RT Buffer | 100029184 | |
| 20X Universal RT Primer | 100029185 | |
| dNTP Mix, 100 mM | 100029186 | |
| 20X miR-Amp Primer Mix | 100029187 | |
| 2X miR-Amp Master Mix | 100029188 | 2°C to 4°C |

Table 3 Other materials and equipment required for the workflow

| Item | Source |
|--|--|
| Real-time PCR instrument, one of the following: | |
| QuantStudio™ 3 or 5 Real-Time PCR System | Contact your local sales office |
| QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System | |
| QuantStudio™ 12K Flex Real-Time PCR System | |
| StepOne™ or StepOnePlus™ Real-Time PCR System | |
| ViiA™ 7 Real-Time PCR System | |
| 7500/7500 Fast Real-Time PCR System | |
| Equipment | |
| Thermal cycler, one of the following (or equivalent): <ul style="list-style-type: none"> GeneAmp™ PCR System 9700 Veriti™ Thermal Cycler | Contact your local sales office |
| Centrifuge, with adapter for 96-well or 384-well plates | MLS |
| Microcentrifuge | MLS |
| Vortex | MLS |
| <i>(Optional)</i> Eppendorf™ MixMate™ (shaker) | Fisher-Scientific 21-379-00 |
| Pipettes | MLS |
| Tubes, plates, and other consumables | |
| Plastics consumables | thermofisher.com/plastics |
| Aerosol-resistant barrier pipette tips | MLS |
| Disposable gloves | MLS |
| Reagents | |
| RNase-free water | MLS |
| Tris-EDTA (TE) Buffer, pH 8.0 | MLS |
| TaqMan® Fast Advanced Master Mix | 4444557 |

Table 4 Recommended RNA isolation kits

| Sample type | Kit | Cat. No. |
|------------------------|---|-----------------|
| Tissue samples | <i>mirVana</i> [™] miRNA Isolation Kit, with phenol | AM1560 |
| | <i>mirVana</i> [™] miRNA Isolation Kit, without phenol | AM1561 |
| | MagMAX [™] <i>mirVana</i> [™] Total RNA Isolation Kit | A27828 |
| Serum / Plasma samples | Total Exosome RNA and Protein Isolation Kit | 4478545 |
| | TaqMan [®] miRNA ABC Purification Kit – Human Panel A | 4473087 |
| | TaqMan [®] miRNA ABC Purification Kit – Human Panel B | 4473088 |
| | MagMAX [™] <i>mirVana</i> [™] Total RNA Isolation Kit | A27828 |
| Cell samples | TaqMan [®] MicroRNA Cells-to-C _T Kit | 4391848 |
| | Single Cell-to-C _T qRT-PCR Kit | 4458236 |
| | Cells-to-C _T [™] 1-Step TaqMan [®] Kit | A25603 |
| FFPE samples | RecoverAll [™] Total Nucleic Acid Isolation Kit | AM1975 |

Options for endogenous and exogenous controls

For information about using endogenous or exogenous controls with TaqMan[®] Advanced miRNA Assays, see “Endogenous and exogenous controls” on page 20.

Table 5 Assay options for endogenous controls

| Assay name ^[1] | Assay ID | Target Sequence |
|---------------------------|------------|--------------------------------|
| hsa-miR-361-5p | 478056_mir | 5' -UUAUCAGAAUCUCCAGGGGUAC-3' |
| hsa-miR-186-5p | 477940_mir | 5' -CAAAGAAUUCUCCUUUUGGGCU-3' |
| hsa-miR-26a-5p | 477995_mir | 5' -UUCAAGUAAUCCAGGAUAGGCU-3' |
| hsa-miR-191-5p | 477952_mir | 5' -CAACGGAAUCCAAAAGCAGCUG-3' |
| hsa-miR-451a | 478107_mir | 5' -AAACCGUUACCAUUACUGAGUU-3' |
| hsa-miR-423-5p | 478090_mir | 5' -UGAGGGGCAGAGAGCGAGACUUU-3' |
| hsa-miR-320a | 478594_mir | 5' -AAAAGCUGGGUUGAGAGGGCGA-3' |

^[1] TaqMan[®] Advanced miRNA Assays do not detect snRNAs or snoRNAs. Do not use snRNAs and snoRNAs as endogenous controls for these assays.

Table 6 Assay options for exogenous controls (for human samples)

| Assay Name | Assay ID | Target Sequence ^[1] |
|----------------|------------|---------------------------------|
| ath-miR159a | 478411_mir | 5' -UUUGGAUUGAAGGGAGCUCUA-3' |
| cel-miR-2-3p | 478291_mir | 5' -UAUCACAGCCAGCUUUGAUGUGC-3' |
| cel-miR-238-3p | 478292_mir | 5' -UUUGUACUCCGAUGCCAUAUCAGA-3' |
| cel-miR-39-3p | 478293_mir | 5' -UCACCGGGUGUAAAUCAGCUUG-3' |
| cel-miR-54-3p | 478410_mir | 5' -UACCCGUAUUCUUAUAAUCCGAG-3' |
| cel-miR-55-3p | 478295_mir | 5' -UACCCGUAUAGUUUCUGCUGAG-3' |

^[1] Oligonucleotides for exogenous controls must be 5'-phosphorylated.

Workflow

Prepare cDNA templates

Input RNA sample



“Perform the poly(A) tailing reaction” on page 12
(55 minutes)



“Perform the adaptor ligation reaction” on page 13
(60 minutes)



“Perform the reverse transcription (RT) reaction” on page 14
(20 minutes)



“Perform the miR-Amp reaction” on page 15
(30 minutes)



Perform real-time PCR

“Prepare PCR reaction plate” on page 17



“Set up and run the real-time PCR instrument” on page 18
(45 minutes)



“Analyze the results” on page 19

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Prepare cDNA templates

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Procedural guidelines

Guidelines for preparing cDNA templates

- Follow best practices when working with RNA samples (see “Best practices for PCR and RT-PCR experiments” on page 24).
- 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 10% overage.
- If using strip tubes, change to a new cap after each step or incubation.
- If using 0.2- or 0.5-mL PCR tubes, use a thermal cycler tray.
- If using plates, use a thermal cycler compression pad.

Guidelines for RNA input

- Prepare samples using a total RNA isolation method that preserves small RNAs. See Table 4 on page 8 for recommended RNA isolation kits.
- For tissue samples: Use 1–10 ng of total RNA per reaction.
Note: Sample concentration before adding to reactions should be ≤ 5 ng/ μ L.
- For blood, serum, or plasma samples: Use 2 μ L of sample eluent (from the sample isolation procedure) 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 (not applicable for double-stranded templates)

IMPORTANT! Do not denature the total RNA.

Perform the poly(A) tailing reaction

1. Thaw samples and cDNA synthesis reagents on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.

Note: Keep the assays in storage until ready for use.

IMPORTANT! The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (next section).

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 | 1 Rxn | 4 Rxns ^[1] | 10 Rxns ^[1] |
|--|---------------|-----------------------|------------------------|
| 10X Poly(A) Buffer | 0.5 µL | 2.2 µL | 5.5 µL |
| ATP | 0.5 µL | 2.2 µL | 5.5 µL |
| Poly(A) Enzyme | 0.3 µL | 1.3 µL | 3.3 µL |
| RNase-free water | 1.7 µL | 7.5 µL | 18.7 µL |
| Total Poly(A) Reaction Mix volume | 3.0 µL | 13.2 µL | 33 µL |

^[1] Volumes include 10% overage.

3. Vortex the Poly(A) Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
4. Add 2 µL of sample to each well of a reaction plate or each reaction tube, then transfer 3 µL of Poly(A) Reaction Mix to each well or tube.

Note: (*Optional*) Before adding the sample to the reaction plate or tube, add RNase Inhibitor Protein to each sample to minimize the effects of RNase contamination. For detailed instructions, see the documentation provided by the RNase Inhibitor Protein manufacturer.

The total volume should be 5 µL per well or tube.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
6. Centrifuge the reaction plate or tubes briefly to spin down the contents and eliminate air bubbles.
7. 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 |

8. Proceed immediately to the adaptor ligation reaction (next section).

Perform the adaptor ligation reaction

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

| Component | 1 Rxn | 4 Rxns ^[1] | 10 Rxns ^[1] |
|---|--------------|-----------------------|------------------------|
| 5X DNA Ligase Buffer | 3 µL | 13.2 µL | 33 µL |
| 50% PEG 8000 ^[2] | 4.5 µL | 19.8 µL | 49.5 µL |
| 25X Ligation Adaptor | 0.6 µL | 2.6 µL | 6.6 µL |
| RNA Ligase | 1.5 µL | 6.6 µL | 16.5 µL |
| RNase-free water | 0.4 µL | 1.8 µL | 4.4 µL |
| Total Ligation Reaction Mix volume | 10 µL | 44 µL | 110 µL |

^[1] Volumes include 10% overage.

^[2] 50% PEG 8000 is very viscous, follow the Important statement below to ensure accurate pipetting.

IMPORTANT! For accurate pipetting of 50% PEG 8000:

- Use 50% PEG 8000 at room temperature.
- Aspirate and dispense solution slowly.
 - Hold the pipette tip in the solution for ~10 seconds after releasing the plunger during aspiration. This action allows the solution to be fully drawn into the pipette tip.
 - Keep the plunger depressed for ~10 seconds to allow the solution to be fully dispensed into the Ligation Reaction Mix.

- Vortex the Ligation Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
- 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.
- 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.

IMPORTANT! Watch for a swirling motion of the adaptor ligation reaction to ensure proper mixing, which is necessary for efficient ligation.

- Centrifuge the reaction plate or tubes briefly to spin down the contents.
- 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 the reverse transcription (RT) reaction (next section).

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 | 1 Rxn | 4 Rxns ^[1] | 10 Rxns ^[1] |
|-------------------------------------|--------------|-----------------------|------------------------|
| 5X RT Buffer | 6 µL | 26.4 µL | 66 µL |
| dNTP Mix (25 mM each) | 1.2 µL | 5.3 µL | 13.2 µL |
| 20X Universal RT Primer | 1.5 µL | 6.6 µL | 16.5 µL |
| 10X RT Enzyme Mix | 3 µL | 13.2 µL | 33 µL |
| RNase-free water | 3.3 µL | 14.5 µL | 36.3 µL |
| Total RT Reaction Mix volume | 15 µL | 66 µL | 165 µL |

^[1] Volumes include 10% overage.

2. Vortex the RT Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
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 spin down the contents.
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 |

7. Proceed to the miR-Amp reaction (next section).

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 | 1 Rxn | 4 Rxns ^[1] | 10 Rxns ^[1] |
|--|--------------|-----------------------|------------------------|
| 2X miR-Amp Master Mix | 25 µL | 110 µL | 275 µL |
| 20X miR-Amp Primer Mix | 2.5 µL | 11 µL | 27.5 µL |
| RNase-free water | 17.5 µL | 77 µL | 192.5 µL |
| Total miR-Amp Reaction Mix volume | 45 µL | 198 µL | 495 µL |

^[1] Volumes include 10% overage.

2. Vortex the miR-Amp Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
3. Transfer 45 µL of the miR-Amp Reaction Mix to each well of a *new* reaction plate or reaction tube.
4. Add 5 µL of the RT reaction product to each reaction well or each reaction tube. The total volume should be 50 µL per well or tube.
5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
6. Centrifuge the reaction plate or tubes briefly to spin down the contents.
7. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings, MAX 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 | 1 |

8. Proceed to performing the real-time PCR (next section).

Store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

3

Perform real-time PCR

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Procedural guidelines for performing real-time PCR

- Follow best practices when performing PCR reactions (see “Best practices for PCR and RT-PCR experiments” on page 24).
- Prepare the real-time PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Keep the assays protected from light and stored at -20°C until ready for use. Excessive exposure to light may affect the fluorescent probe.
- Configure plate documents according to the instructions provided in the real-time PCR instrument resource documents.
- Prepare the real-time PCR Reaction Mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.
- We recommend four replicates of each reaction.
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 10% overage.
- Reaction volumes listed in this section are for 20- μL PCR reactions.
- For reaction volumes that are different from those detailed, scale all components proportionally.
Reaction volumes $< 10 \mu\text{L}$ are not recommended.

Prepare PCR reaction plate

1. Thaw the assays on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.
2. Prepare 1:10 dilution of cDNA template.
For example, add 5 μL of the miR-Amp reaction product to 45 μL 0.1X TE buffer.
3. Gently shake the bottle of TaqMan[®] Fast Advanced Master Mix to thoroughly mix the contents. Do not invert the bottle.
4. In a 1.5-mL microcentrifuge tube, prepare sufficient PCR Reaction Mix for the required number of reactions according to the following table.

| Component | 1 Rxn | 4 Rxns ^[1] |
|---|------------------------------------|------------------------------------|
| TaqMan [®] Fast Advanced Master Mix (2X) | 10 μL | 44.0 μL |
| TaqMan [®] Advanced miRNA Assay (20X) | 1 μL | 4.4 μL |
| RNase-free water | 4 μL | 17.6 μL |
| Total PCR Reaction Mix volume | 15 μL | 66 μL |

^[1] Volumes include 10% overage.

5. Vortex the PCR Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
6. Transfer 15 μL of the PCR Reaction Mix to each well of a PCR reaction plate.
7. Add 5 μL of the diluted cDNA template to each reaction well of the plate.
The total volume should be 20 μL 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 spin down the contents.

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.

The following thermal profiles are optimized for use with TaqMan® Fast Advanced Master Mix and can be used with Fast or Standard reaction plates and the corresponding instrument block configurations.

1. Load the reaction plate in the real-time PCR instrument.
2. Set the appropriate experiment settings and PCR thermal cycling conditions for your instrument. Select the fast cycling mode for all instruments.

Table 7 StepOnePlus™, ViiA™ 7, and QuantStudio™ 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 8 7500 and 7500 Fast systems

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

3. Set the reaction volume appropriate for the reaction plate.
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.

Analyze data generated with TaqMan® Advanced miRNA Assays using any of the following tools:

| Software | Resource |
|--|--|
| Applied Biosystems™ real-time PCR Analysis Modules | thermofisher.com/us/en/home/cloud.html |
| ExpressionSuite™ Software | thermofisher.com/us/en/home/technical-resources/software-downloads/expressionsuite-software.html |

For more information about real-time PCR, go to: thermofisher.com/qpcr/education.



Supplemental information

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Endogenous and exogenous controls

Endogenous controls

An endogenous control shows gene expression that is relatively constant and moderately abundant across tissues and cell types and treatment protocols. Normalization to endogenous control genes is currently the most accurate method to correct for potential biases that are caused by:

- Sample collection
- Variation in the amount of starting material
- Reverse transcription (RT) efficiency
- Nucleic acid (RNA/DNA) preparation and quality

No single control can act as a universal endogenous control for all experimental conditions, so we recommend verifying the chosen endogenous control or set of controls for the sample tissue, cell, or treatment.

See Table 5 on page 9 for available TaqMan® Advanced miRNA Assays that target miRNAs with relatively constant expression levels across many different sample types.

Exogenous controls

An exogenous control is a synthetic RNA oligonucleotide with an miRNA target sequence that is not present in the sample of interest. For example, the target sequence for the miRNA assay ath-miR-159a is not present in humans, so it is a good exogenous control for human samples.

The RNA oligonucleotide is combined with the biological sample during the RNA isolation procedure as a spike-in control to monitor:

- Sample input amount for difficult samples (for example, serum/plasma or other biofluids).
- Extraction efficiency.

When using exogenous controls with TaqMan® Advanced miRNA Assays:

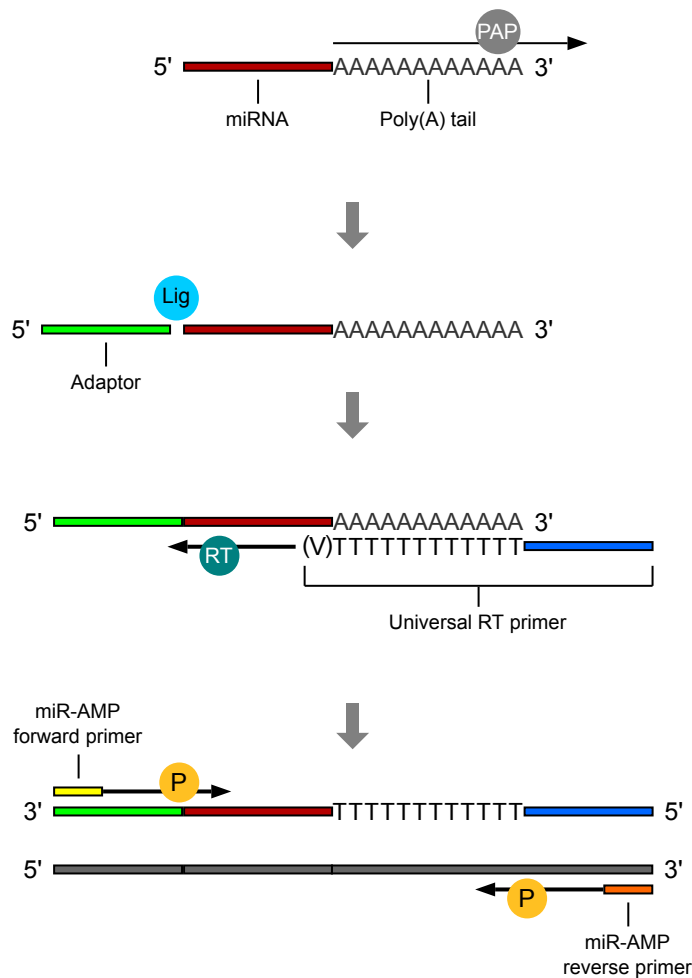
- The assay chemistry requires that exogenous controls be 5'-phosphorylated.
- The final concentration of the spike-in control in the sample should be 1–10 pM.

See Table 6 on page 9 for available TaqMan® Advanced miRNA Assays which target sequences that can be used as exogenous controls with human samples.

cDNA template preparation

Quantification using TaqMan® Advanced miRNA Assays requires the modification of mature miRNAs by the addition of a poly(A) tail (3') and an adaptor (5') to:

- Amplify all miRNAs in a single reverse transcription (RT) reaction.
- Amplify the sample for downstream PCR in a single universal cDNA reaction.



Poly(A) tailing reaction

Starting with a total RNA sample, poly(A) polymerase is used to add a 3'-adenosine tail to the miRNA.

Adaptor ligation reaction

The miRNA with poly(A) tail undergoes adaptor ligation at the 5' end. The adaptor acts as the forward-primer binding site for the miR-Amp reaction.

Reverse transcription (RT) reaction

A Universal RT primer binds to the 3' poly(A) tail and the miRNA is reverse transcribed. The resulting cDNA is suitable for all TaqMan® Advanced miRNA Assays.

miR-Amp reaction

Universal forward and reverse primers increase the number of cDNA molecules.

TaqMan® Advanced miRNA Assays chemistry overview

TaqMan® MGB probes

TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™) at the 5' end of the probe (Afonina et al., 1997; Kutuyavin et al., 1997)
- A non-fluorescent quencher (NFQ) dye at the 3' end of the probe. The NFQ dye does not fluoresce, which allows the real-time PCR system to measure the reporter dye contributions more accurately.
- A minor groove binder (MGB) at the 3' end of the probe that:
 - Increases the melting temperature (T_m) without increasing the probe length.
 - Allows for the design of shorter probes.

About the 5' nuclease assay

Note: The following figures are general representations of real-time PCR with TaqMan® MGB probes and TaqMan® Advanced miRNA Assays. The sequence regions are not necessarily drawn to scale.

The 5' nuclease assay process takes place during PCR amplification. It occurs in every cycle and does not interfere with the exponential accumulation of product.

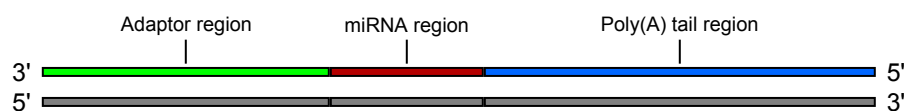


Figure 1 cDNA synthesis product

During the PCR, the forward and reverse primers anneal to complementary sequences along the denatured cDNA template strands (Figure 2). The primer binding sites vary depending on the target miRNA sequence and are designed to maximize specificity. Figure 2 shows an example representation in which the reverse primer is the primer that partially overlaps the miRNA region.

The TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 2). When the probe is intact, the proximity of the reporter dye and quencher dye suppresses the reporter fluorescence, primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

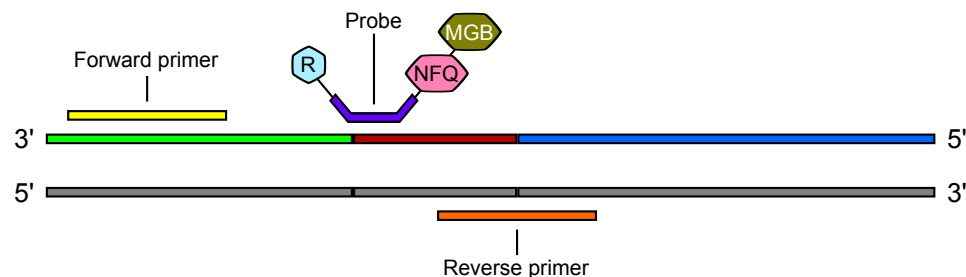


Figure 2 Annealing of probes and primers to cDNA strands

During polymerization, the DNA polymerase only cleaves probes that hybridize to the target sequence. Cleavage separates the reporter dye from the probe. The separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter dye (Figure 3).

This increase in fluorescence occurs only if the probe is complementary to the target sequence and if the target sequence is amplified during PCR. Because of these conditions, nonspecific amplification is not detected.

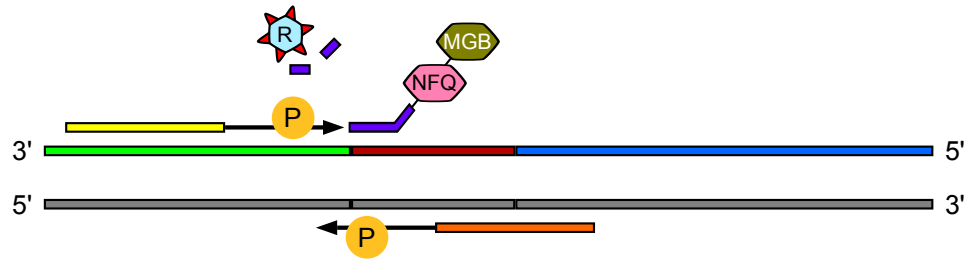


Figure 3 Initial polymerization and cleavage of reporter dye

Polymerization of the strand continues (Figure 4), but because the 3' end of the probe is blocked, no extension of the probe occurs during PCR.

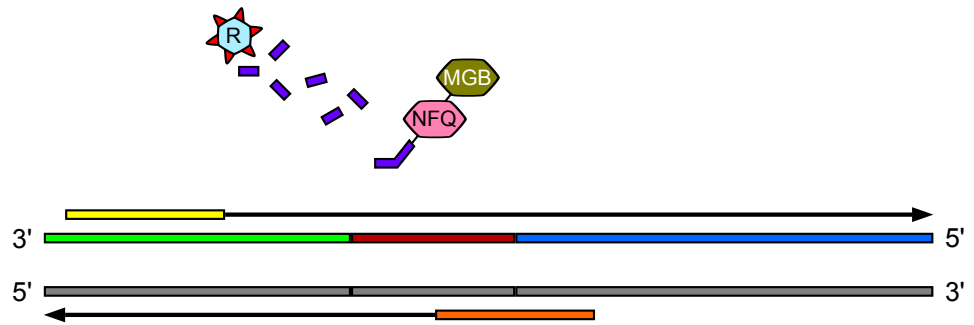






Figure 4 Completion of polymerization

| LEGEND: | | | | | |
|---|--------------------------|---|---------------------|---|------------------------------|
|  | Hot-start DNA polymerase |  | Reporter dye |  | Non-fluorescent quencher dye |
| | |  | Minor groove binder | | |

Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat (not previously worn while handling amplified products or during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
 - Sample preparation and reaction setup.
 - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNAZap™ Solutions (Cat. No. AM9890).

Use UNG to prevent false-positive amplification

Carryover amplicons can result in false-positive amplification during PCR. Use a master mix that contains heat-labile uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)) to degrade many contaminating carryover amplicons.

UNG enzymatic activity occurs during the PCR reaction setup at room temperature; an activation step before thermal cycling is not necessary. Unlike standard UNG, heat-labile UNG is completely inactivated during the first ramp to the high-temperature step for template denaturation and polymerase activation.

To ensure the desired UNG activity:

- Use PCR components and thermal cycling conditions as specified.
UNG-containing master mixes incorporate the optimal concentration of UNG to prevent cross-contamination while not affecting real-time PCR performance.
- Do not attempt to use UNG-containing master mixes in subsequent amplification of dU-containing PCR products, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR products, preventing further amplification.

Although treatment with UNG can degrade or eliminate large numbers of carryover PCR products, use good laboratory practices to minimize cross-contamination from non-dU-containing PCR products or other samples.

Detect fluorescent contaminants

Fluorescent contaminants can generate false positive results. To help detect these contaminants, we recommend including a No-Amplification Control reaction that contains sample, but no master mix.

After PCR, if the absolute fluorescence of the No-Amplification Control is greater than the fluorescence of the no template control (NTC), fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.



Safety



WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
 - Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the “Documentation and Support” section in this document.
-

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the “Documentation and Support” section in this document.
 - Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
 - Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
 - Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
 - Handle chemical wastes in a fume hood.
 - Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
 - After emptying a waste container, seal it with the cap provided.
 - Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
 - Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
 - **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.
-

Biological hazard safety



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
www.cdc.gov/biosafety/publications/bmb15/BMBL.pdf
 - World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf
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Documentation and support

Related documentation

| Document | Pub. No. |
|---|------------|
| <i>TaqMan[®] Advanced miRNA Assays Quick Reference (Single-tube Assays)</i> | 100027898 |
| QuantStudio[™] 3 or 5 Real-Time PCR System | |
| <i>QuantStudio[™] 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide</i> | MAN0010407 |
| <i>QuantStudio[™] Design and Analysis desktop Software User Guide</i> | MAN0010408 |
| QuantStudio[™] 6 / QuantStudio[™] 7 Flex Real-Time PCR System | |
| <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide</i> | 4489821 |
| <i>QuantStudio[™] 6 and 7 Flex Real-Time PCR System Software Getting Started Guide</i> | 4489822 |
| QuantStudio[™] 12K Flex Real-Time PCR System | |
| <i>QuantStudio[™] 12K Flex Real-Time PCR System Maintenance and Administration Guide</i> | 4470689 |
| <i>QuantStudio[™] 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide</i> | 4470050 |
| StepOne[™] or StepOnePlus[™] Real-Time PCR System | |
| <i>StepOne[™] and StepOnePlus[™] Real-Time PCR Systems Installation, Networking and Maintenance User Guide</i> | 4376782 |
| <i>Applied Biosystems[™] StepOne[™] and StepOnePlus[™] Real-Time PCR Systems Relative Standard Curve and Comparative C_t Experiments Getting Started Guide</i> | 4376785 |
| ViiA[™] 7 Real-Time PCR System | |
| <i>Applied Biosystems[™] ViiA[™] 7 Real-Time PCR System User Guide: Calibration, Maintenance, Networking, and Security</i> | 4442661 |
| <i>Applied Biosystems[™] ViiA[™] 7 Real-Time PCR System Getting Started Guide</i> | 4441434 |
| 7500/7500 Fast Real-Time PCR System | |
| <i>Applied Biosystems[™] 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide</i> | 4347828 |
| <i>Applied Biosystems[™] 7500/7500 Fast Real-Time PCR System Getting Started Guide: Relative Standard Curve and Comparative C_t Experiments</i> | 4387783 |

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 - User guides, manuals, and protocols
 - Certificates of Analysis
 - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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- Sninsky and Gelfand, personal communication

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