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TaqMan™ Advanced miRNA Assays

USER GUIDE

Single-tube assays

for use with:
TaqMan™ Advanced miRNA cDNA Synthesis Kit

Catalog Number A25576
Publication Number 100027897
Revision D

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. 100027897

Revision	Date	Description
D	21 August 2023	• The Limited Use Label License was updated. • The guidelines for preparing cDNA templates were updated to refer to the applicable instrument user guide for information about accessories, including trays and compression pads ("Guidelines for preparing cDNA templates" on page 12). • 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 12). • The volumes provided for multiple reactions were updated to 8 reactions from 10 reactions. Eight is a more common factor for setting up reactions. • 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_t value is low ("Perform the miR-Amp reaction" on page 16). • Information was added about algorithms for data analysis ("Algorithms for data analysis" on page 21).
C	2 September 2016	• 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	5 June 2015	• Correct typo • Update software name • Add information about compression pad
A	9 April 2015	New document.

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

Contents

1 Product information

Product description

TaqMan™ Advanced miRNA Assays are preformulated 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 the following sample types:

- 1–10 ng of total RNA from tissue
- 2 µL of sample eluent from total RNA isolation from serum or plasma

For more information about PCR detection with TaqMan™ Advanced miRNA Assays, see page 24.

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

In the first stage of the workflow, mature miRNAs from total RNA are modified by extending the 3' end of the mature transcript through poly(A) addition, then 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 “Overview of cDNA template preparation” on page 23.

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**.

Contents and storage

Predesigned 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" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Table 2 Recommended RNA isolation kits

Sample type	Kit	Cat. No.
Tissue samples	*mir*Vana™ miRNA Isolation Kit, with phenol	AM1560
	*mir*Vana™ miRNA Isolation Kit, without phenol	AM1561
	MagMAX™ *mir*Vana™ 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™ *mir*Vana™ Total RNA Isolation Kit	A27828
Cell samples	TaqMan™ MicroRNA Cells-to-C_T Kit	4391848
	Single Cell-to-C_T qRT-PCR Kit	4458236

Table 2 Recommended RNA isolation kits *(continued)*

Sample type	Kit	Cat. No.
Cell samples	Cells-to-C_T™ 1-Step TaqMan™ Kit	A25603
FFPE samples	RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975

Table 3 TaqMan™ Advanced miRNA cDNA Synthesis Kit

Contents	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	2–4°C

Table 4 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	

Item	Source
Equipment	
Thermal cycler, one of the following (or equivalent): • 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
(Optional) 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

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 22.

Table 5 Endogenous control assays

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′-CAACGGAAUCCCAAAAGCAGCUG-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 Exogenous control assays (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′-UUUGUACUCCGAUGCCAUUCAGA-3′
cel-miR-39-3p	478293_mir	5′-UCACCGGGUGUAAAUCAGCUUG-3′
cel-miR-54-3p	478410_mir	5′-UACCCGUAAUCUUCAUAAUCCGAG-3′
cel-miR-55-3p	478295_mir	5′-UACCCGUAUAAGUUUCUGCUGAG-3′

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

Workflow

Prepare cDNA templates

Input the RNA sample

55 minutes — **Perform the poly(A) tailing reaction** (page 13)

60 minutes — **Perform the adaptor ligation reaction** (page 14)

20 minutes — **Perform the reverse transcription (RT) reaction** (page 15)

30 minutes — **Perform the miR-Amp reaction** (page 16)

Perform real-time PCR

Prepare the PCR reaction plate (page 19)

45 minutes

Set up and run the real-time PCR instrument (page 20)

Analyze the results (page 21)

2 Prepare cDNA templates

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 26).
- 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 strip cap after each step or incubation.
- See your instrument user guide for detailed instructions about using plates, tubes, or strip tubes.

Guidelines for RNA input

- Prepare samples using a total RNA isolation method that preserves small RNAs.
  See Table 2 on page 6 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/µL.
- 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 1–10 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 (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 collect the contents at the bottom of the tube and eliminate air bubbles.

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

   IMPORTANT! The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (see "Perform the adaptor ligation reaction" on page 14).

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[1]	8[1]
10X Poly(A) Buffer	0.5 µL	2.2 µL	4.4 µL
ATP	0.5 µL	2.2 µL	4.4 µL
Poly(A) Enzyme	0.3 µL	1.3 µL	2.6 µL
RNase–free water	1.7 µL	7.5 µL	15.0 µL
Total Poly(A) Reaction Mix volume	**3.0 µL**	**13.2 µL**	**26.4 µL**

[1] Volumes include 10% overage.

3. Vortex the Poly(A) Reaction Mix to thoroughly mix the contents, then centrifuge briefly to collect the contents at the bottom of the tube and eliminate air bubbles.

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. For detailed instructions, see the documentation for the RNase Inhibitor.

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 14.

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[1]	8[1]
5X DNA Ligase Buffer	3 µL	13.2 µL	26.4 µL
50% PEG 8000[2]	4.5 µL	19.8 µL	39.6 µL
25X Ligation Adaptor	0.6 µL	2.6 µL	5.3 µL
RNA Ligase	1.5 µL	6.6 µL	13.2 µL
RNase-free water	0.4 µL	1.8 µL	3.5 µL
Total Ligation Reaction Mix volume	**10 µL**	**44 µL**	**88 µL**

[1] Volumes include 10% overage.
[2] 50% PEG 8000 is very viscous. Follow instructions in 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 the solution slowly.
  a. Hold the pipette tip in the solution for approximately 10 seconds after slowly releasing the plunger during aspiration. This action allows the solution to be fully drawn into the pipette tip.
  b. Keep the plunger depressed for approximately 10 seconds to allow the solution to be fully dispensed into the Ligation Reaction Mix.

2. Vortex the Ligation Reaction Mix to thoroughly mix, then centrifuge briefly to collect the contents at the bottom of the tube and eliminate air bubbles.

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.

   IMPORTANT! If vortexing, watch for a swirling motion of the adaptor ligation reaction to ensure proper mixing. Proper mixing is necessary for efficient ligation.

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 15.

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[1]	8[1]
5X RT Buffer	6 µL	26.4 µL	52.8 µL
dNTP Mix (25 mM each)	1.2 µL	5.3 µL	10.6 µL
20X Universal RT Primer	1.5 µL	6.6 µL	13.2 µL
10X RT Enzyme Mix	3 µL	13.2 µL	26.4 µL
RNase-free water	3.3 µL	14.5 µL	29.0 µL
Total RT Reaction Mix volume	**15 µL**	**66 µL**	**132 µL**

[1] Volumes include 10% overage.

2. Vortex the RT Reaction Mix to thoroughly mix the contents, then centrifuge briefly to collect the contents at the bottom of the tube 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 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 16, 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[1]	8[1]
2X miR-Amp Master Mix	25 µL	110 µL	220 µL
20X miR-Amp Primer Mix	2.5 µL	11 µL	22 µL
RNase-free water	17.5 µL	77 µL	154 µL
Total miR-Amp Reaction Mix volume	**45 µL**	**198 µL**	**396 µL**

[1] Volumes include 10% overage.

2. Vortex the miR-Amp Reaction Mix to thoroughly mix the contents, then centrifuge briefly to collect the contents at the bottom of the tube and eliminate air bubbles.

3. Transfer 45 µL of the miR-Amp Reaction Mix to each well of a reaction plate or reaction tubes.

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

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.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

6. Centrifuge the reaction plate or tubes briefly to collect the contents at the bottom.

7. 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 Chapter 3, “Perform real-time PCR”. Alternatively, store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

3 Perform real-time PCR

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 26).
- 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 as indicated 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 µL are not recommended.

Prepare the PCR reaction plate

1. Thaw the assays on ice, gently vortex to thoroughly mix, then centrifuge briefly to collect the contents at the bottom of the tube.

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	Number of reactions		
	1	4 [1]	8[1]
TaqMan™ Fast Advanced Master Mix (2X)	10 µL	44.0 µL	88.0 µL
TaqMan™ Advanced miRNA Assay (20X)	1 µL	4.4 µL	8.8 µL
RNase-free water	4 µL	17.6 µL	35.2 µL
Total PCR Reaction Mix volume	**15 µL**	**66 µL**	**132 µL**

[1] Volumes include 10% overage.

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

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 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.

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. 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 7 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 8 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.
   - 96-well (0.2-mL) plate: **20 µL**
   - 96-well (0.1-mL) plate: **10 µL**

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.

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/cloud**
ExpressionSuite™ Software	**thermofisher.com/expressionsuite**

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

Algorithms for data analysis

Table 9 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**).

A Supplemental information

Endogenous and exogenous controls

Endogenous controls

An endogenous control shows gene expression that is relatively constant and moderately abundant across treatment protocols, and tissues or cell types. 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.

Overview of 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.

Figure 1 Poly(A) tailing reaction

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

Figure 2 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.

Figure 3 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.

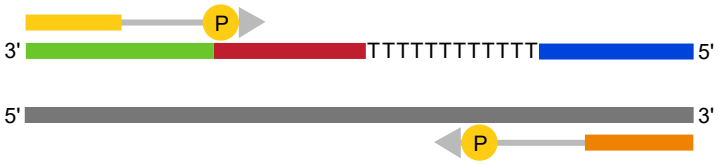

Figure 4 miR-Amp reaction

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

Overview of TaqMan™ Advanced miRNA Assays chemistry

TaqMan™ MGB probes

TaqMan™ MGB probes contain:

- A reporter dye (for example, FAM™ dye) at the 5′ end of the probe.
- 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.

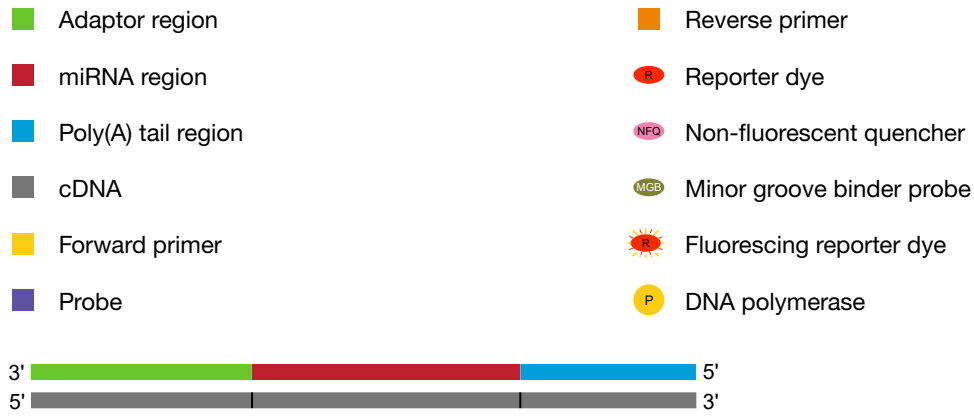

Figure 5 cDNA synthesis product

During the PCR, the forward and reverse primers anneal to complementary sequences along the denatured cDNA template strands (Figure 6). The primer binding sites vary depending on the target miRNA sequence and are designed to maximize specificity. Figure 6 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 6). When the probe is intact, the proximity of the reporter dye and quencher dye suppresses the reporter fluorescence, primarily by Förster-type energy transfer.

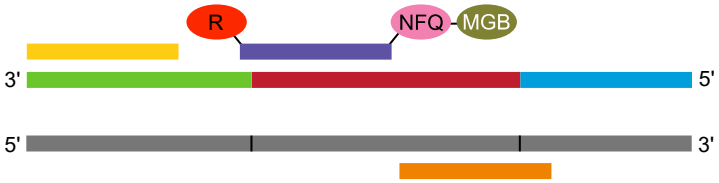

Figure 6 Annealing of probes and primers to cDNA strands

During polymerization, the DNA polymerase cleaves only 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 7).

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.

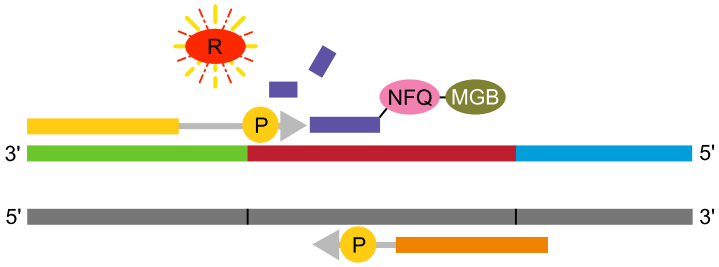

Figure 7 Initial polymerization and cleavage of reporter dye

Polymerization of the strand continues (Figure 8), but because the 3' end of the probe is blocked, no extension of the probe occurs during PCR.

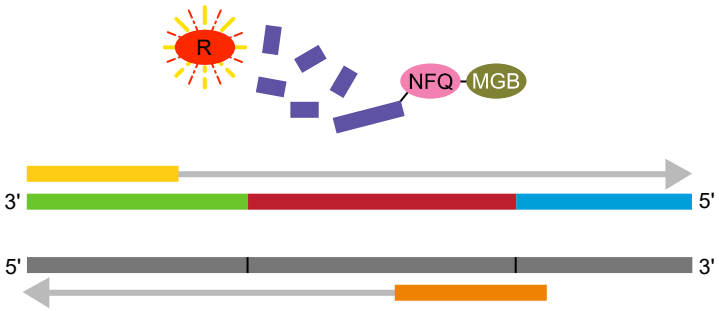

Figure 8 Completion of polymerization

Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if 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 DNA decontamination solution.

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.

B

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, and so on). To obtain SDSs, visit **thermofisher.com/support**.

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 sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer 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 needed) 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.

AVERTISSEMENT ! PRÉCAUTIONS GÉNÉRALES EN CAS DE MANIPULATION DE PRODUITS CHIMIQUES. Pour minimiser les risques, veiller à ce que le personnel du laboratoire lise attentivement et mette en œuvre les consignes de sécurité générales relatives à l'utilisation et au stockage des produits chimiques et à la gestion des déchets qui en découlent, décrites ci-dessous. Consulter également la FDS appropriée pour connaître les précautions et instructions particulières à respecter :

- Lire et comprendre les fiches de données de sécurité (FDS) fournies par le fabricant avant de stocker, de manipuler ou d'utiliser les matériaux dangereux ou les produits chimiques. Pour obtenir les FDS, se reporter à la section « Documentation et support » du présent document.
- Limiter les contacts avec les produits chimiques. Porter des équipements de protection appropriés lors de la manipulation des produits chimiques (par exemple : lunettes de sûreté, gants ou vêtements de protection).
- Limiter l'inhalation des produits chimiques. Ne pas laisser les récipients de produits chimiques ouverts. Ils ne doivent être utilisés qu'avec une ventilation adéquate (par exemple, sorbonne).
- Vérifier régulièrement l'absence de fuite ou d'écoulement des produits chimiques. En cas de fuite ou d'écoulement d'un produit, respecter les directives de nettoyage du fabricant recommandées dans la FDS.
- Manipuler les déchets chimiques dans une sorbonne.

- Veiller à utiliser des récipients à déchets primaire et secondaire. (Le récipient primaire contient les déchets immédiats, le récipient secondaire contient les fuites et les écoulements du récipient primaire. Les deux récipients doivent être compatibles avec les matériaux mis au rebut et conformes aux exigences locales, nationales et communautaires en matière de confinement des récipients.)
- Une fois le récipient à déchets vidé, il doit être refermé hermétiquement avec le couvercle fourni.
- Caractériser (par une analyse si nécessaire) les déchets générés par les applications, les réactifs et les substrats particuliers utilisés dans le laboratoire.
- Vérifier que les déchets sont convenablement stockés, transférés, transportés et éliminés en respectant toutes les réglementations locales, nationales et/ou communautaires en vigueur.
- **IMPORTANT !** Les matériaux représentant un danger biologique ou radioactif exigent parfois une manipulation spéciale, et des limitations peuvent s'appliquer à leur élimination.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

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)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

C

Documentation and support

Related documentation

Document	Pub. No.
TaqMan™ Advanced miRNA Assays Quick Reference—Single-tube Assays	100027898
QuantStudio™ 3 or 5 Real-Time PCR System	
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis Desktop Software User Guide	MAN0010408
QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System	
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems (v1.3) Maintenance and Administration Guide	4489821
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems (v1.6.1 or later) Maintenance and Administration Guide	MAN0018828
QuantStudio™ Real-Time PCR Software Getting Started Guide	4489822
QuantStudio™ 12K Flex Real–Time PCR System	
QuantStudio™ 12K Flex Real–Time PCR System v1.4 Maintenance and Administration Guide	4470689
QuantStudio™ 12K Flex Real–Time PCR System v1.6 or later Maintenance and Administration Guide	MAN0018832
QuantStudio™ 12K Flex Real–Time PCR System: Multi-Well Plates and Array Card Experiments User Guide	4470050
StepOne™ or StepOnePlus™ Real-Time PCR System	
StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking and Maintenance User Guide	4376782
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_t Experiments Getting Started Guide	4376785
ViiA™ 7 Real-Time PCR System	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System User Guide: Calibration, Maintenance, Networking, and Security	4442661
Applied Biosystems™ ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434

(continued)

Document	Pub. No.
7500/7500 Fast Real-Time PCR System	
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Relative Standard Curve and Comparative C_t Experiments	4387783

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- Product documentation
  - 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.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.