TaqMan® Small RNA Assays

USER GUIDE

TaqMan® MicroRNA Assays, Custom TaqMan® Small RNA Assays, and TaqMan® siRNA Assays

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Revision F

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

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Revision	Date	Description
F	12 February 2019	• Removed the optional UNG activation step from PCR protocols. • Added new instruments, Master Mixes, and other applicable products. • Added thermal cycling protocols for all compatible Master Mixes. • Updated options for secondary analysis software. • Added troubleshooting information. • Updated for general style, formatting, and branding.
E	January 2011	Baseline for this revision history.

Contents

1 Product information

Product description

Applied Biosystems™ TaqMan® Small RNA Assays are primer and probe sets designed to detect and quantify mature microRNAs (miRNAs), small interfering RNAs (siRNAs), and other small RNAs. The assays can detect and quantify small RNA in 1 to 10 ng of total RNA with a dynamic range of greater than six logs. When used for microRNA analysis, the assays can discriminate mature miRNA sequences from their precursors.

Note: In this user guide, "small RNA" refers to miRNA, siRNA, or other small RNAs that are less than 200 bases in length.

Predesigned and custom TaqMan® Small RNA Assays are available for a variety of small RNA classes.

- TaqMan® MicroRNA Assays
  - Predesigned assays for the majority of content found in the miRBase miRNA sequence repository.
  - Ideal for targeted quantification, screening, and validation of miRNA profiling results.
- TaqMan® siRNA Assays for *Silencer*™ Select siRNAs
  - Predesigned assays for the quantification of *Silencer*™ Select siRNAs.
  - Ideal for assessing siRNA transfection efficiency, half-life, and bio-distribution.
- TaqMan® Small RNA Controls
  - Predesigned assays for small, non-coding RNAs unrelated to miRNAs used to normalize for differences in sample RNA.
  - TaqMan® Small RNA Controls are available for a number of species.

  For more information on using TaqMan® control assays, see "Select a TaqMan® Small RNA Control" on page 6.

- Custom TaqMan® Small RNA Assays
  - Custom assays designed for any small RNA sequence from 17–200 nucleotides in length.
  - We provide ready-to-use Custom TaqMan® Small RNA Assays based on customer–supplied target sequences for any organism, with optimized primers and probe.

Overview of TaqMan® Small RNA Assays

TaqMan® Small RNA Assays use a stem-looped primer for reverse transcription and a sequence-specific assay to accurately detect mature miRNAs, siRNAs, and other small RNAs.

Each assay includes two tubes:

- A tube of small RNA–specific stem–looped RT Primer.
- A tube containing a mix of small RNA–specific forward PCR Primer, small RNA–specific reverse PCR Primer, and small RNA–specific TaqMan® MGB probe.

For a current list of available assays, use the assay search tool at **thermofisher.com/taqmanmirna**.

TaqMan® Small RNA Controls

We recommend using TaqMan® Small RNA Controls with TaqMan® Small RNA Assays. When quantifying small RNA gene expression levels, variation in the amount of starting material, sample collection, RNA preparation and quality, and reverse transcription (RT) efficiency can contribute to quantification errors. Normalization to endogenous control genes is currently the most accurate method to correct for potential RNA input or RT efficiency biases. An ideal endogenous control generally shows gene expression that is relatively constant and highly abundant across tissues and cell types. You need to verify the chosen endogenous control or set of controls for the target cell, tissue, or treatment because no single control can act as a universal endogenous control for all experimental conditions.

To view a complete list of available controls use the assay search tool at **thermofisher.com/taqmanmirna**.

Contents and storage

Table 1 TaqMan® MicroRNA Assays

Cat. No.	Number of 20–µL reactions	Amount and concentration		Storage
		RT Primer	Assay	
Inventoried predesigned assays				
4427975 (Small)	50 RT 150 PCR	150 µL (5×)	150 µL (20×)	–25°C to –15°C

Cat. No.	Number of 20–µL reactions	Amount and concentration		Storage
		RT Primer	Assay	
Made–to–order predesigned assays				
4440888 (Large)	2900 RT 2900 PCR	725 µL (60×)	967 µL (60×)	–25°C to –15°C
4440887 (Medium)	750 RT 750 PCR	575 µL (20×)	750 µL (20×)	
4440886 (Small)	50 RT 150 PCR	150 µL (5×)	150 µL (20×)	
4440885 (Extra small)	25 RT 75 PCR	75 µL (5×)	75 µL (20×)	

Table 2 Custom TaqMan® Small RNA Assays

Cat. No.	Number of 20–µL reactions	Amount and concentration		Storage
		RT Primer	Assay	
4398989 (Large)	2900 RT 2900 PCR	725 µL (60×)	967µL (60×)	–25°C to –15°C
4398988 (Medium)	750 RT 750 PCR	575 µL (20×)	750 µL (20×)	
4398987 (Small)	50 RT 150 PCR	150 µL (5×)	150 µL (20×)	
4440418 (Extra small)	25 RT 75 PCR	75 µL (5×)	75 µL (20×)	

Table 3 TaqMan® siRNA Assays (predesigned)

Cat. No.	Number of 20–µL reactions	Amount and concentration		Storage
		RT Primer	Assay	
4440880 (Large)	2900 RT 2900 PCR	725 µL (60×)	967 µL (60×)	–25°C to –15°C
4440879 (Medium)	750 RT 750 PCR	575 µL (20×)	750 µL (20×)	
4440878 (Small)	50 RT 150 PCR	150 µL (5×)	150 µL (20×)	
4440877 (Extra small)	25 RT 75 PCR	75 µL (5×)	75 µL (20×)	

How to order TaqMan® Small RNA Assays

Order predesigned and custom assays for miRNAs, siRNAs, and other small RNAs from **thermofisher.com/taqmanmirna**. The website features tools to help select inventoried assays or design custom assays for an unlisted small RNA.

Order Custom TaqMan® Small RNA Assays or predesigned TaqMan® siRNA Assys for *Silencer*™ Select siRNAs from **Silencer Select siRNAs**.

For information on designing custom assays, see the *Custom TaqMan® Small RNA Assays Design and Ordering Guide* (Pub. No. 4412550).

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 4 Recommended products for isolation of RNA

Sample type	Item	Source
Tissue samples	*mir*Vana™ miRNA Isolation Kit, with phenol	AM1560
	*mir*Vana™ miRNA Isolation Kit, without phenol	AM1561
	*mir*Vana™ PARIS™ RNA and Native Protein Purification Kit	AM1556
	MagMAX™ *mir*Vana™ Total RNA Isolation Kit	A27828
	TRI Reagent™ Solution	AM9738
	RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
Cell samples	TaqMan® MicroRNA Cells-to-C_T Kit	4391848
	Cells-to-C_T™ Stop Solution, 1 mL	4402960
	Cells-to-C_T™ Bulk Lysis Reagents	4391851C
	MagMAX™ *mir*Vana™ Total RNA Isolation Kit	A27828
Liquid samples	MagMAX™ *mir*Vana™ Total RNA Isolation Kit	A27828

Table 5 Recommended products for preparation of cDNA

Item	Source
TaqMan® MicroRNA Reverse Transcription Kit[1]	4366596

[1] TaqMan® Small RNA Assays are optimized for the MuLV Reverse Transcriptase contained in the TaqMan® MicroRNA Reverse Transcription Kit. Performance with other RT enzymes cannot be guaranteed.

Table 6 PCR Master Mixes

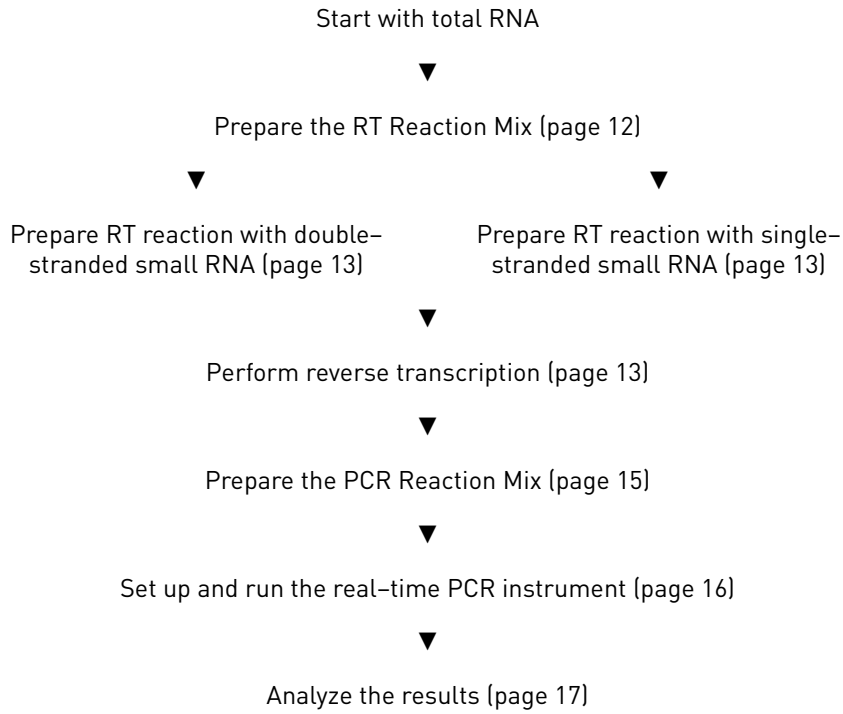
Item	Source
TaqMan® Fast Advanced Master Mix	4444558
TaqMan® Universal Master Mix II, no UNG	4440043
TaqMan® Universal Master Mix II, with UNG	4440042
TaqMan® Universal PCR Master Mix, no AmpErase™ UNG	4364341
TaqMan® Universal PCR Master Mix	4304437

Table 7 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): • Veriti™ Thermal Cycler • SimpliAmp™ Thermal Cycler • ProFlex™ PCR System	Contact your local sales office
Centrifuge, with adapter for 96-well or 384-well plates	MLS
Microcentrifuge	MLS
Vortex mixer	MLS
(Optional) Eppendorf™ MixMate™ (shaker)	Fisher Scientific™ 21-379-00
Pipettes	MLS
Tubes, plates, and other consumables	
Tubes, plates, and film	**thermofisher.com/plastics**
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS

Item	Source
Reagents	
Nuclease-free Water	AM9930
RNase Inhibitor	N8080119
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019
TURBO DNA-*free*™ KitDNase	AM1907
TE, pH 8.0	AM9849

Workflow



2 Perform reverse transcription

Guidelines for isolation of high–quality RNA

- See Table 4 for recommended RNA isolation kits.
- Prepare samples using a method that preserves small RNAs.
- To prevent the loss of the longer control transcripts (such as snoRNAs), size fractionation is *not* recommended.

Guidelines for RNA input

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

  IMPORTANT! Do not denature the total RNA.
- Use 10 to 10,000 cells per sample for the Cells–to–C_t kit.
- When working with double-stranded template such as siRNAs and miRNA mimics, denature the siRNA template with sequence-specific RT Primer before performing the reverse transcription.
- Prepare the RT reactions in an area free of artificial templates, amplified material, and siRNA transfections. High-copy-number templates can easily contaminate the reactions.
- When working with siRNA use total RNA from non-transfected cells as a control in *in-vitro* and *in-vivo* studies. The amount of input total RNA for optimal detection depends on the transfection protocol used.

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

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

Before you begin

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

Prepare the RT Reaction Mix

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

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

[1] Includes 10% overage.

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

Place the RT Reaction Mix on ice. Proceed immediately to "Prepare the RT reaction" on page 12.

Prepare the RT reaction

Use one of the following procedures.

Prepare RT reaction with double–stranded small RNA

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

1. Combine 3 µL of 5× RT Primer and 5 µL of double-stranded template in a reaction tube or in each well of a reaction plate.
2. Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube or wells.
3. Incubate at 85°C for 5 minutes.
4. Incubate at 60°C for 5 minutes, then place on ice.
5. Add 7 µL of RT Reaction Mix to each reaction tube or well.
   RT Reaction Mix was prepared in "Prepare the PCR Reaction Mix" on page 15.
6. Seal the tube or reaction plate.
7. Centrifuge briefly to collect the contents at the bottom of the tubes or wells.

Place on ice and proceed immediately to "Perform reverse transcription" on page 13.

Prepare RT reaction with single–stranded small RNA

1. Combine 7 µL of RT Reaction Mix and 5 µL of total RNA in a reaction tube or in each well of a reaction plate.
   RT Reaction Mix was prepared in "Prepare the RT Reaction Mix" on page 12.
2. Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tubes or wells.
3. Add 3 µL of 5× RT Primer to each reaction tube or each well of a reaction plate.
4. Seal the tubes or reaction plate.
5. Centrifuge briefly to collect the contents at the bottom of the tubes or wells.

Place on ice and proceed immediately to "Perform reverse transcription" on page 13.

Perform reverse transcription

Place the reaction plate or tubes into a thermal cycler, then incubate using standard cycling, a reaction volume of 15.0 µL, and the following settings.

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

The RT reaction product can be stored at –25 to –15°C for up to one week.

3 Perform PCR amplification

Procedural guidelines for performing real–time PCR

- Follow best practices when preparing or performing PCR. See "Good laboratory practices for PCR and RT-PCR" on page 27.
- Prepare the PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Protect the assays from light and store in a freezer. Excessive exposure to light might affect the fluorescent probes.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.
- Perform three replicates of each reaction.
- Include the following reactions:
  - A small RNA assay for each cDNA sample.
  - Endogenous control assays for each cDNA sample.
  - No–template controls (NTCs) for each assay on the plate.

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

Before you begin

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

Prepare the PCR Reaction Mix

1. Mix the PCR Master Mix reagent by gently swirling the bottle.

   See "Required materials not supplied" on page 8 for a list of compatible PCR Master Mixes.

2. Prepare the PCR Reaction Mix in appropriately-sized microcentrifuge tubes, according to the following table. Add 10% overage to account for pipetting loss.

Component	Volume per reaction	
	384–well or 96–well fast (0.1mL) plate	**96–well standard (0.2 mL) plate**
TaqMan® Small RNA Assay (20X)	0.50 µL	1.00 µL
PCR Master Mix[1]	5.00 µL	10.00 µL
Nuclease–free water[2]	3.50 µL	7.00 µL
Total PCR Reaction Mix volume	**9.00 µL**	**18.00 µL**

[1] See Table 6 for recommended PCR Master Mixes.
[2] Adjust quantity of water to account for cDNA template.

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

Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.

2. Add cDNA template (1 pg to 100 ng in nuclease–free water), or nuclease–free water for NTC, to each well.
   - 384–well or 96–well fast (0.1 mL) plate: 1 µL
   - 96–well standard (0.2 mL) plate: 2 µL

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

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

3. Seal the plate with optical adhesive film, then centrifuge the plate briefly to bring the Reaction Mix to the bottoms of the wells.

4. Apply a compression pad to the plate, if required by your real–time PCR system.

Set up and run the real–time PCR instrument

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

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

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

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

2. Set up the thermal protocol for your instrument.

   Table 8 TaqMan® Fast Advanced Master Mix (StepOne™, StepOnePlus™, ViiA™ 7, and QuantStudio™ systems with fast cycling mode)

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

   Table 9 TaqMan® Fast Advanced Master Mix (7500 and 7500 Fast systems with fast cycling mode)

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

   Table 10 TaqMan® Universal Master Mix II, no UNG, TaqMan® Universal Master Mix II, with UNG, TaqMan® Universal PCR Master Mix, no AmpErase™ UNG, or TaqMan® Universal PCR Master Mix (any compatible instrument with standard cycling mode)

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

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

Analyze the results

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

The general guidelines for analysis include:

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

Perform additional analysis using any of the following software:

Software	Resource
Relative Quantification application	thermofisher.com/cloud
Standard Curve application	
ExpressionSuite™ Software[1]	thermofisher.com/expressionsuite
DataAssist™ Software	thermofisher.com/dataassist

[1] Can automatically define the baseline.

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

Algorithms for data analysis

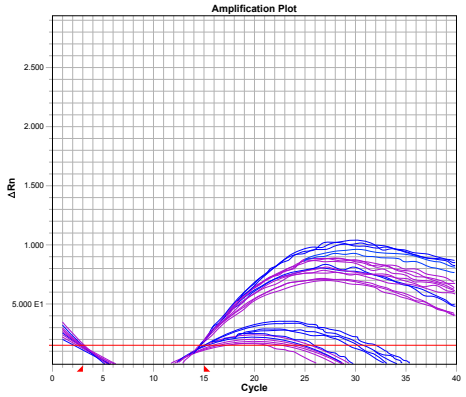
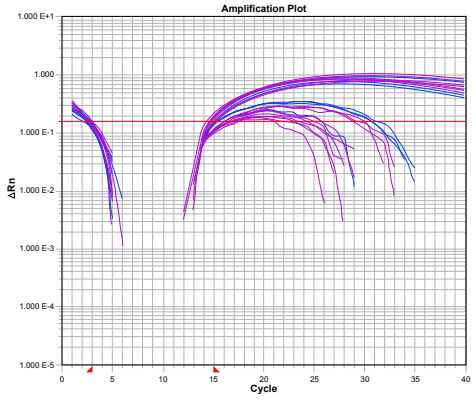
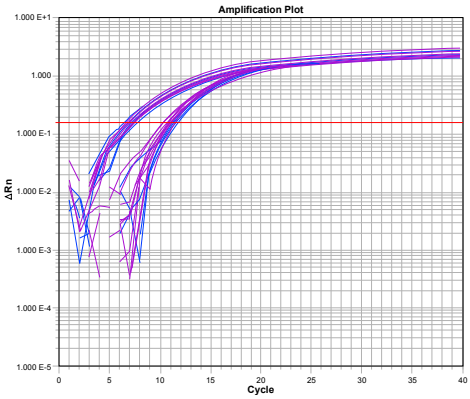
Table 11 Algorithm recommendations for single–tube assays

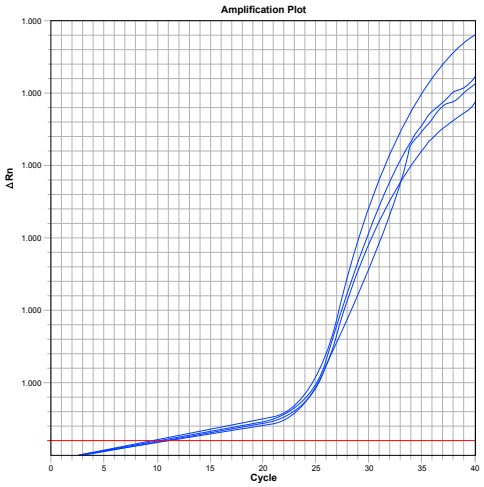
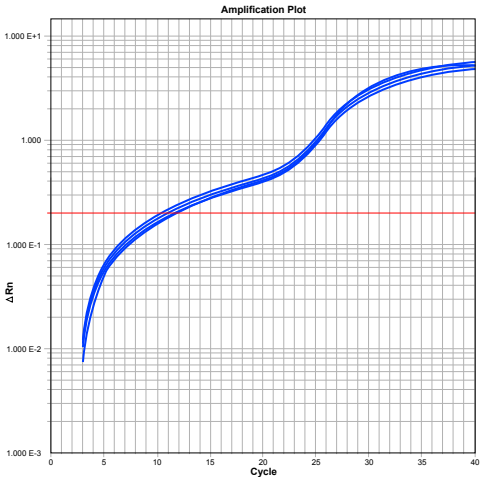
Algorithm	Recommendation
Threshold (C_t)	Recommended.
Relative threshold (C_{rt})	*(Optional)* Use for troubleshooting abnormal or unexpected results.

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

Troubleshooting

Observation	Possible cause	Recommended action
No miRNA is detected	The threshold is set too high to detect miRNA in samples with low expression.	Identify an appropriate NOAMP flag threshold if the relative threshold algorithm (C_{rt}) is used.
		Identify an appropriate threshold if C_t is used.
The C_t value for the no–template control (NTC) is <35 for some assays	There are non–specific interactions between primers.	For NTC information on a specific assay, see the Megaplex™ Assay Performance File.
	The cDNA template is contaminated.	Follow established PCR good laboratory practices.
There is poor reproducibility across technical replicates	The reagents were not adequately mixed.	Ensure that all samples and reagents are mixed well.
The negative control well shows amplification	The reagents or the cDNA template are contaminated.	Follow established PCR good laboratory practices.

Observation	Possible cause	Recommended action
Amplification curve shows abnormal plot and/or low ΔRn values Linear view:  Log view:  	The baseline was set improperly (some samples have C_t values lower than the baseline stop value).	See your real-time PCR system user guide for procedures on setting the baseline. Switch from manual to automatic baselining, or move the baseline stop value to a lower C_t (2 cycles before the amplification curve crosses the threshold). Corrected log view: 
	An amplification signal is detected in the early cycles (no baseline can be set because the signal is detected too early).	Dilute the sample to increase the C_t value.

Observation	Possible cause	Recommended action
Amplification curve shows a rising baseline Linear view:  Log view:  	There is interaction between the primer and probe.	Adjust the threshold manually. Select another assay from the same gene, if available.
Amplification curve shows weak amplification	(*Custom TaqMan® Small RNA Assays only*) Sequence provided for the assay design contains mismatches with sample sequences.	Perform bioinformatics analysis. For more information, see *Custom TaqMan® Small RNA Assays Design and Ordering Guide* (Pub. No. 4412550).
The Multicomponent Plot shows low ROX™ dye (passive reference dye)	Little or no Master Mix is present due to inaccurate pipetting.	Follow accurate pipetting practices.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_t=40) across all assays or in an unusually large number of assays	One or more of the reaction components was not added.	Ensure that the cDNA, the assay and the Master Mix were added to the reaction plate. **Note:** The passive reference fails if the Master Mix is missing.
	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.
	The annealing temperature was too high for the primers and/or probe.	Ensure that the correct annealing and extension temperatures are set.
		Ensure that the real-time PCR instrument is calibrated and maintained regularly.
	Inappropriate reaction conditions were used.	Troubleshoot the real-time PCR optimization.
		Ensure that the properties and the thermal protocol are correct.
	The template is degraded.	Determine the quality of the template.
		Rerun the assay with fresh template.
		Use RNase-free reagents.
		Use an RNase inhibitor.
	Inhibitors are present in the reaction.	Ensure the presence of an inhibitor: 1. Create a serial dilution of your sample. 2. Run the serial dilution with an expressing assay (for example, an endogenous control). If an inhibitor is present, high concentrations yield higher-than-expected C_t values. (High concentration means more inhibition because the sample is not diluted.) 3. Rerun the assay with purified template.
	The baseline and/or threshold was improperly set.	See your real-time PCR system user guide for procedures on setting the baseline and threshold.
		Switch from an automatic baseline to a manual baseline, or from a manual baseline to an automatic baseline.
		Lower the threshold value to within the appropriate range.
	The reverse transcription failed.	Check the RNA integrity and concentration.
		Check for RNase activity.
		Follow the recommended thermal profile.
		Repeat the reverse transcription using new reagents.
	(*Custom TaqMan® Small RNA Assays only*) Design or synthesis failed. The wrong sequence was submitted to Thermo Fisher Scientific.	Ensure that the sequence that you submitted is correct.
		Check for an alternative transcript or a splice variant.

Observation	Possible cause	Recommended action
Amplification curve shows samples targeted by the same assay that have differently shaped curves	The baseline was set improperly.	See your real-time PCR system user guide for procedures on setting the baseline.
		Switch from an automatic baseline to a manual baseline, or from a manual baseline to an automatic baseline.
		Increase the upper or lower value of the baseline range.
	The sample quality was poor.	1. Perform a quality check on the sample. 2. If needed, re-extract the sample.
	There were different concentrations caused by imprecise pipetting.	Follow accurate pipetting practices.
	The reagents or equipment are contaminated.	Ensure that your workspace and equipment are cleaned properly.
Amplification curve shows no amplification of the sample (C_t=40) in the target assay	The sample does not have enough copies of the target RNA.	Confirm the results. 1. Rerun the sample using the same assay. 2. Rerun the assay using more sample. Avoid PCR reaction mix with more than 20% from the reverse transcription reaction. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	One or more of the reaction components was not added.	Check your pipetting equipment and/or technique.
	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.
Decrease in ROX™ dye fluorescence (passive reference dye)	There was precipitation in the buffers.	Mix the Master Mix thoroughly to produce a homogenous solution.
	The reagents are degraded.	Ensure that the kits and reagents have been stored according to the instructions on the packaging and that they have not expired.
There was a simultaneous increase in fluorescence from both the passive reference dye (ROX™ dye) and the reporter dyes	The sample evaporated.	Check the seal of the adhesive film for leaks.
The multicomponent signal for ROX™ dye is not flat	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.
The R_n in the R_n vs. Cycle plot is very high	The ROX™ dye was not set as the passive reference.	Set ROX™ dye as the passive reference, then reanalyze the data.

Observation	Possible cause	Recommended action
The no–template control (NTC) shows amplification	The reagents are contaminated with amplicons.	Rerun the assay using new reagents.
		Ensure that your workspace and equipment are cleaned properly.
		Run no–RT controls to rule out genomic DNA contamination.
		Treat the sample with DNase.
The endogenous control C_t values vary or they do not normalize the sample well	The endogenous control is not consistently expressed across the samples.	Ensure that the endogenous control is consistently expressed in your sample type.
		Use an alternative endogenous control such as a nonvariable miRNA.
	The sample concentrations vary.	Quantify and normalize the PCR samples.
	Pipetting was inaccurate.	Check the calibration of the pipettes.
		Pipet at least 5 µL of sample to prepare the reaction mix.
There is a high standard deviation in the replicates, inconsistent data, or a variable C_t	The reagents were not mixed properly.	Increase the length of time that you mix the reagents.
		Confirm your mixing process by running a replicate assay.
	Pipetting was inaccurate.	Check the calibration of the pipettes.
		Pipette at least 5 µL of sample to prepare the reaction mix.
	The threshold was not set correctly.	Set the threshold above the noise level and where the replicates are tightest. See your real-time PCR system user documentation.
	There was a low concentration of the target of interest.	Rerun the assay with more cDNA template.
	C_t is not the most appropriate analysis for the data.	Use relative threshold (C_{rt}) analysis. See *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239).
The C_t value is lower than expected	Contamination occurred.	Run no-RT control to confirm that there was genomic DNA (gDNA) contamination.
		Use DNase to ensure minimal gDNA contamination of the RNA.
	Too much cDNA template was added.	Reduce the amount of cDNA template.
		Quantitate the RNA before the RT reaction, then adjust the concentration of cDNA from the RT reaction that is added.
	The cDNA template or the amplicon is contaminated.	Follow established PCR good laboratory practices.

Observation	Possible cause	Recommended action
Amplification occurs in the no-RT controls	The cDNA template or amplicon is contaminated.	Follow established PCR good laboratory practices.
There was a shifting Rn value during the early cycles of the PCR (cycles 0 to 5)	Fluorescence did not stabilize to the buffer conditions of the reaction mix. **Note:** This condition does not affect PCR or the final results.	Reset the lower value of the baseline range.
		Use an automatic baseline.
		Use the relative threshold algorithm (C_{rt}). See *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239).
There was a small ΔRn	The PCR efficiency was poor.	Ensure that the reagents were used at the correct concentration.
	The quantity of the cDNA is low (a low copy number of the target).	Increase the quantity of the cDNA.
There is a noisy signal above the threshold	The sample evaporated.	Check the seal of the adhesive film for leaks.
	The well is empty because of inaccurate pipetting.	Check the calibration of the pipettes.
		Pipet at least 5 µL of sample.
	The well is assigned a sample or target in the plate document or experiment, but the well is empty.	Ensure that the plate document or experiment is set up correctly.
		Exclude the well and reanalyze the data.

B Supplemental information

Overview of TaqMan® Small RNA Assay chemistry

Reverse transcription

cDNA is reverse transcribed from total RNA using a small RNA–specific, stem–looped primer.

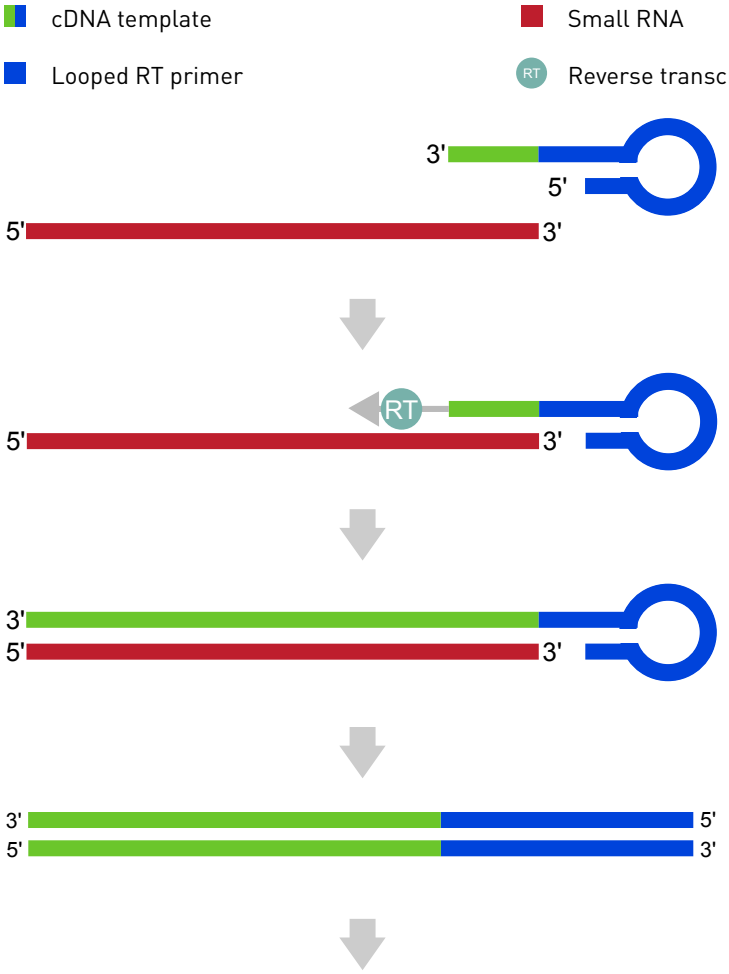


Figure 1 Stem loop primer

TaqMan® MGB probes

TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™) 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® Small RNA 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 cDNA synthesis product.

cDNA template
Forward primer
Probe
Reverse primer
Minor groove binder probe
Non-fluorescent quencher
Reporter dye
Fluorescing reporter dye
DNA polymerase

During the PCR, the forward and reverse primers anneal to complementary sequences along the denatured cDNA template strands.

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

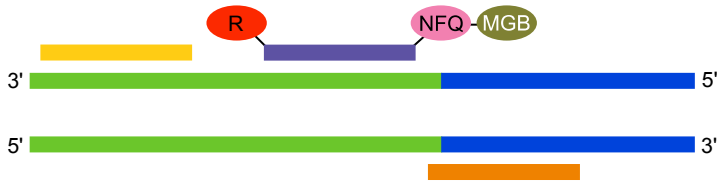


Figure 2 Annealing of probes and primers to cDNA strands or preamplified cDNA strands

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.

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.

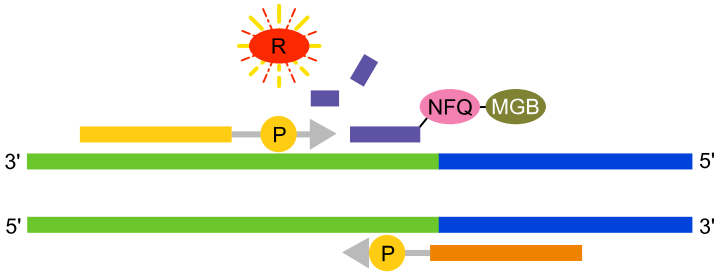


Figure 3 Initial polymerization and cleavage of reporter dye

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

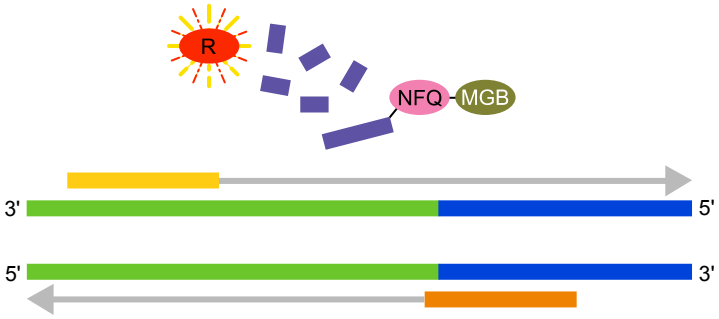


Figure 4 Completion of polymerization

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.

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.

C

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, 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:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.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

Documentation and support

Related documentation

Document	Pub. No.
TaqMan® Small RNA Assay Quick Reference	4412551
Custom TaqMan® Small RNA Assays Design and Ordering Guide	4412550
Custom Reverse Transcription Pools and Custom Preamplification Pools with TaqMan® MicroRNA Assays User Bulletin	4465407
Understanding Your Shipment	MAN0017153

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  - 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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