TOOLS miRNA RT-qPCR assay

User Guide

Contents

Introduction

The TOOLS miRNA RT-qPCR Assay consists of the TOOLS miRNA RT Kit, TOOLS miRNA RT-qPCR primer/probe set, and TOOLS Easy 2×Probe qPCR mix. TOOLS miRNA RT-qPCR Assay System enables the rapid, sensitive, and specific detection of microRNA (miRNA). A single cDNA preparation can be used with the TOOLS miRNA RT-qPCR Assay System to rapidly quantify the expression of mature miRNAs. The TOOLS miRNA RT-qPCR primer/probe set consists of specific primers and probes designed to detect and quantify mature miRNAs. When used for miRNA analysis, the assays can discriminate mature miRNA sequences from their precursors.

Mature miRNAs are a group of short noncoding RNA molecules 18–25 nucleotides long, and they govern various aspects of cellular development and pathological abnormalities. This assay system uses a stem-looped primer to extend the cDNA product in reverse transcription (RT) and a probe-based quantitative polymerase chain reaction (qPCR) assay to increase miRNA detection accuracy. This assay system adopts an innovative RT enzyme that enables the completion of an RT reaction within only 15 min. The TOOLS miRNA RT-qPCR Assay provides a complete and efficient product for miRNA quantification.

Product Overview

- TOOLS miRNA RT Kit
  - The TOOLScript II Enzyme Mix combines an efficient reverse transcriptase and RNase inhibitor.
  - The reverse transcriptase in the kit provides reliable RT to a wide range (from 15 pg to 150 ng) of RNA templates.

- TOOLS miRNA RT-qPCR primer/probe set
  - It consists of predesigned assays for the majority of content found in the miRBase miRNA sequence repository.
  - It is ideal for the targeted quantification and validation of miRNA profiling results.
  - Each RT-qPCR primer/probe set includes two tubes: one tube containing RT primer, and the other tube containing a mix of a qPCR probe and forward and reverse primers.

- TOOLS Easy 2×Probe qPCR Mix
  - The ready-to-load mix contains dNTP/dUTP mix, $Mg2^{+}$, DNA polymerase, dUTP/UDG anticontamination system, specific ROX reference dye, and all components necessary to perform a qPCR.
  - It provides high efficiency and stability.

Assay Components

TOOLS miRNA RT Kit

Contents	TTH-mi50 (50 rxn)	TTH-mi250 (250 rxn)
2× RT buffer	500 µL	1.25 mL × 2
TOOLScript II RT Enzyme Mix	20 µL	100 µL
Nuclease-free ddH_2O	500 µL	1.25 mL × 2
Storage: All components of the kit should be stored at −20°C.		

https://www.tools-biotech.com/product/detail/id/628/name/TOOLS%20miRNA%20RT%20Kit.html

TOOLS miRNA RT-qPCR primer/probe set

Contents	50 rxn	250 rxn
10× RT primer pair	100 µL	500 µL
10× qPCR primer/probe	300 µL	1500 µL
Storage: All components of the kit should be stored at −20°C.		

https://www.tools-biotech.com/product/index/cid/175.html

TOOLS Easy 2×Probe qPCR Mix

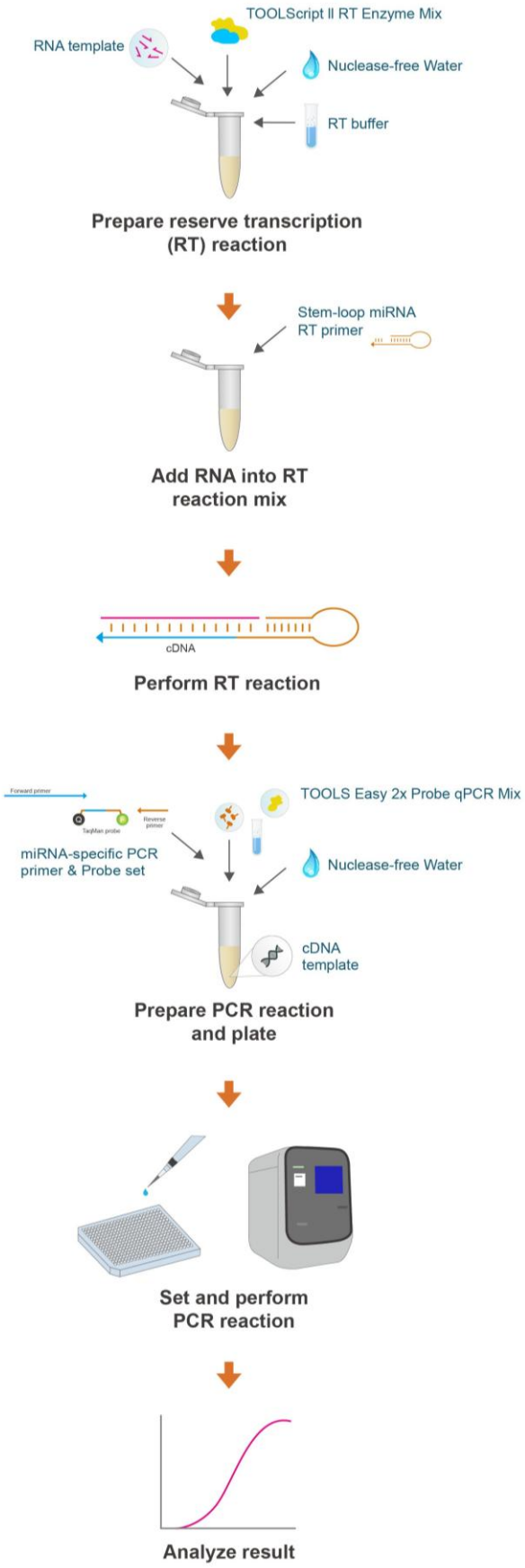
Contents	TTC-QE13 (100 rxn)
TOOLS Easy 2×Probe qPCR Mix	1 mL
Storage: All components of the kit should be stored at −20°C.	

https://www.tools-biotech.com/product/detail/id/627/name/TOOLS%20Easy%202xProbe%20qPCR%20Mix.html

Important Note

TOOLS miRNA RT Kit, TOOLS miRNA RT-qPCR primer/probe set, and TOOLS Easy 2xProbe qPCR mix materials are purchased individually.

Workflow


TOOLScript II RT Enzyme Mix
RNA template
Nuclease-free Water
RT buffer
Prepare reserve transcription (RT) reaction
Stem-loop miRNA RT primer
Add RNA into RT reaction mix
cDNA
Perform RT reaction
TOOLS Easy 2x Probe qPCR Mix
miRNA-specific PCR primer & Probe set
Nuclease-free Water
cDNA template
Prepare PCR reaction and plate
Set and perform PCR reaction
Analyze result

Protocol

Perform RT

Guidelines for RNA input

- When RNA is handled, RNase-free plastic ware and reagents must be used.
- Samples must be prepared using a method that preserves miRNAs.
- RNAs must be aliquoted to minimize freeze–thaw and reduce accidental RNase contamination.
- For most reactions, 15 pg to 150 ng of total RNA per 20 µL of RT reaction is recommended.
- For optimal RT, input RNA should have the following characteristics:
  - Free of inhibitors of RT and PCR.
  - Dissolved in PCR-compatible buffer.
  - Free of RNase activity.

Before you begin

- Prepare pipette, aerosol-resistant pipette tip, cold blocks, and ice.
- Prepare RNase-free EP tube and thermal cycler.
- Thaw 2× and 10× RT primer pair on ice, vortex briefly, and then spin briefly to collect the contents at the bottom of the tube.
- TOOLScript II RT Enzyme Mix contains a high concentration of glycerin. Spin down briefly before use to collect all contents at the bottom without generating air bubbles. Return to −20°C immediately after use.

A. Prepare the RT Reaction Mix

1. Assemble the reaction on ice.
2. Prepare the RT Reaction Mix according to the following components in a sterile RNase-free microfuge tube.

Component	Volume	Final concentration
TOOLScript II RT Enzyme Mix	0.4 µL	-
2× RT buffer	10 µL	1×
Nuclease-free H_2O	2.6 µL	-
Total volume	**13 µL**	

3. Mix the RT Reaction Mix by pipetting gently and spinning down briefly. Place on ice.

B. Prepare the RT Reaction

1. For each 20 µL of RT reaction, combine total RNA with the RT Reaction Mix in a ratio of 5 µL RNA:13 µL RT Reaction Mix. Mix gently and spin down briefly.

Note: 15 pg to 150 ng of total RNA per 20 µL of RT reaction is recommended.

2. Add 2 µL of the RT primer to each reaction tube or each well of a reaction plate.
3. Seal the tubes or reaction plate. Mix gently and spin down briefly. Place on ice.

C. Perform RT Experiment

1. Incubate in a thermal cycler:

Step	Temperature	Time (min)
RT	42°C	15
Stop reaction	95°C	3
Hold	4°C	Hold

2. Store reaction product at −20°C for up to 1 week.

Perform PCR Amplification

Guidelines for real-time PCR

- Follow best practices when preparing or performing PCR and prepare the PCR reactions in a clean area to prevent any artificial templates or high-copy-number templates from causing contamination of real-time PCR reactions.
- Protect the assays from light and store in a freezer. Excessive exposure to light might reduce the efficiency of fluorescent probes.
- Perform three replicates of each cDNA reaction.
- Include no-template control reactions for each target in the PCR experiment to evaluate whether the background signal is appropriate.

Before you begin

- Prepare pipette, aerosol-resistant pipette tip, optical-grade qPCR tubes, plates, and sealing films.
- Thaw 10× qPCR primer/probe, vortex briefly, and then spin briefly to collect the contents at the bottom of the tube.
- Completely thaw the TOOLS Easy 2×Probe qPCR mix before use, mix well through gentle pipetting, and avoid direct light.
- Dilute the RT reaction product to 5-fold volume cDNA by using nuclease-free water (add 80 µL nuclease-free water to 20 µL of RT reaction product).

A. Prepare Real-time PCR Reaction Mix

1. Prepare the reaction mix and assemble reactions according to the following table:

Component	Volume	Final concentration
TOOLS Easy 2×Probe QPCR mix	10 µL	1×
10× QPCR primer/probe	2 µL	1×
cDNA	X µL*	
Nuclease-free H_2O	Up to 20 µL	
Total Volume	**20 µL**	

*cDNA volume must not exceed 4 µL.

2. Prepare reaction mixtures in individual 0.2-mL PCR tubes, an 8-well PCR strip, or a 96-well PCR plate.
3. Gently vortex, and spin down briefly to collect the contents at the bottom.

B. Set up and run the real-time PCR program

Compatible devices
Applied Biosystems 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast; StepOne™, StepOnePlus™ 7500, 7500 Fast, ViiATM7
Qiagen/Corbett Rotor-Gene® Q, Rotor-Gene® 3000, Rotor-Gene® 6000
Stratagene MX4000™, MX3005P™, MX3000P™
Eppendorf Mastercycler® ep realplex, realplex 2 s
Roche Applied Science LightCycler™ 480 and other instruments

1. Set up the thermal cycling parameters according to the following table:

Parameter	Temperature	Time	Cycle
Pollutant digestion	37°C	2 min	1×
Initial denaturation*	95°C	10 min	1×
Denaturation	95°C	15 s	45×
Annealing and extension	60°C	1 min	

*The polymerase included in this mix is a hot-start Taq DNA polymerase, and thus, the predenaturation stage should be set to 95°C for at least 5 min.

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

Analyze the Results

The two strategies for analyzing qPCR data are absolute and relative quantification with respect to the ΔΔCT of the $2^{-\Delta\Delta CT}$ method. Relative quantification uses an internal control (reference gene) or a control group (reference group) to quantify the miRNA of interest relative to these references. At a certain threshold during the linear portion of the PCR reaction, the amount of genes of interest and the control double each cycle. For detailed information about data analysis, please see the appropriate documentation for your instrument on the crucial steps in qPCR data analysis.

The general guidelines for analysis include the following:

- Adjusting your software output.
- View the amplification plot: if needed, adjust the baseline/threshold (recommended 0.2 threshold value as a starting point) and remove outliers on the estimated quantification.
- Compare the Ct values (ΔCt) of all the sample replicates between each replicate group.

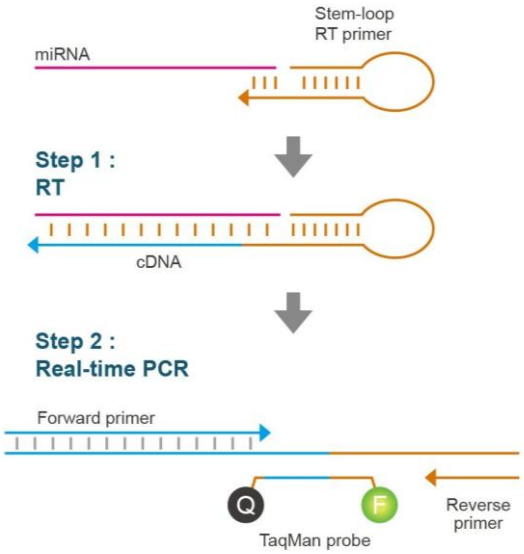
Troubleshooting and FAQs

Symptoms	Possible cause	Solutions
No signal or no amplification product	Template is degraded.	Verify template integrity through electrophoresis.
	Inhibitor is present in sample.	Perform an ethanol precipitation to remove inhibitors.
	Insufficient number of cycles.	Return reactions to thermal cycler for five more cycles.
	One or more of the reaction components were not added.	Make sure all the required components have been added.
The amplification curve shows that the sample was not successfully amplified (Ct = 40)	Too much or too little template was used.	Verify template concentration by comparing the staining intensity of the template after gel electrophoresis and increase sample input.
	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.	Confirm the presence of an inhibitor or return the assay with purified template.
	The baseline or threshold was improperly set.	Refer to your real-time PCR system's user guide for baseline setting procedures and thresholds.
	The RT step failed.	• Check for RNA integrity and concentration. • Follow recommended thermal configuration conditions. • Repeat RT with a new reagent.
The amplification curves of the target samples with the same detection method show different results	The baseline was set improperly.	Please refer to your real-time PCR system's user guide for baseline setup.
	The reagents or equipment are contaminated.	Ensure that your workspace and equipment are cleaned properly.
	The sample quality is poor.	Check the quality of samples.

Supplemental information

Methodology of the TOOLS miRNA RT-qPCR Assay

The TOOLS miRNA RT-qPCR Assay System approaches are based on a stem-loop primer design for the analysis of mature miRNAs; an miRNA-specific TaqMan probe is used, and the resulting fluorescence is detected. It consists of two steps: RT reaction and real-time PCR. First, each stem-loop primer specifically binds to an individual mature miRNA target. Then, it is reverse transcribed to a cDNA template by reverse transcriptase. Finally, the cDNA templates are quantified through probe-based qPCR.

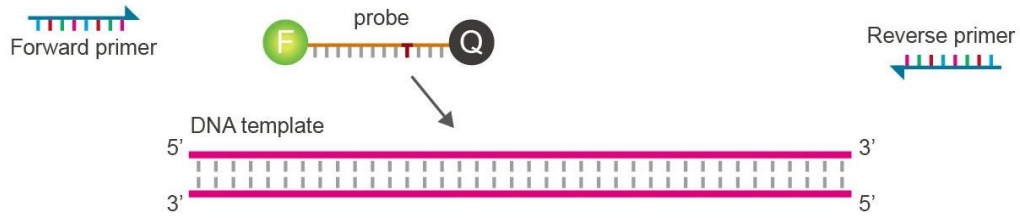


Nucleic acids research 33.20 (2005): e179–e179.

TaqMan minor groove binder probe design

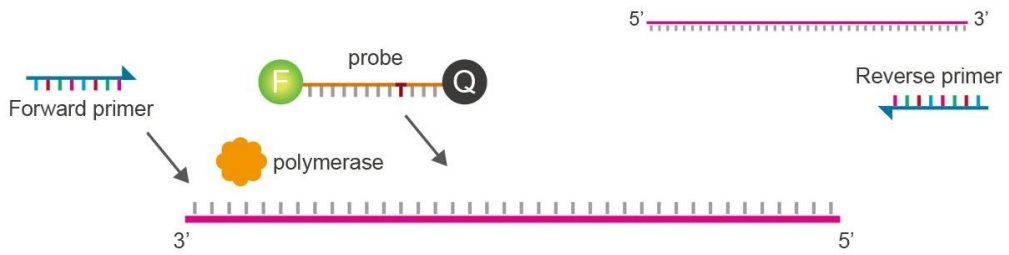
- The fluorescent probe is labeled at 5′ with 6-carboxyfluorescein (FAM) dye as a reporter dye.
- It is labeled at the 3′ end of the probe with a nonfluorescent quencher as a quencher dye that enhances spectral performance and maximizes sensitivity.
- At the 3′ end of the probe, a minor groove binder moiety increases the probe melting temperature and stabilizes probe–target hybridization.

About the 5′-nuclease (TaqMan) assay



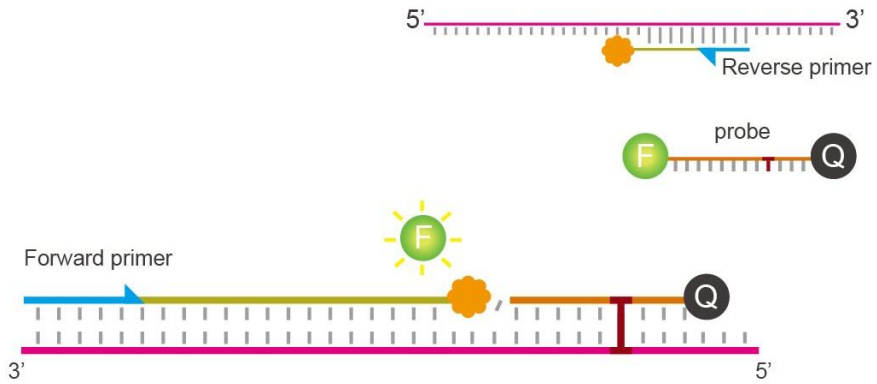
The 5′-nuclease assay is convenient. It consists of a self-contained amplification process. This assay for routine PCR amplification of cDNA uses a temperature-dependent Taq DNA polymerase process that uses the 5′ → 3′ exonuclease activity.

1. Initial denaturation/annealing step:



- Initial denaturation: The temperature is increased to denature the double-stranded cDNA and separate them into a single-strand cDNA pool.
- Annealing: The reaction temperature is reduced such that the primer and probe are annealed to a specific target sequence.

2. Extension/amplification step:

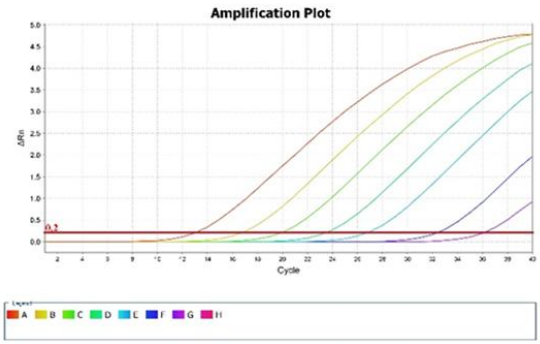


- Extension: The DNA Taq polymerase synthesizes a new cDNA strand and a new DNA strand from the existing strand by adding dNTPs to the growing DNA.
- During the amplification reaction step, when the probe is hybridized with the target, the probe is cleaved through endogenous 5′ nuclease activity, the reporter gene is separated from the quencher, and the resulting FAM fluorescence signal is proportional to the number of amplified products in the sample.

Data Comparison

Amplification plots compare the TOOLS miRNA Assay system with the kit from Company A. Synthetic RNA in 10-fold dilution from 1×10^{10} to 1×10^{4} copies/rxn was used as the template. Identical RT-qPCR programs were run using Applied Biosystems QuantStudio 12K Flex Real-Time PCR System to analyze hsa-miR-140-3p. The TOOLS kit outperformed the kit from Company A with higher detection sensitivity.

TOOLS



Company A