TaqMan® MicroRNA Cells-to-C$_T$™ Kit

Two-Step Real-Time RT-PCR for MicroRNA Directly from Cultured Cell Lysates

Part Numbers 4391848, 4391996

TaqMan® MicroRNA Cells-to-CT™ Kit

(P/N 4391848, 4391996)

Protocol

P/N 4391992 Revision D **Revision Date: August 31, 2010**

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I. Introduction

A. Product Description and Background

The TaqMan® MicroRNA Cells-to-CT™ Kit combines two innovative Applied Biosystems technologies; Cells-to-CT for direct reverse transcription of cultured cell lysates, and TaqMan microRNA (miRNA) amplification. The kit includes Cells-to-CT sample prep reagents and Applied Biosystems TaqMan microRNA reverse transcription reagents. Add a TaqMan MicroRNA Assay or a Multiplex RT for TaqMan MicroRNA Assays primer pool for the target(s) of your choice, and the TaqMan MicroRNA Cells-to-CT includes everything else you need to detect and quantitate mature miRNA directly in cell lysates without isolating RNA.

Traditionally, the first step in gene expression experiments has been to recover pure RNA from experimental samples. Even using the quickest and simplest techniques, however, RNA isolation is fairly time-consuming, often requiring 30 minutes or more of hands-on sample manipulation. Furthermore, with small samples, it can lead to loss of RNA. Cells-to-CT technology enables reverse transcription of lysates from 10–10^5 cultured cells without isolating or purifying RNA. Real-time PCR analysis is carried out directly afterwards. Eliminating the RNA isolation step substantially expedites and simplifies gene expression analysis of cultured cells.

Cells-to-CT lysates exhibit sensitivity and specificity similar to that from purified RNA in real-time RT-PCR using TaqMan® MicroRNA Assays. The lysis procedure simultaneously prepares cell lysates for RT-PCR and removes genomic DNA in ten minutes. The lysis step is simple to automate with robotic platforms for high-throughput processing of 96- or 384-well plates because it takes place entirely at room temperature. The procedure is also economical; there are only a few pipetting steps and, with cells grown in 96- or 384-well plates, no sample transfers.

Procedure overview

The TaqMan MicroRNA Cells-to-CT Kit procedure is shown in Figure 1 and described below.

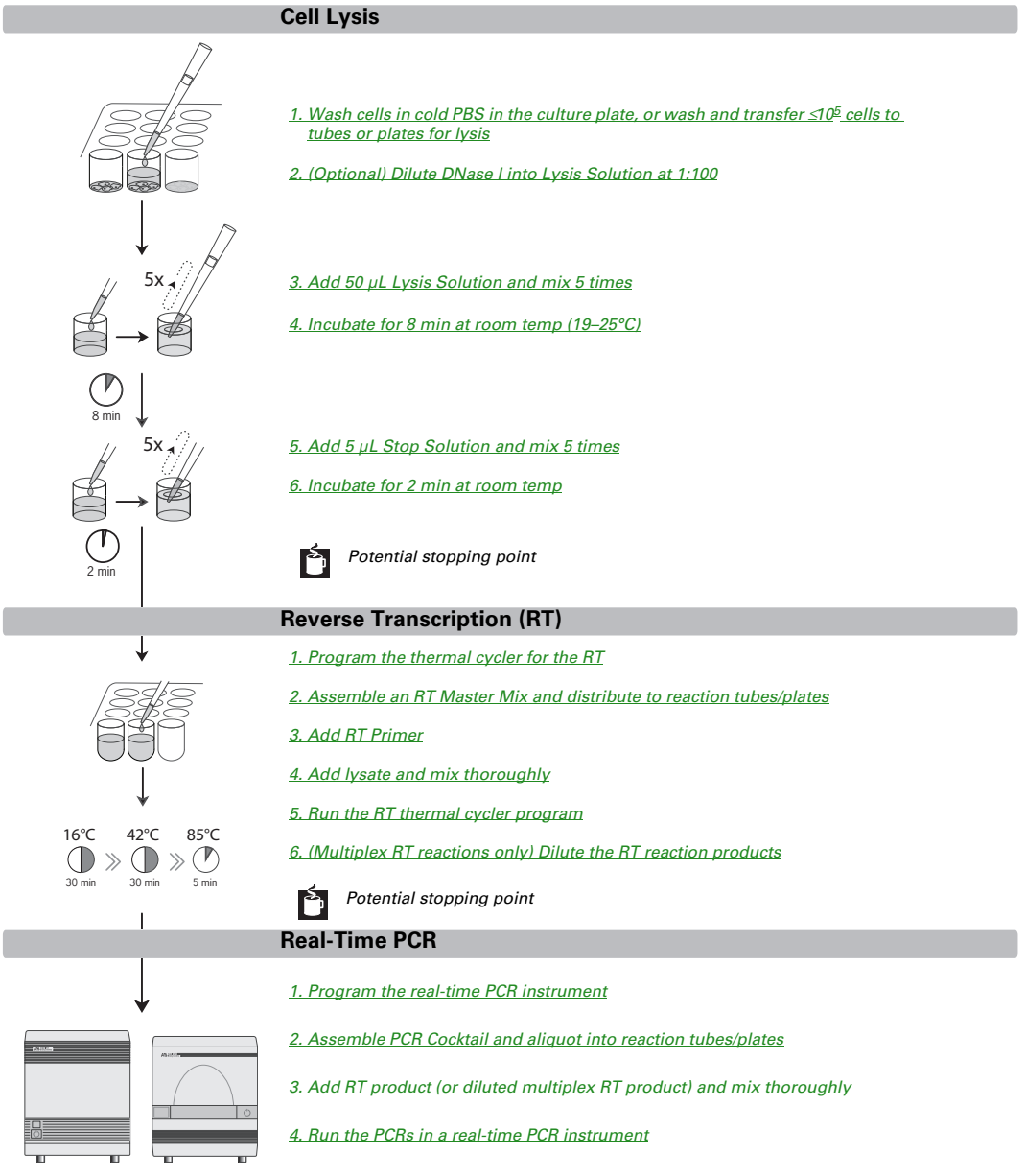
- First, 10–10^5 cultured cells are washed with phosphate-buffered saline (PBS), mixed with Lysis Solution, and incubated at room temperature for 8 min. Cells are lysed during this incubation and RNA is released into the Lysis Solution which contains reagents to inactivate endogenous RNases. If DNase I is added to the Lysis Solution (optional), genomic DNA is also degraded at this step.
- Next, Stop Solution is mixed into the lysate to inactivate the lysis reagents so that they will not inhibit the RT or PCR.

- Cell lysates are then reverse transcribed in reactions primed with the RT primer for the target-of-interest supplied with a TaqMan MicroRNA Assay or with a set of RT primers: Multiplex RT for TaqMan MicroRNA Assays (TaqMan MicroRNA Assays and RT primer pools for multiplex RT sold separately).
- Finally, the RT product is amplified by real-time PCR using the included TaqMan Universal PCR Master Mix No AmpErase® UNG and the TaqMan MicroRNA Assay for your target-of-interest (assays sold separately).

Applications

The TaqMan® MicroRNA Cells-to-CT™ Kit can be used in any real-time RT-PCR application to analyze miRNA from cultured cells. The development process included extensive testing for sensitivity and specificity with a broad selection of TaqMan MicroRNA Assays. The kit is well suited for large experiments, such as real-time PCR analysis of miRNA expression in differentially treated cell cultures or screening experiments using Ambion® Pre-miR™ miRNA Precursors or Anti-miR™ miRNA Inhibitors. Other applications include screening a library of compounds for their effects on miRNA expression, following the regulation of miRNA as cells are treated with increasing concentrations of a particular chemical, or evaluating the expression of miRNA in time course experiments.

Figure 1. TaqMan® MicroRNA Cells-to-C$_T$™ Procedure Overview



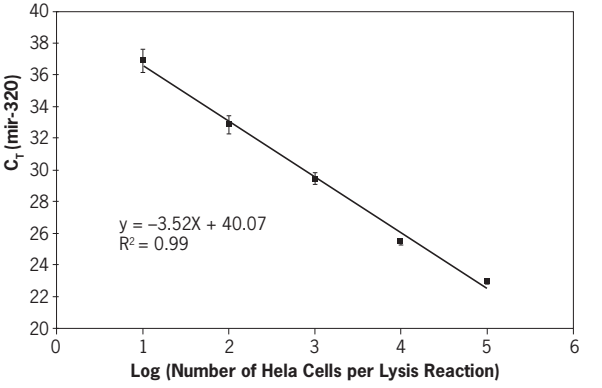


Figure 2. Real-time RT-PCR Using the TaqMan® MicroRNA Cells-to-CT™ Kit

A dilution series of 10–10^5 HeLa cells was processed in triplicate with the TaqMan® MicroRNA Cells-to-CT™ Kit. miR-320 was amplified from the cDNA in triplicate 20 µL reactions. The threshold cycle (C_T) compared to the input number of cells is shown here. Note that amplification was linear over a cell input range of 10–10^5 cells per lysis.

Cell type compatibility

Table 1 lists the cell types that have been shown to be compatible with the Cells-to-CT technology.

Table 1. Cell Types Compatible with Cells-to-CT™ Technology

Cell Line	Growth	Source Species	Source Tissue
A549	adherent	*H. sapiens*	Lung Carcinoma
BJ	adherent	*H. sapiens*	Foreskin fibroblast
CHO-K1	adherent	*C. griseus* (hamster)	Ovary
COS-7	adherent	*C. aethiops* (monkey)	Kidney
DU-145	adherent	*H. sapiens*	Prostate Carcinoma
HEK-293	adherent	*H. sapiens*	Kidney
HeLa	adherent	*H. sapiens*	Cervical Adenocarcinoma
HepG2	adherent	*H. sapiens*	Liver Carcinoma
Huh-7	adherent	*H. sapiens*	Liver carcinoma
Jurkat	suspension	*H. sapiens*	Acute T-Cell Leukemia
K-562	suspension	*H. sapiens*	Chronic Myelogenous Leukemia
ME-180	adherent	*H. sapiens*	Cervical Epidermoid Carcinoma
NCI-H460	adherent	*H. sapiens*	Large Cell Lung Cancer
Neuro 2A	adherent	*M. musculus* (mouse)	Brain blastoma
NIH/3T3	adherent	*M. musculus* (mouse)	Embryonic Fibroblast
PC-12	adherent	*R. norvegicus* (rat)	Adrenal Pheochromocytoma
Primary Hepatocytes	adherent	*H. sapiens*	Liver

Table 1. Cell Types Compatible with Cells-to-Ct™ Technology

Cell Line	Growth	Source Species	Source Tissue
PT-K75	adherent	*S. scrofa* (pig)	Nasal Turbinate Mucosa
Raji	suspension	*H. sapiens*	B Lymphocyte
SK-N-AS	adherent	*H. sapiens*	Brain Neuroblast
SK-N-SH	adherent	*H. sapiens*	Brain Fibroblast
U-87 MG	adherent	*H. sapiens*	Brain Glioblastoma
U-2 OS	adherent	*H. sapiens*	Bone osteosarcoma

B. Materials Provided with the Kit and Storage Conditions

The TaqMan® MicroRNA Cells-to-Ct™ Kit is available in a 100 or 400 lysis-reaction size. The number of RT reactions and PCR assays that can be done with each kit size is listed below:

- ***100 reaction*** (P/N 4391848) TaqMan MicroRNA Cells-to-Ct Kit includes reagents for 100 lysis reactions, and 200 RT reactions using an individual RT Primer or 25 RT reactions using a multiplex RT pool. The real-time PCR master mix can be used for either 200, 50 µL real-time PCR assays or 500, 20 µL reactions.

- ***400 reaction*** (P/N 4391996) TaqMan MicroRNA Cells-to-Ct Kit includes reagents for 400 lysis reactions, and 1000 RT reactions using an individual RT Primer or 125 RT reactions using a multiplex RT pool. The real-time PCR master mix can be used for either 800, 50 µL real-time PCR assays or 2000, 20 µL reactions.

The kits do not include TaqMan MicroRNA Assays, that contain the primers and probe for reverse transcription and real-time PCR.

NOTE

If your research requires more miRNA RT reactions than are provided with the kit, you can purchase the Applied Biosystems TaqMan MicroRNA Reverse Transcription Kit (P/N 4366596, 4366597) for use with Cells-to-Ct lysates.

Cells-to-Ct™ lysis and genomic DNA removal reagents

P/N 4391848 100 rxn	P/N 4391996 400 rxn	Component	Storage
500 µL	2 mL	Stop Solution	–20°C
55 µL	220 µL	DNase I	–20°C
5.5 mL	22 mL	Lysis Solution	4°C

MicroRNA reverse transcription reagents

200 µL	1 mL	MultiScribe™ Reverse Transcriptase	–20°C
1 mL	2 mL	10X RT Buffer	–20°C
200 µL	200 µL	dNTP Mix, 100 mM	–20°C
100 µL	200 µL	RNase Inhibitor (20 U/µL)	–20°C

Real-time PCR master mix

5 mL	4 x 5 mL	TaqMan® Universal PCR Master Mix, No AmpErase® UNG	4°C

The product is shipped on dry ice, but once received, the kit components should be stored at the temperatures indicated in the table above. Storage at –20°C should be in a non-frost-free freezer.

C. Materials Not Provided with the Kit

Lab equipment and supplies

- General laboratory equipment including vortex mixer, microcentrifuge, and pipettors
- Nuclease-free pipette tips, nuclease-free microcentrifuge tubes and/or U-bottom 96-well plates (for cells ***not*** grown in 96- or 384-well culture plates)
- Real-time PCR tubes or 96-well plates appropriate for your instrument
- Thermal cycling instrument: The TaqMan Cells-to-CT Kits were developed using Applied Biosytems thermal cyclers for the RT reaction and real-time PCR instruments for the PCR, however the technology is expected to be compatible with any thermal cycler for the RT reaction and any real-time PCR system compatible with the fluorophores used in TaqMan Gene Expression Assays. TaqMan MicroRNA Cells-to-CT reactions can be run on any currently available Applied Biosystems instrument platforms, including the following:

  Reverse transcription
  - GeneAmp® PCR System 9700
  - Veriti™ 96-Well Thermal Cycler

  Real-Time PCR
  - Applied Biosystems 7900 HT Fast Real-Time PCR System (in "standard" mode)
  - Applied Biosystems 7500 Real-Time PCR System
  - StepOne™ or StepOnePlus™ Real-Time PCR Systems

Reagents

- RT-PCR grade water, for example Ambion P/N AM9935
- Phosphate-buffered saline (PBS), for example diluted from Ambion 10X PBS, P/N AM9624 and AM9625
- TaqMan MicroRNA Assay for reverse transcription and real-time PCR of the target(s) of interest and (optional) Multiplex RT primer pools for TaqMan MicroRNA Assays:
  mirna.appliedbiosystems.com

D. Related Products Available from Applied Biosystems

TaqMan® MicroRNA Assays The current list of available TaqMan MicroRNA Assays is at: mirna.appliedbiosystems.com	TaqMan® MicroRNA Assays quantitate miRNAs with the specificity and sensitivity of TaqMan assay chemistry. Use a simple, two-step strategy of reverse transcription with an miRNA-specific primer, followed by real-time PCR with TaqMan probes. The assays target only mature miRNAs, not their precursors, ensuring biologically relevant results.
Multiplex RT for TaqMan® MicroRNA Assays P/N 4384791 and others	Multiplex RT for TaqMan® MicroRNA Assays are a set of eight predefined reverse transcription primer pools that streamline the process of creating cDNA for TaqMan assay analysis of 365 unique human miRNAs. Each individual Multiplex RT pool contains up to 48 primers per pool, including two endogenous controls for data normalization. Multiplex RT pools are formulated for use with individual TaqMan MicroRNA Assays providing better sensitivity and specificity, reduced sample consumption, and quicker miRNA profiling results compared to microarray-based methods.
Applied Biosystems Real-Time PCR Instruments See web or print catalog for P/Ns	Since pioneering real-time PCR, Applied Biosystems has continued to develop the technology to provide more powerful solutions for labs of all sizes. The Applied Biosystems family of real-time platforms, which includes the Applied Biosystems 7300, 7500, 7500 Fast, 7900HT Fast, StepOne™, and StepOnePlus™ Real-Time PCR Systems, provide cutting-edge tools while making real-time PCR more accessible than ever. These systems are easy to use with next generation software and the flexibility to run the real-time chemistry of your choice.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap*® RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.
RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion® RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com/prod/tubes) for specific information.
RT-PCR Grade Water P/N AM9935	Ambion® RT-PCR Grade Water is certified free of nucleases, and free of nucleic acid contamination that may cause false-positive signals in RT-PCR. The RT-PCR Grade Water is ready to use and requires no preparation, mixing, or autoclaving. Like all Ambion water products, RT-PCR Grade Water is deionized, autoclaved, and sterile filtered. Each lot is subjected to 2 rounds of rigorous quality control testing before being certified nuclease-free.
Plastic Consumables for PCR See web or print catalog for P/Ns	Applied Biosystems MicroAmp® disposables are optimized to provide unmatched temperature accuracy and uniformity for fast, efficient PCR amplification. The plastics are compatible with various Applied Biosystems platforms, including real-time PCR instruments. Choose your format based on throughput requirements or your thermal cycler block.
DNA*Zap*™ Solution P/N AM9890	DNA*Zap*™ PCR DNA degradation solution consists of two solutions that are innocuous separately but, when mixed, are able to degrade high levels of contaminating DNA and RNA from surfaces instantly.

II. TaqMan® MicroRNA Cells-to-CT™ Procedure

A. Suggested Controls and Replicates

Biological and technical replicates

The definitions of biological and technical replicates can have different interpretations; here we define biological replicates as separate lysis reactions and technical replicates as separate PCRs. Our minimum and recommended number of replicates are the following:

- Biological replicates: 2–4
- Technical (PCR) replicates: 2–4

No-template control

No-template controls contain all the PCR components except the cell lysate (substitute water). If the no-template control yields a fluorescent signal, it might indicate that the RT or PCR reagents were contaminated with DNA, for example; PCR product from previous reactions.

B. Cell Lysis

NOTE

These instructions describe using fresh cultured cells. If your experiment requires frozen cultured cells, we recommend washing the cells in cold PBS as described in step 1 (below) before freezing. When you are ready to start the procedure, allow the cells to thaw on ice, and begin the procedure at step 2.

Before you start

- Thaw Stop Solution, invert or flick the tube several times to mix thoroughly (do ***not*** vortex), and place on ice.
- Chill 1X PBS to 4°C.

1. Wash cells in cold PBS in the culture plate, or wash and transfer ≤10^5 cells to tubes or plates for lysis

IMPORTANT

The maximum number of cells that can be used in this procedure varies somewhat according to cell type, but is generally 10^5 cells. Instructions for a pilot experiment to determine the maximum number of cells per reaction is outlined in section IV.A starting on page 17. Using too many cells per lysis reaction may result in incomplete lysis and/or inhibition of RT-PCR.

Adherent cells grown in 96- or 384-well plates

It is important to start with cells that have been cultured until they are fully adhered to the plate, otherwise they will be lost during washing.

a. Estimate (or count) the number of cells per well. Make sure that each well contains 10–10^5 cells.

b. Aspirate and discard the culture medium from the wells.

c. Add 50 µL of cold (4°C) 1X PBS to each well.

d. Aspirate the PBS from the well. Remove as much PBS as possible without disturbing the cells. ***Proceed to step 2.***

Cells grown in other vessels (adherent and suspension cells)

a. Adherent cells only (for suspension cells, start at step b): Detach cells using the subculturing method routinely employed in your laboratory for the cell type. If trypsin is used, inactivate it before proceeding.

b. Count, then gently pellet the cells, aspirate and discard the growth medium, and place cells on ice.

Alternatively, cells can be split into aliquots for individual lysis reactions before washing in PBS.

c. Wash cells in cold (4°C) PBS by resuspending them in ~0.5 mL PBS per 10^6 cells (or ~50 µL PBS for ≤10^5 cells). Gently pellet the cells, then aspirate and discard as much of the PBS as possible without disturbing the cell pellet. Place the cells on ice.

d. Resuspend cells in fresh, cold 1X PBS so that 5 µL will contain the desired number of cells for a single lysis reaction (10–10^5 cells/lysis).

e. Split the cell suspension into individual lysis reactions: distribute 5 µL of the suspension to wells of a U-bottom multiwell plate or microcentrifuge tubes. Place the cells on ice.

2. (Optional) Dilute DNase I into Lysis Solution at 1:100

To remove genomic DNA during cell lysis, determine the volume of Lysis Solution needed for the experiment, and dilute DNase I into the Lysis Solution at 1:100 for use in the next step. Include ~10% overage; examples are shown in Table 2 below:

Table 2. Preparation of Lysis Solution Containing DNase I

Component	each rxn	96 rxns	384 rxns
Lysis Solution	49.5 µL	5.23 mL	20.91 mL
DNase I	0.5 µL	52.8 µL	211 µL

3. Add 50 µL Lysis Solution and mix 5 times

a. Add 50 µL Lysis Solution (with or without DNase I) to each sample.

b. Mix the lysis reaction by pipetting up and down 5 times.
To avoid bubble formation, mix with the pipettor set at 35 µL and expel the solution without emptying the pipette tip completely.

Lysis Solution and Cells-to-CT lysates may appear cloudy at room temperature—this is expected.

4. Incubate for 8 min at room temp (19–25°C)

Incubate the lysis reactions for 8 min at room temperature (19–25°C).

5. Add 5 µL Stop Solution and mix 5 times

a. Pipet 5 µL of Stop Solution directly into each lysis reaction.
Do this by touching the surface of the lysate with the opening of the pipet tip to ensure that all of the Stop Solution is added to the lysate.

NOTE

To dispense Stop Solution using a multichannel pipettor, pipet Stop Solution from a set of strip tubes or adjacent wells in a 96-well plate rather than from a reagent reservoir. If desired, unused Stop Solution can be stored frozen in these tubes when properly sealed.

b. Mix the lysis reaction by pipetting up and down 5 times.
To avoid bubble formation, mix with the pipettor set at 35 µL and expel the solution without emptying the pipette tip completely.

IMPORTANT

It is very important to thoroughly mix the Stop Solution into the lysate.

6. Incubate for 2 min at room temp

Incubate for 2 min at room temp (19–25°C).
Do not allow Cells-to-CT lysates to remain at room temp for longer than 20 min after adding the Stop Solution.

STOPPING POINT

Lysates can be stored on ice for ≤2 hr, or at –20°C or –80°C for ≤5 months.

C. Reverse Transcription (RT)

Before you start

- Thaw the RT reagents on ice.
- Leave the MultiScribe™ Reverse Transcriptase on ice without agitation.

1. Program the thermal cycler for the RT

Use 9600 Emulation Mode to program the thermal cycler as shown in Table 3.

Table 3. Thermal Cycler Settings for RT

	Stage	Reps	Temp	Time
Primer annealing (hold)	1	1	16°C	30 min
Reverse transcription (hold)	2	1	42°C	30 min
RT inactivation (hold)	3	1	85°C	5 min
Hold	4	1	4°C	indefinite

2. Assemble an *RT Master Mix* and distribute to reaction tubes/plates

a. The RT reaction setup recommendations are slightly different for reactions with a single RT primer and for multiplex RT using a pool of RT primers. Calculate the number of RT reactions in the experiment, and assemble an ***RT Master Mix*** for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube on ice. Follow Table 4 for RT using a single RT primer or Table 5 for RT using a multiplex RT pool.

NOTE

If a 15 µL RT reaction will not provide enough material for all of the planned PCR assays, the RT reaction can be scaled up proportionally.

Table 4. RT Master Mix for Single Primer Reactions: 15 µL Final Reaction Volume

Component	Each rxn*	96 rxns	384 rxns
10X RT Buffer	1.5 µL	158.4 µL	633.6 µL
dNTP Mix	0.15 µL	15.84 µL	63.36 µL
RNase Inhibitor	0.19 µL	20.06 µL	80.26 µL
MultiScribe™ RT	1 µL	105.6 µL	422.4 µL
Nuclease-free Water	4.16 µL	439.29 µL	1.757 mL
Final volume RT master mix	7 µL	739.2 µL	2.957 mL

* Each RT reaction will ultimately contain 7 µL RT Master Mix, 3 µL RT Primer, and 5 µL Cells-to-CT sample lysate.

Table 5. RT Master Mix for Multiplex Reaction: 40 µL Final Reaction Volume

Component	Each rxn*	8 rxns
10X RT Buffer	4 µL	35.2 µL
dNTP Mix	0.8 µL	7 µL
RNase Inhibitor	0.5 µL	4.4 µL
MultiScribe™ RT	8 µL	70.4 µL
Nuclease-free Water	12.7 µL	111.8 µL
Final volume RT master mix	26 µL	228.8 µL

* Each RT reaction will ultimately contain 26 µL RT Master Mix, 4 µL Multiplex RT primer pool, and 10 µL Cells-to-CT sample lysate.

b. Mix gently, but thoroughly, centrifuge briefly, then place the RT Master Mix on ice.

c. Distribute RT Master Mix to nuclease-free PCR tubes or wells of a multiwell plate.

3. Add RT Primer


NOTE

If more than one sample will be evaluated with a single TaqMan MicroRNA Assay or will be reverse transcribed with a single multiplex RT pool, the RT primer(s) can be added to the RT Master Mix before distribution to individual RT reaction containers.

Single primer RT reactions: Add 3 µL RT Primer from the TaqMan MicroRNA Assay for the target-of-interest to each aliquot of RT Master Mix.

Multiplex RT reactions: Add 4 µL Multiplex RT Primer Pool to each aliquot of RT Master Mix.

4. Add lysate and mix thoroughly

a. ***Single primer RT reactions:*** Add 5 µL sample lysate to each aliquot of RT Master Mix for a final 15 µL reaction volume.
Multiplex RT reactions: Add 10 µL sample lysate to each aliquot of RT Master Mix for a final 40 µL reaction volume.

b. Once assembled, mix reactions gently, then centrifuge briefly to collect the contents at the bottom of the reaction vessel.


STOPPING POINT

Assembled RT reactions can be stored at 4°C for up to 4 hr.

5. Run the RT thermal cycler program

Using a thermal cycler (or real-time PCR instrument), incubate at 16°C for 30 min, followed by 42°C for 30 min, and then 85°C for 5 min to inactivate the RT enzyme.


STOPPING POINT

Completed RT reactions may be stored at –20°C.

6. (Multiplex RT reactions only) Dilute the RT reaction products

Dilute Multiplex RT reactions 10-fold by adding 360 µL nuclease-free water to each 40 µL RT reaction.

(Do not dilute Single primer RT reactions.)

D. Real-Time PCR

1. Program the real-time PCR instrument

Program the real-time PCR instrument as in Table 6.

IMPORTANT

On Applied Biosystems real-time PCR instruments capable of Fast mode thermal cycling, select Standard mode.

- TaqMan® Universal PCR Master Mix contains ROX™ passive reference dye.
- Specify the fluorescent dye(s) used in the TaqMan MicroRNA Assays for the experiment.

Table 6. Real-Time PCR Cycling Conditions

	Stage	Reps	Temp	Time
Enzyme Activation (hold)	1	1	95°C	10 min
PCR (cycle)	2	40	95°C	15 sec
			60°C	1 min

2. Assemble *PCR Cocktail* and aliquot into reaction tubes/plates

a. The PCR setup recommendations are slightly different for samples that were reverse transcribed using a single RT primer or using a pool of RT primers (multiplex RT). Calculate the number of PCR assays in the experiment, and assemble a ***PCR Cocktail*** for all the reactions plus ~10% overage in a nuclease-free microcentrifuge tube on ice. Follow Table 7 for amplification of RT reactions that used a single RT primer or Table 8 for reactions that used a multiplex RT pool.

NOTE

Before use, mix the TaqMan Universal PCR Master Mix by swirling the bottle. Mix TaqMan Assays by vortexing briefly or flicking the tube a few times, and then centrifuging.

Table 7. PCR Cocktail Setup for Single Primer RT Reactions

Component	20 µL PCRs Each rxn	50 µL PCRs Each rxn
TaqMan® Master Mix (2X)	10 µL	25 µL
TaqMan® MicroRNA Assay*	1 µL	2.5 µL
Nuclease-free water	7.67 µL	19.17 µL
Final volume PCR cocktail	18.67 µL	46.67 µL

* Not included in the TaqMan MicroRNA Cells-to-CT Kit.

Table 8. PCR Cocktail Setup for Multiplex RT Reactions

Component	20 µL PCRs Each rxn	50 µL PCRs Each rxn
TaqMan® Master Mix (2X)	10 µL	25 µL
TaqMan® MicroRNA Assay*	1 µL	2.5 µL
Nuclease-free water	7.3 µL	18.25 µL
Final volume PCR cocktail	18.3 µL	45.75 µL

* Not included in the TaqMan MicroRNA Cells-to-CT Kit.

IMPORTANT

Be sure to use reaction containers and lids that are compatible with your real-time PCR instrument.

b. Distribute the PCR Cocktail into individual PCR tubes or wells of a real-time PCR plate at room temp.

3. Add RT product (or diluted multiplex RT product) and mix thoroughly

a. Add a portion of the RT product, or diluted multiplex RT product, to each aliquot of PCR Cocktail as indicated in Table 9.

Table 9. PCR Setup

Component	Single Primer RT		Multiplex RT	
	20 µL PCRs	50 µL PCRs	20 µL PCRs	50 µL PCRs
PCR cocktail	18.67 µL	46.67 µL	18.3 µL	45.75 µL
RT product from C.5 on page 12	1.33 µL	3.33 µL	– –	– –
Diluted RT product from C.6 on page 12	– –	– –	1.7 µL	4.25 µL

b. Cover the plate or close the tubes, and mix gently. Then centrifuge briefly to remove bubbles and collect the contents at the bottom of the wells/tubes.

4. Run the PCRs in a real-time PCR instrument

Place the reactions in a real-time PCR instrument and start the run using the settings programmed in step D.1 on page 13.

Refer to your real-time PCR instrument guide for information on evaluating the data.

III. Troubleshooting

A. No PCR Product or Unexpected PCR Products

Problems with adding or mixing the Stop Solution

Components in the Lysis Solution may inhibit RT-PCR if they are not fully inactivated by the Stop Solution.

- Be sure to add the Stop Solution directly to the lysate, in other words, touch the lysate with the opening of the pipet tip when adding the Stop Solution to make sure that the entire 5 µL of Stop Solution is added to each sample.
- Also, we recommend mixing by pipetting up and down five times.

RNA was degraded before starting the procedure

To avoid RNA degradation, keep cells in PBS on ice before starting the cell lysis procedure. Take cells off ice just prior to adding Lysis Solution.

RNase in the sample was not completely inactivated

Too many cells were used in the lysis reaction

If too many cells per sample are used in the procedure, the RNase in the sample may not be totally inactivated and/or cellular components or debris could inhibit reverse transcription or PCR.

- Generally ≤10^5 cells can be used successfully in the Cells-to-Ct™ procedure, but if RT or PCR fails, try using fewer cells (e.g., 5–10-fold fewer cells).
- Also, consider performing a pilot experiment to determine the optimal number of cells for your cell type (section IV.A on page 17).

Too much PBS was left on the cells, diluting the Lysis Solution

If >5 µL of PBS remains in samples when the Lysis Solution is added, the Lysis Solution may be too dilute to fully inactivate cellular RNases. To avoid this, remove as much PBS as possible before adding Lysis Solution to the cells, or if you split the cells after the PBS wash, resuspend cells in ≤ µL PBS for each sample of 10–10^5 cells.

Lysates sat too long before going into RT

Do not allow lysates to sit longer than 20 min at room temperature once the Stop Solution has been added: either freeze the lysates at –20°C or –80°C, or start the RT reactions. Alternatively, lysates can be safely stored on ice for up to 2 hr after lysis.

The sample does not contain the target miRNA

Negative results are often difficult to confirm as valid. Consider running the following experiments before concluding that the sample does not contain the miRNA of interest:

- ***For experiments with samples consisting of <100 cells per lysis,*** it may be desirable to verify that each sample did, in fact, contain cells. Check that samples contained cells with intact RNA by real-time RT-PCR with a TaqMan MicroRNA Assay for a highly-expressed endogenous control miRNA such as RNU48.

- ***Check that the PCR for your target works with your PCR primers, reagents, and equipment*** by using cDNA generated from purified RNA from the same source (or a similar one) in PCR. If the amplification does not give good results using cDNA from purified RNA, it will not work with Cells-to-CT lysate.

B. PCR Products in the No-Template PCR Control

Samples were contaminated with PCR product from previous reactions

PCR products in the no-template PCR control most commonly indicate that the sample contains DNA contamination—typically from completed PCRs. Contamination of PCR reagents, pipettors, and benchtops with DNA should be considered.

- Careful laboratory practices are essential to avoid contaminating reactions with PCR products. Keep concentrated DNA solutions (PCR products, plasmid prep, etc.) away from the area where PCRs are assembled. Clean the lab bench and the pipettors routinely with Ambion DNA*Zap*™ Solution (P/N AM9890) or another DNA decontamination product. Use barrier tips to pipette PCR reagents, and store completed PCRs in a different location from the PCR reagents. Unfortunately, the only way to remedy contaminated reagents is to replace them.
- It is always a good idea to routinely include a no-template negative control reaction with experimental PCRs. If no-template controls routinely yield PCR products, more stringent steps may be taken to control contamination.

More than the recommended volume of RT product used in the PCR

Use the recommended volume of RT product or diluted RT product in the PCR (see step II.D.3 on page 14). Using more than the recommended amount can result in a positive signal in no-template PCR controls.

IV. Appendix

A. Pilot Experiment

The purpose of this pilot experiment is to identify the maximum number of cells to use in TaqMan® MicroRNA Cells-to-CT™ reactions. Using too many cells can result in inefficient cell lysis and RT-PCR inhibition, and the maximum number of cells varies somewhat according to the cell type.

In this simple experiment, cells are serially diluted and lysed following the normal protocol. The lysates are then subjected to real-time RT-PCR for an endogenous control microRNA, such as RNU48 (Applied Biosystems P/N 4373383), and the C_T values are plotted against the log of the number of cells in the lysis reaction. The resulting line will be linear for cell numbers that are compatible with the procedure and will deviate from linearity at concentrations that result in incomplete lysis or RT-PCR inhibition.

1. Harvest, count, and wash cells

Follow the instructions for harvesting cells in step II.B.1 on page 8, but follow the instructions in step 2 (next) for resuspending them in PBS.

2. Dilute cells to 2 x 10^5 cells/µL

Prepare a cell suspension containing 2 x 10^5 cells/µL. Keep the cell suspension on ice.

3. Make 5 serial dilutions of the cells in 5-fold increments

a. Prepare 5 tubes containing 45 µL of cold 1X PBS in ice.

b. Transfer 5 µL of the 2 x 10^5 cells/µL to the first tube (1:5 dilution) and mix gently but thoroughly. Continue making the serial dilutions by transferring 5 µL of each solution to the subsequent tube to finish with 5 suspensions containing 2 x 10^4, 2000, 200, 20, and 2 cells per µL.

4. Transfer cells to reaction containers with 3 replicates

Transfer 5 µL of each cell suspension to individual reaction tubes or wells of a multiwell plate. Include 3 biological replicates of each cell concentration.

The final cell counts will be 10^5, 10^4, 1000, 100, and 10 cells.

5. Lyse cells in 50 µL of Lysis Solution and incubate at room temp for 8 min

a. Add 50 µL of Cells-to-CT Lysis Solution to each of the samples prepared in step 4.

b. Mix thoroughly by pipetting up and down 5 times.

c. Incubate at room temperature for 8 min.

6. Add 5 µL Stop Solution, mix, and incubate at room temp for 2 min

a. Add 5 µL Stop Solution to each cell lysate, and mix thoroughly by pipetting up and down 5 times.

b. Incubate at room temp for 2 min.

7. Perform RT-PCR

Follow the protocols described in sections II.C–D to reverse transcribe and perform PCR using all samples.

- Amplify with an endogenous control microRNA, such as RNU48.
- You can also evaluate real-time PCR of the cell titration using the TaqMan MicroRNA Assay for the target-of-interest to help determine the minimum number of cells required for its detection.

8. Evaluate results

Endogenous control

Create a plot of C_T versus the log of the number of cells in the lysis. The C_T values should decrease in a linear fashion as the number of cells increase, for cell numbers that are compatible with the procedure. When the number of cells per lysis reaction exceeds the capacity of the system, resulting in incomplete lysis or inhibition of RT-PCR, the data will not be linear. In future experiments, do not exceed the number of cells per lysis reaction that provided results within the linear range in the pilot experiment.

Target-of-interest

The pilot experiment can provide useful information about the number of cells required to detect the target-of-interest. Examine the results carefully and choose cell numbers that will provide sufficient signal for the experiment.

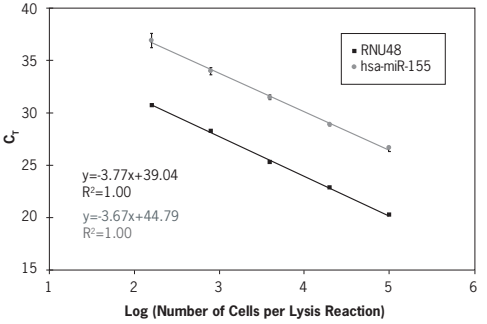


Figure 3. Example Pilot Experiment

HeLa cells were diluted and lysed following the instructions for the pilot experiment. Lysates were reverse transcribed and amplified using the indicated TaqMan MicroRNA Assay in three replicate reactions. The resulting C_T values were plotted against the log of the number of cells used in the lysis reaction (as recommended). This experiment shows that even at 10^5 cells per lysis reaction, RT-PCR is not compromised by reaction inhibitors carried over from the cell lysate. It also shows that 160 cells, the lowest number of cells/lysis reaction tested, is too small a sample size for reliable detection of hsa-miR-155.

B. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

C. Quality Control

Functional testing

Lysis components are functionally tested by producing cDNA from a serial dilution of 10–10^5 cultured cells, detecting PPIA using real-time PCR, and comparing amplification signal from Cells-to-CT lysates to those obtained from purified RNA from the same source.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with super-coiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.