

Megaplex™ Pools

For microRNA Expression Analysis

Protocol

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Preface and Important Safety Information

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Safety Information


Safety Alert Words Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—IMPORTANT, CAUTION, WARNING, DANGER—implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

 **CAUTION** – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

 **WARNING** – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

 **DANGER** – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning



WARNING CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See [“About MSDSs”](#) on page vi.)
- 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 in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to www.appliedbiosystems.com, click **Support**, then click **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
 - **Open** – To view the document
 - **Print Target** – To print the document
 - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide 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.)
- 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.
- Handle chemical wastes in a fume hood.

- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates in your lab.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- 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. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories (stock no. 017-040-00547-4; bmbi.od.nih.gov)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at www.cdc.gov.

How to Obtain More Information

Related Documentation For additional documentation, see “[How to Obtain Support](#)” on [page x](#).

Real-Time PCR System	Document	Part Number
All real-time PCR systems	<i>Real-Time PCR Systems Chemistry Guide</i>	4348358
	<i>TaqMan® PreAmp Master Mix Kit Protocol</i>	4384557
	<i>Applied Biosystems TaqMan® Low Density Array User Bulletin</i>	4371129
7900HT System with TaqMan Array Block	<i>Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide</i>	4347825
	<i>Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide</i>	4364016

Reference URLs

Topic	Web Address
Megaplex Pool Composition	http://miRNA.appliedbiosystems.com
TaqMan MicroRNA Array Layout Information	

How to Obtain Support

For the latest services and support information for all locations, go to www.appliedbiosystems.com, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Megaplex™ Pools Protocol

Product Information

- Purpose of the Product** Megaplex™ Pools are designed to detect and quantitate up to 380 microRNAs (miRNAs) per pool in either human, mouse, or rat species using Applied Biosystems real-time instruments. Megaplex Pools consist of matching primer pools and TaqMan® Arrays:
- **Megaplex™ RT Primers** is a set of two predefined pools (Pool A and Pool B) of up to 380 stem-looped reverse-transcription (RT) primers per pool that enable the simultaneous synthesis of cDNA for mature miRNAs.
 - **Megaplex™ PreAmp Primers (Optional)** is a set of two pools (Pool A and Pool B) of gene-specific forward and reverse primers intended for use with very small quantities of starting material. The primers enable the unbiased preamplification of the miRNA cDNA target by PCR prior to loading the TaqMan® MicroRNA Array.
 - **TaqMan® MicroRNA Arrays** is a set of two 384-well microfluidic cards (Array A and Array B) containing dried TaqMan primers and probes. The array enables quantitation of gene expression levels of up to 380 miRNAs and controls. This is accomplished by loading the cDNA product (with or without preamplification) onto the array for PCR amplification and real-time analysis.
- Each TaqMan MicroRNA Array is shipped with an Information CD that includes:
- Assay Information File (AIF)
 - Card map file (in .html and .xls formats)
 - SDS setup file; SDS.txt
 - *Megaplex™ Pools Protocol* (PN 4399721)
 - *Megaplex™ Pools Quick Reference Card* (PN 4399813)
 - *Applied Biosystems TaqMan® Low Density Array User Bulletin* (PN 4371129)
 - Megaplex™ Assay Performance File

Contents For details on how to order or on the composition of the primer pools, refer to the Megaplex™ products page at <http://miRNA.appliedbiosystems.com>

Megaplex™ RT Primers	Part Number	Contents
Megaplex™ RT Human Pool A	4399966	<ul style="list-style-type: none"> • 50 µL of 10X primers • 1000 µL 25 mM MgCl₂
Megaplex™ RT Human Pool B	4399968	
Megaplex™ RT Rodent Pool A	4399970	
Megaplex™ RT Rodent Pool B	4399972	

Optional Megaplex™ PreAmp Primers	Part Number	Contents
Megaplex™ PreAmp Primers, Human Pool A	4399233	150 µL of 10X primers
Megaplex™ PreAmp Primers, Human Pool B	4399201	
Megaplex™ PreAmp Primers, Rodent Pool A	4399203	
Megaplex™ PreAmp Primers, Rodent Pool B	4399237	

TaqMan® MicroRNA Arrays	Part Number	Contents
TaqMan® Human MicroRNA Array A	4398965	<ul style="list-style-type: none"> • 384-well array • An Information CD
TaqMan® Human MicroRNA Array B	4398966	
TaqMan® Rodent MicroRNA Array A	4398967	
TaqMan® Rodent MicroRNA Array B	4398968	

Storage The TaqMan MicroRNA Arrays are shipped at ambient temperature. When you receive the arrays, store them at 2 to 8 °C and keep them protected from light.

Store the Megaplex RT and PreAmp primer pools at –15 to –25 °C.

Section 1 Prepare the Sample and Choose a Workflow

Prepare the Sample

The starting material is total RNA. To prepare the starting material, use the *mirVana*[™] miRNA Isolation Kit (see [Appendix A on page 23](#) for part numbers).

Note: The method used to isolate the total RNA must preserve the small RNA fraction. However, in order to preserve endogenous control sequences in the total RNA, do *not* enrich for the small RNA fraction. This may cause longer control transcripts (snoRNAs) to be lost.

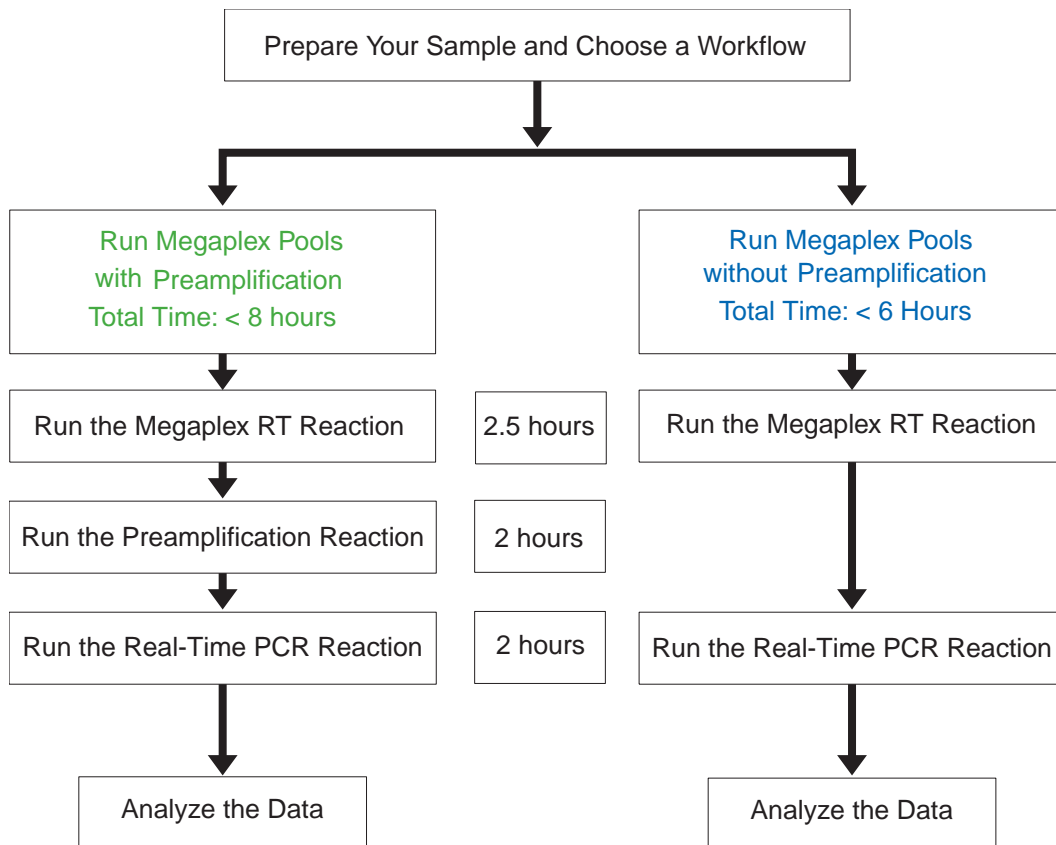
Choose a Workflow

Criteria Two workflows are described in this protocol: one with preamplification of your sample and one without preamplification of your sample.

IMPORTANT! Although a profile can be generated with larger amounts of sample, preamplification is recommended for all samples.




If the total amount of RNA is...	Then choose to...	Described in...
1 – 350 ng	Run Megaplex [™] Pools <i>with</i> preamplification	Section 2 on page 5 .
350 – 1000 ng	Run Megaplex Pools <i>without</i> preamplification	Section 3 on page 15 .

Workflow



Section 2 Run Megaplex™ Pools With Preamplification

This section describes how to:

	<p>Run the Megaplex™ RT Reactions:</p> <ul style="list-style-type: none"> • Prepare the RT Reaction Mix. • Perform Megaplex Reverse Transcription. • (Optional) Stopping Point. 	<p>Use these reagents:</p> <ul style="list-style-type: none"> • Megaplex RT Primers • TaqMan® MicroRNA Reverse Transcription Kit
↓		
	<p>Run the Preamplification Reactions:</p> <ul style="list-style-type: none"> • Prepare the PreAmp Reaction Mix. • Perform the Preamplification. • Dilute the Reaction. • (Optional) Stopping Point. 	<p>Use these reagents:</p> <ul style="list-style-type: none"> • Megaplex PreAmp Primers • TaqMan® PreAmp Master Mix, 2X
↓		
	<p>Run the Real-Time PCR Reactions:</p> <ul style="list-style-type: none"> • Prepare the TaqMan® MicroRNA Array. • Prepare the PCR Reaction Mix. • Load and Run the Array. 	<p>Use these reagents:</p> <ul style="list-style-type: none"> • TaqMan® Universal PCR Master Mix, No AmpErase UNG, 2X • TaqMan® MicroRNA Array

For each sample (and optional no template control), run one Megaplex RT reaction followed by one preamplification reaction per array. This provides enough sample to load one TaqMan MicroRNA array.

For a full microRNA profile, run two Megaplex RT reactions (Pool A and B), two preamplification reactions (Pool A and B), and two TaqMan MicroRNA Arrays (Array A and B) per sample.

Run the Megaplex™ RT Reactions

About the RT Reaction

In this step, use the TaqMan® MicroRNA Reverse Transcription Kit and the Megaplex™ RT Primers to synthesize single-stranded cDNA from total RNA samples. (See [Appendix A on page 23](#) for part numbers.)

The RT reaction with downstream preamplification supports 1 to 1000 ng of input total RNA. For most tissues, 30 ng of total RNA produces a comprehensive microRNA profile with preamplification. Applied Biosystems recommends verifying the optimal quantity of input total RNA for your sample type.

The reverse transcription (RT) reaction has a final volume of 7.5 µL and contains:

- 3 µL (1 to 350 ng) total RNA
- 4.5 µL of RT reaction mix

Prepare the RT Reaction Mix

1. Thaw the following on ice:
 - Megaplex RT Primers
 - TaqMan® MicroRNA Reverse Transcription Kit components
 - MgCl₂ (supplied with the Megaplex™ RT Primers)
2. Combine the following in a 1.5-mL microcentrifuge tube:

RT Reaction Mix Components	Volume for One Sample (μL)	Volume for Ten Samples (μL)‡
Megaplex™ RT Primers (10×)	0.80	9.00
dNTPs with dTTP (100 mM)	0.20	2.25
MultiScribe™ Reverse Transcriptase (50 U/μL)	1.50	16.88
10× RT Buffer	0.80	9.00
MgCl ₂ (25 mM)	0.90	10.12
RNase Inhibitor (20 U/μL)	0.10	1.12
Nuclease-free water	0.20	2.25
Total	4.50	50.62

‡ Includes 12.5% excess for volume loss from pipetting.

3. Invert the tube six times to mix, then centrifuge the tubes briefly.
4. In a 96-well MicroAmp® Optical Reaction Plate or MicroAmp® 8-Tube Strip, pipette 4.5 μL of the RT reaction mix into each well or each tube, respectively.
5. Add 3 μL (1 to 350 ng) total RNA (or 3 μL of water for the No Template Control reactions) into each well or each tube containing RT reaction mix.
6. Seal the plate or tubes using MicroAmp® Clear Adhesive Film or MicroAmp® Optical Strip Caps, respectively. Then invert the plate or tubes six times to mix. Spin briefly.

Note: Do not use MicroAmp® *Optical* Adhesive Film to seal the plate.
7. Incubate the plate on ice for 5 min.

**Perform
Megaplex™
Reverse
Transcription**

1. Set up the run method using the following conditions:
 - Ramp speed or mode: **9700** using **Std** or **Max** ramp speed. **7900HT** using **Std** ramp speed.
 - Reaction volume (µL): **7.5**
 - Thermal-cycling conditions:

Stage	Temp	Time
Cycle (40 Cycles)	16 °C	2 min
	42 °C	1 min
	50 °C	1 sec
Hold	85 °C	5 min
Hold	4 °C	∞

2. Load, then run the plate.

Note: If you are running the plate on a 7900HT System and sealing the plate with MicroAmp Clear Adhesive Film (PN 4306311), then place a MicroAmp® Optical Film Compression Pad (PN 4312639) on top of the plate.

**(Optional)
Stopping Point**

The cDNA can be stored at –15 to –25 °C for at least one week.

Run the Preamplification Reactions

About the Preamplification Reaction

In this step, preamplify specific cDNA targets to increase the quantity of desired cDNA for gene expression analysis using TaqMan® MicroRNA Arrays.

The preamplification reaction has a final volume of 25 µL and contains:

- 2.5 µL RT product
- 22.5 µL PreAmp reaction mix

Prepare the PreAmp Reaction Mix

1. Thaw the Megaplex™ PreAmp Primers on ice and mix by inverting six times. Spin briefly.
2. Mix the TaqMan® PreAmp Master Mix (2X) by swirling the bottle.
3. Combine the following in a 1.5-mL microcentrifuge tube:

PreAmp Reaction Mix Components	Volume for One Sample (µL)	Volume for Ten Samples (µL)‡
TaqMan® PreAmp Master Mix (2X)	12.5	140.62
Megaplex™ PreAmp Primers (10X)	2.5	28.13
Nuclease-free water	7.5	84.37
Total	22.5	253.12

‡ Includes 12.5% excess for volume loss from pipetting.

4. Invert the tube six times to mix, then centrifuge the tubes briefly.
5. In a 96-well MicroAmp® Optical Reaction Plate or MicroAmp® 8-Tube Strips, pipette 2.5 µL of each RT product (from [“Perform Megaplex™ Reverse Transcription” on page 8](#)) into its corresponding well or tube.
6. Dispense 22.5 µL of PreAmp reaction mix into each well of the 96-well plate or MicroAmp Optical 8-Tube Strips containing the RT product.

7. Seal the plate or tubes using MicroAmp® Clear Adhesive Film or MicroAmp® Optical Strip Caps, respectively. Then invert the plate or tubes six times to mix. Spin briefly.
8. Incubate the plate or tubes on ice for 5 min.

Perform the Pre-amplification

1. Set up the run method using the following conditions:
 - Ramp speed or mode: **9700** using **Std** ramp speed.
 - Reaction volume (µL): **25**
 - Thermal-cycling parameters:

Stage	Temp	Time
Hold	95 °C	10 min
Hold	55 °C	2 min
Hold	72 °C	2 min
Cycle (12 Cycles)	95 °C	15 sec
	60 °C	4 min
Hold‡	99.9 °C	10 min
Hold	4 °C	∞

‡ Required for enzyme inactivation.

2. Load, then run the plate.

Dilute the Reaction

1. Remove the 96-well plate or 8-tube strips from the thermal cycler.
2. Briefly centrifuge the tubes or plate.
3. Add 75 µL of 0.1× TE pH 8.0 to each well or tube.
4. Seal the plate or tubes, then invert six times to mix, and spin briefly.
5. Proceed directly to [“Run the Real-Time PCR Reactions” on page 12.](#)

(Optional) Stopping Point

Store the diluted preamplified product at –15 to –25 °C for up to one week, or place the product on ice and proceed directly to [“Run the Real-Time PCR Reactions” on page 12.](#)

Run the Real-Time PCR Reactions


About the Real-Time PCR Reaction

In this step, the DNA polymerase from the TaqMan® Universal PCR Master Mix amplifies the preamplified target cDNA using sequence-specific primers and probe on the TaqMan® MicroRNA Array. The presence of the target is detected in real time through cleavage of the TaqMan probe by the polymerase 5' - 3' exonuclease activity.

Prepare the TaqMan® MicroRNA Array

After the TaqMan® MicroRNA Array has reached room temperature, carefully remove it from its packaging. Refer to the *Applied Biosystems TaqMan® Array User Bulletin* (PN 4371129).

Prepare the PCR Reaction Mix

 **CAUTION CHEMICAL HAZARD.** TaqMan® Universal PCR Master Mix, No Amperase UNG may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Thaw the diluted, stored PreAmp product (from “[Dilute the Reaction](#)” on page 11) on ice. Mix by inverting six times, then centrifuge the tube or plate briefly.
2. Mix the TaqMan Universal PCR Master Mix by swirling the bottle.
3. Combine the following in a 1.5-mL microcentrifuge tube:

Component	Volume for One Array [‡]
TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X	450
Diluted PreAmp product	9
Nuclease-free water	441
Total	900

[‡] Includes 12.5% excess for volume loss from pipetting.

4. Invert the tube six times to mix, then centrifuge the tubes briefly.

Load and Run the MicroRNA Array

For detailed information on how to load, seal, and run the array, refer to the *Applied Biosystems TaqMan® Array User Bulletin* (PN 4371129).

1. Dispense 100 μ L of the PCR reaction mix into each port of the TaqMan MicroRNA Array.
2. Centrifuge, then seal the array.
3. Import the SDS setup file (SDS.txt) located on the Information CD:
 - a. Start the SDS v2.2 or later software.
 - b. In the main menu, select **File ▶ New**
 - c. In the New Document dialog box, select the following from the drop-down menu:
 - **Relative Quantification ($\Delta\Delta C_t$)**
 - **384-well TaqMan Low Density Array**
 - d. In the main menu, select **File ▶ Import** to open the new document.
 - e. In the Open dialog box, navigate to the Setup.txt file specific for the array being run and click **Import**.
 - f. (Optional) Save as an SDS 7900 Template (.sdt) file.
4. Load and run the array using the 384 well TaqMan Low Density Array default thermal-cycling conditions.

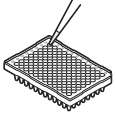
Note: If you are using a 7900HT System with an automation accessory, load a maximum of 24 TaqMan® arrays onto the automation accessory at a time. Arrays containing preamplified product can be kept at room temperature for up to 48 hrs (24 arrays \times 2 hrs/run = 48 hrs).

Analyze the Data


For instructions on how to analyze and troubleshoot the data, proceed to [page 21](#).

Section 3 Run Megaplex™ Pools Without Preamplification

This section describes how to:

	<p>Run the Megaplex™ RT Reactions:</p> <ul style="list-style-type: none"> • Prepare the RT Reaction Mix. • Perform Megaplex Reverse Transcription. • (Optional Stopping Point). 	<p>Use these reagents:</p> <ul style="list-style-type: none"> • Megaplex RT Primers • TaqMan® MicroRNA Reverse Transcription Kit
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	<p>Run the Real-Time PCR Reactions:</p> <ul style="list-style-type: none"> • Prepare the TaqMan® MicroRNA Array. • Prepare the PCR Reaction Mix. • Load and Run the Array. 	<p>Use these reagents:</p> <ul style="list-style-type: none"> • TaqMan® Universal PCR Master Mix, No AmpErase UNG, 2X • TaqMan® MicroRNA Array
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For each sample (and optional no template control), one Megaplex RT reaction provides enough sample to load one TaqMan MicroRNA Array.

For a full microRNA profile, run two Megaplex RT reactions (Pool A and B) and two TaqMan MicroRNA Arrays (Array A and B) per sample.

Run the Megaplex™ RT Reactions

About the RT Reaction

In this step use the TaqMan® MicroRNA Reverse Transcription Kit and the Megaplex™ RT Primers to synthesize single-stranded cDNA from total RNA samples. (See [Appendix A on page 23](#) for part numbers).

The RT reaction supports 350 to 1000 ng of input total RNA. For most tissues, 500 ng of total RNA produces a comprehensive microRNA profile. Applied Biosystems recommends verifying the optimal quantity of input total RNA for your sample type.

The reverse transcription (RT) reaction has a final volume of 7.5 μ L and contains:

- 3 μ L (350 to 1000 ng) total RNA
- 4.5 μ L of RT reaction mix

Prepare the RT Reaction Mix

1. Thaw the following on ice:
 - Megaplex RT Primers
 - TaqMan® MicroRNA Reverse Transcription Kit components
 - MgCl₂ (supplied with the Megaplex RT Primers)
2. Combine the following in a 1.5-mL microcentrifuge tube:

RT Reaction Mix Components	Volume for One Sample (μL)	Volume for Ten Samples (μL)‡
Megaplex™ RT Primers (10×)	0.8	9.00
dNTPs with dTTP (100 mM)	0.2	2.25
MultiScribe™ Reverse Transcriptase (50 U/μL)	1.5	16.88
10× RT Buffer	0.8	9.00
MgCl ₂ (25 mM)	0.9	10.12
RNase Inhibitor (20 U/μL)	0.1	1.12
Nuclease-free water	0.2	2.25
Total	4.5	50.62

‡ Includes 12.5% excess for volume loss from pipetting.

3. Invert the tube six times to mix, then centrifuge the tubes briefly.
4. In a 96-well MicroAmp® Optical Reaction Plate or MicroAmp® 8-Tube Strip, pipette 4.5 μL of the RT reaction mix into each well or each tube, respectively.
5. Add 3 μL (350 to 1000 ng) total RNA (or 3 μL of water for the No Template Control reactions) into each well or each tube containing RT reaction mix.
6. Seal the plate or tubes using MicroAmp® Clear Adhesive Film or MicroAmp® Optical Strip Caps respectively, then invert the plate or tubes six times to mix. Spin briefly.

Note: Do not use *Optical* Adhesive Film to seal the plate.
7. Incubate the plate or tubes on ice for 5 min.

**Perform
Megaplex™
Reverse
Transcription**

1. Set up the run method using the following conditions:
 - Ramp speed or mode: **9700** using **Std** or **Max** ramp speed. **7900HT** using **Std** ramp speed.
 - Reaction volume (µL): **7.5**
 - Thermal-cycling conditions:

Stage	Temp	Time
Cycle (40 Cycles)	16 °C	2 min
	42 °C	1 min
	50 °C	1 sec
Hold	85 °C	5 min
Hold	4 °C	∞

2. Load, then run the plate.

Note: If you are running the plate on a 7900HT System and sealing the plate with MicroAmp Clear Adhesive Film (PN 4306311), then place a MicroAmp® Optical Film Compression Pad (PN 4312639) on top of the plate.

**(Optional)
Stopping Point**

The cDNA can be stored at –15 to –25 °C for at least one week.

Run the Real-Time PCR Reactions

About the Real-Time PCR Reaction

In this step, the DNA polymerase from the TaqMan® Universal PCR Master Mix amplifies the target cDNA using sequence-specific primers and probe on the TaqMan® MicroRNA Array. The presence of the target is detected in real time through cleavage of the TaqMan probe by the polymerase 5' - 3' exonuclease activity.

Prepare the TaqMan® MicroRNA Array

After the TaqMan® MicroRNA Array has reached room temperature, carefully remove it from its packaging. Refer to the *Applied Biosystems TaqMan® Array User Bulletin* (PN 4371129).

Prepare the PCR Reaction Mix



CAUTION CHEMICAL HAZARD. TaqMan® Universal PCR Master Mix, No AmpErase UNG may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Thaw the stored Megaplex RT product on ice and mix by inverting the tube six times.
2. Mix the TaqMan Universal PCR Master Mix by swirling the bottle.
3. Combine the following in a 1.5-mL microcentrifuge tube:

Component	Volume for One Array [‡]
TaqMan® Universal PCR Master Mix, No AmpErase® UNG, 2X	450
Megaplex™ RT product	6
Nuclease-free water	444
Total	900

[‡] Includes 12.5% excess for volume loss from pipetting.

4. Invert the tube six times to mix, then centrifuge the tubes briefly.

Load and Run the TaqMan® MicroRNA Array

For detailed information on how to load, seal, and run the array, refer to the *Applied Biosystems TaqMan® Array User Bulletin* (PN 4371129).

1. Dispense 100 µL of the PCR reaction mix into each port of the TaqMan MicroRNA Array.
2. Centrifuge, then seal the array.
3. Import the SDS setup file (SDS.txt) located on the Information CD:
 - a. Start the SDS v2.2 or later software.
 - b. In the main menu, select **File ▶ New**
 - c. In the New Document dialog box, select the following from the drop-down menu:
 - **Relative Quantification (ΔΔCt)**
 - **384 well TaqMan Low Density Array**
 - d. In the main menu, select **File ▶ Import** to open the new document.
 - e. In the Open dialog box, navigate to the Setup.txt file specific for the array being run and click **Import**.
 - f. (Optional) Save as an SDS 7900 Template (.sdt) file.
4. Load and run the array using the 384 well TaqMan Low Density Array default thermal-cycling conditions.

Analyze the Data

For instructions on how to analyze and troubleshoot the data, proceed to [page 21](#).

Section 4 Analyze and Troubleshoot the Data

Analyze the Data

This section applies to either RT-PCR reactions that were preamplified or not preamplified.

For detailed information on how to analyze Comparative C_T (RQ) and set up an RQ Study, refer to the *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016). Review the chapter on analyzing and viewing RQ study data in RQ Manager.

Review the Results

1. To review the results, transfer the SDS files into an RQ study.

Note: Applied Biosystems recommends analyzing the study with Automatic Baseline and Manual C_T set to 0.2.

2. View the amplification plots, then review the baseline and threshold settings. Adjust the baseline and threshold settings for individual assays if necessary.

IMPORTANT! The same threshold setting must be used across all samples or arrays within a study for a given assay.

3. Review the gene expression plot (for SDS v2.3 software, view the amplification plots in the Plate, Detector, or Sample view).

Comparative C_T uses arithmetic formulas to determine the change in expression of a target in an experimental sample relative to the same target in a reference sample. This method is used for high-throughput measurements of relative gene expression when there are many genes in many samples.

4. In the well table or results table, review C_T values for each well and for each replicate group. Omit outliers if necessary.

For additional analysis the raw C_T and/or ΔC_T values can be exported. If you are using SDS v2.3 software, raw C_T values are exported from the Plate Centric View.

For detailed downstream analysis, Applied Biosystems recommends software such as Integromics Real-Time StatMiner™ Software (www.integromics.com).

Troubleshoot

Observation	Possible Cause	Possible Solution
The C _T value for the No Template Control (NTC) is > 35 for some assays	Non-specific interactions between primers.	For NTC information on a specific assay, refer to the Megaplex™ Assay Performance File on the TaqMan® MicroRNA Array CD.
	Contamination	Work in an amplicon-free environment.
	Improper dilution of the preamplification reaction prior to real-time PCR	Follow the recommendations in the protocol. See “Dilute the Reaction” on page 11 .
The C _T values for the endogenous controls are highly variable across the sample set.	Natural biological variation	Use an alternative endogenous control such as a non-variable miRNA.
No amplification or poor amplification of samples	Low-abundance gene	Use more starting material.
	<ul style="list-style-type: none"> • Use of Non-Applied Biosystems reagents • Sample omitted from RT reaction • Reaction components omitted 	<ul style="list-style-type: none"> • Use Applied Biosystems reagents. • Add sample to RT reaction. • Add all required reaction components. • Follow precise recommendations in protocol.
	Preamplification required but not performed	Perform the protocol with preamplification.
Poor reproducibility across technical replicates	Inadequate mixing of reagents	Make sure that all samples and reactions are mixed well by inverting six times as described in the protocol.
Negative control well shows amplification	Contamination	Work in an amplicon-free environment.

Appendix A Materials Not Included

Reagents

From Applied Biosystems

Reagent	Description and Part Number
<i>mirVana</i> [™] miRNA Isolation Kit	<ul style="list-style-type: none">• 40 purifications (PN AM1560)• 40 purifications without phenol (PN AM1561)
TaqMan [®] MicroRNA Reverse Transcription Kit <ul style="list-style-type: none">• dNTPs with dTTP• Multiscribe[™] reverse transcriptase• 10X RT Buffer• RNase Inhibitor	<ul style="list-style-type: none">• 200 reactions (PN 4366596), sufficient for 130 Megaplex RT reactions• 1000 reactions (PN 4366597), sufficient for 665 Megaplex RT reactions
TaqMan [®] PreAmp Master Mix (2X)	One 1-mL tube (PN 4391128) sufficient for 80 Megaplex preamp reactions Note: The TaqMan PreAmp Master Mix Kit (PN 4384267) contains TaqMan [®] Gene Expression Master Mix which should not be used with Megaplex Pools.
TaqMan [®] Universal Master Mix, No AmpErase [®] UNG, (2X)	<ul style="list-style-type: none">• One 5-mL bottle (PN 4324018), sufficient for 11 TaqMan MicroRNA Arrays• Five 5-mL bottles (PN 4364343) sufficient for 55 TaqMan MicroRNA Arrays• One 50-mL bottle (PN 4326614), sufficient for 111 TaqMan MicroRNA Arrays

Reaction Plates and Accessories

From Applied Biosystems

Use reaction plates and accessories appropriate for your real-time PCR system or thermal cycler.

Real-Time PCR System or Thermal Cycler	Reaction Plates and Accessories
<p>7900HT System (96-Well Block or TLDA Block)</p> <p>Or</p> <p>GeneAmp PCR System 9700</p>	<ul style="list-style-type: none"> • MicroAmp® Optical 96-Well Reaction Plate with Barcode: <ul style="list-style-type: none"> – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Clear Adhesive Films, 100 Films (PN 4306311) • MicroAmp® Optical Film Compression Pad (PN 4312639) for use with one plate • MicroAmp® Optical 8-Tube Strips, 0.2-mL, quantity 1000 tubes in strips of 8 (PN 4316567) (No compression pad required) • MicroAmp® Optical 8-Cap Strips, quantity 300 strips (PN 4323032)

Appendix B Megaplex™ Pools Chemistry

Chemistry Overview

Steps for Megaplex RT Relative quantitation using Megaplex™ Pools is accomplished using RT-PCR, preamplification, and real-time PCR steps.

Reverse Transcription Step [Figure 1](#) shows the reverse transcription (RT) step. In this step, cDNA is reverse transcribed from total RNA samples. The reverse transcription uses specific miRNA primers and reagents from the Megaplex Primers and the TaqMan® MicroRNA Reverse Transcription Kit.



Figure 1 RT Reaction

Preamplification Step

Figure 2 shows the preamplification reaction. In the preamplification step, PCR products are uniformly amplified from cDNA templates using the Megaplex PreAmp Primers and the TaqMan® PreAmp Master Mix.

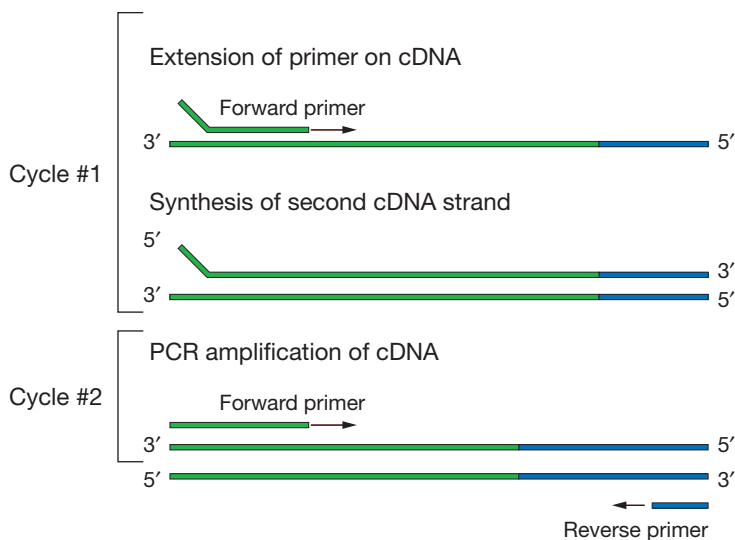


Figure 2 Preamplification Reaction

Real-Time PCR Step

Figure 3 on page 27 shows the steps in real-time PCR (the 5' nuclease assay). During real-time PCR:

- Each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
The TaqMan MGB probes contain:
 - A reporter dye (FAM™ dye) linked to the 5' end of the probe.
 - A minor groove binder (MGB) at the 3' end of the probe to allow the design of shorter probes with greater specificity.
 - A nonfluorescent quencher (NFQ) at the 3' end of the probe.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye causes the reporter dye signal to be quenched.
- AmpliTaq Gold® DNA Polymerase extends the primers bound to the cDNA template.

- AmpliTaq Gold[®] enzyme (containing 5' nuclease activity) cleaves the probes that are hybridized to the target sequence.
- When the hybridized probes are cleaved by AmpliTaq Gold[®] enzyme, the quencher is separated from the reporter dye, increasing the fluorescence of the reporter dye. Therefore, the fluorescence signal generated by PCR amplification indicates the gene expression level in the sample.

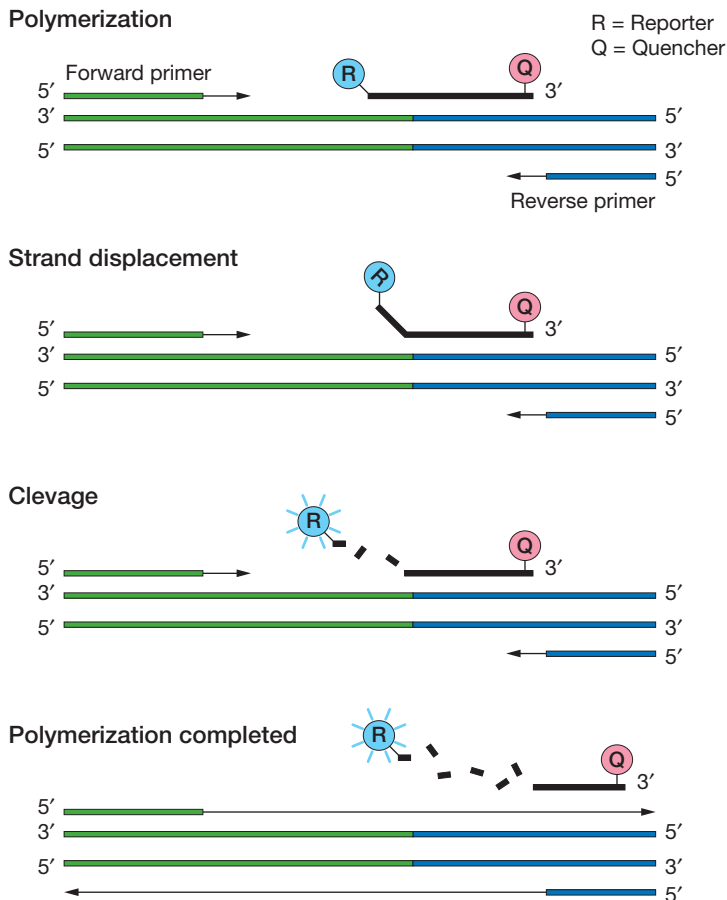


Figure 3 The 5' to 3' nuclease activity of the DNA Polymerase system

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