

# Megaplex™ Primer Pools

For MicroRNA expression analysis with TaqMan® Array Cards

Pub. No. 4399813 Rev. D

**Note:** For safety and biohazard guidelines, see the “Safety” appendix in the *Megaplex™ Primer Pools for MicroRNA expression analysis User Guide* (Pub. No. 4399721). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This Quick Reference is intended as a benchtop reference for experienced users of Megaplex™ Primer Pools and TaqMan® Array Cards. For detailed instructions, supplemental procedures, and troubleshooting, see the *Megaplex™ Primer Pools for MicroRNA expression analysis User Guide* (Pub. No. 4399721).

## Prepare the sample and select the workflow

### Guidelines for total RNA input

- The method used to isolate the total RNA must preserve the small RNA fraction.
- 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.
- Quantify the RNA to determine the appropriate workflow.

### Select the workflow

This document describes two workflows:

- With preamplification of the cDNA:
  - Recommended for all samples
  - Important for samples with 1–350 ng of RNA
  - See “Perform Megaplex™ Primer Pools with preamplification” on page 1
- Without preamplification of the cDNA:
  - Can be used for samples with 350–1000 ng of RNA
  - See “Perform Megaplex™ Primer Pools without preamplification” on page 3

## Perform Megaplex™ Primer Pools with preamplification

### Guidelines for Megaplex™ reverse transcription with preamplification

- Use the TaqMan® MicroRNA Reverse Transcription Kit and the Megaplex™ RT Primers to synthesize single-stranded cDNA from total RNA samples.
- The RT reaction with downstream preamplification supports 1–1000 ng of input total RNA.
- For most tissues, 30 ng of total RNA produces a comprehensive MicroRNA profile with preamplification.

- We recommend verifying the optimal quantity of input total RNA for your sample type.
- Use 3 µL of RNA for each RT reaction, containing 1–350 ng of total RNA.

## Perform Megaplex™ reverse transcription

Thaw the Megaplex™ RT Primers, TaqMan® MicroRNA Reverse Transcription Kit components, and MgCl<sub>2</sub> on ice.

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

Component	Volume (1 sample)	Volume (10 samples) <sup>[1]</sup>
Megaplex™ RT Primers (10X)	0.8 µL	9.00 µL
dNTPs with dTTP (100 mM)	0.2 µL	2.25 µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	1.5 µL	16.88 µL
10X RT Buffer	0.8 µL	9.00 µL
MgCl <sub>2</sub> (25 mM)	0.9 µL	10.12 µL
RNase Inhibitor (20 U/µL)	0.1 µL	1.12 µL
Nuclease-free water	0.2 µL	2.25 µL
<b>Total RT Reaction Mix volume</b>	<b>4.5 µL</b>	<b>50.62 µL</b>

<sup>[1]</sup> Includes 12.5% overage.

2. Invert to mix, then centrifuge briefly to collect the contents at the bottom of the tube.
3. Add 4.5 µL of the RT Reaction Mix to the wells of a reaction plate or to reaction tubes.
4. Add 3 µL of RNA or water (no template control) to each well of a reaction plate or to reaction tubes.  
3 µL of the RNA sample should contain 1-350 ng of total RNA.
5. Seal the reaction plate or tubes, invert to mix, then centrifuge briefly to collect the contents at the bottom.
6. Incubate on ice for 5 minutes.

- Place the reaction plate or tubes into a thermal cycler, then incubate using a standard ramp speed and reaction volume of 7.5  $\mu\text{L}$ .

Step	Temperature	Time	Cycles
Reverse transcription	16°C	2 minutes	40
	42°C	1 minute	
	50°C	1 second	
Stop reaction	85°C	5 minutes	1
Hold	4°C	Hold until stopped	Hold

Proceed to the preamplification reaction or store the RT reaction product at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ .

### Perform the preamplification

Thaw the Megaplex™ PreAmp Primers on ice, invert to mix, then centrifuge briefly to collect the contents at the bottom of the tube.

Thaw the RT reaction product on ice, if necessary.

- Mix the TaqMan® PreAmp Master Mix (2X) by swirling the bottle.
- In an appropriately-sized microcentrifuge tube, prepare Preamplification Reaction Mix according to the following table.

Component	Volume (1 sample)	Volume (10 samples) <sup>[1]</sup>
TaqMan® PreAmp Master Mix (2X)	12.5 $\mu\text{L}$	140.62 $\mu\text{L}$
Megaplex™ PreAmp Primers(10X)	2.5 $\mu\text{L}$	28.13 $\mu\text{L}$
Nuclease-free water	7.5 $\mu\text{L}$	84.37 $\mu\text{L}$
<b>Total Preamplification Reaction Mix volume</b>	<b>22.5 <math>\mu\text{L}</math></b>	<b>253.12 <math>\mu\text{L}</math></b>

<sup>[1]</sup> Includes 12.5% overage.

- Invert to mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.
- Add 2.5  $\mu\text{L}$  of the RT reaction product to the wells of a reaction plate or to reaction tubes.
- Add 22.5  $\mu\text{L}$  of the Preamplification Reaction Mix to each well of a reaction plate or to reaction tubes.
- Seal the reaction plate or tubes, invert to mix, then centrifuge briefly to collect the contents at the bottom.
- Incubate the plate or tubes on ice for 5 minutes.

- Place the reaction plate or tubes into a thermal cycler, then incubate using a standard ramp speed and the following settings.

Step	Temperature	Time	Cycles
Enzyme activation	95°C	10 minutes	Hold
Anneal	55°C	2 minutes	Hold
Extend	72°C	2 minutes	Hold
Denature	95°C	15 seconds	12 <sup>[1]</sup>
Anneal/extend	60°C	4 minutes	
Enzyme inactivation	99.9°C	10 minutes	Hold
Hold	4°C	Hold	Hold

<sup>[1]</sup> The number of cycles can be optimized for extremely low input samples.

Proceed to the next section.

### Dilute the preamplification reaction

- Remove the reaction plate or tubes from the thermal cycler, then briefly centrifuge to collect the contents at the bottom.
- Add 75  $\mu\text{L}$  of 0.1X TE pH 8.0 to each well or tube.
- Seal the plate or tubes, invert to mix, then centrifuge briefly to collect the contents at the bottom.

Place the diluted preamplification reaction product on ice and proceed directly to the real-time PCR reactions (next section), or store at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  for up to one week.

### Prepare PCR reactions

Thaw the RT reaction product on ice, then mix thoroughly by inverting the tube.

- Mix the PCR Master Mix by swirling the bottle.
- In an appropriately-sized microcentrifuge tube, prepare PCR Reaction Mix according to the following table.

Component	Volume (1 card) <sup>[1]</sup>
PCR Master Mix	450 $\mu\text{L}$
Diluted preamplification product	9 $\mu\text{L}$
Nuclease-free water	441 $\mu\text{L}$
<b>Total PCR Reaction Mix volume</b>	<b>900 <math>\mu\text{L}</math></b>

<sup>[1]</sup> Includes 12.5% overage.

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

Proceed to “Perform PCR amplification” on page 4.

## Perform Megaplex™ Primer Pools without preamplification

### Guidelines for Megaplex™ reverse transcription without preamplification

- Use the TaqMan® MicroRNA Reverse Transcription Kit and the Megaplex™ RT Primers to synthesize single-stranded cDNA from total RNA samples.
- The RT reaction supports 350–1000 ng of input total RNA.
- For most tissues, 500 ng of total RNA produces a comprehensive MicroRNA profile.
- We recommend verifying the optimal quantity of input total RNA for your sample type.
- Use 3 µL of RNA for each RT reaction, containing 350–1000 ng of total RNA.

### Guidelines for performing real-time PCR

- Before preparing a TaqMan® Array Card, review *TaqMan® Gene Expression Assays User Guide—TaqMan® Array Cards* (Pub. No. 4400263).
- Prepare the real-time PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Keep the card protected from light and stored as indicated until ready for use. Excessive exposure to light may affect the fluorescent probes of the dried-down assays in the card.
- Use the same quantity of cDNA sample for all reactions. Use a cDNA quantity of 30–1,000 ng per fill reservoir (0.3–10 ng/µL).
- Load each fill reservoir with 100 µL of sample-specific PCR reaction mix.
  - Each fill reservoir contains a single sample as determined by the card layout.
  - The 100-µL volume ensures adequate filling of each reaction well. Volumes smaller than 100 µL result in insufficiently filled cards.
- Equilibrate the card that is loaded with PCR reaction mix to room temperature before loading into the real-time PCR instrument.
- If the card is not run immediately, protect it from light and store at 2–8°C.

### Perform Megaplex™ reverse transcription

Thaw the Megaplex™ RT Primers, TaqMan® MicroRNA Reverse Transcription Kit components, and MgCl<sub>2</sub> on ice.

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

Component	Volume (1 sample)	Volume (10 samples) <sup>[1]</sup>
Megaplex™ RT Primers (10X)	0.8 µL	9.00 µL
dNTPs with dTTP (100 mM)	0.2 µL	2.25 µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	1.5 µL	16.88 µL
10X RT Buffer	0.8 µL	9.00 µL
MgCl <sub>2</sub> (25 mM)	0.9 µL	10.12 µL
RNase Inhibitor (20 U/µL)	0.1 µL	1.12 µL
Nuclease-free Water	0.2 µL	2.25 µL
<b>Total RT Reaction Mix volume</b>	<b>4.5 µL</b>	<b>50.62 µL</b>

<sup>[1]</sup> Includes 12.5% overage.

2. Invert to mix, then centrifuge briefly to collect the contents at the bottom of the tube.
3. Add 4.5 µL of the RT Reaction Mix to the wells of a reaction plate or to reaction tubes.
4. Add 3 µL of RNA or water (no template control) to each well of a reaction plate or to reaction tubes.  
3 µL of the RNA sample should contain 350–1000 ng of total RNA.
5. Seal the reaction plate or tubes, invert to mix, then centrifuge briefly to collect the contents at the bottom.
6. Incubate on ice for 5 minutes.
7. Place the reaction plate or tubes into a thermal cycler, then incubate using a standard ramp speed and reaction volume of 7.5 µL.

Step	Temperature	Time	Cycles
Reverse transcription	16°C	2 minutes	40
	42°C	1 minute	
	50°C	1 second	
Stop reaction	85°C	5 minutes	1
Hold	4°C	Hold until stopped	Hold

Proceed to the real-time PCR, or store the RT reaction product at –25°C to –15°C.

## Prepare PCR reactions

Thaw the RT reaction product on ice, then mix thoroughly by inverting the tube.

1. Mix the PCR Master Mix by swirling the bottle.
2. In an appropriately-sized microcentrifuge tube, prepare PCR Reaction Mix according to the following table.

Component	Volume (1 card) <sup>[1]</sup>
PCR Master Mix	450 µL
cDNA	6 µL
Nuclease-free Water	444 µL
<b>Total PCR Reaction Mix volume</b>	<b>900 µL</b>

<sup>[1]</sup> Includes 12.5% overage.

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

Proceed to “Perform PCR amplification” on page 4.

## Perform PCR amplification

### Prepare a TaqMan® Array Card

**IMPORTANT!** Before preparing a TaqMan® Array Card, review *Megaplex™ Primer Pools for MicroRNA expression analysis User Guide* (Pub. No. 4399721).

1. Load each fill reservoir of the card with 100 µL of prepared PCR reaction mix.
2. Centrifuge, then seal the filled card.

### 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 card.

**Note:** The instrument must be configured with a block appropriate for a card.

1. Import the setup file (SDS in TXT format) into the software specific to your instrument.
2. Set the properties for the run.

Property	Setting
Block	<b>Array Card</b>
Experiment type	<b>Comparative C<sub>t</sub> (ΔΔC<sub>t</sub>)</b>
Reagents or Chemistry	<b>TaqMan® Reagents</b>

**Note:** The default passive reference is set to ROX™ dye and should not be changed.

3. Select the properties.

- TaqMan® Universal Master Mix II, no UNG—**Standard**
- TaqMan® Fast Advanced Master Mix—**Fast**

4. Set up the thermal protocol.

**Note:** Your thermal protocols might differ from the following tables in this user guide.

**Table 1** TaqMan® Fast Advanced Master Mix (ViiA™ 7 Real-Time PCR System, any compatible QuantStudio™ Real-Time PCR System)

Step	Temperature	Time	Cycles
Enzyme activation	92°C	10 minutes <sup>[1]</sup>	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

<sup>[1]</sup> To completely dissolve the assay on the card.

**Table 2** TaqMan® Fast Advanced Master Mix (7900HT Fast Real-Time PCR System)

Step	Temperature	Time	Cycles
Enzyme activation	92°C	10 minutes <sup>[1]</sup>	1
Denature	97°C	1 second	40
Anneal / Extend	60°C	20 seconds	

<sup>[1]</sup> To completely dissolve the assay on the card.

**Table 3** TaqMan® Universal Master Mix II, no UNG (ViiA™ 7 Real-Time PCR System, any compatible QuantStudio™ Real-Time PCR System)

Step	Temperature	Time	Cycles
Enzyme activation	95°C	10 minutes	1
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	60 seconds	

**Table 4** TaqMan® Universal Master Mix II, no UNG (7900HT Fast Real-Time PCR System)

Step	Temperature	Time	Cycles
Enzyme activation	94.5°C	10 minutes	1
Denature	97.0°C	30 seconds	40
Anneal / Extend	59.7°C	60 seconds	

5. Confirm that the reaction volume is set to **1 µL**.
6. Load the reaction card into the real-time PCR instrument.
7. Start the run.

## Analyze the results

For detailed information about data analysis, see the appropriate documentation for your instrument.

Use the relative quantification ( $\Delta\Delta C_t$ ) method to analyze results.

A cutoff of 32 is recommended. If pre-amplification is used, the cutoff can be set to 29 or 30 to reduce the number of false positives.

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 data analysis using the Relative Quantification application or ExpressionSuite™ Software.

$C_t$  ( $C_q$ ) values can be generated using the relative threshold algorithm ( $C_{rt}$ ).

Use the relative threshold algorithm in your software. The relative threshold algorithm is available on the following instruments:

- QuantStudio™ Real-Time PCR Instruments
- ViiA™ 7 instrument

If your software does not have the relative threshold algorithm, use the Relative Quantification application that is available on the Thermo Fisher Cloud.

**Note:** The  $C_t$  algorithm is recommended on the 7900HT Fast Real-Time PCR Instrument.

## Algorithms for data analysis

**Table 5** Algorithm recommendations for TaqMan® Array Cards

Algorithm	Recommendation
Relative threshold ( $C_{rt}$ )	Recommended for the following instruments: <ul style="list-style-type: none"><li>• QuantStudio™ Real-Time PCR Instruments</li><li>• ViiA™ 7 instrument</li></ul> Can correct a variable baseline, which might be due to dried-down assays on the card being reconstituted at different rates.
Threshold ( $C_t$ )	Optional if used for analysis of established protocols. Recommended for 7900HT Fast Real-Time PCR Instrument.

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



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For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](http://thermofisher.com/symbols-definition).

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

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**Revision history:** Pub. No. 4399813

Revision	Date	Description
D	29 November 2018	<ul style="list-style-type: none"><li>• Updated data-analysis algorithms for 7900HT Fast Real-Time PCR Instrument</li><li>• Corrected thermal cycling conditions.</li></ul>
C	1 August 2018	<ul style="list-style-type: none"><li>• Updated recommended Master Mix for real-time PCR.</li><li>• Updated instructions to set up and run the real-time PCR instrument.</li><li>• Updated for general style, formatting, and branding.</li></ul>
B	November 2008	Baseline for this revision history.

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