QUICK REFERENCE

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Protocol for Running Custom RT and Preamplification Pools on Custom TaqMan® Array MicroRNA Cards

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WARNING! For every chemical, read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **lifetechnologies.com/support**.

Purpose

This Quick Reference describes how to use Custom RT and PreAmp Pools with matching Custom TaqMan® Array MicroRNA Cards. This protocol is for RT and PreAmp pools composed of up to 96 individual RT or preamplification primers. For pools containing 192 or 384 individual primers, follow the Megaplex Pools Protocol (Part no. 4399721) or QRC (Part no. 4399813). In order to perform preamplification, you will need to have ordered the optional preamplification pool with your Custom TaqMan® Array MicroRNA Card.

Due to the complexity of the pools, some assays may exhibit less–than–optimal performance. We recommend that you test NTC background and validate pool performance with individual assays.

Choose a workflow

The array card has the possibility of two workflows; one with preamplification of your sample and one without preamplification of your sample.

IMPORTANT! Although results can be generated with larger amounts of sample, preamplification is recommended for detecting low expressing miRNAs.

If you are using a...	And the amount of total RNA is...	Then choose to run the MicroRNA Assays...
Custom TaqMan® Array MicroRNA Card	1–350 ng	With preamplification
	350–1000 ng	Without preamplification

Workflow for using Custom TaqMan® Array MicroRNA Cards

With Preamplification	Without Preamplification
Run the RT reaction (page 2)	Run the RT reaction (page 5)
↓	↓
Run the preamplification reaction (page 3)	Run the real-time PCR reaction (page 5)
↓	
Run the real-time PCR reaction (page 4)	

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Run Custom TaqMan® Array MicroRNA Cards with preamplification

Run the RT reaction

Prepare the RT reaction mix

Note: Excluding the Custom RT Primer Pool, all additional reagents necessary for the multiplex RT step are contained in the *TaqMan® MicroRNA Reverse Transcription Kit* (Part no. 4366596 (200 reactions) or 4366597 (1000 reactions)).

1. Prepare the RT reaction mix on ice in a 1.5-mL microcentrifuge tube:

   Note: Do not vortex the Multiscribe Reverse Transcriptase or the RT reaction mix.

Component	Volume for 1 sample	Volume for 10 samples†
Custom RT Primer Pool	6.00 µL	67.5 µL
dNTPs with dTTP (100 mM)	0.30 µL	3.4 µL
MultiScribe Reverse Transcriptase (50 U/µL)	3.00 µL	33.8 µL
10X RT Buffer	1.50 µL	16.9 µL
RNase Inhibitor (20 U/µL)	0.19 µL	2.1 µL
Nuclease-free water	1.01 µL	11.4 µL
Total	**12.00 µL**	**135.1 µL**

† Includes 12.5% excess for volume loss from pipetting.

2. Mix thoroughly by inverting 6 times. Do not vortex.
3. Centrifuge the tube briefly.
4. In a 96-well plate or 8-tube strip, pipet 12 µL of the RT reaction mix into each well or tube.
5. Add 3 µL sample containing 1–350 ng of total RNA (or 3 µL of water for No Template Control reactions) into each well or tube containing RT reaction mix for a total reaction volume of 15 µL.
6. Seal the plate or tubes, invert 6 times, then spin briefly.
7. Incubate the plate on ice for 5 minutes.

Perform reverse transcription

1. Set up the run method using the following parameters:
   - Ramp speed: Std or Max ramp speed on a GeneAmp® PCR System 9700 Thermal Cycler.
   - Reaction volume: 15 µL
   - Thermal-cycling conditions:

Step	Time	Temperature
Hold	30 min	16°C
Hold	30 min	42°C
Hold	5 min	85°C
Hold	∞	4°C

2. Start the run.

STOPPING POINT (*Optional*): The RT product can be stored at –15 to –25°C for up to one week.

Run the preamplification reaction

Prepare the preamplification reaction mix

1. For a 25-µL final reaction volume, combine:

Component	Volume for 1 sample	Volume for 10 samples†
TaqMan® PreAmp Master Mix, 2X	12.50 µL	140.6 µL
Custom PreAmp Primer Pool	3.75 µL	42.2 µL
Nuclease-free water	6.25 µL	70.3 µL
Total	**22.50 µL**	**253.1 µL**

† Includes 12.5% excess for volume loss from pipetting.

2. Invert the tube 6 times to mix, then centrifuge the tube briefly. Do not vortex.
3. In 96-well plate or 8-tube strip, pipet 2.5 µL of each RT product into its corresponding well or tube.
4. Dispense 22.5 µL of preamplification reaction mix into each well of the 96-well plate or 8-tube strips containing the RT product.
5. Seal the plate or tubes, invert 6 times to mix, then spin briefly.

Run the preamplification reaction

1. Set up the run method:
   - Ramp speed: Std ramp speed on a GeneAmp® PCR System 9700 Thermal Cycler.
   - Reaction volume: 25 µL
   - Thermal-cycling conditions:

Step	Time	Temperature
Hold	10 min	95°C
Hold	2 min	55°C
Hold	2 min	72°C
Cycle (12 Cycles)	15 sec	95°C
	4 min	60°C
Hold†	10 min	99.9°C
Hold	∞	4°C

† Required for enzyme inactivation.

2. Remove the 96-well plate or 8-tube strips from the thermal cycler.
3. Briefly centrifuge the tubes or plate.
4. Add 175 µL of 0.1X TE, pH 8.0 to each well or tube. This is the diluted PreAmp product. (Final Volume = 200 µL).
5. Seal the plate or tubes, invert 6 times to mix, then spin briefly.

STOPPING POINT (*Optional*): The diluted PreAmp product can be stored at –15 to –25°C for up to one week.

Run the real-time PCR reaction

Prepare the Custom TaqMan® Array MicroRNA Card as described in the *TaqMan® Array User Bulletin* (Part no. 4371129).

Prepare the PCR reaction mix

1. Prepare the PCR reaction mix in a 1.5-mL tube:

Component	Volume for 1 sample/ 1 port ‡§	Volume for 1 sample/ 2 ports‡††	Volume for 1 sample/ 4 ports‡‡‡
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)†	56.25 µL	112.50 µL	225.00 µL
Diluted PreAmp Product	1.13 µL	2.25 µL	4.50 µL
Nuclease-free water	55.12 µL	110.25 µL	220.50 µL
Total	**112.50 µL**	**225.00 µL**	**450.00 µL**

† TaqMan® Universal Master Mix, No Amperase® UNG, (2X) may also be used.
‡ Includes a 12.5% excess for volume loss from pipetting.
§ Custom TaqMan® Array Formats 12, 16, 24, and 48 allow 8 unique samples (1 sample, 1 port) on 1 card.
†† Custom TaqMan® Array Formats 32 and 96a allow 4 unique samples (1 sample, 2 ports) on 1 card.
‡‡ Custom Taqman® Array Formats 64, and 96b allow 2 unique samples (1 sample, 4 ports) on 1 card.

2. Invert the tube to mix, then centrifuge briefly.

Perform the real-time PCR reaction

Load and run the array using the Custom MicroRNA TaqMan® Array Card default thermal-cycling conditions. Refer to the *TaqMan® Array User Bulletin* (Part no. 4371129).

Analyze the data

For detailed information on how to analyze Comparative C_T (RQ) and set up an RQ Study, refer to the *Applied Biosystems® 7900 HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (Part no. 4364016).

1. To review the results, transfer the .sds or .eds files into an RQ study or ExpressionSuite.™

   Note: We recommend analyzing the study with Automatic Baseline and manual C_T set to 0.2.

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

   IMPORTANT! Use the same threshold setting for all samples (on arrays or plates) within a study for a given assay.

3. Review the gene expression plot.
4. Review the C_T values for each well and each replicate group in the well table or results table. Omit outliers, if necessary.
5. For additional analysis, the raw C_T and/or ΔC_T values can be exported.
6. For detailed downstream analysis, we recommend software such as ExpressionSuite™ , DataAssist™, or Integromics® RealTime StatMiner® Software available at **www.integromics.com**.

Run Custom TaqMan® Array MicroRNA Cards without preamplification

Run the RT reaction

Prepare the RT reaction mix

Note: Excluding the Custom RT Primer Pool, all additional reagents necessary for the multiplex RT step are contained in the *TaqMan® MicroRNA Reverse Transcription Kit* (Part no. 4366596 (200 reactions) or 4366597 (1000 reactions)).

1. Prepare the RT reaction mix on ice in a 1.5–mL microcentrifuge tube:

   Note: Do not vortex the Multiscribe Reverse Transcriptase or the RT reaction mix.

Component	Volume for 1 sample	Volume for 10 samples†
RT Primer Pool	6.00 µL	67.5 µL
dNTPs with dTTP (100mM)	0.30 µL	3.4 µL
MultiScribe Reverse Transcriptase (50 U/µL)	3.00 µL	33.8 µL
10X RT Buffer	1.50 µL	16.9 µL
RNase Inhibitor (20 U/µL)	0.19 µL	2.1 µL
Nuclease-free water	1.01 µL	11.4 µL
Total	**12.00 µL**	**135.1 µL**

† Includes 12.5% excess for volume loss from pipetting.

2. Mix thoroughly by inverting 6 times. Do not vortex.
3. Centrifuge the tube briefly.
4. In a 96-well plate or 8-tube strip, pipet 12 µL of each RT reaction mix into each well or tube.
5. Add 3 µL of total RNA (350–1000 ng per reaction) or 3 µL of water (for No Template Control reactions) into each well or tube containing RT reaction mix for a total reaction volume of 15 µL.
6. Seal the plate or tubes, invert 6 times, and then spin briefly.
7. Incubate on ice for 5 minutes.

Perform reverse transcription

1. Set up the run method:
   - Ramp speed: Std or Max ramp speed on a GeneAmp® PCR System 9700 Thermal Cycler.
   - Reaction volume: 15 µL
   - Thermal-cycling conditions:

Step	Time	Temperature
Hold	30 min	16°C
Hold	30 min	42°C
Hold	5 min	85°C
Hold	∞	4°C

STOPPING POINT (*Optional*): The RT product can be stored at –15 to –25°C for up to one week.

Run the real-time PCR reaction

Prepare the TaqMan® Array MicroRNA Card as described in the *TaqMan® Array User Bulletin* (Part no. 4371129).

Prepare the PCR reaction mix

1. Prepare the PCR reaction mix in a 1.5–mL tube:

Component	Volume for 1 sample/ 1 port ‡§	Volume for 1 sample/ 2 ports‡††	Volume for 1 sample/ 4 ports‡‡
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)†	56.25 µL	112.50 µL	225.00 µL
RT Product	0.90 µL	1.80 µL	3.60 µL
Nuclease-free water	55.35 µL	110.70 µL	221.40 µL
Total	**112.50 µL**	**225.00 µL**	**450.00 µL**

† TaqMan® Universal Master Mix, No Amperase® UNG, (2X) may also be used.
‡ Includes a 12.5% excess for volume loss from pipetting.
§ Custom TaqMan® Array Formats 12, 16, 24, and 48 allow 8 unique samples (1 sample, 1 port) on 1 card.
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2. Invert the tube 6 times to mix, then centrifuge briefly.

Run the real-time PCR reaction

Load and run the array using Custom TaqMan® Array MicroRNA Card default thermal-cycling conditions. Refer to the *TaqMan® Array User Bulletin* (Part no. 4371129).

Analyze the data

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1. To review the results, transfer the .sds files into an RQ study or ExpressionSuite™.

   Note: We recommend analyzing the study with Automatic Baseline and manual C_T set to 0.2.

2. View the amplification plots, and 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, arrays or plates within a study for a given assay.

3. Review the gene expression plot.
4. Review the C_T values for each well and each replicate group in the well table or results table. Omit outliers, if necessary.
5. For additional analysis, the raw C_T and/or ΔC_T values can be exported.
6. For detailed downstream analysis, we recommend software such as DataAssist™ or Integromics® RealTime StatMiner® Software available at **www.integromics.com**.

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