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Protocol for Creating Custom RT and Preamplification Pools using TaqMan® MicroRNA Assays

Publication Part Number 4465407 **Revision Date** January 2013 (Rev. C)

SUBJECT: Procedure for multiplexing the RT step with or without preamplification while using TaqMan® MicroRNA Assays

In this user bulletin

This user bulletin covers:

Purpose

This user bulletin describes how to pool up to 96 individual reverse transcriptase (RT) primers and/or TaqMan® MicroRNA Assays for preamplification. The standard TaqMan® MicroRNA Assays protocol calls for an individual RT reaction for each target miRNA. This procedure explains a multiplexed RT step for pools composed of up to 96 individual RT primers or TaqMan® miRNA Assays for preamplification. These pools can be used with the matching TaqMan® Array MicroRNA Assays on plates prepared by the user, or through our custom plating service.

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

The following RT-PCR instruments are compatible with the procedures described in this bulletin:

- 7500/7500 Fast Real-Time PCR System
- StepOnePlus™ Real-Time PCR System
- 7900HT/7900HT Fast Real-Time System
- ViiA™ 7 Real-Time PCR System
- QuantStudio™ 12K Flex

Note: For a procedure for using the Custom TaqMan® Array MicroRNA Card format, see *"Protocol for Running Custom RT and Preamplification Pools on Custom TaqMan® Array MicroRNA Cards"*(Part No. 4478705).

Choose a plate format and a workflow

Two plate formats are described in this bulletin:

- 96-well plate (Standard and Fast)
- 384-well plate

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Each format has two alternative workflows: one with preamplification of your sample and one without preamplification of your sample.

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

If the amount of total RNA is...	Then choose to run the MicroRNA Assays...
1–350 ng	with preamplification
350–1000 ng	without preamplification

Required Materials

Item	Description and Part number
Individual MicroRNA Assays	Part no. 4427975
TaqMan® MicroRNA Reverse Transcription Kit	200 reactions (Part no. 4366596) 1000 reactions (Part no. 4366597)
TaqMan® PreAmp Master Mix (2X)	One 1 mL tube (Part no. 4391128) **Note:** Do *not* use the Gene Expression Master Mix contained in the TaqMan® PreAmp Master Mix Kit (Part no. 4384267)
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X) ***or*** TaqMan® Universal Master Mix, No AmpErase® UNG, (2X)	One 5 mL bottle (Part no. 4440040) Five 5 mL bottles (Part no. 4440048) One 50 mL bottle (Part no. 4440041) One 5 mL bottle (Part no. 4324018) Five 5 mL bottles (Part no. 4364343) One 50 mL bottle (Part no. 4326614)
MicroAmp® 96- & 384-Well Optical Adhesive Film	100 covers (Part no. 4311971) 25 covers (Part no. 4360954)
RNase-free Water	MLS
1X TE Buffer	MLS

Workflow using 96- or 384-well plates

Prepare the RT primer pool (page 3) and the preamplification primer pool (page 4)

With Preamplification	Without Preamplification
Run the RT reaction (page 5)	Run the RT reaction (page 10)
↓	↓
Run the Preamplification reaction (page 6)	Run the real-time PCR reaction (page 11)
↓	
Run the real-time PCR reaction (page 8)	

Prepare the custom RT primer pool

Each TaqMan® MicroRNA Assay contains one 5× RT primer. Up to 96 of these primers can be pooled into one RT reaction as follows:

1. In a 1.5 ml microcentrifuge tube, combine 10 µL of each individual 5× RT primer.

   Note: When creating your own pool, we recommend running a No Template Control (use water as sample in the RT reaction) reaction for each assay in the pool to check the background.

2. Add 1× TE to bring the final volume to 1000 µL (refer to this table).

Format	Number of assays	Total pooled volume	Volume of 1× TE	Total volume of RT primer pool
12	12	120 µL	880 µL	1000 µL
16	16	160 µL	840 µL	1000 µL
24	24	240 µL	760 µL	1000 µL
32	32	320 µL	680 µL	1000 µL
48	48	480 µL	520 µL	1000 µL
64	64	640 µL	360 µL	1000 µL
96a / 96b	96	960 µL	40 µL	1000 µL

IMPORTANT! The final concentration of each RT primer in the RT primer pool should be 0.05× each in a final volume of 1000 µL.

Note: The volume is sufficient for 148 × 15 µL RT reactions with 12% allotted for pipetting loss.

3. Store the RT primer pool at –20°C for up to two months.

Prepare the custom preamplification primer pool

TaqMan® MicroRNA Assays contain a 20× mix of forward and reverse primers, and miRNA-specific probe. Prepare the preamplification pool (PreAmp primer pool) so that the final concentration of each assay equals 0.2×.

1. In a 1.5 mL microcentrifuge tube, combine 10 µL of each individual 20× TaqMan® MicroRNA Assay.
2. Add 1× TE to bring the final volume to 1000 µL. Refer to the following table.

Format	Number of assays	Total pooled volume	Volume of 1× TE	Total volume of PreAmp primer pool
12	12	120 µL	880 µL	1000 µL
16	16	160 µL	840 µL	1000 µL
24	24	240 µL	760 µL	1000 µL
32	32	320 µL	680 µL	1000 µL
48	48	480 µL	520 µL	1000 µL
64	64	640 µL	360 µL	1000 µL
96a / 96b	96	960 µL	40 µL	1000 µL

3. Store the PreAmp primer pool at –20°C.

Note: Volume is sufficient for 235 × 25 µL PreAmp reactions.

Run 96- or 384-well plates with preamplification

Prepare the RT primer pool

Prepare the RT primer pool as described on page 3.

Run the RT reaction

Prepare the RT reaction mix

Note: Excluding the RT primer pool, all additional reagents necessary for the multiplex RT step are contained in the TaqMan® MicroRNA RT Kit (Part no. 4366596 or 4366597).

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 1 sample †‡	Volume for 3 samples †§
RT Primer Pool††	6.00 µL	6.75 µL	20.25 µL
dNTPs with dTTP (100 mM)	0.30 µL	0.34 µL	1.01 µL
MultiScribe Reverse Transcriptase (50 U/µL)	3.00 µL	3.38 µL	10.13 µL
10X RT Buffer	1.50 µL	1.69 µL	5.06 µL
RNase Inhibitor (20 U/µL)	0.19 µL	0.21 µL	0.64 µL
Nuclease-free water	1.01 µL	1.14 µL	3.41 µL
Total	12.00 µL	13.5 µL	40.5 µL

† Includes a 12.5% excess for volume loss from pipetting.

‡ Volume includes amount for running up to 96 assays in the PCR step with no replicates.

§ Volume includes amount for running up to 96 assays in the PCR step with 4 replicates of each assay.

†† Prepared on page 3.

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 RT reaction mix into each well or tube.
5. Add 3 µL of total RNA (1–350 ng per reaction) 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 on ice for 5 minutes.

Perform reverse transcription

1. Set up run method using the following parameters:
   - Ramp speed: Std or Max ramp speed on a GeneAmp® 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. Prepare the reaction mix in a 1.5 mL tube:

PreAmp Reaction Mix components	Volume for 1 reaction	1 PreAmp Reaction (96 assays, singleton or 4 replicates)†
RT Product	2.5 µL	2.81 µL
TaqMan PreAmp Master Mix (2X)	12.50 µL	14.06 µL
PreAmp Primer Pool‡	3.75 µL	4.22 µL
Nuclease-free water	6.25 µL	7.03 µL
Total	**25.00 µL**	**28.13 µL**

† Includes a 12.5% excess for volume loss from pipetting.
‡ Prepared on page 4.

2. Combine reagents and mix thoroughly.
3. Centrifuge the tube briefly.
4. In a 96-well plate or 8-tube strip, pipet 25 µL of the PreAmp reaction mix into each well or tube.
5. Seal the plate or tubes.

Run the preamplification reaction

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

Step Type	Time	Temperature
Hold	10 min	95°C
Hold	2 min	55°C
Hold	2 min	72°C
Cycle	15 sec	95°C
(12 Cycles)	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 the plate.
4. Add 175 µL of 0.1× TE, pH 8.0 to each well or tube. This is the diluted PreAmp product (Final Volume = 200 µL).
5. Seal the plates or the 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 PCR reaction mix

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

Component	Volume for 1 reaction†	Volume for 96 reactions†	Volume for 96 reactions × 4 replicates †
384-Well Plates or 96-well Fast Plates			
20X TaqMan® MicroRNA Assays	0.50 µL	—	—
Diluted PreAmp Product	0.10 µL	10.80 µL	43.20 µL
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)‡	5.00 µL	540.00 µL	2160.00 µL
Nuclease-free water	4.40 µL	475.20 µL	1900.80 µL
Total	10.00 µL	1026.00 µL	4104.00 µL
96-Well Plates§			
20X TaqMan® MicroRNA Assays	1.0 µL	—	—
Diluted PreAmp Product	0.20 µL	21.60 µL	86.40 µL
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)‡	10.00 µL	1080.00 µL	4320.00 µL
Nuclease-free water	8.80 µL	950.4 µL	3801.6 µL
Total	20.00 µL	2052.00 µL	8208.00 µL

† Included a 12.5% excess for volume loss from pipetting.

‡ TaqMan® Universal Master Mix, No Amperase® UNG, (2X) may also be used.

§ The total reaction volume per well is adjusted to 20 µL for a 96-well plate. The number of RT reactions may need to be adjusted depending on the number of PCR replicates and assays being run.

2. Add 0.5 µL of TaqMan® MicroRNA Assays (20X) into each well for a 10 µL reaction volume. Add 1.0 µL for a 20 µL reaction volume.
3. Add 9.5 µL of the PCR reaction mix, including the RT product, into each well for a 10 µL reaction volume. Add 19 µL of the PCR reaction mix into each well for a 20 µL reaction volume.
4. Seal the plate with MicroAmp Optical Adhesive Film.
5. Briefly centrifuge the plate.

Run the real-time PCR reaction

Load and run the 96- or 384-well plate using the following thermal cycling parameters:

Step	Time	Temperature
Hold	10 min	95°C
Cycle (40 Cycles)	15 sec	95°C
	60 sec	60°C
Hold	∞	4°C

Analyze the data

Use normalized $\Delta\Delta C_T$ or fold-change for analysis. For detailed information how to analyze Comparative C_T (RQ) refer to the instrument manual:

- *Applied Biosystems® 7300/7500/7500 Fast Real-time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (Part. No. 4347824 Rev. F)
- *Applied Biosystems® StepOne™ and StepOne Plus™ Real-Time PCR Systems, Relative Standard Curve and Comparative C_T Experiments* (Part No. 4376785 Rev. F)
- *Applied Biosystems® 7900 HT Fast Real-Time System Relative Quantitation Using Comparative C_T Getting Started Guide* (Part No. 4364016)
- *Applied Biosystems® ViiA™ 7 Real-Time PCR System Getting Started Guide* (Part No. 4441434 Rev. B)
- *Applied Biosystems® QuantStudio™ 12K Flex Real-Time PCR System Multi-Well Plates and Array Card Experiments* (Part No. 4470050 Rev. A)

For detailed downstream analysis, Life Technologies recommends software such as **ExpressionSuite**, DataAssist, or Integromics Real-Time StatMiner Software available at **www.integromics.com**.

Run 96- or 384-well plates without preamplification

Prepare the RT primer pool

Prepare the RT primer pool as described on page 3.

Run the RT reaction

Prepare the RT reaction mix

Note: Excluding the RT primers, all additional reagents necessary for the multiplex RT step are contained in the TaqMan® MicroRNA RT Kit (Part no. 4366596 or 4366597).

Note: More sample is required when running Custom RT pools (with or without preamplification) when using plates rather than microfluidic cards, therefore additional RT and PreAmp reactions are necessary.

1. Prepare the 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 1 sample †‡	Volume for 3 samples §
RT Primer Pool††	6.00 µL	6.75 µL	20.25 µL
dNTPs with dTTP (100 mM)	0.30 µL	0.34 µL	1.01 µL
MultiScribe Reverse Transcriptase (50 U/µL)	3.00 µL	3.38 µL	10.13 µL
10X RT Buffer	1.50 µL	1.69 µL	5.06 µL
RNase Inhibitor (20 U/µL)	0.19 µL	0.21 µL	0.64 µL
Nuclease-free water	1.01 µL	1.14 µL	3.41 µL
Total	**12.00 µL**	**13.50 µL**	**40.50 µL**

† Includes a 12.5% excess for volume loss from pipetting.
‡ Volume includes amount for running up to 96 assays in the PCR step with no replicates.
§ Volume includes amount for running up to 96 assays in the PCR step with 4 replicates of each assay.
†† Prepared on page 3.

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 RT reaction mix into each well or tube.
5. Add 3 µL of total RNA (350-1000 ng per reaction) 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 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® 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 real-time PCR reaction

Prepare the PCR reaction mix

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

Component	Volume for 1 reaction†	Volume for 96 reactions†	Volume for 96 reactions × 4 replicates †
384-well Plate or 96-well Fast Plate			
20X TaqMan® MicroRNA Assays	0.50 µL	—	—
RT Product	0.08 µL	8.60 µL	34.60 µL
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)‡	5.00 µL	540.00 µL	2160.00 µL
Nuclease-free water	4.42 µL	477.40 µL	1909.40 µL
Total	10.00 µL	1026.00 µL	4104.00 µL
96-well Plate§			
20X TaqMan® MicroRNA Assays	1.0 µL	—	—
RT Product	0.16 µL	17.28 µL	69.12 µL
TaqMan® Universal Master Mix II, No AmpErase® UNG (2X)‡	10.00 µL	1080.00 µL	4320.00 µL
Nuclease-free water	8.84 µL	954.72 µL	3818.88 µL
Total	20.00 µL	2052.00 µL	8208.00 µL

† Included a 12.5% excess for volume loss from pipetting.

‡ TaqMan® Universal Master Mix, No Amperase® UNG, (2X) may also be used.

§ The total reaction volume per well is adjusted to 20 µL for a 96-well plate. The number of RT reactions may need to be adjusted depending on the number of PCR replicates and assays being run.

2. Add 0.5 µL of TaqMan® MicroRNA Assays (20×) into each well for a 10 µL reaction. Add 1.0 µL for a 20 µL reaction.
3. Add 9.5 µL of the PCR reaction mix, including the RT product, into each well for a 10 µL reaction volume. Add 19 µL of the PCR reaction mix into each well for a 20 µL reaction volume.
4. Seal the plate with MicroAmp Optical Adhesive Film.
5. Briefly centrifuge the plate.

Run the real-time PCR reaction

Use the following thermal cycling parameters:

Step	Time	Temperature
Hold	10 min	95°C
Cycle (40 Cycles)	15 sec	95°C
	60 sec	60°C
Hold	∞	4°C

Analyze the data

Use normalized $\Delta\Delta C_T$ or fold-change for analysis. See the recommendations listed on page 9.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) 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 SDS.
- 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 SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

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