Megaplex™ Pools

for microRNA Expression Analysis

For safety and biohazard guidelines, refer to the "Safety" section in the *Megaplex™ Pools Protocol* (PN 4399721). For all chemicals in **bold red** type, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Prepare the Sample and Choose a Workflow

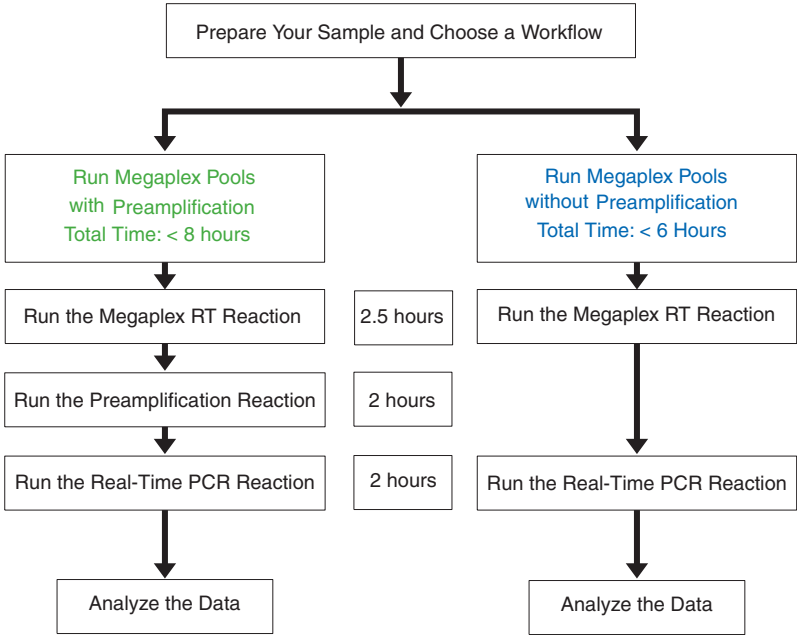
1 **Prepare the sample.**

Prepare total RNA with the *mir*Vana mRNA Isolation Kit. Do *not* enrich for small RNAs.

2 **Choose a workflow.**

Two workflows are described here: one with preamplification of your sample and one without preamplification of your sample. 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 on...
1 – 350 ng	Run Megaplex Pools *with* preamplification	pages 2 to 4
350 – 1000 ng	Run Megaplex Pools without preamplification	pages 5 to 6



Run Megaplex™ Pools *with* Preamplification

1 Run the Megaplex RT Reactions.

a. Prepare the RT reaction mix 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 pippetting.

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

c. In a 96-well plate or 8-tube strips, pipette 4.5 µL of each RT reaction mix into each well or each tube.

d. 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.

e. Seal the plate or tubes, invert six times to mix, and spin briefly.

Note: Do not use MicroAmp® *Optical* Adhesive Film to seal the plate.

f. Incubate the plate on ice for 5 min.

g. Set up the run method:

- Ramp speed or mode: **9700** using **Std** or **Max** ramp speed. **7900HT** using **Std** ramp speed.
- Reaction volume (µL): **7.5** and 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	∞

h. (Optional) stopping point: The cDNA can be stored at –15 °C to –25 °C for at least one week.

2 Run the preamplification reaction.

a. Prepare the PreAmp reaction mix 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, 2×	12.5	140.62
Megaplex™ PreAmp Primers (10×)	2.5	28.13
Nuclease-free water	7.5	84.37
Total	22.5	253.12

‡ Includes 12.5% excess for volume loss from pippetting.

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

c. In a 96-well plate or 8-tube strips, pipette 2.5 µL of each RT product into its corresponding well or tube.

d. Dispense 22.5 µL of PreAmp reaction mix into each well of the 96-well plate or 8-tube strips containing the RT product.

e. Seal the plate or tubes, invert six times to mix, and spin briefly.

f. Incubate the plate or tubes on ice for 5 min.

g. Set up the run method:

- Ramp speed or mode: **9700** using **Std** ramp speed.
- Reaction volume (µL): **25** and thermal-cycling conditions:

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.

h. Remove the 96-well plate or 8-tube strips from the thermal cycler.

i. Briefly centrifuge the tubes or plate.

j. Add 75 µL of 0.1× TE pH 8.0 to each well or tube.

k. Seal the plate or tubes, then invert six times to mix, and spin briefly.

l. (Optional) stopping point: The diluted preamplified product can be stored at −15 to −25 °C for at least one week.

3 **Run the real-time PCR reaction.**

a. Prepare the TaqMan MicroRNA Array.

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

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

‡ Includes 12.5% excess for volume loss from pippetting.

c. Invert the tubes to mix, then centrifuge the tubes briefly.

d. Load and run the array using the 384-well TaqMan Low Density Array default thermal-cycling conditions. Refer to the *Applied Biosystems TaqMan® Array User Bulletin* (PN 4371129).

Analyze the Data

1 **Review the results.**

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

For detailed information, refer to the *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016).

b. 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.

c. Review the gene expression plot.

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

2 **Troubleshoot.**

Refer to the *Megaplex™ Pools Protocol* (PN 4399721).

Run Megaplex™ Pools *Without* Preamplification

1 Run the Megaplex RT Reactions.

a. Prepare the RT reaction mix 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 pippetting.

b. Mix gently, then centrifuge to bring all of the liquid to the bottom of the tube.

c. In a 96-well plate or 8-tube strip, pipette 4.5 µL of the RT reaction mix into each well or each tube, respectively.

d. Add 3 µL (350 to 1000 ng) total RNA (or 3 µL of water for No Template Control reactions) into each well or tube containing RT reaction mix.

e. Seal the plate or tubes, invert six times and spin briefly.

f. Incubate the plate or tubes on ice for 5 min.

g. Set up the run method:

- Ramp speed or mode: **9700** using **Std** or **Max** ramp speed. **7900HT** using **Std** ramp speed.
- Reaction volume (µL): **7.5** and 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	∞

h. Load, then run the plate.

i. (Optional) stopping point: Store the cDNA product at -15 to -25 °C for at least one week.

2 **Run the real-time PCR reaction.**

a. Prepare the TaqMan MicroRNA Array.

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

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

‡ Includes 12.5% excess for volume loss from pippetting.

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

d. Load and run the array using the 384-well TaqMan Low Density Array default thermal-cycling conditions. See the *TaqMan® Array User Bulletin* (PN 4371129).

Analyze the Data

1 **Review the results.**

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

For detailed information, refer to the *Applied Biosystems 7900HT Fast Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016).

b. 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.

c. Review the gene expression plot.

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

2 **Troubleshoot.**

Refer to the *Megaplex™ Pools Protocol* (PN 4399721).

Order Megaplex Products

For details on how to order, refer to the Megaplex™ products page at **http://miRNA.appliedbiosystems.com**

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