Evaluation of normalized miRNA expression in Formalin-Fixed Paraffin-Embedded (FFPE) tissue

Alessandra Bisagni,Stefania Croci.

Abstract

This protocol is provided to synthesize single-stranded cDNA from total RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit and quantification of miRNA using the comparative Cycle threshold (Ct) method. Endogenous controls are used to normalize the expression levels of target miRNAs by correcting differences in the amount of cDNA loaded into the PCR reactions. An appropriate constitutively expressed endogenous control must be selected.

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Guidelines

For the optimal performance of the TaqMan MicroRNA Reverse Transcription Kit and of the TaqMan MicroRNA Assays, use RNA with the following characteristics:

- Free of inhibitors of reverse transcription and PCR,
- Dissolved in PCR-compatible buffer;
- Free of RNase activity;
- Allow the kit components to thaw on ice.

Protocol

Reverse Transcription (RT)

Step 1.

Prepare the RT master mix by scaling the volumes listed below to the desired number of RT reactions:

Component	Master Mix Volume for One 15 µL Reaction (µL)
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10× Reverse Transcription Buffer	1.50
RNase Inhibitor, 20U/µL	0.19

Nuclease-free water	4.16
Total	**7.0**

Reverse Transcription (RT)

Step 2.

Mix gently. Centrifuge to bring solution to the bottom of the tube.

Reverse Transcription (RT)

Step 3.

Place the RT master mix on ice until you prepare the microRNA reaction.

Reverse Transcription (RT)

Step 4.

Combine RT master mix with total RNA and RT primer from each assay set into the corresponding RT reaction tube:

Component	Volume (µL)
RT master mix	7.0
total RNA sample	5.0
miRNA specific RT primer	3.0
Total	**15.0**

Note: We use 10 ng of total RNA per reaction.

Reverse Transcription (RT)

Step 5.

Seal the tube and mix gently. Centrifuge to bring solution to the bottom of the tube.

Reverse Transcription (RT)

Step 6.

Incubate the tube on ice for 5 min and keep on ice until you are ready to load the thermal cycler:

Step Type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

PCR Amplification

Step 7.

Note: Prepare the reactions on ice.

Prepare 17.67 µL of the PCR master mix/water mixture per 20-µL PCR reaction into a eppendorf tube (the PCR reaction tube). Scale the volumes listed below to the appropriate number of RT reactions:

Reagent	Master Mix Volume for One 20 mL Reaction (µL)
TaqMan 2× Universal PCR Master Mix, No AmpErase UNG	10.00
Nuclease-free water	7.67
Total Volume	**17.67**

PCR Amplification

Step 8.

Mix gently. Centrifuge to bring solution to the bottom of the tube.

PCR Amplification

Step 9.

Add 1.0 µL of 20× TaqMan MicroRNA Assay mix into the PCR Reaction tube for appropriate number of RT reactions .

PCR Amplification

Step 10.

Dispense 18.67 µL PCR mix for each cDNA that has to be tested in 96-well PCR reaction plate.

PCR Amplification

Step 11.

Add 1.33 µL of cDNA from the RT reaction tube into each well of the PCR reaction plate.

PCR Amplification

Step 12.

Seal the plate with an optical adhesive cover, then centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.

PCR Amplification

Step 13.

Insert the reaction plate into the instrument and start the Thermal cycling reaction as follow:

Step Type		Time	Temperature (°C)
HOLD		2 min	50
HOLD		10 min	92
CYCLE (40 cycles)	Denature	15 sec	95
	Anneal/Extend	1 min	60

Analyzing Data

Step 14.

Calculate the difference between the Ct of miRNA target and the Ct of the endogenous control miRNA = ΔCt .

Analyzing Data

Step 15.

Calculate the normalized miRNA expression level as $2^{-\Delta Ct}$.

Note: it is recommended to analyzed cDNA sample in triplicate. In this case the mean Ct of miRNA target and of the endogenous control miRNA must be calculated before step 14.

Note: to normalized miRNA expression we suggest the use of microRNA such as miR-191 instead of small RNA.