Chapter 7

MicroRNA Expression Analysis: Techniques Suitable for Studies of Intercellular and Extracellular MicroRNAs

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Abstract

MicroRNAs, the class of small ribo-regulators, have been implicated in the regulation of a range of different biological processes, including development and differentiation, proliferation, and cell death. Only for a small fraction of identified microRNAs has a function been elucidated; therefore, a great deal of research remains to be performed to fully understand the role and implications of microRNAs.

This chapter discusses protocols for the isolation of microRNAs, reverse transcription, PCR, and large scale profiling using TaqMan low density miRNA arrays for analysis of microRNA expression levels.

Key words: MicroRNA, miRNA, TaqMan low density microRNA array, Megaplex RT primers

1. Introduction

MicroRNAs (miRNAs) are a family of endogenous small non-coding RNAs of 19–28 nucleotides in length that regulate gene expression at the post-transcriptional level by binding to complementary sites in the 3'-untranslated region (3'UTR) of target mRNAs. Based on the level of complementarity, miRNAs can direct target mRNAs for degradation or translational repression (1). MiRNAs are estimated to regulate at least one third of all human genes (2) and have been implicated in both normal physiological and pathological conditions, including differentiation and development, metabolism, proliferation, cell death, viral infection, and cancer (3), bringing miRNAs to the forefront of molecular biology interest.

A range of molecular biology tools have been developed in recent years for the identification, quantification, and functional

analysis of miRNAs both in vitro and in vivo. This chapter discusses the techniques used for the isolation and quantification of miRNA expression levels.

Many of the column-based total RNA isolation kits available result in loss of the small RNA fraction (i.e. RNAs of approximately less than 200 nucleotides in length) including miRNAs. Subsequently, many specialist miRNA isolation kits were developed, included the Applied Biosystem MirVana miRNA isolation kit which provides a relatively straightforward, quick procedure (approximately 30 min) for the isolation of total RNA including miRNAs, with the option of further enrichment specifically for small RNAs. For the procedures discussed in this chapter, however, enrichment for small RNAs is not required. Here, the TriReagent method for total RNA isolation (including miRNAs) is described due to the presence of this reagent in most molecular biology labs. This method is based on a guanidine thiocyanate phenol-chloroform extraction for the simultaneous extraction of total RNA, DNA, and protein (4). Total RNA is then precipitated using isopropanol; subsequent washes with 75% ethanol remove any contaminating traces of DNA or protein. The quality and quantity of isolated RNA can be assessed by measuring the ratio of absorbance at 260 and 280 nm. A ratio of 2 indicates good quality RNA, while a lower ratio indicates the presence of protein, phenol, or other contaminants.

The procedure for reverse transcription (RT) of miRNAs as described below involves the use of specific RT primers for the sequence-specific RT of individual miRNAs in a sample. TaqMan chemistries are then employed for real-time relative quantification of miRNA expression levels. TaqMan assays utilise a target-specific probe, containing a fluorescent reporter dye bound to the 5' end, and a non-fluorescent quencher bound to the 3' end. When a probe binds specifically to a target sequence, the fluorescent reporter dye is cleaved off due to the 5'-3' exonuclease activity of *Taq DNA polymerase* enzyme. TaqMan probe-based chemistries provide an extra layer of specificity on SYBR green technology, which fluoresces when bound to double-stranded DNA. Exiqon offers an alternative miRNA PCR technology – miRCURY LNA (locked nucleic acids). miRCURY LNA miRNA PCR primers have incorporated a backbone modification of the sugar residues allowing for high affinity binding to target miRNAs. SYBR green technology is then used for detection of these double-stranded duplexes. miRCURY LNA and SYBR technologies are not discussed in detail in this chapter, for further information see <http://www.exiqon.com/mirna-pcr>.

Relative quantification of miRNA expression levels is calculated based on the $\Delta\Delta C_t$ method (5). An endogenous control, which is expressed constitutively at the same level in all conditions under analysis, is used for normalisation of cycle threshold (C_T) values. A relevant calibrator sample, such as an untreated or time

zero sample, is then used for calculation of relative expression levels in test samples.

Large scale miRNA profiling can be performed using TaqMan low density miRNA arrays (TLDA). TLDA are 384-well microfluidic cards, with each well representing a unique miRNA assay or endogenous control. A set of two cards are available for profiling expression levels of 754 miRNAs plus controls, based on miRBase v 14. Megaplex miRNA RT primer pools are used to reverse transcribe RNA for subsequent analysis using TLDA. These primer pools contain two sets of RT primers, used for RT of the specific miRNAs on each of the TLDA cards. TLDA cards utilise 350–1,000 ng of RNA per card. Alternatively, as little as 1 ng RNA can be used if a pre-amplification step is performed. Exiqon also offer an alternative option for large scale miRNA profiling, their Universal cDNA Synthesis Kit incorporates a polyadenylation step allowing for RT of all miRNAs into cDNA. Ready-to-Use PCR panels are then used to profile expression of 742 miRNAs.

Currently (as of 11/07/2011), 1424 miRNAs have been identified in the human genome (miRBase v 17.0, <http://www.mirbase.org/>) (6–8). However, the function of only a small fraction of these miRNAs has been elucidated. The development of technologies for the analysis of miRNA expression and function, such as those described in this chapter will, undoubtedly, aid in our understanding of miRNAs and elucidating their role in the cell.

2. Materials

2.1. Preparation of Samples

1. Phosphate-buffered saline (PBS).

2.2. Isolation of RNA

1. TriReagent, store at 4°C.
TriReagent is toxic and should be used under a fume cupboard.
2. Chloroform – Chloroform is harmful and should be used under a fume cupboard.
3. Isopropanol – Isopropanol is an irritant and is also highly flammable.
4. 75% Ethanol: Add 75 mL of 100% ethanol to 25 mL of water. Ethanol is highly flammable.
5. RNase-free water.

2.3. Generation of miRNA cDNA

1. RNase-free water.
2. TaqMan miRNA reverse transcription kit (containing 100 mM dNTPs, MultiScribe reverse transcriptase 50 U/μL, 10× RT buffer and RNase inhibitor 20 U/μL) (Applied Biosystems), store at –20°C.

3. TaqMan RT primers, specific for individual miRNAs, from TaqMan miRNA assays (Applied Biosystems), store at -20°C .
4. Thermocycler.

2.4. Real-Time PCR Amplification of miRNAs

1. TaqMan Universal PCR master mix, no amperase UNG (Applied Biosystems), store at 4°C .
2. Nuclease-free water.
3. TaqMan miRNA assays available for each specific miRNA to be analysed (Applied Biosystems), light sensitive, store at -20°C .
4. MicroAmp fast optical 96-well reaction plate with barcode 0.1 mL (Applied Biosystems).
5. MicroAmp optical adhesive film (Applied Biosystems).
6. 7900HT or 7500 fast real-time PCR instrument.

2.5. TaqMan Low Density miRNA Arrays

1. Megaplex RT primers (Applied Biosystems), store at -20°C .
2. TaqMan miRNA reverse transcription kit (containing 100 mM dNTPs, MultiScribe reverse transcriptase 50 U/ μL , 10 $\times$ RT buffer, and RNase inhibitor 20 U/ μL) (Applied Biosystems), store at -20°C .
3. TaqMan universal PCR master mix, no amperase UNG (Applied Biosystems), store at 4°C .
4. TaqMan miRNA array (Applied Biosystems), light sensitive, store at 4°C .
5. 7900HT fast real-time PCR instrument.

3. Methods

3.1. Preparation of Samples

1. Cell pellets are prepared by centrifuging cell suspension at $1,000\times g$ for 5 min.
2. Discard supernatant. Resuspend pellet with 1 mL PBS and centrifuge at $1,000\times g$ for 5 min.
3. Repeat step 2.
4. Discard supernatant. Ensure all traces of PBS are removed. Store pellet at -80°C until required.

3.2. Isolation of RNA

1. Add 1 mL of TriReagent to $5\text{--}10\times 10^6$ cells (see Note 1). Lyse cells by repeat pipetting and allow to stand at room temperature for 5 min.

2. Add 0.2 mL chloroform per mL of TriReagent. Shake vigorously for 15 s and allow to stand at room temperature for 15 min.
3. Centrifuge at $12,000\times g$ for 15 min at 4°C .
4. Three phases should be visible in sample at this stage; the lower organic phase containing protein, an interphase containing DNA, and the upper aqueous phase containing RNA (see Note 2). Remove the upper aqueous phase to a fresh Eppendorf and add 0.5 mL of ice-cold isopropanol per mL of TriReagent used in step 1.
5. Mix sample by inverting and allow to stand at room temperature for 5–10 min (see Note 3).
6. Centrifuge at $12,000\times g$ for 30 min at 4°C to pellet precipitated RNA.
7. Remove supernatant without disturbing the pellet.
8. Wash pellet with 1 mL of 75% ethanol. Vortex sample and centrifuge at $7,500\times g$ for 5 min at 4°C .
9. Remove supernatant and repeat washing step 8.
10. Remove supernatant and allow RNA pellet to air-dry for 5–10 min (see Note 4).
11. Add an appropriate volume of RNase-free water, mix by repeat pipetting, allow to stand at room temperature for 10–15 min to allow dissolution of RNA pellet.
12. Assess RNA quality and quantity using NanoDrop (see Note 5).
13. Store at -80°C until required.

3.3. Generation of microRNA cDNA

1. Dilute total RNA isolated (as described in Subheading 3.2) to 2 ng/ μL with RNase-free water. Add 5 μL of diluted RNA to Eppendorf tube. Use multiple tubes, if multiple miRNAs are to be reverse transcribed from the same sample (see Notes 6 and 7).
2. Prepare RT master mix as follows (volume for one sample):

100 mM dNTPs	0.15 μL
MultiScribe reverse transcriptase 50 U/ μL	1.00 μL
10 $\times$ Reverse transcription buffer	1.50 μL
RNase inhibitor 20 U/ μL	0.19 μL
Nuclease-free water	4.16 μL
Total	7.00 μL

3. Mix RT master mix by flicking, centrifuge briefly to bring solution to the bottom of tube, and place on ice.
4. Add 7 μL of RT master mix to 5 μL of diluted RNA from step 1.
5. Mix gently by flicking and centrifuge to bring solution to bottom of tube (see Note 8).
6. Add 3 μL TaqMan RT primer to RT master mix/RNA tube (see Note 9).
7. Mix gently by flicking and centrifuge to bring solution to the bottom of tube.
8. Place sample in thermocycler and run the following cycle:
 - (a) 16°C for 30 min.
 - (b) 42°C for 30 min.
 - (c) 85°C for 5 min.
9. Store miRNA cDNA at -20°C until required.

3.4. Real-Time PCR Amplification of miRNAs

1. Prepare PCR master mix as follows (volume for one well) (see Notes 10 and 11):

TaqMan 2 $\times$ Universal PCR master mix	10.00 μL
Nuclease-free water	7.67 μL
Total	17.67 μL

2. Mix gently and centrifuge to bring solution to the bottom of the tube.
3. Add 1 μL of TaqMan miRNA assay to PCR master mix, mix gently and centrifuge.
4. Transfer 1.33 μL of RT product from Subheading 3.3 to PCR plate (see Notes 12 and 13).
5. Add 18.67 μL of PCR master mix to each sample of RT product, mix gently by repeat pipetting.
6. Seal the plate with an optical adhesive cover.
7. Run plate at the following cycle parameters on standard mode on 7500 or 7900HT real-time PCR instrument (If using a PCR master mix with Amperase UNG, incorporate a 50°C for 2 minutes step before step (a) to allow activation of Amperase UNG activity):
 - (a) 95°C for 10 min.
 - (b) 95°C for 15 s.
 - (c) 60°C for 60 s.
 - (d) Run 40 cycles of steps (b) and (c).
8. Analyse real-time PCR relative quantification data.

**3.5. Large Scale
miRNA Profiling Using
TaqMan Low Density
MicroRNA Arrays**

1. Dilute RNA, 3 μL RNA to be used per reaction containing 350–1,000 ng (see Note 14).
2. Prepare Megaplex RT master mix as follows (volume for one array):

Megaplex RT primers (10 $\times$)	0.8 μL
dNTPs (100 mM)	0.2 μL
Multiscribe reverse transcriptase (50 U/ μL)	1.5 μL
10 $\times$ RT buffer	0.8 μL
MgCl ₂ (25 mM)	0.9 μL
RNase inhibitor (20 U/ μL)	0.1 μL
Nuclease-free water	0.2 μL
Total	4.5 μL

3. Mix gently and centrifuge to bring solution to the bottom of tube.
4. Add 3 μL of diluted RNA to Megaplex RT master mix.
5. Run sample on thermocycler at the following parameters:
 - (a) 16°C for 2 min.
 - (b) 42°C for 1 min.
 - (c) 50°C for 1 s.
 - (d) Repeat steps (a)–(c) for 40 cycles.
 - (e) 85°C for 5 min.
 - (f) Hold at 4°C.
6. Allow TaqMan miRNA array to warm to room temperature.
7. Prepare PCR reaction mix as follows (volume for one array):

TaqMan universal PCR master mix	450 μL
Megaplex RT product	6 μL
Nuclease-free water	444 μL
Total	900 μL

8. Mix gently and centrifuge to bring solution to the bottom of tube.
9. Dispense 100 μL of PCR reaction mix into each port of the TaqMan miRNA array (see Note 15).
10. Centrifuge TaqMan miRNA array cards at 331 $\times g$ for two consecutive 1 min spins.
11. Seal TaqMan miRNA array cards and load onto 7900HT Fast Real-Time PCR system (see Note 16).

12. Run TLDA cards at the following cycling parameters on standard mode:
 - (a) 50°C for 2 min.
 - (b) 94.5°C for 10 min.
 - (c) 97°C for 30 s.
 - (d) 59.7°C for 1 min.
 - (e) Repeat steps (c) and (d) for 40 cycles.
13. Analyse real-time PCR relative quantification data.

4. Notes

1. If using serum specimens, add 750 μ L TriReagent to 250 μ L serum and continue as per Subheading 3.2 (see Note 3).
2. On removal of upper aqueous phase containing RNA, much care needs to be taken to avoid withdrawing contaminating DNAs from the interphase section. We recommend leaving a little of the upper aqueous phase containing RNA to minimise the possibility of carrying over DNA contamination.
3. If using samples with low levels of RNA, e.g. serum, on adding isopropanol, glycogen can also be added (to a final concentration of 120 μ g/mL), and samples incubated overnight at -20°C to act as a carrier for precipitated RNA.
4. Do not allow pellet to air-dry completely, as this will decrease its solubility. Once pellet begins to change from a white to transparent colour then add RNase-free water to resuspend.
5. RNA purity is assessed by the ratio of absorbance at 260 and 280 nm. A ratio of approximately 2.0 indicates a good quality RNA sample. A lower ratio may indicate the presence of protein, phenol or other contaminants, which absorb at 280 nm, potentially carried over from the RNA isolation procedure.
6. When preparing RT master mix, multiply reagents by the appropriate number of miRNAs to be analysed and samples to be used. Always include 10–12% reagent excess when scaling up.
7. Minus RT controls i.e. without *MultiScribe reverse transcription* enzyme should be included, and used in the subsequent PCR experiments to ensure that the TaqMan miRNA assay does not pick up genomic DNA contaminants.
8. Do not exceed 350 $\times$ g or 5 min when centrifuging.
9. Before opening a tube of TaqMan RT primer, centrifuge to bring solution to the bottom of the tube and so to prevent loss of liquid trapped in the lid.

10. Prepare triplicate wells per sample per miRNA when performing real-time PCR.
11. Always perform endogenous control assay for each different sample used on the plate. "No template" controls should also be performed for each different miRNA assay being used on the assay plate.
12. Always add RT product to PCR plate first. In cases of confusion, it is obvious which wells contain 1.33 μ L of RT product. If PCR master mix is added first, it can be very difficult to decipher to which wells RT product has been added to – as 18.67 μ L and 20 μ L look very similar.
13. When loading 96-well PCR plates, it may help to draw a template table, labelling which wells should contain which samples.
14. If RNA is limited or target miRNAs are expected to be expressed at low levels then very small amounts (1–350 ng) of RNA can be used for megaplex RT followed by a pre-amplification step before the real-time PCR.
15. Take care not to pierce the backing foil of the TLDA when dispensing PCR reaction mix into TaqMan miRNA arrays.
16. Take care that sealer and TaqMan miRNA array card are in the correct orientation before sealing the card, as sealing in the wrong orientation will rip the backing foil from the card and destroy the array.

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