Chapter 13

Profiling Circulating miRNAs from the Plasma of Individuals with Metabolic Syndrome

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Abstract

The technique of RT-qPCR (real time-quantitative polymerase chain reaction) is invaluable in miRNA research both at the profiling and individual RT-qPCR stages. At the profiling stage, numerous miRNAs are looked at in the plasma of numerous individuals from two or more cohorts (*i.e.*, control vs. case). The miRNAs of interest would be either upregulated or downregulated by more than twofold in the case cohort compared to the control cohort. Profiling human specimens for miRNA biomarkers has exploded over the last decade, with researchers profiling plasma, serum, urine, and also the miRNA content of extracellular vesicles, which are also isolated from human specimens. RT-qPCR is a relatively easy technique; however, sample preparation from plasma to RNA to RNA input in RT reaction requires accuracy and precision.

Key words Metabolic syndrome, Blood plasma, RNA isolation, miRNA profiling, RT-qPCR

1 Introduction

Metabolic syndrome (MetS) is the culmination of a number of components, *i.e.*, hypertension, dyslipidemia, abdominal obesity and insulin resistance, resulting in an increased risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and cancer [1]. Obesity is reaching epidemic proportions world-wide and therefore; the rate of MetS is steadily increasing alongside obesity. There is call for a novel method for diagnosis of MetS to subsequently reduce the risk of T2DM, CVD and cancer, which are among the leading causes of death globally [1].

microRNAs (miRNA) are small, approximately 22 nucleotide long noncoding RNA molecules found in animals, plants, and numerous viruses. miRNAs regulate gene expression via their association with target sites on mRNAs and association with effector complexes [2]. Their function is posttranscriptional gene regulation, primarily downregulation of the target protein through translational repression (binding or cleavage) of the target mRNA untranslated regions. However, recent studies have shown

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miRNAs can function to posttranscriptionally stimulate gene expression, by working in concert with their associated proteins, microribonucleoproteins by a direct or indirect mechanism [2]. It is therefore, not surprising that aberrant miRNA expression is associated with numerous disorders, including obesity [3, 4], diabetes [4], and various cancer subtypes [5, 6].

In recent years, miRNA profiling has developed as the preferred method over traditional gene expression profiling due to (1) the stability of miRNAs, this is due to their short length, (2) high sensitivity, (3) reliable as diagnostic tools, and (4) the origin of cancer at a metastatic site can be determined [5]. Additionally, miRNAs are easily assessed in body fluids, *i.e.*, serum and plasma; therefore, they are minimally invasive biomarkers. However, they can also be assessed in tumor or tissue specimens. In this method plasma miRNAs from obese individuals and individuals diagnosed with MetS according to the International Diabetes Federation were analyzed.

Numerous high-throughput miRNA profiling technologies exist, including, Quantitative reverse Transcription PCR (RT-qPCR), miRNA microarray, and RNA sequencing, either smaller scale or high-throughput [5]. In this chapter we discuss the use of the miRCURY LNA™ Universal RT miRNA qPCR Panels. This method was selected as (1) the RT reaction is universal and so supplies templates for all miRNAs in the qPCR reaction and (2) both the reverse and forward amplification primers are specific to the miRNA of interest providing notable sensitivity and specificity.

Blood plasma is a pale yellow liquid and acts as the extracellular matrix of blood cells. It accounts for 55 % of the body's total blood volume. In this method, plasma was chosen for further miRNA analysis as the preparation of plasma is less complex than that for serum. Preparation of plasma involves centrifugation of whole blood to remove white blood cells and red blood cells; however, serum requires blood clotting followed by centrifugation. Plasma isolated from EDTA tubes was, therefore, used to reduce the procedural variation resulting from differences in clotting and subsequent serum collection.

2 Materials

2.1 RNA Isolation and Quality Control

1. TRIReagent™, should be used in the fume hood: store at 4 °C.
2. Chloroform, should be used in the fume hood: store at 4 °C.
3. 120 μg/mL of glycogen: store at −20 °C for up to 1 year.
4. Isopropanol: store at 4 °C.
5. Ethanol: prepare 75 % using ddH_2O and store at room temperature.
6. RNase-free water: store at room temperature.

2.2 Agilent Pico and Small RNA Kits

1. Chips: store at room temperature.
2. Gel matrix, dye concentrate, conditioning medium, and marker (provided with the kit): store at 4 °C.
3. Ladder (provided with the kit): store at –20 °C.

2.3 Reverse Transcription

1. 5× reaction buffer: store at –20 °C.
2. Enzyme mix: store at –20 °C.
3. Spike in RNA (UniSP6) (*see* **Note 1**): store at –20 °C.
4. Nuclease-free water: store at –20 °C.
5. RNA template: store at –80 °C.

2.4 PCR Analysis

1. SYBR Green master mix: store at –20 °C.
2. Profiling plates and individual primer: store at –20 °C.
3. cDNA store at –20 °C.

2.5 Required Equipment

1. NanoDrop.
2. BioAnalyzer.
3. Thermal cycler.
4. Real-time PCR instrument.

3 Methods

3.1 RNA Isolation and Quantification

RNA from plasma is isolated using TRIReagent™ and the resulting RNA is quantified using the NanoDrop. Care is needed when isolating RNA, as RNase enzymes easily degrade RNA. Therefore, benches should be wiped with RNaseZap and 70% ethanol. Eppendorfs and RT-qPCR plates should be RNase-free, RNase-free filter tips should be used and all reagents should be molecular grade.

1. Plasma specimens are procured by standard collection protocols. Specifically, whole blood is procured in EDTA vials and mixed by inversion of the tube (8–10 times). Specimens are immediately centrifuged at 2000 × *g* at room temperature (RT) for 15 min. Following centrifugation the top yellowish layer is collected and stored in 1 mL aliquots in cryovials at –80 °C until required.
2. Plasma specimens are thawed slowly on ice, 250 μL is transferred to an Eppendorf and plasma specimens are returned to –80 °C. 250 μL of plasma and 750 μL of TRIReagent™ (*see* **Note 2**) are combined, mixed, and incubated at RT for 10 min.
3. To this, 200 μL of chloroform is added, mixed vigorously for 15 s and incubated at RT for 10 min.
4. After incubation the resulting mixture is centrifuged at 13,200 × *g* at 4 °C for 15 min to separate the RNA, DNA, and protein layers.

5. The upper colorless aqueous phase is transferred to a fresh Eppendorf tube.
6. To the aqueous phase 1.2 µL of glycogen and 500 µL of ice-cold isopropanol is added, mixed and incubated at RT for 10 min. Samples are then stored at −20 °C overnight to allow maximum precipitation of the RNA.
7. The precipitated RNA is subsequently pelleted by centrifugation at 13,200 × *g* at 4 °C for 30 min.
8. The supernatant is discarded and the pellet is washed with 75% ethanol and vortexed. The pellet is then centrifuged at 7500 × *g* at 4 °C for 5 min.
9. **Step 8** is repeated, however, centrifuged at 13,200 × *g*.
10. The RNA pellet is then allowed to air dry until translucent and resuspended in 10 µL of RNase-free water. The resuspended RNA pellet is incubated on ice for 30 min to allow complete dissolution.
11. Store RNA at −80 °C (*see* **Notes 3** and **4**).
12. Quantification of the RNA is performed using the NanoDrop, with readings taken at 260, 280 and 230 nm (*see* **Notes 2** and **3**).

3.2 Quality Assessment: Agilent Chips (See Notes 4 and 5)

1. Agilent small RNA chips are used to assess the quality and percentage of miRNAs present in the total RNA sample.
2. The chip is placed on the priming station and 9 µL of gel matrix with dye concentrate is added to the appropriate well (Fig. 1a).

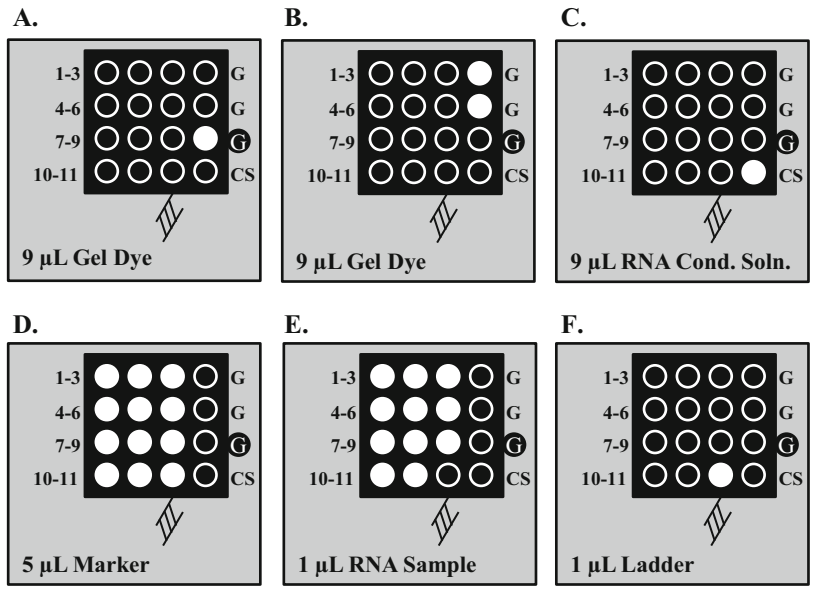

Fig. 1 Diagram of the addition of reagents and samples to the Agilent Small RNA chip for RNA characterization

3. The chip priming station is closed and the syringe plunger pressed until held by the clip for 60 s.
4. The plunger is released and moved to the 1 mL mark.
5. The priming station is opened and 9 μL of gel matrix is added to two additional wells (Fig. 1b).
6. 9 μL of conditioning solution is added to the appropriate well (Fig. 1c).
7. To all sample wells and the ladder well, 5 μL of marker is added (Fig. 1d).
8. Finally, 1 μL of denatured RNA sample is added to wells numbered 1–11 (Fig. 1e).
9. To the ladder well, 1 μL of ladder solution is added (Fig. 1f).
10. The chip is then placed in the IKA vortex for 1 min at 2400 rpm.
11. Subsequently the chip is placed in the BioAnalyzer (*see* **Notes 4** and **5**) and results visualized on an electropherogram (Fig. 2).

3.3 MicroRNA Profiling (Discovery Phase)

There are many techniques available for miRNA profiling as is described in the introduction. Here, we will provide the protocol for the use of miRCURY LNA™ Universal RT miRNA qPCR panels for use with serum/plasma. Under this category of miRNA profiling there are a number of options available to the researcher, *i.e.*, miRNome panels, focus panels and pick and mix custom panels. The basic principal of the RT-qPCR is the same for all options; the choice of panel depends on the researchers focus.

3.3.1 First Strand cDNA Synthesis (RT Reaction) (See Note 6)

Although quantification of the RNA is performed using the NanoDrop, the amount of RNA present in a plasma specimen cannot be accurately determined due to phenol contamination from the TRIReagent™. Therefore, an input volume of RNA rather than an input concentration is the preferred option. Performing a serial dilution of RNA input volumes will determine the correct

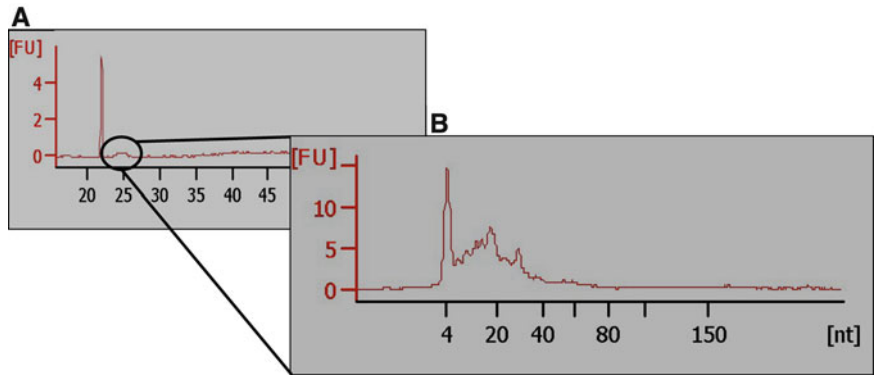

Fig. 2 Agilent electropherograms. A. RNA 6000 Pico kit electropherogram illustrating a peak of small RNAs at ~25 s. B. Small RNA kit illustrating miRNA content (22–26 nt) in the total RNA

RNA input volume to avoid inhibitors of the RT reaction (*see* **Note 7**). Exiqon have recommended the use of 5 potential endogenous control genes (miR-103, 191, 423-5p, 93, 425). miR-93 and miR-425 are usually stably expressed in serum/plasma. One of these miRNAs may be used for determining the input volume for the RT reaction. miR-451 and miR-16 may also be used as controls for hemolysis.

1. From serial dilutions (*see* **Note 7**) 1.6 μL of input RNA was determined as the optimum input volume for our specimens.
2. In an Eppendorf, the RT master mix is set up as follows (volume for one sample):

5× Reaction buffer	2 μL
Enzyme mix	1 μL
Spike ins (optional - *see* **Note 1**)	0.5 μL
Nuclease-free water	4.9 μL

3. Combine all reagents, mix by flicking and centrifuge briefly to bring all reagents to bottom of tube. Place on ice.
4. In a 96-well fast optical reaction plate pipette 8.4 μL of master mix to each well (1 well per RNA sample).
5. Add 1.6 μL of RNA to the appropriate well in the 96-well fast optical plate.
6. Seal the plate with optical sealing film and briefly centrifuge to bring reagents to bottom of plate.
7. Place plate in thermocycler and run the following cycle:
   (a) 42 °C for 60 min.
   (b) 95 °C for 5 min.
   (c) Cool to 4 °C.
8. The cDNA is then stored at −20 °C, until required.

3.3.2 PCR Amplification: miCURY LNA Profiling Panels (See Note 6)

Real-time PCR (qPCR) is performed according to manufacturer's protocol.

1. Thaw cDNA synthesized in Subheading 3.3.1 slowly on ice. Once thawed dilute cDNA 50× in RNase-free water.
2. In an Eppendorf prepare the SYBR Green master mix as follows:

   Serum/plasma focus panels: 1000 μL 2× master mix + 980 μL ddH_2O + 20 μL cDNA.

   miRNome human, mouse, and rat panels: 2000 μL 2× master mix + 1940 μL ddH_20 + 40 μL cDNA.
3. Mix by flicking and spin down to bring contents to bottom of tube.

4. Place solution in a trough and using a multichannel pipette 10 μL into each well of the 384-well plates.
5. Seal the plate and centrifuge for 1 min at 1200 × *g* to bring contents to bottom of well (*see* **Note 8**).
6. Place plate in real-time PCR machine and run the following cycle:
   (a) Hold Stage - 95 °C for 10 min.
   (b) PCR Stage (×40 cycles).
   - 95 °C for 10 s.
   - 60 °C for 1 min.

   (c) Melt Stage.
   - 95 °C for 15 s.
   - 60 °C for 1 min.
   - 95 °C for 15 s.

3.4 Individual RT-qPCR Validation Stage

miRNAs identified as upregulated or downregulated from the miRNA profiling need to be validated in a separate cohort of specimens. The method involves the synthesis of cDNA and qPCR analysis similar to the miRNA profiling, discovery phase.

3.4.1 First Strand cDNA Synthesis

First strand cDNA synthesis is performed as per Subheading 3.3.1 (Fig. 3).

3.4.2 PCR Amplification: Individual Assay (See Note 6) (Fig. 3)

1. Thaw cDNA synthesized in Subheading 3.4.1 slowly on ice. Once thawed, dilute cDNA 40× in RNase-free water.
2. In an Eppendorf prepare the SYBR Green master mix as follows (volume for one reaction):

SYBR Green PCR Master Mix	5 μL
miRNA Primer	1 μL

3. Place 6 μL of the SYBR Green master mix in each well of a 96-well fast optical PCR plate.
4. Add 4 μL of cDNA to each well (in triplicate for each sample) of the 96-well plate.
5. Seal the plate and centrifuge for 1 min at 1200 × *g* to bring contents to bottom of well (*see* **Note 8**).
6. Place plate in real-time PCR machine and run the following cycle:
   (a) Hold Stage - 95 °C for 10 min.
   (b) PCR Stage (×40 cycles).
   - 95 °C for 10 s.
   - 60 °C for 1 min.

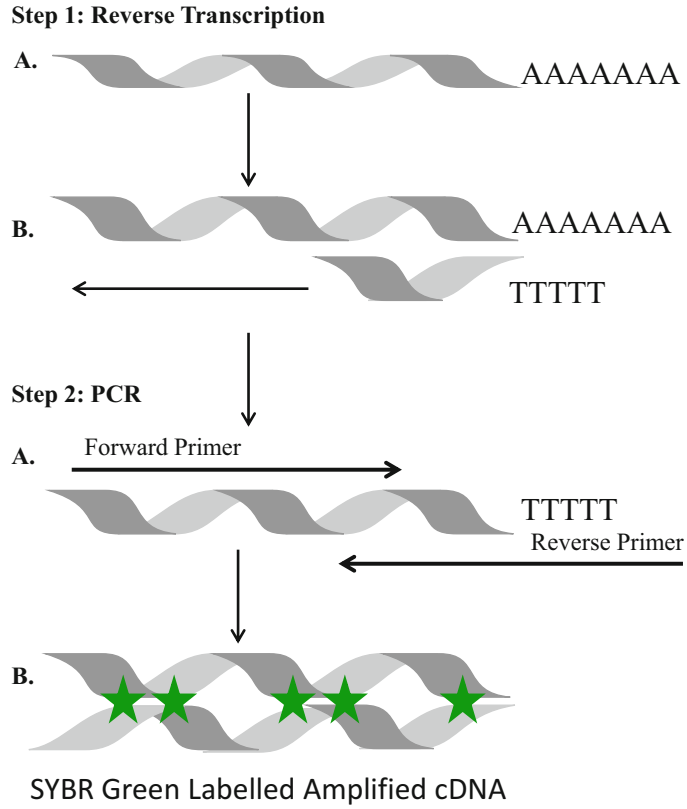

Fig. 3 RT-qPCR Amplification. Step 1 A: a poly-A tail is added to the mature miRNA. Step 1 B: cDNA is synthesized using a poly-T primer with a 5′ universal tag. Step 2 A: The cDNA template is then amplified using forward and reverse primers. Step 2 B: SYBR Green is used for detection of the amplified cDNA

(c) Melt Stage.

- 95 °C for 15 s.
- 60 °C for 1 min.
- 95 °C for 15 s.

3.5 Data Analysis

Each plate contains a plate calibrator UniSp3IPC. The raw C_T values are calibrated for differences between plates using the UniSp3IPC CT values. Specifically,

C_T of sample-(*C_T of UniSp3IPC on plate*)-(*average of UniSp3 IPC for all plates*).

Following calibration miRNA profiling data is analyzed using the $2^{(-\Delta\Delta C_T)}$ method. Specifically:

1. ΔC_T = C_T value of sample – C_T value (mean C_T value) of endogenous control gene(s).
2. $\Delta\Delta C_T$ = ΔC_T case sample – ΔC_T of control sample.
3. Fold change = $\Delta\Delta C_T$ value input into formula $2^{(-\Delta\Delta C_T)}$.

4 Notes

1. RNA spike-ins, such as UniSp6 may be used in the RT reaction and PCR stages to ensure the RT reaction was successful and undetermined values in the PCR are not due to poorly synthesized cDNA.
2. When isolating using TRIReagent™ or TRIzol contamination with phenol may result. Therefore, NanoDrop readings may be inaccurate and RNA input volume may be superior to input concentration in the RT reaction.
3. RNA input volume should be determined by serial dilution, in order to ensure there are no RT reaction inhibitors in the plasma specimens.
4. Isolated RNA should be stored at −80 °C no longer than 30 min after reconstitution in order to ensure integrity of RNA for subsequent RT reaction.
5. Plasma RNA will not show the 18S rRNA or 28S rRNA when using the Agilent Pico kit. Plasma will also only show the miRNA content when using the Agilent Small RNA kit.
6. Use of Agilent BioAnalyzer should be restricted to areas where other instruments are not being used, as vibrations from other instruments may skew results.
7. Setting up of RT and PCR reactions should be done extremely carefully to avoid as much as possible pipetting error.
8. Premade PCR reaction plates may be stored for 24 h at 4 °C.

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References

1. O'Neill S, O'Driscoll L (2015) Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obesity Rev 16(1):1–12. doi:10.1111/obr.12229
2. Vasudevan S (2012) Posttranscriptional upregulation by microRNAs. Wiley Interdiscip Rev RNA 3(3):311–330. doi:10.1002/wrna.121
3. Williams MD, Mitchell GM (2012) MicroRNAs in insulin resistance and obesity. Exp Diabetes Res 2012:484696. doi:10.1155/2012/484696
4. Pescador N, Perez-Barba M, Ibarra JM et al (2013) Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. PLoS One 8(10):e77251. doi:10.1371/journal.pone.0077251
5. Di Leva G, Croce CM (2013) miRNA profiling of cancer. Curr Opin Genet Dev 23(1):3–11. doi:10.1016/j.gde.2013.01.004
6. Blenkiron C, Goldstein LD, Thorne NP et al (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol 8(10):R214. doi:10.1186/gb-2007-8-10-r214