Chapter 3

microRNAs in Cancer Chemoprevention: Method to Isolate Them from Fresh Tissues

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Abstract

microRNAs are 22-nucleotide-long double-strand small RNAs, able to modulate gene expression at posttranscriptional level, degrading mRNA and/or impairing translation. They have been shown to regulate mRNA and protein abundance and to participate in many regulatory circuits controlling developmental timing, cell proliferation and differentiation, apoptosis and stress response. Notably, microRNA activity has been correlated to the pathogenesis of cancer; they are aberrantly expressed in solid and hematological tumors, suggesting that they could function as oncogenes or tumor suppressors. The emerging role of miRNAs in the carcinogenesis and tumor progression has provided opportunities for their clinical application in the capacity of cancer detection, diagnosis, and prognosis prediction. Here, we describe the experimental protocol used to isolate microRNAs from human tissues coming from head and neck, mesothelioma, and thymoma tumors in order to perform microarray and RT-qPCR experiments.

Key words microRNA, Fresh tissues, RNA extraction, HNSCC, Mesothelioma, Thymoma

1 Introduction

microRNAs (miRNAs) are highly conserved small noncoding RNAs (20–22 nucleotides long) that modulate negatively the gene expression by binding to the 3′UTR of multiple target mRNAs [1, 2]. miRNAs play an essential role in many biological processes such as cell proliferation and maturation, cell death, apoptosis, and regulation of chronic inflammation [3, 4]. Based on computational prediction, it has been estimated that more than 60 % of human mRNAs are targeted by at least one miRNA [5]. Many recent studies have shown that miRNAs have specific expression patterns in each cell type and tissues [6] and their aberrantly expression was observed in a wide range of pathologies, including cancer [7, 8]. Moreover, it was recently found that miRNA profiles are more informative than messenger RNA profiles and could classify poorly differentiated tumors since they better reflect the developmental lineage and differentiation state of cancer [9].

Sabrina Strano (ed.), *Cancer Chemoprevention: Methods and Protocols*, Methods in Molecular Biology, vol. 1379,
DOI 10.1007/978-1-4939-3191-0_3,

Therefore, miRNAs' expression profile is emerging among the best markers for diagnosis, staging, and treatment of cancer. Given the significance of miRNAs in modulating gene expression, they could also be used as biomarkers for assessing antineoplastic activity of cancer chemopreventive agents. A key advantage respect to their potential role as biomarkers is their stability due especially to their short length [10]. This stability offers an important privilege in the use of miRNA over mRNA because they are also well preserved in formalin- or paraffin-fixed (FFPE) tissue or fresh tissue with no good quality of RNA and these sample types may be often the only available in a clinical study [10, 11].

The quality of the miRNA expression profiles might depend on the starting RNA material and a robust RNA isolation method is essential for reproducible results. In this chapter, we aim to describe the protocol to isolate total RNA, including miRNA fraction, from head and neck, mesothelioma, and thymoma fresh frozen tissue samples in order to perform miRNA expression analyses by RT-qPCR and/or microarray experiments using Agilent and Affymetrix platforms [12–14].

2 Materials

2.1 RNA Extraction

1. RNaseZapRNase Decontamination Solution (Ambion, Foster City, CA) or similar.
2. RNA later solution (Life Tec Rockville, MD).
3. Phosphate-buffered saline (PBS).
4. Homogenizer for tissues; gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, GE) or similar.
5. miRNeasy Mini kit (Qiagen, Hilden, Germany).
6. Chloroform.
7. Ethanol.
8. DNAse-, RNAse-free set (Qiagen, Hilden, Germany).
9. RNase-free water for molecular biology.

2.2 RNA Quality Control

1. Spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA).
2. Agilent 2100 Bioanalyzer; Total RNA 6000 Nano Kit; Small RNA kit.

3 Methods

3.1 Tissue Sample Collection

The fresh tissue coming from tumor and normal controls may be immediately placed in RNA later stabilization solution (Ambion, Foster City, CA) after tumor removal by surgery (*see* **Note 1**).

This reagent is an aqueous tissue storage solution that rapidly permeates tissue to protect and stabilize its RNA (proteins are also preserved in RNA later solution). The specimen was stored at 4 °C overnight (to allow the solution to thoroughly penetrate the tissue) before to process it. Next the tissue was washed in PBS solution, placed in a cryovial and moved to −80 °C for long-term storage (*see* **Note 2**).

Samples from each case and related control were handled identically and assayed together on the same laboratory session.

3.2 RNA Isolation

The quality of the miRNA expression profiles largely depends on the starting RNA material and a robust RNA isolation method is essential for reproducible results. Before starting, it is important to consider the potential for infection or disease transmission by materials used that contact the sample and/or the homogenate. Wear a lab coat and gloves, RNA extraction may be performed under a chemical hood. In addition, the RNA extraction is complicated by the ubiquitous presence of ribonuclease enzymes which can rapidly degrade RNA. Therefore, the RNase decontamination solution can be applied directly to surfaces and pipettes which will be used during the RNA extraction.

RNA isolation from human tissues was performed by miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of simple modifications to improve essentially the purity of RNA and to enhance RNA yeld.

The miRNeasy mini kit is based on the phenol/guanidine lysis of samples and their purification by the binding of total RNA in silica membrane. Lysis was performed by QIAzol Lysis reagent (included in the miRNeasy mini kit), containing phenol and guanidine thiocyanate which in addition to lysing action prevent the activity of RNase and DNase enzymes to ensure purification of intact RNA. Subsequently, by adding chloroform solution and centrifuging, the homogenate was separated into an upper aqueous phase, containing RNA, a lower interphase and organic phase, containing DNA and proteins. After the addition of absolute ethanol to provide appropriate binding conditions, total RNA from aqueous phase was moved into spin column where RNA binds to the membrane and phenol contaminants are efficiently washed away. Finally, RNA is then eluted in RNase-free water.

With the miRNeasy protocol, all RNA molecules longer than 18 nucleotides are purified.

Carry out all steps at room temperature (15–25 °C) and under chemical hood unless otherwise specified.

1. Homogenize frozen tissue directly in at least 0.7 mL of QIAzol solution and incubate sample for 20 min at room temperature to allow the complete dissociation of nucleoprotein complexes (*see* **Note 3**).

2. Add 140 μL of chloroform. Shake vigorously for 25 s and incubate sample for 3–5 min at room temperature. Centrifuge at 15,700 rcf for 20 min at 4 °C.
3. Draw up carefully the upper aqueous phase of the sample (about 350 μL) by P-200 pipette and transfer it to a new 1.5 mL tube. Be certain that you do not take any of the lower phases when removing the aqueous phase (*see* **Note 4**).
4. Add 1.5 volumes (about 0.525 mL) of absolute ethanol solution and mix by pipetting up and down 8–10 times.
5. Move the obtained solution (about 0.7 mL) into the RNEasy mini spin column in a 2 mL tube. Be certain that the sample does not touch the lid during its closing. Centrifuge the tube at 9300 rcf for 20 s. Discard the flow-through (the 2 mL tube can be used again for next step).
6. Repeat the **step 5** if the solution was more than 0.7 mL.
7. Wash the membrane with the adding of 350 μL of RWT buffer into RNEasy mini spin column and centrifuge for 20 s at 9300 rcf.
8. Use the DNAse-, RNAse-free set (Qiagen, Hilden, Germany) and prepare the DNase mix solution in a 1.5 tube (add 10 μL of DNase stock solution to 70 μL of buffer RDD). Mix gently by inverting the tube (not vortex) and put it in ice before and during the use.
9. Add the DNase mix solution (80 μL) into the RNEasy mini spin column membrane and incubate it at room temperature for 15 min. Do not leave the DNase mix solution into the membrane for more 20 min, the quality of RNA might be compromise.
10. Add 350 μL of RWT wash buffer to the RNEasy mini spin column and centrifuge for 20 s at 9300 rcf. Discard the flow-through; the 2 mL tube can be used again for next step (*see* **Note 5**).
11. Pipette 0.5 mL of the second wash buffer RPE into to the RNEasy mini spin column and close the lid. Wash the column by centrifuging for 20 s at 9300 rcf. Discard the flow-through and use again the 2 mL tube for the next step.
12. Add again 0.5 mL of RPE buffer, close the lid and gently invert the tube 1–2 times to efficiently wash away all contaminants from the lid (*see* **Note 6**). Centrifuge for 2 min the spin column at 9300 rcf to dry the membrane.
13. Move the spin column into a new 2 mL tube and centrifuge for 1 min at full speed to remove any residual of flow-through.
14. Optional: Open the lid of spin column and leave it on the bench for 5 min to be sure to dry any residual of wash buffer.

15. Move the RNEasy mini spin column into a new 1.5 mL tube, elute RNA by pipetting 30–50 μL of RNase-free water directly the membrane without touch it. Close the lid and leave the tube 1 min at room temperature to obtain a higher amount of total RNA. Centrifuge for 1 min at 9300 rcf.
16. Move the RNA sample in ice and quantify its concentration by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville, NC). Do not discard immediately the RNEasy spin column, but move it in a new 1.5 mL tube and leave at room temperature waiting the quantification of RNA.
17. If the concentration of RNA is >about 500 ng/μL, again add water (30 μL) to membrane of spin column and centrifuge for 1 min at 9300 rcf. Move the second aliquot of RNA sample in ice and quantify; you can add the second elute of RNA to the first one, but if the concentration of the second one is too low we suggest you maintain separately the two RNA elutes obtained.
18. Proceed to downstream application, or store at −80 °C (*see* **Note 7**).

To preserve RNA integrity, subdivide the first elute of RNA in at least two sub-aliquots in order to avoid frequent freeze and thaw.

3.3 RNA Quality Control

The expression profiling data can be potentially influenced not only by the method used to isolate the miRNAs, but also by the RNA storage conditions and handling [15]. Studies correlating the RNA quality with the outcome of the microRNA expression experiments have shown contradictory results [16]; several works have reported that a good quality of starting RNA material was essential to obtain reproducible and robust data [17–19], others showed that RNA degradation did not significantly influence the miRNA expression results [20, 21] probably because for their small size, microRNAs are less susceptible to degradation in comparison with mRNAs [22]. In our experience, data obtained by the analysis of microRNAs expression in RNA degraded samples were comparable with the results coming from analysis of intact RNA. However, a good quality of total RNA gives the further advantage to perform expression profile of both mRNAs and miRNAs using the same sample.

Assessment of the purity and quantity of extracted total RNA can be determined by a NanoDrop 2000 Spectrophotometer or similar. Instead, RNA integrity can be assessed by Bioanalyzer 2100 (Agilent Technologies, USA), where total RNA are electrophoretical separated on a chip and detected via laser induced fluorescence detection. Integrity of the RNA may be assessed by visualization of the 18S and 28S ribosomal RNA bands. In order to standardize the RNA integrity interpretation, Agilent technology

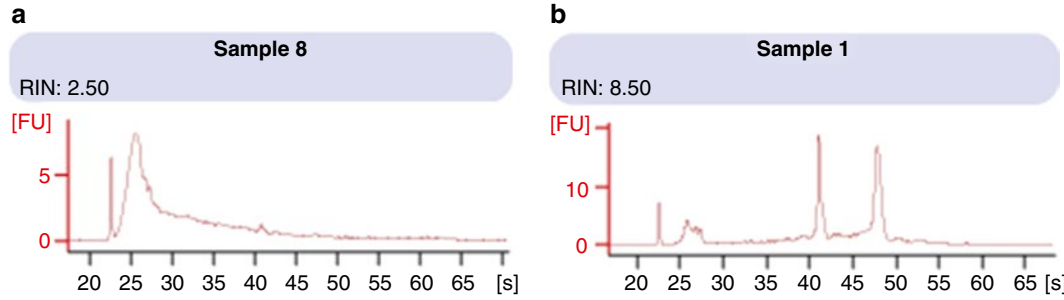


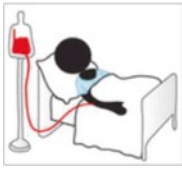
Fig. 1 Example of Agilent Bioanalyzer results. (**a**) Profile of a sample having a completely degraded RNA. (**b**) Profile of a sample with an intact RNA

has developed the RNA Integrity Number (RIN) software algorithm by which total RNA is classified based on numbering system from 1 to 10, according to its integrity; RIN 1 represents the most degraded RNA profile, while RIN 10 represents the most intact RNA (Fig. 1). Typically, values over 7 are good enough for transcriptome analysis using Affymetrix and Agilent platforms. This methodology requires a very small amount of RNA sample (>200 pg). In addition, by Bioanalyzer 2100 instrument can be estimate miRNA abundance expressed as the proportion of RNA in the 15–40 nt window relative to total small RNA abundance (6–150 nt). This information can be useful to evaluate if tissues analyzed have or not similar miRNAs abundance. However, the estimation of miRNA abundance by this method may only be accurate when overall RNA integrity is very high.

The basic workflow of the entire protocol is shown in Fig. 2.

4 Notes

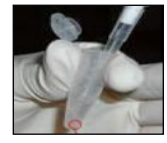
1. This step is critical to obtain an intact RNA from specimen. The piece of tissue may be place immediately into RNA stabilization solution after surgery removal, a delay of a few minutes might cause a rapidly and significant degradation of RNA. The size of tissue should be <0.5 cm and the amount of RNAlater solution may be 8–10 volumes respect to the size of tissue. An incorrect ratio of solution volume on tissue size might be not to ensure the preservation of RNA in all parts of tissue. Usually, we place 1.8 mL of solution in a cryovial where then the surgeon will put the piece of tissue.
2. Do not freeze sample in RNAlater solution immediately, the sample in RNA later solution can be stored at room temperature until 1 week or at 4 °C until 1 month without compromising the quality of RNA. The sample can be freeze at −20 or −80 °C indefinitely also directly in RNA later solution.



Put frozen tissue in RNA later solution and storage at 4° C overnight

Homogenize and lyse frozen tissue in Qlazol solution

Transfer homogenate to an eppendorf, add chloroform solution and shake



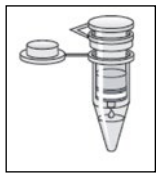
Separate the phases

Move upper aqueous phase to new tube and add ethanol

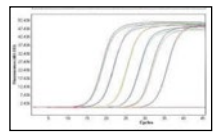
Bind total RNA including small RNA by moving the solution into the column

Wash the column

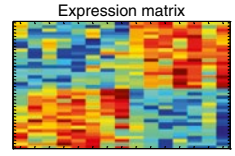
Elute to obtain total RNA including small RNAs



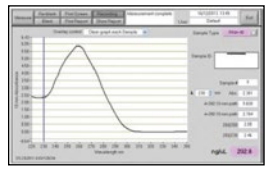
Total and small RNAs are ready to perform miRNA and gene expression analysis



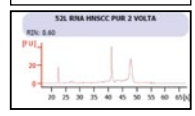
Expression matrix



Quantify and check the quality of total and small RNAs



RNA quality control



Small RNAs control

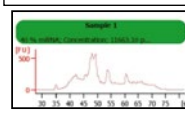


Fig. 2 The basic workflow of the entire protocol of miRNA extraction from tissue

3. To homogenize by gentleMACS Dissociator, transfer the frozen piece into gentleMACS M tube containing QIAzol solution, and set up the program RNA_02 by which the piece will be homogenize in 84 s. If the piece is not completely homogenized, the homogenization can be repeated by adding other 0.7 mL of QIAzol to M tube containing the partially homogenate specimen. After the homogenization, transfer the homogenized sample into to new 1.5 mL tube and proceed with the RNA extraction. Alternatively, the sample in QIAzol solution can be stored at −80 °C for several months without compromising the quality of RNA.
4. If you draw up residuals of lower phase, pull down the aqueous phase contained in the tip of pipette into the 1.5 mL tube, centrifuge it for 5 min at 13,400 rcf in order to separate again the phases and repeat the passage 3. If isolation of DNA or protein is desired, save the interphase and organic phenol chloroform phase. The organic phase can be stored at 4 °C overnight. Follow the protocol of Trizol to proceed with DNA and protein extraction.
5. The necessary to digest sample with DNase enzyme depends on the use that it will be done of RNA. For instance, RNA-seq or other types of gene expression experiments, usually require the removal of DNA from RNA sample. If the treatment of DNase is not necessary, after the passage 7, do not proceed with **steps 8–10**, but add 0.7 mL of RWT wash buffer into the column and centrifuge for 20 s at 9300 rcf. Discard the flow-through, and proceed with the **step 11**.
6. The inversion of tube can increase significantly the purity of RNA, consequently the ratio 260/230 results will be >1.8.
7. Before to freeze the RNA, prepare a small aliquot containing few microliters of sample to perform the quality control by Bionalyzer 2100 (in case of impossibility to check the quality immediately after extraction).

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