Video Article

Cerebrospinal Fluid MicroRNA Profiling Using Quantitative Real Time PCR

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Abstract

MicroRNAs (miRNAs) constitute a potent layer of gene regulation by guiding RISC to target sites located on mRNAs and, consequently, by modulating their translational repression. Changes in miRNA expression have been shown to be involved in the development of all major complex diseases. Furthermore, recent findings showed that miRNAs can be secreted to the extracellular environment and enter the bloodstream and other body fluids where they can circulate with high stability. The function of such circulating miRNAs remains largely elusive, but systematic high throughput approaches, such as miRNA profiling arrays, have lead to the identification of miRNA signatures in several pathological conditions, including neurodegenerative disorders and several types of cancers. In this context, the identification of miRNA expression profile in the cerebrospinal fluid, as reported in our recent study, makes miRNAs attractive candidates for biomarker analysis.

There are several tools available for profiling microRNAs, such as microarrays, quantitative real-time PCR (qPCR), and deep sequencing. Here, we describe a sensitive method to profile microRNAs in cerebrospinal fluids by quantitative real-time PCR. We used the Exiqon microRNA ready-to-use PCR human panels I and II V2.R, which allows detection of 742 unique human microRNAs. We performed the arrays in triplicate runs and we processed and analyzed data using the GenEx Professional 5 software.

Using this protocol, we have successfully profiled microRNAs in various types of cell lines and primary cells, CSF, plasma, and formalin-fixed paraffin-embedded tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/51172/

Introduction

MicroRNAs belong to the family of small (21-23 nt in length) noncoding RNAs that regulate gene expression post-transcriptionally. microRNAs can be secreted in the extracellular space where they appear to be relatively stable. While determining changes in miRNA expression can be an important step toward identification of biomarkers, performing miRNA profiles and handling a large amount of data may be intimidating.

Here, we describe a protocol to determine changes in miRNA expression in the cerebrospinal fluid (applicable to other body fluids) by a sensitive real time PCR. We also describe the use of software for data analysis, including statistical analysis and graphical representation of results. The entire procedure is relatively easy and straightforward and, depending on the number of samples to be profiled and the number of real time PCR machines available, also relatively quick. The experimental part requires accuracy in handling RNA and pipetting into 384-well plates, while the data analysis section using GenEx requires some basic knowledge in informatics and statistics.

Protocol

The following protocol describes the standard procedure to isolate RNA from CSF and profile microRNAs using ready PCR plates. Note that the organic phase extraction is optional, and that a carrier RNA is added to the sample during extraction ensuring maximal recovery of RNA. Consequently, there is no need to quantify the RNA.

Overall, an average of 6-8 plates can be run in one day if the cDNA is synthesized the day before (about 2 hr). Analysis of data is performed when all samples have been profiled and loaded into the software. Depending on the number of samples/groups or their combination for comparison, data analysis may require from one to several hours.

1. RNA Extraction

The RNA extraction was performed from CSF samples, stored frozen at -80 °C in aliquots until analysis. For this procedure the mirVana Paris isolation kit was used, following the manufacturer's instructions for the total RNA isolation. Please note that, although enrichment for small RNA

species is possible with this kit, this step is not done and the RNA extraction procedure is ended after the total RNA isolation step. In addition, although the mirVana Paris isolation kit (like other commercially available RNA isolation kits) does not require organic extraction, a protocol for this procedure can be found below.
The protocol for RNA extraction consists of two steps:
1.1. Organic Extraction (not required if using mirVana Paris RNA extraction kit)
1.2.2. Total RNA isolation

1.1. Organic extraction

1.1.1. Prepare reagents

1. Add 375 µl of 2-mercaptoethanol to 2x Denaturing solution, and store it at 4 °C.

1.1.2. Prepare the CSF

1. Thaw every aliquot of CSF on ice before the RNA extraction. Add 1 µg of MS2 RNA carrier to 0.5 ml of thawed CSF and gently mix. Note that if the organic extraction is omitted, the MS2 carrier will be added to the sample prior RNA isolation described in 1.2.2.1 below.
2. Mix 0.5 ml of 2x Denaturing Solution at room temperature to the CSF.
3. Add 1 ml of acid-phenol:chloroform to the CSF plus the 2x Denaturing Solution: be sure to withdrawn the bottom phase containing acid-phenol:chloroform, not the aqueous buffer that lies on top of the mixture. Additionally, note that acid-phenol:chloroform contains phenol, which is a poison and an irritant, use gloves and other personal protection equipment when working with this reagent.
4. Vortex for 30-60 sec to mix. For convenience, the 2 ml of sample/denaturing solution/acid-phenol:chloroform mix are divided into two 1.5 ml Eppendorf tubes.
5. Centrifuge for 5 min at maximum speed (10,000 x g) at room temperature.
6. Carefully remove the aqueous (upper) phase without disturbing the lower phase or the interphase and transfer it to a fresh tube. Note the volume recovered.

1.2. RNA isolation

It is recommended to have a dedicated bench, set of pipettes, and racks for handling RNA samples. The working area and tools are decontaminated by using RNase Zap spray and wipes prior each experiment.

1.2.1. Prepare reagents:

1. Add 21 ml of 100% ethanol to miRNA Wash Solution 1 and 40 ml of 100 % ethanol to Wash Solution 2/3. Please note that miRNA Wash Solution 1 contains guanidium thiocyanate that is a potentially hazardous substance.

1.2.2. Total RNA isolation

1. Add 1.25 volumes of room temperature 100% ethanol to the aqueous phase from the organic extraction and mix thoroughly.
2. Place a Filter Cartridge into one of the Collection Tubes.
3. Pipette not more than 700 µl of the lysate/ethanol mixture onto the Filter Cartridge.
4. Centrifuge for 30 sec at maximum speed. Apply the mixture exceeding 700 µl in successive applications to the same filter. Discard the flow-through after every centrifugation and save the Collection Tube for the washing steps.
5. Apply 700 µl miRNA Wash Solution 1 to the Filter Cartridge and centrifuge for 15 sec at maximum speed. Discard the flow through from the Collection Tube and replace the Filter Cartridge into the same Collection Tube.
6. Apply 500 µl Wash Solution 2/3 and centrifuge for 15 sec at maximum speed. Discard the flow through from the Collection Tube and replace the Filter Cartridge into the same Collection Tube. Apply a second 500 µl Wash Solution 2/3 and centrifuge for 15 sec at maximum speed. Discard the flow through from the Collection Tube and replace the Filter Cartridge into the same Collection Tube. Spin the assembly for 1 min at maximum speed to remove residual fluid from the filter.
7. Transfer the Filter Cartridge into a fresh Collection Tube. Apply 100 µl of preheated (to 95 °C) nuclease-free water to the center of the filter, and close the cap. Centrifuge for 30 sec at maximum speed to recover the RNA.
8. Collect the eluate containing RNA and store it at -80 °C for subsequent applications.
9. Quantify 1 µl of RNA using the NanoDrop 2000c spectrophotometer. Note that RNA quantification can be skipped if an RNA carrier is added to the sample during RNA extraction. In that case 8 µl of RNA are subjected to cDNA synthesis as detailed below.

2. miRNA Profile: qRT-PCR Protocol

The protocol for miRNA profiling consists of two steps:

1. First-strand cDNA synthesis
2. Real-time PCR amplification

2.1. First-strand cDNA synthesis

2.1.1. Dilute template RNA

1. Adjust each of the template RNA samples to a concentration of 5 ng/µl using nuclease free water. If the RNA has not been quantified (see step 1.2.2.9), then 8 µl of RNA are utilized for cDNA synthesis

2.1.2. Prepare reagents

1. Gently thaw the 5x Reaction buffer and nuclease free water, and immediately place on ice.
2. Mix by vortexing. Resuspend the RNA spike-in by adding 40 µl nuclease free water to the tube and mix by vortexing; leave it on ice for 15-20 min. Immediately before use, remove the enzyme mix from the freezer, mix by flicking the tubes and place on ice. Spin down all reagents.

2.1.3. Reverse transcription reaction setup

1. Prepare the required amount of RT working solution in a 1.5 ml Eppendorf tube, or equivalent (in the proportions indicated in **Table 1**, except for the RNA template), mix by vortexing (1-2 sec at max speed), briefly spin down (we use a mini Eppendorf centrifuge with fixed speed, 10 sec) and place it on ice. Note that the UniSp6 RNA spike-in is added to the sample prior the RT reaction (see **Table 1**).
2. Dispense RT working solution into nuclease free PCR tubes.
3. Dispense template RNA in each tube.
   Note: remember to calculate 10% excess volume/each reagent for pipetting and/or robotic dead volume.

Reagent	Panel I Volume (µl)	Panel I + II Volume (µl)
5x reaction buffer	4.4	8.8
Nuclease free water	9.9	19.8
Enzyme mix	2.2	4.4
UniSp6 RNA Spike-in template	1.1	2.2
Template total RNA (5 ng/µl)	4.4	8.8
Total Volume	**22**	**44**

Table 1. Reverse transcription reaction setup.

2.1.4. Mix and spin reagents

1. Mix the reaction by gentle (1-2 sec) vortexing to ensure that all reagents are thoroughly mixed. After mixing, spin down (mini Eppendorf centrifuge with fixed speed, 10 sec).

2.1.5. Incubate and heat inactivate

1. Program a PCR machine as follow:
2. Incubate for 60 min at 42 °C
3. Heat-inactivate the reverse transcriptase for 5 min at 95 °C
4. Immediately cool to 4 °C
5. Store at 4 °C or freeze
   Note: the protocol can be interrupted at this stage. The undiluted cDNA may be kept at -20 °C for up to 5 weeks (optional store at 4 °C for up to 4 days).

2.2. Real-time PCR amplification

2.2.1. Prepare reagents for Real-time PCR

1. Place cDNA (from step 2.1.5) on ice.
2. Thaw nuclease free water and PCR Master mix on ice. Protect the PCR Master mix vials from light by covering them with aluminum foil. Immediately before use, mix the PCR Master mix by vortexing 1-2 sec in a mini centrifuge and spin down at 1,500 x g for 1 min.

2.2.2. Combine SYBR Green master mix, water, and cDNA and add to PCR plates

The following procedure is recommended to avoid low concentrations of cDNA from adhering to tube surface:

1. Before removing the plate seal, briefly spin down the plate(s) in a refrigerated centrifuge at 1,500 x g for 1 min.
2. In a 15 ml conical tube, combine 2x PCR Master mix and water. Panel I: 2,000 µl 2x master mix and 1,980 µl water; Panel I+II: 4,000 µl 2x master mix and 3,960 µl water.
3. Add 20 µl cDNA (panel I) or 40 µl cDNA (panel I+II) and mix.
4. Mix gently by vortexing 1-2 sec and spin down in a refrigerated centrifuge at 1,500 x g for 1 min.
5. Place the PCR Master mix/cDNA mix in a multichannel pipette reservoir. You may have to identify a reservoir that has the length of the multichannel pipette. This keeps the level of volume high enough allowing for equal volume aliquots throughout the multichannel. This is critical towards the last few aliquots.
6. Dispense 10 µl PCR Master mix/cDNA mix to each well of the 384-well plate using a 16-channel pipette.
7. Seal the plate with optical sealing.
8. Spin plate briefly in a refrigerated centrifuge (1,500 x g for 1 min), to mix the solutions and remove air bubbles.
   Note: the experiment can be paused at this point. Store the reactions protected from light at 4 °C for up to 24 hr.

2.2.3. Real-Time PCR

Perform real-time PCR amplification and melting curve analysis following the parameters detailed in **Table 2**.

Process Step	Settings, Roche LC480
Polymerase Activation/Denaturation	95 °C, 10 min
Amplification	45 Amplification cycles at 95 °C, 10 sec 60 °C, 1 min Ramp-rate 1.6 °C/sec Optical read
Melting curve analysis	Yes

Table 2. Real-time PCR cycle conditions using the Roche LightCycler 480.

2.3. Real-time PCR data analysis

Real time PCR data analysis is done with the Exiqon GenEx Professional 5.0, following the recommended instructions. The GenEx step-by-step manual can be downloaded at http://www.exiqon.com/ls/Documents/Scientific/Exiqon-data-analysis-guide.pdf
Data analysis consists of three steps:

1. Import of data
2. Preprocessing of data
3. Statistical analysis

2.3.1. Import of data

1. In the Roche LightCycler 480 select the 2nd derivative analysis method and calculate the Cq values. Data are exported as a table and saved as text files.
2. To import the data, open GenEx and click on the Exiqon import wizard button and click "Start". Follow the instructions to select format, instrument and plate layout file(s). Note that plate layout files (excel files) are downloaded from the Exiqon web site (http://www.exiqon.com/plate-layout-files).
3. Import Panels I and II and click "Next".
4. The table generated after file import contains predefined columns. Samples names can be edited and classification columns can be added or removed at this step. Click "Next" when you are done.
5. Save data and load to Data Editor.

2.3.2. Preprocessing of data

1. As recommended by Exiqon and GenEx, when comparing multiple plates the first thing to do is to calibrate the data between the plates by choosing inter-plate calibration from the preprocessing menu.
2. It is recommended to run miRNA arrays in triplicate runs. If a miRNA is not present in at least two out of three replicas it will be set as nonexpressed.
3. In the next step in the preprocessing, set a cut off. Defining a cut off value indicates that data with a threshold cycle (C_t) higher than this value are discarded. In the present experiments using cerebrospinal fluid specimen, the cut off was set at 39. Therefore, all samples with a C_t higher than 39 in the three replicas will be discarded. If a miRNA has a C_t > 39 for one probe (one replica out of three), that reading will be replaced with the average of the C_t for the other two probes, provided they are both below 39.
4. Define reference genes. GenEx Professional has the option to utilize geNorm and/or NormFinder to identify reference genes. Select a list of miRNAs that have similar Cq values across all sample and run geNorm and/or NormFinder. According to this analysis, in the example provided here, miR-622 and miR-1266 had the most uniform values across the samples and were chosen as reference genes (**Figure 1**).
5. Next, data are normalized using the reference genes and the values obtained correspond to the ΔC_t
6. If cDNA synthesis reactions were performed in triplicates, at this point the values should be averaged.
7. Validate sheet to remove empty and almost empty columns. In the preprocessing window select "Validate sheet" and than apply on the Remove empty columns line. In the line below, chose the desired percentage of valid data and click apply. For instance, select 100% if you wish to compare only miRNAs common to all samples.
8. Handle missing data.
   Select “Missing data” from the Preprocessing menu and chose one of the options to handle missing data. This step is required. Note that GenEx will give you error if you try to load files that contain unhandled missing data.
9. Determine relative quantification between groups of samples (*i.e.* experiment versus control). Relative quantification among groups is calculated using the $\Delta\Delta$ C_t method. In GenEx, click the preprocessing tab and select relative quantification. In the window select the reference group and hit apply. Note that for graphical representations and/or for further statistical analyses, expression data should be converted to a logarithmic scale. In the preprocessing menu, select Log2.

2.3.3. Statistical Analysis

GenEx software allows a wide range of statistical analyses, including T-test and ANOVA.

1. Load the final mdf file into GenEx and open data manager. It is critical to select the correct samples or groups of samples for which statistical analysis is performed.
2. Select "Statistics" and chose the icon corresponding to the desired test.
3. Click "Run" in the control panel window. Save results as excel or mdf files.

2.3.4. Expression profiling

MicroRNAs and samples can be classified, clustered, and visualized on heat-maps and dendrograms, as follows

1. Load the final mdf file into GenEx and open data manager. Use this window to select and create groups of samples or miRNAs. Click "Apply" when you are done.
2. In the upper left corner select cluster and click on the heatmap icon
3. In the control panel window click "Run". The heatmap can be saved in various formats such as tiff or bmp; it can also be copied and modified as needed.

Representative Results

Results from this study have been previously published[1]. **Figure 1** shows results from the analysis of candidate reference microRNAs using geNorm application. Accordingly, two microRNAs, miR-622 and miR-1266, were identified as reference genes and were used to normalize miRNA values.

For the CSF study we had three groups of samples: HIV- (*n*=10), HIVE (*n*=4), and HIV+ without Encephalitis (HIV+, *n*=5). The two groups, HIVE and HIV+, were compared with the control HIV- group. After statistical analysis (section 2.3.3) expression levels of eleven microRNAs was found statistically significant[1]. **Figure 2** represents a box plot of this eleven microRNAs, miR-1203, -1224-3p, -182*, -19b-2*, -204, -362-5p, -484, -720, -744*, -934, and -937. Each column shows the distribution of data across the samples within the group (green for the HIVE and red for HIV+ without encephalitis). Whiskers indicate the median, the 1st (Q1) and 3rd (Q3) quartile, and the maximum and minimum nonoutliers.

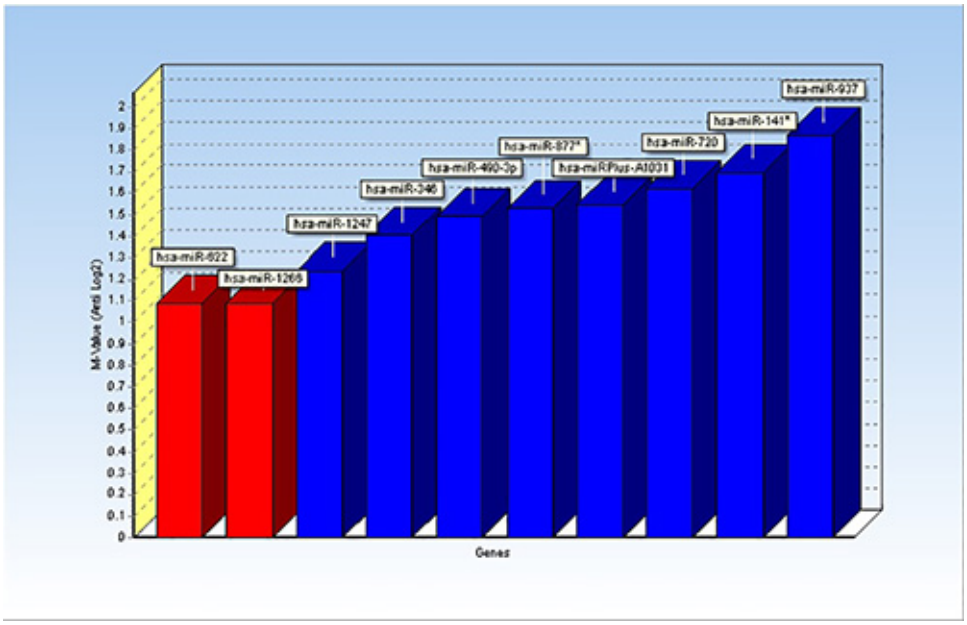

Figure 1. Graphic bar showing results from geNorm application within GenEx. Ten microRNAs were analyzed as possible reference genes and results indicate miR-622 and miR-1266 (red bars) as the most stably expressed miRNAs. Click here to view larger image.

Figure 2. Box plot showing the distribution of data for each of the eleven miRNAs within the groups of HIVE (green) and HIV+ without encephalitis (HIV+, red). The whiskers in each column indicate the median, 1st (Q1, bottom of the box) and 3rd (Q3, top of the box) quartiles, maximum and minimum values that are nonoutliers (black lines). Click here to view larger image.

Discussion

MicroRNAs are small noncoding RNAs that regulate gene expression by inhibiting translation and/or promoting mRNA degradation[2]. Due to their high stability in cell-free conditions, microRNAs have been detected in many body fluids. Furthermore, distinct expression profile of microRNAs has been correlated with stage and/or progression in a variety of human cancers[3-9].

There are several tools available to profile microRNAs, including classical chip arrays or the latest deep sequencing technology. However, we opted to utilize a highly sensitive qPCR platform[10,11], which has the additional advantage of requiring minimal amount of RNAs. The miRCURY LNA Universal RT microRNA PCR protocol is optimized to use 20 ng total RNA per 20 µl cDNA synthesis reaction, 40 ng for the full two-panel array. Having the option of using small amount of RNA is highly important when dealing with valuable and hard-to-obtain clinical specimens such as CSF or formalin-fixed paraffin-embedded tissues[1]. Importantly, utilizing low amounts of RNA minimize the concentration of possible inhibitors present in the sample. Out of range Ct values for the spike-in will likely indicate the presence of some inhibitors in the sample. Spike-in probes can be purchased separately to test the samples for the presence of inhibitors prior to miRNA profiling. For this test, single real time PCR are performed using increasing concentrations of RNA (whether ng or µl).

In the present protocol, we report the organic phase extraction prior to RNA isolation. It should be noted that many new RNA extraction kits do not require this procedure. For instance, we have successfully profiled plasma miRNAs using the miRCURY RNA isolation kit biofluids for RNA extraction directly from 200 µl of plasma (data not shown), without phenol/chloroform extraction.

In general, it is important to design the experiment in advance in order to determine the proper number of replicates needed to obtain statistically significant results. The number of replicates may depend on the number of samples to be analyzed and may depend on the variations within the samples or group of samples. For our study, for which we used a relatively low number of samples, 20 in total, we decided to set up the cDNA synthesis reaction in triplicates. Consulting with a biostatistician before setting up the experiments may be a wise choice and is highly recommended.

Defining the C_t cut off value is important and depends on the type of samples; for highly expressed miRNAs it can be set between 25-35, but for low expressed miRNAs, such as our CSF specimens, can be set higher. Another critical step when it comes to data analysis is the choice of reference gene(s). For some robust data (such as miRNA profiling in cultured cells) a global normalization, which represents the average C_t of all samples, may be used. However, this is not an option for samples like CSF that present variations. Similarly, we couldn't utilize as reference genes those miRNAs that are generally considered unchanged in body fluids. Instead, we selected all miRNAs that showed similar C_t across all samples, including replicas, and ran the geNorm analysis available in GenEx (**Figure 1**). Results indicated miR-1266 and miR-622 as the least variably expressed miRNAs and they were used as reference genes. Comparison of the expression of miRNAs between groups can be determined using the relative quantification tool in GenEx, which follows the standard formula $2^{-(Ct-Ctrel)}$. Finally, results can be visualized as heat map diagrams, graphic bars or other types of plots, such as the box plot in **Figure 2**. Other types of visualization available in GenEx include hierarchical clustering, Self-Organized Map (SOM), and Principal Component Analysis (PCA). In addition to miRNAs, the GenEx software can be used for the analysis of other types of arrays such as long noncoding RNAs (lncRNAs) profiling. The plate layout can be imported in the excel format and data can be handled as described for miRNA analysis. Indeed, we have profiled lncRNAs from primary embryonic mouse cortical neurons using the mirVana miRNA isolation kit as described in the steps above and we analyzed data using GenEx (unpublished results).

In summary, we described a protocol to profile microRNAs in the cerebrospinal fluid. With some modifications related to RNA extraction, this procedure can be adapted to profile miRNAs in other body fluids and/or other type of tissues. Note that in the procedure described here, a step of organic extraction is performed prior to RNA isolation. In general, this is not required when using commercially available kits, such as the miRVANA Paris extraction kit. In addition, when extracting RNA from body fluids and a carrier RNA is added to the samples there is no need to quantify the RNA and 2-8 µl RNA are subjected to cDNA synthesis. From our experience and considering the high costs related to this type of experiments, we recommend testing few samples first, and then consulting with a statistician for the number of samples/replicas to be profiled in order to obtain statistically significant results.

Disclosures

The authors have nothing to disclose.

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