

Chapter 10

Whole-Genome RT-qPCR MicroRNA Expression Profiling

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Abstract

MicroRNAs (miRNAs) are small noncoding RNA molecules that function as negative regulators of gene expression. They are essential components of virtually every biological process and deregulated miRNA expression has been reported in a multitude of human diseases including cancer. Owing to their small size (20–22 nucleotides), accurate quantification of miRNA expression is particularly challenging. In this chapter, we present different RT-qPCR technologies that enable whole genome miRNA expression quantification.

Key words: microRNA, Stem-loop, RT-qPCR, Global mean normalization

1. Introduction

miRNAs represent one of the largest classes of gene regulators. Currently, the miRbase sequence database (Release 16, <http://www.mirbase.org>) contains over 17,000 entries of mature miRNAs in 142 species including 1,223 mature human miRNAs. Their involvement in human disease has important implications for translational research, as miRNA expression signatures have been correlated to diagnosis and prognosis, and are eligible as excellent targets for therapy. Unfortunately, accurate quantification of miRNA expression levels is a major challenge in the field. Several hybridization-based methods, such as microarray and bead-based flow cytometry, have been introduced to quantify the expression of hundreds of miRNAs in a single experiment. However, these approaches require substantial amounts of input RNA, which precludes the use of small biopsies, single cells or body fluids such as serum, plasma, urine, or sputum. While the reverse transcription quantitative PCR (RT-qPCR) in principle has a much higher sensitivity, down to a single molecule, the RT reaction requires

modification to enable the detection of small RNA molecules such as miRNAs (see ref. 1 for a review on all available RT-qPCR platforms for miRNA detection). One approach relies on the use of stem-loop RT primers (2, 3), while another is based on polyadenylation of the mature miRNA prior to oligo-dT primed cDNA synthesis (4). Next to sensitivity, RT-qPCR based approaches have a superior specificity and a high level of flexibility, allowing additional assays to be readily included in the workflow.

1.1. Stem-Loop Reverse Transcription miRNA Profiling

Stem-loop reverse transcription is based on the use of a looped miRNA specific RT-primer that will hybridise to the 3' end of the mature miRNA to initiate cDNA synthesis (2). Upon denaturation, the loop unfolds, providing a longer template for detection in a qPCR reaction (Fig. 1a). Since this process is miRNA specific, multiplex pooling of individual stem-loop primers is necessary to produce cDNA template for multiple miRNAs.

The stem-loop RT-qPCR miRNA profiling platform is provided by Applied Biosystems and uses a miRNA specific forward primer and hydrolysis probe together with a universal reverse primer to measure miRNA expression. Stem-loop primers for more than 700 mature human miRNAs are pooled in two Megaplex primer pools (pool A and pool B) to allow whole genome miRNA expression profiling. An optional limited-cycle preamplification step is introduced to increase the sensitivity of the reaction, enabling miRNA profiling studies of single cells and body fluids. The preamplification procedure uses the same miRNA specific forward and universal reverse primers to amplify the cDNA template in a 12-cycle PCR reaction. As is the case for the stem-loop primers, the forward and reverse preamplification primers are pooled in two pools that match the Megaplex RT primer pools. In order to assess whether the use of a preamplification step introduced a bias in miRNA expression values we compared two workflows, by including or excluding preamplification. We profiled the expression of 430 miRNAs in different neuroblastoma cell lines and evaluated the differential miRNA expression between different cell lines for both procedures. If no bias is introduced, the ΔCq (Cq or quantification cycle according to MIQE-guidelines) (5) for any given miRNA should be similar

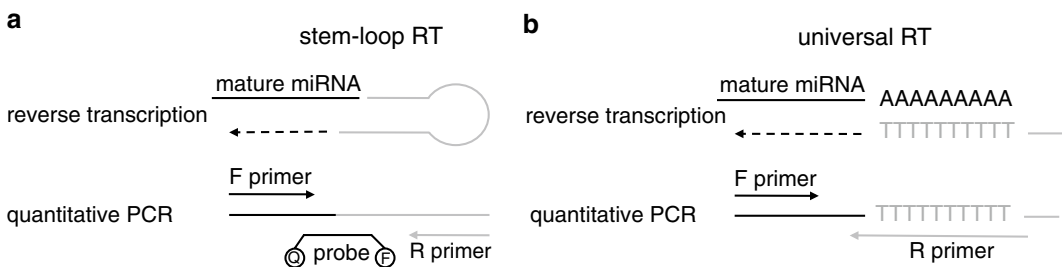


Fig. 1. Schematic overview of the stem-loop RT-qPCR (a) and universal RT-qPCR (b) miRNA profiling platforms.

for both approaches. Therefore, the difference in ΔCq ($\Delta\Delta Cq$) as measured by both approaches should approach zero. We found that 80% of all detected miRNAs had a $\Delta\Delta Cq < 1$ and 75% had a $\Delta\Delta Cq < 0.5$. Following analysis of only the most abundant miRNAs ($Cq < 30$), 94% had a $\Delta\Delta Cq < 1$, suggesting that both procedures give similar results. For low-abundant miRNAs, results should be interpreted with caution. The higher variation observed for the low-abundant miRNAs is partly attributable to increased variation in the RT-reaction, which is typically observed for low copy templates (3).

1.2. Universal Reverse Transcription miRNA Profiling

This approach is based on polyadenylation of the mature miRNA (4). Reverse transcription is initiated using a polyT primer that can be tagged (Fig. 1b). This reaction is universal, providing cDNA template for quantification of any miRNA. Several suppliers provide such a platform, including Exiqon that uses LNA-modified miRNA specific forward and reverse primers to measure miRNA expression. The use of LNA-modified primers precludes the need for a preamplification step and enables the study of miRNA expression when limited amounts of RNA are available.

1.3. Normalizing Whole Genome RT-qPCR miRNA Expression Data

The accuracy of the results obtained through RT-qPCR miRNA expression profiling is largely dependent on proper normalization of the data (Fig. 2). Several parameters inherent to the RT-qPCR reaction need to be controlled for to distinguish technical variation from true biological changes. For normalization of RT-qPCR data, the use of multiple stable reference genes is accepted as the gold standard method (6). As there is no such thing as a set of universal stable reference genes, each individual experiment requires careful selection of the most stable candidates. Typically, a set of ten

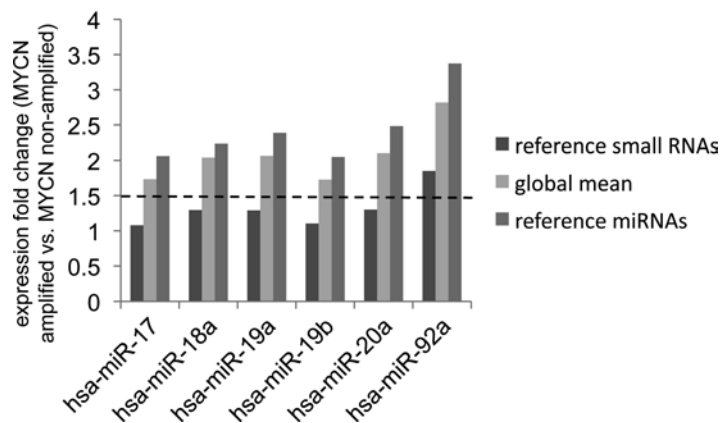


Fig. 2. Expression fold change of miR-17-92 miRNAs in MYCN amplified versus MYCN nonamplified neuroblastoma tumor samples for three different normalization methods: reference small RNAs (i.e., a selection of stable small nuclear/nucleolar RNAs), global mean, and reference miRNAs (i.e., miRNAs that resemble the global mean expression). The dashed line indicates a 1.5-fold change in expression.

candidate reference genes is evaluated in a pilot experiment with representative samples from the different experimental conditions under investigation. The most stable reference genes are subsequently identified using well established algorithms such as geNorm or Normfinder (6, 7). While candidate reference mRNA genes are well established, candidate reference miRNA genes are not. Only few candidate reference miRNA genes have been reported in the scientific literature and all too often, small nuclear or nucleolar RNAs (such as U6, U24, U26) are used instead.

For whole genome miRNA expression profiling, we have successfully introduced the global mean miRNA expression value as a virtual reference gene representing the best normalization factor (8). The global mean expression value is calculated as the average C_q of all expressed miRNAs per sample, where miRNAs with a C_q-value < 32 are considered expressed. Compared to small nuclear and nucleolar RNAs, global mean normalization is by far better in reducing the technical variation and consequently allows a more accurate interpretation of the biological changes (8). This was illustrated by evaluating the differential expression of miRNAs from the miR-17-92 cluster in primary neuroblastoma tumor samples with and without overexpression of the MYCN transcription factor, known to activate the miR-17-92 cluster by binding to its promoter. Neuroblastoma tumor samples with overexpression of the MYCN gene should therefore have increased expression of miR-17-92 miRNAs. Surprisingly, only one of the miRNAs from the miR-17-92 cluster was found to be differentially expressed when normalizing with small nuclear or nucleolar RNAs. Upon normalization using the global mean expression value, all miR-17-92 miRNA were found to be differentially expressed.

1.4. Identification of Stably Expressed Reference miRNAs

Typically, whole-genome miRNA expression studies are followed by focused validation studies for a selection of miRNAs. In this case, the global mean expression can no longer be used for normalization. Our group demonstrated that it is possible to identify miRNAs that resemble the global mean expression value and that the geometric mean of their expression levels can be successfully used to mimic global mean expression value normalization (8). As with the global mean expression, normalization using these miRNAs results in a higher reduction of technical variation and a more accurate interpretation of the biological changes. Alternatively, selection of miRNAs that resemble the global mean expression value can be performed using the miRNA body map Web tool (<http://www.mirnabodymap.org>) (9). The miRNA body map contains whole genome RT-qPCR miRNA expression data for over 700 samples from varying tissue and disease origin (see Note 1). For normalization of experiments in which only a few miRNAs are measured, we recommend to consult the miRNA body map to evaluate whether it contains samples of similar tissue or disease

origin and use the integrated tool to identify stable reference miRNAs. Candidate reference miRNAs for a subset of normal and disease tissues were also identified by Peltier and Latham (10). The authors report that miR-191 and miR-103, among others, were found to be stably expressed across 13 normal tissues and five pair of distinct tumor/normal adjacent tissues (see Note 2). Ultimately, a selection of small nuclear and/or nucleolar RNAs could be applied for miRNA expression normalization, given that these are stably expressed across the samples under investigation. Of note, the use of small nuclear/nucleolar RNAs can, at least in some cases, lead to a misinterpretation of the biological changes (8).

2. Materials

2.1. Stem-Loop RT-qPCR

1. TaqMan microRNA reverse transcription kit (Applied Biosystems) containing dNTPs (100 nM), MultiScribe reverse transcriptase (50 U/ μ l), reverse transcription buffer (10 \times), RNase inhibitor (20 U/ μ l).
2. Human Megaplex primer pools A and B (Applied Biosystems).
3. Human Megaplex PreAmp primer pools A and B (Applied Biosystems). The use of PreAmp primers is optional and depends on the amount of available input RNA (see Methods for details on minimal amounts of input RNA).
4. TaqMan PreAmp master mix (Applied Biosystems). Optional, use in combination with PreAmp primers.
5. TaqMan universal PCR master mix II (2 \times) (Applied Biosystems).
6. TaqMan miRNA assays, either as single tube assays or predisposed in TaqMan array miRNA cards matching Megaplex pool A and B (Applied Biosystems).
7. MgCl₂ (50 mM).
8. Nuclease-free water.

2.2. Universal RT-qPCR

1. Universal cDNA synthesis kit (Exiqon) containing reaction buffer (5 \times) and enzyme mix. The universal reverse transcription primer is included in the reaction buffer.
2. SYBR Green master mix (2 \times) (Exiqon).
3. Forward and reverse LNA primers, either as single tube assays or predisposed (Human panel I and II) in 384-well plates (Exiqon).
4. Nuclease-free water.

3. Methods

3.1. Stem-Loop RT-qPCR

1. Dilute RNA sample to a concentration of 10 ng/ μ l (total RNA) for the workflow with preamplification and 500 ng/ μ l for a workflow without preamplification (see Notes 3 and 4). Sensitivity can be improved by increasing the amount of input RNA. Similarly, decreasing the amount of input RNA will result in a lower sensitivity. Keep RNA on ice at all times to prevent degradation (see Note 3 and Fig. 3).
2. Prepare the reverse transcription reaction mix by combining 0.8 μ l of Megaplex primers pool, 0.2 μ l of dNTPs, 1.5 μ l of Multiscribe reverse transcriptase, 0.8 μ l of reverse transcription buffer, 0.45 μ l of MgCl₂, 0.1 μ l of RNase inhibitor, 0.65 μ l of nuclease-free water, and 3 μ l of the diluted RNA sample. To avoid pipetting volumes below 1 μ l, scale up the individual volumes to process at least 10 samples. Prepare an individual RT reaction for each Megaplex primer pool (pool A and B). Mix reagents by pipetting and spin down (see Note 5).
3. Incubate the reverse transcription mix on ice for 5 min.
4. Run the reverse transcription reaction as follows: (16°C for 2 min, 42°C for 1 min, 50°C for 1 s) \times 40 cycles, 85°C for 5 min, and cooling down to 4°C.
5. In case a preamplification reaction is performed, spin down each sample and add 2.5 μ l of reverse transcription product to 12.5 μ l of TaqMan PreAmp master mix, 2.5 μ l of the matching PreAmp primer pool (pool A or B), and 7.5 μ l of nuclease-free water. Pipette to mix and spin down.

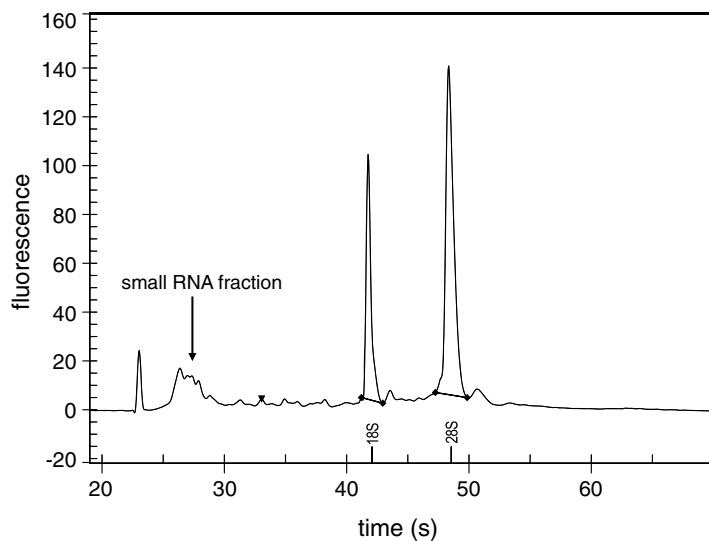


Fig. 3. Experion profile of a good-quality total RNA sample. The small peak represents the small RNA fraction.

6. Run a 12-cycle preamplification reaction as follows: 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, (95°C for 15 s, 60°C for 4 min) × 12 cycles, 99°C for 10 min, and cooling down to 4°C.
7. Dilute the preamplification product 4× by adding 75 µl of nuclease-free water to each sample. Pipette to mix and spin down.
8. For each TaqMan miRNA array, combine 450 µl of TaqMan universal PCR master mix, 441 µl of nuclease-free water, and 9 µl of diluted preamplification product. Pipette to mix and spin down. Increase the fraction of preamplification product to increase sensitivity.
9. For a workflow without preamplification, combine 450 µl of TaqMan universal PCR master mix, 444 µl of nuclease-free water, and 6 µl of Megaplex reverse transcription product.
10. Pipette 100 µl of the PCR reaction mix into each port of the TaqMan miRNA array, centrifuge, and seal.
11. When profiling individual assays, dilute the Megaplex reverse transcription product or the 4× diluted preamplification product 50× and add 2.25 µl of this dilution to 0.25 µl of TaqMan miRNA assay and 2.5 µl of TaqMan universal PCR master mix in a 384-well plate.
12. Run the PCR reaction as follows: 95°C for 10 min, (95°C for 15 s, 60°C for 1 min, optical read) × 40 cycles.

3.2. Universal RT-qPCR

1. Dilute RNA sample to a concentration of 5 ng/µl (total RNA). Sensitivity can be improved by increasing the amount of input RNA (see Notes 3 and 4). Similarly, decreasing the amount of input RNA will result in a lower sensitivity. Keep RNA on ice at all times to prevent degradation. When using the predisposed panels, dilute RNA sample to 5.5 ng/µl to get a 10% excess when preparing the PCR reaction mix.
2. Prepare the reverse transcription reaction mix by combining 4 µl of reaction buffer, 2 µl of enzyme mix, 10 µl of nuclease-free water, and 4 µl of the RNA sample (5 ng/µl). Mix by pipetting and spin down. Prepare two reverse transcription mixes to analyze both human panels.
3. Incubate the reverse transcription mix at 42°C for 60 min, followed by reverse transcriptase heat inactivation at 95°C for 5 min.
4. When profiling individual assays, dilute the reverse transcription product 80× in nuclease-free water and prepare PCR reaction mix by combining 2.5 µl of SYBR Green master mix with 0.5 µl of LNA primer mix and 2 µl of diluted cDNA in a 384-well plate.

5. When using predisposed panels, combine both 20 μ transcription reactions per sample and dilute the reverse transcription product 110 \times by adding 4,360 μ l of nuclease-free water to 40 μ l of reverse transcription product. Prepare the PCR reaction mix for each sample by combining 4,360 μ l of SYBR Green master mix with 4,360 μ l of diluted reverse transcription product and pipette 10 μ l in each well of the predisposed panels.
6. Run the PCR reaction as follows: 95°C for 10 min, (95°C for 10 s, 60°C for 1 min, optical read) \times 40 cycles, melting curve analysis.

3.3. Data Normalization

1. When profiling all (or a substantial subset of) miRNAs, normalize miRNA expression using the global mean expression value (μ) (see Note 6). Given k expressed miRNAs, the normalized relative quantity (in log scale) for miRNA i in sample j is defined as:

$$\text{NRQ}_{i,j} = Cq_{i,j} - \mu_j$$

$$\mu_j = \frac{\sum_{i=1}^k Cq_{i,j}}{k}$$

Alternatively, it is also possible to calculate the normalized relative quantity in linear space (see Note 7). Given k expressed miRNAs in sample j , the normalized miRNA i is defined as:

$$\text{NRQ}_{i,j} = \frac{\text{RQ}_{i,j}}{\sqrt[k]{\prod_{i=1}^k \text{RQ}_{i,j}}}$$

2. To identify a set of reference miRNAs resembling the global mean, calculate the geNorm pairwise variation V value to determine robust similarity in expression of a given miRNA with the global mean expression value. For each miRNA, calculate the difference between its Cq -value and the global mean expression value in each sample. Next, determine the standard deviation of these differences for each miRNA. The miRNAs with the lowest standard deviation most closely resemble the global mean expression value. The optimal number of miRNAs for normalization should be determined through geNorm analysis of the ten best ranked miRNAs. To avoid including miRNAs that are putatively coregulated, exclude those miRNAs that are located within 2 kb of each other. Coregulated miRNAs are replaced by the next best ranked miRNA.

4. Notes

1. The miRNA body map Web tool is available at <http://www.mirnabodymap.org>. To identify stably expressed miRNAs, navigate to the “data analysis” section by clicking the “data analysis” icon in the top left icon bar. Next, choose your species of interest and select a dataset. Under the “miRNA centric analysis” option, choose “Select most stably expressed miRNAs.” Finally, select your samples of interest and click “next” to view stable reference miRNAs for your sample subset.
2. The stability of candidate reference miRNAs depends on the tissue or disease type but also on the experimental conditions (e.g., treatment of the cells with siRNA or compound). When changing experimental conditions, verify the stability of the reference miRNAs by measuring their expression on a representative selection of samples followed by geNorm or Normfinder analysis.
3. miRNA expression profiling will only be successful if the small RNA fraction is retained after RNA isolation. Several commercial kits are available that enable the extraction of total RNA including the small RNA fraction. The presence of the small RNA fraction can be evaluated using microfluidics-based electrophoresis systems such as the Bioanalyser (Agilent) or the Experion (Bio-Rad) (Fig. 3). We strongly encourage to include only RNA samples of sufficient quality. In addition, enrichment of the small RNA fraction is not advised.
4. There is no need to perform a DNase-treatment prior to miRNA expression profiling when using the stem-loop RT-qPCR platform. When using the universal RT-qPCR platform, DNA contamination can be an issue. This can be evaluated by profiling a sample for which the reverse transcription reaction was performed without reverse transcriptase. qPCR signals that are detected in this sample typically indicate a contamination with genomic DNA.
5. The stem-loop RT-qPCR miRNA expression profiling protocol can be adjusted to a multiplex format (both with and without preamplification of the reverse transcription product), which allows to perform reverse transcription (and preamplification) for a limited number of miRNAs as compared to the classical Megaplex format where reverse transcription for all miRNAs is performed. Consult Applied Biosystems for further information on the “Protocol for Custom RT and Preamplification Pools with TaqMan MicroRNA Assays.”
6. Baseline and threshold settings should be carefully evaluated when determining C_q-values. Typically, the baseline should be

set to the cycle interval where no amplification takes place. The threshold is set, with the Y-axis in log-scale, where all assays are in log linear phase.

7. Biogazelle's qbase^{PLUS} software ((11); <http://www.qbaseplus.com>) employs an improved version of the global mean normalization method based on geometric averaging of all expressed miRNAs, as well as an improved version of the geNorm method (enabling identification of the single best reference gene).

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