Chapter 9

RT-qPCR-Based Quantification of Small Non-Coding RNAs

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Abstract

MicroRNAs (miRNAs) are small non-coding RNA molecules that negatively regulate messenger RNA (mRNA) translation into protein. MiRNAs play a key role in gene expression regulation, and their involvement in disease biology is well documented. This has fueled the development of numerous tools for the quantification of miRNA expression levels. These tools are based on three technologies: (microarray) probe hybridization, RNA sequencing, and reverse transcription quantitative polymerase chain reaction (RT-qPCR). In this chapter, we describe a quantification system based on RT-qPCR technology, which is currently considered as the most sensitive, flexible, and accurate method for quantification of not only miRNA but also RNA expression in general. To this purpose, we have divided the protocol in three sections: reverse transcription (RT) reaction, optional preamplification (PA), and finally qPCR. Three quality-control (QC) steps are implemented in this workflow for assessment of RNA extraction efficiency, sample purity (e.g., absence of inhibitors), and inter-run variations, by examining the detection level of different spike-in synthetic miRNAs. We conclude by demonstrating raw data preprocessing and normalization using expression data obtained from high-throughput miRNA profiling of human RNA samples.

Key words MicroRNA, Reverse transcription reaction, Preamplification, qPCR, miScript, Quality control

1 Introduction

MicroRNAs are short non-coding RNA molecules that enable posttranscriptional gene silencing by interacting with the 3′UTR region of a mRNA target sequence [1]. One miRNA may target multiple mRNAs, and one mRNA may be targeted by several miRNAs. With over 2,500 human mature miRNAs described (miRBase release 21), miRNAs constitute one of the largest classes of gene expression regulators [2]. MiRNA-mediated gene expression modulation is an important and vital biological process. This is reflected in the high conservation level of miRNA sequences throughout vertebrates [3]. Since their discovery in 1993, miRNAs have been extensively studied in order to unravel their molecular methods of action and were found to be implicated in almost all

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physiological processes such as cell cycle progression, metastasis, embryogenesis, hematopoiesis, or immune response. Therefore, it was not surprising that aberrantly expressed miRNAs play a role in various diseases such as cancer, neurodevelopmental or cardiovascular diseases, obesity, etc. [4].

The rapidly growing insights in miRNA regulation mechanisms and their implication in health and disease have triggered the development of numerous quantitative miRNA expression platforms. Furthermore, the current popularity of personalized medicine and the search for genetic (cancer) biomarkers in both solid tissue and body fluids have significantly contributed to this development [5, 6].

Most currently used miRNA quantification methods were recently described in a comprehensive miRNA quality-control (miRQC) study published by Mestdagh et al., where 12 commercially available platforms were compared in terms of reproducibility, sensitivity, accuracy, specificity, and concordance of differential expression [7]. Two miRNA sequencing platforms, three miRNA hybridization platforms, and seven RT-qPCR platforms were included in the assessment.

The method described in this book chapter, the miScript PCR System, was also included in the miRQC study, without the optional cDNA preamplification step though. This miRNA expression platform, together with many other qPCR platforms, is characterized by an overall high sensitivity, especially prominent when working with samples with low RNA quantity, which is of particular importance when miRNA expression is studied in body fluids. Other advantages of the miScript platform are the large availability of 2,405 validated human miRNA assays, which cover approximately 93 % of the current miRBase content, and the flexibility to study any subset of these miRNAs using custom miRNA assay plates.

The miScript PCR technology uses a universal reverse transcription step based on polyadenylation, followed by anchored oligodT priming (Fig. 1). Universal RT is less expensive, quicker, and more straightforward than target-specific multiplex RT alternatives. The most important advantage is the flexibility offered by a single RT reaction for the whole miRNome, in contrast to a multiplex reaction where the utility of the RT product is confined to the number of multiplexed assays. cDNA from a universal RT procedure can be reused if the miRNAs under evaluation change throughout the course of the study.

Another key attribute of the miScript PCR platform is the use of internal synthetic controls that are added in the RT Mix (miRTC, miRNA reverse transcription control) or dispensed on pre-spotted qPCR arrays (PPC, positive PCR control). These controls allow evaluation of reverse transcription efficiency, qPCR inhibition, and technical reproducibility.

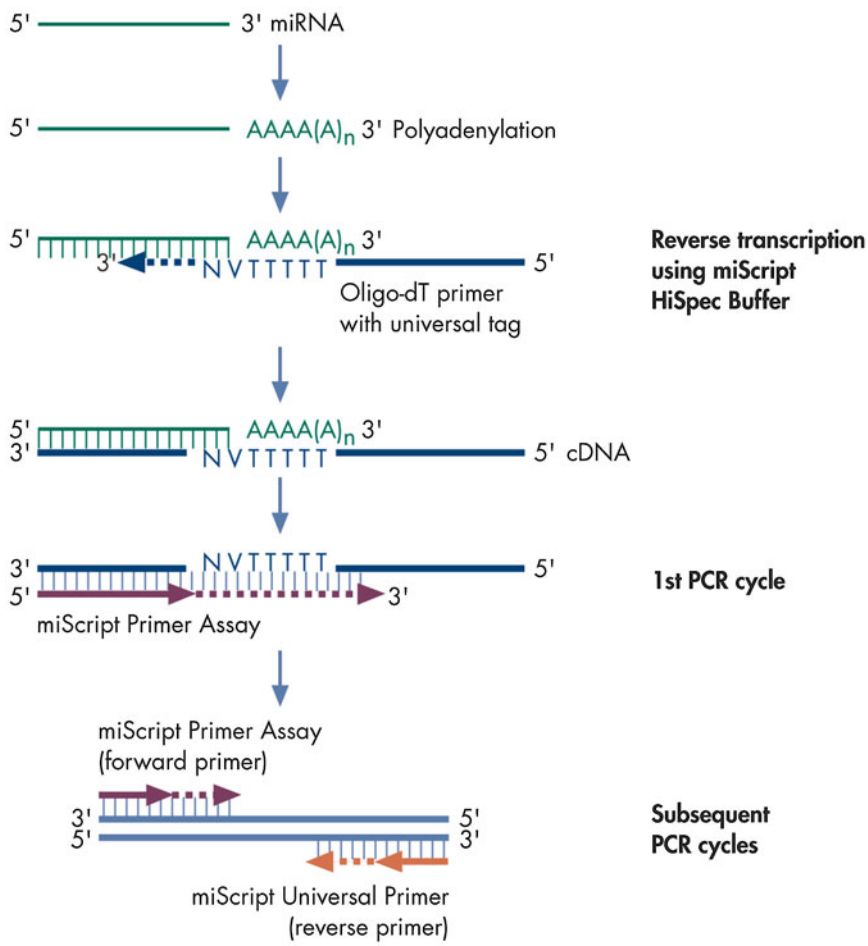

Fig. 1 Selective conversion of mature miRNAs into cDNA in miScript HiSpec Buffer. In a reverse transcription reaction with miScript HiSpec Buffer, mature miRNAs are polyadenylated by a poly-A polymerase and converted into cDNA by a reverse transcriptase with oligodT priming. The cDNA is then used for qPCR quantification of mature miRNA expression

Here, we present a three-step workflow with a quality checkpoint after each step: (1) reverse transcription (RT) and quality control of the RT product by evaluation of miRTC levels, (2) (optional) preamplification (PA) and evaluation of the preamplified miRTC product, and (3) qPCR assessment of spike-in controls miRTC and PPC, together with the endogenous miRNAs of interest. The spike-in controls are evaluated in terms of inter-sample variability and in terms of abundance difference relative to a control sample. Raw data preprocessing, including data filtering and normalization, is demonstrated by using expression data obtained from high-throughput miScript miRNA profiling on human RNA samples. Here, we applied the global mean normalization procedure [8, 9], introduced by Mestdagh et al. and later modified by

D'haene et al. This method was proven to be superior to normalization using a single or even multiple endogenous small RNAs (such as sn(o)RNAs). In Subheading 4, important considerations with respect to RNA quantity are discussed, not only because this influences data quality but also because it affects specific steps during the RT-qPCR workflow.

2 Materials

RNase-free water should be used to prepare all solutions. All PCR preparation steps should be performed in an RNase-free area, separated from the post-PCR product manipulation area. RNase-free tubes and filter tips are required throughout the entire pre-PCR workflow.

2.1 Reverse Transcription (RT) Reaction

1. Total RNA extract (*see* **Notes 1**–**3**).
2. RNase-free water.
3. Carrier RNA (tRNA, MS2 phage RNA) (*see* **Note 4**).
4. Reverse Transcriptase Mix from miScript II RT Kit (Qiagen).
5. 5x miScript Buffer from miScript II RT Kit (Qiagen): HiSpec Buffer if preamplification reaction is performed or HiFlex Buffer if no PA reaction is performed (*see* **Note 5**).
6. 10x Nucleics Mix from miScript II RT Kit (Qiagen) (*see* **Note 6**).
7. Thermal cycler.

2.2 qPCR for Quality Control of the RT Reaction

1. Diluted RT product.
2. RNase-free water.
3. 2× QuantiTect SYBR Green PCR Master Mix from miScript SYBR Green PCR Kit (Qiagen).
4. 10× miScript Universal Primer from miScript SYBR Green PCR Kit (Qiagen).
5. 10× miRTC miScript Primer Assay (Qiagen).
6. qPCR instrument (*see* **Note 7**).
7. 384-well qPCR plate (*see* **Note 8**).

2.3 Preamplification Reaction

1. Diluted RT product.
2. RNase-free water.
3. 5× miScript PreAMP Buffer from miScript PreAMP PCR Kit (Qiagen).
4. HotStarTaq DNA Polymerase from miScript PreAMP PCR Kit (Qiagen).
5. miScript PreAMP Universal Primer from miScript PreAMP PCR Kit (Qiagen).

6. miScript PreAMP Primer Mix (*see* **Note 9**) (Qiagen).
7. Thermal cycler.

2.4 qPCR for Quality Control of the Preamplification Reaction

1. Diluted PA product.
2. RNase-free water.
3. 2× QuantiTect SYBR Green PCR Master Mix from miScript SYBR Green PCR Kit (Qiagen).
4. 10× miScript Universal Primer from miScript SYBR Green PCR Kit (Qiagen).
5. 10× miRTC miScript Primer Assay (Qiagen).
6. qPCR instrument (*see* **Note 7**).
7. 384-well qPCR plate (*see* **Note 8**).

2.5 qPCR Reaction

1. Diluted preamplified cDNA.
2. RNase-free water.
3. 2× QuantiTect SYBR Green PCR Master Mix from miScript SYBR Green PCR Kit (Qiagen).
4. 10× miScript Universal Primer from miScript SYBR Green PCR Kit (Qiagen).
5. Pre-spotted assay multi-well plates (96-well or 384-well plates) or individual assay tubes (*see* **Note 10**).
6. Robotic liquid handler or multichannel pipettes and repetition pipette combined with repetitive pipette syringes.
7. qPCR instrument (*see* **Note 7**).

3 Methods

3.1 Reverse Transcription (RT) Reaction

1. If RNA isolation has not yet been performed, please carefully read **Notes 1–3**. If RNA is available, measure RNA concentration by spectrophotometry or fluorometry for samples with low RNA concentration (<5 ng/μl).
2. If the total RNA concentration is high (>100 ng/μl), dilute the samples to the minimum concentration (*see* **Notes 11** and **12**) and continue with the RT reaction (**step 5**).
3. If total RNA concentration is low (<100 ng/μl), closely evaluate the quality of the samples with the lowest concentrations (<5 ng/μl). Consider their exclusion from the study and avoid equalization of RNA concentrations for the other samples to a suboptimally low concentration (*see* **Notes 11** and **12**). Dilute the samples to the minimum concentration and continue with the RT reaction (**step 5**).
4. If all samples have an RNA concentration varying around 5 ng/μl or if RNA concentration cannot be reliably assessed (e.g.,

Table 1
Reverse transcription reaction mix components

Mix components	Volume per reaction (µl)	10 reactions (µl)	10 % excess (µl)
Reverse Transcriptase Mix	1	10	11
5× miScript RT Buffer	2	20	22
10× Nucleics Mix	1	10	11
RNase-free water	Variable	Variable	Variable
RNA sample	Variable		
Total volume per reaction	10		

from low-volume body fluids), use 1.5 µl of undiluted RNA and continue with the RT reaction (*see* **Notes 11** and **12**).

5. Perform the following steps on ice. Prepare a negative control sample with 10 µl of RNase-free water. If a spike-in control was used during RNA isolation, add the carrier RNA and the spike-in control to the control water sample. If not, continue to step 8.
6. Dilute carrier RNA in RNase-free water. The concentration of carrier RNA in the negative control sample should be in the same range as the RNA concentration of the biological samples.
7. Add the spike-in control solution to the carrier solution in order to obtain the same spike-in concentration as present in the RNA isolates.
8. Prepare one RT Mix for all reactions according to Table 1 (typically one reaction per sample). If needed, RT replicates can be useful to monitor RT variability (*see* **Notes 5**, **13** and **14**).
9. Mix well by gently pipetting up and down and distribute the RT Mix in separate reaction tubes (*see* **Note 15**).
10. Mix the RNA sample by gently pipetting up and down to homogenize the RNA solution and add the RNA sample to each RT tube (*see* **Note 15**).
11. Run the RT reactions on the thermal cycler (Table 2).
12. Spin the RT product briefly to collect the condensed fraction and mix gently by pipetting up and down (*see* **Note 15**).
13. Make one 3 µl aliquot for the RT quality-control step and another 7 µl aliquot for the remaining downstream steps (PA and qPCR) (*see* **Notes 16** and **17**).

3.2 qPCR for Quality Control of the RT Reaction

1. Dilute 2 µl of the RT product (also for the negative control sample) in 20 µl of RNase-free water (*see* **Note 17**).
2. Prepare one qPCR mix for all samples to be measured as shown in Table 3. Prepare a second qPCR mix besides the miRTC

Table 2
Reverse transcription run protocol

Step	Duration (min)	Temperature (°C)
Reverse-strand synthesis	60	37
Enzyme denaturation	5	95
Cool down	1	4

Table 3
qPCR mix components

Mix components	Volume per reaction (µl)	10 × 2 reactions (µl)	10 % excess (µl)
2× QuantiTect SYBR Green PCR Master Mix	5	100	110
10× miScript Universal Primer	1	20	22
10× miScript Primer Assay	1	20	22
RNase-free water	1	20	22
Diluted RT product	2		
Total volume per reaction	10		

Table 4
qPCR run protocol

Step	Duration	Temperature
PCR activation step	15 min	95 °C
40 cycles of:		
Denaturation	15 s	94 °C
Annealing	30 s	55 °C
Extension	30 s	70 °C

qPCR mix for the RNA isolation spike-in control if one was used during RNA isolation. Prepare sufficient mix to perform all reactions in duplicate (*see* **Note 18**).

3. Mix gently by pipetting up and down and distribute the qPCR mix in a 384-well qPCR plate (*see* **Note 15**).
4. Mix the diluted RT sample by gently pipetting up and down and add 2 µl of the product to the qPCR mix (*see* **Note 15**).
5. Run the qPCR protocol (Table 4).

Table 5
Preamplification reaction mix components

Mix components	Volume per reaction (µl)	10 reactions (µl)	10 % excess (µl)
5× miScript PreAMP Buffer	5	50	55
HotStarTaq DNA Polymerase	2	20	22
miScript PreAMP Primer Mix	5	50	55
RNase-free water	7	70	77
miScript PreAMP Universal Primer	1	10	11
Diluted RT product	5		
Total volume	25		

6. Export raw miRTC Cq data and calculate the Cq difference between each RNA sample and the water negative control sample.
7. Exclude samples that show a Cq difference larger than 1 as this likely refers to inefficient RT reaction, RT or PCR inhibition, or technical error during RNA isolation (*see* **Note 19**).

3.3 Preamplification Reaction (Optional, Recommended for Highest Sensitivity)

1. Dilute the 7 µl RT aliquot from the samples and the negative control sample in 28 µl water to obtain a fivefold dilution (*see* **Notes 16** and **17**).
2. Prepare one PA mix for all reactions (one reaction per sample and per primer pool) (Table 5).
3. Mix well by gently pipetting up and down (*see* **Note 15**) and distribute 20 µl in separate reaction tubes.
4. Gently mix the diluted (1:5) RT product by pipetting up and down (*see* **Note 15**) and add 5 µl to each PA reaction tube to obtain a final volume of 25 µl.
5. If multiple PA primer pools are available (e.g., 7 pools for miRBase v.20), multiple PA reactions should be prepared for each sample (*see* **Note 9**).
6. Run the PA protocol on the thermal cycler (Table 6).
7. Centrifuge the PA product briefly to collect the condensed fraction and mix gently by pipetting up and down.
8. If multiple PA reactions per sample were performed, pool the PA reactions prior to dilution.
9. Dilute the pooled PA product five times into the PA reaction to obtain a 1:5 dilution per ng cDNA (*see* **Note 20**).
10. Make a 2 µl aliquot of the diluted PA product for the quality-control step.

Table 6
Preamplification reaction run protocols

For preamplification of ≤96 assays[a]			For preamplification of ≥96 assays[a]		
Step	**Duration**	**Temperature**	**Step**	**Duration**	**Temperature**
PCR activation step	15 min	95 °C	PCR activation step	15 min	95 °C
12 cycles of:			2 cycles of:		
Denaturation	30 s	94 °C	Denaturation	30 s	94 °C
Annealing/extension	3 min	60 °C	Annealing	60 s	55 °C
			Extension	60 s	70 °C
			10 cycles of:		
			Denaturation	30 s	94 °C
			Annealing/extension	3 min	60 °C

[a]Different run protocols are used depending on the number of primer assays in the miScript PreAMP Primer Mix

3.4 qPCR for Quality Control of the Preamplification Reaction

1. Prepare one qPCR mix for all samples (*see* Table 3). Prepare a second qPCR mix besides the miRTC qPCR mix for the RNA isolation spike-in control if one was used during RNA isolation. Prepare sufficient mix to perform all reactions in duplicate (*see* **Note 18**).
2. Mix gently by pipetting up and down and distribute the mix in a 384-well qPCR plate (*see* **Note 15**).
3. Mix the diluted PA sample by gently pipetting up and down and add 2 µl of the product to the qPCR mix (*see* **Note 15**).
4. Run the qPCR experiment (Table 4).
5. Export raw miRTC Cq data and calculate the Cq difference between each RNA sample and the water negative control sample.
6. Exclude samples that show a Cq difference larger than 1 as this likely reflects inefficient PA reaction, qPCR inhibition, or technical failure (*see* **Note 19**).

3.5 qPCR

1. Continue with the samples that passed the quality-control steps after RT reaction and PA reaction.
2. Prepare one qPCR mix per sample and distribute in 384-well plates (Table 7).
3. Run the samples on a qPCR instrument (Table 4).
4. Export raw Cq values from the qPCR experiment.
5. Evaluate PPC variations across samples (*see* **Note 21**).
6. Calculate the global mean miRNA Cq value per sample and evaluate this value together with the Cq values for miRTC and PPC across samples to check for possible occurrence of batch effects during the experiment (*see* **Note 22**).

Table 7
Mix components for 384-well plate[a] qPCR reactions

Mix components	1 plate (µl)	1 plate + 10 % excess (µl)	5 plates + 10 % excess (µl)
2× QuanTitect SYBR Green PCR Master Mix	2,050	2,255	11,275
10× miScript Universal Primer	410	451	2,255
RNase-free water	1,540	1,694	8,470
Template cDNA	100	110	550
Total volume	4,100	4,510	22,550

[a]In this table, mix components are shown for plates pre-spotted with PCR Primer Assays. If non-spotted plates are used then 10× miScript Primer Assay should be added to the PCR Mix (mix composition as shown in Table 3)

7. Determine an upper Cq detection cutoff and remove all values above the cutoff (*see* **Note 23**).
8. Normalize the data by the modified global mean method (*see* **Note 24**).

4 Notes

1. There is no need to isolate pure small RNAs. We recommend total RNA isolation (including small RNAs) which can be used to quantify small RNAs using various methods.
2. The quantity of RNA can have a drastic impact on the reproducibility and reliability of RT-qPCR data. If RNA concentration is sufficiently high (>100 ng/µl), then each sample can easily be diluted to an equal concentration before starting the RT reaction. It is important to perform this step carefully since a variable RNA input contributes to variability in gene expression levels and thus masks true gene expression differences between samples. If the majority of samples have a concentration lower than 5 ng/µl, accurate measurement of RNA concentration by spectrophotometric methods and dilution to equal concentrations before RT reaction is difficult or impossible (as is the case for cell-free body fluids like serum, plasma, sputum, spinal fluid, etc.). The following adjustments can be made to the RNA isolation protocol in order to increase RNA yield and standardize RNA input for the RT reaction.

   Invest sufficient time in choosing an RNA isolation method that is adapted to the starting material type. Different kits have been optimized to increase yield and purity of RNA extracted from specific biomaterials. For example, miRNeasy total RNA isolation Kits (Qiagen) are provided in different formats.

MiRNeasy FFPE Kit is optimized for formalin-fixed paraffin-embedded (FFPE) samples, miRNeasy Serum/Plasma Kit for serum and plasma samples, miRNeasy Micro Kit for low cell numbers, etc. A higher RNA concentration is obtained if the miRNeasy Micro Kit is used for isolation of serum RNA instead of the miRNeasy Mini Kit (elution of the RNA extract in 14 μl water instead of 30 μl water) (Fig. 2).

Start from an equal amount of biomaterial if possible. For example, when working with body fluids, start from equal volumes of the fluid. When working with solid tissue, weigh the tissue or count cells if possible.

Use a spike-in RNA control during RNA isolation in biofluids. The miRNeasy Serum/Plasma Kit provides miRNeasy Serum/Plasma Spike-In Control (cel-miR-39-3p mimic), a synthetic miRNA from *C. elegans*. This will allow you to evaluate RNA isolation efficiency and RNA isolation variability across all samples. Note that it is important to add the spike-in RNA only after lysis (this is after addition of lysis reagent, sample mixing, and incubation at room temperature for 5 min). Synthetic spike-in RNA molecules are sensitive to RNases present in body fluids or tissues. Lysis will cause protein denaturation and inactivation of RNases. As a result, the quantity of the spike-in control RNA will remain stable across samples when added after lysis.

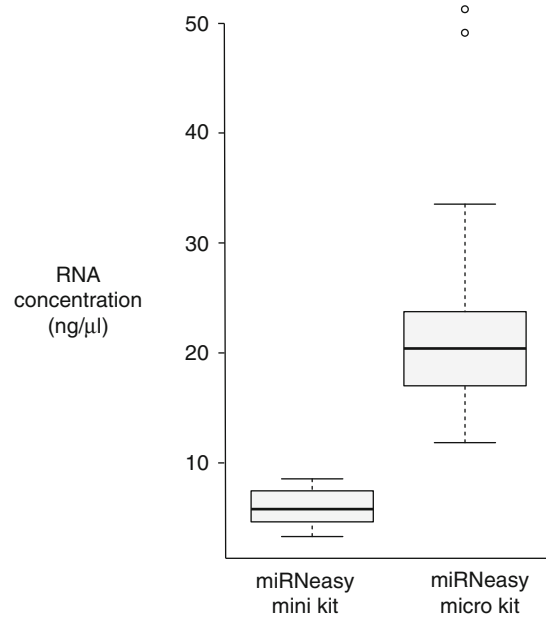

Fig. 2 Purified RNA concentration in serum from healthy individuals measured by NanoDrop 1000, obtained from miRNeasy mini kit (*left*, 30 μl eluate) and miRNeasy micro kit (*right*, 14 μl eluate) ($n = 18$; box plot showing median, first and third quartile, whiskers denote 25th and 75th percentile) (*see* **Notes** 11 and **12**)

A last recommendation is to collect an equal volume of the upper aqueous phase for each sample, even if this is less than the maximum volume you could possibly recover. It might decrease the total RNA yield, but it will contribute to recovery of equal amounts of pure RNA across the sample set.

3. Besides RNA quantity, another important aspect to consider before pursuing with the reverse transcription reaction is RNA quality. Quality is both defined by RNA integrity (i.e., non-degraded) and purity. Impure RNA can inhibit enzymatic reactions such as reverse transcription or PCR. Contaminations can originate from the RNA isolation procedure (ethanol, guanidine salts, contaminating proteins, etc.). In this workflow, miRTC (miRNA reverse transcription control) and PPC (positive PCR control) are used to detect inhibition of RT and/or qPCR reaction. Amplification of these control RNAs in the presence of the RNA extract is compared to its amplification in a negative control sample (e.g., pure water with carrier RNA). If delayed amplification is observed in the RNA isolate (higher Cq values) in comparison to the water sample, inhibitory activity during RT or PCR can be assumed, and the RNA sample should be excluded from downstream experiments (*see* **Note 17**).
4. Higher reproducibility of qPCR measurements is described in literature when carrier RNA is included to RNA samples [10]. Therefore, carrier RNA is used to prepare the RT control sample to which spike-in miRNA is added. This will ensure reproducible data to be obtained for the control sample.
5. The miScript RT Kit provides two types of buffers depending on whether you want to perform cDNA preamplification or not. HiSpec Buffer facilitates the selective conversion of mature miRNAs into cDNA, while HiFlex Buffer promotes conversion of all RNA species (mature miRNA, precursor miRNA, non-coding RNA, and mRNA) into cDNA. Using HiSpec Buffer, the conversion of long RNAs, such as mRNAs, is suppressed. As a result, background signals potentially contributed by long RNAs are nonexistent making HiSpec Buffer highly suitable for miRNA expression profiling. HiSpec Buffer is required when preamplification reactions are performed. HiFlex Buffer or miScript HiSpec buffer may be used if no preamplification reactions are performed.
6. The miScript Nucleics Mix contains the synthetic RNA control miRTC.
7. It is important to use the same qPCR instrument and data analysis settings during the entire experiment. Differences in Cq values can be observed if samples are run on different instruments, and this can lead to increased variability. Ideally, all samples that need to be compared should be analyzed in the same run/plate, corresponding to Hellemans sample maximi-

zation strategy [11]. However, in a screening mode, it is often more convenient to measure one sample per set of assay plates. Using the same instrument and software data analysis settings and including controls (such as the PPC) will greatly help to reduce technical variance.

8. We typically use 384-well plates, but 96-well format, rotor-disc format, and individual tubes work as well, as long as mix composition and volume proportions are respected.
9. The composition of the miScript PreAMP Primer Mix is fully customized. Primer mixes can be ordered for whole miRNome profiling (2,405 miRNAs, v.20 miRBase content) or for a selection of miRNAs. If targets of interest are unknown, whole miRNome profiling can be performed on a reduced number of representative samples. This will allow selection of a set of interesting miRNAs and profiling of only the selected set across a larger sample cohort in a second screening step.
10. We typically use multi-well plates pre-spotted with qPCR primer assays. Since the primer assays are not manually added to the qPCR Mix, this will speed up the preparation and help decrease technical variability. Alternatively, qPCR primer assays can be ordered individually.
11. Make sure to dilute the RNA solution to a concentration and volume within the range of the RT Kit. In this protocol, the recommended RNA input for mature miRNA profiling ranges between 10 ng and 2 µg for a 20 µl RT reaction. In case of preamplification, 10 ng to 100 ng RNA input is recommended in a 10 µl RT reaction. If RNA concentration is unknown (e.g., in the case of body fluids), 1.5 µl of the undiluted RNA input is recommended for a 10 µl RT reaction.
12. The unreliable detection range depends on the RNA concentration measurement device. Spectrophotometry (e.g., NanoDrop 1000) is the commonly used method for RNA concentration measurement. An optimal determination range between 500 and 5 ng/µl was reported for the NanoDrop 1000 [12]. The lowest detection limit is 2 ng/µl, and the precision of the measurement decreases below 5 ng/µl [12]. Therefore, RNA concentration values below 5 ng/µl should be interpreted with caution. Samples with concentration below 5 ng/µl should preferably be excluded, rather than diluting the other samples to the same low concentration.

    The suggested 5 ng/µl cutoff does not apply if methods with lower detection limits are used to measure RNA concentration (e.g., fluorescence-based quantification).
13. The volume of the RT reaction described in the Qiagen miScript handbook differs when preamplification is included in the workflow. In case of preamplification, RT reaction is performed in 10 µl. If preamplification is not included in the workflow, the RT reaction is performed in 20 µl. In our

workflow, we routinely perform 10 μl RT reactions. RT reaction volumes of 20 or 10 μl did not show any discrepancies on the Cq values for cel-miR-39-3p and miRTC.

14. miScript Primer Assays are designed using a proprietary algorithm that promotes efficient and specific detection and amplification of mature miRNA. In addition, the algorithm has been extensively challenged with wet bench data using miRNA families to ensure that primers can distinguish closely related miRNA sequences. Candidate miScript Primer Assays are subjected to a series of wet-lab validation tests with predefined metrics that ensure miRNA-specific amplification. These tests include three background signal tests including a no template control (NTC) real-time PCR reaction, a "water only" reverse transcription reaction, and a poly-A polymerase-minus reverse transcription reaction performed on species-specific universal total RNA. These tests ensure that miScript Primer Assays are not amplifying molecules other than miRNAs. For example, in a poly-A polymerase-minus reaction, a positive real-time PCR signal would indicate that the primer is not specifically amplifying cDNA synthesized from miRNAs, since miRNAs are not naturally polyadenylated. miScript Primer Assays are also tested using a species-specific universal total RNA sample spiked with a synthetic miRNA standard. For this series of tests, each assay must have a Cq within an acceptable range and feature a smooth and sigmoidal amplification curve, single peak dissociation curves, and dissociation curve temperatures indicative of a mature miRNA PCR amplicon. If a miScript Primer Assay fails any of these wet-lab tests, the assay is redesigned and retested.
15. According to miScript qPCR protocols from Qiagen, miScript cDNA samples and mixes should not be mixed on a vortex device. Gentle flicking or pipetting up and down is advised instead. When pipetting up and down, it is important to use a large pipet tip that allows aspiration of at least half of the total mixture volume. Only then will sufficient turbulence be created to allow effective homogenization of the viscous PCR reagents. If not done properly, sample-to-sample variability may be introduced in your data.
16. In this workflow, the RT product is differently diluted depending on whether the RT reaction is followed by a PA reaction or not. If you plan to perform preamplification, divide the RT product in different aliquots, one for QC RT (which is performed directly after RT) and one for the PA reaction. Dilute each of the aliquots accordingly as described in the "Methods" section above.
17. If no PA is performed after the RT reaction, a minimum 11-fold dilution is advised for the RT product, to avoid qPCR inhibition and minimize nonspecific product formation. If the RT reaction is followed by a PA reaction, then a minimum fivefold dilution of the RT product is recommended.

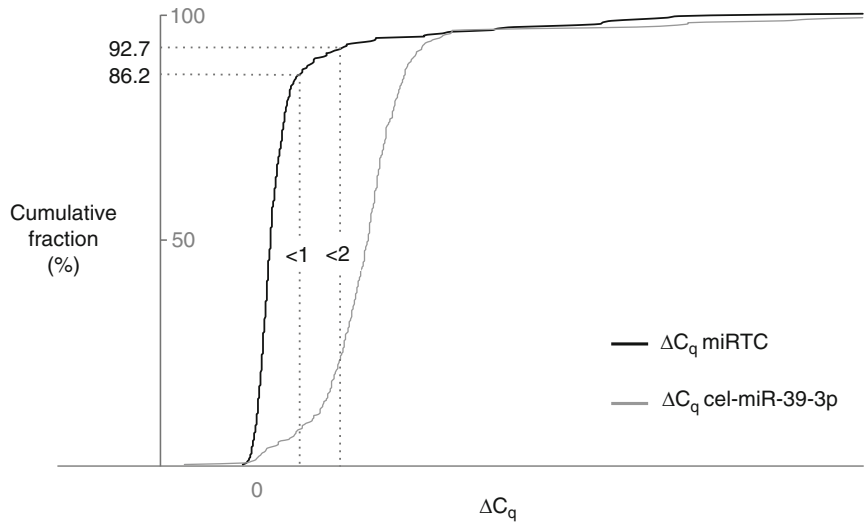

Fig. 3 ΔCq cumulative density for miRTC and cel-miR-39-3p measured in 289 serum samples (ΔCq = Cq RNA sample – Cq negative control sample). 86.2 % of the samples show a miRTC ΔCq smaller than 1, and 92.7 % of the samples show a miRTC ΔCq value smaller than 2

18. For each QC qPCR step, the samples are measured in the same plate according to the sample maximization principle [11]; so run all samples on one plate for as many assays as possible and divide assays over different plates only if needed. If working with a large number of samples (that cannot fit on a single plate), multiple runs per target are required, and inter-run calibration should be performed to avoid technical inter-plate variation.
19. Cumulative density plot for miRTC ΔCq values obtained from 289 serum samples is shown in Fig. 3. Samples showing a miRTC ΔCq value larger than one (13.8 %) were not used in the downstream analyses. A larger variation for spike-in ΔCq (cel-miR-39-3p) was observed. In this study, no sample exclusion was performed based on this criterion, except for one sample where the spike-in was not detected.
20. In this workflow, the minimum dilution factor for preamplified cDNA is fivefold (miRNome arrays and custom arrays for 96–384 assays). If cDNA concentration is known, the dilution factor equals the amount of input cDNA (ng) in the PA reaction multiplied by 5. For example, if 2 ng cDNAs were added to the PA reaction, a tenfold (2×5) dilution should be performed after the PA reaction. If cDNA concentration is unknown (e.g., body fluids), perform a fivefold dilution and prepare an additional QC qPCR test to evaluate the Cq values for some miRNAs (the miScript PreAMP Handbook recommends evaluation of miR-16 or SNORD95). If other miScript array formats are being used (pathway-focused arrays and custom arrays), carefully consult the miScript PreAMP Handbook to determine the optimal dilution factor.

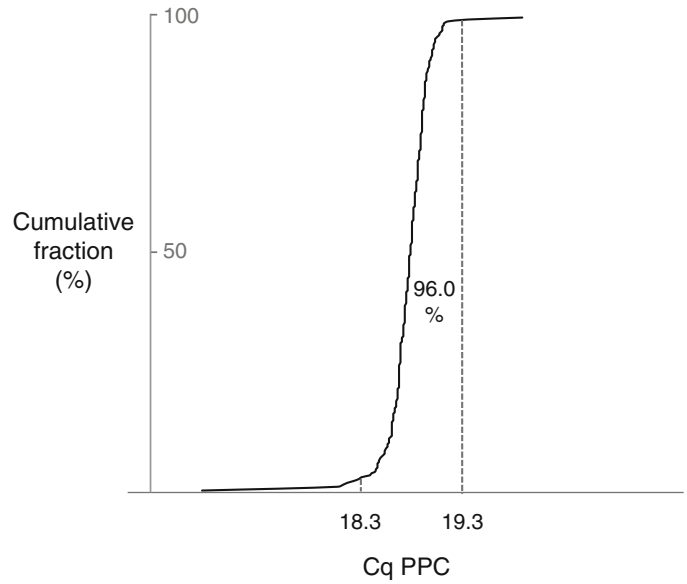

Fig. 4 Cumulative density of PPC (positive PCR control) Cq values measured in 251 serum samples. 96.0 % of the samples are detected within one Cq value around the median value of 18.8

21. Assessments of PPC Cq values will allow monitoring of inter-run variation, batch effect, and technical failures. In Fig. 4, a cumulative density plot is shown for PPC Cq values for 251 RNA samples isolated from human serum. A very tight distribution of PPC Cq values is observed. Approximately 5 % of the samples differ by more than one cycle from the median Cq (18.8).
22. Batch effect refers to a shift in global miRNA expression level that is not caused by the biological effect under evaluation. Batch effects can be explained by effects caused by different RNA isolation batches, RT reaction batches, and PA batches. They can also result from the sample origin, sample transport conditions, tissue type, etc. Global mean, miRTC, PPC, RNA isolation spike-in values, and their trend across the entire sample set can reveal very useful information with respect to the source of global expression shifts (if any are present). If the research objective is to compare two or more sample sets (e.g., treatment groups, survival groups, etc.), it is very important to check whether the differences coincide with a certain sample batch.
23. A robust method to determine the Cq detection cutoff was recently described in the miRQC study [7]. This method is based on the evaluation of single positive signals obtained from replicate miRNA expression data. A single positive is a miRNA for which amplification was obtained in only one of the sample replicates.

The detection cutoff is defined as the Cq value at which the single positive fraction is reduced by 95 %.

24. The modified global mean normalization is a very effective way to eliminate technical variability [9]. The method is implemented in the second version of Biogazelle's qbase + software and is particularly useful in high-throughput miRNA profiling experiments (evaluation of >100 miRNAs). Briefly, this method attributes equal weight to each miRNA by mean centering the miRNA expression values. The arithmetic mean Cq value for one miRNA is calculated across all samples and subtracted from each individual Cq value for that same miRNA prior to calculating the global mean expression value per sample. This value, the normalization factor, is then subtracted from each individual Cq value per sample to obtain normalized miRNA expression values. If only a few miRNAs are being studied, normalization by multiple endogenous small RNAs, such as sno or snRNAs, or by stably expressed miRNAs can be applied [11, 13].

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