

High-Throughput RT-qPCR for the Analysis of Circulating MicroRNAs

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Abstract

Reverse transcription followed by real-time or quantitative polymerase chain reaction (RT-qPCR) is the gold standard for validation of results from transcriptomic profiling studies such as microarray and RNA sequencing. The current need for most studies, especially biomarker studies, is to evaluate the expression levels or fold changes of many transcripts in a large number of samples. With conventional low to medium throughput qPCR platforms, many qPCR plates would have to be run and a significant amount of RNA input per sample will be required to complete the experiments. This is particularly challenging when the size of study material (small biopsy, laser capture microdissected cells, biofluid, etc.), time, and resources are limited. A sensitive and high-throughput qPCR platform is therefore optimal for the evaluation of many transcripts in a large number of samples because the time needed to complete the entire experiment is shortened and the usage of lab consumables as well as RNA input per sample are low. Here, the methods of high-throughput RT-qPCR for the analysis of circulating microRNAs are described. Two distinctive qPCR chemistries (probe-based and intercalating dye-based) can be applied using the methods described here.

Key words High throughput, qPCR, Preamplification, microRNA, TaqMan, miScript

1 Introduction

The throughput of each qPCR run, as indicated by the amount of transcripts and samples that can be studied in one single qPCR plate/chip, can vary up to 100 times between platforms (Fig. 1). The demand for high-throughput qPCR has become very common, especially in studies which need to analyze a large panel of transcripts in a large number of samples. High-throughput qPCR is an answer to studies that utilize low starting input of samples such as laser capture microdissected tissues, single cells and cell-free nucleic acid in biofluid samples. Also, as compared to qPCR platforms of lower throughput, the time and cost needed to complete the experiments are reduced when high-throughput qPCR is utilized.

In high-throughput microfluidic qPCR platform, each qPCR reaction is carried out in a very small volume (range of nanoliter). Preamplification is necessary to increase the target amount prior to

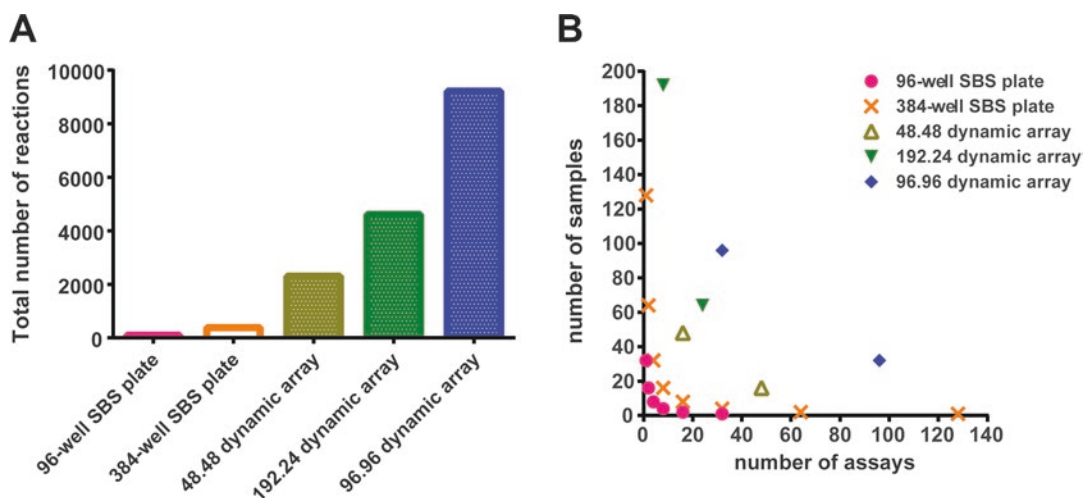


Fig. 1 (a) The numbers of reaction wells/chambers available in each qPCR plate/chip format are different and can vary up to 100 times. **(b)** The maximum numbers of assays and samples that can be analyzed in different format of plates/chips are compared under the setting of singleplex PCR and triplicate qPCR reactions per sample. Standard SBS plate format has lower throughput compared to high-throughput chips such as Fluidigm's dynamic array

qPCR so that targets/samples can be distributed equally among reaction chambers [1]. In order to prevent garbage in garbage out, cautious steps should be taken to evaluate if nonspecific amplifications and over amplification of targets are introduced during the preamplification step. Stringent quality control has to be applied on each primer assay by analyzing data from positive and negative controls to rule out nonlinear and/or nonspecific amplification.

For microRNA (miRNA) expression studies, different reverse transcription (RT) and qPCR chemistries are available. TaqMan is a probe-based system which is designed to specifically detect miRNAs with reference sequence registered in miRBase. On the other hand, miScript is an intercalating dye-based system which detects miRNAs with reference sequence registered in miRBase as well as all isomirs of the said miRNA. At times, miRNA with reference sequence registered in miRBase may not be the most abundant miRNA expressed in the samples of interest [2]. Therefore, the decision on using primer assay which is specific or generic will have to be based on research needs.

In this chapter, the protocols for RT, preamplification, and qPCR of both TaqMan and miScript systems are described. These protocols are optimized for the use with microfluidic chips in BioMark (Fluidigm) but nonetheless, the quality control (QC) principles developed and described here can be applied to all high-throughput RT-qPCR. Details are given on how to identify and exclude data from downstream analysis based on evidences of

nonspecific and/or nonlinear amplifications. Normalization methods for the analysis of circulating miRNAs data are also explained here. It is foreseeable that the lab methods described here might require modifications in the future due to the changes of reagents or protocols by manufacturers. Nonetheless, principle of data QC detailed here is always valid and should be applied to avoid garbage in, garbage out.

2 Materials

2.1 Positive and Negative Controls

1. RNA from pooled cell lines or pooled synthetic oligonucleotides (*see Note 1*).
2. Nuclease-free water.

2.2 TaqMan System

1. TaqMan MicroRNA Reverse Transcription kit: 100 mM dNTPs, RNase Inhibitor (20 U/ μ l), RT Buffer (10 $\times$), Multiscribe Reverse Transcription (50 U/ μ l).
2. TaqMan MicroRNA Assays: RT primer (5 $\times$) and real time primer (20 $\times$).
3. TaqMan PreAmp Master Mix (2 $\times$).

2.3 miScript PCR System

1. miScript II RT kit: miScript Nucleics Mix (10 $\times$), miScript HiSpec Buffer (5 $\times$), miScript Reverse Transcriptase Mix.
2. miScript Primer Assay (10 $\times$).
3. miScript PreAMP Buffer (5 $\times$).
4. HotStarTaqDNA Polymerase.
5. miScript PreAMP Universal Primer.
6. miScript Universal Primer (10 $\times$).
7. Side Reaction Reducer.

2.4 qPCR

1. Assay Loading Reagent (2 $\times$).
2. GE Sample Loading Reagent (20 $\times$) (for TaqMan system only).
3. DNA Binding Dye Sample Loading Reagent (20 $\times$) (for miScript PCR system only).
4. TaqMan Universal PCR Master Mix, no AmpErase UNG (for TaqMan system only).
5. SsoFast EvaGreen Supermix with Low ROX (for miScript PCR system only).
6. Dynamic Array IFC (integrated fluidic circuit) for gene expression (Fluidigm).

2.5 Others

1. Dilution buffer: TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) or nuclease-free water.

3 Methods

3.1 Positive and Negative Controls

3.1.1 Serial Dilution of Positive Controls

1. Pool RNA from different cell lines or synthetic oligonucleotides into a 1.5 ml tube (*see Note 1*).
2. Prepare a serial dilution from the pooled RNA. Suggestion of serial dilution is shown in Table 1 (*see Note 2*).
3. Positive controls, together with experimental samples should be subjected to RT, preamplification and qPCR using the same reagents and conditions.

3.1.2 Negative Controls (See Note 3)

1. Use nuclease-free water as negative control in RT, preamplification and qPCR steps.
2. These negative controls should be analyzed in the same qPCR run together with the positive controls and experimental samples.

Table 1
Recommendations of serial dilutions for positive controls

Tube ^a	Dilution factor	Concentration for pooled total RNA (g/ μ l)	Concentration for each oligonucleotide (mol/ μ l)
1	1	1.0×10^{-8}	5.0×10^{-16}
2	1/2	5.0×10^{-9}	2.5×10^{-16}
3	1/4	2.5×10^{-9}	1.3×10^{-17}
4	1/8	1.3×10^{-9}	6.3×10^{-18}
5	1/16	6.3×10^{-10}	3.1×10^{-18}
6	1/32	3.1×10^{-10}	1.6×10^{-18}
7	1/64	1.6×10^{-10}	7.8×10^{-19}
8	1/128	7.8×10^{-11}	3.9×10^{-19}
9	1/256	3.9×10^{-11}	2.0×10^{-19}
10	1/512	2.0×10^{-11}	9.8×10^{-20}
11	1/1024	9.8×10^{-12}	4.9×10^{-20}
12	1/2048	4.9×10^{-12}	2.4×10^{-20}
13	1/4096	2.4×10^{-12}	1.2×10^{-21}
14	1/8192	1.2×10^{-13}	6.1×10^{-22}
15	1/16,384	6.1×10^{-14}	3.1×10^{-22}
16	1/32,768	3.1×10^{-14}	1.5×10^{-22}

^aIn the case that limited qPCR chambers/wells are available for positive controls, one can omit several tubes intermittently, e.g., tube no. 2, 3, 5, 6, 8, 9, 11, 13. More titration points in the lower end will allow one to maximize the interpolation range towards detection limit.

3.2 TaqMan System

3.2.1 Preparation of Reverse Transcription Primer Pool (See **Note 4**)

1. Thaw RT primers (5×) on ice, and vortex gently to ensure the content of the tube is well-mixed. Centrifuge briefly to bring down the content.
2. To prepare a 32-plex primer pool, transfer equal volume of TaqMan RT primer (intended to be in the RT primer pool) into a 1.5 ml tube. If less primer multiplexing is needed, adjust final volume with dilution buffer accordingly so that final concentration of 0.16× is achieved for each RT primer.
3. Pipette the primer pool up and down to mix well and keep at −20 °C until further use.

3.2.2 Reverse Transcription

1. Thaw the components of TaqMan MicroRNA Reverse Transcription kit, TaqMan RT primer pool and RNA samples on ice.
2. Mix each tube gently and centrifuge briefly to bring down the content in the tubes.
3. Prepare RT reaction master mix by combining 0.2 µl dNTPs (100 mM), 0.15 µl RNase inhibitor (20 U/µl), 1 µl Reverse Transcription Buffer (10×), 1 µl Multiscribe Reverse Transcriptase (50 U/µl), and 3 µl TaqMan RT primer pool for each RT reaction. Preparation of RT reaction master mix should include an additional 10% in volume to compensate for pipetting losses.
4. For each sample, aliquot 5.35 µl of RT reaction master mix to a PCR tube, add 4.65 µl of RNA sample (*see Note 5*) to each RT reaction and mix well.
5. Mix all components well and incubate on ice for 5 min.
6. Run thermal cycling with the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and hold at 4 °C indefinitely until RT products are retrieved.
7. Dilute the RT products 1:4 with dilution buffer and proceed to the preamplification step. If preamplification cannot be performed immediately, diluted RT products can be kept at −20 °C until further use.

3.2.3 Preparation of Preamplification Primer Pool (See **Note 4**)

1. Thaw real time primers (20×) on ice and vortex gently to ensure the content of the tube is well-mixed. Centrifuge briefly to bring down the content.
2. Add equal volume of each real time primer (20×) into a 1.5 ml tube.
3. Dilute the preamplification primer pool to a final concentration of 0.2× for each primer with dilution buffer.
4. Pipette the primer pool up and down to mix well.
5. Keep the TaqMan preamplification primer pool at −20 °C until further use.

3.2.4 Preamplification

1. Allow diluted RT products and TaqMan preamplification primer pool to thaw on ice. Mix the content in each tube by vortexing gently and centrifuge briefly to bring down the contents of the tubes.
2. Mix the content in TaqMan PreAmp Master Mix (2×) by flicking the tube and centrifuge briefly to bring down the content.
3. Prepare the preamplification reaction master mix by mixing 5 µl TaqMan PreAmp Master Mix (2×) and 2.5 µl TaqMan preamplification primer pool for each preamplification reaction. Preparation of preamplification reaction master mix should include an additional 10% in volume to compensate for pipetting losses.
4. Pipette the preamplification master mix up and down to mix well.
5. For each sample, aliquot 7.5 µl of preamplification reaction master mix to a PCR tube, add 2.5 µl diluted RT products to each preamplification reaction and mix well.
6. Incubate the reaction on ice for 5 min.
7. Run thermal cycling with the following conditions: denaturation at 95 °C for 10 min, followed by 16 cycles of preamplification at 95 °C for 15 s and 60 °C for 4 min (*see Note 6*).
8. Dilute the preamplified products 1:4 with dilution buffer and proceed with qPCR or store the preamplified products at −20 °C until further use.

3.2.5 Pre-qPCR Preparation

1. Inject control line fluid into both accumulators on the dynamic array chip.
2. Remove the protective film before inserting the chip into IFC controller and run the “Prime” script.

3.2.6 qPCR

1. For each assay mix, add 3 µl TaqMan Assay (20×) and 3 µl assay loading reagent (2×) into a well of a 96-well plate, and label this plate as plate A.
2. In a 1.5 ml tube, prepare master mix of sample pre-mix by combining 3 µl TaqMan Universal Master Mix (2×) and 0.3 µl GE sample loading reagent for each sample. Prepare an excess of 10% volume to account for pipetting losses.
3. For each sample mix, transfer 2.7 µl diluted preamplified products (Subheading 3.2.4) and 3.3 µl sample pre-mix from **step 2** into a well of another 96-well plate, and label this plate as plate B.
4. Pipette 5 µl assay mix from each well of plate A into the individual assay inlet and 5 µl sample mix from each well of plate B into the individual sample inlet on the dynamic array chips (*see Note 7*). If 48.48 dynamic array is used, it is advised to pipette negative control into inlet 22 (*see Note 8*).

5. Load the assay mix and sample mix by running the “Load Mix” script in the IFC controller.
6. Run the qPCR in BioMark by using the default protocol. Choose the protocol according to the qPCR chemistry and dynamic array chip that is used, e.g., Protocol GE 48×48 Standard v1.pcl if probe-based chemistry and 48.48 dynamic array is used.

3.3 miScript System

3.3.1 Reverse Transcription

1. Thaw miScript Reverse Transcriptase Mix, miScript Nucleics Mix (10×), miScript HiSpec Buffer (5×), and RNA samples on ice.
2. Mix the content of the tubes well and centrifuge briefly to bring down the content.
3. Prepare RT reaction master mix by combining 2 µl miScript Nucleics Mix (10×), 4 µl miScript HiSpec Buffer (5×), and 2 µl miScript Reverse Transcriptase Mix for each RT reaction. Preparation of RT reaction master mix should include an additional 10% in volume to compensate for pipetting losses.
4. For each sample, aliquot 8 µl of RT reaction master mix to a PCR tube, add 12 µl RNA (*see Note 9*) to each RT reaction and mix well.
5. Mix all the components well and incubate on ice for 5 min.
6. Run thermal cycling with the following conditions: 37 °C for 60 min, 95 °C for 5 min, and hold at 4 °C indefinitely until RT products are retrieved.
7. Dilute the RT products 1:5 with dilution buffer and proceed to the preamplification step. If preamplification cannot be performed immediately, diluted RT products can be kept at −20 °C until further use.

3.3.2 Preparation of Preamplification Primer Pool

1. Thaw miScript Primer Assay (10×) on ice. Mix the content of the tube gently and centrifuge briefly to bring down the content.
2. Add equal volume of each miScript primer assay into a 1.5 ml tube.
3. Pipette the primer pool up and down to mix well.
4. Dilute the preamplification primer pool to a final concentration of 0.4× for each primer with dilution buffer.
5. Keep the miScript preamplification primer pool at −20 °C until further use.

3.3.3 Preamplification

1. Thaw miScript PreAMP Buffer, HotStarTaqDNA Polymerase, miScript PreAMP Primer Mix, miScript preamplification primers pool, and diluted RT products on ice.
2. Mix the content of the tubes and centrifuge briefly to bring down the contents.

3. Prepare the preamplification reaction master mix by mixing 5 μ l miScript PreAMP Buffer (5 $\times$), 2 μ l HotStar Taq DNA Polymerase, 1 μ l miScript PreAMP Universal Primer, 5 μ l miScript preamplification primer pool, and 7 μ l nuclease free water for each preamplification reaction. Preparation of preamplification reaction master mix should include an additional 10% in volume to compensate for pipetting losses.
4. For each sample, aliquot 20 μ l of preamplification reaction master mix to a PCR tube, add 5 μ l diluted RT products to each preamplification reaction and mix well.
5. Incubate the reaction on ice for 5 min.
6. Run thermal cycling with the following conditions: denaturation at 95 $^{\circ}$ C for 15 min, followed by 12 cycles of preamplification at 94 $^{\circ}$ C for 30 s and 60 $^{\circ}$ C for 3 min (*see Note 6*).
7. Remove excess primers by adding 1 μ l side reaction reducer to each reaction and heat up the reactions at 37 $^{\circ}$ C for 15 min and 95 $^{\circ}$ C for 5 min.
8. Dilute the preamplified products 1:5 with dilution buffer and proceed to qPCR or store the preamplified products at -20° C until further use.

3.3.4 Pre-qPCR Preparation

1. Inject control line fluid into both accumulators on the dynamic array chip.
2. Remove the protective film before inserting the chip into IFC controller and run the “Prime” script.

3.3.5 qPCR

1. For each assay mix, add 1.5 μ l miScript Primer Assay (10 $\times$), 1.5 μ l miScript Universal Primer (10 $\times$), and 3 μ l assay loading reagent (2 $\times$) into a well of a 96-well plate, and label this plate as plate A.
2. In a 1.5 ml tube, prepare sample pre-mix by combining 3 μ l SsoFast EvaGreen Supermix with low ROX and 0.3 μ l DNA Binding Dye Sample Loading Reagent for each sample. Prepare an excess volume of 10% to account for pipetting losses.
3. For each sample mix, transfer 2.7 μ l diluted preamplified products and 3.3 μ l sample pre-mix from **step 2** into a well of another 96-well plate, and label this plate as plate B.
4. Pipette 5 μ l assay mix from each well of plate A into the individual assay inlet and 5 μ l sample mix from each well of plate B into the individual sample inlet on the dynamic array chips (*see Note 7*). If 48.48 dynamic array is used, it is advised to pipette negative control into inlet 22 (*see Note 8*).
5. Load the assay mix and sample mix by running the “Load Mix” script on the IFC controller.

6. Run the qPCR in BioMark by using the default protocol. Choose the protocol according to the qPCR chemistry and dynamic array chip that is used, e.g., Protocol GE 48×48 PCR+Melt v1.pcl if intercalating dye-based chemistry and 48.48 dynamic array is used.

3.4 Data Analyses and Quality Control

3.4.1 Analysis Settings

1. Open the .bml file in Real Time PCR Analysis software (Fluidigm).
2. Complete the assay (detector) setup and sample setup based on the mapping of assays and samples in the 96-well plates. Set sample type for positive controls as “Standard”. Specify the relative concentration of each positive control based on the serial dilution factor performed earlier.
3. Click on “Analysis Views”. For analysis settings, use default quality threshold, “Linear (Derivative)” for baseline correction, “User (Detectors)” for Ct threshold method. Click on “Ct Thresholds” tab and check on “Initialize with Auto”. As the term Ct is used in Fluidigm’s software, Ct is used in place of Cq (RDML data standard) throughout this article for easy reference.
4. In melt curve analysis, set the T_m range for each miScript assay as average T_m ± 2 standard deviations (*see Note 10*).

3.4.2 Quality Control and Data Analyses

1. For intercalating dye-based qPCR system, data point with T_m value out of the set range is considered invalid due to nonspecific amplification and should be omitted from analysis.
2. Evaluate the results of negative controls for each assay. If amplification signals are detected in these negative controls, data points with equal or higher Ct values than these negative controls should be omitted from analysis (*see Note 11*).
3. View the standard curves (serial dilution of positive controls) by clicking on the “+” sign next to “Analysis Views” and then click on “Calibration View”.
4. Check the standard curve of each assay to ensure that there is indication of linear amplification (linear regression slope, $m \approx -3.32$ and goodness of fit, $R^2 > 0.9$) (*see Note 12*).
5. Remove any assay from further analyses if there is no indication of linear and specific amplification (*see Note 13*).
6. For each primer assay which passes QC in **step 5**, identify the minimum and maximum Ct values in the interpolation range from the standard curve (Fig. 2 and *see Note 14*). Any data point of unknown samples which is beyond the assay interpolation range should be set to an arbitrary value for undetermined expression, e.g., Ct = 30.

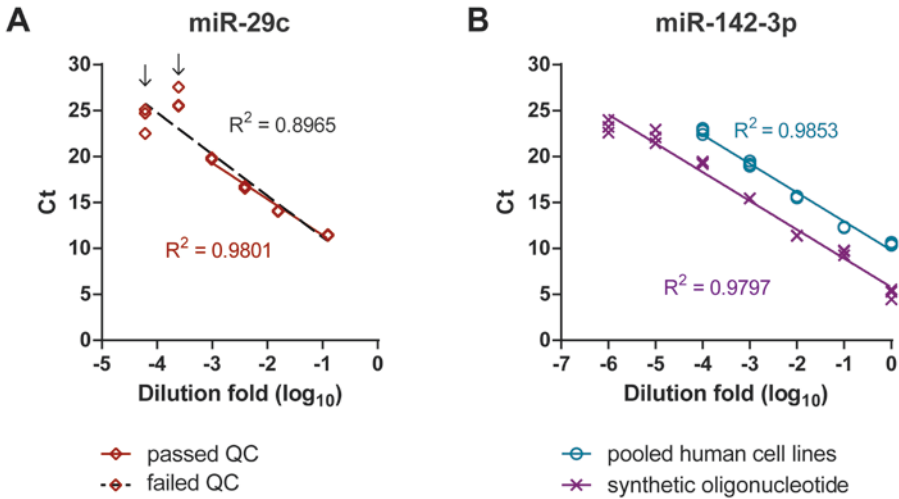


Fig. 2 Linear regression of data points from positive controls. (a) When nonlinear amplification is ignored and an interpolation range of six points from the positive controls are accepted (*dashed line* with $R^2 < 0.9$), fold change between the last two dilution points will be incorrectly calculated based on inaccurate Ct values (as indicated by *arrows*). In this case, for this particular assay miR-29c, the interpolation range for unknown samples should be restricted to the first 4 points of positive controls (*solid line*, $R^2 > 0.9$). (b) Some miRNAs may be less abundant in pooled human cell lines RNA. As a result, the dynamic range of miRNA which can be detected from the serial dilutions of pooled human cell lines RNA is narrower (Ct 10 to 24) than that from the serial dilutions of pooled synthetic oligonucleotides (Ct 5 to 24). Representative graphs shown here are assayed using protocols described in this chapter

7. Technical bias due to extraction can be normalized by using external spike-in controls (*see Note 15*). Normalization can be achieved with these calculations:

Average Ct = average Ct of qPCR replicates

Normalization factor for sample X = median of average Ct_{spike-in} from all samples
– average Ct_{spike-in} of sample X

Normalized Ct_{any assays} for sample X, dCt = average Ct_{any assays} for sample X
+ normalization factor for sample X

8. After data normalization, expression of miRNAs can be presented as fold change to reference sample using the relative quantification formula, 2^{-ddCt} .

4 Notes

1. Positive controls should be used to construct standard curves for each primer assay. This is to ensure that each primer assay can be evaluated for the presence/absence of nonspecific

amplifications as well as over amplification. Positive controls can be created by pooling RNA from different cell lines related to the area of studies. Alternatively, pooled RNA from human cell lines and pooled synthetic oligonucleotides (miRNAs) are commercially available.

2. Typically, a serial dilution needs to show expression with dynamic range of at least five orders of magnitude and the expression levels detected from unknown samples should be within this range. The starting concentration of serial dilution can be as low as 10 ng/ μ l total RNA from pooled human cell lines or 5.0×10^{-16} mol/ μ l of each synthetic oligonucleotide (Table 1). When pooled RNA from human cell lines is used, low abundance transcripts will have a smaller dynamic range (Fig. 2b).
3. Negative controls are needed in all steps of RT-qPCR to rule out cross-contamination. During RT, preamplification and qPCR runs, negative controls should be included in each steps and given specific labels. Negative controls for preamplification (nuclease-free water in preamplification reaction) is especially important, as any positive amplification detected in qPCR from this negative control will indicate nonspecific amplification. Negative control to rule out genomic DNA (no RT enzyme in RT reaction) is not necessary as both TaqMan and miScript systems are not influenced by genomic DNA [3, 4].
4. RT and preamplification primer pools are prepared when custom primer pool is needed. These steps and pools are not required if TaqMan Megaplex primer or the new TaqMan Advanced miRNA Assays are used.
5. Amount of RNA required in the RT reaction is variable. As examples, valid Ct values can be derived from using 4.65 μ l RNA from 25 μ l eluted RNA extracted from 200 μ l plasma/serum [5] or from 10 ng cellular total RNA (data not shown).
6. The recommended amount of preamplification cycle is between 12 and 18 cycles. Preamplification protocols described in this chapter are 16 cycles for TaqMan system and 12 cycles for miScript system. Each primer assay should be evaluated independently during quality control step. Under the preamplification conditions described in this chapter, 1 out of 16 (6.3%) TaqMan assays and 2 out of 16 (12.5%) miScript assays showed nonspecific and/or nonlinear amplification [5].
7. Avoid generating bubbles into the inlets by pressing the plunger of the pipette to the first stop only. Introduction of air bubbles into the inlet will cause part of the reaction chambers to be filled with air instead of qPCR reaction mix. Data acquired from these affected chambers will not be accurate

and can be reflected by negative calls or high CV among qPCR replicates.

8. Reaction from inlet 22 is channeled to the reaction chambers located at the top edge of the IFC where evaporation can occur during the time course of qPCR. All formats of dynamic arrays have hydration inlets around the edges to reduce the effect of sample evaporation around the edge except for the 48.48 dynamic array. When using 48.48 dynamic array, it is recommended to put negative control instead of actual sample in inlet 22.
9. Amount of RNA required in the RT reaction is variable. As an example, valid Ct values can be derived from using 12 μ l RNA from 25 μ l eluted RNA extracted from 200 μ l plasma/serum [5].
10. In qPCR system which utilizes intercalating dye as detection chemistry, melt curve analysis is required for each primer assay in each sample. Any double peaks or unexpected T_m seen in the melt curve analysis is an indication of nonspecific amplification and/or noise from primer-dimer formation. The T_m range is meant to exclude data point with nonspecific amplification from further analysis. If isomirs are the subjects of interest, T_m range can be modified according to the calculation of expected T_m values for all isomirs.
11. When Ct values detected in experimental samples are equal or higher than those detected in negative control samples, one cannot distinguish whether these are real signals or merely noise from false positive.
12. Auto threshold setting may not be optimal for each primer assay. Under auto threshold setting, if linear amplification (linear regression slope between -3.10 and -3.58 and goodness of fit, $R^2 > 0.9$) is not seen in standard curve, one can adjust the threshold line manually in “Ct Thresholds” setting or consider reducing the interpolation range by omitting positive control data points at the end (solid line in Fig. 2a). Data points should not be omitted intermittently. If there is no indication of linear amplification after all these attempts, one needs to omit this primer assay from further analysis. For qPCR studies, calculating fold change between samples is the ultimate goal. If nonlinear amplification is ignored (dash line in Fig. 2a), fold change between samples can be wrongly calculated based on inaccurate Ct values (data points indicated by arrows in Fig. 2a).
13. Primer assays that have been optimized under standard RT-qPCR conditions may not be optimal for RT-preamp-qPCR.
14. In BioMark, low expression is represented by $Ct > 25$. Due to the variation in primer assay efficiencies, the cutoff (minimum and maximum Ct values) for each primer assay has to be based on the linear range of individual standard curve.

15. Commonly used external spike-in controls include synthetic *C. elegans* miRNAs (cel-miR-39 and/or cel-miR-54). During RNA extraction, 500 amol of synthetic oligonucleotide can be spiked-in after the addition of lysis buffer. Exogenous control is advised to be added after sample lysis to avoid degradation by endogenous RNases from plasma/serum.

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