August 2012

miScript® PreAMP Handbook

miScript PreAMP PCR Kit

miScript PreAMP Pathway Primer Mix

miScript PreAMP Pathway HC Primer Mix

miScript PreAMP miRNome Primer Mix

miScript PreAMP Custom Primer Mix

For preamplification of cDNA from samples containing low RNA amounts prior to real-time PCR analysis of microRNA

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

Contents

Kit Contents

miScript PreAMP PCR Kit	**(12)**	**(60)**
Catalog no.	**331451**	**331452**
Number of standard reactions*	**12**	**60**
5x miScript PreAMP Buffer	60 µl	300 µl
HotStarTaq® DNA Polymerase (2 U/µl)	24 µl	120 µl
miScript PreAMP Universal Primer	12 µl	60 µl
miR-16 miScript Primer Assay	300 µl	300 µl
SNORD95 miScript Primer Assay	300 µl	300 µl
miRTC miScript Primer Assay	300 µl	300 µl
C. elegans miR-39 miScript Primer Assay	300 µl	300 µl
RNase-Free Water	1 ml	1 ml
Quick-Start Protocol	1	1

* A standard reaction is 25 µl volume with 1 ng–10 ng cDNA prepared using miScript HiSpec Buffer.

miScript PreAMP Pathway Primer Mix†	**(12)**
Catalog no.	**Varies**
Number of preamplification reactions	**12**
miScript PreAMP Pathway Primer Mix	60 µl

† Each miScript PreAMP Pathway Primer Mix can be used only with the corresponding Pathway-Focused miScript miRNA PCR Array.

miScript PreAMP Pathway HC Primer Mix‡	**(12)**
Catalog no.	**Varies**
Number of preamplification reactions	**12**
miScript PreAMP Pathway HC Primer Mix	60 µl

‡ Each miScript PreAMP Pathway HC Primer Mix can be used only with the corresponding miScript miRNA HC PCR Array.

miScript PreAMP miRNome Primer Mix*	**(12)**
Catalog no.	**Varies**
Number of preamplification reactions	**12**
miScript PreAMP miRNome Primer Mix	60 µl per tube†

* Each miScript PreAMP miRNome Primer Mix can be used only with the corresponding miRNome miScript miRNA PCR Array.

† miScript PreAMP miRNome Primer Mix for a miRNome miScript miRNA PCR Array may be provided in more than one tube. In these cases, separate preamplification reactions must be performed, and the preamplified cDNA pooled prior to real-time PCR, see protocol on page 16.

miScript PreAMP Custom Primer Mix‡	**(80)**
Catalog no.	**Varies**
Number of preamplification reactions	**80**
miScript PreAMP Custom Primer Mix; 4 tubes	100 µl per tube

‡ miScript PreAMP Custom Primer Mixes corresponding to all Custom miScript miRNA PCR Arrays are available.

Shipping and Storage

The miScript PreAMP PCR Kit is shipped on dry ice or cold packs. The kit, including all reagents and buffers, should be stored immediately upon receipt at –20°C in a constant-temperature freezer. If stored under these conditions, the miScript PreAMP PCR Kit is stable for 6 months after receipt.

miScript PreAMP Primer Mixes are shipped frozen or at ambient temperature. They should be stored immediately upon receipt in appropriate aliquots at –20°C in a constant-temperature freezer. Avoid repeated freeze–thaw cycles. If stored under these conditions, miScript PreAMP Primer Mixes can be kept for at least 6 months from the date of receipt without any reduction in performance.

Intended Use

The miScript PreAMP PCR Kit and miScript PreAMP Primer Mixes are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miScript PreAMP PCR Kit and miScript PreAMP Primer Mix is tested against predetermined specifications to ensure consistent product quality.

Introduction

The miScript PCR System consists of the miScript II RT Kit, miScript PreAMP PCR Kit, miScript SYBR® Green PCR Kit, miScript Assays, and miScript miRNA PCR Arrays. The miScript PCR System allows sensitive and specific detection and quantification of microRNA (miRNA). In contrast to other systems, the miScript PCR System enables detection of multiple miRNAs from a single cDNA preparation. The miScript PCR System uses total RNA that contains miRNA as the starting material for cDNA synthesis, and separate enrichment of small RNA is not needed. For more information on miRNA purification, visit www.qiagen.com/miRNA. For general remarks on handling RNA, and information on preparation, quantification, and storage of RNA, see the *miScript miRNA PCR Array Handbook*.

The miScript PreAMP PCR Kit allows researchers to perform miRNA profiling experiments using very limited amounts of starting RNA. This is necessary when working with samples that contain low amounts of RNA, such as body fluids, and formalin-fixed, paraffin-embedded (FFPE) samples, and small cell number samples such as laser capture microdissection (LCM) samples, flow-sorted cells, circulating tumor cells, and fine needle biopsies. The low RNA yields obtained from such samples are often insufficient for reliable miRNA profiling experiments, even when using sensitive techniques such as real-time RT-PCR.

The miScript PreAMP PCR Kit, used with miScript PreAMP Primer Mixes, is a breakthrough technology enabling accurate and comprehensive expression analysis with as little as 10 ng total RNA. The miScript PreAMP PCR Kit uses highly multiplex, PCR-based preamplification of up to 400 miRNA-specific cDNA targets in one reaction. Typically, preamplification results in a 1000–4000-fold amplification in either a 96-plex or 384-plex reaction. miScript PreAMP Primer Mixes are available for every miScript miRNA PCR Array. A single 10 ng cDNA synthesis reaction can be used as template for up to 10 preamplification reactions. This provides sufficient template for multiple Pathway-Focused miScript miRNA PCR Arrays or multiple replicates of a miRNome miScript miRNA PCR Array, depending on the miScript PreAMP Primer Mix used.

Principle and procedure

miRNA profiling from samples containing low RNA amounts requires the following 3 steps: reverse transcription using the miScript II RT Kit, preamplification using the miScript PreAMP PCR Kit and miScript PreAMP Primer Mix, and real-time PCR using the corresponding miScript miRNA PCR Array and the miScript SYBR Green PCR Kit. Control experiments can be performed using either the miScript Primer Assays provided in the miScript PreAMP PCR Kit or the miScript miRNA QC PCR Array.

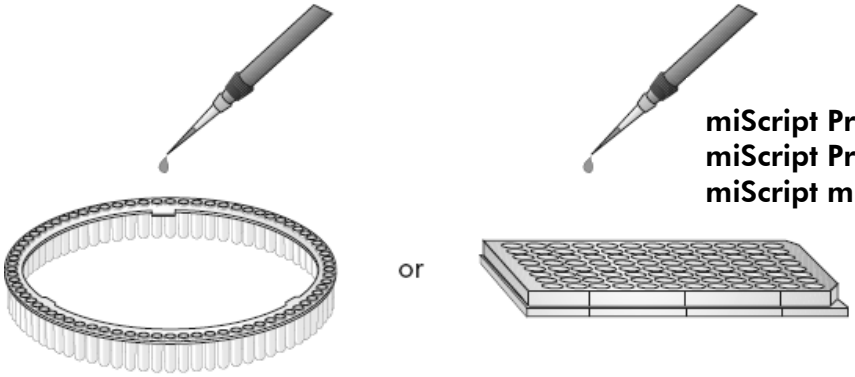
miScript PreAMP workflow

Perform reverse-transcription reaction

miScript II RT Kit with miScript HiSpec Buffer

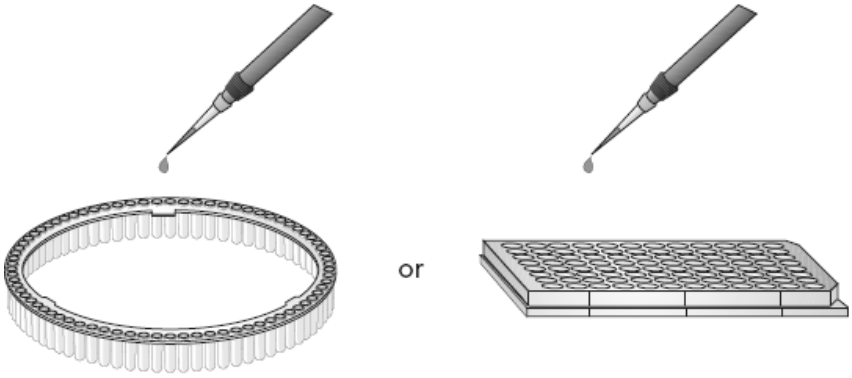
Perform preamplification reaction

Perform control experiments



miScript Primer Assays in miScript PreAMP PCR Kit or miScript miRNA QC PCR Array

Add PCR mix (including preamplified cDNA) to miScript miRNA PCR Array



Perform real-time PCR

Reverse transcription in miScript HiSpec Buffer from the miScript II RT Kit

Two buffers, 5x miScript HiSpec Buffer and 5x miScript HiFlex Buffer, are provided in the miScript II RT Kit. miScript HiSpec Buffer facilitates the selective conversion of mature miRNAs into cDNA. miScript HiSpec Buffer is the only buffer that should be used to prepare cDNA for subsequent preamplification with the miScript PreAMP PCR Kit. **Do not use miScript HiFlex Buffer prior to preamplification with the miScript PreAMP PCR Kit.**

For details of how the miScript II RT Kit works, consult the *miScript miRNA PCR Array Handbook*.

Preamplification

For samples containing low RNA amounts, such as body fluids, FFPE samples, LCM samples, flow-sorted cells, circulating tumor cells, and fine needle biopsies, preamplification using the miScript PreAMP PCR Kit and an appropriate miScript PreAMP Primer Mix enables unbiased amplification of the miRNA targets of interest, ensuring that there is sufficient target for quantification in subsequent real-time PCR. For each miScript miRNA PCR Array, there is a dedicated miScript PreAMP Primer Mix that includes primers to selectively preamplify the miRNAs targeted by the array.

In addition, each miScript PreAMP Primer Mix also includes primers for the miScript Primer Assays recommended as controls, enabling normalization and control experiments using the control wells present on every miScript miRNA PCR Array, the miScript miRNA QC PCR Array, and the miScript PreAMP PCR Kit (see "Preamplification control experiments", below).

Mature miRNA profiling by real-time PCR

Preamplified cDNA serves as the template for real-time PCR analysis using a miScript miRNA PCR Array (containing miRNA-specific forward primers) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect® SYBR Green PCR Master Mix.

For details of how miRNA profiling works, consult the *miScript miRNA PCR Array Handbook*.

Preamplification control experiments

The miScript PreAMP PCR Kit includes 4 miScript Primer Assays for use in preamplification control experiments to test preamplified cDNA (Table 1). These miScript Primer Assays are used in combination with the miScript SYBR Green PCR Kit (see page 24). Preamplification control experiments enable confirmation that there have been no problems with the workflow from sample prep to preamplification, and that the sample is ready for loading onto the

miScript miRNA PCR Array. In addition, preamplification control experiments can be used to determine the optimal dilution factor for preamplified cDNA, which avoids overloading the real-time PCR reaction (leading to reaction inhibition) or over-diluting the preamplified cDNA (leading to loss of sensitivity).

For higher throughput preamplification control experiments, QIAGEN offers the miScript miRNA QC PCR Array (Table 1). This array provides 9 test assays, and can be used to test up to 32 preamplified samples in one PCR run (for more details, see the *miScript miRNA PCR Array Handbook*).

Table 1. Controls included in miScript PreAMP PCR Kit and miScript miRNA QC PCR Array

Control	miScript PreAMP PCR Kit	miScript miRNA QC PCR Array	Purpose
miR-16 miScript Primer Assay	✓	✓	Determination of the optimal dilution factor for preamplified cDNA if the template starting amount is unknown (e.g., for serum, plasma, CSF, and FFPE samples)
miR-21 miScript Primer Assay		✓	
miR-191 miScript Primer Assay		✓	
SNORD95 miScript Primer Assay	✓	✓	Data normalization for tissue and cell samples using the $\Delta\Delta C_T$ method of relative quantification
SNORD61miScript Primer Assay		✓	
SNORD96A miScript Primer Assay		✓	Determination of the optimal dilution factor for preamplified cDNA if the template starting amount is unknown and miR-16 is not present
C. elegans miR-39 miScript Primer Assay	✓	✓	Measurement of miRNeasy Serum/Plasma Spike-In Control (if added during RNA purification) for determination of recovery from serum and plasma samples
miRTC miScript Primer Assay	✓	✓	Assessment of reverse-transcription efficiency
Positive PCR control (PPC)		✓	Assessment of real-time PCR performance

Determination of dilution using the miR-16 miScript Primer Assay or SNORD95 miScript Primer Assay

For some sample types, such as samples containing low RNA amounts, the starting amount of RNA used in reverse transcription is not known. In these cases, we recommend performing a control experiment using either the miR-16 miScript Primer Assay or SNORD95 miScript Primer Assay to determine the correct dilution for the preamplification reaction prior to performing real-time PCR experiments using miScript miRNA PCR Arrays. Perform the control experiment as described in the protocol on page 24. Using the C_T value obtained with the miScript Primer Assay, determine the optimal dilution following the guidelines in Table 9, page 29.

Note: If miR-16 is not present in the sample of interest, the SNORD95 miScript Primer Assay can be used to determine the dilution factor. In this case, it is important to ensure that the sample contains snoRNAs. snoRNAs are present in tissue and cell samples, but are not present in serum, plasma, or other body fluids. Perform the control experiment as described in the protocol on page 24. Using the C_T value obtained with the SNORD95 miScript Primer Assay, determine the optimal dilution using the guidelines in Table 9, page 29.

Normalization using SNORD95 miScript Primer Assay

SNORD95 is conserved in many species and has been verified to have relatively stable expression levels across tissues and cell types. For these reasons, it can serve as a normalization control for relative quantification using the miScript PCR System. cDNA prepared using miScript HiSpec Buffer can be used with the SNORD95 miScript Primer Assay. For more information on normalization controls, see the *miScript PCR System Handbook*.

snoRNAs and long noncoding RNAs are usually not present in serum, plasma, urine, and other body fluids. Therefore, they cannot be used for normalization in these sample types. In these cases, the miRNeasy Serum/Plasma Spike-In Control (ordered separately; cat. no. 219610) together with the *C. elegans* miR-39 miScript Primer Assay or a plate mean C_T of expressed assays can be used for normalization.

Determination of recovery from serum/plasma and an internal control experiment using *C. elegans* miR-39 miScript Primer Assay

QIAGEN recommends the miRNeasy Serum/Plasma Spike-In Control (ordered separately; cat. no. 219610) for use as an internal control for miRNA expression profiling in serum or plasma. The miRNeasy Serum/Plasma Spike-In Control is a *C. elegans* miR-39 miRNA mimic that can be spiked in to the sample during RNA purification and easily detected after purification using the *C. elegans* miR-39 miScript Primer Assay (provided in the miScript PreAMP PCR Kit). These results can then be used for normalization of real-time RT-PCR

results for endogenous miRNAs in the sample. For more information, see the *miRNeasy Serum/Plasma Handbook*.

Assessment of reverse transcription using miRTC miScript Primer Assay

The miRTC miScript Primer Assay assesses the performance of a reverse-transcription reaction performed using the miScript II RT Kit by detecting template synthesized from the kit's built-in control RNA. This control monitors for any variables that may inhibit the reverse-transcription reaction. The expected C_T value for the miRTC miScript Primer Assay, if preamplified cDNA was diluted 20 fold, is 17 ± 3.

If using the miScript miRNA QC PCR Array, C_T values of the reverse transcription control (miRTC) are examined using the values for the positive PCR control (PPC), see step 7 in the protocol on page 33.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For reverse transcription using the miScript II RT Kit and preamplification using the miScript PreAMP PCR Kit

- Thin-walled, DNase-free, RNase-free PCR tubes (for 20 µl and 25 µl reactions)
- Ice
- Thermal cycler
- Microcentrifuge

For quantitative, real-time PCR using miScript miRNA PCR Arrays

- Real-time PCR cycler
- Multichannel pipettor
- Nuclease-free pipet tips and tubes

Protocol: Reverse Transcription Using the miScript II RT Kit Prior to Preamplification

This protocol for reverse transcription using the miScript II RT Kit must be performed prior to preamplification with the miScript PreAMP PCR Kit and subsequent real-time PCR with miScript miRNA PCR Arrays.

Important points before starting

- The miScript II RT Kit includes two 5x buffers: 5x miScript HiSpec Buffer and 5x miScript HiFlex Buffer. Only 5x miScript HiSpec Buffer should be used for reverse transcription prior to preamplification using the miScript PreAMP PCR Kit. **Do not use 5x miScript HiFlex Buffer with the miScript PreAMP PCR Kit**.
- Total RNA containing miRNA should be used as starting material for reverse-transcription reactions. For RNA purification recommendations, visit www.qiagen.com/miRNA. This protocol is for use with 10 ng–100 ng RNA in a 10 µl reaction. Recommended RNA amounts should be used to ensure optimal performance. If the RNA sample concentration is not known, we recommend using 5 µl RNA prep in a 10 µl reaction.
- **IMPORTANT**: In this protocol, reverse transcription is performed in a 10 µl reaction volume to maximize the RNA concentration in the reaction. We highly recommend performing a 10 µl reverse-transcription reaction prior to preamplification. If a reverse-transcription reaction has already been performed using a 20 µl reaction volume, as described in the *miScript PCR System Handbook* or the *miScript miRNA PCR Array Handbook*, and it is not practical to repeat using a 10 µl reaction volume, then this existing reverse-transcription reaction can be used for preamplification. Dilute the 20 µl reaction in 80 µl RNase-free water and continue with the protocol on page 16. It is important not to change this 1:5 dilution factor.
- Set up all reactions on ice to minimize the risk of RNA degradation.
- Do not vortex template RNA or any of the components of the miScript II RT Kit.

Procedure

1. **Thaw template RNA on ice. Thaw 10x miScript Nucleics Mix, RNase-free water, and 5x miScript HiSpec Buffer at room temperature (15–25°C).**

   Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes and then store on ice.

2. **Prepare the reverse-transcription master mix on ice according to Table 2.**

Gently mix and then store on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

Note: miScript Reverse Transcriptase Mix should be removed from the –20°C freezer just before preparation of the master mix, gently mixed, and placed on ice. It should be returned to the freezer immediately after use.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

Table 2. Reverse-transcription reaction components

Component	Volume/reaction
5x miScript HiSpec Buffer	2 µl
10x miScript Nucleics Mix	1 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	1 µl
Template RNA (added in step 3)	Variable*†
Total volume	**10 µl**

* 10 ng–100 ng total RNA can be used in the reaction. This recommended range of RNA amount should be used to ensure optimal performance.

† If using RNA from serum or plasma samples prepared with the miRNeasy Serum/Plasma Kit, we recommend using 1.5 µl RNA preparation as a starting point. If using RNA from serum or plasma samples prepared with the *QIAGEN Supplementary Protocol: Purification of total RNA, including small RNAs, from serum or plasma using the miRNeasy Mini Kit*, we recommend using 5 µl RNA preparation as a starting point.

3. **Add template RNA to each tube containing reverse-transcription master mix. Gently mix, briefly centrifuge, and then store on ice.**
4. **Incubate for 60 min at 37°C.**
5. **Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place on ice.**
6. **If you wish to proceed with preamplification immediately, dilute the 10 µl cDNA in 40 µl RNase-free water.**

   If you wish to store the reverse-transcription reactions prior to preamplification, transfer the undiluted cDNA to a –20°C freezer, or dispense the diluted cDNA into convenient aliquots and transfer them to a –20°C freezer.

Protocol: Preamplification of cDNA Target Templates

This protocol is for preamplification of cDNA generated using the miScript II RT Kit and miScript HiSpec Buffer. The protocol uses the miScript PreAMP PCR Kit and miScript PreAMP Primer Mix. It should be performed prior to miRNA quantification using miScript miRNA PCR Arrays.

If using multiple miScript Primer Assays for preamplification instead of a miScript PreAMP Primer Mix, refer to the Appendix on page 40.

Important points before starting

- **IMPORTANT**: Only cDNA prepared using the miScript II RT Kit with miScript HiSpec Buffer should be used as starting material for this protocol.
- **IMPORTANT**: Use exactly 5 µl diluted cDNA from step 6, page 15 for preamplification as indicated in the protocol. Using any more or less than 5 µl will adversely affect the performance of the preamplification reaction and is not recommended. (If a reverse-transcription reaction has already been performed using a 20 µl reaction volume, as described in the *miScript PCR System Handbook* or the *miScript miRNA PCR Array Handbook*, dilute the 20 µl reaction in 80 µl RNase-free water and use exactly 5 µl diluted cDNA for preamplification.)
- **IMPORTANT**: miScript PreAMP miRNome Primer Mixes may be provided in multiple tubes. In these cases, it is necessary to set up separate preamplification reactions and pool once the reactions are completed. For example, if 3 tubes of miScript PreAMP miRNome Primer Mix are provided, set up 3 preamplification reactions — one for each tube. After the real-time cycler run, pool the 3 reactions into one tube, dilute as described in protocol step 5, and continue with miRNA profiling as described in the protocol "Real-Time PCR for Mature miRNA Expression Profiling" in the *miScript miRNA PCR Array Handbook*.
- The preamplification must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase.

Procedure

1. **Prepare the preamplification master mix at room temperature (15–25°C) according to Table 3.**

   Gently mix and then store on ice.

   Note: Remove 5x miScript PreAMP Buffer and HotStarTaq DNA Polymerase from the –20°C freezer just before preparation of the reaction. Gently mix and place on ice. They should be returned to the freezer immediately after use.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

Table 3. Preamplification reaction components

Component	Volume/reaction
5x miScript PreAMP Buffer	5 µl
HotStarTaq DNA Polymerase	2 µl
miScript PreAMP Primer Mix*	5 µl
RNase-free water	7 µl
miScript PreAMP Universal Primer	1 µl
Diluted template cDNA (added in step 2)	5 µl
Total volume	**25 µl**

* For some miRNome miScript miRNA PCR Arrays, more than one tube of corresponding miScript PreAMP miRNome Primer Mix is provided. Set up separate preamplification reactions for each tube and pool preamplified cDNA prior to use in real-time PCR.

2. **Add diluted template cDNA to each tube containing preamplification master mix. Gently mix, briefly centrifuge, and then store on ice.**
3. **Program the thermal cycler according to either Table 4 (for a 96-plex preamplification reaction) or Table 5 (for a 384-plex preamplification reaction).**

   A 96-plex preamplification reaction amplifies 96 cDNA targets and is used with a miScript PreAMP Pathway Primer Mix or with a miScript PreAMP Custom Primer Mix (up to 96 assays).

   A 384-plex preamplification reaction amplifies 384 cDNA targets and is used with a miScript PreAMP miRNome Primer Mix, miScript PreAMP Pathway HC Primer Mix, or miScript PreAMP Custom Primer Mix (96–384 assays).

Table 4. Cycling conditions for 96-plex preamplification

Step	Time	Temperature
PCR **Initial activation step** HotStarTaq DNA Polymerase is activated by this heating step.	**15 min**	**95°C**
2-step cycling:		
Denaturation	30 s	94°C
Annealing/extension	3 min	60°C
Cycle number	12 cycles	

Table 5. Cycling conditions for 384-plex preamplification

Step	Time	Temperature
PCR **Initial activation step** HotStarTaq DNA Polymerase is activated by this heating step.	**15 min**	**95°C**
3-step cycling:		
Denaturation	30 s	94°C
Annealing	60 s	55°C
Extension	60 s	70°C
Cycle number	2 cycles	
2-step cycling:		
Denaturation	30 s	94°C
Annealing/extension	3 min	60°C
Cycle number	10 cycles	

4. **Place the preamplification reaction in the thermal cycler and start the run.**

5. **After the run has finished, dilute the preamplified cDNA in RNase-free water according to the appropriate formula below. Gently mix and then store on ice.**

   Dilution factor for Pathway-Focused Arrays and Custom Arrays (up to 96 assays) = ng input cDNA x 20-fold/ng

   Dilution factor for miRNome Arrays, Custom Arrays (96–384 assays), and HC Arrays = ng input cDNA x 5-fold/ng

   Example of dilution for Pathway-Focused Array: If 10 ng total RNA was used in the 10 µl reverse-transcription reaction that was subsequently diluted in 40 µl RNase free water, and 5 µl diluted cDNA was used in the preamplification reaction, this results in 1 ng cDNA used as starting material for the preamplification reaction. According to the formula:

   Dilution factor = 1 ng input cDNA x 20-fold/ng = 20-fold

   For a 20-fold dilution, dilute the 25 µl preamplification reaction in 475 µl RNase free water.

   IMPORTANT: The minimum dilution factor for preamplified cDNA is 20-fold for Pathway-Focused Arrays and Custom Arrays (up to 96 assays) and 5-fold for miRNome Arrays, Custom Arrays (96–384 assays), and HC Arrays. Therefore if less than 1 ng input cDNA was used for preamplification, we recommend performing a 20-fold or 5-fold dilution depending on the array type.

   Note: If the input cDNA amount is not known, we recommend performing a 20-fold dilution (for Pathway-Focused Arrays and Custom Arrays [up to 96 assays]) or 5-fold dilution (for miRNome Arrays, Custom Arrays [96–384 assays], and HC Arrays), and then using the miR-16 miScript Primer Assay or SNORD95 miScript Primer Assay (see protocol, page 24) or the miScript miRNA QC PCR Array (see protocol, page 30) to determine the optimal dilution factor.

   Note: The real-time PCR reaction mix composition compensates for the differing dilutions (20-fold or 5-fold), ensuring the same amount of template preamplified cDNA in real-time PCR miRNA quantification.

   IMPORTANT: For some miScript PreAMP miRNome Primer Mixes provided in multiple tubes, separate preamplification reactions were performed. Pool the preamplification reactions prior to dilution.

6. **Proceed with real-time PCR as described in the protocol "Real-Time PCR for Mature miRNA Expression Profiling" in the *miScript miRNA PCR Array Handbook*.**

Protocol: Data Analysis for miScript miRNA PCR Arrays Used with Preamplified cDNA

This protocol describes the steps for analysis of data from miScript miRNA PCR Arrays used with cDNA preamplified with the miScript PreAMP PCR Kit. The first steps should be performed by the user. The latter steps are performed by the free data analysis software. The data analysis software is available at http://pcrdataanalysis.sabiosciences.com/mirna. Either the miScript miRNA PCR Array Web-based software or the miScript miRNA PCR Array Data Analysis Excel® Template can be accessed. Both tools automatically perform quantification using the $\Delta\Delta C_T$ method of relative quantification and interpretation of the control wells. Results are presented in a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).

Important point before starting

■ Text marked with a ■ denotes instructions for 96-well and 384-well plates (Array formats A, C, D, E, F, and G); text marked with a ▲ denotes instructions for 100-well Rotor-Discs (Array format R).

Procedure

Steps performed by the user

1. **Define the baseline.**

   The baseline is the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products.

   ■ Use the "Linear View" of the amplification plot to determine the earliest visible amplification. Set the baseline from cycle 2 to 2 cycles before the earliest visible amplification. Do not use greater than cycle 15. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used. For more information regarding real-time PCR data output, refer to the *miScript miRNA PCR Array Handbook*.

   ▲ For the Rotor-Gene® Q, we recommend using the "Dynamic Tube" setting along with the "Slope Correct" and/or "Ignore First" settings. For more information, refer to the *Rotor-Gene Q User Manual*.

   Note: Ensure that baseline settings are the same across all PCR runs associated with the same experiment to allow comparison of results.

2. **Define the threshold.**

   The threshold should be set using a logarithmic amplification plot so that the log-linear range of the curve can be easily identified. Using the "Log

View" of the amplification plot, place the threshold above the background signal but within the lower half of the log-linear range of the amplification plot. The threshold should never be set in the plateau phase. The absolute position of the threshold is less critical than its consistent position across PCR runs.

■ Various PCR instruments (such as Applied Biosystems® models 7500 and ViiA 7, and Stratagene® models Mx3005P® and Mx3000P®) may require adjustment of the default "Manual C_T" threshold value of 0.2 to a lower value in order to analyze the data properly. Use a value of 0.02 as a starting point.

▲ For the Rotor-Gene Q, we recommend a C_T threshold value of approximately 0.02 in order to analyze the data properly.

Note: Ensure that threshold settings are the same across all PCR runs in the same analysis to allow comparison of results.

3. **Export C_T values according to the manual supplied with the real-time PCR instrument.**
4. **Access the free data analysis tools at http://pcrdataanalysis.sabiosciences.com/mirna.**

   Choose either the Web-based software or the Excel template and follow the instructions provided.

Steps performed by data analysis software

5. **All C_T values reported ■ as greater than 30 or as N/A (not detected) are changed to 30 or ▲ as greater than 28 or as N/A (not detected) are changed to 28.**

   At this point, any C_T value equal to ■ 30 or ▲ 28 is considered a negative call.

   Note: The cutoff C_T values are lower than those used for nonpreamplified cDNA. This decrease has been experimentally determined and is due to the additional 12 PCR cycles performed during preamplification. This has no effect on sensitivity. We recommend carefully checking C_T values of 27–30 using dissociation curve analysis and/or repeat experiments to ensure that they are related to expression of the miRNA of interest.

6. **C_T values of the positive PCR control wells (PPC) are examined.**

   If the RNA sample is of high quality, the cycling program has been correctly run, and the thresholds have been correctly defined, the value of C_T^{PPC} should be ■ 19 ± 2 or ▲ 15 ± 2 across all arrays or samples.

7. **C_T values of the reverse transcription control (miRTC) are examined using the values for the positive PCR control (PPC) by calculating ΔC_T = AVG C_T^{miRTC} – AVG C_T^{PPC}.**

If this value is less than the value indicated in Table 6, then no inhibition of the reverse-transcription reaction is apparent. No action is needed. If this value is greater than the value indicated in Table 6, there is evidence of impurities that may have inhibited the reverse-transcription reaction. See the "Troubleshooting Guide" page 36.

Table 6. Expected values for ΔC_T = AVG C_T^{miRTC} – AVG C_T^{PPC}

Dilution after preamplification reaction (step 5, page 19)	Expected ΔC_T
5-fold	<0
20-fold	<2
40-fold	<3
100-fold	<4
200-fold	<5
1000-fold	<5

Steps performed by data analysis software for miScript miRNA PCR Arrays

1. **ΔC_T value for each mature miRNA profiled in the plate is calculated using the formula $\Delta C_T = C_T^{miRNA} – AVG\ C_T^{SN1/2/3/4/5/6}$.**

   Note: Choose an appropriate snoRNA/snRNA control for normalization. Six snoRNA/snRNA controls (SN1–6) are included on each array. Make sure that the selected controls are not influenced by the experimental conditions. If one or more snoRNA/snRNA have been previously independently identified and if the miScript miRNA PCR Array reproduces those results, use the average of their C_T values in the equation above. If an appropriate snoRNA/snRNA has not been previously identified, use the average C_T value of all the snoRNA/snRNA. When biological and/or technical replicates are performed, calculate the average ΔC_T value of each snoRNA/snRNA (each well) across those replicate arrays for each treatment group.

   Note: If the miRNeasy Serum/Plasma Spike-In Control was added to the sample during RNA purification, the *C. elegans* miR-39 miScript Primer Assay wells can be selected as control genes in the basic setup of the miScript miRNA PCR Array Data Analysis software. The *C. elegans* miR-39 miScript Primer Assay can be used along with, or instead of, the snoRNA/snRNA miScript Primer Assays (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2 miScript Primer Assays) to normalize array data. For serum or plasma samples, the snoRNA/snRNA miScript

Primer Assays do not exhibit robust expression and therefore should not be selected as control genes.

2. **$\Delta\Delta C_T$ for each miRNA across 2 miScript miRNA PCR Arrays or 2 samples is calculated using the formula:**
   $\Delta\Delta C_T = \Delta C_T$ (sample 2) – ΔC_T (sample 1) where sample 1 is the control sample and sample 2 is the experimental sample.
3. **Fold-change for each gene from sample 1 to sample 2 is calculated as $2^{(-\Delta\Delta CT)}$.**

   Optional: If the fold-change is greater than 1, the result may be reported as a fold-upregulation. If the fold-change is less than 1, the negative inverse of the result may be reported as a fold-downregulation.
4. **Fold-changes are presented by the data analysis tool in a variety of formats, including a tabular format, a scatter plot, a three-dimensional profile, and a volcano plot (when replicates are included).**

Protocol: Real-Time PCR for Preamplification Control Experiments

Four miScript Primer Assays are provided in the miScript PreAMP PCR Kit for control experiments: miR-16 miScript Primer Assay, SNORD95 miScript Primer Assay, miRTC miScript Primer Assay, and *C. elegans* miR-39 miScript Primer Assay. The purpose of each control assay is detailed on page 9.

This protocol describes control experiments using these miScript Primer Assays, together with the miScript SYBR Green PCR Kit, and preamplified cDNA generated using the protocol on page 16.

For quality control of preamplified cDNA using the miScript miRNA QC PCR Array, see the protocol "Real-Time PCR for Quality Control and Determination of Preamplified cDNA Dilution Using the miScript miRNA QC PCR Array" on page 30.

Important points before starting

- If performing an experiment with multiple samples of the same type (e.g., multiple 100 µl serum samples), it is not necessary to perform control experiments for each individual sample. We recommend performing control experiments for a small number of representative samples.
- cDNA prepared using the miScript II RT Kit with miScript HiSpec Buffer and preamplified using the miScript PreAMP PCR Kit is the appropriate starting material for this protocol.
- Ensure that preamplified cDNA has been diluted appropriately (see protocol step 5, page 19).
  For Pathway-Focused Arrays: minimum dilution 20-fold
  For Custom Arrays (up to 96 assays): minimum dilution 20-fold
  For HC Arrays: minimum dilution 5-fold
  For miRNome Arrays: pool preamplification reactions, minimum dilution 5-fold
  For Custom Arrays (96–384 assays): minimum dilution 5-fold
- The PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green PCR Master Mix).
- Do not vortex template cDNA or the components of the miScript SYBR Green PCR Kit.
- If using the iCycler iQ®, iQ5, or MyiQ™, well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or *Technical Information: Using QuantiTect SYBR Green Kits on Bio-Rad® cyclers* available at www.qiagen.com.

Procedure

1. **Thaw 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, template cDNA, and RNase-free water at room temperature (15–25°C). Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 7 for either a 10 µl per well reaction volume (used in 384-well plates), a 25 µl per well reaction volume (used in 96-well plates), or a 20 µl per well reaction volume (used in the Rotor-Disc® 100). Mix gently and thoroughly.**

   Use the same plate format for the control reactions as will be used for miRNA profiling by real-time PCR.

   Reaction mix contains everything except the template cDNA. Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 7. Reaction setup for real-time PCR

Component	Volume/reaction (384-well)	Volume/reaction (96-well)	Volume/reaction (Rotor-Disc 100)
2x QuantiTect SYBR Green PCR Master Mix*	5 µl	12.5 µl	10 µl
10x miScript Universal Primer (provided in the miScript SYBR Green PCR Kit)	1 µl	2.5 µl	2 µl
10x miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	1 µl	5.5 µl	4 µl
Template cDNA (added at step 4)	2 µl	2 µl	2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration provided by 2x QuantiTect SYBR Green PCR Master Mix gives optimal results.

3. **Perform a further dilution of diluted preamplified cDNA from step 5, page 19.**
   For cDNA to be used with Pathway-Focused Arrays and Custom Arrays (up to 96 assays), dilute 3 µl in 24 µl RNase-free water.
   For cDNA to be used with miRNome Arrays, HC Arrays, and Custom Arrays (384–96 assays), dilute 4 µl in 92 µl RNase-free water.
   This additional dilution provides sufficient cDNA to test with all 4 controls in triplicate reactions. This dilution is only used for control experiments.
4. **Dispense 2 µl diluted cDNA into the individual plate/Rotor-Disc wells**
5. **Mix the reaction mix thoroughly but gently, and dispense appropriate volumes into the plate/Rotor-Disc wells containing template cDNA.**
6. **Carefully, tightly seal the plate or disc with caps, film, or Rotor-Disc Heat-Sealing Film.**
7. **Centrifuge for 1 min at 1000 x *g* at room temperature (15–25°C) to remove bubbles.**
   Note: This step is not necessary for reactions set up in Rotor-Discs.
8. **Program the real-time cycler according to Table 8.**

Table 8. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension§	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles¶		

* For Bio-Rad models CFX96™ and CFX384™: adjust the ramp rate to 1°C/s.

† For Eppendorf® Mastercycler® ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche® LightCycler® 480, adjust the ramp rate to 1°C/s.

§ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM® 7000 or 34 s with the Applied Biosystems 7300 and 7500.

¶ If using a Roche LightCycler 480, use 45 cycles.

9. **Place the plate/Rotor-Disc in the real-time cycler and start the cycling program.**

   Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

10. **When the run is finished, analyze the data. First, define the baseline.**

    The baseline is the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products.

    Use the "Linear View" of the amplification plot to determine the earliest visible amplification. Set the baseline from cycle 2 to 2 cycles before the earliest visible amplification. Do not use greater than cycle 10. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used.

    Note: Ensure that baseline settings are the same across all PCR runs associated with the same experiment to allow comparison of results.

11. Define the threshold.

The threshold should be set using a logarithmic amplification plot so that the log-linear range of the curve can be easily identified. Using the "Log View" of the amplification plot, place the threshold above the background signal but within the lower half of the log-linear range of the amplification plot. The threshold should never be set in the plateau phase. The absolute position of the threshold is less critical than its consistent position across PCR runs.

Note: Ensure that threshold settings are the same across all PCR runs in the same analysis to allow comparison of results.

12. Export the C_T values according to the manual supplied with the real-time PCR cycler.

13. Examine the C_T values for the preamplification controls.

The expected C_T value for the miRTC miScript Primer Assay, if preamplified cDNA was diluted 20 fold, is 17 ± 3.

14. If desired, use results from the miR-16 miScript Primer Assay or SNORD95 miScript Primer Assay to determine the dilution factor of the preamplification reaction for use with miScript miRNA PCR Arrays.

Recommendations for the dilution to use depending on the C_T value achieved are shown in Table 9.

Table 9. Recommended dilution factor for miRNA quantification

C_T miR-16 /SNORD95 miScript Primer Assay	Dilution of preamplified cDNA
>28	The sample does not contain RNA, or the sample does not contain small RNA, or the RNA concentration of the sample is very low. Use more RNA sample if possible. Check that the RNA purification protocol was performed correctly and repeat if necessary.
>25	The RNA concentration of the sample is low, and low abundant miRNAs may not be detected. Check that the RNA purification protocol was performed correctly and repeat if necessary.
>10	No further dilution needed
9–10	Dilute 25-fold (1:25)
7–8	Dilute 50-fold (1:50)
5–6	Dilute 100-fold (1:100)

Protocol: Real-Time PCR for Quality Control and Determination of Preamplified cDNA Dilution Using the miScript miRNA QC PCR Array

This protocol describes quality control of preamplified cDNA samples and determination of the appropriate dilution for real-time PCR using the miScript SYBR Green PCR Kit and miScript miRNA QC PCR Array prior to miRNA profiling using a miScript miRNA PCR Array. In total, 32 cDNA samples can be analyzed on one 384-well miScript miRNA QC PCR Array, 8 cDNA samples can be analyzed on one 96-well miScript miRNA QC PCR Array, and 8 cDNA samples can be analyzed on one Rotor-Disc 100 miScript miRNA QC PCR Array.

Important points before starting

- If performing an experiment with multiple samples of the same type (e.g., multiple 100 µl serum samples), it is not necessary to perform control experiments for each individual sample. We recommend performing control experiments for a small number of representative samples.
- cDNA prepared using the miScript II RT Kit with miScript HiSpec Buffer and preamplified using the miScript PreAMP PCR Kit is the appropriate starting material for this protocol.
- Ensure that preamplified cDNA has been diluted appropriately (see protocol step 5, page 19).
  For Pathway-Focused Arrays: minimum dilution 20-fold
  For Custom Arrays (up to 96 assays): minimum dilution 20-fold
  For HC Arrays: minimum dilution 5-fold
  For miRNome Arrays: pool preamplification reactions, minimum dilution 5-fold
  For Custom Arrays (96–384 assays): minimum dilution 5-fold
- The PCR must start with an **initial incubation step of 15 minutes at 95°C** to activate HotStarTaq DNA Polymerase (included in 2x QuantiTect SYBR Green PCR Master Mix).
- Do not vortex template cDNA or the components of the miScript SYBR Green PCR Kit.
- If using the iCycler iQ, iQ5, or MyiQ, well factors must be collected at the beginning of each experiment. Well factors are used to compensate for any system or pipetting nonuniformity. For details, refer to the user manual supplied with the instrument or *Technical Information: Using QuantiTect SYBR Green Kits on Bio-Rad cyclers* available at www.qiagen.com.

Procedure

1. **Thaw 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, template cDNA, and RNase-free water at room temperature (15–25°C). Mix the individual solutions.**
2. **Prepare a reaction mix according to Table 10 for either a 10 µl per well reaction volume (used in 384-well plates), a 25 µl per well reaction volume (used in 96-well plates), or a 20 µl per well reaction volume (used in the Rotor-Disc 100). Mix gently and thoroughly.**

   Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.

Table 10. Reaction mix for miScript miRNA QC PCR Arrays

Array format: Component	384-well Formats E, G*	96-well Formats A, C, D, F*	Rotor-Disc 100 Format R*
2x QuantiTect SYBR Green PCR Master Mix†	75 µl	175 µl	150 µl
10x miScript Universal Primer	15 µl	35 µl	30 µl
RNase-free water	45 µl	125 µl	105 µl
Template cDNA	15 µl	15 µl	15 µl
Total volume	**150 µl**	**350 µl**	**300 µl**

* Volumes shown are sufficient for one cDNA template. In total, 8 cDNA samples can be analyzed on one 96-well miScript miRNA QC PCR Array, 8 cDNA samples can be analyzed on one Rotor-Disc 100 miScript miRNA QC PCR Array, and 32 cDNA samples can be analyzed on one 384-well miScript miRNA QC PCR Array.

† No optimization of the Mg^{2+} concentration is required. The final Mg^{2+} concentration provided by 2x QuantiTect SYBR Green PCR Master Mix gives optimal results.

3. **Carefully remove the miScript miRNA QC PCR Array from its sealed bag.**
4. **Add reaction mix to the wells of the miScript miRNA QC PCR Array as follows.**

   For 384-well miScript miRNA QC PCR Array: add 10 µl per well.
   For 96-well miScript miRNA QC PCR Array: add 25 µl per well.
   For Rotor-Disc 100 miScript miRNA QC PCR Array: add 20 µl per well.

5. **Carefully, tightly seal the miScript miRNA PCR Array with Optical Thin-Wall 8-Cap Strips (Formats A and D), Optical Adhesive Film (Formats C, E, F, and G), or Rotor-Disc Heat-Sealing Film (Format R).**
6. **Centrifuge the PCR plate for 1 min at 1000 x *g* at room temperature (15–25°C) to remove bubbles.**

   Note: This step is not necessary for reactions set up in Rotor-Discs.
7. **Program the real-time cycler according to Table 11.**

   Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 11. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension§	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles¶		

* For Bio-Rad models CFX96 and CFX384: adjust the ramp rate to 1°C/s.

† For Eppendorf Mastercycler ep *realplex* models 2, 2S, 4, and 4S: for the Silver Thermoblock, adjust the ramp rate to 26%; for the Aluminum Thermoblock, adjust the ramp rate to 35%.

‡ If using a Roche LightCycler 480, adjust the ramp rate to 1°C/s.

§ Due to software requirements, the fluorescence detection step must be at least 30 s with the ABI PRISM 7000 or 34 s with the Applied Biosystems 7300 and 7500.

¶ If using a Roche LightCycler 480, use 45 cycles.

8. **Place the plate/Rotor-Disc in the real-time cycler and start the cycling program.**
9. **Perform data analysis.**

   For more details, see "Data Analysis for miScript miRNA QC PCR Arrays Used with Preamplified cDNA", page 33.

Protocol: Data Analysis for miScript miRNA QC PCR Arrays Used with Preamplified cDNA

This protocol describes the steps for analysis of data from miScript miRNA QC PCR Arrays used with cDNA preamplified with the miScript PreAMP PCR Kit. The first steps should be performed by the user. The later steps are performed by the free data analysis Excel template. A free miScript miRNA PCR Array Data Analysis Excel Template specific for the miScript miRNA QC PCR Array is available at http://pcrdataanalysis.sabiosciences.com/mirna.

Important point before starting

■ Text marked with a ■ denotes instructions for 96-well and 384-well plates (formats A, C, D, E, F, and G); text marked with a ▲ denotes instructions for 100-well Rotor-Discs (format R).

Procedure

Steps performed by the user

1. Define the baseline.

The baseline is the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products.

■ Use the "Linear View" of the amplification plot to determine the earliest visible amplification. Set the baseline from cycle 2 to 2 cycles before the earliest visible amplification. Do not use greater than cycle 15. The number of cycles used to calculate the baseline can be changed and should be reduced if high template amounts are used. For more information on real-time PCR data output, refer to the *miScript miRNA PCR Array Handbook*.

▲ For the Rotor-Gene Q, we recommend using the "Dynamic Tube" setting along with the "Slope Correct" and/or "Ignore First" settings. For more information, refer to the *Rotor-Gene Q User Manual*.

Note: Ensure that baseline settings are the same across all PCR runs in the same analysis to allow comparison of results.

2. Define the threshold.

The threshold should be set using a logarithmic amplification plot so that the log-linear range of the curve can be easily identified. Using the "Log View" of the amplification plot, place the threshold above the background signal but within the lower half of the log-linear range of the amplification plot. The threshold should never be set in the plateau phase. The absolute position of the threshold is less critical than its consistent position across PCR runs.

■ Various PCR instruments (such as Applied Biosystems models 7500 and ViiA 7, and Stratagene models Mx3005P and Mx3000P) may require

adjustment of the default "Manual C_T" threshold value of 0.2 to a lower value in order to analyze the data properly. Use a value of 0.02 as a starting point.

▲ For the Rotor-Gene Q, we recommend a C_T threshold value of approximately 0.02 in order to analyze the data properly.

Note: Ensure that threshold settings are the same across all PCR runs in the same analysis to allow comparison of results.

3. **Export C_T values according to the manual supplied with the real-time PCR cycler.**
4. **Access the free data analysis tools at http://pcrdataanalysis.sabiosciences.com/mirna.**

   Choose the Excel template and follow the instructions provided.

Steps performed by Excel template

5. **All C_T values reported ■ as greater than 30 or as N/A (not detected) are changed to 30 or ▲ as greater than 28 or as N/A (not detected) are changed to 28.**

   At this point, any C_T value equal to ■ 30 or ▲ 28 is considered a negative call.

   Note: The cutoff C_T values are lower than those used for nonpreamplified cDNA. This decrease has been experimentally determined and is due to the additional 12 PCR cycles performed during preamplification. This has no effect on sensitivity. We recommend carefully checking C_T values of 27–30 using dissociation curve analysis and/or repeat experiments to ensure that they are related to expression of the miRNA of interest.

6. **C_T values of the positive PCR control wells (PPC) are examined.**

   If the RNA sample is of high quality, the cycling program has been correctly run, and the thresholds have been correctly defined, the value of C_T^{PPC} should be ■ 19 ± 2 or ▲ 15 ± 2 across all arrays or samples.

7. **C_T values of the reverse transcription control (miRTC) are examined using the values for the positive PCR control (PPC) by calculating ΔC_T = AVG C_T^{miRTC} – AVG C_T^{PPC} – correction factor.**

   If this value is less than the value indicated in Table 12, then no inhibition of the reverse-transcription reaction is apparent. No action is needed. If this value is greater than the value indicated in Table 12, there is evidence of impurities that may have inhibited the reverse-transcription reaction. See the "Troubleshooting Guide" page 36.

Table 12. Expected values for ΔC_T = AVG C_T^{miRTC} – AVG C_T^{PPC}

Dilution after preamplification reaction (step 5, page 19)	Expected ΔC_T
5-fold	<0
20-fold	<2
40-fold	<3
100-fold	<4
200-fold	<5
1000-fold	<7

8. **Recovery from serum/plasma using the *C. elegans* miR-39 miScript Primer Assay is determined.**

   Note: The *C. elegans* miR-39 miScript Primer Assay should only result in C_T values above the threshold if the miRNeasy Serum/Plasma Spike-In Control was added to the sample during RNA purification. For more information, see page 11 and the *miRNeasy Serum/Plasma Handbook*.

9. **The optimal dilution factor of preamplified cDNA prior to analysis on a miScript miRNA PCR Array is determined.**

   From the miScript Primer Assays for miR-16, miR-21, and miR-191, select the assay that shows the lowest C_T value and determine the dilution factor according to Table 9, page 29.

10. **C_T values of the SNORD61, SNORD95, and SNORD96A miScript Primer Assays are examined.**

    Note: These small RNAs have been verified to have relatively stable expression levels across tissues and cell types. Nevertheless, snoRNA control C_T values remain sample dependent and should be checked to determine whether their expression is consistent across the samples that are being analyzed. If the expression of a particular control is not consistent across experimental samples, that control should not be used for data normalization. For examples of C_T values associated with various tissue types, refer to the miScript PCR Controls application data at www.qiagen.com/miRNAControls.

11. **Once the appropriate preamplified cDNA dilution is chosen, and if all additional criteria described above are met, cDNA samples are ready for analysis. Proceed with miScript miRNA PCR Array experiments.**

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Evidence of poor reverse-transcription efficiency (value of AVG C_T^{miRTC} – AVG C_T^{PPC} higher than expected)	
Poor quality RNA	Check the A_{260}:A_{280} and A_{260}:A_{230} ratios of the RNA samples. Be sure to perform the dilutions for spectrophotometry in RNase-free 10 mM Tris·Cl, pH 7.5. If necessary, repurify RNA with a spin-column based clean up method, such as the miRNeasy Mini Kit (cat. no. 217004).
C_T value for miRTC miScript Primer Assay is higher than expected	
a) Reverse-transcription inhibitors present in RNA sample	Check the A_{260}:A_{280} and A_{260}:A_{230} ratios of the RNA samples. Be sure to perform the dilutions for spectrophotometry in RNase-free 10 mM Tris·Cl, pH 7.5. If necessary, repurify RNA with a spin-column based clean up method, such as the miRNeasy Mini Kit (cat. no. 217004).
b) Inefficient preamplification due to incorrect real-time cycling	Ensure that cycling conditions in Table 4, page 18 are used for 96-plex preamplification reactions and cycling conditions in Table 5, page 18 are used for 384-plex preamplification reactions.
No product, or product detected late in real-time PCR (indicative of problems occurring during reverse transcription)	
a) Pipetting error or missing reagent when setting up reverse-transcription reaction	Check the pipets used for experimental setup. Mix all reagents well after thawing and repeat the reverse-transcription reaction.
b) Incorrect setup of reverse-transcription reaction	Be sure to set up the reaction on ice.

	Comments and suggestions
c) Poor quality or incorrect amount of template RNA for reverse-transcription reaction	Check the concentration, integrity, and purity of the template RNA before starting the protocol. Mix well after thawing the template RNA. Even minute amounts of RNases can affect synthesis of cDNA and sensitivity in RT-PCR, particularly with small amounts of RNA.
d) RNA concentration too high or too low	The miScript II RT Kit in combination with the miScript PreAMP PCR Kit are intended for use with 10–100 ng RNA.
e) RNA denatured	Denaturation of the template RNA is not necessary. If denaturation was performed, the integrity of the RNA may be affected.
f) Incubation temperature too high	Reverse transcription should be carried out at 37°C. Higher temperatures may reduce the length of cDNA products or the activity of miScript Reverse Transcriptase Mix. Check the temperature of your heating block or water bath.

No product, or product detected late in real-time PCR, or only primer–dimers detected (indicative of problems occurring during real-time PCR)

a) Incorrect storage of QuantiTect SYBR Green PCR Master Mix	QuantiTect SYBR Green PCR Master Mix should be stored immediately upon receipt at –20°C in a constant-temperature freezer.
b) Volume of reverse-transcription reaction added to the preamplification reaction was too high or too low	It is important to add the exact recommended volume of reverse-transcription reaction to the preamplification reaction to guarantee optimal specificity and efficiency. Repeat the preamplification reaction using the correct volume of reverse-transcription reaction (5 µl of a 10 µl reverse-transcription reaction, see "Important points before starting", page 16).
c) PCR annealing time too short	Use the annealing time specified in the protocol.
d) PCR extension time too short	Use the extension time specified in the protocol.

	Comments and suggestions
e) Pipetting error or missing reagent when setting up PCR	Check the concentrations and storage conditions of reagents, including primers and cDNA.
f) HotStarTaq DNA Polymerase not activated with a hot start	Ensure that the cycling program includes the hot start activation step for HotStarTaq DNA polymerase; for details, check the protocol.
g) Primer concentration for real-time PCR not optimal	Use the primer concentrations recommended in the protocol for the real-time PCR kit.
h) PCR annealing temperature too high	Decrease annealing temperature in 3°C steps.
i) PCR annealing temperature too low	Increase annealing temperature in 2°C steps.
j) No detection activated	Check that fluorescence detection was activated in the cycling program.
k) Wrong detection step	Ensure that fluorescence detection takes place during the extension step of the PCR cycling program.
l) Real-time PCR primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
m) Wrong dye layer/filter chosen on real-time cycler	Ensure that the appropriate layer/filter is activated.
n) Insufficient starting template	Increase the amount of template cDNA.
No linearity in ratio of C_T value/crossing point to log of the template amount	
a) Template amount too high	Do not exceed maximum recommended amounts of template cDNA. For details, see the protocol.
b) Template amount too low	Increase amount of RNA in reverse-transcription reaction or decrease the dilution factor of the preamplification reaction.

	Comments and suggestions
High fluorescence in "No Template" control	
a) Contamination of reagents	Discard reaction components and repeat with new reagents.
b) Contamination during reaction setup	Take appropriate safety precautions (e.g., use filter tips).
Varying fluorescence intensity	
a) Real-time cycler contaminated	Decontaminate the real-time cycler according to the supplier's instructions.
b) Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the supplier's instructions.
Signal appears in "No RT" control	
DNA present in RNA sample	Ensure that no genomic DNA is present in the RNA sample (e.g., perform an on-column DNase digestion if using miRNeasy Kits).

Appendix: Preamplification Using up to 25 miScript Primer Assays

miScript PreAMP Primer Mixes are available for all miScript miRNA PCR Arrays. Alternatively, if desired preamplification can be performed using the miScript PreAMP PCR Kit together with a mix of miScript Primer Assays prepared by the user as described in this protocol, instead of a miScript PreAMP Primer Mix.

Procedure

A1. Perform cDNA synthesis as described in the protocol on page 14.

A2. Dilute miScript Primer Assay(s) for up to 25 miRNAs of interest as described in Table 13.

Note: We highly recommend including control assays in the miScript Primer Assay mix. For details of recommended controls, see page 9.

Table 13. miScript Primer Assay mix

Number of miRNAs for expression analysis	miScript Primer Assay*	RNase-free water
1 miRNA	1 assay x 10 µl = 10 µl	240 µl
2 miRNAs	2 assays x 10 µl each = 20 µl	230 µl
3 miRNAs	3 assays x 10 µl each = 30 µl	220 µl
Up to 25 miRNAs	Up to 25 assays x 10 µl each = 250 µl	0 µl

* If using miScript Primer Assays for the first time, be sure to reconstitute before use according to the instructions in the *miScript PCR System Handbook*.

A3. Prepare the preamplification master mix at room temperature (15–25°C) according to Table 14.

Gently mix and then store on ice.

Note: 5x miScript PreAMP Buffer and HotStarTaq DNA Polymerase should be removed from the –20°C freezer just before preparation of the reaction, gently mixed, and placed on ice. They should be returned to the freezer immediately after use.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed.

Table 14. Preamplification reaction components

Component	Volume/reaction
5x miScript PreAMP Buffer	5 µl
HotStarTaq DNA Polymerase	2 µl
miScript Primer Assay mix from step 2	5 µl
RNase-free water	7 µl
miScript PreAMP Universal Primer	1 µl
Diluted template cDNA (added in step 4)	5 µl
Total volume	**25 µl**

A4. Add diluted template cDNA to each tube containing preamplification master mix. Gently mix, briefly centrifuge, and then store on ice.

A5. Program the thermal cycler according to Table 15.

Table 15. Cycling conditions for preamplification

Step	Time	Temperature
PCR **Initial activation step** HotStarTaq DNA Polymerase is activated by this heating step.	**15 min**	**95°C**
2-step cycling:		
Denaturation	30 s	94°C
Annealing/extension	3 min	60°C
Cycle number	12 cycles	

A6. Place the preamplification reaction in the thermal cycler and start the run.

A7. After the run has finished, dilute the preamplified cDNA in RNase-free water according to step 5, page 19. Gently mix and then store on ice.

The minimum dilution is 20-fold.

A8. Proceed with real-time PCR for detection of mature miRNA using 1 µl diluted cDNA per reaction.

Perform real-time PCR according to protocols provided in the *miScript PCR System Handbook*.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Ordering Information

Product	Contents	Cat. no.
miScript PreAMP PCR Kit (12)	For 12 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, miScript PreAMP Controls, RNase-Free Water	331451
miScript PreAMP PCR Kit (60)	For 60 preamplification reactions: 5x miScript PreAMP Buffer, HotStarTaq DNA Polymerase (2 U/µl), miScript PreAMP Universal Primer, miScript PreAMP Controls, RNase-Free Water	331452
miScript PreAMP Pathway Primer Mix	60 µl primer mix for preamplification; for use with a Pathway-Focused miScript miRNA PCR Array	Varies
miScript PreAMP Pathway HC Primer Mix	60 µl primer mix for preamplification; for use with a miScript miRNA HC PCR Array	Varies
miScript PreAMP miRNome Primer Mix	60 µl/tube primer mix for preamplification; for use with a miRNome miScript miRNA PCR Array	Varies
miScript PreAMP Custom Primer Mix	100 µl/tube primer mix for preamplification; for use with a Custom miScript miRNA PCR Array	Varies
miScript II RT Kit (12)	For 12 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218160
miScript II RT Kit (50)	For 50 cDNA synthesis reactions: miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, 5x miScript HiFlex Buffer, RNase-Free Water	218161
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218073

Product	Contents	Cat. no.
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript Primer Assay (100)	10x miScript Primer Assay (contains one miRNA-specific primer)	Varies*
Pathway-Focused miScript miRNA PCR Array	Array of assays for human, mouse, rat, or dog miRNAs; available in 96-well, 384-well, or Rotor-Disc format	Varies
miRNome miScript miRNA PCR Array	Array of assays for the complete human, mouse, rat, or dog miRNome; available in 96-well, 384-well, or Rotor-Disc 100 format	Varies
miScript miRNA HC PCR Array	High-content array of assays for human, mouse, rat, or dog miRNAs; available in 384-well format	Varies
Custom miScript miRNA PCR Array	Custom-designed array of assays for human, mouse, rat, or dog miRNAs; available in 96-well, 384-well, or Rotor-Disc format	Varies
miScript miRNA QC PCR Array	Array of quality control assays for human, mouse, rat, or dog miRNAs; available in 96-well, 384-well, or Rotor-Disc 100 format	Varies
Related products		
miRNeasy Micro Kit (50)	For 50 total RNA preps: 50 RNeasy® MinElute® Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol® Lysis Reagent, RNase-Free Reagents and Buffers	217084
miRNeasy Mini Kit (50)	For 50 preps: 50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217004

* Visit www.qiagen.com/GeneGlobe to search for and order these products.

Product	Contents	Cat. no.
miRNeasy 96 Kit (4)	For 4 x 96 preps: 4 RNeasy 96 plates, Collection Microtubes (racked), Elution Microtubes CL, Caps, S-Blocks, AirPore Tape Sheets, QIAzol Lysis Reagent, RNase-Free Reagents and Buffers	217061
miRNeasy Serum/Plasma Kit (50)	For 50 total RNA preps: 50 RNeasy MinElute Spin Columns, Collection Tubes (1.5 ml and 2 ml), QIAzol Lysis Reagent, Ce_miR-39_1 miScript Primer Assay, RNase-free Reagents and Buffers	217184
miRNeasy Serum/Plasma Spike-In Control	10 pmol lyophilized *C. elegans* miR-39 miRNA mimic	219610
miRNeasy FFPE Kit (50)	50 RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	217504
PAXgene® Tissue miRNA Kit (50)	For 50 RNA preps: PAXgene RNA MinElute Spin Columns, PAXgene Shredder Spin Columns, Processing Tubes, Microcentrifuge Tubes, Carrier RNA, RNase-Free DNase, RNase-Free Buffers; to be used with PAXgene Tissue Containers	766134
PAXgene Tissue Containers (10)	For collection, fixation, and stabilization of 10 samples: 10 Prefilled Reagent Containers, containing PAXgene Tissue Fix and PAXgene Tissue Stabilizer	765112
PAXgene Blood miRNA Kit (50)	For 50 RNA preps: PAXgene Spin Columns, PAXgene Shredder Spin Columns, Processing Tubes, Microcentrifuge Tubes, RNase-Free DNase, RNase-Free Reagents and Buffers; to be used with PAXgene Blood RNA Tubes (available from BD, cat. no. 762165)	763134

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Notes

Notes

Notes

Trademarks: QIAGEN®, QIAzol®, HotStarTaq®, miScript®, QuantiTect®, RNeasy®, Rotor-Gene®, Rotor-Disc®, MinElute® (QIAGEN Group); PAXgene® (PreAnalytiX GmbH); ABI PRISM®, Applied Biosystems®, (Life Technologies Corporation); SYBR® (Molecular Probes, Inc.); Stratagene®, Mx3005P®, Mx3000P® (Stratagene); Bio-Rad®, CFX96™, CFX384™, iCycler iQ®, MyiQ™ (Bio-Rad Laboratories, Inc.); Roche®, LightCycler® (Roche Group); Eppendorf®, Mastercycler® (Eppendorf AG); Excel® (Microsoft Corporation). Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

Limited License Agreement for miScript PreAMP PCR Kit and miScript PreAMP Primer Mixes

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see www.qiagen.com.

www.qiagen.com

Australia ■ techservice-au@qiagen.com

Austria ■ techservice-at@qiagen.com

Belgium ■ techservice-bnl@qiagen.com

Brazil ■ suportetecnico.brasil@qiagen.com

Canada ■ techservice-ca@qiagen.com

China ■ techservice-cn@qiagen.com

Denmark ■ techservice-nordic@qiagen.com

Finland ■ techservice-nordic@qiagen.com

France ■ techservice-fr@qiagen.com

Germany ■ techservice-de@qiagen.com

Hong Kong ■ techservice-hk@qiagen.com

India ■ techservice-india@qiagen.com

Ireland ■ techservice-uk@qiagen.com

Italy ■ techservice-it@qiagen.com

Japan ■ techservice-jp@qiagen.com

Korea (South) ■ techservice-kr@qiagen.com

Luxembourg ■ techservice-bnl@qiagen.com

Mexico ■ techservice-mx@qiagen.com

The Netherlands ■ techservice-bnl@qiagen.com

Norway ■ techservice-nordic@qiagen.com

Singapore ■ techservice-sg@qiagen.com

Sweden ■ techservice-nordic@qiagen.com

Switzerland ■ techservice-ch@qiagen.com

UK ■ techservice-uk@qiagen.com

USA ■ techservice-us@qiagen.com

QIAGEN

1073620 08/2012

Sample & Assay Technologies