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miScript® Single Cell qPCR System Handbook

Universal quantification solution for precision miRNA profiling from individual cells

Sample to Insight

QIAGEN

Contents

Kit Contents 3
Storage 4
Intended Use 5
Safety Information 6
Quality Control 6
Introduction 7
Principle and Procedure 9
Equipment and Reagents to Be Supplied by User 14
Important Notes 15
Protocol: Cell Lysis 16
Protocol: 3′ Ligation 18
Protocol: 5′ Ligation 20
Protocol: Reverse-Transcription 22
Protocol: "Beads + Bind" Mixture Preparation 24
Protocol: cDNA Cleanup 26
Protocol: Preamplification 28
Protocol: Real-time PCR Quality Control 34
miScript Handbook Recommendations for Real-Time PCR Applications and Data Analysis . 40
Troubleshooting Guide 42
Appendix: General Remarks on Handling RNA 44
Ordering Information 47

Kit Contents

miScript Single Cell qPCR Kit		(24)	(96)
Catalog no.		**331053**	**331055**
Number of reactions		**24**	**96**
Bottle	Buffer FCPL	10 ml	10 ml
Brown	Buffer FCPM	2.5 µl	2.5 µl
Vial	gDNA Wipeout Buffer 2	1 vial	1 vial
Pink	miScript SC 3′ Ligation Buffer	24 µl	96 µl
Pink	miScript SC Ligation Activator	192 µl	768 µl
Pink	miScript SC 3′ RNA Ligase	12 µl	48 µl
Blue	Nuclease-free water	1 ml	2 x 1 ml
Orange	miScript SC 5′ Ligation Buffer	96 µl	384 µl
Orange	miScript SC 5′ RNA Ligase	24 µl	96 µl
Green	miScript SC 5x RT Buffer	192 µl	768 µl
Green	miScript SC 10x RT Nucleics	96 µl	384 µl
Green	miScript SC Reverse Transcriptase	24 µl	96 µl
Lavender	miScript SC Cleanup Beads	1.6 ml	1.6 ml
Bottle	miScript SC Cleanup Bind	7.5 ml	7.5 ml
Lavender	Buffer EB	1 ml	2 x 1 ml
Red	miScript SC PreAMP Buffer	120 µl	480 µl
Red	miScript SC PreAMP Universal Primer	48 µl	192 µl
Red	HotStarTaq DNA Polymerase 2 U/µl	48 µl	192 µl
Red	Side Reaction Reducer	48 µl	192 µl
Yellow	miC3′ 10x Primer Assay	60 µl	240 µl
Yellow	miC5′ 10x Primer Assay	60 µl	240 µl
Yellow	miCRT 10x Primer Assay	60 µl	240 µl
Yellow	miSCPA 10x miScript Primer Assay	60 µl	240 µl
Yellow	PPC	60 µl	240 µl
Quick-Start Protocol		2	2

miScript SYBR Green PCR Kit Catalog no. Number of reactions	(200) 218073 200	(1000) 218075 1000	(2000) 218076 2000
2x QuantiTect® SYBR Green PC Master Mix, containing: HotStarTaq® DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix, including dUTP, SYBR Green I, ROX™ passive reference dye, 5 mM $MgCl_2$	3 x 1.7 ml	25 ml	2 x 25 ml
10x miScript Universal Primer	1 ml	5 x 1 ml	10 x 1 ml
RNase-free water	2 x 2 ml	20 ml	2 x 20 ml
Quick-Start Protocol	2	2	2

Storage

The miScript Single Cell qPCR Kit is shipped in two boxes. Box 1 is shipped on blue ice bricks or dry ice, and Box 2 is shipped at room temperature (15–25°C). Upon receipt, all components in Box 1, *except* for Buffer FCPM, should be stored immediately at –20°C in a constant-temperature freezer. Buffer FCPM should be stored at 4°C. From Box 2, Buffer FCPL and miScript SC Cleanup Bind should be stored at 4°C, and miScript SC Cleanup Beads should be stored at room temperature.

Intended Use

All miScript products 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 safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miScript Single Cell qPCR Kit, miScript SYBR Green PCR Kit, miScript Microfluidics PCR Kit, miScript miRNA PCR Array and miScript Primer Assay is tested against predetermined specifications to ensure consistent product quality.

Introduction

The miScript Single Cell qPCR Kit includes reagents for cell lysis, cDNA synthesis and unbiased preamplification of all microRNAs (miRNAs), facilitating the expression analysis of miRNAs from individual cells. One to 100 cells or 25 pg to 1 ng of purified RNA is required as template for each miScript Single Cell qPCR Kit reaction.

Unlike other single-cell miRNA expression products, the miScript Single Cell qPCR Kit is a completely universal kit. Starting from a single cell, *all* miRNAs, both annotated and non-annotated, are selectively converted into cDNA and subsequently amplified. There is no fractionation of the sample or selection of specific miRNAs required. From cell lysis through preamplification, the miScript Single Cell qPCR Kit is a robust, universal solution for precision single-cell miRNA expression analysis. An entire miRNome expression profile can be generated from an individual cell.

Tissues are heterogeneous mixtures of different cell populations with each cell contributing a unique proteome and transcriptome. For example, normal and disease-biology are both inherently heterogeneous, and cells can respond individually and in concert to internal and external stimuli. Further, individual cells can differ due to epigenetics, circadian clock, cell cycle, microenvironments, cell-to-cell contacts and intrinsic transcriptional "noise." While bulk transcriptomic analysis of mRNA, lncRNA and miRNA expression is critical for understanding biological systems, the consequential "cellular averages" mask intrinsic transcriptional variability across individual cell subpopulations. In fact, the contribution of rare cell subtypes may be completely obscured when cells are assessed in bulk. Single-cell expression analysis brings into focus the individual contribution of every cell providing a complete, granular understanding of a specific biological response.

A Sample to Insight®, universal quantification solution is critical for the complete understanding of single-cell miRNA expression analysis. Of utmost importance is a system that facilitates universal cDNA synthesis and universal amplification of all miRNAs in one workflow without

introducing, bias, artificial selection or dilution via sample fractionation. This enables the complete miRNome profile of an individual cell to be archived, facilitating future discoveries. For real-time PCR, having access to the most complete portfolio of miRNA expression assays permits the analysis of either all or select miRNAs tailored for your biological system. In addition, data analysis and interpretation solutions should enable the rapid assessment and grouping of similar cells as well as the identification of miRNA signatures underlying the groupings. QIAGEN's Sample to Insight miScript Single Cell qPCR System embraces this philosophy, consisting of the miScript Single Cell qPCR Kit, miScript miRNA PCR Arrays, miScript Primer Assays, miScript SYBR Green PCR Kit, miScript Microfluidics PCR Kit and the GeneGlobe® Data Analysis Center.

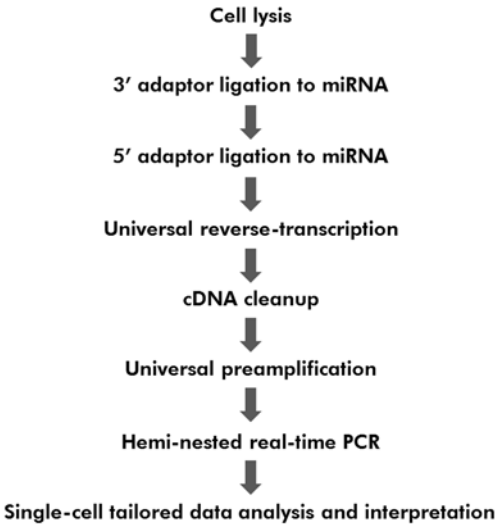

Figure 1. QIAGEN's Sample to Insight miScript Single Cell qPCR System Workflow.

Principle and Procedure

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate posttranscriptional gene regulation. Animal mature miRNAs possess a 3′ hydroxyl group and a 5′ phosphate group (unlike most other cellular RNAs). Due to this, adapters can be ligated to both the 3′ end and 5′ end of mature miRNAs enabling universal reverse-transcription and preamplification, while minimizing the background from other RNA species. The miScript Single Cell qPCR Kit includes all of the reagents required to perform cell lysis through preamplification in a streamlined, integrated workflow.

Cell lysis

Individually isolated cells are lysed using a cell processing mix (Buffer FCPL supplemented with Buffer FCPM and gDNA Wipeout Buffer 2). During cell lysis:

- **Cellular RNA is stabilized:** This ensures that the RNA accurately reflects the in vivo miRNA expression profile.
- **Genomic DNA is eliminated:** The cell processing mix effectively removes contaminating genomic DNA.
- **Enzymatic inhibitors are blocked or inactivated:** This allows efficient cDNA synthesis.

Universal cDNA synthesis and preamplification of miRNA

In an unbiased reaction, adapters are ligated sequentially to the 3′ and 5′ ends of miRNAs. Subsequently, universal cDNA synthesis, cleanup and universal preamplification are performed. The following reactions are part of the workflow (Figure 2):

- **3′ Ligation:** A pre-adenylated DNA adapter is ligated to the 3′ end of all miRNAs. The miScript SC 3′ Ligation Buffer contains the 3′ adapter and an internal synthetic control (miScript SC 3′ Ligation Control [miC3′]) that is used to monitor 3′ ligation performance.

The miScript SC 3′ Ligase is optimized for the 3′ ligation of low RNA amounts found in single cells.

- **5′ ligation:** An RNA adapter is ligated to the 5′ end of the mature miRNAs. The miScript SC 5′ Ligation Buffer contains the 5′ adapter, ATP and an internal synthetic control (miScript SC 5′ Ligation Control [miC5′]) that is used to monitor 5′ ligation performance. The miScript SC 5′ Ligase is optimized for the 5′ ligation of low RNA amounts found in single cells.
- **cDNA synthesis:** The reverse-transcription (RT) primer binds to a region of the 3′ adapter and facilitates conversion of the 3′ / 5′ ligated miRNAs into cDNA. During reverse-transcription, a universal sequence is also added as part of the RT primer. The unique, patent-pending formulation of 5x miScript SC RT Buffer facilitates the selective conversion of mature miRNAs into cDNA by suppressing the ability of the enzyme to process longer RNA species. 10x miScript SC Nucleics contains dNTPs, RT primer and an internal synthetic control (miScript SC Reverse-Transcription Control [miCRT]) that is used to monitor reverse-transcription performance. The miScript SC Reverse Transcriptase is optimized for the reverse-transcription of low RNA amounts.
- **cDNA cleanup:** After reverse-transcription, a cleanup of the cDNA is performed using a streamlined magnetic bead-based method.
- **Preamplification:** Preamplification uses a single universal primer pair that targets the sequence of the 5′ adapter and the universal sequence added during reverse-transcription. This unbiased amplification of all miRNAs in a single reaction ensures that sufficient target is present for quantification in subsequent real-time PCR. The miScript SC PreAMP Universal Primer contains an internal synthetic control (miScript SC PreAMP Control [miSCPA]) that is used to monitor preamplification performance.

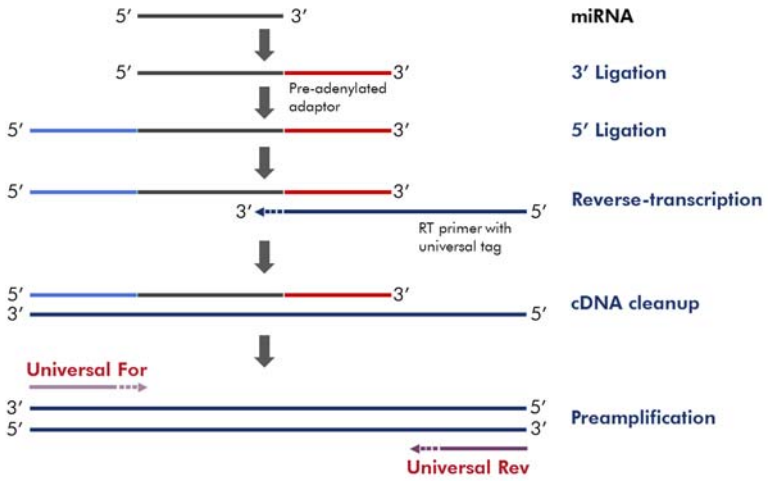

Figure 2. Universal reverse-transcription and preamplification of mature miRNAs using the miScript Single Cell qPCR Kit. After cell lysis, 3′ and 5′ adapters are ligated to mature miRNAs. The ligated miRNAs are then reverse-transcribed to cDNA. Following cleanup, the cDNA is preamplified in a universal reaction. The preamplified product is then used for real-time PCR expression analysis with either miScript miRNA PCR Arrays or miScript Primer Assays.

Real-time PCR for the Fluidigm® BioMark™ System

Preamplified product from the miScript Single Cell qPCR Kit can serve as the template for real-time PCR on the Fluidigm BioMark System using either miScript miRNA PCR Arrays or miScript Primer Assays and the miScript Microfluidics PCR Kit. This specialized real-time PCR kit contains the miScript Microfluidics Universal Primer (reverse primer) and Microfluidics qPCR Master Mix (specifically optimized for the Fluidigm BioMark System; contains EvaGreen® dye for detection and ROX™ passive reference dye). For more information, please refer to the *miScript Microfluidics Handbook*.

Real-time PCR for plate-based instruments

miScript miRNA PCR Arrays: Preamplified product from the miScript Single Cell qPCR Kit serves as the template for real-time PCR analysis using a miScript miRNA PCR Array in combination with the miScript SYBR Green Kit, which contains the miScript Universal Primer

(reverse primer) and QuantiTect SYBR Green PCR Master Mix. For more information, please refer to the *miScript miRNA PCR Array Handbook*.

miScript Primer Assays: Preamplified product from the miScript Single Cell qPCR Kit serves as the template for real-time PCR analysis using a miScript Primer Assay and the miScript SYBR Green Kit. For more information, please refer to the *miScript PCR System Handbook*.

Integrated reaction controls

The miScript Single Cell qPCR Kit contains integrated reaction controls to monitor the 3′ ligation, 5′ ligation, reverse-transcription, preamplification and real-time PCR performance (Table 1). Together, the controls monitor every critical step of the workflow and enable determination of the overall workflow's performance.

Table 1. miScript Single Cell qPCR Reaction Controls

Control	Purpose
miScript SC 3′ Ligation Control (miC3′)	Assessment of 3′ ligation performance
miScript SC 5′ Ligation Control (miC5′)	Assessment of 5′ ligation performance
miScript SC Reverse-Transcription Control (miCRT)	Assessment of reverse-transcription performance
miScript SC PreAMP Control (miSCPA)	Assessment of preamplification performance
Positive PCR Control (PPC)	Assessment of real-time PCR performance

Data analysis

Free data analysis software tailored for single-cell miRNA expression analysis is available at **www.qiagen.com/GeneGlobe.** The analysis software performs principle component analysis (PCA) and hierarchical clustering, and results are presented in a variety of visual formats.

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 safety data sheets (SDSs) available from the product supplier.

- **When processing 1–24 cells:** 8-well PCR Strip with attached Optically Clear Cap (VWR cat. no. 93001-118) or equivalent
- **When processing 24–96 cells:** 96 x 0.2 ml Plate (BIOplastics cat. no. AB17500) with EU Optical Wide Area 8-Cap Strip (BIOplastics cat. no. B57801B) or equivalent
- Nuclease-free pipet tips and tubes
- Microfuge tubes
- Multichannel pipettor
- Ice
- Microcentrifuge
- Centrifuge with swing-bucket rotor
- Thermal cycler
- Eppendorf MixMate® with MixMate PCR 96 Adapter (or comparable instrument)
- MagneSphere® Technology Magnetic Separation Stand (Promega cat. no. Z5342)
- DynaMag™-96 Side Magnet (Life Technologies cat. no. 12331D)
- miScript SYBR Green PCR Kit (QIAGEN cat. no. 218073, 218075 or 218076)

Important Notes

- Any method for single cell isolation is compatible with the miScript Single Cell qPCR Kit as long as the cells are in 2 µl or less of a neutral buffer like PBS. To avoid any impact on the efficiency of the lysis reaction, carryover of chelators or large amounts of divalent ions should be avoided. We recommend to wash the cell with a neutral buffer such as 1x PBS prior to lysis if your isolation protocol requires such chelators or a high concentration of divalent ions.
- Ensure reactions are thoroughly mixed, prepared at recommended temperatures and incubated at recommended temperatures.
- If the workflow is not expected to be completed in one day, convenient stopping points are indicated at the end of particular sections, including *Protocol: Cell Lysis*, *Protocol: Reverse-Transcription*, *Protocol: cDNA Cleanup*, *Protocol: Preamplification* and *Protocol: Real-time PCR Quality Control*.
- It is highly recommended to perform *Protocol: Real-time PCR Quality Control* prior to performing real-time PCR with miScript miRNA PCR Arrays or miScript Primer Assays.
- Preamplified miRNAs prepared with the miScript Single Cell qPCR Kit are compatible with all miScript miRNA PCR Arrays and miScript Primer Assays.
- When using the GeneGlobe Data Analysis Center to analyze data associated with purified total RNA samples rather than individual cells, data should be analyzed using the “Standard Samples” option instead of the “Single Cell Samples” option.

Protocol: Cell Lysis

Before starting

- Add 2.5 µl Buffer FCPM to Buffer FCPL, mix well and store at 2–8°C. This only needs to be added once. Briefly shake or vortex the FCPL / FCPM mixture before each use.
- Add 750 µl RNase-free water to lyophilized gDNA Wipeout Buffer 2. Mix by gently inverting the vial, divide into single-use aliquots and store at –20°C. To avoid loss of lyophilized gDNA Wipeout Buffer 2, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe.

Important points before starting

- Use 1–100 cells as starting material. The cells should be in 2 µl or less of neutral buffer like PBS.
- Ensure that Buffer FCPM has been added to Buffer FCPL.
- Do not vortex the lysed cells.

Procedure

1. Prepare reagents required for cell lysis. Ensure that Buffer FCPM has been previously added to Buffer FCPL. Briefly shake or vortex the FCPL / FCPM mixture and place on ice. Mix gDNA Wipeout Buffer 2 by flicking the tube briefly to collect residual liquid from the sides of the tube. Place on ice.
2. Thaw cells on ice.
3. Prepare the cell processing master mix on ice according to Table 2. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   The cell processing master mix contains all components required for cell lysis (10% greater than required for the total number of reactions).

Table 2. Preparation of cell processing master mix

Component	Master mix 24 samples	Master mix 96 samples
Buffer FCPL / FCPM	124 µl	496 µl
gDNA Wipeout Buffer	8 µl	32 µl
Total volume	**132 µl**	**528 µl**

4. Add 3 µl of cell processing master mix to each well of the PCR strip tubes or 96-well plates containing a cell. Do not mix the cells / cell processing master mix.

   Important: The cell processing master mix volume assumes the cells are in a volume of 2 µl PBS, which is the maximum allowable PBS volume. If the cells are in less PBS, make up the volume difference by adding additional cell processing master mix. For example, if the cells are in 1 µl PBS, add 4 µl of cell processing master mix.
5. Incubate at room temperature for 5 min.
6. Incubate for 5 min at 75°C and hold at 4°C.
7. Proceed to *Protocol: 3′ Ligation*. Alternatively, the lysed cells can be stored at –80°C.

Protocol: 3′ Ligation

Important points before starting

- Use 5 µl cell lysates from *Protocol: Cell Lysis* as the starting materials for the 3′ ligation reactions.
- This protocol can also be used with low amounts of purified RNA (25 pg to 1 ng).
- The 3′ ligation components are added directly to the wells of the PCR strip tubes or 96-well plates containing cell lysates. New strip tubes or 96-well plates are not required.
- Set up the 3′ ligation reactions at room temperature.
- The 3′ ligation reactions are very viscous. Pipet slowly and thoroughly (pipet up and down 12 times) to mix.
- Do not vortex miScript SC 3′ RNA Ligase, cell lysates or the 3′ ligation reactions.
- Upon completion of the 3′ ligation reactions, proceed immediately to *Protocol: 5′ Ligation*.

Procedure

1. If previously frozen, thaw PCR strip tube(s) or 96-well plate containing cell lysate on ice. Centrifuge briefly to collect residual liquid from the sides of the tubes and return to ice.
2. Prepare reagents required for the 3′ ligation reactions. Thaw miScript SC 3′ Ligation Buffer, miScript SC Ligation Activator and nuclease-free water at room temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.

   miScript SC 3′ RNA Ligase should be removed from the –20°C freezer just before preparation of the master mix, gently mixed and placed on ice. miScript SC 3′ RNA Ligase should be returned to the freezer immediately after use.
3. Prepare the 3′ ligation reaction master mix at room temperature (15–25°C) according to Table 3. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

The 3′ ligation master mixes contain all components required for 3′ ligation (10% greater than required for the total number of reactions) except the cell lysate and miScript SC Ligation Activator.

Table 3. Setup of 3′ ligation reactions

Component	Volume one sample	Master mix 24 samples	Master mix 96 samples
Cell lysate (already in well of PCR strip tube or 96-well plate)	5 µl	–	–
miScript SC 3′ Ligation Buffer	1 µl	27 µl	108 µl
Nuclease-free water	0.5 µl	13.5 µl	54 µl
miScript SC 3′ RNA Ligase	0.5 µl	13.5 µl	54 µl
miScript SC Ligation Activator (added in step 5)	8 µl	–	–
Total volume	**15 µl**	**54 µl**	**216 µl**

4. At room temperature, aliquot 2 µl of the 3′ ligation master mix into each well of the PCR strip tubes or 96-well plates containing 5 µl cell lysate.

   Note: The 3′ ligation master mix is added directly to the wells of the PCR strip tubes or 96-well plates containing cell lysates. New strip tubes or 96-well plates are not required.

5. Slowly add miScript SC Ligation Activator to each well containing ligation mix and cell lysate. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   Important: Pipet slowly to mix. miScript SC Ligation Activator is very viscous.

6. Incubate for 1 hour at 16°C.
7. Hold at 4°C.
8. Proceed immediately to *Protocol: 5′ Ligation*.

Protocol: 5′ Ligation

Important points before starting

- The completed 15 µl 3′ ligation reactions from *Protocol: 3′ Ligation* are the starting materials for the 5′ ligation reactions.
- The 5′ ligation components are added directly to the wells of the PCR strip tubes or 96-well plates containing completed 3′ ligation reactions. New strip tubes or 96-well plates are not required.
- Set up the 5′ ligation reactions at room temperature.
- The 5′ ligation reactions are very viscous. Pipet slowly and thoroughly (pipet up and down 12 times) to mix.
- Do not vortex the miScript SC 5′ RNA Ligase or 5′ ligation reactions.
- Upon completion of the 5′ ligation reactions, proceed immediately to *Protocol: Reverse-Transcription*.

Procedure

1. Prepare reagents required for the 5′ ligation reactions. Thaw miScript SC 5′ Ligation Buffer at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tube briefly to collect residual liquid from the sides of the tube and keep at room temperature.

   miScript SC 5′ RNA Ligase should be removed from the –20°C freezer just before preparation of the master mix, gently mixed and placed on ice. miScript SC 5′ RNA Ligase should be returned to the freezer immediately after use.
2. Immediately prior to setting up the 5′ ligation reactions, allow the completed 3′ ligation reactions to equilibrate to room temperature for 2 min. Centrifuge briefly to collect residual liquid from the sides of the wells.
3. Prepare the 5′ ligation reaction master mix at room temperature (15–25°C) according to Table 4. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

The 5′ ligation master mixes contain all components required for 5′ ligation (10% greater than required for the total number of reactions) except the completed 3′ ligation reactions.

Table 4. Setup of 5′ ligation reactions

Component	Volume one sample	Master mix 24 samples	Master mix 96 samples
3′ ligation reaction (already in well of PCR strip tube or 96-well plate)	15 µl	–	–
miScript SC 5′ Ligation Buffer	4 µl	108 µl	432 µl
miScript SC 5′ RNA Ligase	1 µl	27 µl	108 µl
Total volume	**20 µl**	**135 µl**	**540 µl**

4. At room temperature, aliquot 5 µl of the 5′ ligation master mix into each well of the PCR strip tubes or 96-well plates containing the completed 3′ ligation reaction. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   Note: The 5′ ligation master mix is added directly to the wells of the PCR strip tubes or 96-well plates containing the completed 3′ ligation. New strip tubes or 96-well plates are not required.

   Important: Pipet slowly to mix. miScript SC Ligation Activator from 3′ ligation is very viscous.

5. Incubate for 5 min at 37°C.
6. Incubate for 15 min at 65°C to inactivate the miScript SC 5′ RNA Ligase and hold at 4°C.

   Important: Hold at 4°C for at least 5 min.

7. Proceed immediately to *Protocol: Reverse-Transcription*.

Protocol: Reverse-Transcription

Important points before starting

- The completed 20 µl 5′ ligation reactions from *Protocol: 5′ Ligation* are the starting materials for the reverse-transcription reactions.
- The reverse-transcription components are added directly to the wells of the PCR strip tubes or 96-well plates containing completed 5′ ligation reactions. New strip tubes or 96-well plates are not required.
- Set up reverse-transcription reactions on ice.
- Do not vortex the miScript SC Reverse Transcriptase or reverse-transcription reactions.
- Upon completion of the reverse-transcription reactions, proceed to *Protocol: "Beads + Bind" Mixture Preparation*. Alternatively, the completed reverse-transcription reactions can be stored at –20°C in a constant-temperature freezer.

Procedure

1. Prepare reagents required for the reverse-transcription reactions. Thaw miScript SC 5x RT Buffer and miScript SC 10x RT Nucleics at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.

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

2. Prepare the reverse-transcription reaction master mix on ice according to Table 5. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   The reverse-transcription master mixes contain all components required for reverse-transcription (10% greater than required for the total number of reactions) except the completed 5′ ligation reactions.

Table 5. Setup of reverse-transcription reactions

Component	Volume one sample	Master mix 24 samples	Master mix 96 samples
5′ ligation reaction (already in well of PCR strip tube or 96-well plate)	20 µl	–	–
miScript SC 5x RT Buffer	8 µl	216 µl	864 µl
miScript SC 10x RT Nucleics	4 µl	108 µl	432 µl
Nuclease-free water	7 µl	189 µl	756 µl
miScript SC Reverse Transcriptase	1 µl	27 µl	108 µl
Total volume	**40 µl**	**540 µl**	**2160 µl**

3. On ice, aliquot 20 µl of the reverse-transcription master mix into each well of the PCR strip tubes or 96-well plates containing the completed 5′ ligation reaction. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   Note: The reverse-transcription master mix is added directly to the wells of the PCR strip tubes or 96-well plates containing the completed 5′ ligation. New strip tubes or 96-well plates are not required.

4. Incubate for 2 hour at 37°C.
5. Incubate for 5 min at 95°C to inactivate the miScript SC Reverse Transcriptase and hold at 4°C.

   Important: Hold at 4°C for at least 5 min.

6. Proceed to *Protocol: "Beads + Bind" Mixture Preparation*. Alternatively, the completed reverse-transcription reactions can be stored at –20°C in a constant-temperature freezer.

Protocol: “Beads + Bind” Mixture Preparation

Important points before starting

- miScript SC Cleanup Beads need to be thoroughly mixed and homogeneous. This necessitates working quickly and vortexing the beads immediately before use. If a delay in the protocol occurs, simply re-vortex the beads.
- **Important:** Ensure the miScript SC Cleanup Bind is ice cold at all times.
- **Note:** The “Beads + Bind” mixture provides enough mixture for the cleanup of 8 cDNA syntheses. Prepare enough “Beads + Bind” mixtures to accommodate your number of reverse-transcription reactions.

  For example, if you have 24 cDNA syntheses, prepare three separate “Beads + Bind” mixtures. As a second example, if you have 96 cDNA syntheses, prepare 12 separate “Beads + Bind” mixtures.
- **Important:** After preparation, the “Beads + Bind” mixture needs to be placed on ice.

Procedure

1. Prepare reagents required for the “Beads + Bind” mixture. Ensure the miScript SC Cleanup Bind is ice cold (4°C) and the miScript SC Cleanup Beads are at room temperature (15–25°C). Mix both reagents by vortexing. Do not centrifuge the reagents.

   Important: miScript SC Cleanup Beads need to be thoroughly mixed and homogeneous. If a delay in the protocol occurs, simply re-vortex the beads.

   Important: Ensure the miScript SC Cleanup Bind is ice cold at all times.
2. Determine how many “Beads + Bind” mixtures need to be prepared, and repeat steps 3 through 8 for each mixture.

   Important: Each “Beads + Bind” mixture provides enough mixture for the cleanup of 8 cDNA syntheses.

3. Thoroughly resuspend the miScript SC Cleanup Beads by vortexing.
4. Pipet 13.5 µl of the miScript SC Cleanup Beads into a microfuge tube.
5. Pipet 100 µl of ice cold miScript SC Cleanup Bind onto the beads and vortex to resuspend.
6. Immediately separate the beads from suspension on a magnet stand.
7. Using a pipet, remove the supernatant while taking care not to disturb the beads.
8. Pipet 360 µl of ice cold miScript SC Cleanup Bind onto the beads and vortex to resuspend.
9. The "Beads + Bind" mixture for the cleanup of eight cDNA syntheses is now prepared and must be stored on ice.
10. Proceed immediately to *Protocol: cDNA Cleanup*.

Protocol: cDNA Cleanup

Important points before starting

- The completed 40 µl cDNA syntheses from *Protocol: Reverse-Transcription* are the starting materials for the cleanup procedure.
- The "Beads + Bind" mixture prepared in *Protocol: "Beads + Bind" Mixture* is required for the cleanup procedure. Each "Beads + Bind" mixture provides enough mixture for the cleanup of eight cDNA syntheses. Prepare enough "Beads + Bind" mixtures to accommodate your number of cDNA syntheses.

- **Important:** The "Beads + Bind" mixture and the synthesized cDNA need to be ice cold.
- **Important:** 80% ethanol must be freshly prepared.
- **Note:** Using the DynaMag–96 Side Magnet, it is best to process the reactions in "columns" to ensure that the bead pellets are all on the same side of the PCR strip tubes or 96-well plate wells.

Procedure

1. Ensure the "Beads + Bind" mixture is thoroughly mixed at all times. This necessitates working quickly and resuspending the beads immediately before use. If a delay in the protocol occurs, simply vortex the beads.
2. Add 40 µl of the "Beads + Bind" mixture to each cDNA synthesis and vortex.

   Note: Using the DynaMag–96 Side Magnet, it is best to process the reverse-transcription reactions in "columns" to ensure that the bead pellets are all on the same side of the PCR strip tubes or 96-well plate wells.
3. Using an Eppendorf MixMate, shake at 1100 rpm for 10 min.
4. Separate the beads from suspension on a magnet stand. Remove and discard supernatant while taking care not to disturb the beads.
5. Add 150 µl of freshly prepared 80% ethanol to the beads. Mix by vortexing.

6. Separate the beads from suspension on a magnet stand. Remove and discard supernatant while taking care not to disturb the beads.
7. Add 150 µl of freshly prepared 80% ethanol to the beads. Mix by vortexing.
8. Separate the beads from suspension on a magnet stand. Remove and discard supernatant while taking care not to disturb the beads.
9. Ensure all residual ethanol has been completely removed. If needed, use a pipettor to remove remaining ethanol.
10. Air dry the beads for 10 minutes.
11. Add 20 µl Buffer EB to the beads. Mix by vortexing.
12. Using an Eppendorf MixMate, shake at 1100 rpm for 5 min.
13. Separate the beads from suspension on a magnet stand.
14. Taking care not to disturb the beads, pipet 16 µl from each well into the wells of a new PCR strip tube or 96-well plate.
15. Proceed to *Protocol: Preamplification*. Alternatively, the cleanup products can be stored at –20°C in a constant-temperature freezer.

Protocol: Preamplification

Important points before starting

- The 16 µl cleanup products from *Protocol: cDNA Cleanup* are the starting materials for the preamplification procedure.
- The preamplification components are added directly to the wells of the PCR strip tubes or 96-well plates containing the cleanup products. New strip tubes or 96-well plates are not required.
- Set up preamplification reactions on ice.
- Do not vortex the HotStarTaq DNA Polymerase or preamplification reactions.
- Upon completion of the preamplification, proceed to *Protocol: Real-time PCR Quality Control*. Alternatively, the completed preamplification reactions can be stored at –20°C in a constant-temperature freezer.

Procedure

1. Thaw PCR strip tube(s) or 96-well plate containing cleanup product. Centrifuge briefly to collect residual liquid from the sides of the tubes and place on ice.
2. Prepare reagents required for the preamplification reactions. Thaw miScript SC PreAMP Buffer and miScript SC PreAMP Universal Primer at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.

   HotStarTaq DNA Polymerase should be removed from the –20°C freezer just before preparation of the master mix, gently mixed and placed on ice. HotStarTaq DNA Polymerase should be returned to the freezer immediately after use.
3. Prepare the preamplification reaction master mix on ice according to Table 6. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

The preamplification master mixes contain all components required for reverse-preamplification (10% greater than required for the total number of reactions) except the cleanup products.

Table 6. Setup of preamplification reactions

Component	Volume one sample	Master mix 24 samples	Master mix 96 samples
Cleanup product (already in well of PCR strip tube or 96-well plate)	16 µl	–	–
miScript SC PreAMP Buffer	5 µl	135 µl	540 µl
miScript SC PreAMP Universal Primer	2 µl	54 µl	216 µl
HotStarTaq DNA Polymerase	2 µl	54 µl	216 µl
Total volume	**25 µl**	**243 µl**	**972 µl**

4. On ice, aliquot 9 µl of the preamplification master mix into each well of the PCR strip tubes or 96-well plates containing cleanup product. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   Note: The preamplification master mix is added directly to the wells of the PCR strip tubes or 96-well plates containing the cleanup product. New strip tubes or 96-well plates are not required.

5. Program the thermal cycler according to Table 7, Table 8 or Table 9. The correct program depends on the intended real-time PCR experiment.

   Important: Table 7 (12 cycles) should be used when qPCR will be performed on plate-based cyclers / Rotor-Discs when 1–8 96-well plates or 1–2 384-well plates will be used.

   Important: Table 8(13 cycles) should be used when qPCR will be performed on the Fluidigm BioMark HD or on plate-based cyclers / Rotor-Discs when 9–16 96-well plates or 3–4 384-well plates will be used.

Important: Table 9 (14 cycles) should be used when qPCR will be performed on plate-based cyclers / Rotor-Discs when 17–32 96-well plates or 5–8 384-well plates will be used.

Table 7. Twelve cycles of preamplification

Step	Time	Temperature
PCR initial activation step	15 min	95°C
HotStarTaq DNA Polymerase is activated by this heating step		
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	
Hold at 4°C	∞*	

* Hold at 4°C for at least 5 min.

Table 8. Thirteen cycles of 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	11 cycles	
Hold at 4°C	∞*	

* Hold at 4°C for at least 5 min.

Table 9. Fourteen cycles of 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	12 cycles	
Hold at 4°C	∞*	

* Hold at 4°C for at least 5 min.

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

   Important: Upon completion of the protocol, hold at 4°C for at least 5 min.

7. Immediately upon completion of the preamplification cycling, add 2 µl Side Reaction Reducer to each well of the PCR strip tubes or 96-well plates containing amplified product. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge again.

   Important: Ensure reactions are thoroughly mixed while taking care to avoid excess bubbles while mixing.

8. Incubate for 30 min at 37°C.

9. Incubate for 5 min at 95°C to inactivate the Side Reaction Reducer and hold at 4°C.

   Important: Hold at 4°C for 5 min.

10. Dilute preamplified product to 127 µl using 100 µl nuclease-free water. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge.
11. Proceed to *Protocol: Real-time PCR Quality Control*. Alternatively, the diluted preamplified product can be stored in at –20°C in a constant-temperature freezer.

Protocol: Real-time PCR Quality Control

Five control miScript Primer Assays are provided to assess reaction performance: miC3′ 10x Primer Assay, miC5′ 10x Primer Assay, miCRT 10x Primer Assay, miSCPA 10x miScript Primer Assay and PPC. The purpose of each control assay is detailed on page 12.

Important points before starting

- It is not necessary to perform control experiments for all individual samples. We recommend performing control experiments for a small number of representative samples.
- The diluted preamplified products from *Protocol: Preamplification* are the starting materials for the quality control procedure.
- Ensure the preamplified products have been diluted to 127 µl.
- The miScript SYBR Green PCR Kit is required for this quality control procedure.
- 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).
- **Important:** When amplified products have been prepared using the miScript Single Cell qPCR Kit, the recommended number of real-time PCR cycles is 35, not 40.
- For the real-time PCR quality control cycling program, there is no need to perform dissociation curve analysis of the PCR product(s).
- Do not vortex diluted, amplified products 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. Prepare reagents required for the real-time PCR quality control. Thaw control primers, components of the miScript SYBR Green PCR Kit (2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, and nuclease-free water) at room temperature (15–25°C). Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.
2. Thaw diluted preamplification product from *Protocol: Preamplification.* Mix by flicking the tube. Centrifuge the tubes briefly to collect residual liquid from the sides of the tubes and keep at room temperature.

   For each sample, prepare a master mix 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) according to
3. Table 10. Mix gently and thoroughly.

   Important: Reaction mix contains everything except the control primers. These are added in step 5.

Table 10. Setup of real-time PCR quality control

Component	Master mix (for 384-well)	Master mix (for 96-well)	Master mix (for Rotor-Disc 100)
2x QuantiTect SYBR Green PCR Master Mix	30 µl	75 µl	60 µl
10x miScript Universal Primer (provided in the miScript SYBR Green PCR Kit)	6 µl	15 µl	12 µl
Control Primer Assay (added in step 5)	–	–	–
Nuclease-free water	12 µl	39 µl	30 µl
Diluted preamplification product	6 µl	6 µl	6 µl
Total volume	**54 µl**	**135 µl**	**108 µl**

4. For each sample, dispense master mix into five individual wells of an empty plate / Rotor-Disc (9 µl for 384-well plates, 22.5 µl for 96-well plates, 18 µl for Rotor-Disc 100).
5. Into each sample's five wells containing master mix, dispense the five control primers (1 µl for 384-well plates, 2.5 µl for 96-well plates, 2 µl for Rotor-Disc 100).

   Note: Order of primers should be: 1. miC3, 2. miC5, 3. miCRT, 4. miSCPA, 5. PPC.
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 11.

   Note: For the real-time PCR quality control cycling program, there is no need to perform dissociation curve analysis of the PCR product(s).

Table 11. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	15 min	95°C	HotStartTaq 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¶	**35 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.
10. When the run is finished, analyze the data. First, define the baseline:

    Use the "Linear View" of the amplification plot to determine the earliest visible amplification. Set the baseline from cycle 2 to two cycles before the earliest visible amplification. 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 of the positive PCR control (PPC).

    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 (all plate-based PCR instruments) or 15 ± 2 (Rotor-Gene Q).
14. Interpret the C_T values for the miC3′, miC5′, miCRT and miSCPA controls using the values for the PPC control by calculating $\Delta C_T = C_T^{Control} - C_T^{PPC}$.

    If this value is less than 5 for all of the controls, then no inhibition of the reaction is apparent. No action is needed. If this value is greater than 5 for even one particular control, there is evidence of inhibition. See the “Troubleshooting Guide,” page 42.

15. If the representative samples have passed quality control, dilute all samples being assessed in accordance with the intended real-time PCR experiment listed in Table 12. Briefly centrifuge, mix by pipetting up and down 12 times and briefly centrifuge.

Important: Experiments intended for the Fluidigm BioMark HD, 1–4 96-well plates / Rotor-Discs or one 384-well plate do not require further dilution.

Table 12. Additional dilution of 125 µl preamplification product

Real-time PCR Application	Reaction Dilution
Fluidigm BioMark HD	No further dilution recommended
1–4 96-well plates/Rotor-Discs 1 384-well plate	No further dilution recommended
5–8 96-well plates/Rotor-Discs 2 384-well plates	Add 110 µl of diluted, preamplification product to 110 µl of nuclease-free water.
9–12 96-well plates/Rotor-Discs 3 384-well plates	Add 110 µl of diluted, preamplification product to 220 µl of nuclease-free water.
13–16 96-well plates/Rotor-Discs 4 384-well plates	Add 110 µl of diluted, preamplification product to 330 µl of nuclease-free water.
17–20 96-well plates/Rotor-Discs 5 384-well plate	Add 110 µl of diluted, preamplification product to 440 µl of nuclease-free water.
21–24 96-well plates/Rotor-Discs 6 384-well plates	Add 110 µl of diluted, preamplification product to 550 µl of nuclease-free water.
25–28 96-well plates/Rotor-Discs 7 384-well plates	Add 110 µl of diluted, preamplification product to 660 µl of nuclease-free water.
29–32 96-well plates/Rotor-Discs 8 384-well plates	Add 110 µl of diluted, preamplification product to 770 µl of nuclease-free water.

16. Proceed to *miScript Handbook Recommendations for Real-Time PCR Applications and Data Analysis*. Alternatively, the diluted samples can be dispensed into 110 µl aliquots and stored in at –20°C in a constant-temperature freezer.

miScript Handbook Recommendations for Real-Time PCR Applications and Data Analysis

Important points before starting

- The preamplified products diluted according to Table 12 are the starting materials for real-time PCR.
- **Important:** When amplified products have been prepared using the miScript Single Cell qPCR Kit, the recommended number of real-time PCR cycles is 35, not 40.

Protocol

1. For real-time PCR setup, proceed to the recommended miScript handbook in Table 13.

   Important: When amplified products have been prepared using the miScript Single Cell qPCR Kit, the required number of real-time PCR cycles is 35, not 40.

Table 13. miScript Handbook recommendations for real-time PCR

Real-time PCR application	Handbook recommendation for real-time PCR setup
Fluidigm BioMark HD	*miScript Microfluidics Handbook* (Page 20 or Page 24)
384-well plates, 96-well plates, or Rotor-Discs	Individual Assays: *miScript PCR System Handbook* (Page 22) PCR Arrays: *miScript miRNA PCR Array Handbook* (Page 30)

2. After performing the real-time PCR experiment, define the baseline and threshold and export the C_T values according to what is described in the *miScript Microfluidics Handbook* (Page 28, Steps 1–5), *miScript PCR System Handbook* (Page 34, Steps A1 and A2) or *miScript miRNA PCR Array Handbook* (Page 35–36, Steps 1–3).
3. Access the GeneGlobe Data Analysis Center at **www.qiagen.com/GeneGlobe** to perform automatic data analysis.

Note: When analyzing data associated with single cells, choose the "Single Cell Samples" option.

Note: When analyzing data associated with purified total RNA samples, choose the "Standard Samples" option.

Note: The cel-miR-39-3p assay, snoRNA/snRNA panel of assays and miRTC control assay found on miScript miRNA PCR Arrays (or as individual miScript Primer Assays) are not compatible with the miScript Single Cell qPCR Kit. Results of these assays should be ignored and not chosen for normalization or quality control purposes.

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 / or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

		Comments and suggestions
When performing real-time PCR quality control, evidence of inhibition of all control reactions (value of $C_T^{Control}$ – C_T^{PPC} > 5).		
a)	Quality control was not conducted on properly diluted preamplification product.	Expected ΔC_T is based on the preamplified product being diluted to 127 µl using 100 µl nuclease-free water.
b)	Incorrect programming of the real-time PCR instrument.	Compare the temperature profile with the protocol. See the cycling protocol in Table 11.
c)	Cell lysis was not performed correctly.	Ensure Buffer FCPM has been added to Buffer FCPL. Ensure the "cell processing mix" consists of FCPM + Genomic DNA Wipeout Buffer 2. Ensure cell lysis protocol has been performed as described.
d)	cDNA cleanup was not performed.	Cleanup ensures reverse-transcription components are not carried in excess and prevents inhibition of the preamplification reaction.
e)	Preamplification was not performed.	Preamplification ensures that there is sufficient target for quantification in subsequent real-time PCR. Ensure that preamplification was performed using the appropriate cycling conditions (Table 7, Table 8 or Table 9).
When performing real-time PCR quality control, evidence of 3′ ligation inhibition (value of $C_T^{miC3'}$ – C_T^{PPC} > 5) while all other controls (miC5′, miCRT, miSCPA) show no inhibition.		
a)	3′ ligation reaction was not set up correctly.	8 µl of the miScript SC Ligation Activator must be added to each 3′ ligation. In addition, the miScript SC Ligation Activator is very viscous. Therefore, it is important to set up the reactions at room temperature and pipet slowly, carefully, and thoroughly (up and down 12 times) to mix the reaction appropriately. Further, ensure the 3′ ligation occurs at 16°C for 1 hour.

	Comments and suggestions
When performing real-time PCR quality control, evidence of 3′ ligation inhibition and 5′ ligation inhibition (value of $C_T^{miC3'} - C_T^{PPC} > 5$ and $C_T^{miC5'} - C_T^{PPC} > 5$) while the other controls (miCRT, miSCPA) show no inhibition.	
a) 5′ ligation reaction was not set up correctly.	The miScript SC Ligation Activator (from the 3′ ligation reaction) is very viscous. Therefore, it is important to set up the 5′ ligation reactions at room temperature and pipet slowly, carefully and thoroughly (up and down 12 times) to mix the reaction appropriately. In addition, the signal of the 3′ ligation is dependent on a successful 5′ ligation. As a result, if the 5′ ligation fails, the 3′ ligation control will show failure as well. Last, ensure the 5′ ligation occurs at 37°C for 5 min followed by a heat kill at 65°C for 15 min to achieve optimal results.
When performing real-time PCR quality control, evidence of 3′ ligation inhibition, 5′ ligation inhibition and reverse-transcription inhibition (value of $C_T^{miC3'} - C_T^{PPC} > 5$, $C_T^{miC5'} - C_T^{PPC} > 5$, and $C_T^{miCRT} - C_T^{PPC} > 5$) while the preamplification control (miSCPA) show no inhibition.	
a) Reverse-transcription reaction was not set up correctly.	Ensure the reverse-transcription reaction is set up on ice and mixed thoroughly by pipetting up and down 12 times. Also, ensure the correct components have been added to the reverse-transcription reaction. In addition, the signal of the 3′ and 5′ ligation controls are dependent on a successful reverse-transcription reaction. As a result, if the reverse-transcription reaction fails, both the 5′ ligation and 3′ ligation controls will show failure as well. Last, ensure the reverse-transcription occurs at 37°C for 2 hour followed by a heat kill at 95°C for 5 min to achieve optimal results.
b) cDNA cleanup was not performed as described.	It is critical for the miScript SC Cleanup Beads to be prepared as described using ice-cold miScript SC Cleanup Bind. In addition, the exact amount of the “Beads + Bind” mixture must be added to the synthesized cDNA.

Appendix: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice.

To remove RNase contamination from bench surfaces, nondisposable plasticware and laboratory equipment (e.g., pipets and electrophoresis tanks), use of RNaseKiller (cat. no 2500080) from 5 PRIME (**www.5prime.com**) is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 45), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate

* When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate Safety Data Sheets (SDS) available from the product supplier.

electrophoresis tanks, clean with detergent* (e.g., 0.5% SDS), rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant) and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent*, thoroughly rinsed and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Note: QIAGEN solutions, such as the components found in the miScript Single Cell qPCR Kit, are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. DEPC is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react

* When working with chemicals, always wear a suitable lab coat, disposable gloves and protective goggles. For more information, consult the appropriate Safety Data Sheets (SDS) available from the product supplier.

with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA–RNA or RNA–RNA hybrids is not seriously affected unless a large fraction of the purine residues has been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

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

Ordering Information

Product	Contents	Cat. no.
miScript Single Cell qPCR Kit (24)	For 24 reactions: Cell Lysis, 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup and preamplification reagents; quality control primers	331053
miScript Single Cell qPCR Kit (96)	For 96 reactions: Cell Lysis, 3′ ligation, 5′ ligation, reverse-transcription, cDNA cleanup and preamplification reagents; quality control primers	331055
miScript SYBR Green PCR Kit (200)	For 200 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218073
miScript SYBR Green PCR Kit (1000)	For 1000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218075
miScript SYBR Green PCR Kit (2000)	For 2000 reactions: QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer	218076
miScript Microfluidics PCR Kit (5)	For 5 x 96. 96 Dynamic Array IFCs: Microfluidics qPCR Master Mix, miScript Microfluidics Universal Primer	331431
miScript Primer Assay (100)	10x miScript Primer Assay (contains one miRNA-specific primer)	Varies*

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

Product	Contents	Cat. no.
miScript Primer Assay 96 Plate	Customer-configured miScript Primer Assays in 96-well plates. Content may be chosen by the customer at **www.qiagen.com/GeneGlobe.** Two synthesis scales are available	218540
miScript miRNA PCR Array	Array of assays for a miRNome, pathway, disease or gene family; available in 96-well, 384-well or Rotor-Disc 100 format	Varies
miScript miRNA PCR Array, Format M	Single-use miRNome or biology focused panels of miRNA primer assays ready for use with the Fluidigm BioMark System	Varies
RT^2 PCR Array Loading Reservoir	12 x 5 ml capacity, irradiation-sterilized loading reservoirs	338162
384 EZLoad Covers	Pack of 4 color-coded covers for loading 384-well plates	338125

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.

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Notes

Notes

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Technical assistance

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