Fourth Edition **October 2011**

miScript PCR System Handbook

miScript II RT Kit

miScript SYBR® Green PCR Kit

miScript Primer Assays

miScript Precursor Assays

miScript PCR Starter Kit

For real-time PCR analysis of microRNA using SYBR Green detection

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Contents

Kit Contents

miScript II RT Kit	**(12)**	**(50)**
Catalog no.	**218160**	**218161**
Number of standard reactions*	**12**	**50**
miScript Reverse Transcriptase Mix	24 µl	100 µl
10x miScript Nucleics Mix	50 µl	200 µl
5x miScript HiSpec Buffer	100 µl	400 µl
5x miScript HiFlex Buffer	100 µl	400 µl
RNase-Free Water	1.9 ml	1.9 ml
Quick-Start Protocol	1	1

* A standard reaction is 20 µl in volume with 10 pg–2 µg total RNA (when using miScript HiSpec Buffer) or 10 pg–1 µg total RNA (when using miScript HiFlex Buffer).

miScript Primer Assay	**(100)**
Catalog no.	**Varies**†
Number of 50 µl reactions	**100**
10x miScript Primer Assay (contains one miRNA-specific primer)‡	1 vial

† Visit www.qiagen.com/GeneGlobe to search for and order an assay.

‡ The 10x miScript Primer Assay is supplied lyophilized and must be reconstituted according to the instructions in "Shipping and Storage" (page 6).

miScript Precursor Assay	**(100)**
Catalog no.	**Varies**§
Number of 50 µl reactions	**100**
10x miScript Precursor Assay (contains one precursor-miRNA–specific forward primer and one precursor-miRNA–specific reverse primer)¶	1 vial

§ Visit www.qiagen.com/GeneGlobe to search for and order an assay.

¶ The 10x miScript Precursor Assay is supplied lyophilized and must be reconstituted according to the instructions in "Shipping and Storage" (page 6).

miScript SYBR Green PCR Kit	**(200)**	**(1000)**
Catalog no.	**218073**	**218075**
Number of 50 µl reactions	**200**	**1000**
2x QuantiTect® SYBR Green PCR 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
10x miScript Universal Primer	1 ml	5 x 1 ml
RNase-Free Water	2 x 2 ml	20 ml
Quick-Start Protocol	2	2

miScript PCR Starter Kit	**(80)**
Catalog no.	**218193**
Number of 20 µl RT reactions	**10**
Number of 25 µl PCRs	**80**
miScript Reverse Transcriptase Mix	24 µl
10x miScript Nucleics Mix	50 µl
5x miScript HiSpec Buffer	100 µl
5x miScript HiFlex Buffer	100 µl
2x QuantiTect SYBR Green PCR Master Mix	1 ml
10x miScript Universal Primer	1 ml
Human RNU6B (RNU6-2) miScript Primer Assay*	200 µl
Human miR-15a miScript Primer Assay*	200 µl
RNase-Free Water	1.9 ml
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* miScript Primer Assays in the miScript PCR Starter Kit are provided as ready-to-use solutions and do not need to be reconstituted.

Shipping and Storage

The miScript II RT Kit, miScript SYBR Green PCR Kit, and miScript PCR Starter Kit are shipped on dry ice. The kits, including all reagents and buffers, should be stored immediately upon receipt at –20°C in a constant-temperature freezer.

miScript Primer Assays and miScript Precursor Assays are shipped lyophilized at room temperature. Store them at –20°C, either lyophilized or reconstituted (see next paragraphs). Avoid repeated freeze–thaw cycles. When stored under these conditions and handled correctly, the product can be kept for at least 18 months from date of receipt without reduction in performance.

miScript Primer Assays in the miScript PCR Starter Kit are provided as ready-to-use solutions and do not need to be reconstituted.

To reconstitute 10x miScript Primer Assay or 10x miScript Precursor Assay, briefly centrifuge the vial, add 550 µl TE, pH 8.0,† and mix by vortexing the vial 4–6 times. This will provide sufficient primer for 100 x 50 µl reactions. We

† For details, see "Equipment and Reagents to Be Supplied by User", page 17.

recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miScript II RT Kit, miScript SYBR Green PCR Kit, miScript PCR Starter Kit, miScript Primer Assay, and miScript Precursor Assay is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

The miScript II RT Kit, miScript SYBR Green PCR Kit, miScript PCR Starter Kit, miScript Primer Assays, and miScript Precursor Assays 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.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any

difficulties regarding the miScript II RT Kit, miScript SYBR Green PCR Kit, miScript PCR Starter Kit, miScript Primer Assays, miScript Precursor Assays, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

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/Support/MSDS.aspx 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

Introduction

The miScript PCR System consists of the miScript II RT Kit, miScript SYBR Green PCR Kit, miScript Primer/Precursor Assay, and miScript miRNA PCR Array. The miScript PCR System allows sensitive and specific detection and quantification of microRNA (miRNA). 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.

In contrast to other systems, the miScript PCR System enables detection of multiple miRNAs from a single cDNA preparation. The miScript PCR System can also be used for the detection of precursor miRNAs, other noncoding RNAs, and mRNAs.

miScript II RT Kit

The expanded miScript II RT Kit includes miScript Reverse Transcriptase Mix, 10x miScript Nucleics Mix, 5x miScript HiSpec Buffer, and 5x miScript HiFlex Buffer. miScript Reverse Transcriptase Mix is an optimized blend of poly(A) polymerase and reverse transcriptase. 10x miScript Nucleics Mix contains dNTPs, rATP, oligo-dT primers, and an internal synthetic RNA control (miRNA reverse transcription control [miRTC]) that is used to assess reverse transcription performance during profiling experiments with miScript miRNA PCR Arrays (for more information, see the *miScript miRNA PCR Array Handbook*). Two buffers, 5x miScript HiSpec Buffer and 5x miScript HiFlex Buffer, are provided in the miScript II RT Kit to meet the distinctive needs of miRNA quantification studies using real-time PCR (Figure 1). The unique, patent-pending formulation of 5x miScript HiSpec Buffer facilitates the selective conversion of mature miRNAs into cDNA, which can then be used for miRNA quantification with either miScript Primer Assays or miScript miRNA PCR Arrays. 5x miScript HiFlex Buffer promotes conversion of all RNA species (mature miRNA, precursor miRNA, noncoding RNA, and mRNA) into cDNA, which can then be used in real-time PCR to quantify each RNA species using appropriate primer assays.

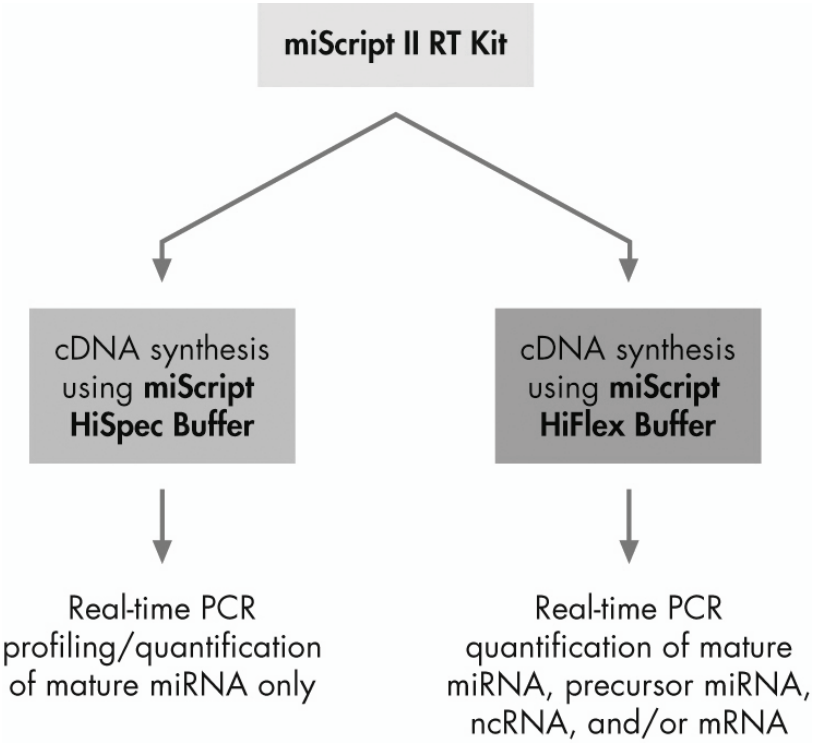


Figure 1. Mature miRNA, precursor miRNA, other noncoding RNA, and mRNA detection. Two buffers are supplied with the miScript II RT Kit. Use miScript HiSpec Buffer for cDNA synthesis to enable either mature miRNA profiling (using miScript miRNA PCR Arrays) or mature miRNA quantification using individual miScript Primer Assays. For protocols using miScript HiSpec Buffer in combination with miScript miRNA PCR Arrays, see the *miScript miRNA PCR Array Handbook*. Use miScript HiFlex Buffer for cDNA synthesis to enable quantification of mature miRNA, precursor miRNA, noncoding RNA (ncRNA), and/or mRNA from the same cDNA. cDNA prepared using either miScript HiSpec Buffer or miScript HiFlex Buffer can be used with miScript PCR Controls.

Principle and procedure

Mature miRNAs are naturally occurring, 22-nucleotide, noncoding RNAs that mediate posttranscriptional gene regulation. Unlike mRNAs, miRNAs are not polyadenylated in nature.

Reverse transcription in miScript HiSpec Buffer

When reverse transcription reactions are performed using miScript HiSpec Buffer, mature miRNAs and certain small nucleolar RNAs and small nuclear RNAs (snoRNAs and snRNAs, see “Normalization controls”, page 15) are selectively converted into cDNA. Mature miRNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers (Figure 2). Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time PCR step. miScript Primer Assays, used in combination with the miScript SYBR Green PCR Kit, enable quantification of mature miRNA by real-

time PCR. The combination of polyadenylation and the universal tag addition ensures that miScript Primer Assays do not detect genomic DNA.

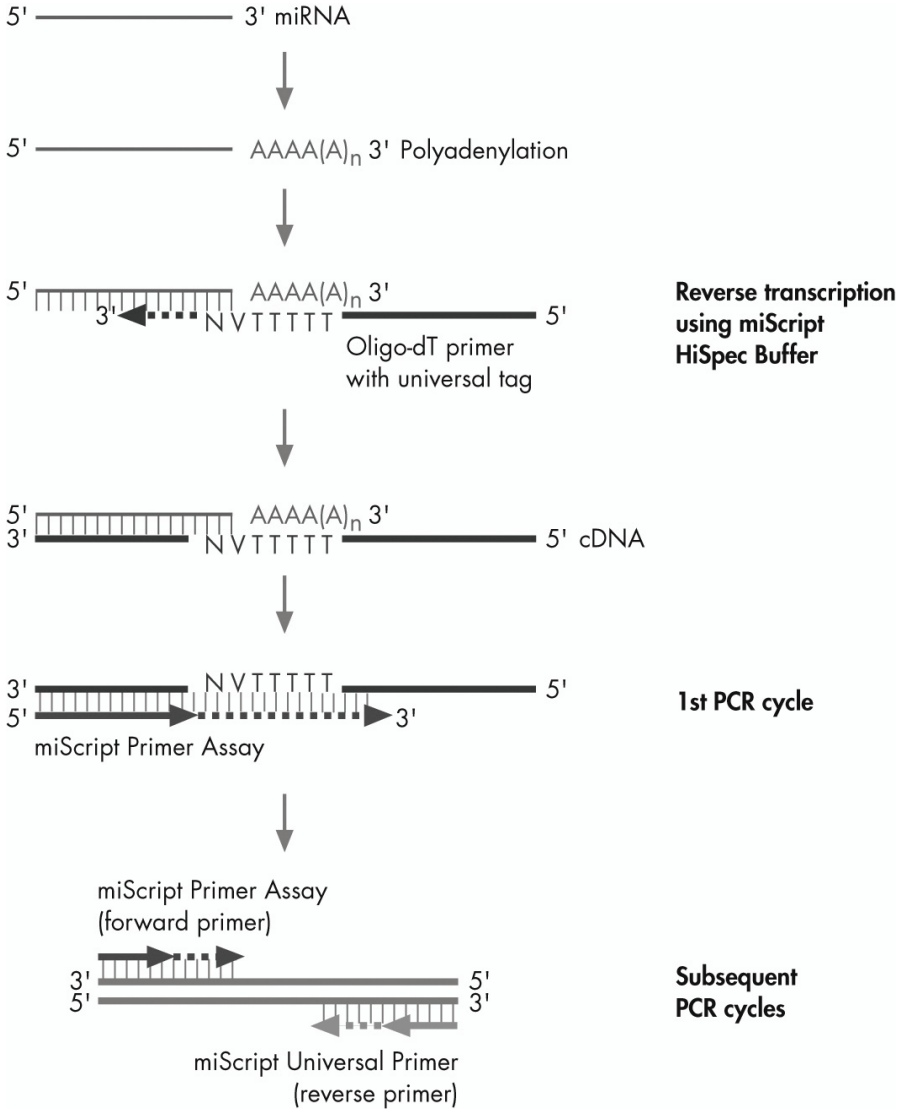


Figure 2. Selective conversion of mature miRNAs into cDNA in miScript HiSpec Buffer. In a reverse transcription reaction with miScript HiSpec Buffer, mature miRNAs are polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. The cDNA is then used for real-time PCR quantification of mature miRNA expression.

Reverse transcription in miScript HiFlex Buffer

When reverse transcription reactions are performed using miScript HiFlex Buffer, all RNA species are converted into cDNA (Figure 3). Mature miRNAs are polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end allowing amplification of mature miRNA in the real-time PCR step. miScript Primer Assays, used in combination with the miScript SYBR Green PCR Kit, enable quantification of mature miRNA

by real-time PCR. The combination of polyadenylation and the universal tag addition ensures that miScript Primer Assays do not detect genomic DNA.

All other RNA species (including precursor miRNA, other noncoding RNA, and mRNA) are also converted into cDNA using oligo-dT and random primers. Real-time PCR detection of these RNAs can then be performed using the appropriate assays (e.g., miScript Primer Assays for noncoding RNA detection, miScript Precursor Assays for precursor miRNA detection, and QuantiTect Primer Assays for mRNA detection) in combination with the miScript SYBR Green PCR Kit (Figure 3).

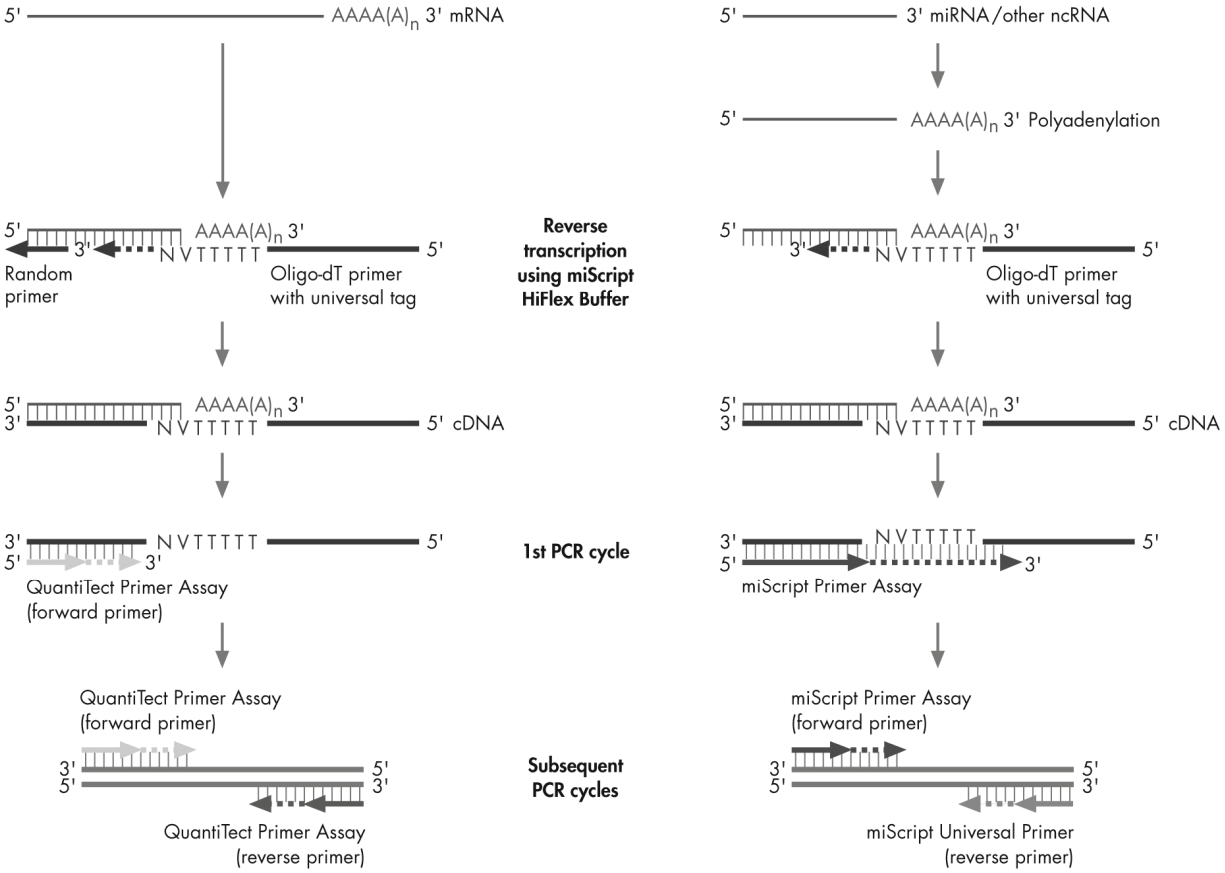


Figure 3. Simultaneous conversion of all RNA species into cDNA in miScript HiFlex Buffer. In a reverse transcription reaction with miScript HiFlex Buffer, miRNAs and other noncoding RNAs (ncRNAs) are polyadenylated by poly(A) polymerase and converted into cDNA by reverse transcriptase with oligo-dT priming. mRNAs are converted into cDNA by reverse transcriptase using both oligo-dT and random priming. Detection of mature miRNA, precursor miRNA, other ncRNA, and mRNA can be performed using the appropriate assays.

Real-time PCR

For detection of mature miRNA, cDNA prepared in a reverse transcription reaction using miScript HiSpec Buffer or miScript HiFlex Buffer serves as the template for real-time PCR analysis using an miRNA-specific miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix.

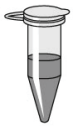
For detection of precursor miRNA, cDNA prepared in a reverse transcription reaction using miScript HiFlex Buffer serves as the template for real-time PCR analysis using a miScript Precursor Assay in combination with QuantiTect SYBR Green PCR Master Mix. QuantiTect SYBR Green PCR Master Mix is provided in the miScript SYBR Green PCR Kit. miScript Precursor Assays consist of a precursor-miRNA–specific forward and reverse primer targeting the stem-loop sequence of the precursor miRNA under study. The miScript Universal Primer is not used. The stem-loop sequence targeted by the miScript Precursor Assay is present in both primary miRNA (pri-miRNA) and precursor miRNA, therefore miScript Precursor Assays provide quantification of both primary and precursor miRNA simultaneously.

For detection of mRNA, cDNA prepared in a reverse transcription reaction using miScript HiFlex Buffer serves as the template for real-time PCR analysis using a QuantiTect Primer Assay in combination with QuantiTect SYBR Green PCR Master Mix. QuantiTect SYBR Green PCR Master Mix is provided in the miScript SYBR Green PCR Kit. QuantiTect Primer Assays consist of a forward and reverse primer targeting the mRNA of interest. The miScript Universal Primer is not used. Reference genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), potential target messenger RNAs of miRNAs, or any other mRNAs of interest can be quantified.

For detection of other noncoding RNA, cDNA prepared in a reverse transcription reaction using miScript HiFlex Buffer serves as the template for real-time PCR analysis using a miScript Primer Assay (forward primer) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. If a miScript Primer Assay for the small RNA of interest is not already available, one can be custom-designed (www.qiagen.com/miDesign).

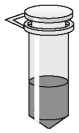
miScript PCR System Workflow

Prepare reverse transcription reaction

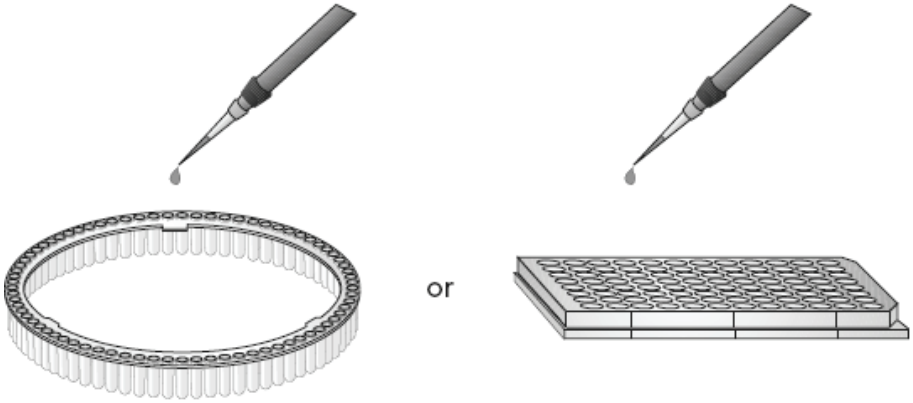


Incubate at 37°C for 60 min, then at 95°C for 5 min

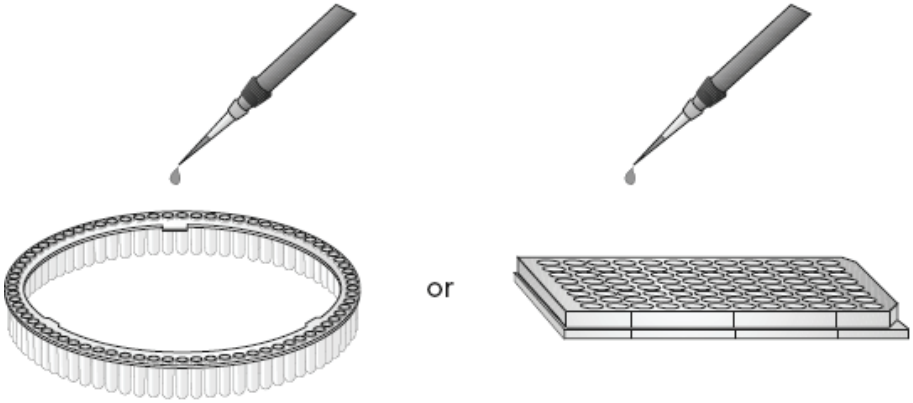
Prepare PCR mix



Add cDNA to tubes, Rotor-Disc, or plate



Add PCR mix to tubes, Rotor-Disc, or plate



Perform real-time PCR

Normalization controls

For accurate and reproducible results in miRNA quantification by real-time PCR, it is necessary to normalize the amount of target miRNA by using a suitable endogenous reference RNA. This approach is known as relative quantification. Normalization corrects for factors that could otherwise lead to inaccurate quantification. These factors include variation in quantity of input RNA, possible RNA degradation or presence of inhibitors in the RNA samples, and differences in sample handling. Normalization also allows results from different experiments and samples to be compared directly.

miScript PCR Controls are primers designed to quantify a panel of 5 snoRNAs (SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A) and the snRNA RNU6B (RNU6-2). These controls take into consideration sequence homologies in human, mouse, rat, and dog so that the same controls can be used for all 4 species. In addition, these RNAs have been verified to have relatively stable expression levels across tissues and cell types. cDNA prepared using either miScript HiSpec Buffer or miScript HiFlex Buffer can be used with miScript PCR Controls. As a result, miScript PCR Controls act as normalization controls for relative quantification using the miScript PCR System. All the controls have amplification efficiencies close to 100%. For more information and to view data, visit www.qiagen.com/miRNAControls.

A miScript PCR Control is provided in the miScript PCR Starter Kit. The control targets the snRNA RNU6B (RNU6-2).

Template RNA requirements

Total RNA containing miRNA is the starting material required for the miScript PCR System. It is not necessary to enrich for small RNA. QIAGEN provides a range of solutions for purification of total RNA containing miRNA (Table 1). For more information on miRNA purification, visit www.qiagen.com/miRNA.

Table 1. Kits for purification of RNA including miRNA

Kit	Cat. no.	Starting material
miRNeasy Mini Kit*	217004	Animal/human tissues, cells, and serum/plasma†
miRNeasy 96 Kit*	217061	Animal/human tissues and cells
miRNeasy FFPE Kit	217504	Formalin-fixed paraffin-embedded (FFPE) tissue samples
PAXgene® Tissue miRNA Kit	766134	Animal/human tissues that have been fixed and stabilized in PAXgene Tissue Containers
PAXgene Blood miRNA Kit	763134	Human blood that has been stabilized in PAXgene Blood RNA Tubes

* For the quantification of precursor miRNA and mRNA, we recommended performing the on-column DNase digestion step, using the RNase-Free DNase Set (cat. no. 79254), when performing the protocol for total RNA purification using the miRNeasy Mini and miRNeasy 96 Kits. This ensures that any minute traces of genomic DNA are removed from the sample and is especially recommended for quantification of precursor miRNA due to the low levels of this miRNA species.

† miRNA can be isolated from serum or plasma samples using the *QIAGEN Supplementary Protocol: Purification of total RNA, including small RNAs, from serum or plasma using the miRNeasy Mini Kit*. For more information, visit www.qiagen.com/miRNeasyMiniResources.

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 reconstitution of 10x miScript Primer Assay or 10x miScript Precursor Assay

TE, pH 8.0 contains 10 mM Tris/HCl and 1 mM EDTA. To prepare 100 ml TE, pH 8.0, mix the following stock solutions:

- 1 ml of 1 M Tris/HCl, pH 8.0 (autoclaved)
- 0.2 ml of 0.5 M EDTA, pH 8.0 (autoclaved)
- 98.8 ml of distilled water

Alternatively, ready-made TE can be purchased from chemicals suppliers.

To reconstitute 10x miScript Primer Assay or 10x miScript Precursor Assay, briefly centrifuge the vial, add 550 µl TE, pH 8.0, and mix by vortexing the vial 4–6 times. We recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

For reverse transcription

- Thin-walled, DNase-free, RNase-free PCR tubes (for 20 µl reactions)
- Ice
- Thermal cycler, heating block, or water bath (capable of reaching 95°C)
- Microcentrifuge

For quantitative, real-time PCR

- miScript PCR Controls for normalization of real-time PCR results (visit www.qiagen.com/GeneGlobe to select and order controls; a control is provided in the miScript PCR Starter Kit)

Protocol: Reverse Transcription for Quantitative, Real-Time PCR

Important points before starting

- The miScript II RT Kit and the miScript PCR Starter Kit include 2 different 5x buffers: **5x miScript HiSpec Buffer** and **5x miScript HiFlex Buffer**. Only one of these buffers should be used in each reverse-transcription reaction. The correct buffer to use depends on the subsequent PCR application (see Table 2, page 19). The 10x miScript Nucleics Mix is used together with either 5x buffer.
  - **Use 5x miScript HiSpec Buffer** to prepare cDNA for the subsequent quantification of mature miRNA using miScript Primer Assays and the miScript Universal Primer. 5x miScript HiSpec Buffer should also be used for mature miRNA profiling using miScript miRNA PCR Arrays (for more information, see the *miScript miRNA PCR Array Handbook*).
  - **Use 5x miScript HiFlex Buffer** to prepare cDNA for the subsequent quantification of mature miRNAs, precursor miRNAs, mRNAs, and/or other noncoding RNAs from the same sample using miScript Primer Assays, miScript Precursor Assays, and/or QuantiTect Primer Assays. Quantification of the different RNA species may be performed simultaneously or at separate times.
- Total RNA containing miRNA should be used as starting material for reverse-transcription reactions. For RNA purification recommendations, see page 16. This protocol is for use with 10 pg–2 µg RNA in 5x miScript HiSpec Buffer, or for 10 pg–1 µg RNA in 5x miScript HiFlex Buffer. If using higher RNA amounts, scale up the reaction linearly. For quantification of precursor miRNA, the maximum amount of input RNA recommended is 500 ng. Recommended amounts of template RNA to use depend on the downstream PCR application and are shown in Table 2 (page 19). If working with RNA for the first time, read Appendix D (page 45).
- miScript miRNA Mimics can be used as an internal positive control during miRNA quantification (for a protocol, see Appendix C, page 43).
- For detection of precursor miRNA or mRNA, it is important to avoid the presence of contaminating genomic DNA in the RNA samples. In these cases, we recommend performing the optional on-column DNase digestion using the RNase-Free DNase Set (cat. no. 79254) when using the miRNeasy Mini or miRNeasy 96 Kits. In addition, a "No RT" control should be included. This is a sample which has gone through the reverse-transcription procedure without addition of miScript Reverse Transcriptase Mix.
- 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.

Table 2. Recommended RNA starting amounts and buffers for reverse transcription reactions for various PCR applications

PCR application	Assay	Buffer	Recommended RNA input
Mature miRNA quantification only	miScript Primer Assays	5x miScript HiSpec Buffer	Depends on abundance and number of target miRNAs to be quantified; from 10 ng up to a maximum of 2 µg
Parallel quantification of mature miRNAs, precursor miRNAs, mRNAs, and/or other noncoding RNAs	miScript Primer Assays, miScript Precursor Assays, and/or QuantiTect Primer Assays	5x miScript HiFlex Buffer	Depends on abundance and number of target miRNAs to be quantified; up to a maximum of 1 µg
Precursor miRNA detection	miScript Precursor Assays	5x miScript HiFlex Buffer	Depends on abundance and number of target miRNAs to be quantified; up to a maximum of 0.5 µg

Procedure

1. **Thaw template RNA on ice. Thaw 10x miScript Nucleics Mix, RNase-free water, and either 5x miScript HiSpec Buffer or 5x miScript HiFlex 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 3.**

   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 3. Reverse transcription reaction components

Component	Volume/reaction
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer*	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA (added in step 3)	Variable (see Table 2 for recommendations)†
Total volume	**20 µl**

* The correct buffer to use depends on the subsequent PCR application, see Table 2, page 19.

† If using RNA from serum or plasma samples prepared using 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 of the 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 real-time PCR immediately, dilute the cDNA in RNase-free water according to Table 4.**

   If you wish to store the reverse transcription reactions prior to real-time PCR, 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.

Table 4. cDNA dilution prior to PCR

PCR application	Assay	Reaction dilution
Mature miRNA quantification only	miScript Primer Assays	Depends on abundance of miRNAs of interest; ensure 50 pg–3 ng cDNA per PCR by adding at least 200 µl RNase-free water, or more if necessary, to the 20 µl reverse-transcription reaction
Parallel quantification of mature miRNAs, precursor miRNAs, mRNAs, and/or other noncoding RNAs	miScript Primer Assays, miScript Precursor Assays, and/or QuantiTect Primer Assays	Depends on abundance of RNAs of interest; for parallel detection of mature miRNA with either precursor miRNA and/or mRNA, ensure 10–20 ng cDNA per PCR; for parallel detection of mature miRNA and other noncoding RNAs, ensure 50 pg–3 ng cDNA per PCR
Precursor miRNA detection	miScript Precursor Assays	Depends on abundance of precursor miRNA of interest; ensure 10–20 ng cDNA per PCR

Protocol: Real-Time PCR for Detection of Mature miRNA or Noncoding RNA

cDNA prepared using the miScript II RT Kit with either miScript HiSpec Buffer (mature miRNA detection only) or miScript HiFlex Buffer (detection of mature miRNA and other noncoding RNA) is the appropriate starting material for this protocol. This protocol enables real-time PCR quantification of mature miRNA or noncoding RNA using target-specific miScript Primer Assays (forward primers) and the miScript SYBR Green PCR Kit, which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. This protocol should also be used with the miScript PCR Starter Kit using either the human RNU6B (RNU6-2) miScript Primer Assay or the human miR-15a miScript Primer Assay along with the miScript Universal Primer. In addition, we recommend that this protocol should be used with miScript PCR Controls.

Important points before starting

- 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).
- Ensure that the 20 µl cDNA synthesis reaction has been diluted appropriately. See Table 4, page 21 for recommendations.
- If quantifying mature miRNA and precursor miRNA in parallel, we recommend using the higher amount of cDNA template as recommended for precursor miRNA detection for all the reactions (10–20 ng). This enables direct comparison of results.
- Do not vortex template cDNA or the components of the miScript SYBR Green PCR Kit.
- If using the miScript Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 6. (miScript Primer Assays in the miScript PCR Starter Kit and the miScript Universal Primer are provided as ready-to-use 10x solutions and do not need to be reconstituted).
- 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 5 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).**

   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 5. 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	1 µl	2.5 µl	2 µl
10x miScript Primer Assay	1 µl	2.5 µl	2 µl
RNase-free water	Variable	Variable	Variable
Template cDNA (added at step 3)‡	≤1 µl	≤2.5 µl	≤2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene® Q 72-Well Rotor.

† 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.

‡ The volume of diluted cDNA should not exceed 10% of the final reaction volume. Volumes refer to cDNA prepared using the miScript II RT Kit with **miScript HiSpec Buffer** (mature miRNA detection only) or **miScript HiFlex Buffer** (detection of mature miRNA and other noncoding RNA), and diluted according to Table 4. The final concentration of cDNA should be within 50 pg–3 ng per reaction.

3. **Dispense template cDNA into the individual plate/Rotor-Disc wells.**
4. **Mix the reaction mix thoroughly but gently, and dispense appropriate volumes into the tubes or plate/Rotor-Disc wells containing template cDNA.**

5. **Carefully, tightly seal the plate or disc with caps, film, or Rotor-Disc Heat-Sealing Film.**
6. **Centrifuge for 1 min at 1000 *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 6.**

Table 6. 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® Mastercyler® 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.**

   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.

Protocol: Real-Time PCR for Detection of Precursor miRNA

cDNA prepared using the miScript II RT Kit with miScript HiFlex Buffer is the appropriate starting material for this protocol. This protocol enables real-time PCR quantification of precursor miRNAs using target-specific miScript Precursor Assays and QuantiTect SYBR Green Master Mix, which is provided in the miScript SYBR Green PCR Kit. miScript Precursor Assays consist of precursor-miRNA–specific forward and reverse primers. The miScript Universal Primer is not used.

Important points before starting

- 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).
- For detection of precursor miRNA, the cDNA can be diluted to an appropriate volume if necessary with RNase-free water (see Table 4, page 21).
- If quantifying mature miRNA and precursor miRNA in parallel, we recommend using the higher amount of cDNA template as recommended for precursor miRNA detection for all the reactions (10–20 ng). This enables direct comparison of results.
- **Do not use the miScript Universal Primer.**
- Do not vortex template cDNA or the components of the miScript SYBR Green PCR Kit.
- Genomic DNA (1 ng/reaction) can be used as a positive control. Amplification of genomic DNA in parallel with the precursor miRNA under study allows comparison of dissociation curves from both samples and verification that the dissociation curves overlap.
- If using the miScript Precursor Assay for the first time, be sure to reconstitute it before use according to the instructions in "Shipping and Storage", page 6.
- 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 Precursor 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 100-well Rotor-Discs).**

   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 Precursor Assay‡	1 µl	2.5 µl	2 µl
RNase-free water	Variable	Variable	Variable
Template cDNA (added at step 3)§	≤1 µl	≤2.5 µl	≤2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene Q 72-Well Rotor.

† 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.

‡ The miScript Precursor Assay contains both a forward and a reverse primer. Do not add miScript Universal Primer.

§ The volume of diluted cDNA should not exceed 10% of the final reaction volume. Volumes refer to cDNA prepared using the miScript II RT Kit with miScript HiFlex Buffer. The final concentration of cDNA should be 10–20 ng per reaction.

3. **Dispense template cDNA into the individual plate/Rotor-Disc wells.**
4. **Mix the reaction mix thoroughly but gently and dispense appropriate volumes into the plate/Rotor-Disc wells containing template cDNA.**

5. **Carefully, tightly seal the plate/Rotor-Disc with caps, film, or Rotor-Disc Heat-Sealing Film.**
6. **Centrifuge for 1 min at 1000 *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 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 Mastercyler 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.**

   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.

Protocol: Real-Time PCR for Detection of mRNA

cDNA prepared using the miScript II RT Kit with miScript HiFlex Buffer is the appropriate starting material for this protocol. This protocol enables real-time PCR quantification of mRNA using target-specific QuantiTect Primer Assays and QuantiTect SYBR Green Master Mix, which is provided in the miScript SYBR Green PCR Kit. QuantiTect Primer Assays consist of mRNA-specific forward and reverse primers. The miScript Universal Primer is not used. For more detailed information on mRNA detection by real-time PCR refer to the *QuantiTect Primer Assay Handbook* (www.qiagen.com/HB/PrimerAssay).

Important points before starting

- 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).
- For detection of mRNA, the cDNA can be diluted to an appropriate volume if necessary with RNase-free water (see Table 4, page 21).
- **Do not use the miScript Universal Primer.**
- Do not vortex template cDNA or the components of the miScript SYBR Green PCR Kit.
- If using the QuantiTect Primer Assay for the first time, be sure to reconstitute it before use according to the instructions in the *QuantiTect Primer Assay Handbook*.
- If using the iCyler 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 QuantiTect 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 9 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 100-well Rotor-Discs).**

   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 9. 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 QuantiTect Primer Assay‡	1 µl	2.5 µl	2 µl
RNase-free water	Variable	Variable	Variable
Template cDNA (added at step 3)§	≤1 µl	≤2.5 µl	≤2 µl
Total volume	**10 µl**	**25 µl**	**20 µl**

* These volumes can also be used for reactions set up in Strip Tubes for use with the Rotor-Gene Q 72-Well Rotor.

† 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.

‡ The QuantiTect Primer Assay contains both a forward and a reverse primer. Do not add miScript Universal Primer.

§ The volume of diluted cDNA should not exceed 10% of the final reaction volume. Volumes refer to cDNA prepared using the miScript II RT Kit with miScript HiFlex Buffer. The final concentration of cDNA should be 10–20 ng per reaction.

3. **Dispense template cDNA to the individual plate/Rotor-Disc wells.**
4. **Mix the reaction mix gently and thoroughly, and dispense appropriate volumes into the plate/Rotor-Disc wells containing template cDNA.**
5. **Carefully, tightly seal the plate/Rotor-Disc with caps, film, or Rotor-Disc Heat-Sealing Film.**
6. **Centrifuge for 1 min at 1000 *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 10.**

   Data acquisition should be performed during the extension step.

Table 10. 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 Mastercyler 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.

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.

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
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.
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	miScript Reverse Transcriptase Mix used with miScript HiSpec Buffer is intended for use with 10 pg–2 µg RNA. miScript Reverse Transcriptase Mix used with miScript HiFlex Buffer is intended for use with 10 pg–1 µg RNA. If using >1 µg RNA, scale up the reaction linearly to the appropriate volume. Do not use less than 10 pg RNA. See also Table 2, page 19.
e) RNA denatured	Denaturation of the template RNA is not necessary. If denaturation was performed, the integrity of the RNA may be affected.

	Comments and suggestions
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 real-time PCR was too high	Adding a high volume of reverse-transcription reaction to the PCR mix may reduce amplification efficiency and the linearity of the reaction. Generally, the volume of reverse-transcription reaction added should not exceed 10% of the final PCR volume.
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.
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.

	Comments and suggestions
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	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 template cDNA.

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 A: Real-Time PCR Data Capture

In a typical amplification plot resulting from a real-time PCR reaction, fluorescence is plotted against the number of cycles, producing sigmoidal-shaped plots (when using a linear scale). The threshold cycle (C_T) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable increase in fluorescence. Determination of C_T values is described below. There may be variation in how determination of C_T values is carried out depending on the real-time PCR cycler that is used.

A1. 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 (Figure 4). 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. Typical amplification plots of a mature miRNA are shown (Figure 5).

Note: 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.

A2. Define the threshold.

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

Note: Various PCR instruments (such as Applied Biosystems 7500, Stratagene® models Mx3005P® and Mx3000P®, Rotor-Gene Q) may require adjustment of the default "Manual C_T" threshold value of 0.2 to a lower value (e.g., 0.02) in order to analyze the data properly.

A3. Once data capture (determination of C_T values) is complete, perform expression analysis by relative quantification as described in Appendix B.

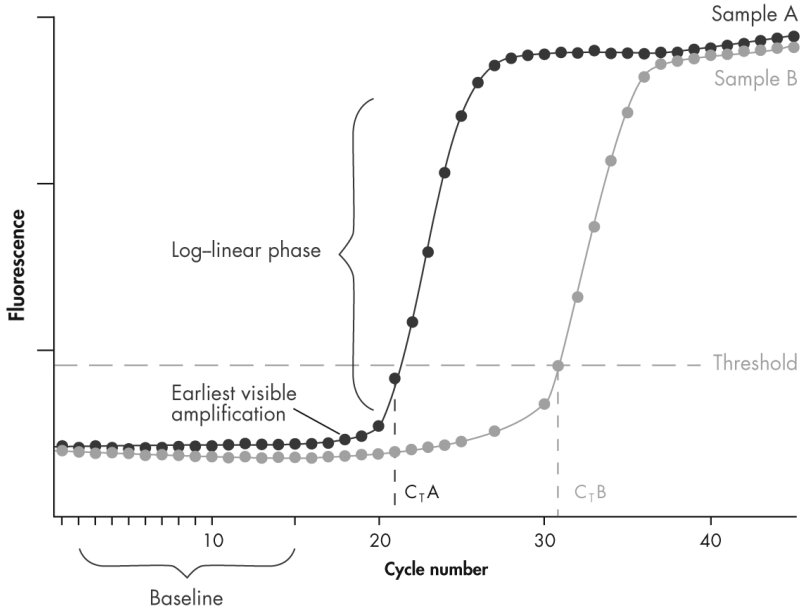


Figure 4. Amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B.

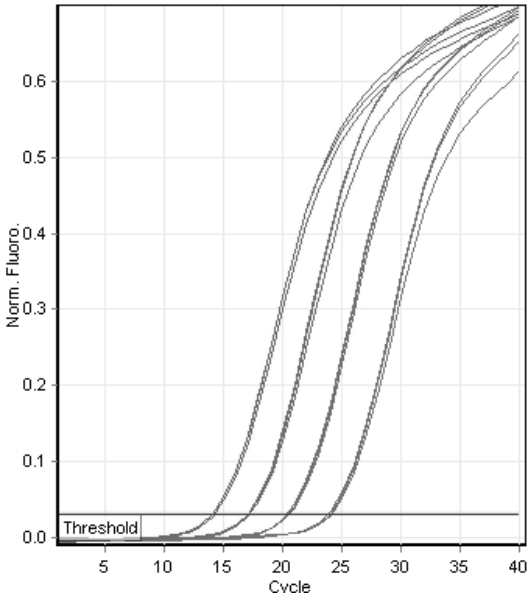


Figure 5. Typical amplification plot. Amplification plot after quantification of a range of amounts of miR-21. Real-time PCR was performed using the Rotor-Gene Q.

Dissociation curves and gel analysis

A dissociation curve analysis may be optionally performed to aid in verifying the specificity and identity of PCR product(s). Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow instructions provided by the supplier.

To carry out dissociation curve analysis, the temperature is increased very slowly from a low temperature (e.g., 65°C) to a high temperature (e.g., 95°C). At low temperatures, PCR products are double stranded, so SYBR Green I dye binds to

them and fluorescence is high. However at high temperatures, PCR products are denatured, resulting in rapid decreases in fluorescence.

The fluorescence is measured continuously as the temperature is increased and the fluorescence values are plotted against temperature. A curve is produced, because fluorescence decreases slightly through the lower end of the temperature range, but decreases much more rapidly at higher temperatures as the dissociation temperatures of nonspecific and specific PCR products are reached. The detection systems calculate the first derivatives of the curves, resulting in curves with peaks at the respective T_ms (Figures 6 and 7). Curves with peaks at a T_m lower than that of the specific PCR product indicate the formation of primer–dimers, while diverse peaks with different T_ms or plateaus indicate production of nonspecific products or a smear. Typical dissociation curves for mature miRNA and precursor miRNA are shown (Figures 6 and 7).

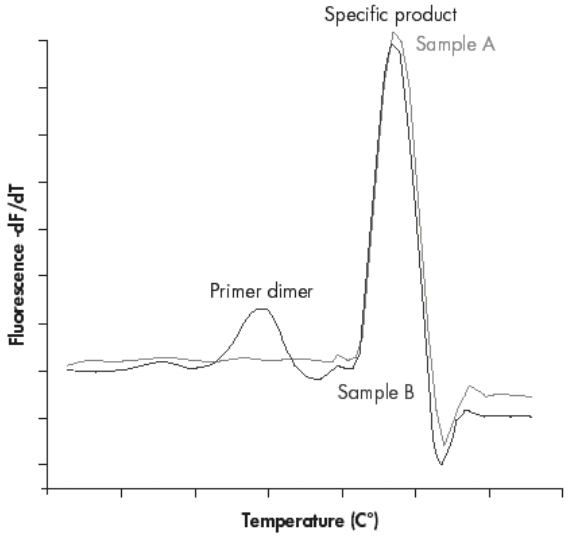


Figure 6. Dissociation curve analysis. Dissociation curve analysis of 2 samples (A and B). Sample A yields only one peak, resulting from the specific amplification product (primer–dimers not coamplified). Sample B shows a peak from the specific product and a peak at a lower temperature from amplification of primer–dimers.

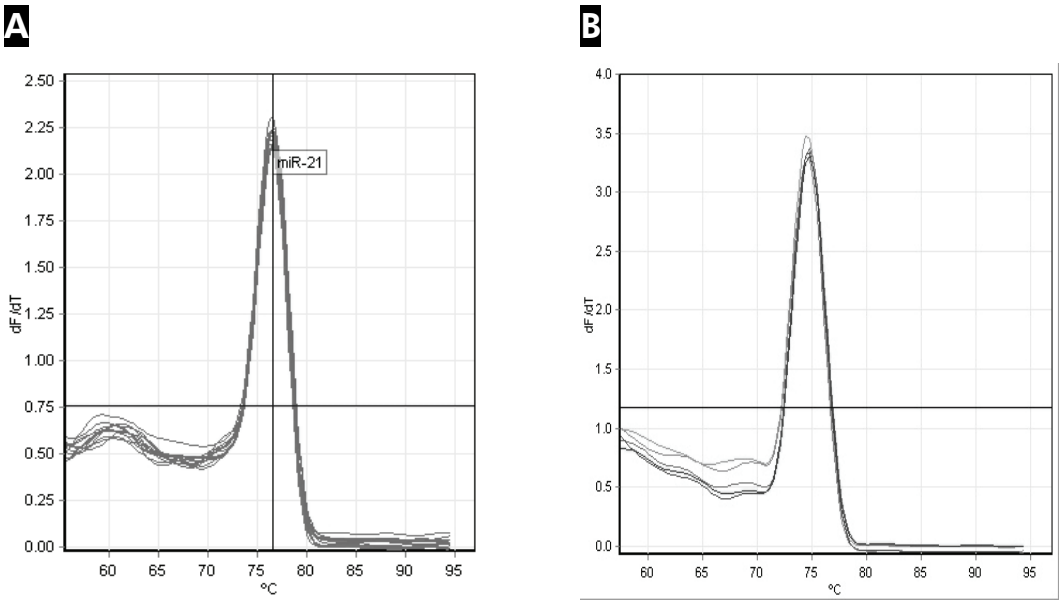


Figure 7. Dissociation curves. A Dissociation curve analysis of mature miRNA miR-21 PCR products showing single peaks from the specific amplification products. B Dissociation curve analysis of precursor miRNA miR-7-1 PCR products show single peaks from the specific amplification products. Dissociation curve analysis was performed using the Rotor-Gene Q.

In addition to dissociation curve analysis, if desired, PCR products can be run on a gel to verify specificity. A single band should be observed after gel electrophoresis. The size of the observed band depends on whether quantification of mature miRNA or precursor miRNA was performed (Figure 8 and Table 11). Table 11 shows the expected T_m after dissociation curve analysis and the expected band size after gene electrophoresis for a mature miRNA and a precursor miRNA PCR product.

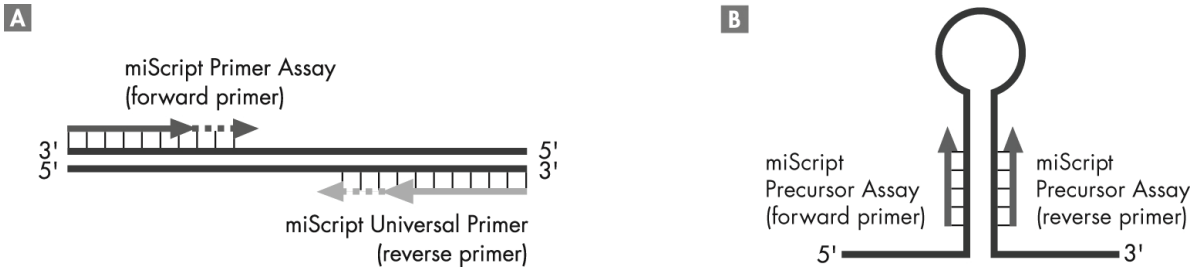


Figure 8. PCR amplification of mature and precursor miRNA. A Mature miRNA is quantified using a miScript Primer Assay and the miScript Universal Primer, resulting in a PCR product of 85–87 base pairs. B Precursor miRNA is quantified using a miScript Precursor Assay resulting in a PCR product of 45–100 base pairs, depending on the size of the stem-loop.

Table 11. Dissociation curve and gel analysis after mature and precursor miRNA quantification

	Mature miRNA	Precursor miRNA
Expected T_m*	74.5–76°C	70–90°C
Expected PCR product size	85–87 bp	45–100 bp†

* T_m values vary depending on the cycler used and on the base composition of the miRNA.

† The size of the PCR product for precursor miRNA varies depending on the size of the stem-loop.

Appendix B: Determination of PCR Efficiency and Quantification of miRNA for Single-Assay Experiments

The following paragraphs provide information on quantification of target nucleic acids. Further information can be found in *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/brochures to download a PDF.

Number of cycles

It is sufficient to use 40 cycles of PCR when using miScript or QuantiTect Primer Assays in combination with miScript or QuantiTect SYBR Green PCR Kits, regardless of the number of copies of starting template and the type of instrument used for real-time PCR.

Relative quantification

Gene expression levels can be normalized to the amount of RNA in the reaction. However, this relies on accurate determination of RNA concentration. Relative quantification is an alternative approach which determines the ratio between the amount of target RNA and a reference molecule that is present in all samples. In this approach, the normalized value can be used to compare, for example, differential expression of the target RNA in different tissues. The expression level of the reference RNA molecule, such as a housekeeping gene, must not vary under different experimental conditions, or in different states of the same tissue (e.g., "disease" versus "normal" samples). The level is therefore used as a reference value for quantification. The quantification procedure differs depending on whether the target nucleic acid and the reference molecule are amplified with comparable efficiencies. For determination of PCR efficiency, see page 41. miScript Primer Assays, miScript Precursor Assays, and QuantiTect Primer Assays are tested for high amplification efficiency. However, since amplification efficiencies are dependent on a number of factors (e.g., instrumentation, variation in pipetting and handling, presence of inhibitors in the sample, and the effect of RT reaction volume on PCR efficiency), we recommend quantifying target and reference amounts by using standard curves for both the target and reference genes (page 42).

miScript PCR Controls are miScript Primer Assays for several snoRNAs and snRNAs which are ideal for use as control reference RNAs to normalize the expression of miRNAs of interest in a given sample and to perform relative quantification of miRNAs (visit www.qiagen.com/GeneGlobe to order miScript PCR Controls). Housekeeping genes (e.g., GAPDH, β2-microglobulin, or peptidylprolyl isomerase A) or any other appropriate reference mRNA can be used to normalize the expression of mRNAs.

Different amplification efficiencies

An RNA standard should be used for gene expression studies, since this takes into account the variable efficiency of reverse transcription. The RNA standard, for example, total RNA prepared from a reference cell line or tissue, is used as a reference for all experiments. Two standard curves, one for the target gene and one for the reference molecule (such as a housekeeping gene), are prepared by accurately diluting the reference sample in 5-fold or 10-fold dilutions. Since the resulting standard curves may not be completely parallel, the differences in C_T values between the target and reference will not be constant when the amounts of target and reference are varied. The amounts of reference and target are calculated using the C_T values obtained from the sample of interest and the corresponding standard curve. The ratio of the resulting amounts of target and reference in the sample of interest can then be determined.

Guidelines for relative quantification with different amplification efficiencies of target and reference

- Choose an appropriate endogenous reference RNA whose expression level does not change under the experimental conditions or between different tissues (e.g., miScript PCR Controls [SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6B] for miRNAs/noncoding RNAs and GAPDH, β2-microglobulin, or peptidylprolyl isomerase A for mRNAs).
- Prepare a dilution series (e.g., 5-fold or 10-fold dilutions) of a cDNA control sample to construct standard curves for target and reference.
- Perform real-time PCR.
- Determine C_T values for the standards and samples of interest.
- Construct standard curves for both target and reference by plotting C_T values (y axis) against the log of template amount or dilution (x axis).
- Calculate amount of target and reference in samples of interest using C_T value and corresponding standard curve.
- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use the average value).
- Set the normalized target amount of one sample to 1 (calibrator sample). Compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator.

Comparable amplification efficiencies

If the amplification efficiencies of target and reference are comparable, gene expression levels can be determined by 2 methods. A single standard curve can

be generated for the reference sequence only. Unknown amounts of target and reference in each sample are calculated by comparing the resulting C_T values with the standard curve for the reference sequence. Before using this method, make sure that the amplification efficiencies of both target and reference are comparable (see page 42).

Guidelines for relative quantification with comparable amplification efficiencies of target and reference

- Choose an appropriate endogenous reference RNA whose expression level does not change under the experimental conditions or between different tissues (e.g., miScript PCR Controls [SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6B] for miRNAs/noncoding RNAs and GAPDH, β2-microglobulin, or peptidylprolyl isomerase A for mRNAs).
- Prepare a dilution series (e.g., 5-fold or 10-fold dilutions) of a cDNA control sample to construct a standard curve for the reference.
- Perform real-time PCR.
- Determine C_T values for the standards and samples of interest.
- Construct a standard curve for the reference by plotting C_T values (y axis) against the log of template amount or dilution (x axis).
- Calculate amount of target and reference in samples of interest using C_T value and standard curve.
- To calculate the normalized amount of target, divide the amount of target by the amount of reference (if replicate reactions were performed, use average value).
- Set the normalized target amount of one sample to 1 (calibrator sample). Compare relative expression level of target gene in samples of interest by dividing the normalized target amounts by the value of the calibrator.

An alternative approach is the comparative method ($\Delta\Delta C_T$ method), which relies on comparing differences in C_T values. Preparation of standard curves is only needed to determine the amplification efficiencies in an initial experiment, but not in each subsequent real-time PCR. However, this method can only be used if amplification efficiencies of target and reference sequence are nearly equivalent and close to 1. For more detailed information, refer to Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25**, 402.

Determination of PCR efficiency

Amplification efficiency should be determined by performing real-time PCR with a dilution series of nucleic acid that contains the sequence of interest. The C_T values obtained are used for construction of a standard curve. The amplification

efficiency (E) for the sequence of interest can be calculated according to the following equation:

$E = 10^{(-1/S)} - 1$ (S is the slope of the standard curve)

Comparison of PCR efficiencies

To compare the amplification efficiencies of 2 target sequences, prepare a dilution series for each target (targets A and B). Amplify each dilution series by real-time two-step RT-PCR. Subtract the C_T values/crossing points of target A from the C_T values of target B. Plot the differences in C_T values/crossing points against the logarithm of the template amount. If the slope of the resulting straight line is <0.1, amplification efficiencies are comparable.

Generating standard curves

To generate a standard curve, at least 5 different concentrations of the standard should be measured, and the amount of unknown target should fall within the range tested. Reactions should be carried out in sufficient number of replicates (usually 3 or more).

Appendix C: Protocol for Use of miScript miRNA Mimic as a Positive Control in miRNA Detection

This protocol describes the use of miScript miRNA Mimic as an internal positive control during miRNA quantification. Amplification of miScript miRNA Mimic in parallel with the miRNA under study allows comparison of dissociation curves from both samples. In addition, cDNA synthesized using miScript miRNA Mimic can be used to construct a standard curve which can be used for determination of copy number of the miRNA under study.

Things to do before starting

- Dilute miScript miRNA Mimic to a concentration of 10^{10} copies/µl (based on the molecular extinction coefficient or molecular weight and A_{260} reading.
- Prepare carrier bacterial RNA. For example, use the RNeasy® Protect Bacteria Mini Kit (cat. no. 74524) to isolate RNA from *E. coli* (any laboratory strain).

Procedure

C1. Prepare a 20 µl reverse-transcription reaction according to Table 3, page 20, using 5 µl miScript miRNA Mimic (10^{10} copies/µl) and 50 ng carrier bacterial RNA.

C2. Incubate for 60 min at 37°C.

C3. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix and place the cDNA preparation on ice.

C4. Add 480 µl carrier bacterial RNA (1 ng/µl concentration) to the 20 µl reaction. Mix gently by pipetting up and down and centrifuge briefly.

Note: This dilution yields 10^8 copies cDNA/µl (assuming an efficiency of 100%).

C5. Using the diluted cDNA mix from step C4 and carrier bacterial RNA (1 ng/µl), prepare serial dilutions as shown in Table 12.

Table 12. Serial dilutions of miScript miRNA Mimic

Dilution tube	Volume	Carrier RNA	Copies/µl	Use in PCR
1	5 µl diluted cDNA mix	45 µl	1×10^7	2 µl (2×10^7 copies)
2	5 µl from tube 1	45 µl	1×10^6	2 µl (2×10^6 copies)
3	5 µl from tube 2	45 µl	1×10^5	2 µl (2×10^5 copies)
4	5 µl from tube 3	45 µl	1×10^4	2 µl (2×10^4 copies)
5	5 µl from tube 4	45 µl	1×10^3	2 µl (2×10^3 copies)
6	5 µl from tube 5	45 µl	1×10^2	2 µl (2×10^2 copies)

C6. Use 2 µl from each of dilution tubes 2–6 to set up 5 separate real-time PCR reactions according to Table 5, page 23. Proceed with the protocol from step 3 onwards (page 23).

Template copy numbers in the PCRs range from 2×10^6 to 2×10^2.

Results from the PCRs can be used to plot a standard curve of C_T values (y axis) against log copy number (x axis).

Appendix D: 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 when aliquots are pipetted for downstream applications.

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 46), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate 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.

* 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.

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 miScript Nucleics Mix, miScript HiFlex Buffer, miScript HiSpec Buffer, and RNase-free water, 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. It 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 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 have 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.

Appendix E: Preparation, Quantification, and Storage of RNA

RNA preparation and quality

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, and EDTA than single-step enzyme-catalyzed reactions. Purity of nucleic acid templates is particularly important for real-time PCR, since contaminants can interfere with fluorescence detection. See Table 1, page 16 for kits recommended for the purification of total RNA that includes miRNA. For more information about kits for miRNA purification, visit www.qiagen.com/miRNA.

Determining concentration and purity of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The sample should be diluted in water since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA in water. To ensure significance, readings should fall between 0.15 and 1.0.

Note that absorbance measurements cannot discriminate between DNA and RNA. Depending on the method used for RNA preparation, RNA may be contaminated with DNA, and this will result in misleadingly high A_{260} values.

The ratio between the absorbance values at 260 nm and 280 nm and at 260 nm and 230 nm give an estimate of RNA purity. To determine RNA purity, we recommend measuring absorbance in 10 mM Tris/HCl, pH 7.5.* Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 and an A_{260}/A_{230} ratio of 2.0–2.2.† Lower ratios indicate the presence of contaminants such as proteins.

Storage of RNA

Purified RNA should be stored at –20°C or –70°C in RNase-free water. When purified using QIAGEN systems, no degradation is detectable for at least 1 year under these conditions. Diluted solutions of RNA (e.g., dilution series used as standards) should be stored in aliquots and thawed once only. We recommend storage of aliquots in siliconized tubes where possible. This avoids adsorption of the RNA to the tube walls, which would reduce the concentration of RNA in solution.

* 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.

† A_{260}:A_{280} values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris/HCl, pH 7.5) with some spectrophotometers.

DNA contamination in RNA samples purified from different tissues

Depending on the type of tissue used as starting material for RNA purification, a fluorescent signal may also be generated in "No RT" control reactions. When using QuantiTect Primer Assays, pseudogenes that are almost identical to the gene transcript sequence and also genes lacking introns may generate a signal from contaminating genomic DNA in late PCR cycles (see assay specifications). When RNA is purified from tissues that contain large amounts of DNA, such as spleen or thymus, the level of DNase treatment required may be higher than for other tissues. For such tissues, we recommend performing a DNase digestion (using the QIAGEN RNase-Free DNase Set, cat. no. 79254) when using the miRNeasy Mini and miRNeasy 96 Kits for RNA purification.

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 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
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*
miScript Precursor Assay (100)	10x miScript Precursor Assay (contains one miRNA-precursor–specific forward primer and one miRNA-precursor–specific reverse primer)	Varies*
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
Related products		
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 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, and 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
QuantiTect Primer Assay (200)	10x QuantiTect Primer Assay (contains a mix of forward and reverse primers for a specific target)	Varies*

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

Additional kit for DNase digestion during miRNA purification

RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer RDD, and RNase-Free Water	79254

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

www.qiagen.com

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Austria ■ Orders 0800-28-10-10 ■ Fax 0800-28-10-19 ■ Technical 0800-28-10-11

Belgium ■ Orders 0800-79612 ■ Fax 0800-79611 ■ Technical 0800-79556

Brazil ■ Orders 0800-557779 ■ Fax 55-11-5079-4001 ■ Technical 0800-557779

Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

China ■ Orders 86-21-3865-3865 ■ Fax 86-21-3865-3965 ■ Technical 800-988-0325

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Spain ■ Orders 91-630-7050 ■ Fax 91-630-5145 ■ Technical 91-630-7050

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