miRNA 1st-Strand cDNA Synthesis Kit

Instruction Manual

Catalog #600036

Revision D

This document contains two instruction manuals for use with Catalog # 600584. See the 600036-12 manual, shown first, for reverse transcription protocols. See the 600583-12 manual, shown second, for QPCR detection protocols.

Research Use Only. Not for Use in Diagnostic Procedures.

600036-12

Agilent Technologies

Limited Product Warranty

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Ordering Information and Technical Services

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit www.agilent.com/genomics/contactus

miRNA 1st-Strand cDNA Synthesis Kit

CONTENTS

Materials Provided 1
Storage Conditions 1
Additional Materials Required 1
Notices to Purchaser 2
Introduction 3
Overview of the miRNA 1st-Strand cDNA Synthesis Protocol 3
Preprotocol Considerations 4
Preparation of Total RNA 4
Quantity of RNA to Use as Template 4
Use of Manganese Chloride 4
No-PAP Control 4
QPCR Forward Primer Selection 5
General Notes 5
Protocols 6
Polyadenylation Reaction 6
RNA Purification 7
1st-Strand cDNA Synthesis 9
Troubleshooting 10
References 10
Endnotes 10
MSDS Information 10

miRNA 1st-Strand cDNA Synthesis Kit

MATERIALS PROVIDED

Catalog #600036

Materials Provided	Concentration	Quantity
E. coli Poly A Polymerase (PAP)	2 U/µl	100 U
E. coli Poly A Polymerase Buffer	5×	200 µl
Manganese chloride	25 mM	50 µl
rATP	10 mM	250 µl
Glycogen	20 mg/ml	25 µl
AffinityScript RT Buffer	10×	100 µl
RT Adaptor Primer	10 µM	50 µl
100 mM dNTP mix	25 mM each	40 µl
AffinityScript RT/RNase Block Enzyme Mixture	—	50 µl
Universal Reverse Primer	3.125 µM	200 µl

[a] The kit provides sufficient reagents for fifty 20-µl polyadenylation reactions and fifty 20-µl cDNA synthesis reactions.

STORAGE CONDITIONS

All materials: Store at –20°C upon receipt.

ADDITIONAL MATERIALS REQUIRED

Nuclease-free water

Revision D

© Agilent Technologies, Inc. 2012.

NOTICES TO PURCHASER

This product is licensed for research and development only under patents and patent applications jointly owned by BIOTIUM and ALLELOGIC.

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155, 5,677,152 (claims 1 to 23 only), 5,773,258 (claims 1 and 6 only), 5,407,800, 5,322,770, 5,310,652, 5,994,056, 6,171,785, and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser’s own internal research. No right under any other patent claim (such as apparatus or system claims in US Patent No. 6,814,934) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Notice to Purchaser: Limited License

Purchase of this product includes an immunity from suit under patents specified in the product insert to use only the amount purchased for the purchaser’s own internal research. No other patent rights are conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

INTRODUCTION

The miRNA 1st-Strand cDNA Synthesis Kit provides the reagents to elongate miRNAs in a polyadenylation reaction and then reverse transcribe the polyadenylated RNA into QPCR-ready cDNA. The cDNA may then be amplified using the provided universal reverse primer and a unique forward primer that is specific to the miRNA target of interst (not provided).

Overview of the miRNA 1st-Strand cDNA Synthesis Protocol

Elongation of miRNA with Poly A Polymerase

Because of their short length, miRNAs are difficult to detect with standard QRT-PCR protocols. To elongate the miRNAs, total RNA is first treated with *E. coli* poly A polymerase (PAP) to generate a poly-A tail at the 3´ end of each RNA molecule. The miRNA 1st-strand cDNA synthesis kit provides all the reagents needed for the polyadenylation reaction.

Manganese chloride is also included in the cDNA synthesis kit as an optional reagent in the polyadenylation reaction. Adding $MnCl_2$ to the reaction may improve the efficiency of poly A polymerase (see *Use of Manganese Chloride* in *Preprotocol Considerations* for further information). If $MnCl_2$ is included, the polyadenylated RNA will need to be purified with a phenol-chloroform extraction and ethanol precipitation before the cDNA synthesis step. The 20 mg/ml stock of glycogen is provided in the kit for this purpose.

Synthesis of 1st-Strand cDNA

Following polyadenylation, the RNA is used as template to synthesize 1st-strand cDNA. The cDNA synthesis protocol is optimized for reverse transcription of mRNA and miRNA templates. The reverse transcriptase (RT) provided with the 1st-strand cDNA synthesis kit is Agilent's AffinityScript RT, a genetically engineered version of Moloney murine leukemia virus RT. AffinityScript RT is provided in combination with RNase block as a safeguard against contaminating RNases, and is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis, particularly from small input RNAs.

The cDNA synthesis reaction is primed using the RT adaptor primer. This carefully designed primer anneals to the 3´ poly-A tail that was added during the polyadenylation reaction. In addition to this poly-A binding sequence, the RT adaptor primer also contains additional bases that create a universal sequence tag on each cDNA strand that is synthesized. This universal tag is incorporated at the 5´ end of the cDNA.

PREPROTOCOL CONSIDERATIONS

Preparation of Total RNA

The miRNA 1st-strand cDNA synthesis kit uses total RNA as the starting material in the protocol. This RNA sample may be prepared from any source or cell type using most standard RNA purification procedures. In some cases, the RNA isolation protocol may need to be modified to ensure recovery of small RNAs. The quality of the RNA preparation may impact the sensitivity of miRNA detection. RNA samples with $OD_{260/280}$ ratios of 1.8–2.0 are optimally pure. Enriching the RNA sample for miRNA is not necessary, but may improve detection of some difficult targets.

Quantity of RNA to Use as Template

The miRNA 1st-strand cDNA synthesis kit can accommodate a range of total RNA input amounts from 30 ng to 1 μg. The optimal quantity of RNA template depends on the RNA purity and the expression level of the particular miRNA of interest.

Use of Manganese Chloride

Manganese chloride is provided in the miRNA 1st-strand cDNA synthesis kit as an optional reagent in the polyadenylation reaction. Adding $MnCl_2$ to the reaction may improve the efficiency of the poly A polymerase enzyme. However, the presence of $MnCl_2$ during the subsequent cDNA synthesis and PCR reactions could lead to errors in nucleotide incorporation, creating mutations in the DNA sequence.[1, 2] In order to prevent $MnCl_2$ from interfering with these downstream steps, if $MnCl_2$ is included in the polyadenylation reaction, purify the polyadenylated RNA with a phenol-chloroform extraction and ethanol precipitation to remove the $MnCl_2$ before cDNA synthesis. A protocol is provided under *RNA Purification* in the *Protocols* section of the manual. If $MnCl_2$ is omitted, skip the purification protocol and proceed directly to the *1st-Strand cDNA Synthesis* section following polyadenylation.

No-PAP Control

To screen for contamination, consider including with the QPCR reactions a *no-PAP* control cDNA template. The *no-PAP* control cDNA is prepared from a polyadenylation reaction in which the poly A polymerase is omitted.

QPCR Forward Primer Selection

The primers used to perform QPCR with the miRNA-derived cDNA are the universal reverse primer provided in the miRNA 1st-strand cDNA synthesis kit and a unique forward primer that allows specific amplification of the target of interest. The universal reverse primer anneals to the cDNA sequence tag that was added to the 5´ end of all cDNA species by the RT adaptor primer during 1st-strand cDNA synthesis.

For the unique forward primer, use a custom primer specific to the target of interest. Ensure the custom primer is designed to be complementary to the 3´ end of the cDNA strand. Generally, the forward primer should be identical in sequence and length to the miRNA itself. Further guidelines on designing a custom forward primer for miRNA detection are available online at *www.genomics.agilent.com/files/LitItems/miRNA_primer_design_guidelines.pdf*. Once the sequence of the forward primer has been determined, the primer should be ordered through a custom oligo supplier. Dilute the primer stock to a concentration of 3.125 μM in TE (5 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) and store at –20°C.

General Notes

Preventing Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Preparing a Master Mix for Multiple Samples

If analyzing multiple samples, a master mix of reaction components may be prepared by combining the desired multiple of each component. Using a master mix facilitates accurate dispensing of reagents, minimizes loss of reagents during pipetting, and makes repeated dispensing of each reagent unnecessary, all of which help minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Enzymes (e.g. poly A polymerase) should be mixed gently without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

PROTOCOLS

Polyadenylation Reaction

1. Prepare the polyadenylation reactions by adding the following components *in order* to separate RNase-free 0.5-ml microcentrifuge tubes:

   RNase-free water to bring final volume to 20 µl (including polymerase added in step 2)
   4.0 µl of 5× poly A polymerase buffer
   1.0 µl of rATP (10 mM)
   1.0 µl of 25 mM $MnCl_2$ (optional)
   x µl of total RNA (30 ng – 1 µg)

   Note *Including $MnCl_2$ in the reaction may improve the efficiency of the PAP enzyme. If $MnCl_2$ is included, the polyadenylated RNA will need to be purified with a phenol-chloroform extraction and ethanol precipitation before cDNA synthesis.*

2. Add 1 µl of *E. coli* poly A polymerase to each reaction and mix gently (do not vortex). Briefly centrifuge the reactions to collect the contents at the bottoms of the tubes.

3. Incubate the reactions at 37°C for 30 minutes.

4. Incubate the reactions at 95°C for 5 minutes to terminate adenylation, then immediately transfer the tubes to ice.

5. Proceed to either *RNA Purification* (if $MnCl_2$ was included) or *1st-Strand cDNA Synthesis* (if $MnCl_2$ was omitted), or store the reactions at –20°C. For long term storage, store the reactions at –80°C.

RNA Purification

Note *If $MnCl_2$ was included in the polyadenylation reaction, the RNA needs to be purified to remove the $MnCl_2$ before proceeding to cDNA synthesis. Follow the protocol below to purify the RNA with a phenol-chloroform extraction and ethanol precipitation. Alternatively, RNA may be purified by gel filtration using a ready-to-use chromatography column packed with a polyacrylamide gel matrix. When using a chromatography column, the volume of the column filtrate may need to be reduced down to 10 µl by drying the filtrate in a vacuum centrifuge.*

If $MnCl_2$ was omitted, skip the RNA purification step and proceed directly to the 1st-Strand cDNA Synthesis *section.*

Additional Materials Required

The RNA purification protocol requires the following materials not included with the miRNA 1st-strand cDNA synthesis kit:

Siliconized 1.5-ml microcentrifuge tubes, RNase-free
Phenol:chloroform:isoamyl alcohol [25:24:1 (v/v/v)]
Chloroform
3.0 M Sodium acetate, pH 5.2
95% Ethanol prepared with RNase-free water (bring to 4°C before use)
80% Ethanol prepared with RNase-free water (bring to 4°C before use)
Vacuum centrifuge (e.g. SpeedVac® concentrator)
RNase-free water

Phenol-Chloroform Extraction and Ethanol Precipitation

1. To each polyadenylation reaction sample, add 80 µl of RNase-free water. Mix well.

2. Transfer each sample into a siliconized 1.5-ml microcentrifuge tube.

   Note *The use of low-retention, siliconized tubes helps prevent the loss of small RNAs during the purification procedure.*

3. Add 100 µl of phenol:chloroform:isoamyl alcohol to each sample and vortex.

4. Spin the samples in a microcentrifuge at 14,000 × g for 10 minutes at 4°C.

5. For each sample, transfer the aqueous upper-phase (containing the RNA) into a fresh 1.5-ml siliconized tube. Be careful to avoid disturbing the interface between the two layers.

6. Add an equal volume of chloroform to each sample and vortex the mixture.

7. Spin the samples in a microcentrifuge at 14,000 × g for 10 minutes at 4°C.

8. Again, transfer the aqueous upper-phase (containing the RNA) of each sample into a fresh 1.5-ml siliconized tube. Be careful to avoid disturbing the interface between the two layers.

9. Add the following to each sample:

   0.5 µl of 20 mg/ml glycogen
   10 µl of 3.0 M NaOAc, pH 5.2

10. Invert the tubes to mix contents then add 300 µl of cold 95% ethanol to each sample to precipitate the RNA.

    Note *Allowing the RNA to precipitate overnight at –20°C may improve yield.*

11. Spin the samples in a microcentrifuge at 14,000 × g for 10 minutes at 4°C to pellet the RNA precipitate. A white pellet should be visible near the bottom of the tube following centrifugation.

12. Carefully decant the supernatant without disturbing the pellet.

13. Wash the RNA pellet by adding 300 µl of cold 80% ethanol to each tube. Invert the tubes to mix contents.

14. Spin the samples in a microcentrifuge at 14,000 × g for 10 minutes at 4°C to pellet the washed RNA precipitate. A white pellet should be visible near the bottom of the tube.

15. Carefully decant the supernatant without disturbing the pellet

16. Dry the RNA pellets under vacuum centrifugation for 2–5 minutes. Do not overdry the sample.

17. Resuspend each RNA pellet in 10 µl of RNase-free water.

18. If proceeding directly to *1st-Strand cDNA Synthesis*, keep the tubes on ice. For long term storage, store the RNA at –80°C.

1st-Strand cDNA Synthesis

1. For each RNA sample, prepare a cDNA synthesis reaction by adding the following components *in order* to a RNase-free microcentrifuge tube:

   RNase-free water to bring final volume to 20 µl
   2.0 µl of 10× AffinityScript RT buffer
   10 µl of polyadenylated and purified RNA
   or 4 µl of the polyadenylation reaction
   0.8 µl of dNTP mix (100 mM)
   1.0 µl of RT adaptor primer (10 µM)
   1.0 µl of AffinityScript RT/RNase Block enzyme mixture

2. Gently mix the reactions (do not vortex) and briefly centrifuge the tubes.

3. Incubate the reactions at 55°C for 5 minutes.

4. Transfer the reactions to 25°C and incubate for 15 minutes.

5. Transfer the reactions to 42°C and incubate for 30 minutes to allow reverse transcription of 1st-strand cDNA.

6. Incubate the reactions at 95°C for 5 minutes to terminate reverse transcription.

7. Add up to 280 µl of RNase-free water to each reaction.

8. Place the completed 1st-strand cDNA synthesis reactions on ice for immediate use in QPCR. For long-term storage, keep the reactions at –20°C.

TROUBLESHOOTING

Observation	Suggestion
No or low yield of 1st-strand cDNA	Ensure the correct stock of dNTP mix (100 mM) was added to the cDNA synthesis reaction.
	Increase the concentration of polyadenylated template RNA added to the cDNA synthesis reaction.
	If $MnCl_2$ was not included in the polyadenylation reaction, try adding it to improve the efficiency of the reaction.
	As a positive control, carry out the entire protocol using the Agilent HeLa-S3 Cell Line Total RNA as the input RNA sample.

REFERENCES

1. Beckman, R. A., Mildvan, A. S. and Loeb, L. A. (1985) *Biochemistry* 24(21):5810-7.
2. Leung, D. W. (1989) *Journal of Methods in Cell and Molecular Biology* 1(1):11-15.
3. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

SpeedVac® is a registered trademark of Savant Instruments, Inc.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

miRNA 1st-Strand cDNA Synthesis Kit

Catalog #600036

Quick-Reference Protocol

Polyadenylation Reaction

1. Prepare the polyadenylation reactions by adding the following components *in order* to separate RNase-free 0.5-ml microcentrifuge tubes:

   RNase-free water to bring final volume to 20 μl (including polymerase added in step 2)
   4.0 μl of 5× poly A polymerase buffer
   1.0 μl of rATP (10 mM)
   1.0 μl of 25 mM $MnCl_2$ (optional)
   x μl of total RNA (30 ng–1 μg)

2. Add 1 μl of *E. coli* poly A polymerase to each reaction and mix gently (do not vortex).

3. Incubate the reactions at 37°C for 30 minutes.

4. Incubate the reactions at 95°C for 5 minutes to terminate adenylation, then immediately transfer the tubes to ice. To store the reactions, keep the tubes at –20°C or –80°C.

5. **If $MnCl_2$ was included in the reaction, purify the RNA with a phenol-chloroform extraction and ethanol precipitation before proceeding to cDNA synthesis.** A protocol for RNA purification is provided in the *Protocols* section of the manual.

cDNA Synthesis

1. For each RNA sample, prepare a cDNA synthesis reaction by adding the following components *in order* to a RNase-free microcentrifuge tube:

   RNase-free water to bring final volume to 20 μl
   2.0 μl of 10× RT buffer
   10 μl of polyadenylated and purified RNA or 4 μl of polyadenylation reaction
   0.8 μl of 100 mM dNTP mix (from the 1st-Strand cDNA Synthesis Kit)
   1.0 μl of RT adaptor primer (10 μM)
   1.0 μl of AffinityScript RT/RNase Block

2. Gently mix the reactions (do not vortex) and incubate at 55°C for 5 minutes.

3. Transfer the reactions to 25°C and incubate for 15 minutes.

4. Transfer the reactions to 42°C and incubate for 30 minutes to allow reverse transcription.

5. Incubate the reactions at 95°C for 5 minutes to terminate reverse transcription.

6. Add 280 μl of RNase-free water to each reaction. Proceed to QPCR or store at –20°C.

miRNA QPCR Master Mix

Instruction Manual

Catalog #600583

Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600583-12

Agilent Technologies

Limited Product Warranty

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Ordering Information and Technical Services

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.agilent.com/genomics/contactus*

miRNA QPCR Master Mix

CONTENTS

Materials Provided **1**

Storage Conditions **1**

Additional Materials Required **1**

Notices to Purchaser **2**

Introduction **3**

Universal Reverse Primer 3

EvaGreen® Dye 3

Preprotocol Considerations **4**

QPCR Forward Primer Selection 4

Recommended Controls for QPCR 4

Use of the Reference Dye 4

Data Acquisition with a Spectrofluorometric Thermal Cycler 5

Protocol **6**

QPCR Amplification 6

Troubleshooting **8**

References **9**

Endnotes **9**

MSDS Information **9**

miRNA QPCR Master Mix

MATERIALS PROVIDED

Catalog #600583

Materials Provided	**Concentration**	**Quantity** [a]
miRNA QPCR Master Mix [b]	2×	2.5 ml
Reference Dye [b]	1 mM	100 µl

[a] The miRNA QPCR master mix provides sufficient reagents for two hundred 25-µl QPCR reactions.
[b] The master mix and reference dye are light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All materials: Store at –20°C upon receipt. After thawing, the master mix may be stored at 4°C for up to six months.

Note *The master mix and reference dye are light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Agilent miRNA 1st-Strand cDNA Synthesis Kit (Catalog #600036)
miRNA-specific forward primer for QPCR
Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

Revision C © Agilent Technologies, Inc. 2012.

NOTICES TO PURCHASER

This product is licensed for research and development only under patents and patent applications jointly owned by BIOTIUM and ALLELOGIC.

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155, 5,677,152 (claims 1 to 23 only), 5,773,258 (claims 1 and 6 only), 5,407,800, 5,322,770, 5,310,652, 5,994,056, 6,171,785, and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as apparatus or system claims in US Patent No. 6,814,934) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Notice to Purchaser: Limited License

Purchase of this product includes an immunity from suit under patents specified in the product insert to use only the amount purchased for the purchaser's own internal research. No other patent rights are conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

INTRODUCTION

The miRNA QPCR Master Mix provides the reagents for quantitative PCR amplification of cDNA templates derived from micro RNAs (miRNAs) within a total RNA population. Because of their short length, miRNAs are difficult to detect with standard QRT-PCR protocols. As a first step, use the Agilent miRNA 1st-Strand cDNA Synthesis Kit (Catalog #600036) to elongate miRNAs in a polyadenylation reaction and then reverse transcribe the poly(A) RNA into QPCR-ready cDNA. The target of interest may then be amplified and detected using the miRNA QPCR master mix. The reagents in the master mix are sensitive enough to detect even low-abundance miRNAs and can discriminate between homologous miRNA species that differ by only a single nucleotide.

Universal Reverse Primer

The universal reverse primer* serves as the downstream primer in the QPCR reaction. This primer anneals to the universal tag that was added to the cDNA sequence during reverse transcription, making it capable of annealing to all cDNA targets. The specificity of the QPCR reaction is provided by the miRNA-specific forward primer. See *QPCR Forward Primer Selection* under *Preprotocol Considerations* for further guidelines.

EvaGreen® Dye

The miRNA QPCR master mix contains EvaGreen® dye, a double-stranded DNA-binding dye similar to SYBR® Green I dye, but with increased fluorescence, better stability, lower inhibition of PCR and increased specificity.[1, 2] With an excitation maximum at 500 nm and an emission maximum at 530 nm, EvaGreen is spectrally similar to SYBR Green, making it compatible with any spectrofluormetric thermal cycler that is equipped to detect SYBR Green fluorescence.

* The universal reverse primer is provided in the Agilent miRNA 1st-strand cDNA synthesis kit (Catalog #600036).

PREPROTOCOL CONSIDERATIONS

QPCR Forward Primer Selection

The primers used in the QPCR protocol are the universal reverse primer and a unique forward primer that allows specific amplification of the target of interest. The universal reverse primer anneals to the cDNA sequence tag that was added to the 5´ end of all cDNA species during reverse transcription with the Agilent 1st-strand cDNA synthesis kit.

For the unique forward primer, use a custom primer specific to the target of interest. Ensure the custom primer is designed to be complementary to the 3´ end of the cDNA strand. Generally, the forward primer should be identical in sequence and length to the miRNA itself. Further guidelines on designing a custom forward primer for miRNA detection are available online at *www.genomics.agilent.com/files/LitItems/miRNA_primer_design_guidelines .pdf*. Once the sequence of the forward primer has been determined, the primer should be ordered through a custom oligo supplier. Dilute the primer stock to a concentration of 3.125 µM in TE (5 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) and store at –20°C.

Recommended Controls for QPCR

No Template Control (NTC)

Include no-template control reactions for each experimental sample to screen for contamination of reagents or false amplification.

No-PAP Control

When performing the polyadenylation reaction with the RNA sample, include a *no-PAP* control reaction by omitting the poly A polymerase. The cDNA prepared from this reaction may then be used in a no-PAP control QPCR reaction to screen for contamination.

Use of the Reference Dye

The passive reference dye is provided as an optional reagent that may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Providing the reference dye in a separate tube makes it adaptable for many real-time QPCR platforms. Although addition of the reference dye is optional when using the Mx3000P, Mx3005P or Mx4000 system, with other instruments (including the ABI 7900HT and ABI PRISM® 7700) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If you are using the Agilent Mx3000P or Mx3005P real-time PCR system or Mx4000 multiplex quantitative PCR system, use the reference dye at a final concentration of 30 nM. If you are using the ABI 7900HT real-time PCR instrument or the GeneAmp® 5700 instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

Data Acquisition with a Spectrofluorometric Thermal Cycler

To detect EvaGreen dye fluorescence, the instrument should be set to collect SYBR Green or FAM emission. Collect fluorescence data in real-time at the annealing step of each cycle. How this is accomplished will depend on the software that commands the particular instrument you are using. Consult the instrument manufacturer's instruction manual to ensure the correct settings are used. If using the ABI 7900HT real-time PCR instrument, select the channel for SYBR Green detection.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

PROTOCOL

QPCR Amplification

Notes *The master mix is light-sensitive; solutions containing the master mix should be protected from light whenever possible.*

The protocol for generating cDNA from miRNA (using total RNA as the starting material) is provided in the manual for the Agilent miRNA 1st-strand cDNA synthesis kit, Catalog #600036.

Setting Up the QPCR Reactions

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI 7900HT real-time PCR instrument or GeneAmp 5700 instrument)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in *Use of the Reference Dye* under *Preprotocol Considerations*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the 1:500 dilution and 300 nM for the 1:50 dilution. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture for replicate experimental reactions and controls (plus at least one reaction volume excess), using multiples of each component listed below.

   Nuclease-free, PCR-grade H_2O to adjust the final volume to 25 µl (including cDNA added in step 4)
   12.5 µl of 2× miRNA QPCR master mix
   0.375 µl of diluted reference dye (optional)
   1.0 µl of 3.125 µM universal reverse primer*
   1.0 µl of 3.125 µM miRNA-specific forward primer

3. Gently mix the reactions without creating bubbles (do not vortex), then distribute to individual PCR reaction tubes.

* The universal reverse primer is provided in the Agilent miRNA 1st-strand cDNA synthesis kit (Catalog #600036).

4. Using cDNA generated from the miRNA 1st-strand cDNA synthesis kit, add 0.5–1.0 µl of cDNA sample to each reaction and gently mix without creating bubbles (do not vortex).

   Note *The optimal quantity of cDNA may vary depending on target abundance.*

5. Centrifuge the reactions briefly.

PCR Cycling

6. Place the reactions in the instrument and run the PCR program outlined in the table below. Use the instrument's optical setting designed for SYBR Green or FAM detection. Consult the instrument manufacturer's instruction manual to ensure the correct settings are used.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	10 seconds	95°C
	15 seconds[a]	60°C
	20 seconds[b]	72°C

[a] Set the temperature cycler to detect and report fluorescence during the annealing step of each cycle

[b] When detecting a mRNA (rather than a miRNA) increase the extension time as appropriate. Generally, an extension time of 1 minute/kb is recommended.

Note *This protocol has been designed for maximum performance on the Agilent Mx3000P and Mx3005P real-time PCR systems. Some optimization in cycling parameters may be required when using another real-time PCR instrument.*

Dissociation Program

Mx3000P and Mx3005P Instruments

Use the default EvaGreen experiment type (default cycling parameters and dissociation curve). The default profile of the dissociation curve begins with a 1-minute incubation at 95°C to melt the DNA and then a 30-second incubation at 55°C. This is followed by a ramp up to 95°C with *Allpoints data collection* performed during the ramp.

Mx4000 Instrument

Incubate the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec. For the dissociation curve, complete 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set the duration of each cycle to 30 seconds.

Other Instruments

Follow the manufacturer's guidelines for generating dissociation curves.

TROUBLESHOOTING

Observation	Suggestion
No or low yield of amplification product in QPCR	As a positive control, carry out the entire protocol using the Agilent HeLa-S3 Cell Line Total RNA as the input RNA sample and a validated miR-23a miRNA forward primer for QPCR.
	Analyze the PCR products on a gel to determine if there was successful amplification.
	Titrate the amount of cDNA template in the QPCR reaction. To optimize the amount of template, make serial dilutions of the 1st-strand cDNA to add to the QPCR reaction.
	Ensure the annealing temperature and extension temperature are set as described in the *Protocols* section.
	If the target is a cDNA from a mRNA (rather than a miRNA) optimize the annealing temperature and increase the extension time as appropriate for the primer Tm and amplicon length.
	Increase the number of thermal cycles.
	Make sure the miRNA-specific forward primer is not self-complementary. Verify that the forward primer is designed to be complementary to the appropriate strand.
	Ensure that the cycling program begins with a 10-minute incubation at 95°C in order to activate the PCR enzyme.
	Verify that the correct dilution of reference dye was used based on the type of QPCR instrument used.
	Ensure the instrument has been properly programmed for EvaGreen dye detection. The dye has an excitation maximum at 500 nm and emission maximum at 530 nm.
	If poor RNA quality is suspected, verify the integrity of the RNA sample by denaturing agarose gel electrophoresis or Agilent Bioanalyzer analysis to ensure it is not degraded. Isolate the RNA in the presence of a ribonuclease inhibitor and ensure that all reagents and labware are free of RNases.
	Replace the total RNA. Use the Agilent Absolutely RNA miRNA kit to isolate intact total RNA (which includes miRNA).
Increasing fluorescence in no-template control (NTC) reactions with cycling	The reaction has been contaminated; follow the procedures outlined in reference [3] to minimize contamination.
	To check for the formation of primer-dimers, analyze the QPCR products on a gel in addition to the dissociation curve analysis.
Increasing fluorescence in no-PAP control reactions with cycling	The reaction has been contaminated; use gel analysis or a dissociation curve to ensure that the experimental reactions amplify the correct product and that the no-PAP control reactions amplify nonspecific products.
cDNA titration curve is not linear	Prepare fresh dilutions of the cDNA and store at 4°C in low-binding tubes; avoid repeated freeze-thaw cycles.
	The reaction has been contaminated. Follow the procedures outlined in reference [3] to minimize contamination.
An abundance of nonspecific PCR products are observed on a gel	Re-design the miRNA-specific forward primer. For design guidelines, see *www.genomics.agilent.com/files/LitItems/miRNA_primer_design_guidelines.pdf*.

REFERENCES

1. Mackay, J. and Landt, O. (2007) *Methods Mol Biol* 353:237-61.
2. Sang, F. and Ren, J. (2006) *J Sep Sci* 29(9):1275-80.
3. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

ABI PRISM® is a registered trademark of Applied Biosystems.
EvaGreen® is a registered trademark of Biotium.
GeneAmp® is a registered trademark of Roche Molecular Systems, Inc.
SYBR® is a registered trademark of Molecular Probes.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

miRNA QPCR Master Mix

Catalog #600583

QUICK-REFERENCE PROTOCOL

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx3005P, Mx3000P, or Mx4000 instruments) or 1:50 (ABI PRISM® 7700 or GeneAmp® 5700 instruments). Keep all solutions containing the reference dye protected from light. *If using a system other than the Mx3005P, Mx3000P or Mx4000 instruments, the use of the reference dye may be required for optimal results.*

2. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture using multiples of each component below.

   Nuclease-free, PCR-grade H_2O to adjust the final volume to 25 μl (including cDNA)
   12.5 μl of 2× miRNA QPCR master mix
   0.375 μl of diluted reference dye (optional)
   1.0 μl of 3.125 μM universal reverse primer
   1.0 μl of 3.125 μM miRNA-specific forward primer

3. Gently mix the reactions without creating bubbles (do not vortex), then distribute to individual PCR reaction tubes.

4. Add 0.5–1.0 μl of the cDNA sample to each reaction and gently mix without creating bubbles (do not vortex). *Bubbles interfere with fluorescence detection*.

5. Centrifuge the reactions briefly and place in the instrument. Run the PCR program outlined in the table below. Use the instrument's optical setting for SYBR Green or FAM detection.

Cycles	Duration of cycle	Temperature
1	10 minutes	95°C
40	10 seconds	95°C
	15 seconds [a]	60°C
	20 seconds [b]	72°C

[a] Set the cycler to detect and report fluorescence during the annealing step of each cycle
[b] When detecting a mRNA (rather than a miRNA) increase extension time to 1 minute/kb

Dissociation Program

Mx3000P and Mx3005P Instruments: Use the default EvaGreen experiment type with dissociation curve.

Mx4000 Instrument: Incubate the amplified product for 1 minute at 95°C, ramping down to 55°C at a rate of 0.2°C/sec. For the dissociation curve, complete 81 cycles of incubation where the temperature is increased by 0.5°C/cycle, beginning at 55°C and ending at 95°C. Set the duration of each cycle to 30 seconds.

Other Instruments: Follow the manufacturer's guidelines for generating dissociation curves.