High-Specificity miRNA QPCR Core Reagent Kit

Instruction Manual

Catalog #600545

Revision H.0

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MATERIALS PROVIDED

Catalog #600545

Materials Provided	Concentration	Quantity [a]
High-Specificity PCR Enzyme Blend	4 U/µl	400 U
Core PCR Buffer	10×	0.5 ml
Reference Dye[b]	1 mM	100 µl
EvaGreen® Dye[b]	20×	250 µl
Magnesium chloride	50 mM	0.55 ml
20 mM dNTP mix	5 mM each	200 µl

[a] Sufficient reagents are provided for two hundred 25-µl QPCR reactions.

[b] The EveGreen dye 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. Once thawed, store the EvaGreen dye at 4°C. Continue storing remaining components at –20°C.

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

ADDITIONAL MATERIALS REQUIRED

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

Revision H.0

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INTRODUCTION

The High-Specificity miRNA QPCR Core Reagent Kit provides the reagents for quantitative PCR amplification of cDNA templates derived from 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 polyadenylated RNA into QPCR-ready cDNA. The target of interest is then amplified and detected using the high-specificity miRNA QPCR core reagent kit. The reagents are sensitive enough to detect even low-abundance miRNAs and can discriminate between homologous miRNA species that differ by only a single nucleotide.

QPCR Primers

The universal reverse primer (provided in the Agilent miRNA 1st-strand cDNA synthesis kit) 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. With a properly designed forward primer, the reagents in the core reagent kit can discriminate between homologous miRNA sequences, even those that differ by a single nucleotide. See *QPCR Forward Primer Selection* under *Preprotocol Considerations* for further guidelines.

EvaGreen® Dye

The high-specificity miRNA QPCR core reagent kit includes 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.

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 polyadenlyation 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.

Endogenous Control

Consider performing an endogenous control reaction to normalize variations in the amount of cDNA template across samples. For example, the U6 small nuclear RNA (snRNA) may be a good candidate for an endogenous control target when detecting miRNAs.[3] (Note that the nucleotide sequence of the U6 snRNA varies between organisms.)

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, be sure to 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 EvaGreen dye is light-sensitive; solutions containing the dye should be protected from light whenever possible.*

Before pipetting the EvaGreen dye, vortex the solution for several seconds to ensure the dye is not adhering to the tube.

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 no-template-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)
   2.5 µl of 10× core PCR buffer
   2.75 µl of 50 mM $MgCl_2$
   1.0 µl of 20 mM dNTP mix
   0.375 µl of diluted reference dye (optional)
   1.25 µl of 20× EvaGreen dye
   1.0 µl of 3.125 µM universal reverse primer
   1.0 µl of 3.125 µM miRNA-specific forward primer
   0.5 µl of High-Specificity PCR enzyme blend

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

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.

QPCR 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 dissociation curve for SYBR Green experiments and adjust the default cycling parameters of the amplification segments to match those described in the table above. 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 forward primer for QPCR.
	Ensure the stock of EvaGreen dye is well-mixed before adding it to the reaction. Vortexing the dye for several seconds is recommended. Solutions containing EvaGreen dye should be protected from light as much as possible.
	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 or complementary to the universal primer. Verify that the forward primer is designed to be complementary to the appropriate strand.
	Ensure that the cycling program includes the 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 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 Agilent RNA isolation kits to isolate intact total RNA.
Increasing fluorescence in no-template control (NTC) reactions with cycling	The reaction has been contaminated; follow the procedures outlined in reference [4] 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 4 to minimize contamination.
An abundance of nonspecific PCR products are observed on a gel	Re-design the miRNA-specific forward primer. For guidelines, see *www.genomics.agilent.com/files/LitItems/miRNA_primer_design_guidelines.pdf*.

REFERENCES

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2. Sang, F. and Ren, J. (2006) *J Sep Sci* 29(9):1275-80.
3. Thomson, J. M., Newman, M., Parker, J. S., Morin-Kensicki, E. M., Wright, T. *et al.* (2006) *Genes Dev* 20(16):2202-7.
4. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

MSDS INFORMATION

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

High-Specificity miRNA QPCR Core Reagent Kit

Catalog #600545

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)
   2.5 µl of 10× core PCR buffer
   2.75 µl of 50 mM $MgCl_2$
   1.0 µl of 20 mM dNTP mix (from the QPCR Core Reagent Kit)
   0.375 µl of diluted reference dye (optional)
   1.25 µl of 20× EvaGreen dye (vortex the dye before pipetting)
   1.0 µl of 3.125 µM universal reverse primer
   1.0 µl of 3.125 µM miRNA-specific forward primer
   0.5 µl of PCR enzyme blend

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 QPCR 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 dissociation curve for SYBR Green experiments. Adjust the default amplification segments to match the program in the table above.

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.