miRNA 1st-Strand cDNA Synthesis Kit

Instruction Manual

Catalog #600036

Revision E.0

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600036-12

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miRNA 1st-Strand cDNA Synthesis Kit

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miRNA 1st-Strand cDNA Synthesis Kit

MATERIALS PROVIDED

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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 E.0

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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.

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

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MSDS INFORMATION

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

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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.