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rtStar™ PreAMP cDNA Synthesis for Small RNA

Cat#: AS-FS-006-02

Instruction Manual version 1.0

Product Summary

Product description

rtStar™ PreAMP cDNA Synthesis Kit is designed to create cDNA libraries from low input small RNAs for qPCR. This method sequentially ligates 3'-Adaptor to the 3'-end of the RNAs, and 5'-Adaptor to the 5'-end of the RNAs. The first-strand cDNA is then reverse transcribed and preamplified by PreAMP PCR SuperMix from the priming sites in the 5' and 3' adaptor regions. The PreAMP PCR SuperMix contains an ultra-pure, highly processive, thermostable DNA polymerase. Residual primers are removed by PreAMP Primer Remover to eliminate their adverse impact on qPCR reaction. Spike-in control RNA is provided to monitor the cDNA synthesis efficiency and used as a quantitative reference.

Kit components

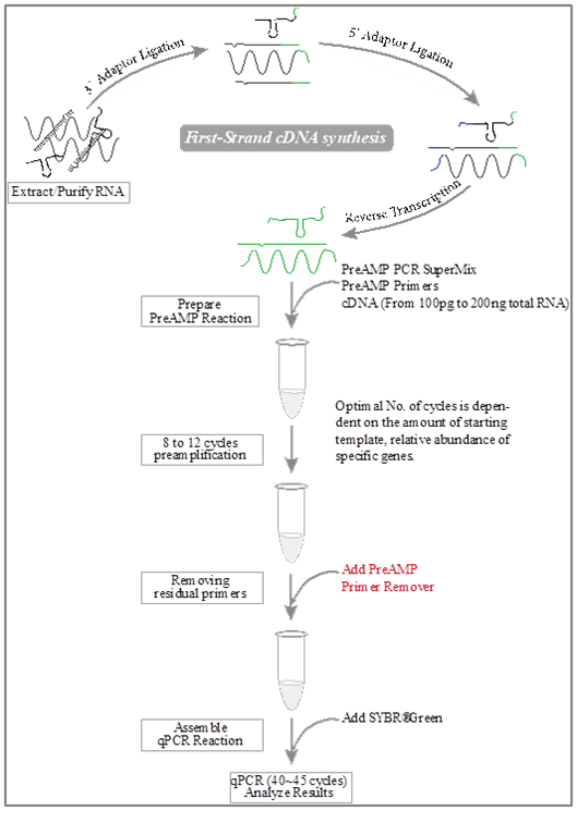
The provided kit components are sufficient for up to 12 cDNA synthesis and 48 preamplification reactions. The recommended range of total RNA sample input amount is 100 pg ~ 200 ng.

Kit component	Amount	Storage	Shipping
3' Adaptor	12 µL	-20°C	dry ice
3' Ligation Enzyme Mix	12 µL	-20°C	dry ice
3' Ligation Reaction Buffer	88 µL	-20°C	dry ice
5' Adaptor	12 µL	-80°C	dry ice
5' Ligation Enzyme Mix	12 µL	-20°C	dry ice
5' Ligation Reaction Buffer	6 µL	-20°C	dry ice
10 mM ATP	30 µL	-20°C	dry ice
First-Strand Synthesis Reaction Buffer	96 µL	-20°C	dry ice
0.1 M DTT	36 µL	-20°C	dry ice
2.5 mM dNTP Mix	24 µL	-20°C	dry ice
Reverse Transcription Primer	powder	-20°C	dry ice
Reverse Transcriptase	12 µL	-20°C	dry ice
RNase Inhibitor	24 µL	-20°C	dry ice
RNA Spike-in	powder	-20°C	dry ice
RNA spike-in qPCR Primer Mix	powder	-20°C	dry ice
Nuclease-free water	1 mL	-20°C	dry ice
PreAMP PCR SuperMix	480 µL	-20°C	dry ice
PreAMP Primers	powder	-20°C	dry ice
PreAMP Primer Remover	12 µL	-20°C	dry ice

Additional required materials

- RNase-free 200 µL PCR tubes
- Thermal cycler
- Microcentrifuge for 200 µL tubes
- Pipettors and tips

Workflow



Protocol

3' Adaptor Ligation

Note *To reconstitute the RNA Spike-in and Reverse Transcription Primer, add 80 µL and 60 µL Nuclease-free water to the corresponding tube respectively.*

For smaller input amounts of total RNA at ~100 ng, reduce the use of 3'Adaptor by diluting 1:2 in Nuclease-free water.

1. Combine the reagents in a RNase-free 200 µL PCR tube according to the order in the table:

Nuclease-free Water	variable
Input RNA	1~7.3 µL
3' Adaptor	1 µL
RNA Spike-in	0.5 µL
Total volume	8.8 µL

2. Incubate the mix at 70°C for 2 min. Transfer the tube to ice.
3. Ligate 3' Adaptor to the RNA by adding the Components:

3' Ligation Reaction Buffer	7.2 µL
3' Ligation Enzyme Mix	1 µL
RNase Inhibitor	1 µL
Total volume	9.2 µL

4. Incubate at 25°C for 1 hour.

Note *To improve the ligation efficiency for end-methylated RNA such as piRNA, longer incubation at a lower temperature (e.g.16°C for 18 hrs) may be used. However, some concatemerization products may occur.*

Hybridization of Reverse Transcription Primer

The excess of the unligated 3' Adaptor is blocked by hybridization with Reverse Transcription Primer to prevent adaptor-dimer formation. The double stranded DNA duplexes are not a substrate for T4 RNA Ligase 1 and will not ligate to 5' Adaptor in the 5' Adaptor Ligation step.

Note *For smaller input amounts of total RNA at ~100 ng, reduce the use of Reverse Transcription Primer by diluting 1:2 in Nuclease-free water.*

5. Add following reagents to the tube from Step 4 and mix well:

Nuclease-free water	1 µL
Reverse Transcription Primer	1 µL
Total volume	20 µL

6. Hybridize the primers at 75°C for 5 min, 37°C for 15 min and 25°C for 15 min.

5' Adaptor Ligation

Note *For smaller input amounts of total RNA at ~100 ng, reduce the use of 5' Adaptor by diluting 1:2 in Nuclease-free Water. Store unused 5' Adaptor at -80°C.*

7. Aliquot 1.1 × number of samples (µL) of 5' Adaptor in a Nuclease-free 200 µL PCR tube.
8. Denature the adaptor at 70°C for 2 min and then immediately chill on ice. Use the denatured 5' Adaptor within 30 minutes.
9. Add the following components to the Reverse Transcription Primer hybridized 3' Adaptor ligation product from Step 6 and mix well:

5' Adaptor (denatured)	1 µL
10 mM ATP	2.5 µL
5' Ligation Reaction Buffer	0.5 µL
5' Ligation Enzyme Mix	1 µL
Total volume	25 µL

10. Incubate at 25°C for 1 hr.

Reverse Transcription

11. Mix the components in a RNase-free 200 µL PCR tube:

Adaptor ligated RNA	25 µL
First-Strand Synthesis Reaction Buffer	8 µL
0.1 M DTT	3 µL
2.5 mM dNTP Mix	2 µL
RNase Inhibitor	1 µL
Reverse Transcriptase	1 µL
Total volume	40 µL

12. Incubate at 45°C for 60 min followed by heat inactivation at 70 ℃ for 15 min, then chill on ice.

Pre-amplification of Low Input Samples

Note *To reconstitute PreAMP Primers, add 50 µL Nuclease-free water to its tube and mix well.*
The optimal number of pre-amplification cycles depends on the amount of starting template, and the transcript abundance. 10~12 cycles are recommended for 100 pg of total RNA.

13. Mix the components in a RNase-free 200 µL PCR tube:

PreAMP PCR SuperMix (2×)	10 µL
PreAMP Primers	1 µL
cDNA	9 µL
Total volume	20 µL

14. Incubate assembled reactions in a conventional thermal cycler as follows:

Cycles	Temperature	Time
1	94°C	30 sec
8~12	94°C	15 sec
	62°C	30 sec
	70°C	15 sec
1	70°C	5 min
Hold	4°C	

15. Add following reagents to the tube from Step 14 and mix well:

PreAMP Primer Remover	0.25 µL
Nuclease-free water	4.75 µL
Total volume	25 µL

16. Incubate at 37°C for 30 min followed by heat inactivation at 80°C for 15 min.
17. Immediately add 75 µL Nuclease-free water. Mix well.
18. Place on ice prior to qPCR or it can be store overnight at -20°C.

Optional *To reconstitute the RNA Spike-in qPCR Primer Mix for the RNA Spike-in, add 100 µL Nuclease-free water to its tube. Use 1 µL primer in a 10 µL qPCR reaction.*

Troubleshooting

Low 5' Adaptor ligation efficiency

- Degradation of the labile 5'RNA adaptor. Keep it cold at all times and store in single-use aliquots.
- Free 3'Adaptor from Step 4 ligated with 5'Adaptor. Ensure 3' Adaptor and Reverse Transcription Primer are used in the indicated amounts.

PCR signal even without reverse transcriptase in the first-strand cDNA synthesis

- Genomic DNA contamination in the template RNA. Perform DNase treatment of the RNA sample. If this does not solve the problem, RNA samples or other reagents may have been contaminated with PCR products.

PCR signal in no template control

- Contamination of the cDNA template or PCR reagents by amplified PCR product.
- Exposing the reactions to elevated temperatures (*i.e.* room temperature) during any part of the protocol increases the risk of background signals. It is important that the reagents and assembled reactions are kept cold (on ice or 4°C) at all times.

Weak PCR signals

- Verify PCR positive control has good signals
- Adjust real-time PCR cycler gain settings if available
- RNA samples may contain PCR inhibitors. Re-purify or use an alternative RNA extraction method. Less cDNA volume may be used to lower overall PCR inhibitor.
- Verify the PreAMP Primer Remover is inactivated by heat inactivation and nuclease-free water is immediately added to dilute the reaction mix.

No fluorescent signal detected during qPCR

- Check the presence of PCR product by gel electrophoresis.
- Check any procedural errors during first-strand cDNA synthesis.

No fluorescent signal during qPCR, but PCR product is present in gel electrophoresis

- Check qPCR cycler settings are correct for SYBR® Green or FAM/FITC.
- Check qPCR optical reading is at the correct step of qPCR cycles.
- Adjust the baseline in the qPCR cycler software.

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