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rtStar™ tRNA-optimized First-Strand cDNA Synthesis Kit

Cat#: AS-FS-004

Instruction Manual version 1.0

Product summary

Product description

rtStar™ tRNA-optimized First-Strand cDNA Synthesis Kit is designed to create cDNA libraries from tRNAs. As it is well known, tRNAs undergo by far the greatest number of and the most chemically diverse post-transcriptional modifications, which hinder the cDNA synthesis of the tRNA. To overcome the problem, we use demethylase to efficiently remove m1A, m3C and m1G modifications in the tRNAs to greatly improve cDNA synthesis. The kit uses reverse transcriptase at high temperature and without RNase H activity, ensuring efficient reverse transcription of tRNA templates with strong secondary structures. In addition, the RNA Spike-in can be used for monitoring the cDNA synthesis efficiency and as a reference for qPCR data comparison.

Kit components

The components provided in the kit are sufficient for up to 12 reactions.

Kit component	Amount	Storage	Shipping
RT Reaction Buffer (5×)	50 µl	-20°C	Dry Ice
Reverse Transcriptase	5 µl	-20°C	Dry Ice
RNase Inhibitor	20 µl	-20°C	Dry Ice
2.5 mM dNTP Mix	25 µl	-20°C	Dry Ice
Random Primers	15 µl	-20°C	Dry Ice
0.1 M DTT	15 µl	-20°C	Dry Ice
Demethylation Reaction Buffer (5×)	500 µl	-20°C	Dry Ice
Demethylase	60 µl	-20°C	Dry Ice
Demethylation Stop Buffer (5×)	500 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	5 ml	-20°C	Dry Ice

Additional required materials

- RNase-free 1.5 ml microcentrifuge tubes, 200 µl PCR tubes
- Microcentrifuge for 200 µl/1.5 ml tubes
- Thermostatic Water Bath
- Thermal cycler
- Pipettors and tips
- Phenol, chloroform
- Isopropanol
- 75% ethanol

Protocol

RNA demethylation

1. Prepare reagents

   Gently thaw the Demethylation Reaction Buffer (5×) and Nuclease-free Water. Briefly vortex and spin down the solutions and keep on ice. Before its immediate use, remove the Demethylase from the freezer, mix by flicking the tube, do not vortex. Briefly spin down the content and place on ice.

2. Set up demethylation mix

   Combine reagents per demethylation reaction according to the following table in the order shown. To account for pipetting losses, 10% excess of all reagents is recommended for calculating multiple samples.

Nuclease-free Water	Variable
Demethylation Reaction Buffer (5×)	40 µl
Demethylase	5 µl
RNase Inhibitor	1 µl
Input RNA	≤5 µg
Total volume per reaction	200 µl

3. Perform demethylation reaction

   Incubate the above mix at 37°C for 100 min. Then orderly add 160 µl Nuclease-free Water and 40 µl Demethylation Stop Buffer (5×) to terminate the reaction.

4. RNA precipitation

   1) Add 400 µl phenol:chloroform to the sample. Mix well by inverting. Incubate at room temperature for 10 min. Centrifuge at 12,000 rpm for 10 min.
   2) Carefully transfer the top layer by a micropipette to a RNase-free tube. Discard the bottom liquid phase to waste.
   3) Add 400 µl chloroform to sample, mix well then microfuge at 12,000 rpm for 10 min.
   4) Carefully transfer the top layer to a RNase-free tube. Discard the bottom phase to waste.
   5) Add 1 ml isopropanol to the aqueous solution. Mix well by inverting. Incubate at room temperature for 10 min. Centrifuge at 12,000 rpm for 10 min.

6) Remove the supernatant from the tube, leaving only the RNA pellet.
7) Add 1 ml 75% ethanol (in DEPC-treated water). Mix well by inverting.
8) Centrifuge the tube at 7,500 rpm for 5 min at 4°C. Discard the wash.
9) Vacuum dry under centrifugation or air dry the RNA pellet for 5–10 min.
10) Resuspend the RNA pellet in 12 µl Nuclease-free Water.
11) Incubate at 55–60°C for 10–15 min to fully dissolve the RNA.

First-Strand cDNA synthesis

Note *The recommended amount of starting material can vary from 10 pg to 5 µg of total RNA according to the abundance of RNA of interest.*

5. Prepare reagents
   Gently thaw all of the kit components except for the Reverse Transcriptase. Mix by brief vortexing, spin down all reagents, and immediately place on ice.

Note *The first time to use this kit, please reconstitute the RNA Spike-in by adding 200 µl Nuclease-free Water to the tube. Mix by vortexing and spin down. Leave on ice for 20 ~ 30 min to fully dissolve the RNA Spike-in. Vortex again, then spin down.*

6. Set up Annealing Mix
   Combine the reagents below in the same order according to the table. It is recommended to prepare a 10% excess volume for pipetting losses to account for pipetting loss.

Random Primers	1 µl
dNTP Mix	1.5 µl
RNA Spike-in	1 µl
Template Total RNA	10.5 µl
Total volume per reaction	14.0 µl

7. Incubate in a thermal cycler at 65°C for 5 min, and then immediately place on ice for at least 1 min. Briefly spin down the contents in the tube.

8. Set up cDNA Synthesis Mix
   Combine the reagents in the order according to the table below. 10% excess volume for pipetting losses is recommended.

RT Reaction Buffer (5×)	4 µl
0.1 M DTT	1 µl
RNase Inhibitor	0.6 µl
Reverse Transcriptase	0.4 µl
Total volume per reaction	6.0 µl

9. Add the cDNA Synthesis Mix to the tube from step 7. Vortex briefly to mix, and spin down. Incubate at 25°C for 5–10 min, followed by 50 min at 50°C.

10. Terminate the reaction at 85°C for 5 min. Chill on ice.

11. OPTIONAL. To check the synthesized cDNA quality, reconstitute the RNA Spike-in qPCR Primer Mix in 100 µl Nuclease-free Water. Use 1 µl RNA Spike-in qPCR Primer Mix with 2 µl cDNA, 5 µl SYBR Green Master Mix, and 2 µl Nuclease-free Water. Run the PCR program described in "Running Real-Time PCR Detection" in the Manual of nrStar™ Human tRNA Repertoire PCR Array. A *Ct* value < 30 for the RNA Spike-in indicates a successful tRNA cDNA synthesis.

Note *The cDNA synthesis product can proceed directly to PCR, or can be stored at -20°C.*

Troubleshooting

Problem	Suggestion
PCR signal detected in samples from the first-strand synthesis reactions even without reverse transcriptase	• This typically indicates genomic DNA contamination in the template RNA. Perform DNase treatment of the RNA sample. If this does not solve the problem, the RNA sample or reagents may have been contaminated with PCR products.
PCR signal in no-template PCR reaction	• This typically indicates contamination of the cDNA template or PCR reagents with amplified PCR product. • Exposing the reaction to an elevated temperature (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 cool (on ice or 4°C) at all time.
Signals too weak	• In some real-time PCR cyclers, gain-settings are adjustable. Make sure the gain settings of your real-time PCR cycler have been set to accommodate the signals generated from the specific assay. • The RNA samples may contain PCR inhibitors. Further purification or an alternative RNA extraction method may be necessary to remove the inhibitors. Check the positive controls. You may try less cDNA volume in real-time PCR to reduce the inhibitor level.
No fluorescent signal is detected during the PCR	• Confirm whether there had been a procedural error during the first-strand cDNA synthesis. • Check whether a PCR product can be detected by agarose gel electrophoresis.
No fluorescent signal detected during the PCR, but a PCR amplicon can be detected by agarose gel electrophoresis	• Check if the filter in the real-time PCR cycler is set to either SYBR® Green or FAM/FITC. • Check if the optical measurement is reading at the correct step of the real-time PCR cycles. • Adjust the baseline in the real-time PCR cycler software.