Arraystar Inc., 9430 Key West Avenue #128, Rockville, MD 20850, USA
Tel: 888-416-6343 • Fax: 240-314-0301 • Email: info@arraystar.com • www.arraystar.com

# rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit

Cat#: AS-FS-004

## Instruction Manual version 3.0

## Product summary

### Product description

rtStar™ tRNA Pretreatment & First-Strand cDNA Synthesis Kit is designed to efficiently create cDNA libraries from tRNAs of any species by employing enzymatic pretreatment to remove reverse-transcription-blocking modifications. 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 pretreat the RNA with 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. If stored properly, the kit is stable for 6 months after receipt. We recommend a maximum of 6 freeze-thaw cycles.

| Kit component | Amount | Storage | Shipping |
|---|---|---|---|
| RT Reaction Buffer (5×) | 50 µl | -20°C | Dry Ice |
| Reverse Transcriptase | 12 µl | -20°C | Dry Ice |
| RNase Inhibitor | 36 µl | -20°C | Dry Ice |
| 2.5 mM dNTP Mix | 15 µl | -20°C | Dry Ice |
| Random Primers | 15 µl | -20°C | Dry Ice |
| DRB-C1 | 100 µl | -20°C | Dry Ice |
| DRB-C2 | 100 µl | -20°C | Dry Ice |
| DRB-C3 | 100 µl | -20°C | Dry Ice |
| DRB-C4 | 100 µl | -20°C | Dry Ice |
| DRB-C5 | 100 µl | -20°C | Dry Ice |
| Demethylase | 60 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
- Isopropanol
- Phenol, chloroform
- 75% ethanol

## Protocol

The recommended amount of starting material can vary from 50 ng to 5 µg of total RNA according to the abundance of tRNA in samples of interest. The condition in the RNA demethylation step may cause some RNA degradation and loss.

### RNA Demethylation Pretreatment

1. Prepare reagents

   Gently thaw all of the kit components except for the Reverse Transcriptase and Demethylase. 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.*

2. Prepare Demethylation Reaction Buffer (4×)

   Prepare fresh Demethylation Reaction Buffer (4×) by combine the DRB-C1, C2, C3, C4, C5 with 1:1:1:1:1 volume and keep on ice. The total required volume of Demethylation Reaction Buffer (4×) is 1.1 × 25 × number of samples (µl).

**Note** *Prepare fresh Demethylation Reaction Buffer (4×) each time before use.*

3. Set up Demethylation Master Mix

   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. If performing demethylation on multiple RNA samples, it is recommended to prepare a Demethylation Master Mix of the Demethylation Reaction Buffer (4×), Demethylase, and RNA Inhibitor (in the proportion indicated in Table). To account for pipetting losses, 10% excess of all reagents is recommended for calculating multiple samples.

| | |
|---|---|
| Demethylation Reaction Buffer (4×) | 25 µl |
| Demethylase | 5 µl |
| RNase Inhibitor | 2 µl |
| **Total volume per reaction** | **32 µl** |

4. Perform demethylation reaction

Combine the reagents per demethylation reaction according to the following table in the order shown. Vortex briefly to mix, and spin down.

| | |
|---|---|
| Demethylation Master Mix | 32 µl |
| Input RNA | ≤5 µg |
| Nuclease-free water | x µl |
| **Total volume per reaction** | **100 µl** |

Incubate the above mix at 25°C for 2h. Then orderly add 100 µl Nuclease-free water and 200 µl phenol:chloroform to terminate the reaction and perform the RNA purification procedure immediately.

5. RNA purification
   1) Mix well the tube form step 4 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) Precipitate the RNA from the aqueous phase by mixing with equal volume of Isopropanol. Vortex the mixture and place the tube for 30 min at -80°C. Centrifuge at 12,000 rpm for 10 min.
   4) Remove the supernatant from the tube, leaving only the RNA pellet.
   5) Add 1 ml 75% ethanol (in Nuclease-free Water). Mix well by inverting.
   6) Centrifuge the tube at 7,500 rpm for 5 min at 4°C. Discard the wash.
   7) Vacuum dry under centrifugation or air dry the RNA pellet for 5–10 min.
   8) Resuspend the RNA pellet in 11 µl Nuclease-free Water.
   9) Incubate at 55–60°C for 10–15 min to fully dissolve the RNA.

## First-Strand cDNA synthesis

6. Set up Annealing Mix
   Combine the reagents below in the same order according to the table. If performing first-strand cDNA synthesis on multiple RNA samples, it is recommended to prepare an Annealing Mix of the Primer, dNTP Mix and RNA Spike-in (in the proportion indicated in Table). 10% excess volume for pipetting losses is recommended.

| | |
|---|---|
| Random Primers | 1 µl |
| 2.5 mM dNTP Mix | 1 µl |
| RNA Spike-in | 1 µl |
| Template Total RNA | 10 µl |
| **Total volume per reaction** | **13 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
   cDNA Synthesis Mix is recommended to prepare for multiple RNA samples. It includes the components in the following table. And 10% excess volume for pipetting losses should be well advised to take into account. Before its immediate use, remove the Reverse Transcriptase from the freezer, mix by flicking the tube, do not vortex. Briefly spin down the content and place on ice.

| | |
|---|---|
| RT Reaction Buffer (5×) | 4 µl |
| RNase Inhibitor | 1 µl |
| Reverse Transcriptase | 1 µl |
| Nuclease-free Water | 1 µl |
| **Total volume per reaction** | **7 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 10 min, followed by 50 min at 45°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 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.<br>• 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. |
| Low demethylation efficiency | • Prepare a new demethylation reaction buffer |