RPF Recovery, Adapter Ligation, and cDNA Synthesis

Materials

ribosome-protected fragments
RNase-free water
Filtered pipette tips
Streptavidin magnetic beads
T4 PNK
Murine RNase inhibitor
Pre-adenylated L1 adapter
T4 RNA Ligase 2 truncated
Deadenylase
RecJf
T4 RNA Ligase 1
L2 adapter
Antisense LNA oligonucleotide
RT primer
SuperScript III
First-Strand Buffer
DTT
1 M Tris-HCl (pH 7.0)

Buffer Preparation
Prepare the following buffers before starting the experiment.

BW Buffer

Component	Final Concentration
Tris, pH 7.0	10 mM
NaCl	500 mM
EDTA	1 mM
Tween-20	0.05%

Mild Wash Buffer

Component	Final Concentration
Tris, pH 7.0	10 mM
Tween-20	0.05%

Procedure

Adapter Ligation and Reverse Transcription

RPF 3′-End Dephosphorylation

1. Resuspend the recovered RPF pellet in 4 μL PNK reaction mixture.
2. Prepare the mixture as follows.

Component	Final Amount
PNK buffer	1×
T4 PNK	3.2 U
Murine RNase inhibitor	16 U

3. Incubate at 37 °C for 1 h.
4. Heat at 75 °C for 10 min to inactivate PNK.

L1 Adapter Ligation

1. Add 4 μL L1 ligation mixture.
2. Prepare the mixture as follows.

Component	Final Concentration/Amount
RNA ligase buffer	1×
Pre-adenylated L1 adapter	1 μM
T4 RNA Ligase 2 truncated	80 U
PEG-8000	30%

3. Incubate at 22 °C for 2 h.
4. Add 25 U deadenylase and 45 U RecJf.
5. Incubate at 37 °C for 30 min.
6. Heat at 75 °C for 10 min to terminate the reaction.

Streptavidin Bead Capture

1. Add 2 μL streptavidin magnetic beads to the reaction.
2. Incubate at room temperature for 10 min.
3. Place the tube on a magnetic rack for 1 min.
4. Discard the supernatant.
5. Wash the beads twice with 200 μL BW buffer.
6. Wash the beads twice with 200 μL mild wash buffer at 50 °C for 1 min.
7. Remove residual wash buffer completely.

RPF 5′-End Phosphorylation

1. Prepare a 4 μL phosphorylation mixture with the components listed below and use it to resuspend the beads.

Phosphorylation mixture (4 μL total)

Component	Final Concentration/Amount

Component	Final Concentration/Amount
PNK buffer	1×
T4 PNK	3.2 U
ATP	1 mM
Mild wash buffer	0.6 ×

2. Incubate at 37 °C for 1 h.

L2 Adapter Ligation

1. Prepare 4 µL of L2 ligation mixture using the components listed below, then add it to the beads.

Component	Final Concentration/Amount
RNA ligase buffer	1×
L2 adapter	1 µM
ATP	1 mM
T4 RNA Ligase 1	12 U
PEG-8000	30%
Antisense LNA	10 µM final

2. Incubate at 37 °C for 1 h.
3. Magnetically separate the beads.
4. Remove the supernatant.
5. Wash once with 50 µL mild wash buffer.
6. Resuspend the beads in 8 µL mild wash buffer.

Reverse Transcription

1. Prepare 3 µL of RT1 mixture using the components listed below, then add it to the beads.

Component	Final Concentration
RT primer	6.7 µM
dNTPs	3.3 mM
Antisense LNA	10 µM final

2. Incubate at 65 °C for 5 min.
3. Immediately place the beads on ice.
4. Prepare 5 µL of RT2 mixture using the components listed below, then add it to the reaction.

Component	Volume

Component	Volume
First-Strand Buffer	3 μL
100 mM DTT	1 μL
SuperScript III	1 μL

5. Incubate at 50 °C for 30 min.

Post-RT Washing
1. Place the tube on the magnetic rack for 1 min.
2. Remove the supernatant.
3. Wash the beads with 50 μL mild wash buffer.
4. Magnetically separate the beads again.
5. Remove the wash buffer.
6. Resuspend the beads in 30 μL mild wash buffer.

cDNA Release
1. Add NaOH to a final concentration of 0.1 M.
2. Incubate at 95 °C for 15 min.
3. Place the tube on the magnetic rack for at least 2 min.
4. Transfer the cDNA-containing supernatant into a new tube.
5. Add 0.1 volume of 1 M Tris, pH 7.0 immediately to neutralize the sample.
6. Store the cDNA at −20 °C.

Oligonucleotides
All oligonucleotides except L2 were synthesized by GenScript. L2 was synthesized by GENEWIZ.

Oligonucleotide	Sequence
L1 adapter	5’-phosphate-AGATCGGAAGAGCACACGTCT-GAACTCCAGTCAC-3’-TEG-biotin
L2 adapter	5’-FAM-CTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNNNNNNCrCrCrCrCrC-3’-OH
Antisense LNA	5’-[C][T][C][T][T][C]CGATCT[G][G][G][G]-3’
RT primer	5’-FAM-GTGACTGGAGTTCAGACGTGTGCT-3’-OH

[N] indicate LNA base.
N in the L2 sequence represent random nucleotides.