m^1A-MAP protocol

1. m^1A immunoprecipitation

   a. Adjust the volume of 40 μg polyA$^+$ RNA to 72 μL with RNase free water. Add 8 μL RNA Fragmentation Buffer (10×) and mix well by pipetting up and down. Incubate in a preheated thermal cycler for 5 minutes at 94 °C and then chill on ice immediately.

   b. Add 8 μL 10× RNA Fragmentation Stop Solution to the mixture and fragmented RNA is purified by ethanol precipitation. Dissolve the RNA pellets with 105 μL RNase free water completely.

   c. Save 5 μL of fragmented RNA as input sample. Subject the remaining 100 μL of fragmented RNA to m^1A immunoprecipitation.

   d. Add 40 μL 10× IPP buffer (1.5 M NaCl, 1% NP-40, 100 mM Tris, pH 7.4), 10 μL RiboLock RNase Inhibitor (40 U/μL), 5 μg anti-m1A antibody to the 100 μL fragmented RNA and adjust the volume to 400 μL with RNase free water. Rotate at 4 °C overnight.

   e. Resuspend 40 μL Protein A/G UltraLink Resin into 400 μL 1× IPP buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4) and then add to the RNA antibody mixture followed by rotating at 4 °C for additional 3 h.

   f. Resins are washed with twice with 1 mL IPP buffer, once with 1 mL low salt buffer (75 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4), once with 1 mL high salt buffer (200 mM NaCl, 0.1% NP-40, 10 mM Tris, pH 7.4) and twice with 1 mL TEN buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 0.05% NP-40).

   g. Add 200 μL of 3 mg/mL N^1-methyladenosine in IPP buffer and rotate at room temperature for 1 h to elute the m^1A containing RNA from resins. Repeat this step once and combine the supernatants together as immunoprecipitated sample and further purify the immunoprecipitated RNA by phenol-chloroform isolation.

   h. Dissolve the RNA pellets with 30 μL RNase free water completely. Save 10 μL of immunoprecipitated RNA as (-) demethylase sample and subject the remaining 20 μL of immunoprecipitated RNA to demethylase treatment.

2. Demethylase treatment

   a. Heat 20 μL of immunoprecipitated RNA at 65°C for 5 min and then chill on ice immediately to disrupt the RNA secondary strucuture. .

   b. Prepare the demethylation mix as follow:

Reagents	Volume (μL)
0.5 M MES, pH 6.5	4
28.3 mM of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	0.4
30 mM 2-ketoglutarate	0.4
200 mM L-ascorbic acid	0.4
RNase inhibitor (40 U/μL)	0.4
1 μM AlkB	0.4
RNase free water	14
Total	40

   Mix the demethylation mix well by pipetting.

   c. Add the demethylation mix into denatured RNA and incubate at 37 °C for 2 h.

   d. Quench the demethylation reaction by the addition of 4 μL 50 mM EDTA and purify the

demethylated RNA by phenol-chloroform isolation.

e. Dissolve the RNA pellets with 10 μL RNase free water completely [as (+) demethylase sample].

3. Library construction

Input sample, (-) demethylase sample and (+) demethylase sample are subjected to library construction.

a. PNK treatment

Reagents	Volume (μL)
10× PNK buffer	2
T4 PNK (NEB)	1
RNase inhibitor (40 U/μL)	1
RNA	10
RNase free water	6
Total	20

Mix well and incubate at 37 °C for 1 h and then heat-inactivate of PNK at 65 °C for 20 min. Dephosphorylated RNAs are purified by ethanol precipitation and the RNA pellets are dissolved in 7 μL RNase free water completely.

b. 3’ RNA linker ligation

Add 1 μL 20 μM 3’ RNA linker (5’rAPP-AGATCGGAAGAGCGTCGTG-3SpC3) into 7 μL RNA sample, and mix well. Heat at 70°C for 2 min and then chill on ice immediately.

Prepare the ligation mix as follow:

Reagents	Volume (μL)
10× RNA ligation buffer	2
0.1M DTT	2
50% PEG8000	6
T4 RNA ligase2, truncated KQ (NEB)	1
RNase inhibitor (40 U/μL)	1
Total	12

Add the ligation mix into the RNA mix and mix well.

Incubate at 25 °C for 2 h.

Add 1 μL 5' Deadenylase into the ligation mix followed by incubating at 30 °C for 1 h and then add 1 μL RecJf followed by incubating at 37 °C for another 1 h to remove excess 3’ RNA linker. These enzymes are then heat-inactivated at 70 °C for 20 min. RNAs are purified by ethanol precipitation and dissolved in 11 μL RNase free water.

c. Reverse transcription

Add 1 μL 2 μM RT primer (ACACGACGCTCTTCCGATCT) into 10 μL RNA sample, and mix well. Heat at 80°C for 2 min and then chill on ice immediately.

Prepare the reverse transcription mix as follow:

Reagents	Volume (μL)
10×reverse transcription buffer (500 mM Tris, pH 8.3, 750 mM KCl,)	2

30 mM $MgCl_2$	2
10 mM dNTP mix	2
0.1M DTT	1
TGIRT	1
RNase inhibitor (40 U/μL)	1
Total	9

Add the reverse transcription mix into RNA and mix well.
Incubate at 57 °C for 2 h.
Add 1 μL Exonuclease I (NEB) and incubate at 37 °C for 30 min to digest excess RT primers.
The cDNAs are purified using silane beads.

d. 5' adaptor ligation
Add 0.8 μL 80 μM 5' adaptor (5Phos-NNNNNNNNNNAGATCGGAAGAGCACACGTCTG-3SpC3) and 1 μL DMSO into 5 μL cDNA sample, and mix well. Heat at 75°C for 2 min and then chill on ice immediately.
Prepare the ligation mix as follow:

Reagents	Volume (μL)
10× RNA ligation buffer	2
0.1M ATP	0.2
50% PEG8000	9
T4 RNA ligase 1, high concentration (NEB)	1.5
H_2O	1.1
Total	13.8

Add the ligation mix into cDNA and mix well.
Incubate at 25 °C overnight. The cDNAs are purified using silane beads.

e. PCR amplification
Prepare PCR mix as follow:

Reagents	Volume (μL)
High-Fidelity 2X PCR Master Mix (NEB)	25
Universal primer (NEB)	1
Index primer (NEB)	1
cDNA	23
Total	50

Mix well and run the PCR amplification as follow:

Temperature	Time	Cycle
98 °C	30 s	1
98 °C	10 s	12-15
65 °C	30 s	
72 °C	30 s	
72 °C	5 min	1
4 °C	Hold	

PCR products are purified by 1.8× XP beads (Beckman) and then run on the 8% TBE gel. The

DNA bands according to 160-300 bp are excised. The gels are crushed to small pieces and DNAs are eluted from gel slices at 37 °C for 4h in 400 μL 1×TE buffer. DNAs are purified by ethanol precipitation and subjected to high throughput sequencing on Illumina Hiseq X10 with paired-end 2×150 bp read length.