**m6Am-seq protocol**

Hanxiao Sun1,5, Kai Li1,2,3,5, Xiaoting Zhang1, Jun’e Liu1, Meiling Zhang1, Haowei Meng1 & Chengqi Yi1,3,4,\*

*1State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, Peking University, Beijing 100871, China.*

*2Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China.*

*3Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China.*

*4Department of Chemical Biology and Synthetic and Functional Biomolecules Center, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China.*

*5These authors contributed equally to this work.*

*\*Correspondence:* [*chengqi.yi@pku.edu.cn*](mailto:chengqi.yi@pku.edu.cn) *(C. Y.)*

**Materials**

**Reagents**

1x m7G-IPP buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40

1x m7G-Low-salt IP buffer: 10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.05% NP-40

1x m7G-High-salt IP buffer: 10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.05% NP-40

1x m7G-TET buffer: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.05% NP40

1x m6A-IPP buffer: 10 mM Tris-HCl, pH 7.4,150 mM NaCl, 0.1% NP-40

1x m6A-Low-salt IP buffer: 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1% NP-40

1x m6A-High-salt IP buffer: 10 mM Tris-HCl, pH 7.4, 500 mM NaCl,0.1% NP-40

protein A/G UltraLink Resin (ThermoFisher Scientific, 53132)

RiboLock RNase inhibitor (ThermoFisher Scientific, EO0381)

protein A magnetic beads (ThermoFisher Scientific, 10002D)

protein G magnetic beads (ThermoFisher Scientific, 10004D)

NEBNext Magnesium RNA fragmentation Module (NEB, E6150S)

RNasin Plus RNase Inhibitor (Promega, N2615)

SUPERase·In RNase inhibitor (Invitrogen, AM2694)

N6-methyladenosine (Sigma-Aldrich, M2780)

Anti-m7G-cap monoclonal antibody (MBL, RN016M)

Anti-m6A polyclonal antibody (Millipore, ABE572)

MEGAclear Transcription Clean-Up Kit (Ambion, AM1908)

TRIzol Reagent (Invitrogen, 15596018)

SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara, 634413)

**Procedure**

**cap-m7G RNA immunoprecipitation**

1. The total RNA is extracted from cells using TRIzol, according to the manufacturer’s instructions.

2. Small RNA is depleted using MEGAclear Transcription Clean-Up Kit.

3.100 μg >200 nt RNA is fragmented using RNA fragmentation buffer at 94°C for 4 min, followed by chilling in ice.

4. Add fragment stop solution buffer to stop the reaction.

5. Add 2μl 3M sodium acetate (pH 5.2), 4 μl glycogen and 50 μl ice-cold 100%(vol/vol) ethanol to fragmented RNA mixture from step 4, vortex and precipitate for 2 h at -20°C; Next, centrifuge at 14,000 rpm for 30 min at 4°C in a precooled centrifuge. Wash the pellet twice with 1ml of 75%(vol/vol) ethanol at 14,000 rpm for 5 min. Centrifuge and carefully remove 75% ethanol. Air-dry the pellets and dissolve them in 100μl nuclease-free water.

6. 10 ng of fragments are used as “input” and stored at -80°C.

7. The remaining fragmented RNA is denatured at 65°C for 5 min, followed by chilling in ice.

8. Prepare RNA-antibody incubation buffer as the following table.

|  |  |  |
| --- | --- | --- |
| Component | Amount per reaction (μl) | Total amount |
| denatured RNA | 100 | / |
| 10x m7G-IPP buffer | 20 | 1x |
| cap-m7G antibody | 2 | 1:100 dilution |
| RiboLock RNase inhibitor | 5 | 1:40 dilution |
| nuclease-free water | 73 | / |

9. Mix gently with a pipette. The RNA-antibody mixture was incubated overnight at 4°C.

10. Transfer 20 μl of protein A/G UltraLink Resin into 1.5ml nuclease-free centrifuge tube. Resin is washed twice with 1 ml of 1x m7G IPP buffer by gently pipetting.

11. Resuspended protein A/G UltraLink Resin with 200 μl 1x m7G IPP buffer, add 5 μl RiboLock RNase inhibitor.

12. Add 200μl of the antibody-RNA mixture into Resin from step 11. Incubate the mixture by rotator at 4°C for 3 h.

13. After incubation, centrifuge at 800 rpm for 5s at 4°C, remove the supernatant.

14. Add 1ml of IPP buffer into the pellet from step 13. Wash the pellet by rotator at 4°C for 3 min. Centrifuge at 800 rpm for 2min at 4°C. Remove the supernatant.

15. Repeat step 14.

16. Add 1ml of 1x m7G-Low-salt IP buffer into the pellet from step 15. Wash the pellet by rotator at 4°C for 3 min. Centrifuge at 800 rpm for 2min at 4°C. Remove the supernatant.

17. Add 1ml of 1x m7G-High-salt IP buffer into the pellet from step 16. Wash the pellet by rotator at 4°C for 3 min. Centrifuge at 800 rpm for 2min at 4°C. Remove the supernatant.

18. Add 1ml of 1x m7G-TET buffer into the pellet from step 17. Wash the pellet by rotator at 4°C for 3 min. Centrifuge at 800 rpm for 2min at 4°C. Remove the supernatant.

19. Repeat step 18.

20. The resin-antibody-RNA pellet is resuspended in 1 ml Trizol and rotated for 10 min at room temperature.

21. Add 200μl chloroform into the Trizol from step 20 and mix the mixture by vortexing. Centrifuge at 14,000 rpm for 30 min at 4°C and transfer around 600 μl of supernatant into a new 1.5ml nuclease-free centrifuge tube.

22. Add 60μl 3M sodium acetate (pH 5.2), 4 μl glycogen and 660 μl ice-cold 100%(vol/vol) Isopropanol to supernatant from step 21, vortex and precipitate for 2 h at -20°C; Next, centrifuge at 14,000 rpm for 30 min at 4°C in a precooled centrifuge. Wash the pellet twice with 1ml of 75%(vol/vol) ethanol at 14,000 rpm for 5 min. Centrifuge and carefully remove 75% ethanol. Air-dry the pellets and dissolve them in 16.5 μl nuclease-free water.

23. Take 1μl of cap-m7G-containing RNA fragments and quantify by Qubit. Around 150 ng cap-m7G-immunoprecipitated RNA fragments could be obtained.

***In vitro* demethylation treatment:**

1. 1 ng of the cap-m7G-immunoprecipitated RNA is used as “m7G-IP” and store at -20°C.

2. RNA is subjected to [“FTO (+)”] or [“FTO (-)”] treatment. About 10 μl of RNA (around 100ng) is used for [“FTO (+)”] treatment. RNA is denatured at 65°C for 5 min, followed by chilling in ice.

3. Prepare the demethylation assay buffer as the following table.

|  |  |  |
| --- | --- | --- |
| Component | Amount per reaction (μl) | Total amount |
| denatured RNA | 10 | / |
| 500mM MES pH6.5 | 2 | 50mM |
| 2M KCl | 1 | 100mM |
| 30mM MgCl2 | 1.33 | 2mM |
| SUPERase·InRNase inhibitor | 1 | 0.4 U/mL |
| 3mM 2-ketoglutarate | 2 | 300μM |
| 1mM (NH4)2Fe(SO4)2·6H2O | 2 | 100 μM |
| purified FTO | / | 1 μM |
| nuclease-free water | Add nuclease-free water to 20μl | |

4. Incubation at 37°C for 20 min.

5. Add 180μl nuclease-free water into demethylation reaction. Add 200μl phenol : chloroform : isoamyl alcohol (25:24:1) and mix. Centrifuge at 16,000 g for 15min at 4°C. Add 200μl chloroform : isoamyl alcohol (24:1) and mix. Centrifuge at 16,000 g for 15min at 4°C. Transfer 200μl of supernatant into a new 1.5ml nuclease-free centrifuge tube.

6. Add 20μl 3M sodium acetate (pH 5.2), 4 μl glycogen and 550 μl ice-cold 100%(vol/vol) ethanol to supernatant from step 5, vortex and precipitate for 2 h at -20°C; Next, centrifuge at 14,000 rpm for 30 min at 4°C in a precooled centrifuge. Wash the pellet twice with 1ml of 75%(vol/vol) ethanol at 14,000 rpm for 5 min. Centrifuge and carefully remove 75% ethanol. Air-dry the pellets and dissolve them in 155 μl nuclease-free water.

7. Add 150 μl nuclease-free water into the 5 μl of remaining cap-m7G-immunoprecipitated RNA from step 2.

**m6A RNA immunoprecipitation:**

1. Transfer 10 μl of protein A magnetic beads and 10 μl of protein G magnetic beads into a 1.5ml nuclease-free centrifuge tube.

2. Beads are washed with 500 μl of 1x m6A-IPP buffer by gently pipetting. Keep the tube on the magnetic separation device, pipette out the supernatant.

3. The beads are resuspended in 500 μl of 1x m6A-IPP buffer and add 2 μg of anti-m6A polyclonal antibody. Mix the beads-antibody mixture by gently pipetting. Incubation on the rotator at 4°C for at least 6 h.

4. After incubation, keep the beads-antibody mixture on the magnetic separation device, pipette out the supernatant.

5. The beads are resuspended in 500 μl of 1x m6A-IPP buffer, mix by gently pipetting. Keep the tube on the magnetic separation device, pipette out the supernatant.

6. 155 μl [“FTO (+)”] or [“FTO (-)”] RNA fragment is denatured at 65°C for 5 min, followed by chilling in ice.

7. Prepare the incubation mixture as the follow table.

|  |  |  |
| --- | --- | --- |
| Component | Amount per reaction (μl) | Total amount |
| denatured RNA | 155 | / |
| 5×m6A-IPP buffer | 40 | 1x |
| RNasin Plus RNase Inhibitor | 5 | 1:40 dilution |

8. Transfer 200 μl mixture from step 7 into beads from step 5. Mix the beads-antibody-RNA mixture by gently pipetting. Incubation on the rotator at 4°C for 2h.

9. Keep the tube on the magnetic separation device, pipette out the supernatant.

10.The beads are resuspended in 500 μl of 1x m6A-IPP buffer. Mix the mixture by gently pipetting. Keep the tube on the magnetic separation device, pipette out the supernatant.

11. Repeat step 10.

12. The beads are washed twice with 500 μl of 1x m6A-Low-salt IP buffer as the same methods.

13. The beads are washed twice with 500 μl of 1x m6A-High-salt IP buffer as the same methods.

14.Prepare the elution buffer as the follow table：

|  |  |  |
| --- | --- | --- |
| Component | Amount per reaction (μl) | Total amount |
| 5x m6A-IPP buffer | 40 | 1x |
| 20mM N6-methyladenosine | 67 | 6.7 mM |
| RiboLock RNase inhibitor | 5 | 1:40 dilution |
| nuclease-free water | 88 | / |

15. Transfer the elution buffer into the beads from step 13. Incubation on the rotator at room temperature for 1h.

16. Keep the tube on the magnetic separation device, transfer 200 μl of supernatant into a new 1.5ml nuclease-free centrifuge tube.

17. Add 195 μl of 1x m6A-IPP buffer and 5 μl of RiboLock RNase inhibitor into the beads from step 16. Incubation on the rotator at room temperature for 20 min.

18. Keep the tube on the magnetic separation device, transfer 200 μl of supernatant into 1.5ml nuclease-free centrifuge tube from step 16.

19. Add 400μl phenol : chloroform : isoamyl alcohol (25:24:1) and mix. Centrifuge at 16,000 g for 15min at 4°C. Add 400μl chloroform : isoamyl alcohol (24:1) and mix. Centrifuge at 16,000 g for 15min at 4°C. Transfer 400μl of supernatant into a new 1.5ml nuclease-free centrifuge tube.

20. Add 40μl 3M sodium acetate (pH 5.2), 4 μl glycogen and 1 ml ice-cold 100%(vol/vol) ethanol to supernatant from step 19, vortex and precipitate for 2 h at -20°C.

21.Centrifuge at 14,000 rpm for 30 min at 4°C in a precooled centrifuge. Wash the pellet twice with 1ml of 75%(vol/vol) ethanol at 14,000 rpm for 5 min. Centrifuge and carefully remove 75% ethanol. Air-dry the pellets and dissolve them in 8 μl nuclease-free water.

22. The elut RNA is called [“FTO (+) m6A-IP”] and [“FTO (-) m6A-IP”].

**Library preparation:**

The “input”, “m7G-IP”, [“FTO (+) m6A-IP”] and [“FTO (-) m6A-IP”] samples are subjected to library construction using SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian according to the manufacturer's protocol (<https://www.takarabio.com/assets/a/113361>).

There are two details to be noted as following list:

1. For the cDNA synthesis step, we performed the first step according to Option 2 (without fragmentation).

2. For the final RNA-seq library amplification, we perform PCR cycles according to the following list

|  |  |
| --- | --- |
| RNA | PCR cycles |
| 10ng input | 11 |
| 1ng m7G-IP | 13 |
| FTO (+) m6A-IP | 14 |
| FTO (-) m6A-IP | 14 |

Finally, determine the concentration by the Qubit and assess the size distribution by the analyzer.

