

Tailor-made Antibodies and Tools for Life Science

IP: Immunoprecipitation Protocol - m6A-sequencing / MeRIP-Sequencing

In immunoprecipitation (IP) approaches, antibodies are employed to specifically pull down the corresponding target molecule from a complex sample like a tissue or cell lysate.

General considerations

Use RNase-free molecular biology-grade water for all buffers and RNase-free tips and tubes.

Materials and reagents

- Total RNA or mRNA preparation kit
- RNase-free molecular biology-grade water
- Fragmentation buffer 10x: 100 mM Tris-HCl pH 7.0, 100 mM ZnCl₂
- 0 EM EDTA
- 3M Sodium acetate, pH 5.2
- Ethanol, 100%
- Ethanol, 75%
- Glycogen
- IP buffer 5x: 50 mM Tris-HCl pH 7.4, 750 mM NaCl and 0.5% (vol/vol) Igepal CA-630
- m6A Stock solution 20 mM: Dissolve 10 mg of m6A in 1.3 ml of molecular biology-grade, RNase-free water. Store aliquots of 150 µl at -20°C.
- Elution buffer: Mix 90 μl (5x stock) of IP buffer, 150 μl (20 mM stock) of m6A, 7 μl of RNasin Plus and 203 μl of water (use molecular biology–grade, RNase-free water)
- m6A antibody stock solution: 0.5 mg/ml. Reconstitute 50 μg of lyophilized affinity-purified m6A-specific antibody in 100 μl of molecular biology-grade,
 RNase-free water.
- Ice
- RNase inhibitor (e.g. RNasin)
- Nuclease inhibitor (e.g. ribonucleoside vanadyl complexes, RVC)



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Procedure

- 1. Prepare total RNA or mRNA according to the kit manufacturer's instructions and adjust the RNA concentration to approx. 1 μg/μl with RNase-free water.
- 2. Set up the fragmentation reaction in a thin-walled 200 μ l PCR tube. 18 μ l RNA solution, 2 μ l 10x fragmentation buffer. Vortex and spin down the tube.
- 3. Incubate the tubes at 94°C for 5 min in a preheated thermal cycler block with the heated lid closed. Remove the tubes from the block and immediately add 2 μl of 0.5 M EDTA. Vortex and spin down the tubes and place them on ice. **Note:** Stick to the specified amounts and volumes, as scaling may affect fragmentation efficiency and the resulting size distribution. If higher amounts are processed use several tubes.
- 4. Collect contents of all tubes, add one-tenth volume of 3 M sodium acetate, glycogen (100 μg/ml final concentration) and 2.5 volumes of 100% ethanol. Mix the contents and incubate at -80°C overnight.
- 5. Centrifuge the tubes at 15,000x g for 25 min at 4°C. Discard the supernatant. Do not to disrupt the pellet, which is easily visible because of the presence of glycogen. Wash the pellet with 1 ml of 75% ethanol and centrifuge again at 15,000x g for 15 min at 4°C.
- 6. Aspirate the supernatant and let the pellet air-dry. Resuspend the pellet in 300 μl of RNase-free water.
- 7. Validate RNA postfragmentation size distribution by measuring RNA concentration with a spectrophotometer and running 0.5 µg of RNA on 1.5% (wt/vol) agarose gel for approx. 30 min. The outlined fragmentation procedure should produce a distribution of RNA fragment sizes centered around 100 nt.
- 8. Save several µg of untreated fragmented RNA to serve as input control in RNA-seq.
- 9. Adjust the volume of the remaining RNA to 755 μl with RNase-free water. Prepare the reaction mixture by adding 10 μl RNasin (200-400 U), 10 μl RVC (2 mM final concentration), 200 μl 5x IP buffer and 25 μl of m6A antibody (12.5 μg) to a final volume of 1 ml in a low-binding microcentrifuge tube. Vortex and spin down the tube. Set up a parallel reaction that includes the same amount of fragmented RNA, but without the antibody. It will serve as a bead-only control to assess background levels and efficiency of RNA elution.
- 10. Incubate with head-over-tail rotation for 2 h at 4°C.
- 11. While the samples are incubating, wash 200 µl of protein A bead slurry twice in 1 ml of 1x IP buffer supplemented with RNasin and RVC. Resuspend the beads in 1 ml of 1x IP buffer supplemented with BSA (0.5 mg/ml), RNasin and RVC and incubate on a rotating wheel for 2 h. Spin down, remove and discard the supernatant and wash twice in 1 ml of 1x IP buffer supplemented with RNasin and RVC. Equally divide the beads between two 1.7 ml microcentrifuge tubes (one for the IP sample and one for the bead-only control).
- 12. Transfer the IP-reactions into the bead-containing tubes prepared and incubate the reaction mixtures for 2 h head-over-tail at 4°C.
- 13. Spin down the beads and carefully remove and retain the supernatant. Wash the beads with 1 ml of 1x IP buffer three times.
- 14. Add 100 µl of elution buffer to the sedimented beads. Incubate the mixture for 1 h with continuous shaking at 4°C.
- 15. Spin down the beads and carefully remove and retain the supernatant that contain the eluted RNA fragments.
- 16. Add 100 µl of **1x IP buffer** to the sedimented beads and gently tap the tube to mix. Spin down the beads and carefully remove and retain the supernatant (repeat steps 14-16).
- 17. Combine all eluates from the same sample (IP or bead-only control) and add one-tenth volumes of 3 M sodium acetate (pH 5.2), and 2.5 volumes of 100% ethanol. Mix and incubate the sample at -80°C overnight.
- 18. Centrifuge the tube at 15,000x g for 25 min at 4°C. Discard the supernatant. Do not disrupt the pellet, which is not visible at the bottom of the tube. Wash the pellet with 1 ml of 75% (vol/vol) ethanol and centrifuge it again at 15,000x g for 15 min at 4°C. Aspirate the supernatant and let the pellet air-dry. Resuspend the pellet in 15 µl of RNase-free water and quantify the RNA.

The RNA can now be used for library preparation.

For more background information and protocol details, refer to <u>Dominissini D et al., 2013</u>.