# 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 $ZnCl_2$
- **0.5 M 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)

## 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 µl PCR tube. 18 µl RNA solution, 2 µl **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 Dominissini D et al., 2013.*