Magna MeRIP™ m^{6}A Kit

Transcriptome-wide Profiling of *N*^{6}-Methyladenosine

Magna MeRIP™ m^{6}A Kit (Catalog No. 17-10499)

10 reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

N6-methyladenosine (m^6A) represents an abundant RNA modification that is conserved across many different species, ranging from plants, to yeast, to mammals. Similar to the 5-methylcytosine (5-mc) modification of DNA, m6A is a reversible chemical modification of RNA. m^6A RNA modifications are an emerging topic in controlling cell fate transitions in mammalian embryonic stem cells. m^6A is one of the most prevalent modifications on both mRNAs and noncoding RNAs in eukaryotes with an estimated 12,000 m6A sites in over 7,000 genes. The mark is deposited by a heterodimer of methyltransferase-like 3 and 14 (Mettl3 and Mettl14) and can be removed by the RNA demethylase enzymes FTO and ALKBH5. Obesity risk gene FTO encodes the first identified m^6A demethylase. Mutations in FTO have been associated with increased risk for obesity and type II diabetes. Recently, impairment of the status of m^6A regulation has lead to prolonged expression of Nanog in ES cells and the inability to exit self-renewal stages toward differentiation. m^6A RNA immunoprecipitation (MeRIP) is a method to monitor the status of m^6A and map the location of m^6A modifications transcriptome-wide. We have optimized the method by using a monoclonal antibody against m^6A modifications and magnetic A/G beads. The streamlined protocol is easy to process and can produce results with high SN ratio.

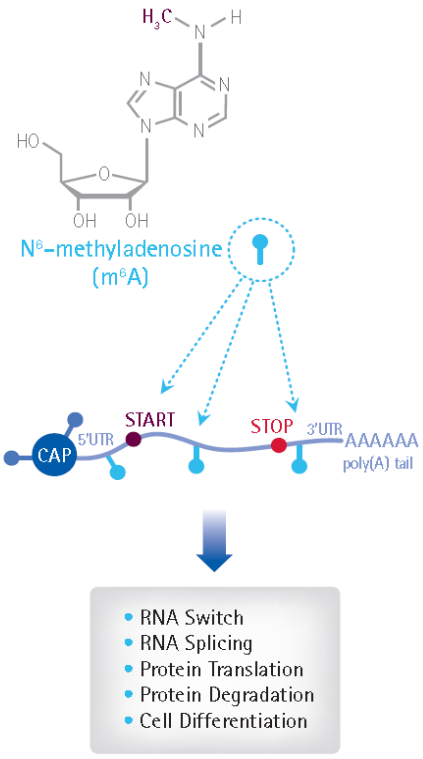


Figure 1. m6A modification of RNA

Overview of the Magna MeRIP Method

The Magna MeRIP M^6A Assay Kit uses the MeRIP method to enable identification and transcriptome-wide profiling of m^6A. In the MeRIP assay, RNA is chemically fragmented into 100 nucleotides or smaller fragments followed by magnetic immunoprecipitation with a monoclonal antibody toward m^6A. After immunoprecipitation, isolated RNA fragments can be subjected with qRT-PCR or RNA sequencing (RNA-seq).

Overview of Magna MeRIP Kit Workflow

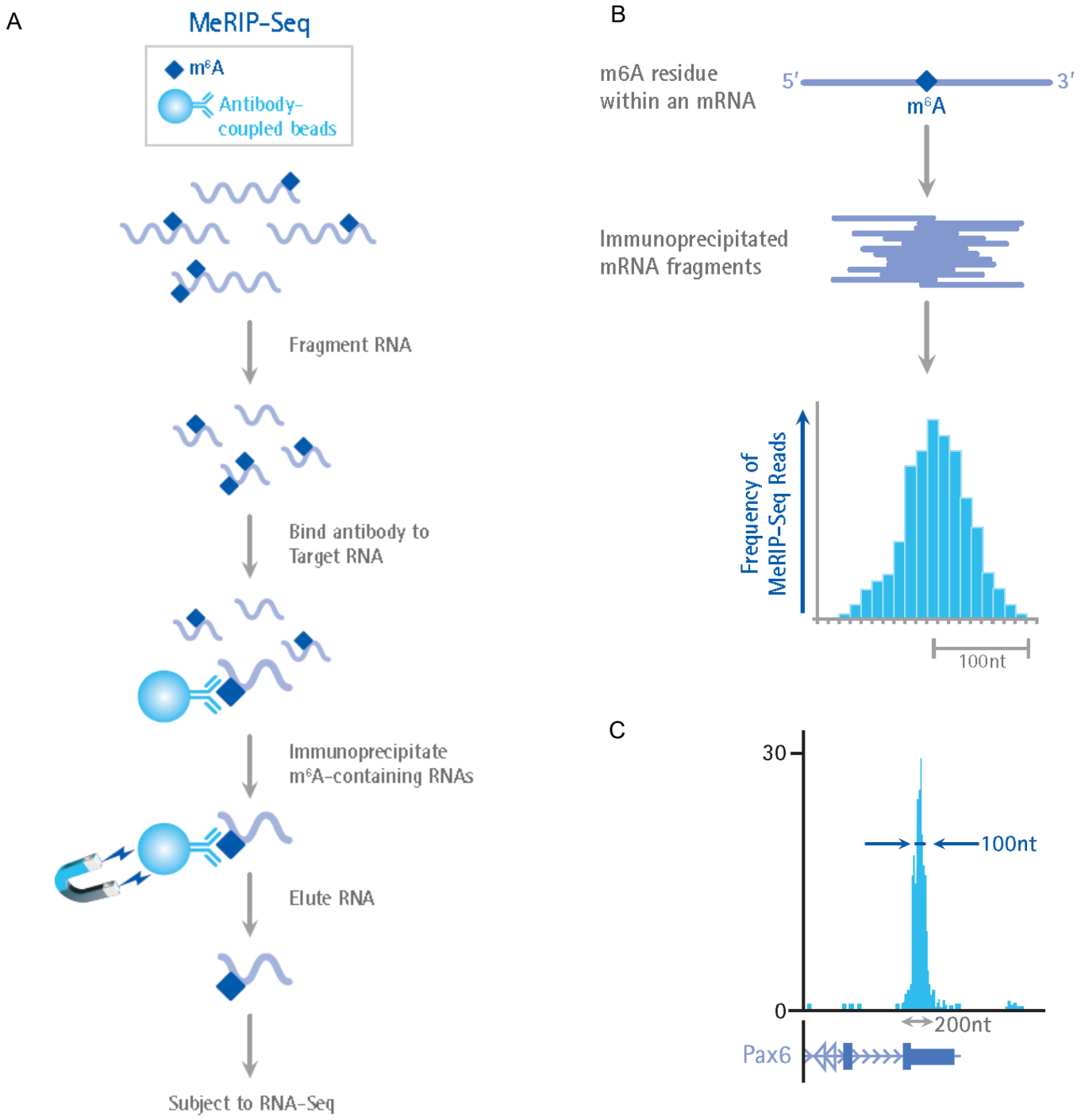


Figure 2. Magna MeRIP m6A Assay kit process

Materials Provided (Kit Configurations)

The MeRIP kit provides sufficient reagents for 10 MeRIP reactions using 5 µg of mRNA or 5 MeRIP reactions when 300 µg of total RNA is used.

17-10499-1 (Component Box for Magna MeRIP, Store at 2° to 8°C)		
Component	**Part #**	**Quantity**
RNA Fragmentation Buffer 10X	CS220011	0.5 mL
0.5M EDTA	CS203175	0.5 mL
IP Buffer 5X	CS220009	15 mL
Magnetic Beads A/G Blend	CS203152	315 µL
Anti-N6-methyladenosine (m^6A), clone 17-3-4-1	MABE1006	100 µg

17-10499-2 (Component Box for Magna MeRIP, Store at -20°C)		
RNase Inhibitor	CS216138	90 µL
N6-Methyladenosine, 5′-monophosphate sodium salt (m^6A)	CS220007	10 mg
Normal Mouse IgG	CS200621	125 µg
MeRIP Primers Human EEF1A1 Positive F: CGG TCT CAG AAC TGT TTG TTT C R: AAA CCA AAG TGG TCC ACA AA	CS220017	75 µL
MeRIP Primers Human EEF1A1 Negative F: GGA TGG AAA GTC ACC CGT AAG R: TTG TCA GTT GGA CGA GTT GG	CS220018	75 µL

Materials Required But Not Supplied

Reagents

- Total RNA or Messenger RNA
- 100% Ethanol (molecular biology grade)
- 3M Sodium Acetate (EMD Cat. # 567422-100ML)
- Nuclease Free Water
- RNeasy® MiniElute® Cleanup kit (QIAGEN, Cat. # 74204) or similar RNA purification column kit
- Glycogen if total RNA is used
- Conical tube (15 mL and 50 mL)
- Nuclease-free Microcentrifuge tubes, 1.5 mL, 2.0 mL
- PCR plate, 0.2 mL

Reagents for qRT-PCR Analysis

- One-Step RT-PCR Reagent (e.g. Bio-Rad iTaq™ Universal SYBR® Green One-Step Kit Cat. # 172-5150)

Reagents for MeRIP-Seq Library Construction

- Sequence library preparation kit
  low input RNA next generation sequencing library construction kit

Equipment

- Magnetic Separation Rack
  - Magna GrIP™ Rack (8 well, Millipore Cat. # 20-400) or PureProteome™ Magnetic Stand, (Millipore Cat. # LSKMAGS08 and LSKMAGS15)
- Vacuum Aspirator
- Vortex mixer
- Microcentrifuge
- Ultra low temperature freezer (below -80°C)
- Thermomixer® (4°C capable)
- Variable temperature water bath or incubator
- Rotating microtube mixer
- Pipette (2 mL, 5 mL, 10 mL, 25 mL)
- Variable volume (5-1000 µL) pipettes
- Real-time PCR thermal cycler
- NanoDrop™ or spectrophotometer, or Bioanalyzer

Important Notes Before Starting

Please read through the entire protocol and carefully plan your work before starting. The MeRIP method requires multiple steps and can be done over a two-day period or over multiple days. There are several stopping points to allow the method to be carried out over multiple days. The approximate time required for each step and potential stopping points are provided in the tables below.

Fragmentation, Immunoprecipitation, and Elution

Protocol Step	Time Required	Stopping Points and Protocol Notes
Fragmentation	~3 hours	Can freeze fragmented RNA in ethanol (total RNA) or after column purification (mRNA) and store at -80°C
Immunoprecipitation	~2.5 hours	Continue to elution step
Elution	~1 hours	Can store eluted RNA at -80°C

Recovery and Analysis of RNA

Protocol Step	Time Required	Stopping Points and Protocol Notes
RNA Isolation	~2.5 hours	Can store column purified RNA -80°C
RNA Analysis	~4 hour for RT-qPCR	Note that analysis can also be performed by RNA-seq

RNase control

Throughout this method, all standard precautions should be taken to minimize RNase contamination. Gloves should be worn at all steps of the procedure. All instruments, glassware and plastics that touch cells or cell lysates should be certified nuclease-free or should be pretreated using DEPC or other RNase inactivation reagents according to established protocols for working with RNA. RNase inhibitor (Part# CS216138) is included as a component in this kit. All solutions utilized that are not kit components should be certified DNase-free and RNase-free from the manufacturer wherever possible.

Detailed Protocol

If you are new to the Magna MeRIP method, please read and understand this entire protocol before starting. This version of the protocol contains important details and helpful tips to facilitate a successful result. For advanced users, a summary protocol is presented starting on page 15.

A. Planning RNA Requirements for MeRIP Experiments

- MeRIP experiments can be started from total RNA, mRNA, or rRNA depleted RNA. We recommend starting with 5 ug of mRNA or rRNA depleted RNA per immunoprecipitation since there are considerable amount of m6A in rRNA (18S). mRNA can be purified by using commercially available kits like GenElute mRNA miniprep kit (SIGMA, Cat. No.MRN10) and rRNA depletion can also be done by various kits. If starting with total RNA, 300 ug of total RNA per immunoprecipitation is recommend.

    - *Although we recommend starting the experiment with enough RNA, we were able to obtain 10 to 20 ng of sample (enough for preparing RNA-seq library) from 0.5 ug of mRNA or 30 ug of total RNA from HEK293 cells in our internal evaluation.*

- Use of intact RNA is critical. We recommend to use column purification and check the RNA integrity by Bioanalyzer. Degraded RNA affect to the quality of the assay.

- DNase treatment is highly recommended. This is more important working with RNA from organism known to contain m6A in their DNA. The anti-m6A antibody recognize m6A in single strand DNA.

Table 1. Approximate number of the cells and expected total RNA yield per cell culture vessel (HeLa cells)

Type of vessel	Surface Area (cm^2)	Cell Number	total RNA Yield (µg)
T-75	75	~0.5 x 10^7	70
T-225	225	~1.3 x 10^7	180
10 cm plate	78.5	~0.5 x 10^7	70
15 cm plate	176.6	~1.0 x 10^7	140

B. RNA fragmentation

Important: Optimal conditions need to be determined to shear RNA to ~100 nt in length. See Appendix A for a typical protocol. Once shearing conditions have been optimized, proceed with the steps below. The protocol presented below describes typical examples for mRNA or total RNA.

B1 Process with mRNA (polyadenylated RNA), Skip this section and process section B2 if started with total RNA

1. Adjust the RNA concentration to ~1 µg/µL and the volume to 18 µL with nuclease-free water in a thin-walled 200-µl PCR tube. Add 2 µL of Fragmentation Buffer 10X (part # CS220011). Mix well by pipetting and spin down the tube if necessary.

    - *Adherence to the specified amounts and volumes is highly recommended, as scaling may affect fragmentation efficiency and the resulting size distribution.*

2. Preheat a thermal cycler block at 94 °C. Incubate the tube in the thermal cycler block for 4 min with the heated lid closed. Remove the tube from the block and immediately add 2 µl of 0.5 M EDTA (part # CS203175). Vortex and spin down the tube and place it on ice.

    - *Work quickly at this stage and immediately proceed to the next Step.*
    - *Substituting metal ion–induced fragmentation with physical fragmentation by sonication is not advised, as it yields fragments >200 nt and might not be entirely random.*

3. Transfer the fragmented RNA to a new 1.5 mL microcentrifuge tube.
4. Purity the fragment RNA by RNase MiniElute Kit or similar RNA purification
    a. Add 78 µl of Nuclease-free water to the fragmented RNA from Step 3 to adjust to a volume of 100 µl. Add 350 µl of Buffer RLT, and mix well.
    b. Add **700 µl** of 96–100% ethanol to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to step c.
    c. Transfer 700 µl of the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Transfer the remaining 450 µl of sample to the centrifugation. Discard the flow-through.
    d. Place the spin column in a new 2 ml collection tube (supplied in the RNA purification kit). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step e.
    e. Add 500 µl of 80% ethanol to the spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.
    f. Place the spin column in a new 2 ml collection tube (supplied in the RNA purification kit). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
    g. Place the spin column in a new 1.5 ml collection tube (supplied in the RNA purification kit). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

    - *Eluted RNA can be stored at −80 °C for up to 1 year.*

B2 Process with total RNA (Skip this section and continue to section C if started with mRNA)

1. Adjust the RNA concentration to ~1 µg/µL with nuclease-free water. Dispense 18 µL (~18 µg) of total RNA to each thin-walled 200-µl PCR tube. Add 2 µL of Fragmentation Buffer 10X (part # CS220011). Mix well by pipetting and spin down the tube if necessary.

    - *Adherence to the specified amounts and volumes is highly recommended, as scaling may affect fragmentation efficiency and the resulting size distribution.*
    - *For 300 µg of total RNA 17 tubes are required.*

2. Preheat a thermal cycler block at 94 °C. Incubate 5 tubes at a time in the thermal cycler block for 5 min with the heated lid closed. Remove the tubes from the block and immediately add 2 µl of 0.5 M EDTA (part # CS203175) to each tube. Vortex and spin down the tube and place it on ice. Repeat this step for each batch of five tubes until all of the RNA is fragmented.

    - *Work quickly at this stage and immediately proceed to next step.*
    - *We advise working in batches of five tubes at a time (300 µg of RNA require ~17 tubes).*

3. Collect contents of all tubes, add one-tenth volumes of 3 M sodium acetate (pH 5.2), glycogen (100 µg ml−1 final) and 2.5 volumes of 100% ethanol. Mix the contents and incubate at −80 °C overnight.

    - *Do not use nucleic acids as carriers for precipitation, as they will interfere with downstream IP and sequencing.*
    - *RNA is stable in the precipitation mixture when stored at −80 °C for up to 1 year.*

4. Centrifuge the tubes at 15,000g for 25 min at 4 °C. Discard the supernatant, taking care not to disrupt the pellet, which is easily visible because of the presence of glycogen. Wash the pellet with 1 ml of 75% (vol/vol) ethanol and centrifuge again at 15,000g for 15 min at 4 °C.
5. Carefully aspirate the supernatant and let the pellet air-dry. Resuspend the pellet in 300 µl of RNase-free water.

    - *RNA can be stored at −80 °C at this stage until further use for up to 1 year.*

C. Validation of post-fragmentation size distribution

Validate RNA post-fragmentation size distribution and concentration by Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit. Alternatively, fragmented RNA concentration can be measured by a NanoDrop spectrophotometer and the size distribution can be checked by running 0.5 µg of RNA on 1.5% (wt/vol) agarose gel. The outlined fragmentation procedure should produce a distribution of RNA fragment sizes centered on ~100 nt.

Validate RNA size distribution only after it has been column purified or ethanol-precipitated, as the presence of salts may affect gel migration of the fragments. We advise using an RNA-dedicated electrophoresis apparatus.

D. Preparation of magnetic beads for immunoprecipitation

Key Considerations Before Starting This Section

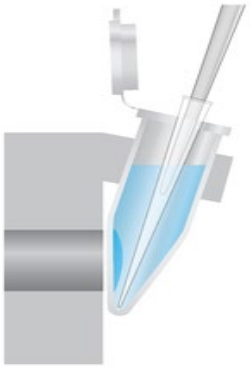
As a starting point 2.5 to 5 µg of anti-M6A antibody for 5 µg of fragmented mRNA or 10 µg of antibody for 300 µg of fragmented total RNA is suggested as a guideline, but the quantity may need to be optimized.

When performing wash steps with magnetic beads, the use of a vacuum aspirator is recommended. To avoid introduction of RNases, use an aspirator pipette with the addition of RNase free sterile microtips when possible.

1. Prepare **4 mL** per sample for mRNA (or **5 mL** per sample for total RNA) 1X IP buffer in a new conical tube by diluting 800 µL for mRNA (or 1 mL for total RNA) IP buffer, 5X (part # CS220009) with 3.2 mL for mRNA (or 4 mL for total RNA) of nuclease free water. Place the tube on ice.
2. Label the appropriate number of 1.5 mL microcentrifuge tubes for the number of desired MeRIP reactions.
   - *Label one microcentrifuge tube for anti-m6A antibody and one negative control Normal Mouse IgG (Part # CS200 621).*
3. Completely disperse and re-suspend Magna ChIP Protein A/G Magnetic Beads (Part # CS203152) by end over end rotation or by pipetting. No clumps of beads should be visible
4. For each reaction planned in step 2 above, transfer 25 µL for mRNA (or 50 µL for total RNA) of Magna ChIP protein A/G Magnetic Beads to a microcentrifuge tube.
5. Add ten times the original bead volume of **1X** IP Buffer prepared at step 1 for the number of MeRIP samples (250 µL MeRIP Dilution Buffer per 25 µL of original volume of the magnetic beads) and mix the beads by gently pipetting up and down several times to completely resuspend beads. Place the tube on the magnetic separator (e.g. Millipore Cat. # 20-400) for 1 minute.

Magna GrIP™ Rack
Millipore Cat. # 20-400

6. Remove the supernatant making sure not to aspirate any magnetic beads. Remove the tubes from the magnet.
7. Repeat Step 4 and Step 5 for one additional wash.
8. Re-suspend the beads in 100 µL of the **1X** IP Buffer per 25 µL of original volume of magnetic beads. If multiple reactions are being performed, transfer 100 µL for mRNA (or 200 µL for total RNA) of the beads suspension to each microcentrifuge tube. Add 5 µg for mRNA (or 10 µg for total RNA) of the anti m6A antibody (Part # MABE1006) to each microcentrifuge tube.
   - *It is highly recommended you perform a negative control RIP reaction using normal Mouse IgG (Part # CS200621).*


Supernatant can be removed gently and easily with no sample loss

9. Incubate with rotation for 30 minutes at room temperature.
10. Centrifuge the tubes briefly and place on the magnetic separator for 1 minute and remove the supernatant.

11. Remove the microcentrifuge tubes from the magnet. Add 0.5 mL of **1X** IP Buffer to each tube and mix the beads by gently pipetting several times to completely resuspend beads. Place the microcentrifuge tube on a magnetic separator for 1 minute then remove supernatant.
12. Repeat step 10 for two additional washes. Be sure to remove final wash leaving only beads.
13. Remove the microcentrifuge tubes from the magnet and place them on ice. Close tube caps to avoid drying out of beads. These samples will be used in section E step 3.

E. Immunoprecipitation (MeRIP)

1. Remove 0.5 µg of fragmented mRNA or 30 µg of total RNA (10% of Input), and place it into a new microcentrifuge tube labeled as "**RNA input**". Keep the input sample at −80 °C. The sample will be used to generate a standard curve or for comparison in RT-PCR methods, or input control in RNA-seq.
2. Prepare the MeRIP reaction mixture tabulated below. Prepare 500 µL reaction mixture for 5 µg of mRNA or 1000 µL reaction mixture for 300 µg of total RNA.

Table 2. MeRIP Reaction Mixture

Component	mRNA	Total RNA
Fragmented RNA in Nuclease free water	395 µL	790 µL
RNase Inhibitor	5 µL	10 µL
IP buffer, 5x	100 µL	200 µL
Total	**500** µL	**1000** µL

3. Add 500 µL of MeRIP reaction mixture to each beads-antibody tube from section **D** step 12**.** Mix by gently pipetting several times to completely resuspend beads. Place on ice.
4. Incubate all tubes with rotation for 2 hours at 4°C.
5. Centrifuge the MeRIP reactions briefly to remove liquid from cap and sides of the microcentrifuge tube. Place on a magnetic separator for 1 minute.
6. Discard the supernatant, being careful not to disturb the magnetic beads.
7. Remove tubes from the magnet. Add 500 µL cold **1X** IP Buffer prepared at section D step 1, and mix the beads by gently pipetting several times to completely resuspend beads.
8. Place tubes on a magnetic separator for 1 minute then discard supernatant.
9. Repeat the above wash procedure (steps 7 to step 8) twice and total 3 wash.
10. Place the tubes on ice and immediately proceed to Elution

F. Elution

1. Prepare 20 mM m^6A by dissolving 10 mg of N6-Methyladenosine, 5′-monophosphate sodium salt (m^6A) (part # CS220007) in 1.3 mL nuclease free water. Store aliquots of 150 µL at -20 °C.
2. Prepare 225 µL Elution Buffer per sample by mixing 45 µL of IP Buffer 5X (part # CS220009), 75 µL of 20 mM m6A prepared at step 1, 3.5 µL of RNase Inhibitor (part # CS216138) and 101.5 uL of nuclease free water.
3. Add 100 µL of elution buffer to the beads from section E step10. Mix by gently pipetting several times to completely resuspend beads.
4. Incubate all tubes for 1 h with continuous shaking at 4 °C.

- Remember that Steps 3–4 should be carried out on the MeRIP samples, as well as negative control normal mouse IgG sample.

5. Centrifuge the MeRIP reactions briefly to remove liquid from cap and sides of the microcentrifuge tube. Place on a magnetic separator for 1 minute.
6. Transfer the supernatant containing eluted RNA fragments to a new 1.5 mL microcentrifuge tube.Take special care not aspirate the beads, as it will increase background noise.
7. Repeat step 3-6 and combine all elutes from the same sample. Total elution volume should be 200 μL.
8. Purify the eluted RNA by RNeasy mini kit or similar RNA purification column
    a. Transfer the 200 μL of sample to a new 15 mL conical tube. Add 700 µl of Buffer RLT, and mix well.
    b. Add **1,400 µl** of 96–100% ethanol to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step c.
    c. Transfer 700 µl of the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Transfer the another 700 µl of sample to the centrifugation. Discard the flow-through. Repeat the process until all sample has loaded to the column.
    d. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied in the RNA purification kit). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step e.
    e. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.
    f. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied in the RNA purification kit). Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
    g. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied in the RNA purification kit). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

- *Do not add glycogen (or other carrier) to the precipitation mixture at this stage, as it can precipitate free m6A, possibly interfering with downstream reactions and measurements.*
- *RNA can be stored at − 80 °C*.

G. Analysis of MeRIPed RNA

RNAs isolated using the Magna MeRIP kit can be analyzed by quantitative RT-PCR. Once successful methylated RNA retrieval (if methylated and unmethylated regions are known) by MeRIP can be confirmed, further transcriptome wide interrogation can be pursued by deep sequencing.

Presented below are illustrative methods for performing real time quantitative measurement of MeRIP experiments using the control primers. Verification of retrieval of methylated RNA can be performed using the relative standard curve method of qPCR analysis to compare RNA from control normal Mouse igG vs. anti-m6A, or can alternatively be compared using the comparative Ct (ΔΔCt) method with two PCR amplicons, a positive control methylated region (Human EEF1A positive), and a negative control unmethylated region (Human EEF1A1 negative). Input RNA is required whether using relative standard curve method or the comparative Ct (ΔΔCt) method. An examples of successful RNA retrievals are shown in Figure 3 to Figure 5.

1-Step Real-time Quantitative RT-PCR for RNA

1. Add 2 µL of the RNA sample to the PCR plate suitable for your real-time instrument of choice (Performing a triplicate of qPCR reactions per MeRIP sample is recommended).
   - *12 µL from all MeRIP or input sample are available.*
   - *2.0 µL or less MeRIP RNA is recommended for a 20 µL RT-qPCR reaction.*
   - *Performing triplicate of qPCR reactions per MeRIP sample is also recommended.*
   - *If using the relative standard curve method, perform four 5- or 10-fold serial dilutions using the RNA from the 10% input sample, and use these samples to build a standard curve. Concentration of the MeRIP samples can be calculated as percent of input using the standard curve. Alternatively, data can be calculated in relation to cell equivalents or mass of purified RNA, if desired.*
2. Prepare a master reaction mix as shown in Table 3. Dispense enough reagents for one extra tube to account for loss of volume. For reagents other than iTaq™ Universal one-Step Kits (Bio-Rad) follow manufactures recommendations.
3. Add 18 µL of qPCR mix to 2 µL of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.

Table 3. 1-Step qRT-PCR setup and running parameters using iTaq Universal One-Step Kit

1-Step qRT-PCR reagent assembly for 1 reaction:

SYBR® Green Master Mix	10.0 µL
Reverse Transcriptase	0.25 µL
ddH_2O	6.75 µL
Primer mix	1.0 µL
Total	18 µL

qPCR parameters:

cDNA Synthesis	50°C 10 min	
Polymerase Inactivation	95°C 1 min	
Denature	95°C 10 sec	40 cycles
Anneal and Extend:	60°C 30 sec	

I. Data Analysis

There are many algorithms to analyze MeRIP result; the two most common methods are the relative standard curve method and the ΔΔCt method.

a. Normalize RNA concentration to percent of input using relative standard curve

1. For each RNA of interest, make four 5- or 10-fold serial dilutions with the 10% (RNA) input sample, perform quantitative real-time RT-PCR with these input samples, antibody the sample with anti-m6A, and the sample with negative control mouse IgG.
2. Calculate the threshold cycle (Ct) values using real-time detection system software from qPCR equipment manufacturer.
3. Use the threshold cycle (Ct) values of these input samples to build a standard curve.
4. Determine the concentration (C) of the sample with anti-m6A and the sample with control mouse IgG as percent of input using the standard curve.

5. Determine the fold enrichment by calculating the ratio of C_{m6A} and $C_{\text{negative control mouse IgG}}$.
    - *For each independent experiment, we suggest that you perform the following MeRIP qPCR assays in triplicates in the same plate, if possible.*
    - *For control experiments, the positive control region is stop codon of human EEF1A1 (primers provided), and the negative control region is exon 5 of human EEF1A1 (primers provided).*

b. ΔΔCt method

1. Perform quantitative real-time RT-PCR with antibody the sample with anti-m6A, the sample with negative control mouse IgG, and input sample in triplicates.
2. Perform quantitative real-time RT-PCR with primer set targeting a positive control and primer set targeting a negative control separately.
3. Calculate the threshold cycle (Ct) values using a real-time software from qPCR equipment manufacturer.
4. Normalize Ct values of the sample with anti-m6A and the sample with negative control mouse IgG to input (ΔCt) by subtracting the Ct value obtained for the input from the Ct value for IP sample: $\Delta Ct = Ct_{IP} - (Ct_{input}\text{-Log2 [Input Dilution Factor]})$ (Input dilution factor is 10 if using 10% input sample).
5. Calculate the percent of input for each IP sample: $\%Input = 2^{(-\Delta Ct\ [\text{normalized IP}])}$.
6. Normalize the ΔCt values of sample with anti-m6A to the sample with negative control IgG (ΔΔCt) by subtracting the ΔCt value obtained for anti-m6A antibody from the ΔCt value for negative control mouse IgG ($\Delta\Delta Ct = \Delta Ct_{positive} - \Delta Ct_{negative}$).
7. Estimate the fold enrichment of the sample with anti-m6A antibody over the negative control mouse IgG: Fold enrichment $= 2^{-\Delta\Delta Ct}$.

MeRIP-seq (NGS Analysis)

RNAs isolated using the Magna MeRIP kit can produce RNA-seq library using standard RNA-seq library construction products, such as mRNA-seq or TruSeq sample preparation kits (Illumina) or other library preparation kits. As RNA purification and fragmentation are already performed in the meRIP assay, these steps are skipped during library preparation. MeRIP library preparation can be started from first-strand cDNA synthesis step. We recommend size-selecting the library by gel excision (adjusting the expected size range of the desired band according to the average size of fragmented RNA and considering the added length of adapter or sequencing primers) instead of Agencourt beads purification. It is important to validate the successful preparation of each library before proceeding to sequencing using an Agilent 2100 Bioanalyzer (or equivalent). Libraries can be prepared from input and IP samples. The bead-only control sample usually cannot produce a library because of very few RNA recovery (background). The analysis of MeRIP RNA-Seq library can be performed between a MeRIP and input RNA (total RNA). It usually is difficult to perform analysis using the mock IgG control since the amounts of material that are recovered from this sample is very little.

Summary Protocol for Experienced Users

If this is your first time using this kit please follow detailed protocol above for best results. Once you are comfortable with all of the steps of the protocol this summarized version can be used.

I. RNA Fragmentation

IA. Process with mRNA (polyadenylated RNA), Skip this section and process section IB if started with total RNA

1. **Adjust the mRNA concentration** to ~1 µg/µL and total volume to 18 µL with nuclease-free water. Add 2 µL of Fragmentation buffer 10x (part # CS220011) in a thin-walled 200-µl PCR tube.
2. **Incubate the tube in the thermal cycler block at 94 °C for 4 min** with the heated lid closed. Remove the tube from the block and immediately add 2 µl of 0.5 M EDTA (part # CS203175). Vortex and spin down the tube and place it on ice.
3. **Transfer the fragmented RNA** to a new 1.5 mL microcentrifuge tube.
4. Purity the fragment RNA by RNase MiniElute Kit or similar RNA purification
    a. Add 78 µl of Nuclease-free water to the fragmented RNA from Step 3. Add 350 µl of Buffer RLT, and mix well.
    b. Add **700 µl** of 96–100% ethanol and mix well by pipetting. Proceed immediately to step c.
    c. Transfer 700 µl of the sample to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Transfer the remaining 450 µl of sample to the centrifugation. Discard the flow-through.
    d. Place the spin column in a new 2 ml collection. Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Reuse the collection tube in step e.
    e. Add 500 µl of 80% ethanol to the spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm). Discard the flow-through and collection tube.
    f. Place the spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
    g. Place the spin column in a new 1.5 ml collection tube. Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

IB. Process with total RNA, Skip this section and continue to section II if started with mRNA

6. **Adjust the RNA concentration** to ~1 µg/µL with nuclease-free water. Dispense 18 µL (~18 µg) of total RNA to each thin-walled 200-µl PCR tube. Add 2 µL of Fragmentation buffer 10x (part # CS220011). Mix well by pipetting and spin down the tube if necessary.
7. **Preheat a thermal cycler block** at 94 °C. Incubate 5 tubes at a time in the thermal cycler block for 5 min with the heated lid closed. Remove the tubes from the block and immediately add 2 µl of 0.5 M

EDTA (part # CS203175) to each tube. Vortex and spin down the tube and place it on ice. Repeat this step for each batch of five tubes until all of the RNA is fragmented.

8. **Collect contents of all tubes,** add one-tenth volumes of 3 M sodium acetate (pH 5.2), glycogen (100 µg ml−1 final) and 2.5 volumes of 100% ethanol. Mix the contents and incubate at −80 °C overnight.
9. **Centrifuge the tubes at 15,000g for 25 min at 4 °C.** Discard the supernatant, taking care not to disrupt the pellet, which is easily visible because of the presence of glycogen. Wash the pellet with 1 ml of 75% (vol/vol) ethanol and centrifuge again at 15,000g for 15 min at 4 °C.
10. **Carefully aspirate the supernatant and let the pellet air-dry.** Resuspend the pellet in 300 µl of RNase-free water.

II. Validation of post-fragmentation size distribution

Validate RNA post-fragmentation size distribution and concentration by Agilent 2100 Bioanalyzer with an Agilent RNA 6000 Pico kit. Alternatively, fragmented RNA concentration can be measured by a NanoDrop spectrophotometer and the size distribution can be checked by 1.5% (wt/vol) agarose gel.

III. Preparation of magnetic beads for immunoprecipitation

1. **Prepare 4 mL per sample for mRNA (or 5 mL per sample for total RNA) 1X IP buffer** in a new conical tube by diluting 800 µL for mRNA (or 1 mL for total RNA) IP buffer, 5X (part # CS220009) with 3.2 mL for mRNA (or 4 mL for total RNA) of nuclease free water. Place the tube on ice.
2. **Label the appropriate number of 1.5 mL microcentrifuge tubes** for the number of desired IP reactions.
3. **Completely disperse and re-suspend Magna ChIP Protein A/G Magnetic Beads (Part # CS203152)** by end over end rotation or by pipetting. No clumps of beads should be visible.
4. **For each reaction planned in step 2 above, transfer 25 µL (for mRNA) or 50 µL (for total RNA) of Magna ChIP protein A/G Magnetic Beads** to a microcentrifuge tube.
5. **Add ten times the original bead volume of 1X IP Buffer prepard at step 1** for the number of MeRIP samples and mix the beads by gently pipetting up and down several times to completely resuspend beads. Place the tube on the magnetic separator for 1 minute.
6. **Remove the supernatant** making sure not to aspirate any magnetic beads. Remove the tubes from the magnet.
7. **Repeat Step 4 and Step 5** for one additional wash.
8. **Re-suspend the beads in 100 µL of the 1X IP Buffer** per 25 µL of original volume of magnetic beads. If multiple reactions are being performed, transfer 100 µL for mRNA (or 200 µL for total RNA) of the beads suspension to each microcentrifuge tube. Add 5 µg for mRNA (or 10 µg for total RNA) of the anti m6A antibody (Part # MABE1006) to each microcentrifuge tube.
9. **Incubate with rotation** for 30 minutes at room temperature.
10. **Centrifuge the tubes briefly** and place on the magnetic separator for 1 minute and remove the supernatant.
11. **Remove the microcentrifuge tubes** from the magnet. Add 0.5 mL of 1X IP Buffer to each tube and mix the beads by gently pipetting several times to completely resuspend beads. Place the microcentrifuge tube on a magnetic separator for 1 minute then remove supernatant.

12. **Repeat step 10 for two additional washes.** Be sure to remove final wash leaving only beads.
13. **Remove the microcentrifuge tubes** from the magnet and place them on ice. Close tube caps to avoid drying out of beads. These samples will be used in section E step 3.

IV. Immunoprecipitation (MeRIP)

1. **Remove 0.5 µg of fragmented mRNA or 30 µg of total RNA (10% of Input),** and place it into a new microcentrifuge tube labeled as "RNA input". Keep the input sample at −80 °C.
2. **Prepare the MeRIP reaction mixture** tabulated below. Prepare 500 µL reaction mixture for 5 µg of mRNA or 1000 µL reaction mixture for 300 µg of total RNA.

Component	mRNA	Total RNA
Fragmented RNA in Nuclease free water	395 µL	790 µL
RNase Inhibitor	5 µL	10 µL
IP buffer, 5x	100 µL	200 µL
Total	**500** µL	**1000** µL

3. **Add 500 µL of MeRIP reaction mixture** to each beads-antibody tube from section D step 12. Mix by gently pipetting several times to completely resuspend beads. Place on ice.
4. **Incubate all tubes with rotation** for 2 hours at 4°C.
5. **Centrifuge the MeRIP reactions briefly** to remove liquid from cap and sides of the microcentrifuge tube. Place on a magnetic separator for 1 minute.
6. **Discard the supernatant,** being careful not to disturb the magnetic beads.
7. **Remove tubes from the magnet.** Add 500 µL cold 1X IP Buffer prepared at section III step 1, and mix the beads by gently pipetting several times to completely resuspend beads.
8. **Place tubes on a magnetic separator** for 1 minute then discard supernatant.
9. **Repeat the above wash procedure** (steps 7 to step 8) twice and total 3 wash.
10. **Place the tubes on ice** and immediately proceed to Elution

V. Elution

1. **Prepare 20 mM m6A** by dissolving 10 mg of N6-Methyladenosine, 5′-monophosphate sodium salt (m6A) (part # CS220007) in 1.3 mL nuclease free water. Store aliquots of 150 µL at -20 °C.
2. **Prepare 225 µL Elution Buffer per sample** by mixing 45 µL of IP Buffer 5X (part # CS220009), 75 µL of 20 mM m6A prepared at step 1, 3.5 µL of RNase Inhibitor (part # CS216138) and 101.5 uL of nuclease free water.
3. **Add 100 µL of elution buffer** to the beads from section E step10. Mix by gently pipetting several times to completely resuspend beads.
4. **Incubate all tubes** for 1 h with continuous shaking at 4 °C.
5. **Centrifuge the MeRIP reactions briefly** to remove liquid from cap and sides of the microcentrifuge tube. Place on a magnetic separator for 1 minute.
6. **Transfer the supernatant** containing eluted RNA fragments to a new 1.5 mL microcentrifuge tube.Take special care not aspirate the beads, as it will increase background noise.

7. **Repeat step 3-6** and combine all elutes from the same sample. Total elution volume should be 200 µL.
8. **Purify the eluted RNA** by RNeasy mini kit or similar RNA purification column
    a. **Transfer the 200 µL of sample** to a new 15 mL conical tube. Add 700 µl of Buffer RLT, and mix well.
    b. **Add 1,400 µl of 96–100% ethanol** to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step c.
    c. **Transfer 700 µl of the sample** to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. Transfer the another 700 µl of sample to the centrifugation. Discard the flow-through. Repeat the process until all sample has loaded to the column.
    d. **Place the RNeasy MinElute spin column** in a new 2 ml collection tube. Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step e**.**
    e. **Add 500 µl of 80% ethanol** to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.
    f. **Place the RNeasy MinElute spin column** in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the flow-through and collection tube.
    g. **Place the RNeasy MinElute spin column** in a new 1.5 ml collection tube. Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

VI. Analysis of MeRIPed RNA

Purified RNA can be analyzed by quantitative RT-PCR. Once succesful methylated RNA retrival can be confirmed, further transcriptome wide interrogation can be pursued by deep sequencing.

Example of Magna MeRIP m6A Assay Data

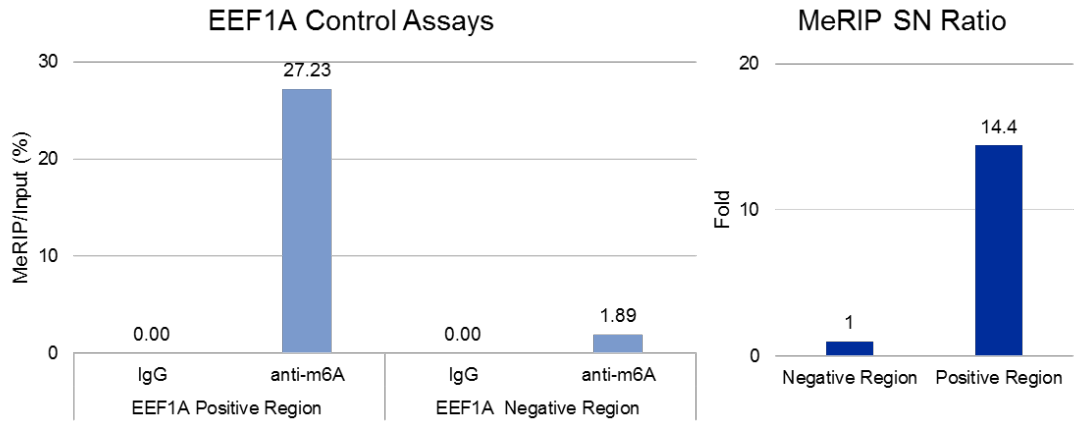


Figure 3: Successful retrieval of methylated RNA by MeRIP m6A Assay (mRNA)

MeRIP was performed using mRNA from HEK293 cells. Purified RNA was then analyzed by qRT-PCR using Positive Control Primers (MeRIP Primers Human EEF1A1 Positive, Part # CS220017) and Negative Control Primers (MeRIP Primers Human EEF1A1 Negative, Part # CS220017).

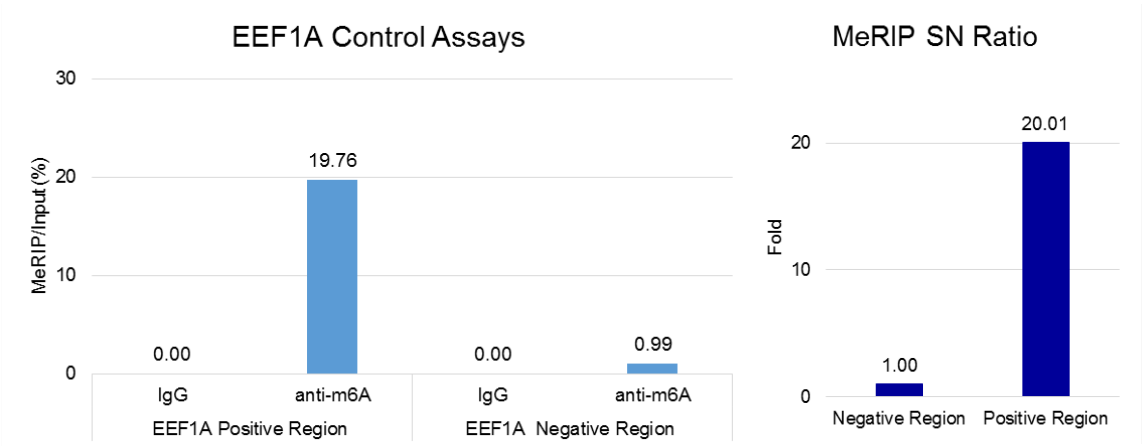


Figure 4. Successful retrieval of methylated RNA by MeRIP m6A Assay (total RNA)

MeRIP was performed using total RNA from HEK293 cells. Purified RNA was then analyzed by qRT-PCR using Positive Control Primers (MeRIP Primers Human EEF1A1 Positive, Part # CS220017) and Negative Control Primers (MeRIP Primers Human EEF1A1 Negative, Part # CS220017).

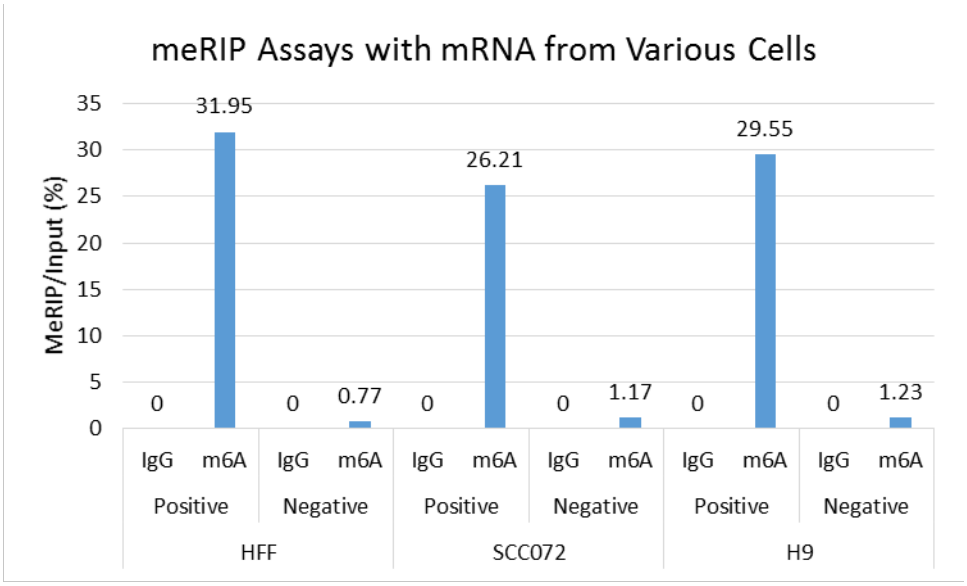


Figure 5. Successful retrieval of methylated RNA by MeRIP m6A Assay with mRNA from various cells

MeRIP was performed using mRNA from xeno-free human foreskin fibroblast cells (HFF), human neck squamous carcinoma cells (SCC072), and human embryonic stem cells (H9). Purified RNA was then analyzed by qRT-PCR using Positive Control Primers (MeRIP Primers Human EEF1A1 Positive, Part # CS220017) and Negative Control Primers (MeRIP Primers Human EEF1A1 Negative, Part # CS220017).

Appendix A: Optimization of RNA Fragmentation

Optimal conditions for chemical fragmentation of RNA to 100 nt in length depend on concentration of RNA, kinds of RNA (mRNA or total RNA), and the specific thermal cycler and the equipment settings (incubation time and temperature), and presence of residual EDTA and salts.
Approaches for optimizing sonication may include the following:

A. A fixed concentration of RNA (~1 µg/µL) and temperature 94 °C.
B. Varying the concentration of RNA with constant time and temperature parameters.
C. A combination of both approaches. However changing more than one parameter at a time is not recommended.

The protocol presented below describes optimization by option A with a thermal cycler (S1000 Thermal Cycler, Bio-Rad) as a specific example.

I. Adjust the RNA concentration to ~1 µg/µL for mRNA and the volume to 18 µL with nuclease-free water in a thin-walled 200-µl PCR tube. Add 2 µL of Fragmentation buffer 10x (part # CS220011). Mix well by pipetting and spin down the tube if necessary.

II. Prepare 100 µL of 0.05 M EDTA by diluting 10 µL of 0.5M EDTA (part # CS203175) with 90 µL of nuclease free water in a new micro centrifuge tube.

III. Preheat a thermal cycler block at 94 °C. Incubate the tube in the thermal cycler block min with the heated lid closed.

IV. Remove 2 µL fragmented RNA to a new micro centrifuge tube every minute. Add 2 µl of 0.05 M EDTA prepared at step II. Mix them and spin down the tube and place it on ice.

- *Optimum fragmentation time is usually around 4 minutes for mRNA and 5 to 6 minutes for total RNA.*
- *An example of total RNA from HEK293 cells suitably fragmented for use with MeRIP kit is shown in Figure 6.*

V. Analyze fragmented RNA by Agilent 2100 Bioanalyzer with an Agilent RNA kit or load 0.5 µg of fragmented RNA and analyze 10 µL on a 1-2% agarose gel.

VI. Observe which of the shearing conditions gives a peak of fragmented RNA centered 100 nt. See Figure 6 as an example.

VII. Repeat optimization of the shearing conditions if the resulting RNA fragments are not in the desired size range. Increase the process time if the RNA fragments are too large.

RNA Fragmentation: Sheared RNA Peak Should be 100 nt in Length

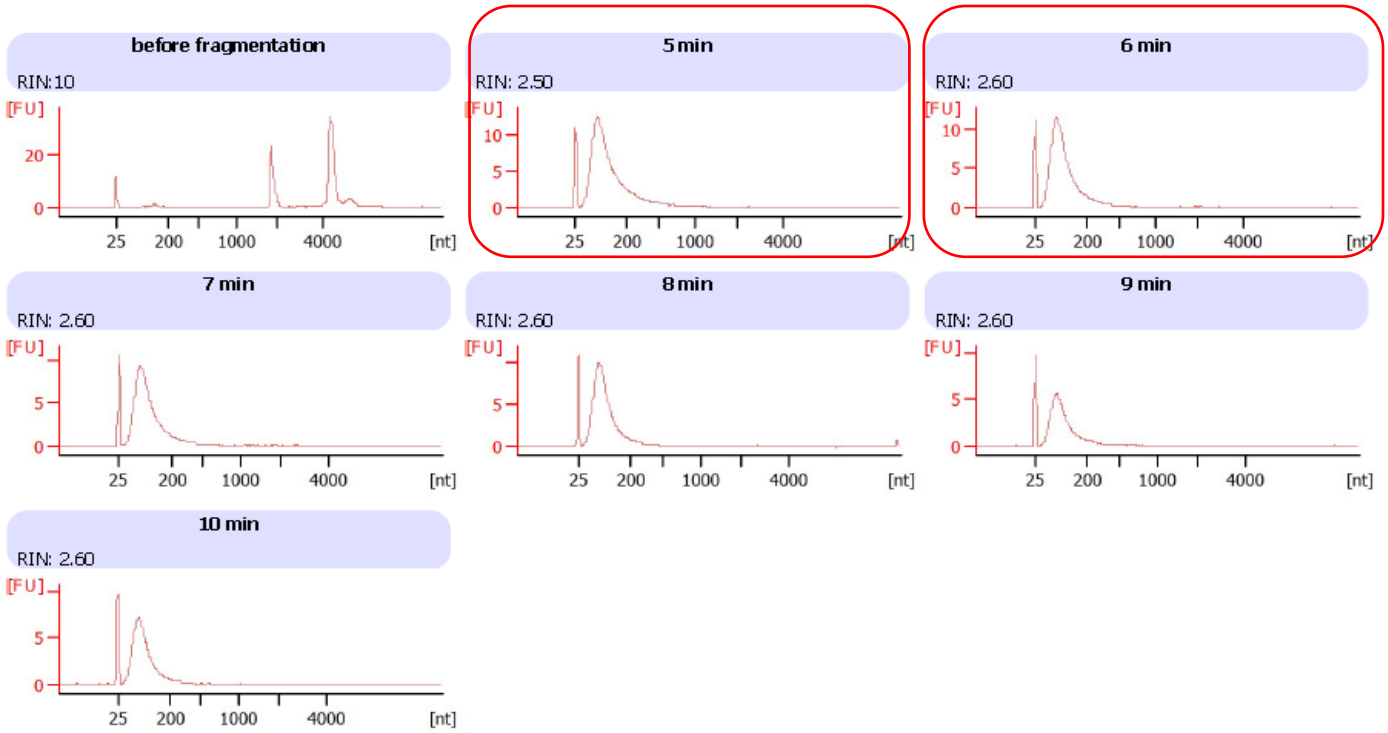


Figure 6: RNA fragmentation

Total RNA from HEK293 cells were fragmented. Time course experiments were performed with S1000 Thermal Cycler (Bio-Rad). The fragmented RNA was analyzed by Agilent 2100 Bioanalyzer with an Agilent RNA kit. Total RNA fragmented for 5 and 6 minutes resulted in the best shearing for MeRIP experiments.

MeRIP Optimization and Troubleshooting

Step	Potential Problems	Experimental Suggestions
RNA fragmentation	Not enough/too much fragmentation	If fragments are too large or too small, optimize fragmentation conditions using approach outline in appendix A to obtain appropriate size fragments.
Immuno-precipitation	Antibody doesn't immune-precipitate methylated RNA	• Titrate antibody and RNA to determine most effective immunoprecipitation conditions by performing IP using a dilution series of antibody with a fixed amount of RNA. • Increase incubation time of the antibody of interest with RNA to overnight at 4°C.
	Insufficient quantity of magnetic beads in immune-precipitation	• The magnetic beads settle to the bottom of the tube over time. Verify that the Magna ChIP Protein A/G magnetic beads are well mixed prior to removing the appropriate volume for IP. • Carefully aspirate liquids when using vacuum aspirator and use a high strength neodymium magnetic rack such as the Millipore Cat. # 20-400 Magna GrIP Rack to ensure Magna ChIP Protein A/G magnetic beads are tightly held against the wall of the microcentrifuge tube.
RNA Purification	Low RNA yield	• Most MeRIP reactions can expect yields on the order of tens to hundreds of nanograms from 5 µg of mRNA or 300 µg of total RNA depends on the cell line of tissue of origin. • If RNAs are not detectable consult immunoprecipitation step troubleshooting above.
	RNA degraded	• Use RNase inhibitor in solutions as recommended in this protocol. Make certain that all work conditions are RNase-free and RNases are not being introduced. • Follow the guidelines in the RNase control section before the Detailed Protocol section. • Use RNase-inactivating reagents to ensure work area and materials are RNase-free.
	No RNA detected	• Confirm the quality of RNA before and after fragmentation
PCR	No PCR product from Positive Control assay	• Increase amount of MeRIP sample used for PCR reaction up to 10% of total reaction volume. • Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct Tm. (If own primers are designed) • Perform PCR reaction with melting curve assessment to confirm amplification conditions and ability of primers to generate a single DNA product.
	High background level with negative control assay	Insufficient wash after immunoprecipitation. Increase the time of beads washing. Too long immunoprecipitation incubation time. Shorten the incubation time.

Related Products

Product	Description	Catalog Number
Magna ChIRP RNA Interactome Kit	Complete set of reagents for performing 12 ChIRP assays	17-10494
EZ-Magna ChIRP RNA Interactome Kit	Complete set of reagents for performing 12 ChIRP assays, plus positive and negative control primers and positive control probes	17-10495
Magna ChIRP™ Negative Control Probe Set	Negative control probe set for ChIRP	03-307
Magna ChIRP™ NEAT lncRNA Probe Set	Pre-designed even and odd probe set for ChIRP	03-308
Magna ChIRP™ TERC lncRNA Probe Set	Pre-designed even and odd probe set for ChIRP	03-309
Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays	17-10520
EZ-Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10521
Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays	17-10522
EZ-Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10523
Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10460
Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays	17-700
EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays, plus positive control antibody and control primers	17-701
EZ-Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions plus positive and negative control antibodies and validated qPCR primer set. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10461
RIPAb+™ Validated Antibody Primer Set	Proven for RIP or ChIP and lot tested for performance. See the complete selection at www.emdmillipore.com/chipab+ www.emdmillipore.com/ripab+	Multiple
ChIPAb+™ Validated Antibody Primer Set		Multiple

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2. D. Dominissini, S. Moshitch-Moshkovitz, S. Schwartz, et al. *Nature*. 2012 May 10; 484, 201-206
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