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High-Resolution Mapping of *N*^{6}-Methyladenosine Using m^{6}A Crosslinking Immunoprecipitation Sequencing (m^{6}A-CLIP-Seq)

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Abstract

N^6-Methyladenosine, an abundant chemical modification in mRNA, plays crucial roles in regulating gene expression and biological processes. Research on m^{6}A and its functions has progressed rapidly in the past few years, aided substantially by advances in high-throughput sequencing-based methods to profile m^{6}A along the transcriptome. We present here a protocol for m^{6}A crosslinking immunoprecipitation sequencing (m^{6}A-CLIP-seq), which profiles m^{6}A on mRNA at high resolution from as little as 1 μg of poly(A)-selected mRNA.

Keywords

N^6-Methyladenosine; Transcriptome; Methylome; Affinity purification; Sequencing

1 Introduction

RNA contains reversible chemical modifications that play essential roles in regulating gene expression. *N*^{6}-Methyladenosine (m^{6}A) is the most abundant posttranscriptional modification in eukaryotic mRNA, and was recently characterized as a reversible RNA mark [1, 2]. This discovery, along with recent advances in mass spectrometry, has allowed research on m^{6}A to progress at a rapid pace. m^{6}A has been shown to play critical roles in a wide range of biological processes including cancer, the viral life cycle, and stem cell differentiation [3–5].

Methods to map the locations of m^{6}A across the transcriptome are crucial for investigating its biological functions. Several high-throughput sequencing methods have been developed, with varying levels of sensitivity and required starting material. m^{6}A affinity purification and sequencing (m^{6}A-seq or MeRIP-seq, hereafter referred to as m^{6}A-seq) was the first to be developed [6, 7], and optimized protocols have allowed starting material as low as approximately 1 μg of poly(A)-selected mRNA [8]. m^{6}A-sequtilizes an m^{6}A-specific antibody to perform affinity purification of m^{6}A-marked mRNA fragments, profiling m^{6}A at a resolution of 200–400 nucleotides. Techniques with greater resolution have also been developed. Photo-crosslinking-assisted m^{6}A-sequencing (PA-m^{6}A-seq) utilizes 4-thiouridine and 365 nm UV irradiation to crosslink the m^{6}A antibody onto mRNA, thus achieving a resolution of approximately 23 nucleotides [9, 10]. The published PA-m^{6}A-seq protocol utilizes 12 μg of poly(A)-selected mRNA as starting material. m^{6}A iCLIP (miCLIP), which also uses UV-induced antibody-RNA crosslinking, allows single-nucleotide resolution profiling of m^{6}A, while requiring 20 μg of Poly(A)-selected mRNA as starting material [11].

Our laboratory has developed m^{6}A crosslinking immunoprecipitation sequencing (m^{6}A-CLIP-seq), which profiles m^{6}A at a resolution of approximately 100 nucleotides using as little as 1 μg of poly(A)-selected mRNA as starting material [12]. m^{6}A-CLIP-seq, which uses 254 nm UV irradiation, provides greater resolution than m^{6}A-seq, while using significantly less starting material than PA-m^{6}A-seq and miCLIP. In addition, m^{6}A-CLIP-seq can be performed on RNA extracted from freshly isolated tissue specimens, whereas PA-m^{6}A-seq requires cells grown in culture medium containing 4-thiouridine.

All existing high-throughput sequencing methods to map m^{6}A rely on antibodies that specifically enrich for m^{6}A. Although various commercial antibodies have been developed, many suffer from poor specificity to m^{6}A, and may enrich for other similar modifications such as N^1-methyladenosine (m^{1}A) or N^6,2′-O-dimethylade-nosine (m^{6}A$_m$). Moreover, the affinity purification step of antibody-based methods requires a substantial amount of starting material, thus restricting use of these methods to high-abundance biological samples. It will be important to develop methods that allow m^{6}A profiling in low-abundance starting materials, such as human patient samples. Chemical reagent-based methods that mutate either m^{6}A or adenine (unmethylated at the 6 position), but not both, could provide single-nucleotide resolution profiling of m^{6}A from low-abundance samples.

Here we describe the procedure used to perform m^{6}A-CLIP-seq. Several of the steps, such as RNA isolation, antibody binding, and size selection using gel electrophoresis, are identical to those used in our method for m^{6}A-seq [8], and as such are printed here without alteration. Bioinformatics analysis is performed using PAR-alyzer [13], using the same parameters as those used for PA-m^{6}A-seq, with the addition of using all mutations rather than simply mutations of T to C [9].

2 Materials

Prepare all solutions using DEPC-treated nuclease-free water and molecular grade reagents. Be sure to freshly prepare all reagents supplemented with SUPERase inhibitor or BSA, as reagent integrity may be compromised if these reagents are left at 4 °C or room temperature (RT).

2.1 Reagents

1. Cultured cells or tissues as a source of RNA. Any cell line or tissue is suitable for this procedure. As a reference, 1 confluent 10 cm plate of HeLa cells provides around 100 μg of total RNA, of which 2–5% is mRNA. At least 1 μg of poly(A)-selected mRNA is required.
2. RNeasy Plus Mini Kit (*see* Note 1).
3. mRNA Miniprep Kit.
4. RNA Clean & Concentrator-5.
5. Gel Extraction Kit.
6. RNase T1 (1000 U/μL).

7. T4 Polynucleotide Kinase.
8. Adenosine 5′-triphosphate (ATP).
9. NEBNext Small RNA Library Prep Set for Illumina.
10. FastStart Essential DNA Green Master.
11. β-Mercaptoethanol.
12. 100% ethanol.
13. 3% low melting point agarose gel with 0.5 μg/mL ethidium bromide.
14. 1× TAE buffer: Dilute 50× TAE buffer with double distilled autoclaved water. To prepare 1 L of 50× TAE buffer dissolve Tris free base 242 g, 18 g disodium EDTA, glacial acetic acid 57.1 mL, and add double distilled autoclaved water to make the volume to 1 L.
15. 50 mg/mL UltraPure BSA.
16. PBS, sterile.
17. Protein A beads for Immunoprecipitation.
18. SUPERase in RNase Inhibitor (20 U/μL).
19. Agencourt AMPure XP Beads.
20. FastStart Essential DNA Green Master.
21. 4–20%TBE Gels, 10 well
22. 0.5× TBE buffer: Dilute 10× TBE buffer 20 times with autoclaved double distilled water to obtain 0.5× TBE. To make 1 L of 10× TBE dissolve 121.1 g Tris-base, 61.8 boric acid, and disodium salt EDTA 7.4 g into a total volume of 1 L of double distilled water.
23. RNA Loading Dye (2×).
24. Low Range ssRNA Ladder.
25. SYBR Gold Nucleic Acid Gel Stain (10,000× Concentrate in DMSO).
26. 5× IP buffer: 50 mM Tris–HCl, pH7.4, 750 mM NaCl, and 0.5% NP-40.
27. 1 × IP buffer (kept on ice, prepared fresh): 5 × IP buffer diluted to 1 ×, supplemented with 0.1% SUPERase inhibitor.
28. High salt wash buffer (kept on ice, prepared fresh): 50 mM HEPES pH 7.4, 500 mM KCl, 0.05% NP-40, supplemented with 0.1% SUPERase inhibitor.
29. PNK buffer (no DTT) (kept on ice, prepared fresh): 50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, supplemented with 0.1% SUPERase inhibitor.
30. 2× proteinase K buffer: 100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 12.5 mM EDTA, 2% w/v SDS.
31. Proteinase K solution.

32. 1× Proteinase K mixture (prepared fresh during 5′ phosphate end repair, kept at room temperature): 2× Proteinase K buffer diluted to 1 × in 1 × IP buffer, with Proteinase K at a final concentration of 2 mg/mL (e.g., 100 μL 2× Proteinase K buffer, 20 μL 20 mg/mL Proteinase K, 80 μL 1× IP buffer).

33. m^6A-specific antibody solution, 0.5 mg ml^{-1}: Reconstitute 50 μg of affinity purified anti-m^6A rabbit polyclonal antibody in 100 μL of DEPC-treated nuclease-free water. Aliquot and store at −20 °C; avoid multiple freeze-thaw cycles; use within 12 months.

34. Blocking buffer (Wash buffer supplemented with 0.5 mg/mL UltraPure BSA).

2.2 Equipment

1. Low-adhesion microcentrifuge tubes (1.5–1.75 mL).
2. Thin-walled PCR tubes with flat cap.
3. 0.65 mL Bioruptor Pico microtubes.
4. Heating block.
5. Refrigerated benchtop microcentrifuge (capable of >16,000 × *g*).
6. Cell scrapers.
7. Magnetic rack for 1.6 mL tubes.
8. Head-over-tail rotator.
9. 254 nm UV crosslinker (i.e., UV Stratalinker 2400).
10. Thermal cycler.
11. Vortex mixer.
12. Spectrophotometer (e.g., NanoDrop Technologies ND-1000 or equivalent).
13. Sonication device.
14. Pipettes.
15. Pipette tips with filters.
16. Gel electrophoresis system.
17. Weigh boats.
18. Weighing scale.
19. Transilluminator.
20. Gel imager.
21. Cell lifters.

3 Methods

3.1 RNA Isolation

1. Remove medium from the cells by pouring or pipetting, and wash the cells gently with 10 mL of ice-cold PBS (*see* Note 2). Add 2 mL of ice-cold PBS to the cells, and scrape the cells from the plate using a cell lifter. Pipette the suspended cells into a 15 mL tube, and centrifuge at 4 °C for 5 min at 300 × *g.* Carefully remove the supernatant, and proceed immediately to **step 2**.
2. Isolate the RNA using the RNeasy kit following the manufacturer's protocol, being sure to use the gDNA Eliminator Columns to remove genomic DNA. Elute using 100 μL of DEPC-treated nuclease-free water.
3. (Recommended): Determine RNA integrity using an Agilent 2100 Bioanalyzer or by agarose gel electrophoresis.
4. If desired, isolate the mRNA using the GenElute mRNA mini-prep kit (*see* Note 3). Perform both the first and second elution using 50 μL of DEPC-treated nuclease-free water rather than the supplied elution buffer, as the components of the elution buffer may interfere with downstream steps. Measure the concentration of the mRNA via spectrophotometer.

3.2 Antibody Binding, UV Crosslinking,and Affinity Purification

1. Make the IP mixture (200 μL per reaction): 1 μg of poly(A)-enriched mRNA (*see* Note 4), 100 μL of 5 × IP buffer, 5 μL of m^6A-specific antibody (0.5 mg mL^{-1}), 5 μL of SUPERase inhibitor, 0.5 mM EDTA (optional), DEPC-treated nuclease-free water to 200 μL.
2. Incubate the IP mixture on a head-over-tail rotor at 4 °C for 2 h (*see* Note 5).
3. While the IP mixture is incubating, gently resuspend the Protein A beads using a vortex mixer. Wash 20 μL of Protein A beads per reaction three times in ice-cold 1 × IP buffer using a magnetic rack. Resuspend the Protein A beads in 500 μL of blocking buffer and rotate for at least 1 h (*see* Note 6).
4. UV-crosslink the IP mixture in a clear flat-bottom 96-well plate (Nalgene) on ice at 254 nm with 0.15 J three times. (*see* Note 7). Use multiple wells (50–100 μL per well).
5. Transfer the sample back into a 1.5 mL tube.
6. First RNase T1 digestion: Add RNase T1 to a final concentration of 1 U/μL to fragment the mRNA. Incubate for exactly 6 min at 22 °C on a shaking incubator, shaking at maximum speed. Quench on ice for 5 min.
7. Using the magnetic rack, remove the blocking buffer from the Protein A beads, and wash twice with 500 μL of 1 × IP buffer. Remove the wash buffer, add the crosslinked IP mixture to the washed Protein A beads, and rotate the mixture on a head-over-tail rotor at 4 °C for 2 h.

8. Second RNase T1 digestion: Add RNase T1 to a final concentration of 10 U/μL. Incubate for exactly 6 min at 22 °C on a shaking incubator, shaking at maximum speed. Quench on ice for 5 min (*see* Note 8).

9. Wash the beads-RNA mixture in 200 μL of ice-cold high salt wash buffer 6 times using a magnetic rack. (This step removes any cleaved fragments.)

10. Wash the beads-RNA mixture in 200 μL of ice-cold PNK buffer (no DTT) 2 times using a magnetic rack. (This step changes the buffer to accommodate the PNK enzyme).

3.3 5′ Phosphate End Repair, RNA Elution,and RNA Purification

1. Perform 5′ phosphate end repair: Resuspend the beads-RNA mixture in 100 μL of 1 × NEB T4 polynucleotide kinase buffer and 10% (v/v) NEB T4 PNK. Incubate at 37 °C for 20 min on a shaking incubator, shaking at maximum speed.

2. Add ATP to a final concentration of 100 μM. Incubate at 37 °C for 20 min on a shaking incubator, shaking at maximum speed. While this incubation is occurring, prepare 1 × Proteinase K mixture: 2 × Proteinase K buffer diluted to 1 × in 1 × IP buffer, with Proteinase K at a final concentration of 2 mg/mL (e.g., 100 μL 2 × Proteinase K buffer, 20 μL 20 mg/mL Proteinase K, 80 μL 1× IP buffer).

3. Wash the beads-RNA mixture in 200 μL of ice-cold PNK buffer (no DTT) 2 times using a magnetic rack.

4. Wash the beads-RNA mixture in 200 μL of ice-cold 1× IP buffer three times using a magnetic rack.

5. First elution: Resuspend the beads-RNA mixture in 50 μL of 1× proteinase K mixture. Incubate at 55 °C for 20 min on a shaking incubator, shaking at maximum speed. Using a magnetic rack, remove and save the eluent in a separate tube.

6. Second elution: Resuspend the beads-RNA mixture in another 50 μL of 1× Proteinase K mixture. Incubate at 55 °C for 10 min on a shaking incubator, shaking at maximum speed. Using a magnetic rack, remove and save the eluent, combining it with the eluent from the first elution. The total volume of eluent should now be 100 μL.

7. Purify the RNA using RNA clean and concentrator-5, following the manufacturer's protocol. Perform two elutions using 7 μL of DEPC-treated nuclease-free water each time. Combine the two eluents (*see* Note 9).

3.4 Library Preparation

1. Use 6 μL of the eluted RNA as starting material for RNA library preparation, saving the remaining material as backup in case library preparation fails. Prepare the RNA library using the NEBNext Small RNA Library Prep Set for Illumina (NEB) following the manufacturer's instructions through reverse transcription. In

all steps when adapter dilution is recommended for total RNA inputs of 100 ng, dilute adapters 1:10 in nuclease-free water. This applies for the 3′ SR Adaptor, SR RT Primer, and 5′ SR adaptor. This helps reduce the amount of adapter dimers that form. Be sure to heat inactivate the RT reaction at 70 °C for 15 min, as PCR amplification will not be immediately performed.

2. Determine the number of PCR cycles needed to amplify the cDNA library using qPCR. For each sample, combine: 10 μL FastStart Essential DNA Green Master, 0.5 μL SR Primer for Illumina, 0.5 μL Index 1 Primer, 1 μL RT reaction product, and 8 μL DEPC-treated nuclease-free water. Run the qPCR reaction as recommended by the manufacturer. Determine the Ct at which the amplification curve is at its half maximum. The number of PCR cycles needed to amplify the cDNA library is three fewer cycles than this Ct (e.g., if the half maximum of the amplification curve occurs at a Ct of 16, use 13 PCR cycles).

3. Perform PCR Amplification following the manufacturers' instructions, using the PCR cycle number determined in Subheading 3.4, **step 2** (*see* Note 10).

4. After the PCR amplification step, perform size selection using gel electrophoresis. Run the PCR product on a 3% low melting point agarose gel with 0.5 μg/mL ethidium bromide in 1× TAE buffer at 90 V for 40 min along with the DNA marker provided in the kit.

5. Use a transilluminator to image the gel. Use a clean, sharp scalpel to isolate the higher band around 140–180 bp, which contains the cDNA library, being careful to avoid the lower band at 127 bp, which consists of adapter dimers (*see* Note 11).

6. Weigh the gel fragment in a colorless tube, and add 6 volumes of Buffer QG from the MinElute Gel Extraction Kit to the gel slice.

7. Let the gel slice completely dissolve in the Buffer QG at room temperature (around 10 min).

8. Isolate the cDNA library by following the remaining steps of the MinElute Gel Extraction Kit's manufacturer's protocol.

9. Elute with 10 μL of DEPC-treated nuclease-free water.

10. Analyze RNA size on an Agilent 2100 Bioanalyzer. Ensure that fragments are 140–180 bp in length, and that few or no dimers are present (dimers are seen at ~127 bp). If the Bioanalyzer analysis indicates that primer dimers are present, repeat size selection using gel electrophoresis:

11. Deep sequence the cDNA library using an Illumina HiSeq platform (or similar).

4. Notes

1. Although the RNeasy Plus Mini Kit is an efficient method to purify total RNA from limited samples of animal cells or tissues, it isolates only RNA molecules longer than 200 nt. This procedure enriches mRNA species, since RNAs below

the <200 nucleotide cutoff, such as miRNAs, tRNAs, and smaller rRNAs, are excluded. If the user wishes to analyze RNA species shorter than 200 nt, an alternative is to use the direct-zol™ RNA Miniprep plus kit (Zymo research) with on-column DNAse I digestion, following the manufacturer's protocol.

2. Do not detach the cells from the plate using trypsin, as this may compromise RNA integrity. If using easily detached cells, such as HEK293T, pipette the ice-cold PBS gently onto the edge of the cell culture dish when washing to minimize loss of cells. Work quickly when scraping cells; scrape the cells in a 4 °C cold room if possible to minimize RNA degradation. Be sure to keep all samples on ice throughout the entirety of the procedure.

3. It is generally not necessary to remove any trace amounts of ribosomal RNA left over from the mRNA purification step, as the majority of the reads will be of mRNA. However, if the sequencing results show that there is too much ribosomal RNA, it is possible to perform a second round of poly (A) selection using the GenElute kit, or to further deplete ribosomal RNA using RiboMinus Eukaryote System v2 (Ambion). If you wish to analyze pre-mRNA as well as mRNA, it is possible to extract total RNA using the RNeasy kit, which will remove small RNAs, and then use the RiboMinus Eukaryote System v2 directly to remove ribosomal RNAs.

4. Be sure to use at least 1 μg of mRNA as starting material, or it may not be possible to construct the library. If library construction fails, consider starting with more mRNA, i.e., 2–10 μg.

5. Be sure to use Low-Adhesion tubes to allow more thorough washing and mixing, and to avoid sample loss. Be sure to wrap the caps of the tubes in Parafilm to prevent them from accidentally popping open. Longer incubations times (of up to 5 h) may increase IP efficiency.

6. Blocking the Protein A beads is optional but recommended. Minimize bead loss by using low retention pipette tips. For each wash, remove the tube from the magnetic stand, and make sure the beads are well resuspended. Place the tube back on the magnetic rack, and make sure that all beads have cleared from solution before removing the supernatant by waiting 30–60 s.

7. Placing the plate as close to the bulb as possible, and readjust the plate to make sure it's firmly on the ice each time.

8. It is recommended to verify RNA fragmentation before library preparation. RNA fragmentation can be verified using an Agilent 2100 Bioanalyzer or by TBE gel electrophoresis. RNA fragments should be 25–30 nucleotides long, but may be as low as 15 nucleotides long. Shorter RNA fragments may cause difficulties in mapping reads to the transcriptome during data analysis. If RNA fragments are too long or too short, it is recommended to increase or decrease, respectively, the second RNAse T1 digestion time.

   If using TBE gel electrophoresis: Wash the wells of a 4–20% TBE gel, thoroughly with 0.5× TBE buffer, and prerun for 10 min at 180 V at 4 °C in 0.5×

TBE buffer. Dilute 15 ng (1.5 μL) of fragmented RNA to 10 μL, and add 10 μL of RNA Loading Dye, (2×). Denature the RNA at 70 °C for 3 min. Carefully load the RNA onto the gel along with 15 ng of NEB Low Range ssRNA ladder in an adjacent lane, and run at 180 V for 50 min at 4 °C. Add 3 μL of SYBR Gold Nucleic Acid Gel Stain to 30 mL fresh 0.5 × TBE buffer, and mix well. Carefully remove the gel and allow it to soak in the SYBR Gold Nucleic Acid Gel Stain diluted in 0.5× TBE buffer for 10 min at room temperature. Image using a gel imager.

9. There is no need to measure mRNA concentration after the IP step, as the concentration is likely too low to be measured. This low concentration is sufficient to construct the libraries.

10. It is recommended to perform the PCR reaction at half scale, saving half of the template in case more or fewer PCR cycles are needed.

11. If major adapter dimer bands are present, it is possible to dilute the adapters 1:50 or 1:100.

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