bio-protocol

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Analysis of N^6-methyladenosine RNA Modification Levels by Dot Blotting

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Abstract

N^6-methyladenosine (m^{6}A) is the most prevalent internal modification of eukaryotic messenger RNAs (mRNAs), affecting their fold, stability, degradation, and cellular interaction(s) and implicating them in processes such as splicing, translation, export, and decay. The m^{6}A modification is also extensively present in non-coding RNAs, including microRNAs (miRNAs), ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs). Common m^{6}A methylation detection techniques play an important role in understanding the biological function and potential mechanism of m^{6}A, mainly including the quantification and specific localization of m^{6}A modification sites. Here, we describe in detail the dot blotting method for detecting m^{6}A levels in RNA (mRNA as an example), including total RNA extraction, mRNA purification, dot blotting, and data analysis. This protocol can also be used to enrich specific RNAs (such as tRNA, rRNA, or miRNA) by isolation technology to detect the m^{6}A level of single RNA species, so as to facilitate further studies of the role of m^{6}A in biological processes.

Keywords: Dot blot; N^6-methyladenosine; RNA m^{6}A; RNA modification; mRNA; Non-coding RNAs; METTL3

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Background

N^6-methyladenosine (m^6A) is the most prevalent internal RNA modification in eukaryotic mRNAs and long non-coding RNAs (Jia et al., 2013). The m^6A modification refers to the methylation of the nitrogen atom at position 6 of the RNA adenosine, mainly located on the common motif of RRm^6ACH (R denotes A or G, H denotes A, C, or U) (Csepany et al., 1990). The reversible activity of m^6A modification is regulated by the combined action of methylases and demethylases (Niu et al., 2013). The m^6A *writers* with methyltransferase activity consist of three individual proteins: METTL3, METTL14, and WTAP (Wiedmer et al., 2019); FTO and ALKBH5 are m^6A demethylases (Nachtergaele and He, 2018). Another protein family is the m^6A *readers*, which can recognize the m^6A modification to modulate the fate of mRNA (Harcourt et al., 2017). Recent studies suggest that m^6A plays a pivotal role during various biological processes including virus infection (Winkler et al., 2019), stress (Engel et al., 2018), heat shock (Zhou et al., 2015), and DNA damage (Xiang et al., 2017; E. Li et al., 2022). In mammals, m^6A methylation plays a variety of key roles including embryonic development, neurogenesis, circadian rhythm, stress responses, sex determination, and tumorigenesis (Sun et al., 2019). The m^6A modification is vital during stem cell proliferation, with METTL3 depletion reducing the differentiation of embryonic stem cells (Batista et al., 2014). The correlation between the level of m^6A modification and clinicopathological features has been shown in diverse tumors, which may provide prognostic value in these diseases.

Detection of m^6A modification in vitro can help identify the precise regulatory forms and synergistic roles of m^6A modifications in cancer and other diseases. Also, detection of m^6A is important for studying its biological functions and mechanisms. Currently, a variety of methods have been developed to identify m^6A modifications in cells, which can be divided into three categories: semi-quantitative, quantitative, and precise location detection. Semi-quantitative methods include dot blot technology (Z. Li et al., 2017), methyl-sensitivity of MazF RNA endonucleases (Imanishi et al., 2017), and immuno-northern blotting (Mishima et al., 2015). Quantification methods include RNA photo-crosslinkers and quantitative proteomics (Arguello et al., 2017), electrochemical immunosensors (Yin et al., 2017), and support vector machine–based methods (Chen et al., 2016). Precise location detection includes methylated RNA immunoprecipitation sequencing (MeRIP-seq) (Liu et al., 2018), m^6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP) (Linder et al., 2015), and high-performance liquid chromatography (HPLC) (Rana and Tuck, 1990). Although many methods have been developed to detect m^6A methylation, in many cases dot blot hybridization remains the method of choice for analyzing the global changes of m^6A levels in total RNA or single RNA species. The dot blotting technique significantly saves time because it does not require chromatography, gel electrophoresis, or complex gel closure procedures, and is relatively low in cost (Wang et al., 2018). In this protocol, we describe in detail how to detect m^6A content in mRNA by dot blot (Figure 1).

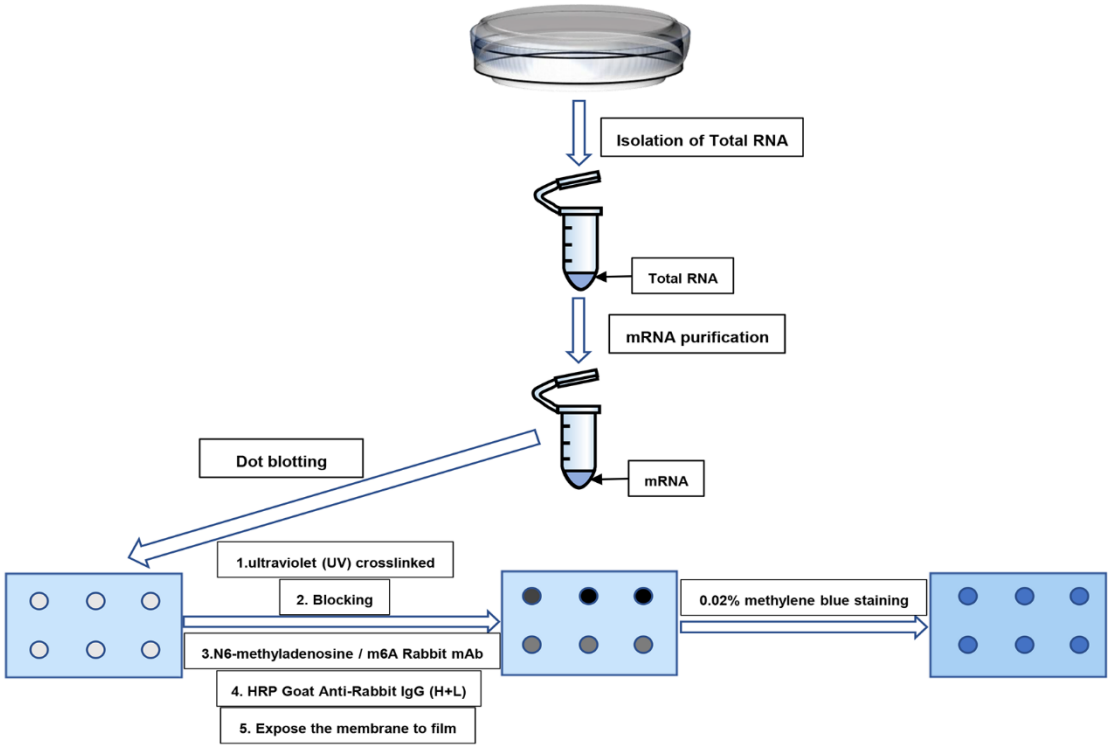

Figure 1. Schematic diagram of the major steps of dot blot analysis for the separation and purification of m^6A in mRNA

Materials and Reagents

1. 100 mm culture dish (Corning, Falcon, catalog number: 353003)
2. Pipette tips (Axygen, catalog numbers: T-300, T-200Y, T-1000B)
3. 1.5 mL RNase/DNase-free microcentrifuge tube (Axygen, catalog numbers: MCT-50-C)
4. Cell scraper (any brand)
5. TRIzol reagent (Invitrogen, catalog number:15596026)
6. Chloroform (General-Reagent, catalog number: G75915B)
7. Isopropanol (Sangon Biotech, catalog number: A507048)
8. Ethanol absolute (Sangon Biotech, catalog number: A500737)
9. RNase-free water
10. GenElute mRNA miniprep kit (Sigma, catalog number: DMN10)
11. Amersham^{TM} Hybond^{TM}-N+ membrane (GE Healthcare, catalog number: RPN203B)
12. N^6-methyladenosine/m^6A rabbit mAb (ABclonal, catalog number: A19841)
13. HRP goat anti-rabbit IgG (H+L) (ABclonal, catalog number: AS014)
14. ECL western blotting substrate (Thermo Fisher Scientific, catalog number: 32106)
15. Saran wrap (any brand)
16. Tween-20 (Sigma-Aldrich, catalog number: P1379)
17. Non-fat milk (any brand)
18. Na_2HPO_4 (Sangon Biotech, catalog number: A501727)
19. KH_2PO_4 (Sangon Biotech, catalog number: A100781)
20. KCl (Sangon Biotech, catalog number: A100395)
21. NaCl (Sangon Biotech, catalog number: A501218)
22. 10× phosphate-buffered saline (PBS) (see Recipes)

23. 1× phosphate-buffered saline (PBS) (see Recipes)
24. 1× phosphate-buffered saline/0.1% Tween-20 (PBST) (see Recipes)
25. Blocking buffer (see Recipes)
26. Antibody dilution buffer (see Recipes)
27. Methylene blue dye buffer (Solarbio, catalog number: G1301)

Equipment

1. Refrigerated centrifuge (Eppendorf, model: 5424 R)
2. NanoDrop (Thermo Fisher Scientific, model: ND-1000 spectrophotometer)
3. UV cross-linker or UV torch with 254 nm wavelength UV (UVP, model: CL1000)
4. Chemiluminescent imaging system (Tanon, model: Tanon 5200)
5. Camera (any brand)

Software

1. GraphPad Prism (GraphPad Software)
2. ImageJ (https://imagej.nih.gov/ij)

Procedure

A. Isolation of total RNA

1. For mammalian cells we recommend using approximately 1×10^7 cells for mRNA purification.
2. Remove the medium and rinse the cells twice with 1–2 mL of ice-cold PBS.
3. Remove PBS and lyse the cells directly in the culture dish by adding 1 mL of TRIzol reagent per 100 mm culture dish and scraping with a cell scraper.
4. Pass the cell lysate through a pipette several times. Then, transfer to 1.5 mL RNase-free microcentrifuge tubes. Vortex thoroughly.
5. Incubate the homogenized samples for 5 min at room temperature.
6. Add 0.2 mL of chloroform, vortex for 30 s, and incubate for 2–3 min at room temperature.
7. Centrifuge at 12,000 × *g* and 4 °C for 15 min.
8. Carefully transfer the upper, aqueous phase to a fresh tube without disturbing the interface.
9. Add 0.5 mL of isopropanol to the aqueous phase and mix well. Incubate the samples for 10 min at room temperature.
10. Centrifuge at 12,000 × *g* and 4 °C for 10 min.
11. Decant the supernatant and completely remove any traces of liquid by aspiration.
12. Wash the pellet with 1 mL of ice-cold 75% ethanol.
13. Centrifuge at 12,000 × *g* and 4 °C for 15 min.
14. Remove all traces of ethanol. Air-dry or vacuum-dry the RNA pellet for 5–10 min.
15. Resuspend the pellet in 10–30 μL of RNase-free water.

B. mRNA purification

1. Isolate mRNA from total RNA using the GenElute mRNA miniprep kit following the manufacturer's instructions (other brands can also be used for the purification of mRNA).
2. Mix the oligo (dT) beads thoroughly by vortexing and inverting until resuspended and homogenous.

3. Add the resuspended oligo (dT) beads to the total RNA, cap the tube, and mix thoroughly by vortexing.
4. Incubate the mixture at room temperature for 10 min. No mixing or rocking is necessary.
5. Resuspend the pellet in 350 µL of wash solution by vortexing.
6. Transfer the suspension into a GenElute spin filter–collection tube assembly by pipetting. Spin for 1–2 min at maximum speed.
7. Remove the spin filter, discard the flow through liquid, then place the spin filter back into the same collection tube.
8. Pipette 350 µL of low salt wash solution into the column. Spin for 1–2 min at maximum speed.
9. Remove the spin filter, discard the flow through liquid, then place the spin filter back into the same collection tube.
10. Pipette another 350 µL of low salt wash solution into the column. Spin for 1–2 min at maximum speed.
11. Transfer the spin filter into a fresh collection tube. Pipette 10–20 µL of preheated (65 °C) elution solution onto the spin filter, ensuring that it comes into contact with the bead–mRNA complex. Incubate for 2–5 minutes at 65 °C. Spin for 1–2 min at maximum speed. Save the flow through liquid; it contains most of the purified mRNA.
12. Determine the concentration of purified mRNA with NanoDrop.
13. Dilute different concentrations of mRNA to 50 ng/µL.

C. Dot blotting

1. The isolated mRNA is first denatured by heating at 95 °C for 5 min to disrupt the secondary structure.
2. Chill on ice for 5 min immediately after denaturation to prevent re-formation of the mRNA secondary structure.
3. Have the nitrocellulose membrane ready.
4. Using a narrow-mouth pipette tip, drop 2 µL of mRNA sample directly onto the Hybond-N+ membrane at the center of the grid (Figure 2).

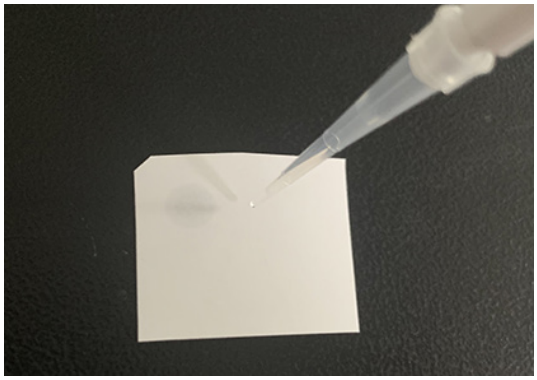

Figure 2. Dot sample example

5. Air dry at room temperature for 5 min.
6. Crosslink spotted RNA to the membrane using the UV cross linker (irradiate under ultraviolet lamp with 254 nm wavelength for 5 min).
7. Incubate the membrane with blocking buffer for 1 h at room temperature.
8. After blocking, incubate the membrane with anti-m6A antibody (1:1,000 dilution) in 5 mL of antibody dilution buffer overnight at 4 °C with gentle shaking.
9. Wash three times with PBST (3 × 5 min).
10. Incubate the membrane with HRP goat anti-rabbit IgG (1:10,000 dilution) in 5 mL of antibody dilution buffer for 1 h at room temperature with gentle shaking.
11. Wash three times with PBST (3 × 5 min).
12. Incubate with ECL substrate for 1 min (according to the manufacturer's instructions, 0.125 mL ECL

solution per cm^2 of the membrane is recommended), cover with Saran wrap aiming to remove the excess solution from the surface, and expose the film in the darkroom (Figure 3B). Try different exposure times to get clear results.

13. After exposure, transfer the membrane to a solution containing 10 mL of 0.02% methylene blue dye buffer, gently shake the membrane at room temperature, and incubate for 30 min.
14. Lastly, clean the membrane with dH_2O until the background is clean (Figure 3C).

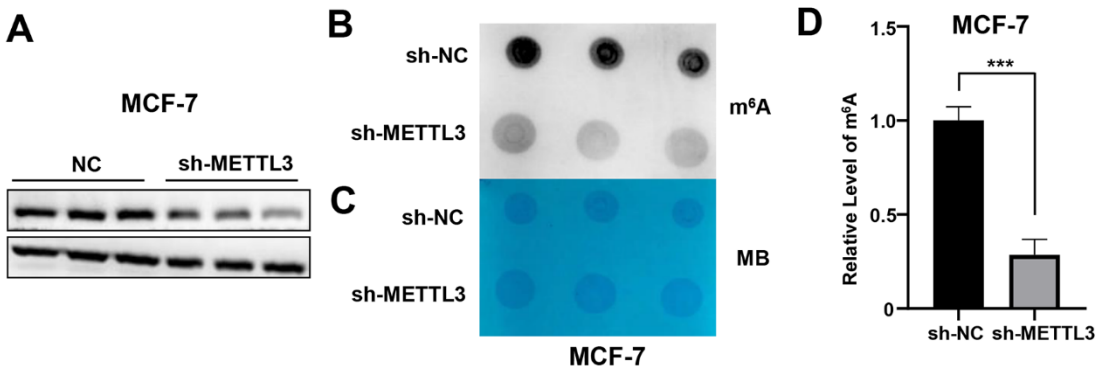

Figure 3. Representative data of m^6A level detected by dot blot. (A) Western blotting assay of METTL3 in the METTL3-KD stable cell lines in MCF-7. (B) Dot blot to detect the m^6A level of mRNA isolated from total RNA of METTL3-KD MCF-7 cells. (C) Methylene blue (MB) staining served as a loading control. (D) Measurement and normalization of m^6A levels in ImageJ.

Data analysis

1. Perform densitometric analysis using ImageJ (result calculation method: m^6A grayscale value/MB grayscale value). Use a minimum of three biological replicates to perform statistical analysis of the m^6A levels between samples (Figure 3D).
2. For statistical analysis, use GraphPad Prism software.

Notes

1. This protocol can be modified for individual species of RNA, such as messenger RNA, tRNA, rRNA, or microRNA, by employing isolation techniques for the enrichment of the specific RNAs to be analyzed.
2. For the preparation of wash buffer, blocking buffer, and antibody dilution buffer, the use of nuclease-free water is not necessary.

Recipes

1. 10× phosphate-buffered saline (PBS) (1,000 mL)

a. Weigh 14.4 g of Na_2HPO_4, 2.4 g of KH_2PO_4, 2.0 g of KCl, and 80.0 g of NaCl
b. Add 800 mL of sterile water
c. Stir until dissolved
d. Bring the final volume to 1,000 mL using sterile water

2. **1× phosphate-buffered saline (PBS) (500 mL)**
   50 mL of 10× PBS
   Bring final volume to 500 mL using sterile water

3. **1× phosphate-buffered saline/0.1% Tween-20 (PBST) (500 mL)**
   50 mL of 1× PBS
   1 mL of 100% Tween-20
   Bring final volume to 500 mL using sterile water

4. **Blocking buffer and antibody dilution buffer**
   50 mL of PBST
   5 g non-fat milk

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Competing interests

The author declares there are no competing interests.

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