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N6-methyladenosine regulates maternal RNA maintenance in oocytes and timely RNA decay during mouse maternal-to-zygotic transition

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Method Article

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Abstract

N^6-methyladenosine (m^6A) on RNA and its regulatory components play critical roles in various developmental processes in mammals. However, the landscape and function of m^6A in early embryos remain unclear due to limited materials. Current methods typically need total RNA greater than microgram amount to map m^6A positions, which prevents us from revealing the crucial role of m^6A in early embryonic development in mice. Here, we developed an ultra-low-input(ULI) MeRIP-seq method to reveal the transcriptome-wide m^6A landscape in limited biological materials which contain as low as 50ng total RNA. Antibody-based ULI MeRIP-seq reveal the high efficiency of immunoprecipitation and reduced RNA loss during the experiments. Sequencing data reveal the m^6A enrichment of mRNAs as well as non-coding RNAs which are highly recapitulated the results generated by 2μg of total RNA.To further promote the application on limited RNA materials, we describe a step-by-step protocol about how to construct a successful ULI MeRIP-seq library.

Introduction

In eukaryotes, N^6-methyladenosine (m^6A) is found on messenger RNAs (mRNAs), repeat RNAs and long noncodingRNAs (lncRNAs) and participates in various important biological events by playing roles in RNA-related processes and chromatin state regulation.A previous study in *zebrafish* found that m^6A can promote maternal RNA degradation during the maternal-to-zygotic transition (MZT). In mice, different studies have revealed the essential roles of m^6A writer and reader proteins in oogenesis and early embryogenesis. For instance, depletion of METTL3 in embryos from crossed heterozygous mice results in embryonic lethality at the early stage in postimplantation embryos , which is similar to the lethality phenotype of *Mettl14*- and *Mettl16*-knockout (KO) embryos from crossed heterozygotes . All these findings from mouse models reveal that regulating RNA m^6A and determining the m^6A-marked (m^6A+) RNA fate is quite important for oocyte maturation, ZGA and postimplantation development. However, the molecular dynamics of m^6A on RNA in oocytes and early embryos remain unknown due to limited materials, and the detailed regulatory model is largely ambiguous.In this study, we developed ULI-MeRIP-seq for m^6A mapping with as little as 50 ng of total RNA, and the data quality was highly repeatable and consistent with that of the published data. With this method, we revealed the dynamics of the m^6A landscape of the transcriptome during MZT progression from GV oocytes to 4-cell (4C)-stage embryos. Here we provided the detail protocol for a greater application on other biological field.

Reagents

RNAiso Plus reagent (Takara, 9109)

MaXtract high density tube (Qiagen, 129046)

NaAc、ethanol、isopropanol

glycogen (5 mg/mL, Roche, 10901393001)

Qubit RNA HS Assay (Thermo Fisher Scientific, Q32852)

anti-m^{6}A antibody (Millipore, ABE572)

protein A Dynabeads (Thermo Scientific,10002D)

protein G Dynabeads (Thermo Scientific,10004D)

NaCl (Sigma-Aldrich,S5150)

Trizma® hydrochloride solution,pH 7.4, 1 M, (Sigma-Aldrich,T2663-1L)

Nonidet™ P 40 Substitute solution,BioXtra, ampule, ~10% (Thermo Fisher Scientific, 85124)

Epimark N6-Methyladenosine Enrichment Kit (NEB, E1610S)

Anti-N6-methyladenosine (m6A) Antibody（Millipore, ABE572）

Invitrogen Ambion RNase Inhibitor (Cloned)(Thermo Fisher Scientific, AM2684)

SUPERase-In RNase Inhibitor (Thermo Fisher Scientific, AM2696)

All-in-One Reverse Transcription Kit (ABM, G490)

SMARTer Stranded Total RNA-Seq Kit version 2 (Takara, 634411)

IP buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP40 in nuclease-free H_2O)

low-salt buffer (50 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP40 in nuclease-free H_2O)

high-salt buffer (500 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.1% NP40 in nuclease-free H_2O)

Equipment

Qubit fluorometer (Thermo Scientific)

Agilent High Sensitivity RNA ScreenTape (G2964AA)

Covaris S220

microTUBE-50 AFA Fiber Screw-Cap (covaris 520166)

1.5 ml DNA LoBind tubes (Eppendorf, 022431021)

Thermal cycler (Bio-Rad, T100)

Procedure

1) RNA extraction and purification.

1. low number of cells or embryos (about 150 mouse oocytes for one reaction) with high viability were washed with BSA/PBS solution (0.5% BSA in 1×PBS) 3 times using mouth pipette under a microscope.

2. Cell or embryo samples were picked through mouth pipette and transfer into 200 μL RNAiso Plus reagent on ice. (Samples can be stored in -80°C for 1 month)

3. 40 μL chloroform was added to the samples.

4. Prepare a MaXtract high density tube and centrifuge at full speed for 5 min to make sure the gel was in the bottom of the tube.

5. The mixture was transferred to a MaXtract high density tube and vortex for 5 seconds to make sure the samples are mixed thoroughly.

6. Centrifuge at full speed for 10 min at 4°C.

7. The aqueous phase was recovered and transferred to a new tube.

8. Add 12μL 3 M NaAc, 2 μL of glycogen (5 mg/mL) to the samples and mixed briefly.

9. Add 120 μL isopropanol to the samples and mixed thoroughly through vortex for 10 seconds.

10. The mixture was incubated at -80°C for 30 min for RNA precipitation.

11. The RNA was precipitated after centrifugation for 15 min at full speed at 4°C.

12. The supernatant was removed carefully to avoid touch the white pellet.

13. Washing twice with 1mL 75% ethanol, centrifuge for 5 min each time.

14. Spin down for another time and remove all the ethanol.

15. Dry the pellet by opening the cap of the tube for several minutes.

14. The pellet was resolved in RNase-free water and mixed thoroughly.

15. 1μL samples can be used to assess the concentration via Qubit RNA HS Assay, or run the Agilent High Sensitivity RNA ScreenTape. (To make sure the extracted RNA are more than 50 ng. RNA with high degradation level would not be recommended.)

2) Antibody-coated bead preparation.

1. 10 μL protein A Dynabeads and 10 μL of protein G Dynabeads was washed twice with 200 μL IP buffer. For each time, adding the IP buffer and mix the beads with pepitte thoroughly, then placing the microcentrifuge tube into a magnetic stand for 1 min and discard the supernatant.

2. Add 200 μL IP buffer to the beads.

3. Add 0.5 μL anti-m^6A antibody to the tube and rotate for 2 h in 4°C. (the dose of anti-m^6A antibody from different companies need to test before in order to get the highest IP efficiency).

3) RNA fragmentation.

1. Dilute the m6A+ control RNA (GLuc) and m6A- control RNA (CLuc) for 1:1000 using RNase-free water on ice. (control RNA are from Epimark N6-Methyladenosine Enrichment Kit).

2. Prepare the sample mixture on ice:

Sample RNA: 50ng-500ng

diluted m^6A+ control RNA (GLuc) : 1μL

diluted m^6A- control RNA (CLuc) : 1μL

RNase inhibitor (40 U/μL): 0.5 μL

SUPERase inhibitor (20 U/μL): 0.5 μL

IP buffer without NP-40 : to 50 μL

3. Mixture are transfer into a new microTUBE-50 AFA Fiber Screw-Cap, and

fragmented using a Covaris S220 (peak power: 50, duty factor: 20, cycles/burst: 200, duration: 120 sec).

4) MeRIP and purification.

1. 2.5 μL fragmented RNA mixture was kept as input.

2. Wash the antibody-coated beads twice in 200 μL IP buffer and suspend in 200 μL IP buffer.

3. The remaining fragmented RNA(47.5 μL) was transferred to the antibody-coated beads.

4. Rotated at 20 RPM at 4°C for 4-5 h.

5. Spin briefly and place the tube into a magnetic stand for 1 min. Discard the supernatant and wash the beads with 200μL IP buffer twice, 200μL low-salt buffer twice and 200μL high-salt buffer twice. Each time rotate the tube at 4°C for 5 min.

6. Spin briefly and place the tube into a magnetic stand for 1 min. Discard the supernatant and resuspend the beads with 200 μL of RNAiso Plus reagent. (Stop time point. Samples can be stored in -80°C overnight)

7. The RNA was extracted with the same method as that used for RNA extraction(first part).

8. Elute the RNA with 4μL RNase-free water. The eluted RNA was used for RT-qPCR or library preparation.

5) RT-qPCR

(if the samples are precious，this step can be skipped, and S/N ratio test by qPCR can be done with the library products)

1. Eluted RNA and input RNA was reverse-transcribed using an All-in-One Reverse Transcription Kit directly after ULI-MeRIP. Add the following reagents in a PCR tube:

Eluted RNA and input RNA

5X All in one mix: 4μL

RNase-free water: to 20μL

2. Put the mixture in Thermal cycler, procedure is 25°C 5min, 42°C 50min, 85°C 5min, 4°C ∞

3. cDNA of input and IP samples are used for qPCR to test the IP efficiency.

Calculating the S/N ratio as following equation is important to evaluate the sample quality and whether the experiment is successful.

S/N ratio = $2^\wedge(-Ct_{\text{GLuc or m6A-positive gene in IP}} + Ct_{\text{GLuc or m6A-positive gene in input}})/2^\wedge(-Ct_{\text{CLuc or m6A-negative gene in IP}} + Ct_{\text{CLuc or m6A-negative gene in input}})$.

4. Usually we will test the GLuc, CLuc, endogenous gene A(m6A positive genes such like *Sox2, c-Myc* in ESCs) and *Gapdh* or *Actin* (m6A-negative genes) to evaluate the S/N ratio. For 50 ng total RNA starting, the S/N ratio of GLuc/CLuc is about 100.

6) Library preparation

1. Library preparation of ULI-MeRIP samples was performed using a SMARTer Stranded Total RNA-Seq Kit

version 2 according to the manufacturer’s protocol.

2. Fragmented input RNA and IP-extracted RNA was reverse-transcribed into cDNA without fragmentation option.

3. The libraries of IP and input samples were amplified for 15-19 cycles and 14-15 cycles, respectively.

4. Quantify the library by Qubit dsDNA HS Assay Kit.

5. Step5)-3 can be done with the library products.

Troubleshooting

step:5)-4

problem:The S/N ratio are lower than 20 is not a successful result.

Possible reason:

1.The sample RNA are degraded or degraded during the procedure.

2.Antibody dosage need to be tested.

solution:

1.RNase free in the environment.

2.Use an high quality RNA samples qualified by gel confirmation as an positive control.

3.Gradient dilution of the antibody from 1μL to 0.01μL per IP reaction.

4. After sonication, the fragment size was analyzed with Agilent High Sensitivity RNA ScreenTape.
The total RNA need to be fragmented to 200-600 nt.

Time Taken

Approximate time for the whole procedure are about 3 days.

1) RNA extraction and purification: 2 hours

2) Antibody-coated bead preparation and 3) RNA fragmentation: 2 hours

4) MeRIP and purification: 7 hours

5) RT-qPCR:4 hours

6) Library preparation: 1 day

Anticipated Results

Usually we will test the GLuc, CLuc, endogenous gene A(m6A positive genes such like *Sox2, c-Myc* in ESCs) and *Gapdh* or *Actin* (m6A-negative genes) to evaluate the S/N ratio. For 50 ng total RNA starting, the S/N ratio of GLuc/CLuc is about 100 (value=50-200 is quite normal).