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The Effect of Elution Volume for Immunoprecipitation on m⁶A-Seq Analysis

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Abstract: ***Objective***: To develop a cost-effective method to reduce the time consumption of elution in immunoprecipitation. ***Method***: Two volumes (125 μL for Group C and 100 μL for Group T) of elution buffer were used to explore whether smaller volume could save testing time. ***Result:*** Time consumption of elution in Group T was significantly shorter than that in Group C, while the efficiency of eluted m^{6}A-containing fragments and the performance of m^{6}A-Seq as indicated by m^{6}A peak distributions showed no difference between the two groups. ***Conclusion:*** A smaller volume of elution buffer was an economical way to reduce time consumption in immunoprecipitation.

Keywords: m^{6}A-Seq; Immunoprecipitation; Elution buffer

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1. Introduction

Many studies have shown that N6-methyladenosine (m^{6}A) RNA modification plays a key role in biological process, especially in the initiation and progression of different types of human cancers [1]. In 2012, the m^{6}A landscape in humans and mice at whole transcriptional level was revealed by an immunoprecipitation-based next generation sequencing method called m^{6}A-Seq (also known as MeRIP-Seq) [2-3]. However, m^{6}A-Seq were still limited by time consumption of immunoprecipitation, higher amount of total RNA input for immunoprecipitation, bacterial contamination, etc. In this study, we reduced the elution volume of buffer in order to develop a more cost-effective method for immunoprecipitation and reduce time consumption during the whole experiment cycle.

2. Materials and methods

2.1. Cell line

The Mouse Renal Carcinoma cells (RenCa) were cultured in DMEM medium (Invitrogen) containing 10% FBS, 2 mM L-Glutamine and 1% penicillin-streptomycin. RenCa cell line was purchased from Allcells Biotechnology (Shanghai, China) and were routinely tested for mycoplasma contamination.

2.2. Equipment

Equipment/resource	Source	Identifier
Water bath	Shanghai Boxun	DK-8D
Room temperature centrifuge	Eppendorf	5415D
Refrigerated centrifuge	Eppendorf	5418R

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Equipment/resource	Source	Identifier
Vertical mixer	Ningbo Xinzhi	HS-3
NanoDrop ND-1000	NanoDrop	ND-1000
Bioanalyzer 2100	Agilent	G2939BA
Pipettor	Eppendorf	L17490H

2.3. Reagent and buffer

Reagent/resource	Source	Identifier	Storage (°C)
TRIzol	Invitrogen	15596026	2-8°C
Dynabeads® Oligo (dT)	Thermo Fisher	25-61005	2-8°C
m^6A antibody	Synaptic Systems (SYSY)	202003	-20°C
Magnesium RNA Fragmentation Module	New England Biolabs, Inc	E6150S	-20°C
Dynabeads Antibody Coupling Kit	Thermo Fisher	14311D	2-8°C
SuperScript™ II Reverse Transcriptase	Invitrogen	1896649	-20°C
E. coli DNA polymerase I	New England Biolabs, Inc	m0209	-20°C
RNase H	New England Biolabs, Inc	m0297	-20°C
dUTP Solution	Thermo Fisher	R0133	-20°C
UDG enzyme	New England Biolabs, Inc	m0280	-20°C
Truseq stranded mRNA library prep kit	Illumina	15031047	-20°C

Buffer	Components
Fragment buffer	10 mM ZnCl2, 10 mM Tris-HCl (pH7.0)
Fragment stop buffer	0.5 M EDTA
m^6A binding buffer	50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1% NP-40，2 mM EDTA
Low salt buffer	0.2×SSPE, 0.001 M EDTA, 0.05% Tween-20
High salt buffer	0.2×SSPE, 0.001 M EDTA, 0.05%, Tween-20, 137.5 mM NaCl
TET buffer	10 mM Tris–HCl (pH8.0), 1 mM EDTA (pH8.0), 0.05% Tween-20
Elution buffer	0.02 M DTT, 0.150 M NaCl, 0.05 M Tris–HCl (pH7.5), 0.001 M EDTA, 0.10% SDS

2.4. RNA extraction and mRNA enrichment

Total RNA of cell line was isolated and purified using TRIzol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. More than 300 µg total RNA was yielded by RenCa cell (3×10^8) in each sample. The RNA amount and purity were quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Bioanalyzer 2100 (Agilent, CA, USA) with RNA integrity number (RIN) >7.0 and confirmed by electrophoresis with denaturing agarose gel. Poly(A) RNA was purified from 300 µg total RNA using Dynabeads Oligo (dT) 25-61005 (Thermo Fisher, CA, USA) in four rounds of purification. Then, the poly(A) RNA was fragmented into small pieces using Magnesium RNA Fragmentation Module (NEB, cat.e6150, USA) under 86°C for 7 min. Amount of fragmented Poly(A) enriched RNA was 3458.4 ng and we equally separated into six tubes named with C1, C2, C3, T1, T2, and T3.

2.5. Immunoprecipitation

500 ng of poly(A) RNA was used for m^6A-Seq in each biological replicate. 140 µL of pre-equilibrated m^6A-Dynabeads was added to the 360 µL of fragmented RNA to a final volume of 500 µL. The fragmented

RNA was allowed to bind the m^{6}A-Dynabeads at room temperature for 1 h. The tubes containing the samples were placed on a magnet allowing the bead complexes to cluster until the solution became clear. The liquid phase or supernatant in this 500 μL was discarded as this fraction represented the m^{6}A negative fragments, which was not captured by the antibody. The m^{6}A-Dynabeads-RNA complexes was resuspended and incubated in 500 μL of m^{6}A Binding Buffer for 3 min at room temperature, and clear supernatant was removed after placing the beads in the magnet. Finally, high salt buffer, low salt buffer and TET buffer were added subsequently.

For immunoprecipitation, each biological replicate in Group T (T1, T2, T3) was treated with 100 μL elution buffer, while each biological replicate in Group C (C1, C2, C3) was treated with 125 μL elution buffer.

2.6. Library construction and sequencing

The eluted m^{6}A-containing fragments (IP) and untreated input control fragments were reverse-transcribed to create cDNA libraries by SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, USA), which were then used to synthesize U-labeled second-stranded DNAs with *E. coli* DNA polymerase I (NEB, cat.m0209, USA), RNase H (NEB, cat.m0297, USA) and dUTP Solution (Thermo Fisher, cat.R0133, USA). An A-base was then added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. Each adapter contained a T-base overhang for ligating the adapter to the A-tailed fragmented DNA. Single- or dual-index adapters were ligated to the fragments, and size selection was performed using AMPureXP beads. After the heat-labile UDG enzyme (NEB, cat.m0280, USA) treatment of the U-labeled second-stranded DNAs, the ligated products were amplified with PCR by the following conditions: initial denaturation at 95°C for 3 min, 8 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 15 sec, extension at 72°C for 30 sec, and final extension at 72°C for 5 min. The average insert size for the final cDNA library was 300±50 bp. At last, 2×150bp paired-end sequencing (PE150) was performed on an Illumina Novaseq™ 6000 (LC-Bio Technology Co., Ltd., Hangzhou, China) following the manufacturer's recommended protocol.

2.7. Bioinformatic analysis of m^{6}A-Seq

The fastp software (https://github.com/OpenGene/fastp) were used to remove the reads containing adaptor contamination, low quality bases and undetermined bases with default parameters. Then sequence quality of IP and Input samples were also verified using fastp. We used HISAT2 (http://daehwankimlab.github.io/hisat2) to map reads to the reference genome of *Mus musculus* (Ensembl Version: v96). The mapped reads of IP and input libraries were loaded into R package exomePeak (https://bioconductor.org/packages/exomePeak), which could identify m^{6}A peaks with bed or bigwig format that could be adapted for visualization on the IGV software (http://www.igv.org). Called peaks were annotated by intersection with gene architecture using R package ChIPseeker (https://bioconductor.org/packages/ChIPseeker).

2.8. Statistical methods

Student's *t* test (*t.test* function) was utilized for data processing. The *P* value less than 0.01 (P <0.01) was considered statistically significant.

3. Result

3.1. Less elution volume saves elution time

The IP efficiency of m^{6}A modified RNA eluted by Group T (6.267%±0.64) and Group C (5.923%±1.07) was not significant difference ($P > 0.1$) (**Figure 1A**). However, the time consumption of elution in Group

T (109.67±3.05 min) was significantly shorter ($P < 0.001$) than that in Group C (194±8.89 min) (**Figure 1B**).

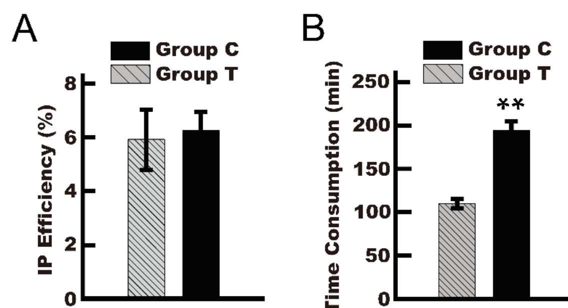

Figure 1. Less elution volume saves elution time. The immunoprecipitation (IP) efficiency **(A)** and time consumption **(B)** of elution in Group C (n=3) and Group T (n=3). Data were presented as mean±SEM, and ** represented $P < 0.001$ based on student's t test.

3.2. Less elution volume does not affect the distribution of m^{6}A peaks in m^{6}A-Seq

We next characterized the distribution of m^{6}A peaks in the whole transcriptome of all samples. The metagenomic profiles of m^{6}A peaks in both Group C and Group T indicated that m^{6}A modifications were highly enriched around the 5' stop codon untranslated region (UTR) and 3' UTR (**Figure 2A**).

To confirm the distribution of m^{6}A within the transcript, we divided the transcript into four non-overlapping segments: 5' UTR, 3' UTR, the first Exon and other Exons. Each m^{6}A peak was assigned into one of the four transcript segments. The 3' UTR appeared to be greatly enriched in m^{6}A peaks, and 49.82% to 50.13% of the peaks from the two groups fell into this segment (**Figure 2B**).

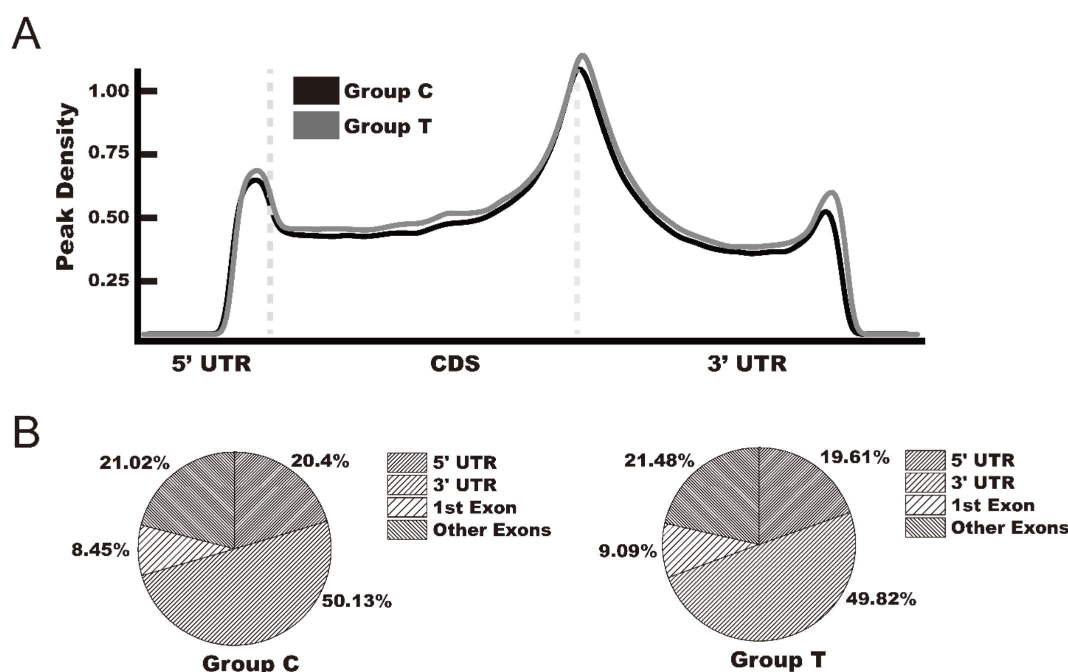

Figure 2. Lower elution volume does not affect the distribution of m^{6}A peaks in m^{6}A-Seq. Epi-Transcriptome analysis of total m^{6}A peaks in Group C (n=3) and Group T (n=3). **(A)** Metagenomic profiles of m^{6}A peak summit distributions along the transcripts composed of 5' UTR, CDS, and 3' UTR. **(B)** Pie charts depicting the distribution of m^{6}A peak within four non-overlapping gene regions (5' UTR, 3' UTR, first exon, other exons).

These results were consistent with the m^6A distribution in previous studies of m^6A modification landscape in human and mouse [2-3], suggesting that the modified IP assay and library construction were successful.

4. Discussion

A classic protocol for m^6A-Seq [2-3] included RNA extraction, poly(A) RNA enrichment, RNA binding to m^6A-Dynabeads, washing of m^6A-Dynabeads, elution of m^6A-positive RNA, library construction, and Illumina sequencing. These steps may account for more than 36 hours, of which more than 2 hours will be spent on the elution step. The content of eluted m^6A-containing fragments for library construction is no more than 50 ng. On the basis of canonical method for m^6A immunoprecipitation, we reduced elution volume buffer from 125 μL to 100 μL. Lower volume of elution buffer could save almost 100 min than higher volume, but the performance of m^6A-Seq proved that m^6A peaks distributed on gene regions had no difference between the two conditions. In conclusion, 100 μL elution buffer could save almost half of the working time in IP elution than the step using 125 μL while maintaining similar performance in m^6A-Seq.

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Disclosure statement

The authors declare no conflict of interest.

Author contributions

Yuhui Xu, Lize Shen, Guolin Li designed the experiments, performed all the experiments, and wrote the manuscript. Lize Shen designed and performed the bioinformatics analysis. All the authors commented and approved the paper.

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