Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Steric hindrance of *N*^{6}-methyl in m^{6}A and the application for specific loci detection

Xiaoyi Xie,[a,‡] Qi Wei,[a,‡] Jingyu Han,[a,‡] Xin Fang,[a] Wei Yang,[a] Xiang Zhou,[a] Yafen Wang[b,*] and Xiaocheng Weng[a,*]
[a]College of Chemistry and Molecular Science, Wuhan University, Wuhan 430072, Hubei, China
[b]School of Public Health, Wuhan University, Wuhan 430071, Hubei, China
[‡]Co-first author
*Correspondence: xcweng@whu.edu.cn; yfwang@whu.edu.cn

This PDF file includes:

Table of contents

Experimental section

Materials: SuperScript™ III Reverse Transcriptase purchased from invitrogen, DNA Clean & Concentrator®-5 provided by Zymo Research, Micro Bio-Spin® Chromatography Columns from Bio-Rad. Oligonucleotide sequences containing primers are provided in Table S1 in the Supporting Information. Gel Imaging was performed on Pharos FX Molecular imager and ChemiDoc™ MP Imaging System (Bio-Rad, USA). HPLC from Agilent Technologies. Micro Bio-Spin® Chromatography Columns by Bio-Rad. Hieff® qPCR SYBR® Green Master Mix (No Rox) from Yeasen. TRIzol™ Reagent by invitrgen.

Condition of DMS reaction and whole procedure. Dimethyl sulfate (DMS) first attenuation in ethanol to 1 M for further use. 1 M Sodium cacodylate-HCl buffer (pH 7.45) to be attenuated. In the HPLC experiment, 2 μL RNA oligos (100 μM) are incubated with 4 ℃ 1 M Sodium cacodylate buffer, 2 μL 1 M DMS solution and 12 μL RNase-free water in the total 20 μL system to react at 25 ℃ or 37 ℃ for 1 h or 2 h.

In the PAGE analysis, 2 μL RNA oligo A and RNA oligo m^6A (100 μM) are first annealed with up probe, down probe and annealing buffer in total 10 μL, incubate in 65 ℃ for 5 min and then quickly chilled on the ice to 0 ℃. Then mix the whole system with 250 mM sodium cacodylate buffer and DMS solution to react in 25 ℃ for 1 h, followed by purification using Micro Bio-Spin® Chromatography Columns to centrifuge in 1000 g for 2 min and repeat one more time to ensure complete elution. After that, add FS buffer and SuperScript™ III to reverse transcript in 50 ℃ for 10 min.

The 28s qPCR analysis follow the same procedure except for changing the probe to m6A_4190_up_qPCR and m6A_4190_down_qPCR and using qPCR_RP_univ and qPCR_FP_univ for qPCR instead.

HPLC program. In this assay, we use 3 series of oligo RNA with or without m^6A to simulate real sample, which are purchased from Gene Create and Takara. The HPLC program use gradient elution: 0-5min 5% organic phase, 25min 40% organic phase,

26-35min 100% organic phase. Organic phase with chromatographic pure acetonitrile and aqueous phase with 50mM TEAA buffer pH=7±0.5. Dimethyl Sulfate (DMS) is mixed with ethanol to 1M, and 1M Sodium cacodylate-HCl buffer(pH=7.45). 2μL of nucleotides(100μM) are incubated with 4μL 1M Sodium cacodylate buffer, 2μL 1M DMS solution and 12μL RNase-free water in the total 20μL system. Then inject the total system into HPLC and record the chromatogram to confirm the possibility of reaction.

PAGE analysis and qPCR to distinguish A and m^6A. 2 μL RNA oligo A / RNA oligo m^6A (100 μM) are annealed with up probe and down probe in total 10 μL, first in 65 ℃ for 5 min and then quickly incubate on the ice. Then mix the whole system with Sodium cacodylate buffer and DMS solution to react in 25 ℃ for 1 h. Then followed by purification using Micro Bio-Spin® Chromatography Columns, followed by centrifuge in 1000 g for 2 min and repeat one more time to ensure complete elution. After that, add FS buffer and SuperScript™ III to reverse transcript in 50 ℃ for 10 min. Finally, mix half of the residual with deionized formamide to denature and followed by PAGE analysis without Gel-Rad. Directly use the Alexa-488 channel to detect and record the signal from FAM decorated primer. The other half is then attenuation and mixed with SYBR Green Mix, motif RP, motif FP and RNase-free water. Finally run the universal qPCR program: denature in 95 ℃ for 5 min, then cycle the amplification program (denature in 95 ℃ for 10 sec, anneal in 55 ℃ for 20 sec and extend in 72 ℃ for 20 sec) 40 times.

FTO treat of total RNA. Total RNA is extracted from Hela Cells by Trizol. The demethylation assay was performed in a 100 μl scale reaction containing 500 ng total RNA, 0.2 nmol purified FTO, 50 mM HEPES pH 7.0, 75 μM $(NH_4)_2Fe(SO_4)_2•6H_2O$, 2 mM L-ascorbic acid, 300 μM 2- ketoglutarate and 50 μg/ml BSA. For the control reaction, EDTA was added to 5 mM prior to the addition of the enzyme. The demethylation reactions were incubated at 37 °C for 1h before being quenched by the addition of EDTA to 5 mM. RNA from the reaction was purified using the RNA clean & concentrator kit (Zymo Research) for subsequent use.100 ng of RNA from the

demethylation reaction was first digested by nuclease P1 from Penicillium citrinum (Sigma, >1U) in 25 μL 1× P1 digestion buffer containing 10 mM NH_4HCO_3, pH 5.3 at 42 °C for 2 h. Then, 1 μl FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher, 1 U/μl) and 3 μl 10× FastAP buffer (ThermoFisher) were added to the reaction and incubated at 37 °C for 2 h.

LC-MS/MS. Series of m^6A nucleoside from 1nM-50nM and A 0.1μM-5μM are prepared to draw the standard line for the quantify if m^6A is totally demethylated. Filtered samples were injected into a C18 reverse phase column (Agilent) on a HPLC coupled to an Agilent 6460 Triple Quad Mass Spectrometer in positive electrospray ionization mode. Quantitation was performed on the basis of peak areas of characteristic nucleoside-to-base ion mass transitions of 268-to-136 (A) and 282-to-150 (m6A, retention time 2.8 min).

Table S1. DNA and RNA sequences used in the current study

Name	Sequence (from 5' to 3')	Construct
12 mer m^6A-1	5'-UAUUUUm^6AUUUUU-3'	RNA
12 mer m^6A-2	5'- UUUUUUm^6AUUUUU-3'	RNA
RNA_oligo_A	5'-GCCGUUCAUCUGCUAAAAGGACUGCUUUUGGGGCUUGUAA-3'	Motif A
RNA_oligo_m^6A	5'-GCCGUUCAUCUGCUAAAAGGm^6ACUGCUUUUGGGGCUUGUAA-3'	Motif m^6A
Primer_FAM	5'-FAM-TTACAAGCCCCAAAAGCAG-3'	Primer for reverse transcription
Block_up	5'-CCTTTTAGCAGATGAACGGC-3'	Down probe
Motif RP	5'-TTACAAGCCCCAAAAGCAG-3'	The reverse primer
Motif FP	5'-GCCGTTCATCTGCTAAAAG-3'	The forward primer
m6A_4190_up_FAM	5'-FAM-CGCCTTAGGACACCTGCG-3'	Up probe
m6A_4190_down	5'-TACCGTTTGACAGGTGTA-3'	Up probe
A_4194_up_FAM	5'-FAM-AGCTCGCCTTAGGACACC-3'	The forward primer
A_4194_down	5'-GCGTTACCGTTTGACAGGT-3'	Down probe
qPCR_RP_univ	5'-GCCAGGTGGGGAGTTTGAC-3'	The reverse primer
qPCR_FP_univ	5'-CACGGGAGGTTTCTGTCCTCC-3'	The forward primer
m6A_4190_up_qPCR	5'-TACCGTTTGACAGGTGTACCGCCCCAGTCAAACTCCCCACCTGGCACTGTCCCCGGAGCG-3'	Up probe
m6A_4190_down_qPCR	5'-TTGCCCTTCTGCTCCACGGGAGGTTTCTGTCCTCCCTGAGCTCGCCTTAGGACACCTGCG-3'	Down probe

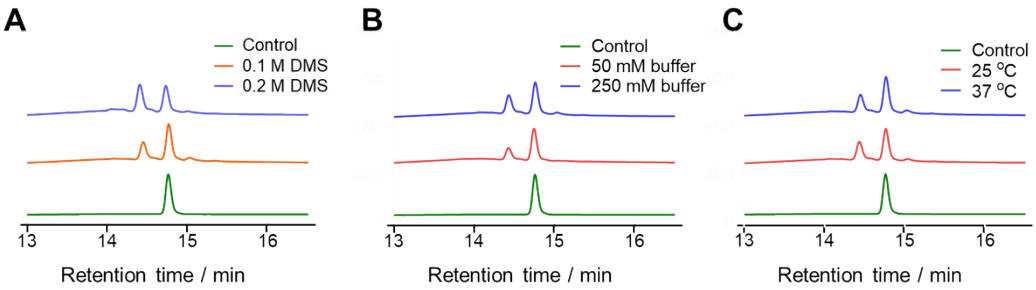

Figure S1. HPLC analysis was performed to optimize DMS labeling conditions. (**A**) Different concentration of DMS (250 mM buffer, 25 ℃). (**B**) Different concentration of buffe (0.1 M DMS, 25 ℃). (**C**) Different temperature (0.1 M DMS, 250 mM buffer).

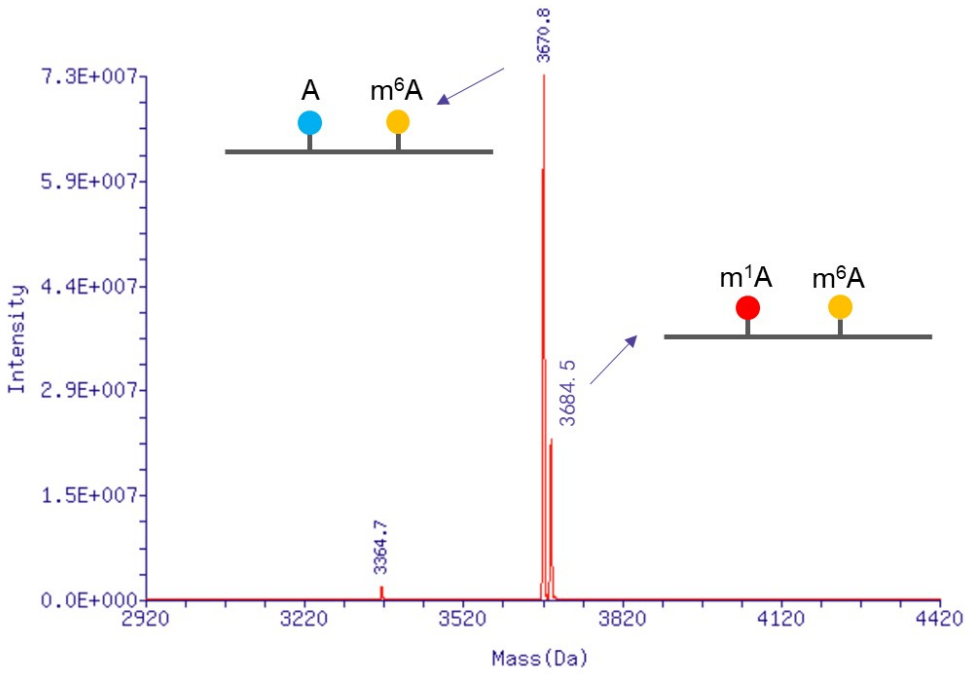

Figure S2. ESI mass spectrometry for nucleotides after DMS reaction using RNA oligo containing both A and m^6A (12 mer m^6A-1). 3670.8 represents the mass before the reaction and 3684.5 represents the mass after the reaction.

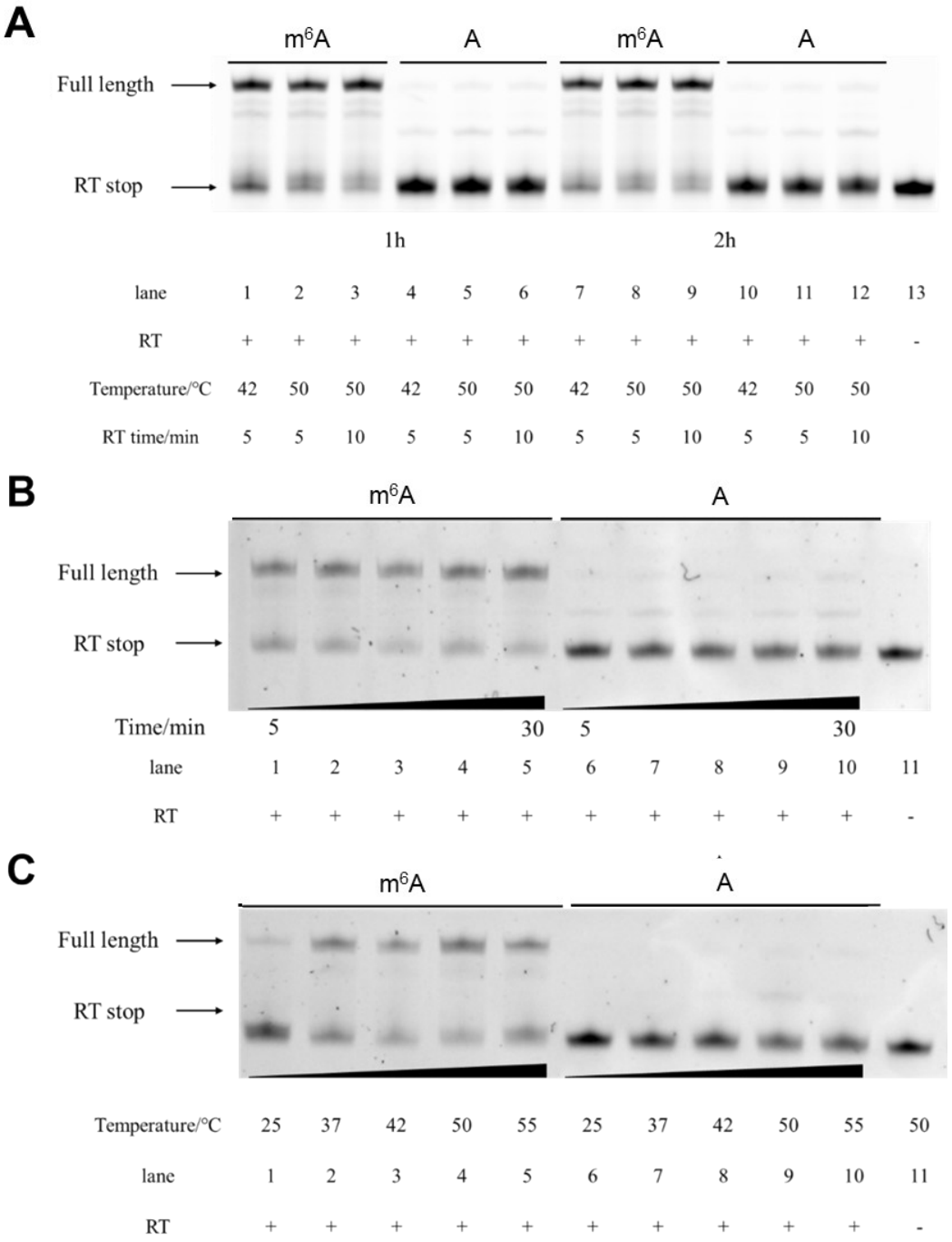

Figure S3. **The effect of temperature, time and concentration**. Reactions were performed as described in the Experimental (**A**)using the same condition besides the DMS reaction time (reverse transcription in 37℃ for 10 min). lane 1-3,7-9 for m^6A react for 1 and 2 hours, respectively. Lane 4-6,10-12 for A react for 1or 2h, respectively. Lane 13 for only primer with FAM. (**B**) using the same condition besides the reverse transcription time (reverse transcription in 37℃, DMS react for 1h). lane1-5 m^6A reverse transcribe for 5,10,15,20,25,30 min, respectively. Lane6-10

A reverse transcribe for 5,10,15,20,25,30 min, respectively. Lane 11 for only primer with FAM. (**C**)using the same condition besides the reverse transcription temperature (reverse transcription for 10min, DMS react for 1h). lane 1-5 m6A reverse transcribe for 25,37,42,50,55℃, respectively. Lane 6-10 A reverse transcribe for 25,37,42,50,55℃ respectively. Lane 11 for only primer with FAM.

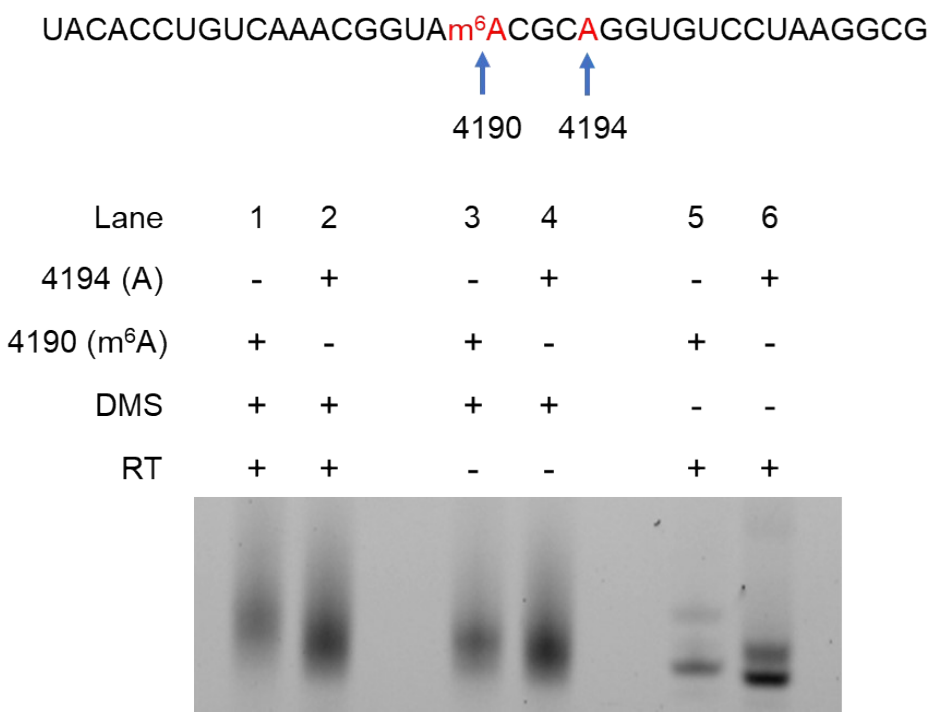

Figure S4. PAGE analysis of the primer extension results with only dTTP of m^{6}A4190 and A4194 in 28S rRNA. Without DMS treatment, the m^{6}A4190 can extend 2 base pairs (lane 5), while the 4194A can only extend 1 base pair. Due to the low amount of m^{6}A in the sample, the primer did not extend completely. After DMS labeling, the sample smeared and could not be seen clearly (lanes 3, 4). Subsequent reverse transcription, m^{6}A4190 was extended by 1 base pair but not by 2 base pairs due to the DMS marking having adjacent A (lanes 1 and 3, lane 1 above lane 2), while A4194 was not extended (lane 2 and lane 4 are almost the same height).