Enzyme-assisted chemical labelling enables precise identification of an RNA methyltransferase's substrate modification sites

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Materials and instruments

All chemicals and reagents were used as purchased, without further purification. *Se*-allyl-L-selenohomocysteine (SeAHC) was synthesized following the protocol previously described[1]. Recombinant human methyltransferases, METTL3/METTL14 heterodimer, were co-expressed in insect cells according to a previously published procedure[2]. UHPLC-QQQ-MS/MS results were acquired using a Waters TQ MS triple-quadrupole LC spectrometer.

Molecular cloning

The gene coding for full-length (FL) human MAT2A (hMAT2A; 395 aa; Accession number NP_005902) and full-length (FL) human METTL16 (hMETTL16; 562 aa; Accession number NP_076991) were amplified from HeLa cell cDNA by PCR (polymerase chain reaction). MAT2A coding gene was cloned into the pET28a vector with 6 × his-tag in N-terminal. The recombinant pET28a based plasmid encoding MTN (*E. coli* MTN; 232 aa; Accession number: NP_414701) was constructed similarly. hMETTL16 sequence was cloned into the pcDNA3.0 vector to provide eukaryotic expression plasmid bearing CMV enhancer and N-terminal flag tag. For MAT2A I117A, we use KOD-Plus-Mutagenesis Kit to generate a mutation sequence from wild type (MAT2A wt). Constructs covering the core methyltransferase domain of hMETTL16[3] (METTL16-MTD; 1-291 aa) was cloned into the pET28a vector for bacterial expression.

Expression and purification of proteins

(1) Prokaryotic expression and purification of MAT2A (wt and I117A), METTL16-MTD and MTN Purification of recombinant MAT2A and MTN was performed as previously described[4, 5]. For MAT2A (wt and I117A) and MTN, plasmids were transferred into *E. coli* BL21(DE3), while *Rosetta* DE3 strain was chosen for the preparation of METTL16-MTD. Strains harboring abovementioned plasmids were grown in 10 mL of LB broth containing corresponding antibiotic at 37°C overnight.

Then culture was inoculated into new LB media for mass cultivation with a volume ratio 1:100 and incubated at 37 °C to reach an OD 600 of 0.6 to 0.8. MAT2A wt and I117A expressions were induced with 0.5 mM IPTG for 16 h at 16 °C. The induced expression condition of METTL16-MTD was 0.7 mM IPTG, 16 h at 20 °C. For MTN, cultures were induced by the addition of 1 mM ITPG and were incubated at 16 °C for 20 h. Cultures were harvested via centrifugation at 6000 g for 20 min. The resulting cells were resuspended in the corresponding lysis buffer and followed by 70% intensity sonication for 1 h. The target proteins were then purified by Ni-NTA (GE Healthcare) and followed by gel filtration chromatograph (Superdex 75 prep grade). The pure fractions retained with N-terminal 6 × his-tag were verified by SDS-PAGE gel and then stored at -20℃ in the buffer containing 20 mM Tris-HCl pH 8.0, 200 mM KCl and 20% glycerol. Compositions of the lysis buffer used in the purification of above proteins is shown in **Supplementary Table 1**.

(2) Eukaryotic expression and purification of METTL16-FL

For the expression of hMETTL16-FL, pcDNA3 plasmid harboring METTL16 was transfected into HEK293T cells. Cells were grown at 37 ℃ with 5% CO_2 and harvested after 48 h. Cultures were resuspended in lysis buffer and hold for 15~30 min on ice. After that, cells were disrupted with 45% intensity super-sonication for 2 min. The target protein was purified by ANTI-FLAG M2 Affinity Gel (Sigma) followed by corresponding protocol. The eluted protein was concentrated with an ultrafiltration tube and then confirmed by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis).

Enzymatic labelling assay

The *in vitro* enzymatic labelling reactions were carried out in a volume of 50 μl with 2 mM SeAHC, 1 mM ATP (NEB, 10 mM), 1–5 μg RNA probes, 5 μM MAT2A (wt or I117A), 10 μM MTN, 2–5 μM RNA methyltransferase (METTL3/METTL14 or METTL16) in reaction buffer containing 25 mM Tris buffer (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 0.05 mM $ZnCl_2$ and 0.2 U/μL RNasin (TaKaRa, 40 U/μL). Prior to the reaction, the RNA probes were denatured and annealed with a program of (i) 90 °C for 3 min, (ii) −2 °C/cycle for 40 cycles within 30 min and (iii) 4 °C for 5 min. After that, other components were added and reactions were incubated at 37 °C for 4 h. Reactions were quenched by inactivating the enzyme at 70 ℃ for 20 min. RNA probes were precipitated from the reaction solution with isopropyl alcohol. About 100 ng product was measured through UHPLC-QQQ-MS/MS after enzymatic digestion to quantify enzymatic labelling efficiency while others were used for immunoprecipitation and subsequent TA cloning assay.

Quantitative analysis of enzymatic labelling efficiency by UHPLC-QQQ-MS/MS

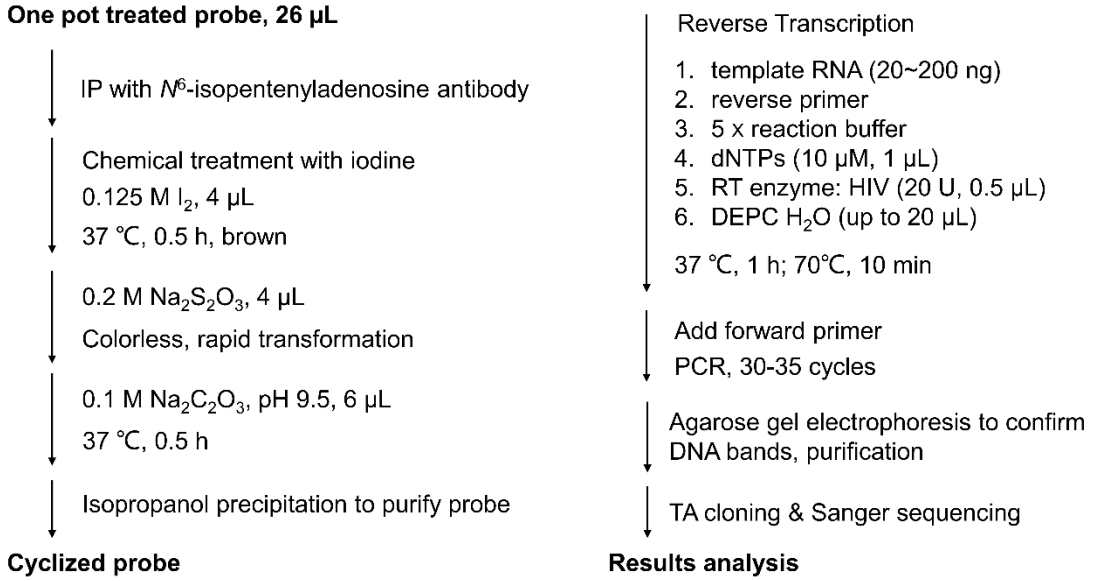
RNA probes enzymatic labelled were digested into nucleosides and measured by reverse-phase UHPLC on a C18 column with online MS detection using a Waters TQ MS triple-quadrupole LC spectrometer in positive electrospray ionization mode. The modification efficiency was based on the ratio of a^6A (N^6-allyladenosine) to A, while the amounts of nucleosides from sample were calculated on the base of the standard curve generated by pure standards. For each sample, 100~300 ng RNA was digested by using 1 U nuclease P1 (Wako) in 40 μL reaction containing 20 mM NH_4OAc at 42 ℃ for 2 h. Afterwards, 1 μL BAP (Bacterial Alkaline Phosphatase, Toyobo), 5 μL 10 × BAP Buffer (Toyobo) and 4 μL DEPC H_2O were added and the reaction was incubated at 37℃ for 2 h. Samples were then filtered by 0.22 μm filter (Millipore) and diluted to 80 μL. A 10 μL

volume of the sample was injected into the UHPLC-QQQ-MS/MS system. The a^6A nucleoside was quantified by using the nucleoside to base ion mass transitions of 308 to 176.

Immunoprecipitation (IP) and biochemical mutation assay

After the completion of enzymatic reactions, we used immunoprecipitation to enrich the a^6A-containing RNA probes. N^6-isopentenyladenosine antibody which can specifically recognize a^6A was utilized. The IP procedures followed the previous work[1]. The enriched a^6A-containing probes were treated by iodine to induce the formation of 1, N^6-cylized adenosine (cyc-A). After purification, cyclized RNA probes were used as templates for reverse transcription (RT) to get complementary DNA (cDNA) following the protocol[6] in **Scheme S1**. The HIV reverse transcriptase was selected due to its higher read-through rate for cyc-A. The cDNA was amplified by polymerase chain reaction (PCR). The PCR product was cloned into pClone007 Simple Vector by a sticky TA end annealing and then the recombinant plasmid was transformed into competent cells. Single colony was picked for Sanger sequencing.

Scheme S1



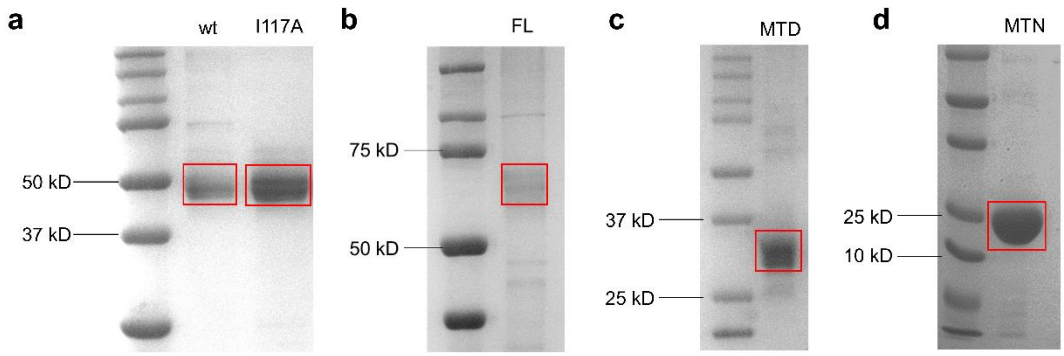


Figure S1. Coomassie staining results of purified a) MAT2A wt & I117A, b) METTL16-FL, c) METTL16-MTD and d) MTN. The red boxes indicate the target proteins.

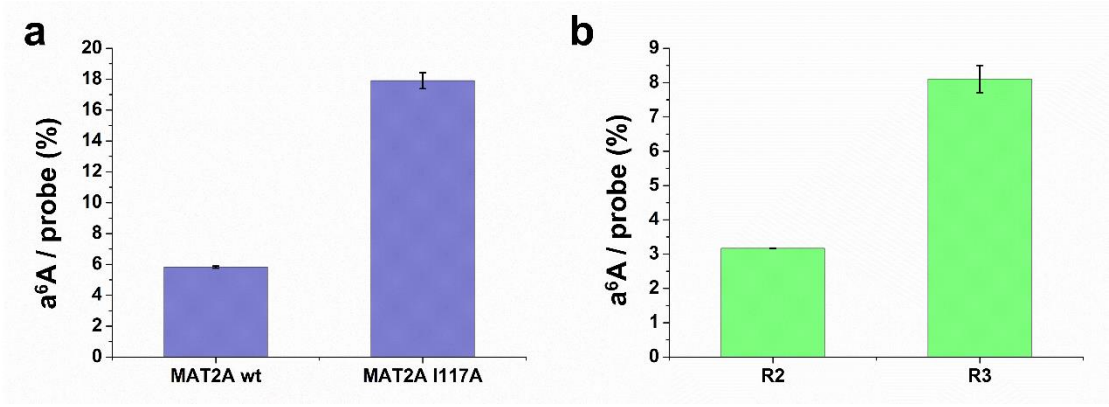


Figure S2. The a^{6}A modification ratio of RNA probes measured by UHPLC-QQQ-MS/MS. a) The a^{6}A modification ratio of RNA probe R1 catalyzed by METTL3/METTL14 in the presence of MAT2A wt or I117A. b) The a^{6}A modification ratios of probe R2 and R3 catalyzed by METTL16-FL through enzymatic labelling assay.

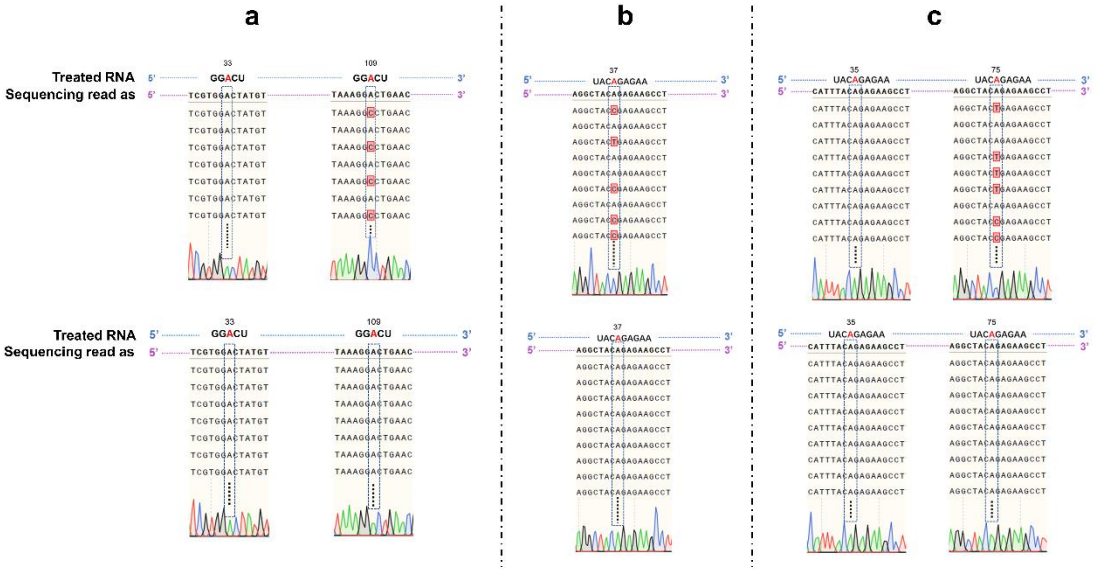


Figure S3. Examples of Sanger sequencing data showing the cDNA mutation results of the RNA probes R1 (a), R2 (b), and R3 (c) with (Top panel) and without (Bottom panel) a cascade of enzymatic treatments as illustrated in our developed method.

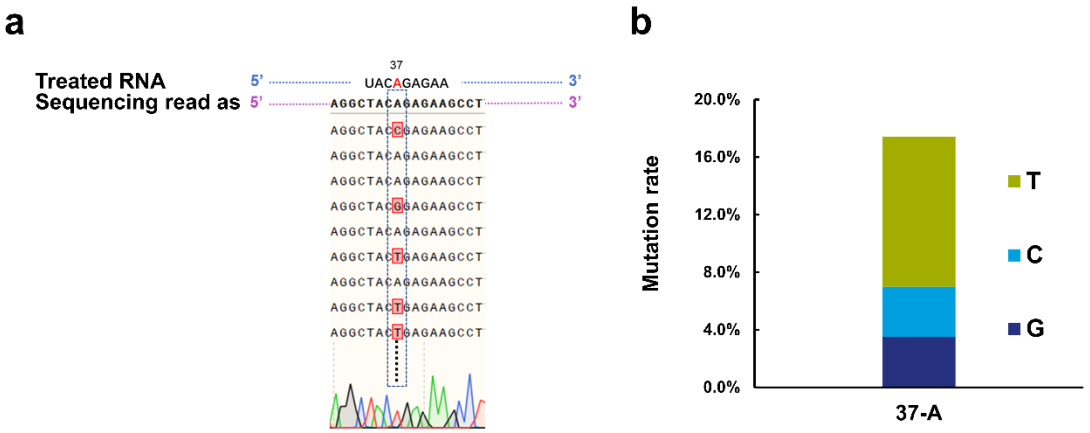


Figure S4. The METTL16-MTD-catalysed labelling of R2 probe and characterization of its modification site. a) The Sanger sequencing results for cDNA of allyl-modified and iodinated R2 with adenosine (37-A) located in UACAGAGAA motif. b) Statistics of mutation (A to T/C/G) rates of 37-A in R2 probe.

Supplementary Table 1. The bacterial strain or cell line for expressing the listed enzymes and their corresponding lysis buffer used in this work.

	Bacterial strain or cell line	Lysis buffer
MAT2A wt & MAT2A I117A	*E. coli* BL21(DE3)	50 mM Tris-HCl, pH 8.0; 50 mM NaCl; 5 mM *β*- Mercaptoethanol; 25 mM Imidazole; 5% Glycerol
METTL 16 FL	HEK293T	50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 0.5% CA-630; 0.5 mM DTT; 1/100 protease inhibitor cocktail
METTL 16-MTD	*Rosetta* DE3	25 mM Tris-HCl, pH 8.0; 400 mM NaCl; 5 mM *β*-Mercaptoethanol; 10 mM Imidazole; 5% Glycerol; 0.5% Tween-20
MTN	*E. coli* BL21(DE3)	20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM β-Mercaptoethanol, 5 mM Imidazole

Supplementary Table 2. The sequences of RNA probes and DNA primers used in this work.

R1 probe sequence	GGGAAAGUCCAUGACCGAGAAGGAGAUCG UGGACUAUGUGGCCAGCCAGGUUACAACC GCCAAGAAGCUGCGCGGUGGUGUUGUGUU CGUGGACGAGGUGCCUAAAGGACUGAACG GCACGUUGGACGC
Primer-R1-F	GGGAAAGTCCATGACCGAGAAGGAG
Primer-R1-R	GCGTCCAACGTGCCG
R2 probe sequence	GGGCCUUUUUUCCCCAGACUUGUUGGCGU AGGCUACAGAGAAGCCUUCAAGCUCUGAG GGAAAGGGC
Primer-R2-F	GGGCCTTTTTTCCCCAGACTTGTTGG
Primer-R2-R	GCCCTTTCCCTCAGAGCTTGAAG
R3 probe sequence	GGGUACAGACAUACAUACAGACAGAUACA UUUACAGAGAAGCCUUUUUUCCCCAGACU UGUUGGCGUAGGCUACAGAGAAGCCUUCA AGCUCUGAGGGAAAGGGC
Primer-R3-F	GGGTACAGACATACATACAGACAGATAC
Primer-R3-R	GCCCTTTCCCTCAGAGCTTGAAG

References

1. X. Shu, J. Cao, M. Cheng, S. Xiang, M. Gao, T. Li, X. Ying, F. Wang, Y. Yue, Z. Lu, Q. Dai, X. Cui, L. Ma, Y. Wang, C. He, X. Feng and J. Liu, *Nat. Chem. Biol.*, 2020, **16**, 887-895.
2. J. Liu, Y. Yue, D. Han, X. Wang, Y. Fu, L. Zhang, G. Jia, M. Yu, Z. Lu, X. Deng, Q. Dai, W. Chen and C. He, *Nat. Chem. Biol.*, 2014, **10**, 93-95.
3. K. E. Pendleton, B. Chen, K. Liu, O. V. Hunter, Y. Xie, B. P. Tu and N. K. Conrad, *Cell*, 2017, **169**, 824-835 e814.
4. R. Wang, K. Islam, Y. Liu, W. Zheng, H. Tang, N. Lailler, G. Blum, H. Deng and M. Luo, *J. Am. Chem. Soc.*, 2013, **135**, 1048-1056.
5. M. T. Banco, V. Mishra, A. Ostermann, T. E. Schrader, G. B. Evans, A. Kovalevsky and D. R. Ronning, *Proc. Natl. Acad. Sci.* 2016, **113**, 13756-13761.
6. X. Shu, Q. Dai, T. Wu, I. R. Bothwell, Y. Yue, Z. Zhang, J. Cao, Q. Fei, M. Luo, C. He and J. Liu, *J. Am. Chem. Soc.*, 2017, **139**, 17213-17216.