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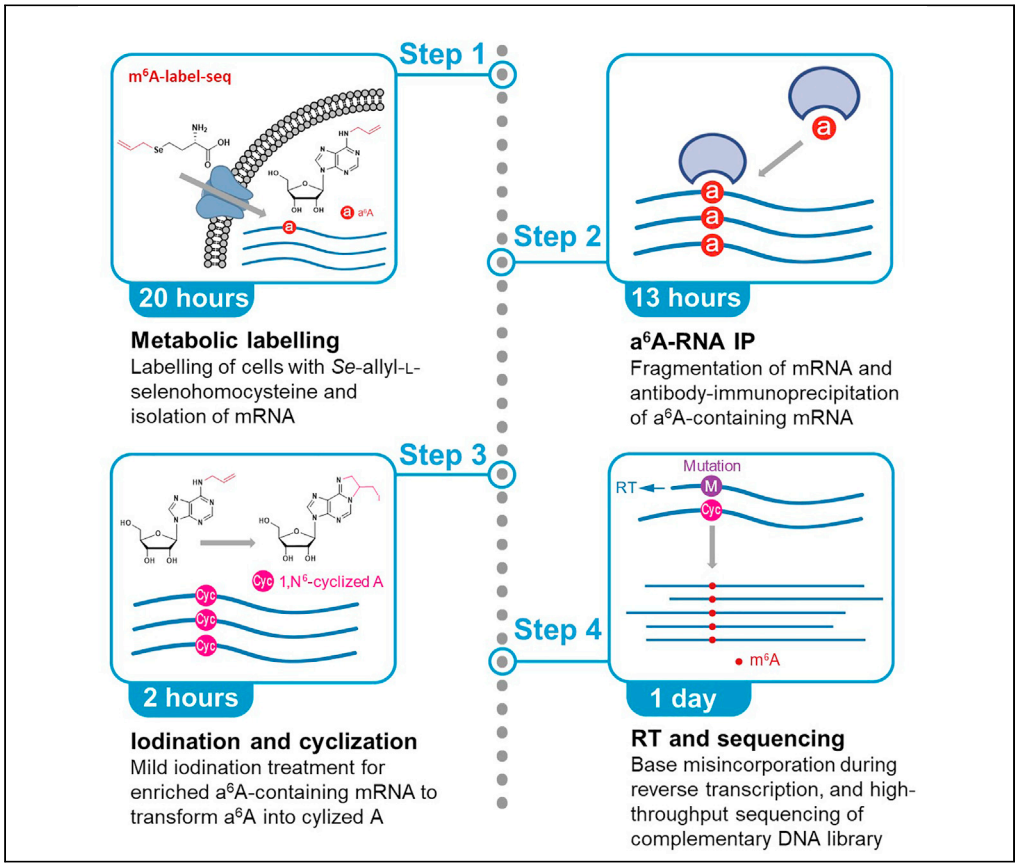
m^{6}A-label-seq: A metabolic labeling protocol to detect transcriptome-wide mRNA N^6-methyladenosine (m^{6}A) at base resolution



We describe here a metabolic labeling protocol for detecting cellular transcriptome-wide mRNA N^6-methyladenosine (m^{6}A) at base resolution. By feeding cells an analog of methionine, potential mRNA m^{6}A forming sites are replaced with the N^6-allyladenosine (a^{6}A). A mild chemical iodination of a^{6}A in RNA results in its opposite base misincorporation during RNA reverse transcription, and thus m^{6}A locations could be precisely identified in the high-throughput sequencing. m^{6}A-label-seq provides a strategy to label and identify cellular epitranscriptomic modification sites.

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Highlights

Detection of cellular mRNA m^{6}A modification sites at single base resolution

m^{6}A-forming site labeling via an allyl-substituted methyltransferase cofactor

Replacement of RNA m^{6}A by N^6-allyl adenosine via RNA metabolism

N^6-allyl adenosine is a chemical sequencing nucleoside analog

Shu et al., STAR Protocols 3, 101096
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m^{6}A-label-seq: A metabolic labeling protocol to detect transcriptome-wide mRNA *N*^{6}-methyladenosine (m^{6}A) at base resolution

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SUMMARY

We describe here a metabolic labeling protocol for detecting cellular transcriptome-wide mRNA *N*^{6}-methyladenosine (m^{6}A) at base resolution. By feeding cells an analog of methionine, potential mRNA m^{6}A forming sites are replaced with the *N*^{6}-allyladenosine (a^{6}A). A mild chemical iodination of a^{6}A in RNA results in its opposite base misincorporation during RNA reverse transcription, and thus m^{6}A locations could be precisely identified in the high-throughput sequencing. m^{6}A-label-seq provides a strategy to label and identify cellular epitranscriptomic modification sites.

For complete details on the use and execution of this profile, please refer to Shu et al., 2020.

BEFORE YOU BEGIN

Synthesis of *Se*-allyl-L-selenohomocysteine

Se-allyl-L-selenohomocysteine is the precursor of allyl-modified methyltransferases' cofactor, enabling the replacement of endogenous mRNA modification *N*^{6}-methyladenosine (m^{6}A) with *N*^{6}-allyladenosine (a^{6}A) through metabolic pathways. We synthesized it by two steps of chemical reactions followed by HPLC purification (Bothwell and Luo, 2014; Hartstock et al., 2018).

Timing: 3 days

1. Synthesis of L-selenohomocystine.
   a. Under a nitrogen atmosphere, in a 250 mL round-bottom flask, selenium powder (316 mg, 4 mmol) and sodium borohydride (151.2 mg, 4 mmol) are suspended in 120 mL ethanol.
   b. The mixture is heated to reflux at 80°C for 2 h, while the solution became dark maroon in color (Figure 1A).
   c. After that, (S)-(+)-2-amino-4-bromobutyric acid hydrobromide (526 mg, 2 mmol) is added, resulting in a rapid formation of an opaque yellow mixture (Figure 1B).
   d. The reaction is allowed to proceed under reflux at 80°C for 12–24 h and then quenched with 10 mL 2 M HCl.
   e. After the solvent is removed by Rotary Evaporator, the resulting residue is mixed with 50 mL 5% v/v HCl, and then centrifuged at 10000×*g* for 10 min to remove precipitation.
   f. The supernatant aqueous solution is then washed three times with 75 mL diethyl ether and $NaHCO_3$ is added to adjust pH = 7 approximately.

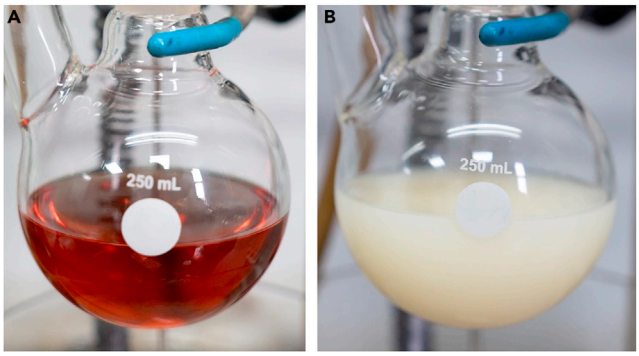

Figure 1. Color change in the synthesis of L-selenohomocystine

(A and B) The images in the synthesis of L-selenohomocystine showing the color change of the reaction mixture from dark maroon (A) to opaque yellow (B).

Note: The pH could not be less than 6, or the acidic conditions will lead to failure of subsequent experiments.

g. After removing bulk solvent by Rotary Evaporator, the resulting solid is directly taken to next reaction step.

2. Synthesis of *Se*-allyl-L-selenohomocysteine.
   a. Under a nitrogen atmosphere, in a 250 mL round bottom flask, the solid obtained above and sodium borohydride (226.8 mg, 6 mmol) are dissolved in 120 mL ethanol.
   b. The mixture is stirred for 15 min at 20°C–30°C.
   c. Then, $NaHCO_3$ (252 mg, 3 mmol) and allyl bromide (328 μL, 460 mg, 3.8 mmol) are added and stirred for 12–24 h at 20°C–30°C.
   d. After the reaction is finished, the solvent is removed by Rotary Evaporator and the crude product is dissolved in 10 mL 2 M HCl (Figure 2, Table 1).
   e. The solution above is purified by reverse phase HPLC using a gradient of 95% H_2O and 5% acetonitrile to 0% H_2O and 100% acetonitrile in 30 min (Figure 3, Table 2).
   f. The fractions containing the desired product are lyophilized to afford 80 mg white solid, yield = 18%.

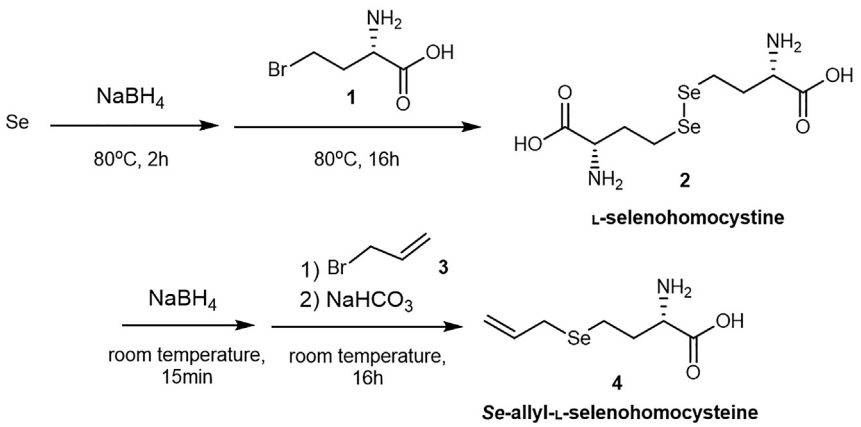

Figure 2. A synthetic route of L-selenohomocystine (2) and *Se*-allyl-L-selenohomocysteine (4)

The details are provided in Table 1.

Table 1. Synthesis of L-selenohomocystine and *Se*-allyl-L-selenohomocysteine

Synthetic steps	Compound	Label	Mole number	Final concentration
Synthesis of L-selenohomocystine	Selenium powder	Se	4 mmol	33.3 mM
	Sodium borohydride	$NaBH_4$	4 mmol	33.3 mM
	(S)-(+)-2-amino-4-bromobutyric acid hydrobromide	1	2 mmol	16.7 mM
Synthesis of *Se*-allyl-L-selenohomocysteine	L-selenohomocystine	2	1 mmol	8.3 mM
	Sodium borohydride	$NaBH_4$	6 mmol	50 mM
	Allyl bromide	3	3.8 mmol	31.7 mM
	Sodium bicarbonate	$NaHCO_3$	3 mmol	25 mM

Synthesis of N^6-allyladenosine-5′-triphosphate (a^6ATP)

Timing: 1 day

N^6-allyladenosine-5′-triphosphate (a^6ATP) is the competitive elution reagent for a^6A-containing RNAs, and can be synthesized and purified according to the published protocol with modified procedures (Ottria et al., 2010; Grammel et al., 2011).

3. Synthesis of a^6A.
   a. Under a nitrogen atmosphere, in a 250 mL round-bottom flask, 6-chloropurine riboside (573 mg, 2 mmol) and allylamine hydrochloride (561 mg, 6 mmol) are suspended in 120 mL ethanol.
   b. Then, triethylamine (1.2 g, 1.7 mL, 12 mmol) is added, and the mixture is heated to reflux at 80°C for 3 h, while the solution became clear.
   c. After the reaction is finished, the solution is cooled to 25°C for 10 min and the solvent is removed under vacuum by Rotary Evaporator to leave syrupy residue.
   d. The crude residue is then recrystallized from methanol, and a white solid is obtained with a yield of 73%.
4. Synthesis of a^6ATP.
   a. Under a nitrogen atmosphere, trimethyl phosphate (20 mL) is dried for 18 h over molecular sieves (3A).
   b. Under a nitrogen atmosphere, a^6A (61.4 mg, 0.2 mmol) and trimethyl phosphate (0.5 mL) are added into a flame-dried round-bottom tube in a sequential manner (Figure 4A).
   c. After that, phosphoryl chloride (39.8 mg, 24.2 μL, 0.26 mmol) is added, and the reaction mixture is stirred for 1.5 h at 0°C until clear (Figure 4B).

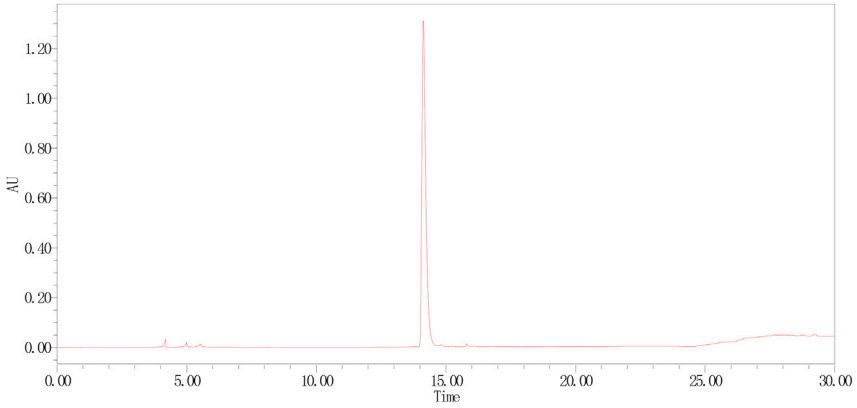

Figure 3. An analytical HPLC trace for *Se*-allyl-L-selenohomocysteine by Waters e2695 module with an Atlantis T3 OBD Prep Column (100 Å, 5 μm, 10 mm × 250 mm, 1/pkg; cat. no. 186008205)

The absorbance was measured at 214 nm. The details are provided in Table 2. Characterization data: ^{1}H NMR (500 MHz, D_2O) δ 5.84 (ddt, J = 17.6, 9.9, 7.7 Hz, 1H), 5.10–4.90 (m, 2H), 3.72 (dd, J = 6.7, 5.7 Hz, 1H), 3.29–3.06 (m, 2H), 2.62–2.41 (m, 2H), 2.24–1.93 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ 174.02 (s), 134.73 (s), 116.75 (s), 54.78 (s), 31.19 (s), 25.33 (s), 17.17 (s). HRMS (ESI), $C_7H_{13}NO_2Se$, m/z 224.0183 ([M+H]$^+$, calcd 224.0184).

Table 2. HPLC gradient for purification of *Se*-allyl-L-selenohomocysteine

Time (min)	Flow rate (mL/min)	H_2O (%)	Acetonitrile (%)
0	2.5	95	5
5	2.5	95	5
10	2.5	70	30
20	2.5	50	50
25	2.5	0	100
30	2.5	0	100

d. Then, tributylammonium pyrophosphate (549 mg, 1 mmol) in 2 mL anhydrous dimethylformamide is added, and the reaction mixture is stirred for 20 min at 0°C.
e. The reaction mixture is further stirred for 5 min at 20°C–30°C, and 2 mL of triethylammonium bicarbonate (1 M) is added (Figure 5, Table 3).
f. The crude product is purified by reverse-phase HPLC using a gradient of 95% 20 mM triethylammonium bicarbonate and 5% acetonitrile to 0% 20 mM triethylammonium bicarbonate and 100 % acetonitrile in 20 min (Figure 6, Table 4).
g. Fractions containing the desired product are lyophilized to afford 40 mg of a^{6}ATP as a triethylammonium salt with a yield of 37%.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-cytokinin | N^6-isopentenyladenosine (10 µg/µL)	Agrisera	Cat#AS09 415
Chemicals, peptides, and recombinant proteins		
Trizol Reagent	Invitrogen	Cat#15596018
Reverse transcriptase recombinant HIV	Worthington Biochemical Corporation	Cat#LS05003
Reverse transcriptase RevertAid	Thermo Scientific	Cat#EP0441
5x RT reaction buffer	Thermo Scientific	Cat#EP0441
Recombinant RNase Inhibitor	Takara Bio	Cat#2313A
Igepal CA-630	Sigma-Aldrich	Cat#I8896
Bovine Serum Albumin (BSA)	Roche	Cat#10711454001
Dynabeads™ Protein A	Invitrogen	Cat#10002D
Glycogen	Thermo Scientific	Cat#R0561
Sodium Acetate Solution (pH 5.2)	Thermo Scientific	Cat#R1181
Trizma® base	Sigma-Aldrich	Cat#V900483
0.5 M EDTA solution (pH = 8.0)	Invitrogen	Cat#AM9260G
Diethyl pyrocarbonate	Sigma-Aldrich	Cat#40718
Iodine	Sigma-Aldrich	Cat#207772
Potassium iodide	Sigma-Aldrich	Cat#221945
Sodium thiosulfate	Sigma-Aldrich	Cat#217263
Sodium carbonate	Sigma-Aldrich	Cat#222321
Dexamethasone	Sigma-Aldrich	Cat#D4902
L-Cysteine	Sigma-Aldrich	Cat#C7352
$ZnCl_2$	Sigma-Aldrich	Cat#208086
NaCl	Sigma-Aldrich	Cat#S7653
Na_2CO_3	Sigma-Aldrich	Cat#S7795
$NaHCO_3$	Sigma-Aldrich	Cat#S5761
6-Chloropurine riboside	Sigma-Aldrich	Cat#852481
Allylamine hydrochloride	Sigma-Aldrich	Cat#735132
Triethylamine	Sigma-Aldrich	Cat#471283
HCl	Sigma-Aldrich	Cat#258148

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Isopropanol	Sigma-Aldrich	Cat#190764
Chloroform	Sigma-Aldrich	Cat#C2432
Phosphoryl chloride	Energy-Chemical	Cat#W610782
Tributylammonium pyrophosphate	Sigma-Aldrich	Cat#P8533
Anhydrous dimethylformamide	Sigma-Aldrich	Cat#V900809
Triethylammonium bicarbonate (1 M)	Sigma-Aldrich	Cat#18597
Acetonitrile	Sigma-Aldrich	Cat#34851
Selenium	Sigma-Aldrich	Cat#209651
Sodium borohydride	Sigma-Aldrich	Cat#71320
(S)-(+)-2-amino-4-bromobutyric acid hydrobromide	Sigma-Aldrich	Cat#476986
Allyl bromide	Sigma-Aldrich	Cat#A29585
Experimental models: Cell lines		
HeLa	Our Lab	n/a
HEK293T	Our Lab	n/a
H2.35	Our Lab	n/a
Critical commercial assays		
GenElute™ mRNA Miniprep Kit	Sigma-Aldrich	Cat#MRN10-1KT
NEBNext Ultra II Directional RNA Library Prep Kit	New England BioLabs	Cat#E7765
Software and algorithms		
Fastqc	Babraham Institute	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Fastp	(Chen et al., 2018)	https://github.com/OpenGene/fastp
Hisat2	(Kim et al., 2019)	http://daehwankimlab.github.io/hisat2/
Samtools	(Li et al., 2009)	http://www.htslib.org/
Exomepeak	(Meng et al., 2013)	https://bioconductor.riken.jp/packages/3.1/bioc/html/exomePeak.html
Others		
RPMI 1640/methionine-deficient	Gibco	Cat#A1451701
DMEM/high-glucose medium	HyClone	Cat#SH30243.01
Fetal Bovine Serum (FBS)	Gibco	Cat#10270
Penicillin/Streptomycin Solution	HyClone	Cat#SV30010

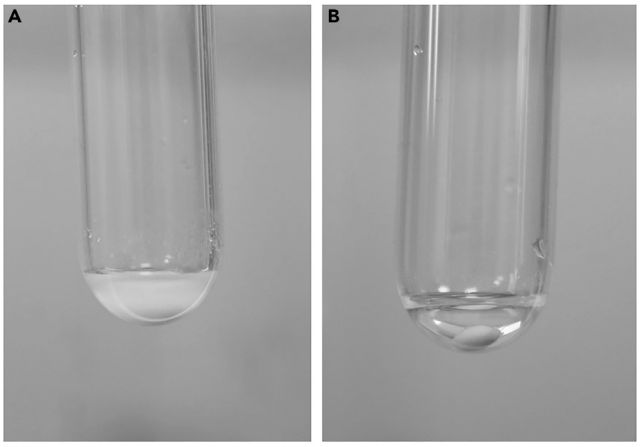

Figure 4. Color change in the synthesis of a^{6}ATP

(A and B) The images in the synthesis of N^6-allyladenosine-5′-triphosphate (a^{6}ATP) showing the initial (A) and final (B) states of the reaction mixture.

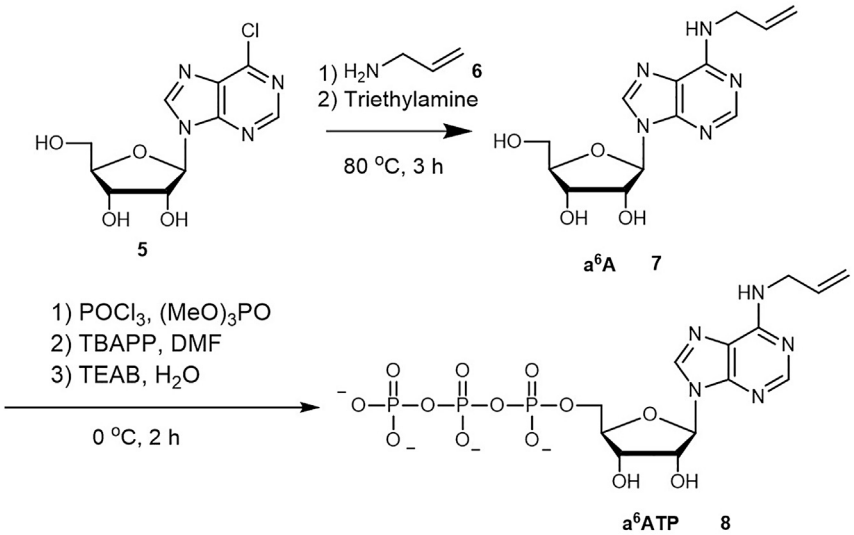

Figure 5. A synthetic route of a^6A (N^6-allyladenosine, 7) and a^6ATP (N^6-allyladenosine-5′-triphosphate, 8). The details are provided in Table 3.

MATERIALS AND EQUIPMENT

Purification of synthesized chemical compounds

Critical equipment: HPLC profiles are acquired using a Waters e2695 module with an Atlantis T3 OBD Prep Column (100 Å, 5 μm, 10 mm × 250 mm, 1/pkg; Cat#186008205).

Solutions for cell culture

Complete DMEM medium		
Reagent	Final concentration	Amount
DMEM, high glucose	n/a	44.5 mL
FBS	10%	5 mL
Penicillin/Streptomycin Solution	1 ×	500 μL
Total	**n/a**	**50 mL**

Note: For H2.35 culturing, dexamethasone (200 nM in final concentration) is added to all medium whenever used.

Pretreating medium		
Reagent	Final concentration	Amount
RPMI 1640, methionine-deficient	n/a	44 mL
FBS	10%	5 mL
Penicillin/Streptomycin Solution	1 ×	500 μL
L-cysteine (100 mM in sterile water)	1 mM	500 μL
Total	**n/a**	**50 mL**

Feeding medium		
Reagent	Final concentration	Amount
RPMI 1640, methionine-deficient	n/a	43 mL
FBS	10%	5 mL
Penicillin/Streptomycin Solution	1 ×	500 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
L-cysteine (100 mM in sterile water)	1 mM	500 μL
Se-allyl-L-selenohomocysteine (50 mM in sterile water)	1 mM	1 mL
Total	n/a	50 mL

Purification of mRNA

RNA precipitation		
Reagent	Amount ("X" is 40–500 μL)	Final concentration
RNA	X μL	n/a
3 M sodium acetate solution (pH = 5.2)	0.1× μL	300 mM
Isopropanol	1.1× μL	50% v/v
Glycogen (20 mg/mL)	1μL (0.011× μL if less than 1μL)	No more than 0.1 mg/mL
Total	2.2× μL	

Antibody immunoprecipitation of mRNA

10× Fragmentation buffer		
Reagent	Final concentration	Amount
$ZnCl_2$ (1 M)	100 mM	1 μL
Tris-HCl pH=7.0 (1 M)	100 mM	1 μL
RNase-free water	n/a	8 μL
Total	n/a	10 μL

Note: The solution could be stored at 4°C for up to one week.

5× IP buffer		
Reagent	Final concentration	Amount
Tris-HCl pH=7.4 (1 M)	50 mM	0.5 mL
NaCl (5 M)	750 mM	1.5 mL
10% vol/vol Igepal CA-630	0.05%	0.5 mL
RNase-free water	n/a	7.5 mL
Total	n/a	10 mL

Note: The solution was freshly prepared just before use, and kept on ice for one day.

1× IP buffer	
Reagent	Amount
5× IP buffer	2 mL
RNase-free water	8 mL
Total	10 mL

Note: The solution was freshly prepared just before use, and kept on ice for one day.

Chemical treatment of a^6A-containing RNA

Iodination solution		
Reagent	Final concentration	Amount
Iodine (I_2)	0.125 M	31.8 mg
Potassium Iodide (KI)	0.25 M	41.5 mg
RNase-free water	n/a	1 mL
Total	n/a	1 mL

Note: The solution could be stored at 4°C keeping away from light for up to one month.

STEP-BY-STEP METHOD DETAILS

⚠ CRITICAL: Use nuclease-free H_2O (DEPC H_2O, 1‰ diethyl pyrocarbonate in ddH_2O, sterile) at all steps whenever H_2O is needed in biochemical experiments.

Metabolic labeling and isolation of cellular mRNA

Timing: 17 h for step 1

Timing: 2 h for step 2

Timing: 1 h for step 3

We start from the metabolic labeling process by feeding human HeLa, HEK293T and mouse H2.35 cells with a methionine analog, *Se*-allyl-L-selenohomocysteine, which substitutes the methyl group on the enzyme cofactor *S*-adenosyl methionine (SAM) with allyl group. Accordingly, cellular RNAs are expected to be metabolically modified with a^6A at the supposed m^6A-forming sites which are manipulated by cellular methyltransferase. Then, we isolate mRNA from these metabolic labeled cells, which is prepared for further isolation by selective immunoprecipitation.

HeLa, HEK293T, and mouse H2.35 cells have been used. HeLa and HEK293T cells are first cultured in DMEM/high-glucose medium supplemented with 10% FBS and 1% 100 × Penicillin/Streptomycin Solution at 37°C with 5% CO_2 with a confluency of 80%.

1. Cell culture and metabolic labeling for HeLa, HEK293T, and mouse H2.35 cells
   a. Dissolve *Se*-allyl-L-selenohomocysteine in sterile water to a final concentration of 50 mM.
   b. Dissolve L-cysteine in sterile water to a final concentration of 100 mM.
   c. Prepare pretreating medium. The methionine-deficient medium (RPMI 1640, methionine-deficient) is supplemented with 10% FBS, 1% 100 × Penicillin/Streptomycin Solution, and L-cysteine at a final concentration of 1 mM.

Table 3. Synthesis of a^6A and a^6ATP

Synthetic steps	Compound	Label	Mole number	Final concentration
Synthesis of a^6A	6-chloropurine riboside	5	2 mmol	16.7 mM
	Allylamine hydrochloride	6	6 mmol	50 mM
	Triethylamine	Triethylamine	12 mmol	100 mM
Synthesis of a^6ATP	N^6-allyladenosine	7	0.2 mmol	400 mM
	Trimethyl phosphate	$(MeO)_3PO$		n/a
	Phosphoryl chloride	$POCl_3$	0.26 mmol	520 mM
	Tributylammonium pyrophosphate	TBAPP	1 mmol	400 mM
	Dimethylformamide	DMF		n/a
	Triethylammonium bicarbonate (1 M)	TEAB	2 mL	440 mM

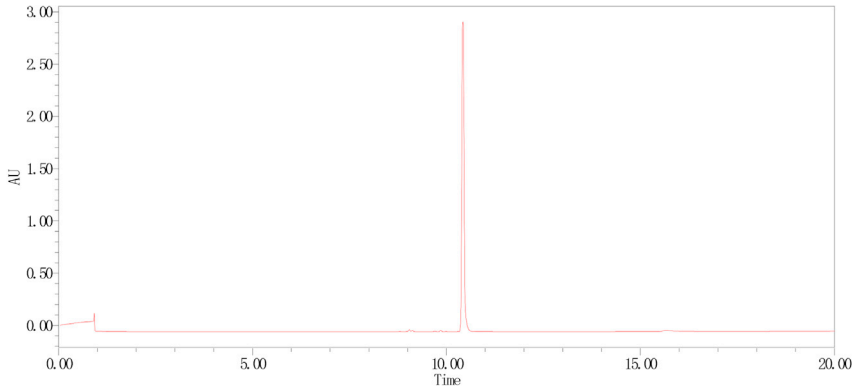

Figure 6. An analytical HPLC trace for N^6-allyladenosine-5′-triphosphate by Waters e2695 module with an Atlantis T3 OBD Prep Column (100 Å, 5 μm, 10 mm × 250 mm, 1/pkg; cat. no. 186008205)

The absorbance was measured at 260 nm. The details are provided in Table 4. Characterization data: HRMS (ESI), $C_{13}H_{20}N_5O_{13}P_3$, m/z 546.0192 ([M-H]$^-$, calcd 546.0198).

d. Prepare feeding medium. Add *Se*-allyl-L-selenohomocysteine prepared above into the pretreating medium to a final concentration of 1 mM.
e. Remove the regular culture medium (complete DMEM medium) from cells, wash once with PBS, remove the PBS, and then add equal volume of pretreating medium for 30 min of incubation at 37°C. The purpose of this step is to deplete cellular and intracellular methionine.
f. Remove the pretreating medium and add equal volume of feeding medium. The cells are further cultured at 37°C for another 16 h.

Note: The plate size is determined by the mRNA needed. If 150 mm plate size is used, 20 mL pretreating medium and feeding medium will be added.

2. Isolation of total RNA
   a. After metabolic labeling for 16 h, remove the feeding medium, wash once with cold PBS, remove the PBS, and then add 5 mL TRIzol™ Reagent to 150 mm culture dish to lyse the cells.
   b. Incubate the homogenized cell lysate of TRIzol at 20°C–30°C for 5 min, and transfer it to a 15 mL centrifuge tube.
   c. Add 1 mL of chloroform (1:5 of TRIzol) to the tube, shake the tube vigorously for 15 s, and incubate the mixture for 3 min at 20°C–30°C.
   d. Centrifuge at 12,000×*g* for 15 min at 4°C. The mixture is separated into three phases: a colorless upper aqueous, a white interphase, and a lower red phenol-chloroform phase.
   e. Carefully transfer the upper aqueous phase containing RNA to a new tube by angling the tube at 45° and pipetting the solution out.

⚠ CRITICAL: Avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.

   f. Add an equal volume of isopropanol (about 3 mL) to the above aqueous solution, and mix thoroughly and incubate them on ice for 15 min. Centrifuge the mixture at 15,000×*g* for 15 min at 4°C to obtain total RNA precipitate, a white gel-like pellet, at the bottom of the tube.

Table 4. HPLC gradient for purification of a^6ATP

Time (min)	Flow rate (mL/min)	Triethylammonium bicarbonate 20 mM (%)	Acetonitrile (%)
0	2.5	95	5
4	2.5	70	30
16	2.5	30	70
20	2.5	0	100

g. Discard the supernatant but not disturb the pellet, wash once with 6 mL ice-cold 75% ethanol, and centrifuge at 15,000×*g* for 15 min at 4°C.
h. Discard the supernatant, and air dry the RNA pellet at 20°C–30°C for 5 min.
i. Dissolve total RNA pellet in 260 μL RNase-free water, incubate at 60°C for 10 min, and 1 μL could be used to analyze with 1.5% agarose gel to confirm the RNA product.

Pause Point: Total RNA can be stored at −80°C for one month at this point.

3. Isolation of a^{6}A-labeled mRNA
   a. Use GenElute™ mRNA Miniprep Kit to isolate mRNA from total RNA.

Note: Preheat the Elution Solution at 70°C in a heating block.

i. Pipette 250 μL total RNA (200–300 μg) into RNase-free 1.5 mL microcentrifuge tube, add 250 μL of 2× Binding Solution, and vortex briefly to mix.
ii. Add 20 μL of oligo(dT) beads and vortex thoroughly to mix the contents.

Note: Increase volume to 25 μL of oligo(dT) when more than 300 μg total RNA is started.

iii. Incubate the mixture at 70°C for 3 min in a heating block to denature the RNA.
iv. Remove the sample from the heating block and allow it to stand at 20°C–30°C for 10 min.
v. Spin at 16,000×*g* for 2 min at 20°C–30°C, carefully remove the supernatant, leaving approximately 50 μL to avoid disturbing the white pellet containing oligo(dT) beads/mRNA complex.
vi. Resuspend the pellet in 500 μL Wash Solution, and use pipette to mix.
vii. Pipet all of the suspension into a GenElute spin filter/collection tube assembly.
viii. Spin at 16,000×*g* for 2 min at 20°C–30°C, and discard the flow-through.
ix. Pipet 500 μL Wash Solution onto the spin filter, and spin at 16,000×*g* for 2 min at 20°C–30°C.
x. Discard the flow-through and the original collection tube.
xi. Transfer the spin filter into a fresh collection tube, add 50 μL Elution Solution (70°C) into the center of the spin filter, and incubate for 5 min at 70°C.
xii. Spin at 16,000×*g* for 2 min at 20°C–30°C, and transfer the flow-through into a new 1.5 mL microcentrifuge tube to collect polyadenylated RNA.
xiii. Add another 30 μL Elution Solution (70°C) into the center of the spin filter, incubate for 5 min at 70°C, and then spin at 16,000×*g* for 2 min at 20°C–30°C. Afterwards, transfer the flow-through into the same 1.5 mL microcentrifuge tube.

Pause Point: The purified polyadenylated RNAs can be stored at −80°C for two weeks at this point.

Enrichment of a^{6}A-containing mRNA

Timing: 14 h for step 4

Timing: 10 min for step 5

Timing: 14 h for step 6

Timing: 10 h for step 7

Timing: 14 h for step 8

To increase the signal-to-noise ratio of the result, a specific antibody is selected to enrich the metabolically labeled a^6A-containing mRNAs. Isolated mRNA is fragmented into around 100–300 nt pieces, and an antibody which recognizes a^6A instead of m^6A and A, is applied to enrich a^6A-containing fragments. Afterwards, a^6A-containing mRNAs are eluted by competitive elution with a^6ATP, and purified for the next step.

⚠ CRITICAL: We use a commercial antibody which targets N^6-isopentenyladenosine (Cytokinin, Rabbit, IgG). Due to the geometrical similarity between isopentenyl and allyl, this antibody is also able to recognize a^6A but not m^6A or A (Shu et al., 2020).

4. Concentrating a^6A-labeled mRNA
   a. Transfer 200 μL mRNA (about 25 μg) products obtained from the above mRNA preparation kit into a 1.5 mL microcentrifuge tube, add 20 μL of 3 M sodium acetate solution (pH = 5.2), 220 μL of isopropanol, and no more than 2 μL glycogen. Then pipet to mix and incubate at −20°C about 12–16 h for precipitation.
   b. Centrifuge at 16,000×*g* for 45 min at 4°C.
   c. Discard the supernatant but not disturb the mRNA pellet, wash once with 440 μL 80% ethanol, and centrifuge at 16,000×*g* for 15 min at 4°C.
   d. Discard the supernatant again, and air dry the mRNA pellet for 5 min at 20°C–30°C.
   e. Dissolve mRNA with 36 μL RNase-free water to recover mRNA at a concentration of around 550 ng/μL.

Note: The recovery yield from isopropanol precipitation is about 80%. Add RNase-free water if needed to adjust the concentration of mRNA to be around 500–600 ng/μL, which is the best RNA concentration for our fragmentation by $ZnCl_2$. If the concentration is too high, there will be some larger size RNA remnants. If the concentration is too low, the size of fragments will be less than 150 bp.

5. Fragmentation of a^6A-labeled mRNA
   a. Add 4 μL 10 × fragmentation buffer to the above mRNA solution, pipet to mix, and incubate at 70°C for 7 min.
   b. Add 8 μL 0.5 M EDTA solution (pH = 8.0) to stop the reaction, and adjust the volume of mixture into 100 uL by RNase-free water.
6. Purification of mRNA fragments
   a. Add 1/10 volume (10 μL) of 3 M sodium acetate solution (pH = 5.2), equal volume (110 μL) of isopropanol, and no more than 1 μL glycogen. Then pipet to mix and incubate for precipitation about 12–16 h at −20°C.
   b. Centrifuge at 16,000×*g* for 45 min at 4°C.
   c. Discard the supernatant but not disturb the mRNA pellet, wash once with 220 μL 80% ethanol, and centrifuge at 16,000×*g* for 15 min at 4°C.
   d. Discard the supernatant again, and air dry mRNA pellet for 5 min at 20°C–30°C.
   e. Dissolve mRNA pellet with 100 μL RNase-free water, and 1 μL could be used to analyze with 1.5% agarose gel to confirm the size of RNA fragment.

Pause Point: Take out about 100 ng mRNA fragments to save as input for library construction at −80°C for one week. The rest are used for immunoprecipitation immediately or stored at −80°C for no longer than one week.

Note: The following steps are performed for antibody-immunoprecipitation from 5 μg mRNA fragments. If a larger quantity is used, amplify the system proportionally. The procedures are performed according to the protocol of m^6A-seq (Dominissini et al., 2013).

7. Antibody enrichment of a^6A-labeled mRNA fragments

a. Prepare 1.5 mL low-binding microcentrifuge tube, and transfer 5 μg mRNA fragments into it.
b. Prepare 5× IP buffer freshly before experiment and keep it on ice.
c. Mix the listed reagents as indicated below.

Reagent	Amount
5× IP buffer	80 μL
RNase inhibitor (40 Units/μL)	2 μL
anti-cytokinin | N^6-isopentenyladenosine (10 μg/μL)	1 μL
RNA fragments (200 ng/μL)	25 μL
RNase-free water	292 μL
Total	**400 μL**

d. Incubate with head-over-tail rotation (10 rpm, crystalindustries TR-02U model) for 4 h at 4°C.
e. Prepare another 1.5 mL low-binding microcentrifuge tube for ProteinA beads incubation.
f. Prepare 1× IP buffer just before use.

⚠ CRITICAL: Keep it on ice.

g. Transfer 40 μL ProteinA beads (Dynabeads™ Protein A for Immunoprecipitation) into the tube, and place the tube on a magnetic rack to separate beads from the supernatant. After the supernatant becomes clear (about 2 min later), carefully remove and discard it.
h. Resuspend the beads in 200 μL 1× IP buffer, pipet to mix and wash, and place the tube on a magnetic rack to separate beads from the supernatant. After the supernatant becomes clear (about 2 min later), carefully remove and discard it.
i. Repeat 2 times for the above wash step.
j. Add the following reagents into proteinA beads prepared above as indicated below.

Reagent	Amount
5× IP buffer	80 μL
BSA (20 mg/mL)	10 μL
RNase-free water	310 μL
Total	**400 μL**

k. Incubate with head-over-tail rotation for 2 h at 4°C.

⚠ CRITICAL: It should be properly scheduled to make sure that the antibody-RNA incubation and ProteinA beads blocking are finished at about the same time.

l. Spin down the tube gently and place it on a magnetic rack to separate beads from the supernatant (Figure 7A). After the supernatant becomes clear (about 2 min later at 20°C–30°C, Figure 7B), carefully remove and discard it.
m. Resuspend the beads in 200 μL 1× IP buffer, pipette to mix and wash, and place the tube on a magnetic rack to separate beads from the supernatant (Figure 7A). After the supernatant becomes clear (about 2 min later, Figure 7B), carefully remove and discard it.
n. Repeat 2 times for the above wash step.
o. Take off the tube for antibody-RNA incubation, spin down gently, and transfer all the solution into the tube containing ProteinA beads. Resuspend the beads, and pipet to mix.
p. Incubate with head-over-tail rotation for another 2 h at 4°C, in order to allow ProteinA beads to sufficiently capture the antibody-RNA complex.
q. Prepare IP wash solution freshly just before use and keep it on ice.

IP wash solution

Note: This should be prepared freshly, and do not prepare more than you need, as you only need 400 μL for each wash reaction.

Reagent	Amount
5× IP buffer	0.8 mL
RNase inhibitor (40 Units/μL)	20 μL
RNase-free water	3.2 mL
Total	**4 mL**

r. Prepare a^{6}A elution buffer freshly just before use and keep it on ice.

a^{6}A elution buffer

Note: This should be prepared freshly, and do not prepare more than you need, as you only need 200 μL for each elution reaction

Reagent	Final concentration	Amount
5 × IP buffer	1 ×	90 μL
N^6-allyl adenosine-5′-triphosphate (75 mM)	6.67 mM	40 μL
RNase inhibitor (40 Units/μL)	0.27 Units/μL	3 μL
RNase-free water	n/a	317 μL
Total	**n/a**	**450 μL**

s. When the incubation of antibody-RNA and proteinA beads are finished, spin down the mixture gently and place it on a magnetic rack to separate beads from the supernatant. After the supernatant is clear (about 2 min later), carefully remove and discard it.

Note: The supernatant could be saved as control for quantifying a^{6}A level compared with the enriched a^{6}A-containing RNA.

t. On the magnetic rack, add 400 μL IP wash solution to immerse the beads, wait for 30 s and discard the supernatant.
u. Repeat the wash step once.

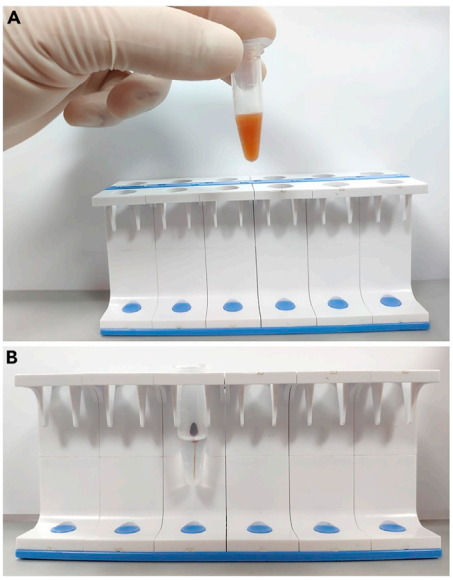

Figure 7. A separation process of proteinA beads and supernatant
(A and B) Images of the mixture before (A) and after (B) placed on a magnetic rack.

v. Resuspend proteinA beads bound with antibody-RNA complex in 100 μL a^6A elution buffer.

w. Incubate by head-over-tail rotation (10 rpm, crystalindustries TR-02U model) with occasional shaking for 3 h at 4°C to elute the a^6A-containing RNA from antibody-RNA complex.

x. Spin down the tube gently and place it on a magnetic rack to separate beads from the supernatant (Figure 7A). After the supernatant becomes clear (about 2 min later at 20°C–30°C, Figure 7B), carefully transfer it into a new 600 μL microcentrifuge tube.

8. Purification of enriched a^6A-labeled RNA

a. Add 1/10 volume (10 μL) of 3 M sodium acetate solution (pH = 5.2), equal volume (110 μL) of isopropanol, and 1 μL glycogen into the above tube. Then pipet to mix and incubate for precipitation about 12–16 h at −20°C.

b. Centrifuge at 16,000×*g* for 45 min at 4°C.

c. Discard the supernatant but not to disturb the mRNA pellet, wash once with 220 μL 80% ethanol, and centrifuge at 16,000×*g* for 15 min at 4°C.

d. Discard the supernatant again, and air dry RNA pellet for 5 min at 20°C–30°C.

e. Dissolve RNA pellet (around 40 ng from 5 μg mRNA fragments) with 10–20 μL RNase-free water.

Pause Point: The obtained a^6A-containing RNA can be directly used for next step or stored at −80°C for three days.

Chemical treatment of a^6A-containing mRNA

Timing: 1 h for step 9

Timing: 14 h for step 10

In our previous work, we found that the iodination of N^6-allyl group of a^6A under alkaline conditions spontaneously induces the formation of 1, N^6-cyclized adenosine (cycA), which generates base misincorporation at its opposite site during complementary DNA (cDNA) synthesis via reverse transcription (Shu et al., 2017). Therefore, the above a^6A-enriched mRNAs are treated with iodine for iodination reaction, and purified for the next step.

9. Chemical treatment of enriched a^6A-labeled RNA

a. Transfer around 80 ng a^6A-enriched RNA into a 200 μL PCR tube, and add RNase-free water to a final volume of 26 μL.

b. Prepare iodination solution.

c. Add 4 μL iodination solution and pipet to mix. The solution turns brown (Figure 8A) and is incubated at 37°C for 30 min.

d. Transfer the solution into a new tube (Figure 8B), add sodium thiosulfate solution ($Na_2S_2O_3$, 0.2 M, RNase-free water), and pipet to mix until the solution turns colorless and clear (around 2–4 μL 0.2 M $Na_2S_2O_3$ added, Figure 8C).

e. Add 6 μL sodium carbonate solution (Na_2CO_3, 0.1 M, pH = 9.5), pipet to mix, and incubate at 37°C for 30 min.

Note: Sodium bicarbonate solution is obtained by adjusting the pH of sodium carbonate and hydrochloric acid.

10. Purification of iodinated a^6A-labeled RNA

a. Transfer the reaction mixture into a 600 μL microcentrifuge tube, and add equal volume (40 μL) of isopropanol and 1 μL glycogen. Then pipet to mix and incubate for precipitation for 12–16 h at −20°C.

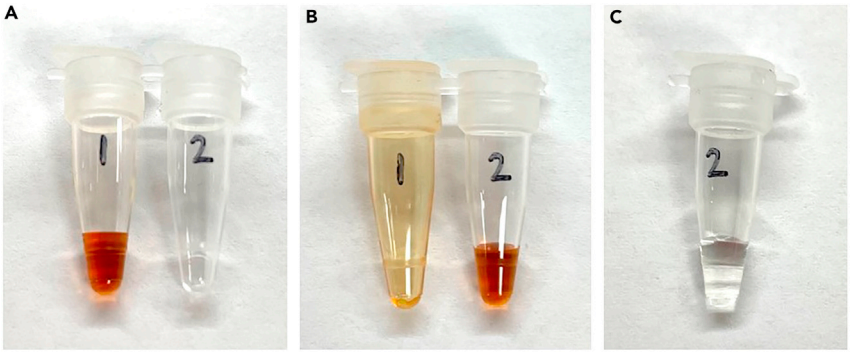

Figure 8. An iodination reaction process of a^{6}A-enriched RNA

(A–C) (A) The solution turns brown in color after addition of iodine; (B) The solution has been transferred into a new tube; (C) The solution turns colorless and clear after addition of sodium thiosulfate.

⚠ CRITICAL: Do not add acidic sodium acetate because the chemical reactions occur under alkaline conditions.

b. Centrifuge at 16,000×*g* for 45 min at 4°C.
c. Discard the supernatant but not to disturb the mRNA pellet, wash once with 80 μL 80% ethanol, and centrifuge at 16,000×*g* for 15 min at 4°C.
d. Discard the supernatant, and air dry RNA pellet for 5 min at 20°C–30°C.
e. Dissolve cycA-containing RNA pellet with 10 μL RNase-free water, and use it directly for next step or store it at −80°C.

Pause Point: The obtained cycA-containing RNA can be stored at −80°C for three days.

Library preparation, next-generation sequencing, and data analysis

Timing: 1 day for step 11

Timing: 3 days for step 12

Library preparations are conducted following the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina E7760 E7765 manual, section 5, except the step of first strand cDNA synthesis.

⚠ CRITICAL: There are three types of RNA samples which all have been fragmented:

RNA fragments without antibody immunoprecipitation, named input-RNA;
RNA fragments enriched by antibody immunoprecipitation, named IP-RNA;
RNA fragments enriched by antibody immunoprecipitation and subjected to chemical treatments as above, named cyc-RNA.

During library construction, different reverse transcription (RT) enzymes are used for different purposes. For the sample of IP-RNA, RevertAid RT enzyme is used in order to improve the overall read-through rate and thus enhance the a^{6}A-IP peak calling. For the sample of cyc-RNA, HIV is used because it can induce base misincorporation at opposite site of cyc-A during cDNA synthesis.

11. Reverse transcription of a^{6}A-labeled RNA and cDNA library construction
    a. Transfer the RNA samples (10–50 ng) into new PCR tubes.
    b. Priming of the RNA samples following the protocol.

Priming reaction	Volume
Fragmentated RNA (10–50 ng)	5 μL
Random Primers	1 μL
Total	6 μL

c. Mix thoroughly by pipetting up and down several times and briefly spin down.
d. Incubate the sample in a preheated thermal cycler at 65°C for 5 min and then stay at 4°C with lid setting to 105°C.
e. Mix reagents in the tube for Synthesizing First Strand cDNA as indicated below.
   i. Input- and IP-RNA samples.

First strand synthesis reaction	Amount
Primed input-RNA/IP-RNA	6 μL
NEBNext First Strand Synthesis Reaction Buffer (5X)	4μL
NEBNext Strand Specificity Reagent	8 μL
RevertAid reverse transcriptase (200 Unites/μL)	1 μL
5× RT buffer (Thermo)	5 μL
RNase-free water	1 μL
Total	25 μL

   ii. Cyc-RNA sample.

First strand synthesis reaction	Amount
Primed cyc-RNA	6 μL
NEBNext First Strand Synthesis Reaction Buffer (5X)	4μL
NEBNext Strand Specificity Reagent	8 μL
Recombinant HIV (20 Unites/μL)	0.5 μL
5× RT buffer (Thermo)	5 μL
RNase-free water	1.5 μL
Total	25 μL

f. Mix thoroughly by pipetting up and down several times.
g. Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 100°C as follows.
   i. Input- and IP-RNA samples.

Step 1	10 min at 25°C
step 2	60 min at 42°C
step 3	15 min at 70°C
step 4	Hold at 4°C

   ii. Cyc-RNA sample.

Step 1	10 min at 25°C
step 2	60 min at 37°C
step 3	15 min at 70°C
step 4	Hold at 4°C

h. Follow the protocol for Second Strand cDNA Synthesis and other continuing steps to complete the library construction.
i. All libraries are analyzed with Agilent 2100 bioanalyzer and sequenced by Hiseq X10 in paired-end mode with read length of 150 bp using primers listed below.

Sequencing primer	Sequences
Read1	5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′
Read2	5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′

12. Bioinformatics analysis of sequencing data
    a. Raw data are quality controlled with FastQC (version: v0.11.8).
    Command: fastqc *gz -o ./fastqc/ -t 8.
    b. Adapters are trimmed by fastp (version: 0.19.7).
    Command: fastp -x –detect_adapter_for_pe -l 25 -i R1.fastq.gz -I R2.fastq.gz -o ./fastp/R1.trim.fastq.gz -O ./fastp/R2.trim.fastq.gz -h fastp.html -j fastp.json.
    c. Clean data are mapped to corresponding genomes (hg38 for human and mm10 for mouse) with HISAT2 (version: 2.1.0).
    Command: hisat2 -x index_flie_path/index -p 10 –rna-strandness RF –no-unal –no-mixed –no-discordant -t -1 R1.trim.fastq.gz -2 R2.trim.fastq.gz -S sample.sam –summary-file sample-align-summary.
    d. Unique mapped data are extracted by samtools (version: 1.9).
    Command: samtools view -S -h -q 30 -f 2 sample.sam > sample.unique.sam.
    e. Unique mapped Sam files are converted and sorted into bam files by Samtools.
    Command: samtools sort -@ 4 sample_unique.sam -o sample_unique.bam.
    f. For a^6A-peak calling, the commonly used software such as MACS, exomePeak and MeTPeak can be used following their pipelines. We use exomepeak for peak calling, details can be found from public resources (https://github.com/ZW-xjtlu/exomePeak).
    g. For cycA-induced mutation analysis, the commonly used tools such as GATK and Samtools can be used following corresponding guidance. We use samtools for mutation calling.
    Command: samtools mpileup -BQ0 -d 10000000 -o output.pileup.xls -f ref.fa sample.bam.

EXPECTED OUTCOMES

We used a concentration of 1 mM *Se*-allyl-L-selenohomocysteine to feed cells, and obtained labeled mRNAs with a^6A/A levels of 0.005%–0.015% in different cell lines. mRNAs were fragmented into 100–300 nucleotides in length. Starting from 5 μg of mRNA fragments as input, about 40 ng of a^6A-containing RNAs could be immunoprecipitated, with an enrichment fold of 10–25 quantified by a^6A/A level. For the iodination treatment, the recovery yield is approximately 80%, and thus more than 20 ng of a^6A-mRNA is suggested. For cDNA library construction, the starting amount of RNA was 10–50 ng of each sample. No less than 15 ng of DNA libraries were used for high-throughput sequencing. For each m^6A-label-seq library, we aim to obtain at least 100 million reads for human cells and 70 million reads for mouse cells. The high-throughput sequencing results of a^6A-RNA immunoprecipitation yielded thousands of peaks located on polyadenylated RNA targets in HeLa, HEK293T and H2.35 cells, and the mutation analysis identified a few to more than ten thousand m^6A sites depending on the fold change cutoff of the mutation rate (for example, we used fold change > 3) between labeled and control samples.

LIMITATIONS

m^6A-label-seq is an in vivo metabolic labeling method, and shows major limitations on the RNA labeling yield and methionine analog-induced moderate cellular stress. To overcome the limitation of low labeling yield, antibody immunoprecipitation technology is used to specifically select the a^6A-labeled mRNA. However, immunoprecipitation requires a relatively large quantity of RNA input materials. One future solution can be engineering the methionine adenosyl methyltransferase or m^6A methyltransferases to better fit and accommodate the allyl group and thus increase the labeling yield. Alternatively, a method with low input or free of immunoprecipitation needs to be developed. We are working along this line. To reduce cellular stress and extend the application to tissue labeling, optimum metabolic labeling conditions need to be screened. Finally, the synthesis of amino acid precursor and adenosine-5′-triphosphate analogs is time-consuming, and simple and efficient methods are greatly appreciated.

TROUBLESHOOTING

Problem 1

The *Se*-allyl-L-selenohomocysteine shows poor water solubility (step 1.a).

Potential solution

Every time using the stock solution of 50 mM *Se*-allyl-L-selenohomocysteine, please vortex the tube thoroughly until the solution becomes clear without precipitation.

Problem 2

In the synthesis of L-selenohomocystine, selenium will slowly sublime when the solution is heated to reflux at 80°C (before you begin 1.b).

Potential solution

Fill cotton in the neck of round-bottom flask containing reaction mixture in order to prevent the selenium sublimation.

⚠ CRITICAL: The sublimate selenium has a strong smell of rotten eggs, please be careful and wear protective equipment.

Problem 3

During RNA precipitation, an excess or insufficient amount of glycogen is used (steps 4.a and 6.a).

Potential solution

If an excess amount of glycogen is used, the glycogen may affect the IP process or iodination step. If insufficient amount of glycogen is used, the yield of RNA product will be low.

Problem 4

During chemical treatment of a^6A-containing mRNA, the color of solution is not brown, but tends to be yellow after adding iodine (step 9.c and Figure 8).

Potential solution

Reprepare fresh Iodination solution, and no more than 4 μL in volume is gradually added until the solution turns brown.

Problem 5

The sublimation of iodine solution will make the walls of PCR tubes turn brown, and the color change of the reaction mixture is hardly observed (step 9.d and Figure 8).

Potential solution

Remember to transfer the solution of iodination reaction into a new PCR tube before adding sodium thiosulfate.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jianzhao Liu (liujz@zju.edu.cn).

Materials availability

Please contact Prof. Liu (liujz@zju.edu.cn) for the requests of N^6-allyladenosine (a^6A), N^6-allyladenosine-5′-triphosphate (a^6ATP) and *Se*-allyl-L-selenohomocysteine.

Data and code availability

The published article (Shu et al., 2020) includes all datasets generated and analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.S., J.C. and J.L.; Methodology, X.S. and J.C.; Investigation, X.S. and J.C.; Writing – Original Draft, X.S. and J.C.; Writing – Review & Editing, X.S and J.L.; Funding Acquisition, J.L.; Resources, J.L.; Supervision, J.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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