Supporting Information

Selective and Sensitive Fluorescence "Turn-On" Detection of 4-Thiouridine in Nucleic Acids via Oxidative Amination

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Materials and Instruments

Chemicals

All chemicals were used as received without further purification unless specified. Ethylenediamine tetraacetic acid disodium salt (EDTA-2Na), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium chloride (NaCl), potassium hydroxide (KOH), perchloric acid ($HClO_4$), glacial acetic acid (HAc), ammonia solution, 95% ethanol, chloroform, methanol and isopropanol were purchased from Beijing Chemical Works (Beijing, China). Sodium periodate ($NaIO_4$), m-chloroperbenzoic acid (mCPBA) and methyl tertiary butyl ether (MTBE) were purchased from Aladdin (Shanghai, China). Ammonium acetate (NH_4Ac) was purchased from Macklin (Shanghai, China). Dimethylformamide (DMF), urea and tri(hydroxymethyl)aminomethane (Tris) were purchased from Amresco (Solon, USA). 4-thiouridine (s^4U) was purchased from Sigma-Aldrich (St. Louis, USA). Adenosine, guanosine, cytidine and uridine were purchased from Alfa Aesar (Shanghai, China). Fluorescein (FAM) and fluoresceinamine (FAM-NH_2) were purchased from Adamas-beta (Shanghai, China) and Heowns (Tianjin, China) respectively. Cysteine (Cys), 3 M NaAc-HAc buffer (pH 5.2), 30% PAGE pre-solution (29:1), ampicillin, LB medium powder and DEPC-treated water were purchased from Solarbio (Beijing, China). 10000×SYBR Gold and 10×TBE were purchased from Biolite (Tianjin, China) and Leagene (Beijing, China) respectively. Gradient grade acetonitrile was purchased from Hipure Chem (Elmsford, USA).

Model nucleic acids, tRNA samples, proteins and other materials

U-17nt DNA:
TCACGCT/{deoxyuridine}/TCACCGTGG
s^2U-17nt DNA:
TCACGCT/{2-Thiothymidine}/TCACCGTGG
s^4U-17nt DNA:
TCACGCT /{4-Thio-2'-deoxyuridine}/ TCACCGTGG
s^4U-60nt DNA:
CTGGCTATCTAACGCTTATAGACTGGTACA/{4-Thio-2'-deoxyuridine}/CAGATAACCCTCAAGGATCGATTGTGAAG

U-17nt DNA were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China) and Tsingke Biotech Co., Ltd. (Beijing, China). The other model nucleic acids were synthesized and purified by Trilink Biotech Co., Ltd. (San Diego, USA).

Transfer ribonucleic acid (tRNA) from *E. coli* MRE 600 was purchased from Roche (Mannheim, Germany). tRNA from brewer's yeast (10 mg/mL) was purchased from Ambion (Carlsbad, USA). tRNA from wheat

germ and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). Calf intestinal alkaline phosphatase was purchased from NEB (Ipswich, USA).

Amicon-3k and Amicon-10k centrifugal filters were purchased from Millipore (Billerica, USA). Borosilicate glass culture tube were purchased from VWR (West Chester, USA). TRIzol™ Max™ Bacterial RNA Isolation Kit was purchased from Ambion (Carlsbad, USA).

Instruments

The reaction and digestion of tRNAs were carried on a ProFlex™ Base PCR system (Singapore). All absorbance spectra were measured on a JASCO V-550 UV-visible spectrometer (Tokyo, Japan), and all fluorescence spectra were recorded on a JASCO FP-8600 fluorescence spectrometer (Tokyo, Japan). All the gels were imaged on a Bio-Rad ChemiDoc™ XRS+ system. The NMR spectra was recorded on a JOEL JNM-ECA400 spectrometer (Tokyo, Japan). Mass spectra of model nucleic acids was obtained on a SHIMADZU MALDI-TOF mass spectrometer (Kyoto, Japan). All the HPLC analysis were conducted on a JASCO LC-Net II/ADC series equipped with a UV-2070 UV/Vis detector and a FP-2020 fluorescence detector, and the separation was using an Inertsil ODS-SP 250×4.6 column.

Experimental Section

Fluorescence Derivatization of s^4U in Nucleic Acids Using Oxidative Amination Reaction by FAM-NH_2 and IO_4^-

To unfold their folded structures, the nucleic acids especially tRNA solutions were heated to 95 ℃ for 5 min before mixed with the reactants, and immediately placed on ice for 5 min. Solutions of 5 μM model nucleic acids or 250 μg/mL tRNA samples were incubated with 1 mM EDTA, 10 mM $NaIO_4$, 250 mM FAM-NH_2, 100 mM NaAc-HAc buffer (pH 5.2) and 50% DMF at 80 ℃ for 3 h. After the reaction, 1/10 volume of 3 M NaAc-HAc buffer (pH 5.2) and 3 volumes of 95% ethanol were added to the reaction mixture. Then, the mixture was placed at −80 ℃ for 3 h. The nucleic acids were precipitated by centrifugation at 12000 rpm at −8 ℃ for 30 min and the precipitates were washed with 1 mL pre-chilled 75% ethanol twice and re-dissolved in 0.1 M sodium phosphate buffer (pH 8.0). After that, the solutions were purified by ultrafiltration to remove the co-precipitated $NaIO_4$. The solutions were washed by 0.1 M sodium phosphate buffer (pH 8.0) for eight times before PAGE and optical analysis (or washed with Millipore water to remove salts for MALDI-TOF MS analysis).

Fluorescence Measurement of FAM-NH_2-derived Nucleic Acids

Solutions of 200 μL nucleic acids (after the above oxidative amination reaction) in 0.1 M sodium phosphate buffer (pH 8.0) were used to measure the fluorescence spectra. Excitation was performed at 490 nm. The band width of excitation and emission was 5 nm. The concentration of nucleic acids was determined by its UV absorbance at 260 nm.

PAGE Analysis of FAM-NH_2-derived Nucleic Acids

For denaturing PAGE analysis, 10 pmol FAM-NH_2-derived model nucleic acids or 250 ng derived tRNAs were mixed with equal volume of 8 M urea, and the mixture were heated to 95 ℃ for 2 min and immediately placed on ice for 2 min. Then, the 17 nt model nucleic acids were loaded on 20% denaturing PAGE, and the 60 nt model nucleic acids and tRNA samples were loaded on 10% denaturing PAGE for electrophoresis. The gels were imaged using the SYBR Green image mode. The gels were then stained by SYBR Gold in 1×TBE for 8 min and imaged using SYBR Gold image mode.

Synthesis of FAM-C

The reagent mCPBA 102.98 mg (450 μmol) was added to a solution of 39.54 mg (150 μmol) s^4U nucleotide and 163.43 mg (450 μmol) FAM-NH_2 dissolved in 30 mL ethanol. The reaction mixture was stirred at 45 °C

for 5 h in the dark. The reaction was monitored by TLC with $CHCl_3$: MeOH : HAc = 100:50:1 (v:v:v). The reaction mixture was recrystallized in MTBE for three times. FAM-C was obtained as an orange solid (65.51 mg, yield 75.2%) from MeOH solution by drying in vacuo for 1 h. ^{1}H NMR (400 MHz, MeOH-d_4), δ (ppm): 8.40 (m, 2H), 8.14 (s, 1H), 7.33 (d, J = 8.2 Hz , 1H), 7.15 (d, J = 8.9 Hz , 2H), 7.02 (d, J = 1.7 Hz, 2H), 6.87 (d, J = 8.7 Hz, 2H), 6.29 (d, J = 7.3 Hz, 1H), 5.88 (d, J = 2.9 Hz, 1H), 4.21 (m, 1H), 4.17 (m, 1H), 4.05 (m, 1H), 3.92 (dd, J = 12.3, 2.1 Hz, 1H) 3.77 (dd, J = 12.3, 2.6 Hz, 1H). ^{13}C-NMR (100 MHz, MeOH-d_4), δ (ppm): 167.29, 166.66, 161.16, 156.67, 153.67, 147.56, 143.32, 139.17, 131.90, 131.28, 130.45, 128.69, 127.43, 122.40, 116.83, 114.05, 102.15, 95.31, 91.14, 84.79, 75.08, 69.14, 63.74, 60.28, 14.06. HR ESI: calc. for $C_{29}H_{23}O_{10}N_3$, $[M+H]^+$ 574.1462, found 574.1483; $[M-H]^-$ 572.1305, found 572.1292.

Relative Fluorescence Quantum Yield of FAM-C

About 2 μM FAM, FAM-C and FAM-NH_2 were prepared in 10 mM sodium phosphate buffer (pH 7.3) and placed in a 1 cm cuvettes. The absorbance spectrum was recorded from 800 nm to 220 nm. Making sure that the absorbance of each solution at 450 nm is below 0.05, the emission spectrum from 470 nm to 650 nm was recorded with excitation at 450 nm. The band width of excitation and emission was 2.5 nm and 10 nm respectively. The relative fluorescence quantum yield was calculated as follow:

$$\Phi_u = \Phi_s \times \frac{F_u}{F_s} \times \frac{A_s}{A_u}$$

where Φ_s is the quantum yield of standard sample, F_s and F_u are integrated fluorescence intensity of standard sample and unknown samples, A_s and A_u are absorbance at 450 nm of standard sample and unknown samples. FAM in 10 mM sodium phosphate buffer (pH 7.3) was chosen as the standard (Φ = 0.85).

UV330 and UV260 Absorption Measurement of the s^{4}U Content in Commercial tRNA Samples

The 10 mg/mL commercial tRNA stock solutions were prepared with DEPC-treated water and diluted with 25 mM sodium phosphate buffer (pH 7.2) and 5 mM NaCl to give a 1.2 mg/mL solution. The samples were placed in a 1 cm cuvettes. The absorbance spectra were recorded from 400 nm to 310 nm. The solutions were then diluted to 75-fold with the same buffer, and the absorbance spectra were recorded from 400 nm to 260 nm.

HPLC Analysis of Digested tRNA

The tRNA samples were hydrolyzed with 1/10 volume of 3 M KOH at 37 ℃ for 24 h. The samples were neutralized with 1/10 volume of 3 M $HClO_4$, and the precipitated $KClO_4$ was removed by centrifugation. The

supernatants were collected and mixed with 1/10 volume of 1 M Tris-HAc (pH 8.8) and 10 U alkaline phosphatase. The samples were incubated at 37 ℃ for 16 h and then diluted to 4-fold with Millipore water. The proteins were removed by ultrafiltration. The standard sample was prepared by mixing adenosine, guanosine, cytidine, uridine and 4-thiouridine each at 40 μM in 25 mM Tris-HAc buffer (pH 8.8).

The digested tRNAs or standard sample were analyzed on a HPLC system equipped with a UV detector and a fluorescence detector. A C18 column (5 μM particle size, 120 Å pore size, 250 mm length, 4.6 mm inner diameter) was used at 35 ℃ with 5 mM NH_4Ac-NH_3 (pH 7.5) as eluent A and eluent A-acetonitrile 50:50 (v:v) as eluent B. The flow rate was 1.0 mL/min. The gradient elution procedure was as following: 0 min 100% eluent A, 3 min 100% eluent A, 10 min 96% eluent A, 25 min 80% eluent A, 30 min 75% eluent A, 40 min 75% eluent A, 45 min 0% elution A, 60 min 0% elution A, 65 min 100% elution A, 75 min 100% elution A.

Exposure of *E. coli* to UVA Irradiation

Overnight cultures of ampicillin-resistant *E. coli* were diluted to 100-fold in 40 mL LB cultures containing 50 μg/mL ampicillin and shaken at 37 ℃ until reaching the log phase. The bacteria were collected by centrifugation and resuspended in 50 mL physiological saline solution (0.15 M NaCl). The suspensions of bacteria were divided into two fractions. One fraction was kept in the dark with ice bath, and the other fraction was placed in four borosilicate glass culture tubes with ice bath and irradiated with UVA at the average fluence rate of ~33 W/m^2 for 40 min. The UVA irradiation was obtained from a 300 W xenon lamp with a 365 nm cut-off filter, and the fluence rate was measured at the surface of culture tube from various sides with a radiometer.

Extraction of Whole tRNA from *E. coli*

After the irradiation or dark, *E. coli* cultures were immediately harvested by centrifugation at 4 ℃. Whole tRNAs were extracted by a TRIzol™ Max™ Bacterial RNA Isolation Kit according to the manufacturer's protocol. To isolate the total tRNAs, about 200 μg total RNAs were loaded in each well on 10% denaturing PAGE and electrophoresed at 10 W for 40 min. The gels were stained by SYBR Gold in 1×TBE for 8 min and placed under 254 nm hand-held UV lamp to check the tRNA bands. The tRNA bands were excised and shredded in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA prepared with DEPC-treated water. The gel fragments were placed in 4 ℃ overnight and the total tRNAs were precipitated with three volume of 95%

ethanol and washed with 75% ethanol, and then dissolved in buffers or DEPC-treated water for subsequent analysis.

Additional Figures

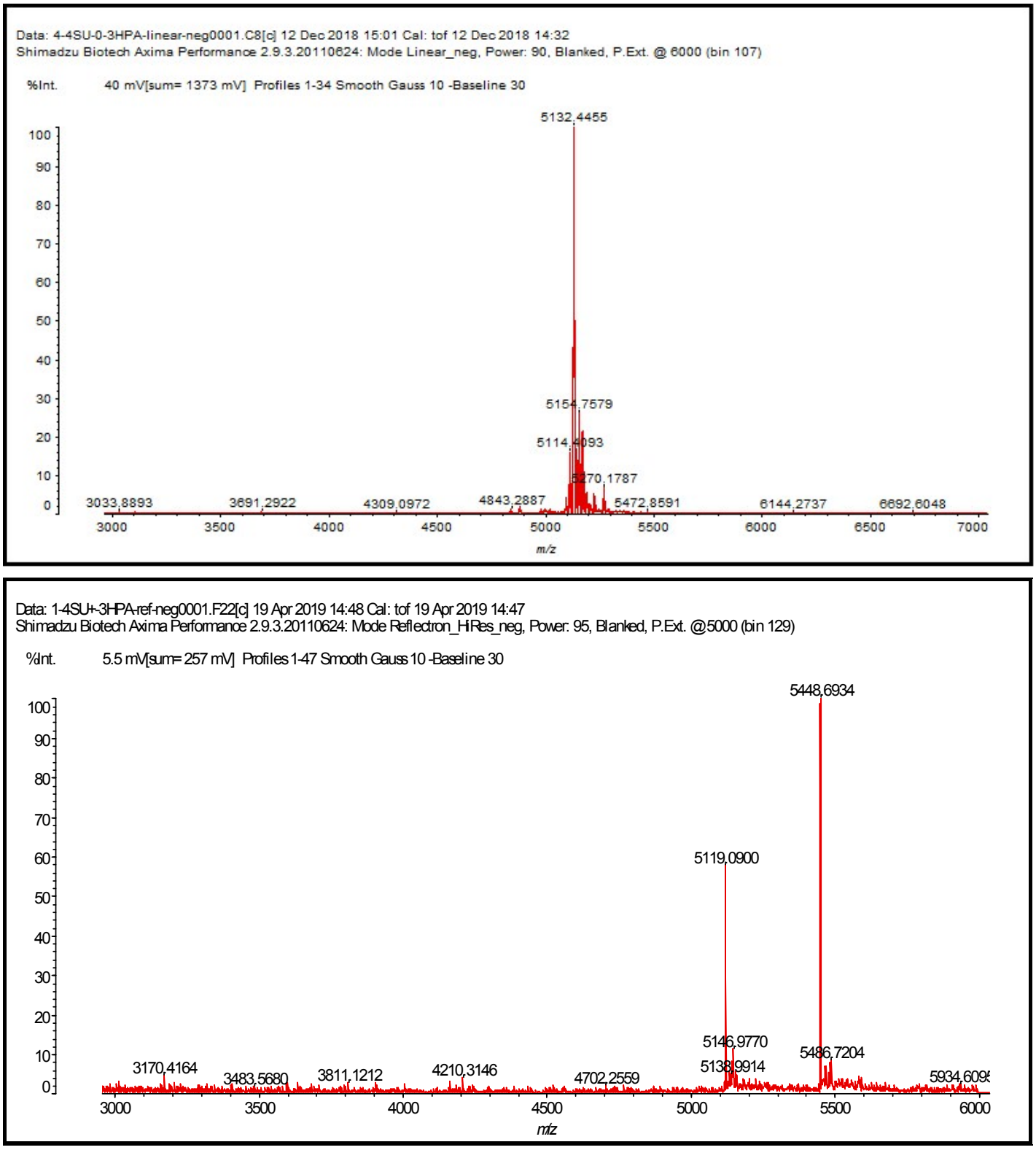

Figure S1. Enlarged MALDI-TOF MS of s^4U-17nt DNA (originally Figure 1a), before (top: calc. 5139, found 5132) and after (bottom: amination product FAM-C-17nt, calc. 5452, found 5449; hydrolysis product

U-17nt, calc. 5123, found 5119) the oxidative amination reaction. The yield of FAM-C-17nt formation from s^4U-17nt was found about 59%, according to the MS peak intensities.

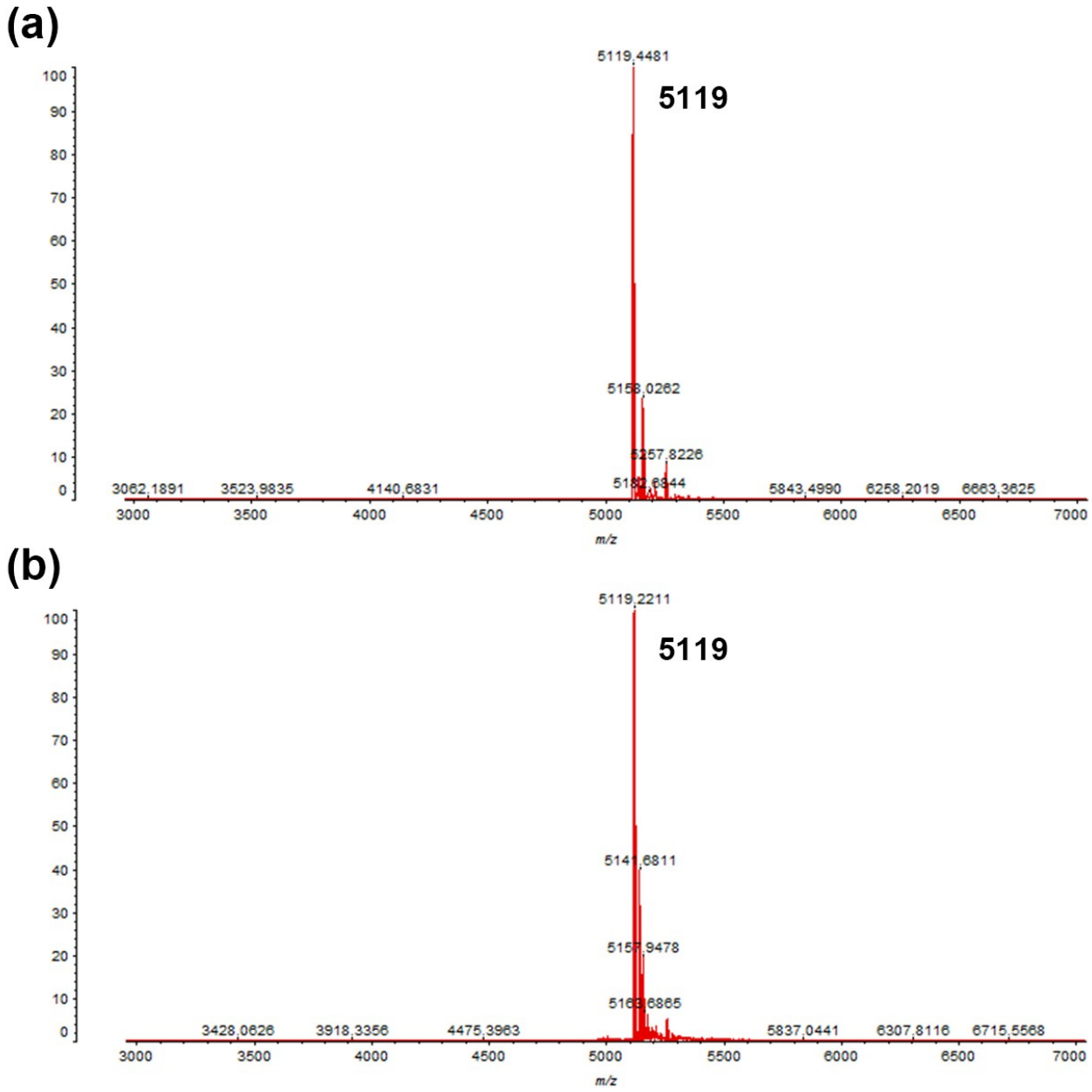

Figure S2. MALDI-TOF MS characterizations of U-17nt DNA before (top: calc. 5123, found 5119) and after (bottom: calc. 5123, found 5119) the oxidative amination reaction.

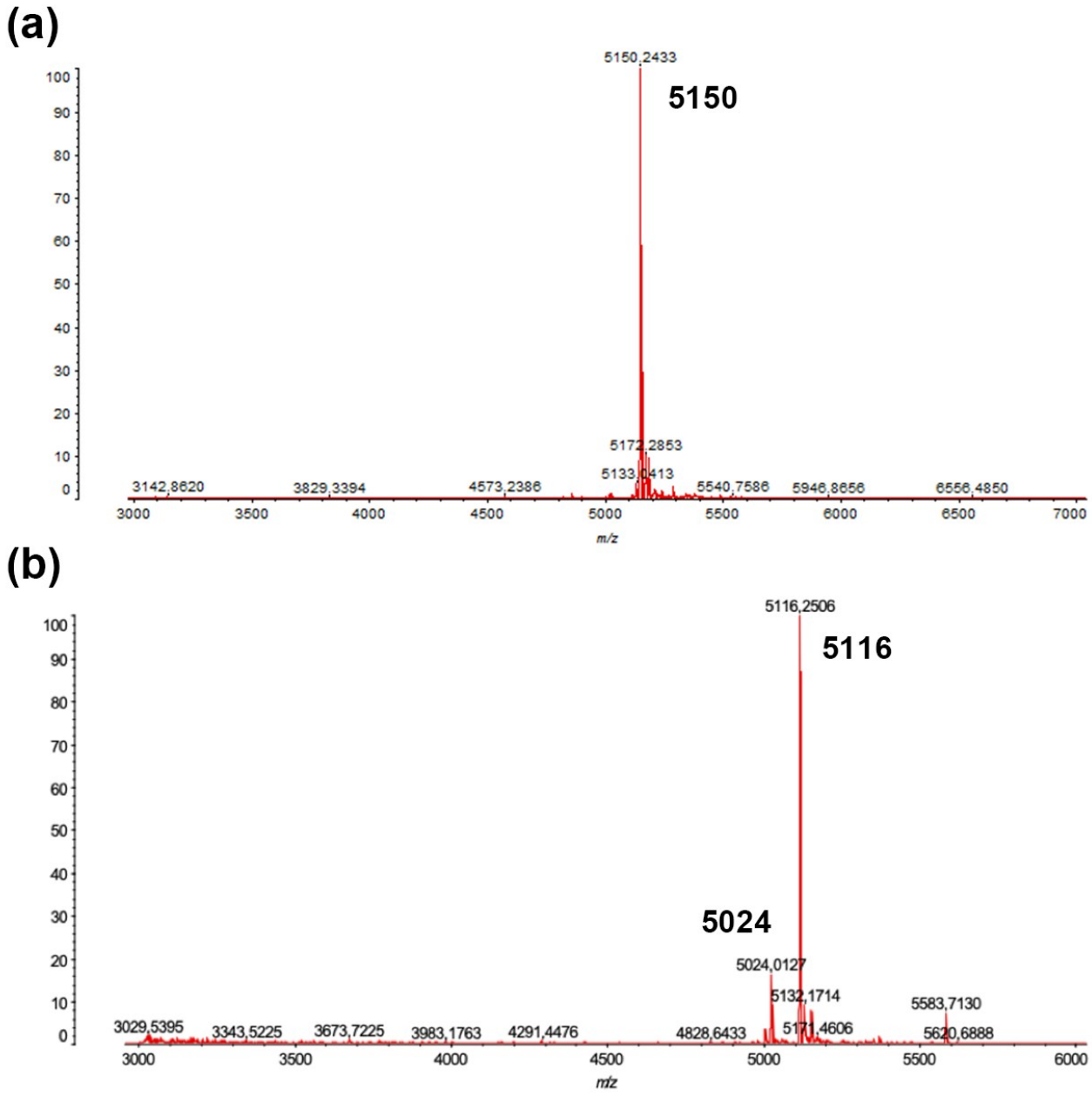

Figure S3. MALDI-TOF MS characterizations of s^2U-17nt DNA before (top: calc. 5153, found 5150) and after (bottom: amination product, calc. 5463, not found; desulfurization product, calc. 5122, found 5116; depyrimidine product, calc. 5029, found 5024) the oxidative amination reaction.

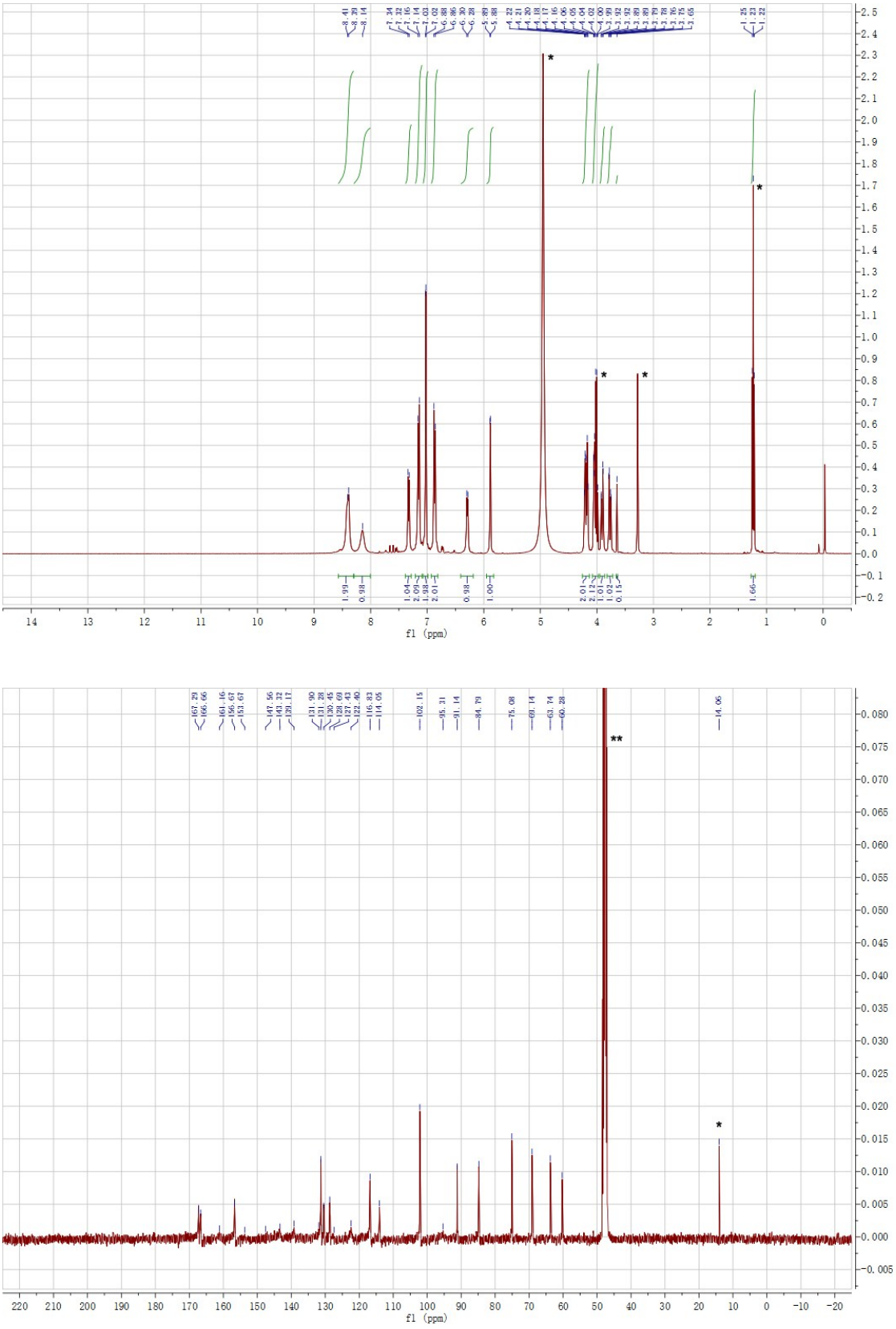

Figure S4. ^{1}H-NMR and ^{13}C-NMR (400 MHz, MeOH-d_4) characterizations of the product FAM-C yielded from the oxidative amination of s^4U nucleoside by FAM-NH_2 and mCPBA. The starred peaks are solvent

signals from trace CH_3OH, CH_3CH_2OH and H_2O. See the synthesis details in the experimental section for more details.

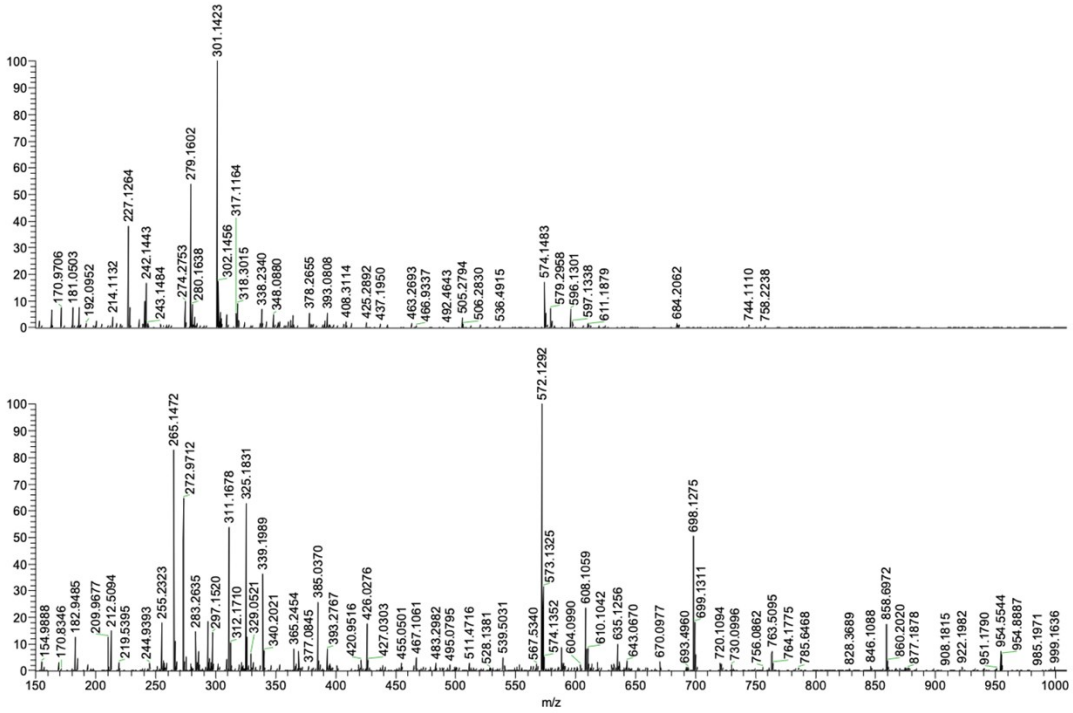

Figure S5. HR ESI-MS characterizations of the product FAM-C yielded from the oxidative amination of s^4U nucleoside by FAM-NH_2 and mCPBA. Positive mode (top): $[M+H]^+$ = 574.1483, calc. 574.1462. Negative mode (bottom): $[M-H]^+$ = 572.1292, calc. 572.1305.

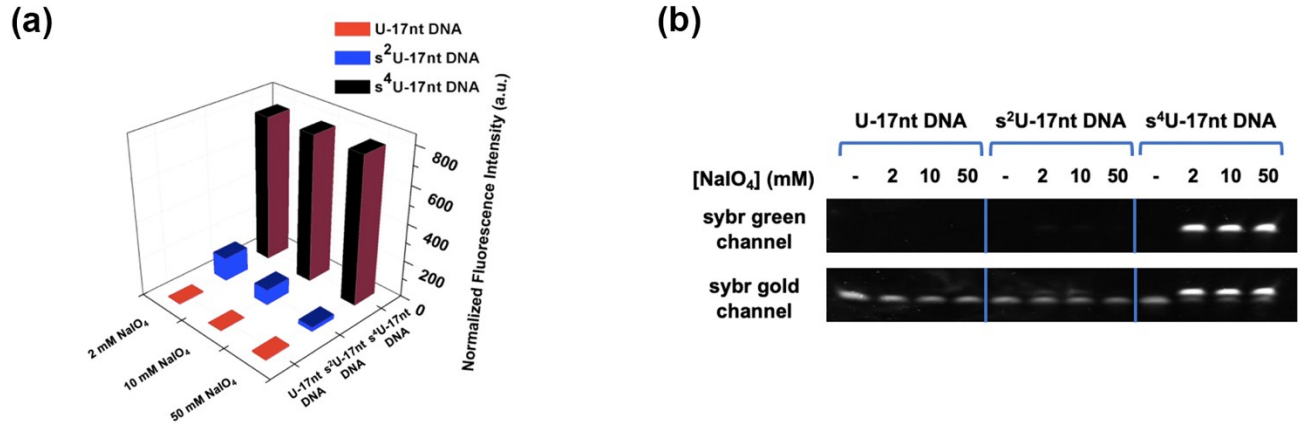

Figure S6. Higher concentration of IO_4^- contributed to reduce the fluorescence signal of s^2U-17nt DNA after the oxidative amination reaction at 45 °C for 14 h, without affecting the fluorescence signal of s^4U-17nt DNA

treated with same reaction condition. Fluorescence (a) and PAGE analysis (b) showed 50 mM IO_4^- promoted selectivity to s^4U over s^2U.

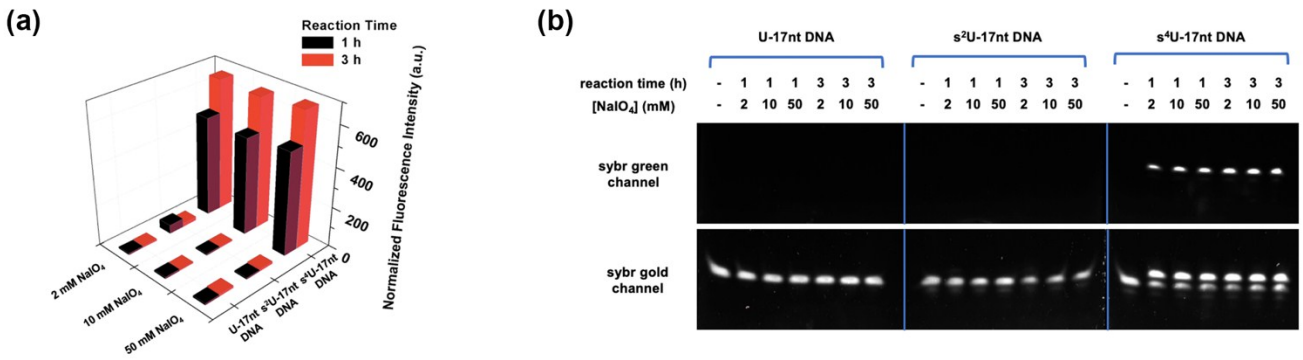

Figure S7. Higher reaction temperature and concentration of IO_4^- both contributed to reduce the fluorescence signal of s^2U-17nt DNA after the oxidative amination reaction at 80 °C for 3 h, without significantly affecting the fluorescence signal of s^4U-17nt DNA treated with same reaction condition. Fluorescence (a) and PAGE analysis (b) showed 10 mM IO_4^- was enough for s^2U-17nt DNA to avoid fluorescent derivatization.

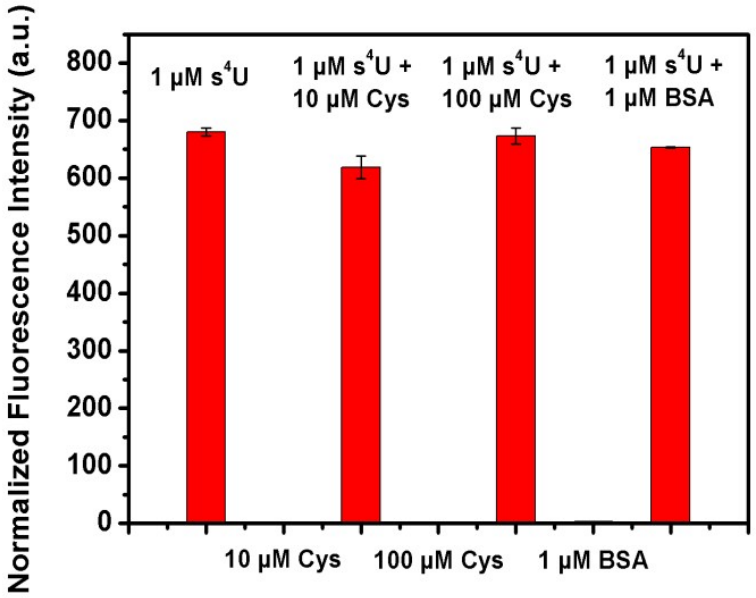

Figure S8. Effect of cysteine (Cys) or BSA on the fluorescence signal of s^4U-60nt DNA after the oxidative amination reaction. 10 μM Cys, 100 μM Cys or 1 μM BSA exhibited little fluorescence signal after the

derivatization reaction. When added to 1 μM s^4U and subjected to oxidative amination reaction, little influences on the fluorescence signal of s^4U-60nt DNA were observed from these biological thiol species.

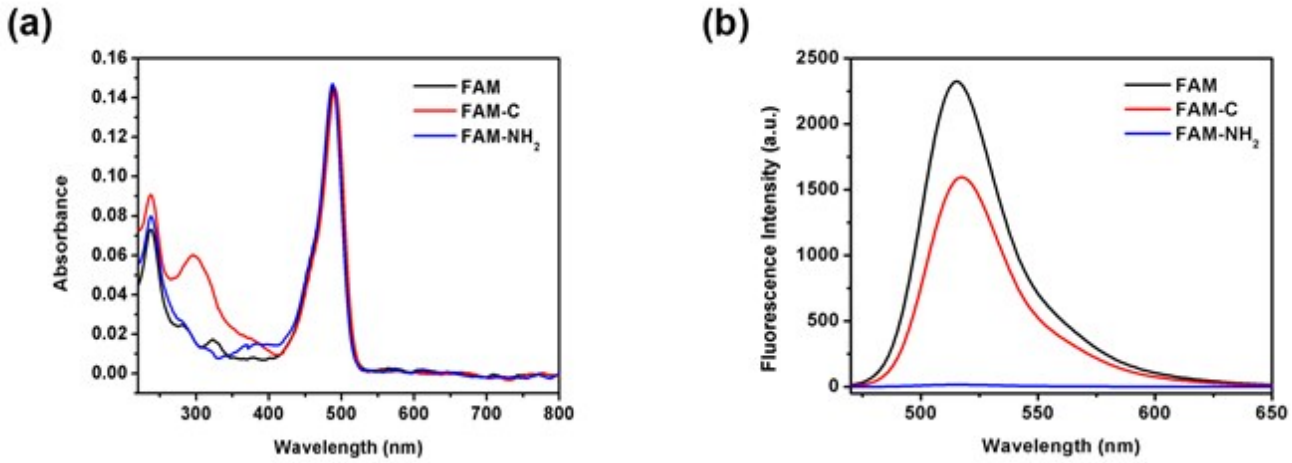

Figure S9. Absorption (a) and fluorescence spectra (b) of 2 μM fluorescein (FAM), FAM-C and $FAM\text{-}NH_2$. FAM-C showed strong fluorescence enhancement over $FAM\text{-}NH_2$.

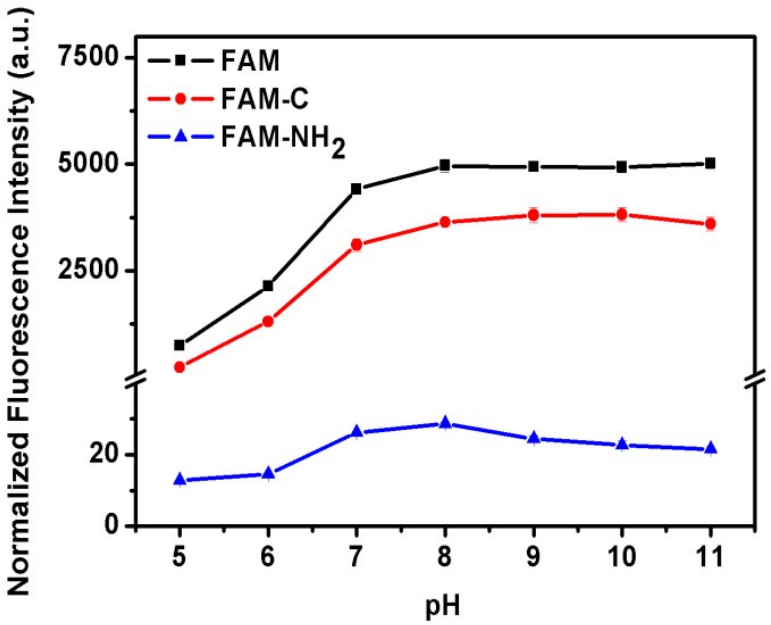

Figure S10. The pH-dependent fluorescence emission (λ_{ex} = 490 nm, λ_{em} = 520 nm) of both 1.1 μM FAM-NH2 and FAM-C in the range of pH 5~11 (100 mM sodium phosphate buffer). In any situation, FAM-C has

a much stronger (>100 fold) fluorescence than FAM-NH_2, which is in agreement with the quenching effect from photoinduced electron transfer (PET) for FAM-NH_2 but not for FAM-C.

Sample	Absorbance at 450 nm	Integrated Fluorescence Intensity	Fluorescence Quantum Yield
2 μM FAM	0.04122	73715.8225	0.85 (standard)
2 μM FAM-C	0.04060	50839.0472	0.60
2 μM FAM-NH_2	0.04696	536.06070	0.0054

Table S1. Relative fluorescence quantum yield of FAM-C and FAM-NH_2 calculated by formula mentioned in experimental section.

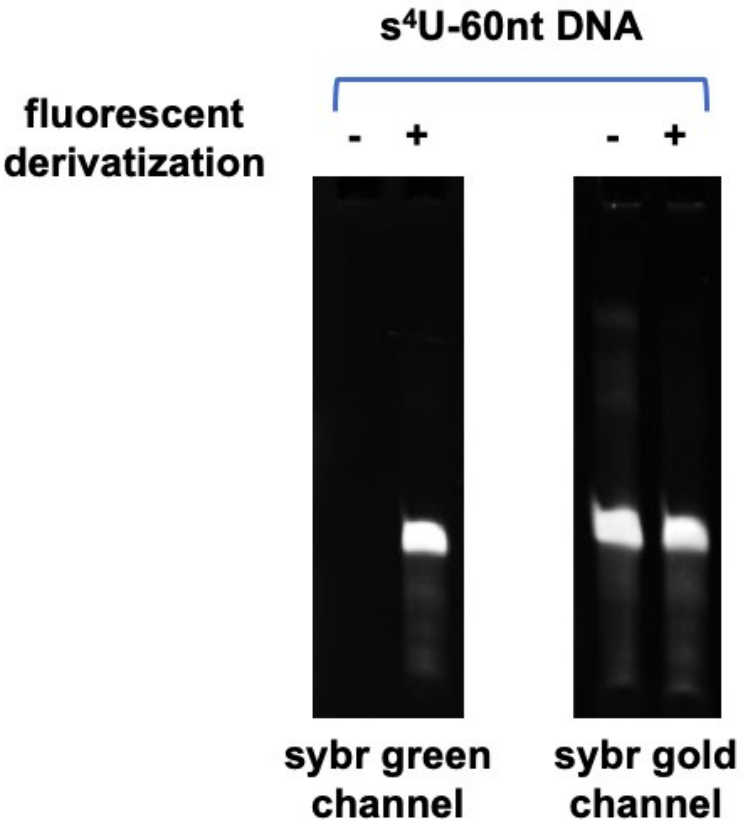

Figure S11. PAGE images of the s^4U-60nt DNA before and after oxidative amination reaction. *Left:* SYBR Green image mode without staining. *Right:* SYBR Gold channel with SYBR Gold staining.

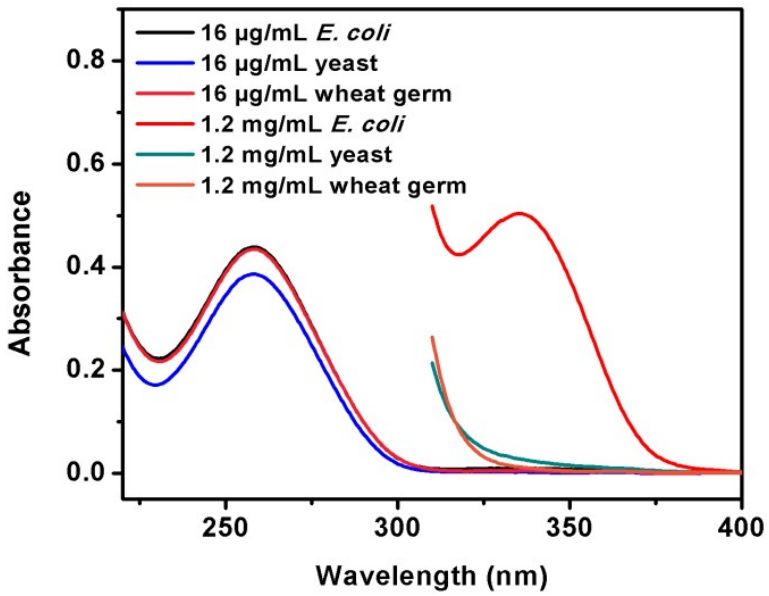

Figure S12. Absorption spectra of 16 μg/mL and 1.2 mg/mL different tRNA samples. *E. coli* tRNA exhibited an absorption peak near 330 nm belonging to s^4U.

Sample	Absorbance at 260 nm	Absorbance at 330 nm
E. coli tRNA	0.436 × 75 = 32.7	0.503
yeast tRNA	0.384 × 75 = 28.8	0.027
wheat germ tRNA	0.432 × 75 = 32.4	0.012

Table S2. The absorbance of 1.2 mg/mL *E. coli*, yeast and wheat germ tRNA samples at 260 nm and 330 nm.

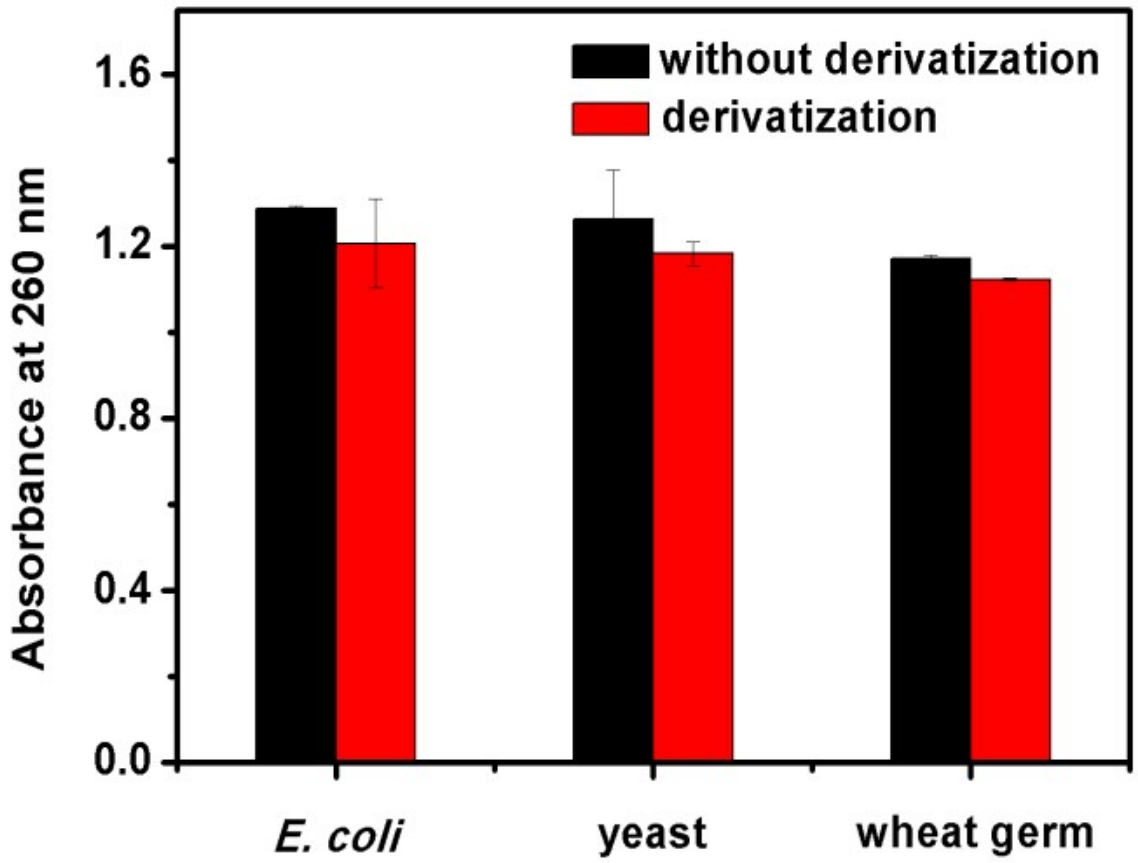

Figure S13. Stability of RNA samples during the oxidative amination reaction. Samples of ~10 μg tRNAs were each treated by the oxidative amination reaction, followed by ultrafiltration using Amicon-10k to remove small molecules including potential hydrolysis product, and then diluted to 200 μL before measured by UV absorption at 260 nm. Only little change in absorbance at 260 nm was observed for the RNA samples regardless of the oxidative amination derivatization, indicating the stability of RNA well maintained in the oxidative amination reaction. Good reservation of RNA stability upon oxidative amination was also supported by ref 14~16 in the main text.

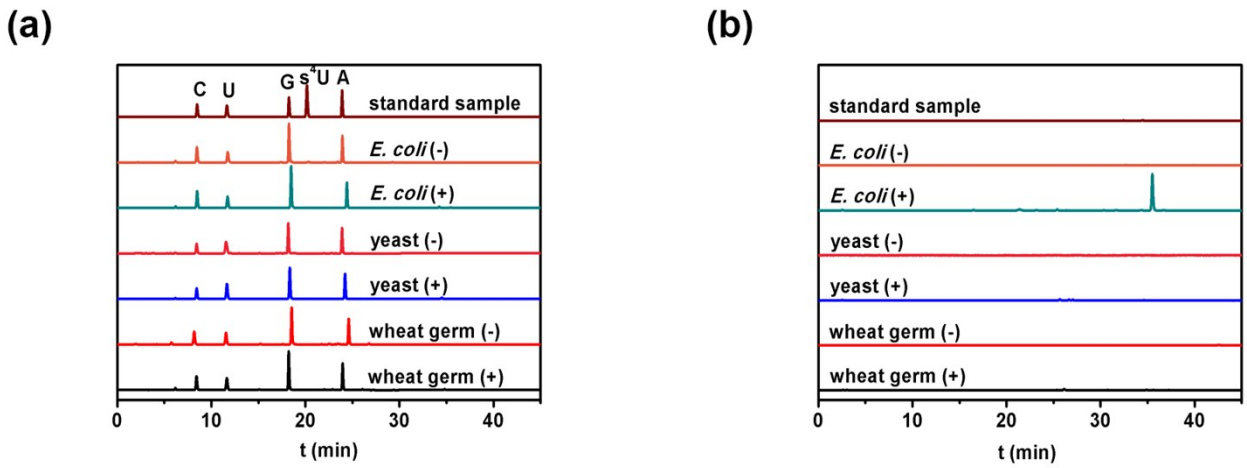

Figure S14. Fluorescence HPLC analysis of three digested tRNA samples before and after the oxidative amination reaction. The standard sample at the top consisted of adenosine, guanosine, cytidine, uridine and 4-thiouridine at each 40 μM. Total nucleosides (a) and FAM-NH_2-derived nucleosides (b) were monitored by UV detector (A_{260}) and fluorescence detector (λ_{ex} = 490 nm, λ_{em} = 520 nm), respectively.

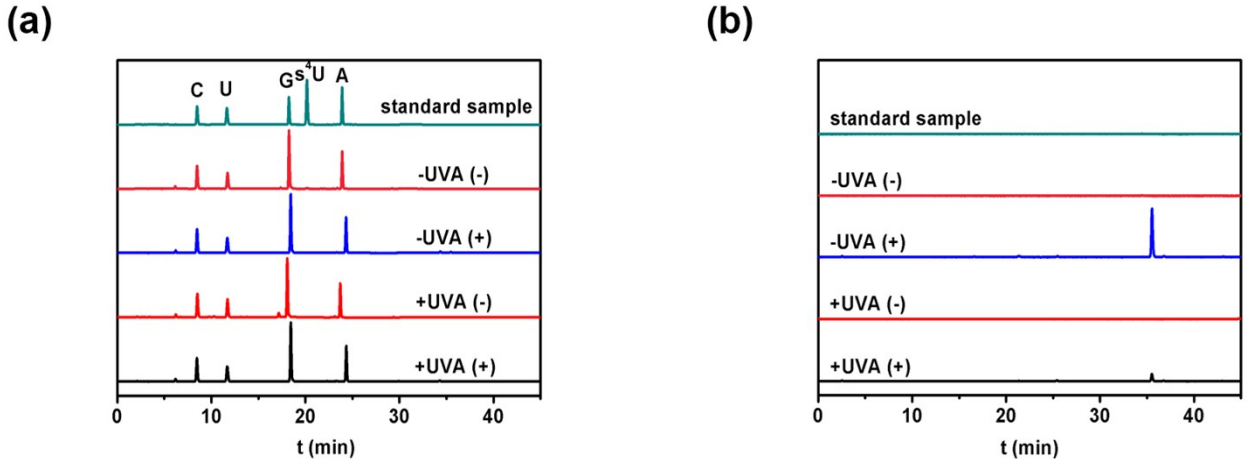

Figure S15. Fluorescence HPLC analysis of digested tRNA samples extracted from *E. coli* with or without UVA irradiation before and after derivatization reaction. Standard sample consisted of adenosine, guanosine, cytidine, uridine and 4-thiouridine each 40 μM. Total nucleosides (a) and FAM-NH_2-derived nucleosides (b) were monitored by UV detector (A_{260}) and fluorescence detector (λ_{ex} = 490 nm, λ_{em} = 520 nm), respectively.

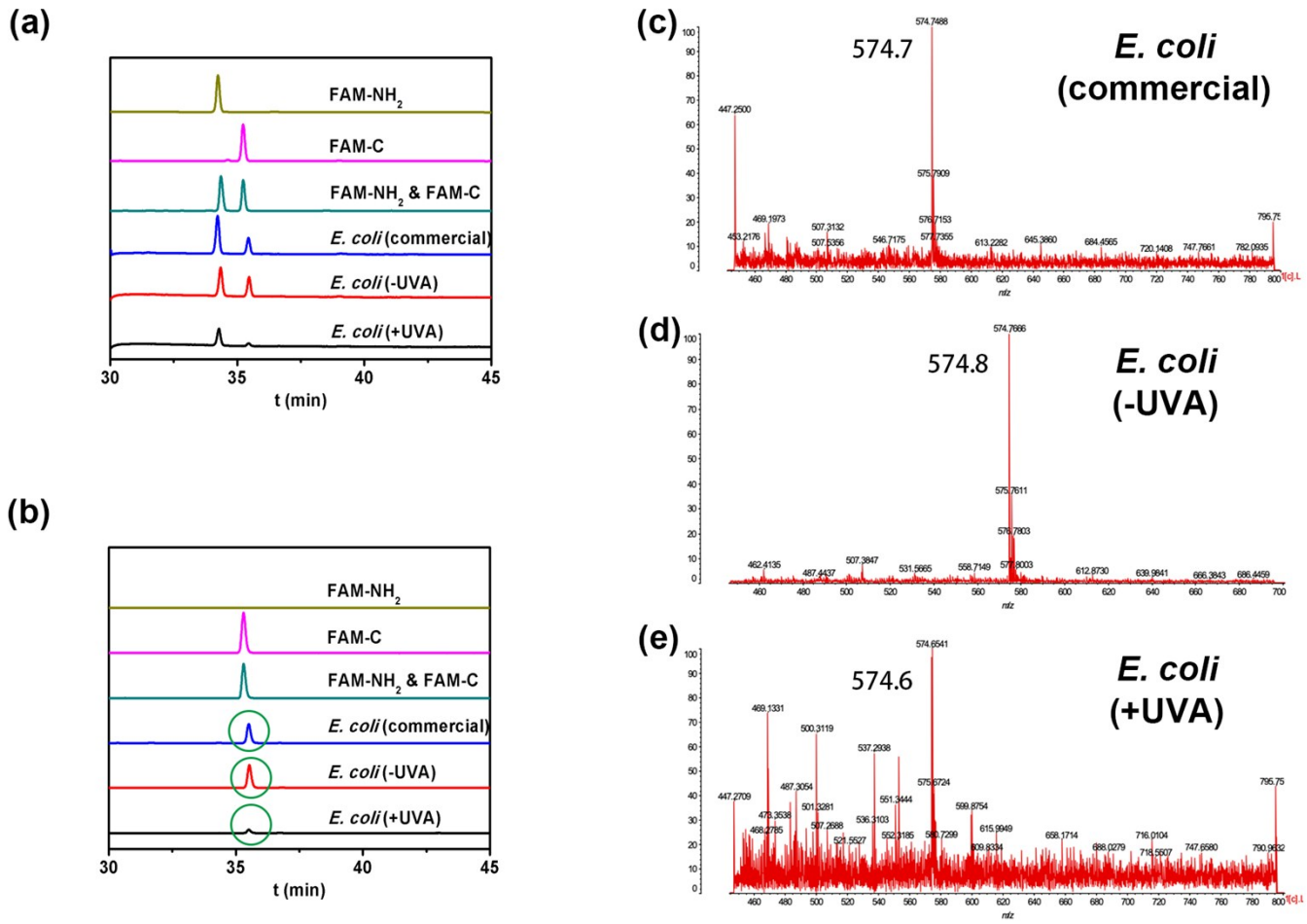

Figure S16. Fluorescence HPLC analysis of FAM-NH_2, FAM-C, mixture of same equivalent FAM-NH_2 and FAM-C, and digested *E. coli* tRNA samples after derivatization reaction. FAM-NH_2 and its derived nucleosides were monitored by UV detector (A_{490}) (a) and fluorescence detector (λ_{ex} = 490 nm, λ_{em} = 520 nm) (b). Fluorescent bands presented in green circles were collected and concentrated for MALDI-TOF MS analysis. (c), (d) and (e) were MALDI-TOF MS characterization of fluorescent bands collected from digested purchased *E. coli* tRNA samples and extracted tRNA samples from *E. coli* treated with darkness and irradiation, respectively. The main peak (*m/z* = 574) indicated the fluorescent bands collected were FAM-C (M = 573).

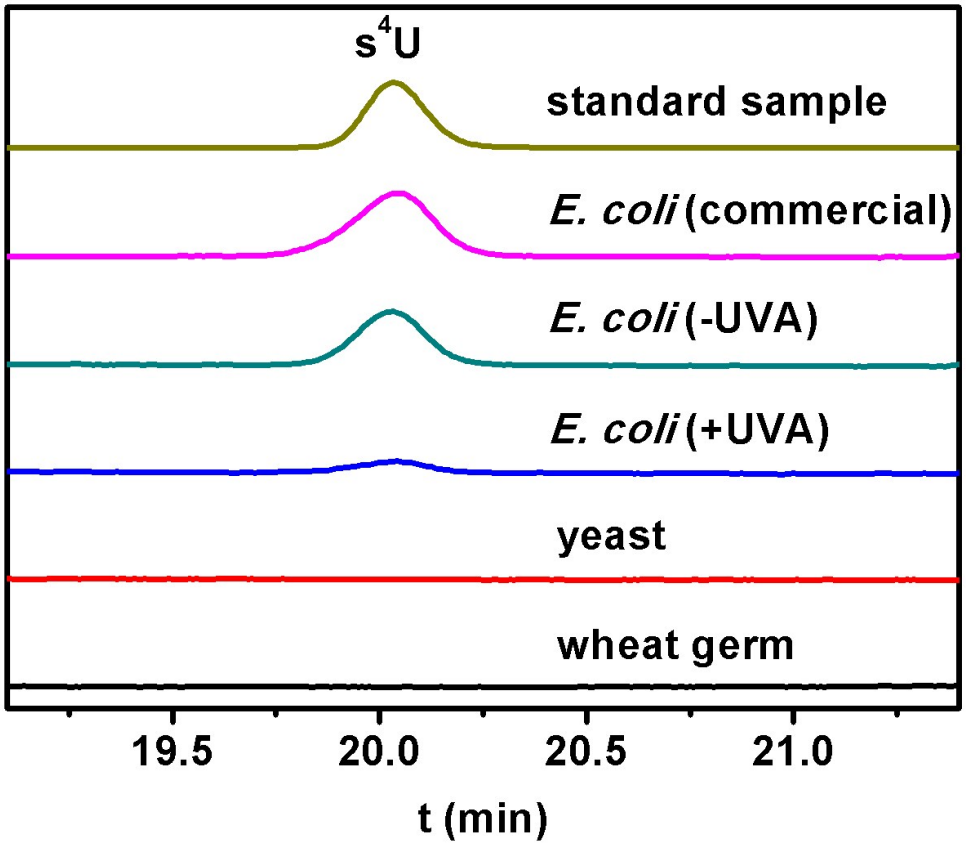

Figure S17. HPLC-UV330 analysis of digested tRNA samples without oxidative amination reaction. s^4U was monitored by UV detector (A_{330}).