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Supplementary Information

A Detection System Using Sensing Motif-tethered Oligodeoxynucleotides for the Multiplex Biomolecular Analysis

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General Methods

Reagents were purchased from Wako pure chemical industries, Sigma Aldrich, Nacalai Tesque, Takara Bio, Dojindo, Tokyo chemical industries, New England BioLabs, and eurofins Genomics. Analytical and preparative high-performance liquid chromatographies were performed on a D2000 HPLC system and L7000 HPLC system (HITACHI) with reversed phase column (InerrtsilvODS-3, GL Science Inc., ϕ 10 mm×250 mm (particle size; 5 μm), ϕ 4.6 mm×250 mm (particle size; 5 μm)). MALDI-TOF mass spectrometry were recorded on an AXIMER-LNR spectrometer (SHIMADZU). DNA hybridization and quantitative PCR (qPCR) analysis was carried out with MyGo Mini S Real Time PCR (IT-IS Life Science Ltd.).

1-1. Synthesis

Synthesis of Mal-DNA1 (Figure S1)

Sulfo-SMCC (10 μmol) were added the solution of NH_2-DNA**1** (5.0 nmol) in 100 mM pH8.7 carbonate-bicarbonate buffer and incubated at room temperature for 5 min. The reaction mixture was purified using Micro Bio-Spin™ 6 Columns (Bio-Rad) and milliQ water as an elution solution. The concentration of the oligomer was determined by complete digestion with alkaline phosphatase (AP), nuclease P1 (PI) and phosphodiesterase I at 37°C for overnight. Identities of synthesized oligomers

were confirmed by MALDI-TOF MS spectrometry ([M-H]- calcd. 6525.2, found. 6524.5). The purity of synthesized oligomer was confirmed by monitoring of UV absorbance at 260 nm at a flow rate of 0.6 (analysis) mLmin-1 with a 0–25% (over 50 min) gradient of acetonitrile (ACN) / triethylammonium acetate buffer (TEAA buffer) (100 mM, pH 7.0).

Synthesis of PB-DNA, Biotin-DNA and Biotin-SS-DNA

PB-DNA**1**, PB-DNA**2**,[1] Biotin-DNA**1**, Biotin-DNA **IS**,[2] and Bitoin-SS-DNA**1**[2] was synthesized according to previous reports.

1-2. Reaction monitoring of Mal-DNA1, PB-DNA1 and Biotin-SS-DNA1

The reaction mixtures were analyzed by HPLC. The column eluents were monitored by the UV absorbance at 260 nm at a flow rate of 0.6 (analysis) $mLmin^{-1}$ with a 0–25% (0–50 min) and 25–100% (50-60 min) gradient of ACN/TEAA buffer (100 mM, pH 7.0).

Reaction monitoring of Mal-DNA1 (Figure 2a, b)

5 μM Mal-DNA**1** was reacted with 0, 25, 50, or 150 μM GSH in phosphate buffer (PB, 250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC as described above.

Reaction monitoring of PB-DNA1 (Figure 2c, d)

5 μM PB-DNA**1** was reacted with 0, 25, 50, or 150 μM H_2O_2 in PB (250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC as described above.

Confirmation of NH_2-DNA1 production using authentic sample (Figure S2)

5 μM PB-DNA**1** was reacted with 1 mM H_2O_2 in PB (250 mM, pH 7.4) for 1 h at room temperature. A half of the reaction mixture (10 μL) was mixed with 5 μM NH_2-DNA**1** (10 μL, 50 pmol) and analyzed by HPLC as described above.

Selectivity of PB-DNA for H_2O_2 (Figure S3)

5 μM PB-DNA**1** was reacted with 0 or 50 μM H_2O_2, potassium superoxide, NaOCl, NOC7 or AAPH in PB (250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC as described above.

Sample preparation

H_2O_2. The H_2O_2 solution was diluted with milliQ water.

ROO˙. 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was dissolved in milliQ water. The solution was mixed with the PB-DNA solution immediately.

$O_2^{\bullet-}$. Potassium superoxide was dissolved in milliQ water. The solution was mixed with the PB-DNA solution immediately.

NO. 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazone (NOC7) was dissolved in milliQ solution. The solution was mixed with the PB-DNA solution immediately.

$^-$OCl. NaOCl solution was diluted with milliQ solution.

Purification and recovery of biotin-DNA using the EtOH precipitation (Figure S5)

0 or 150 pmol NH_2-DNA**2** was incubated with 0 or 1 μmol biotin-AC5 sulfo-OSu in carbonate-bicarbonate buffer (200 mM, pH8.7, 20 μL) and incubated for 1 h at room temperature. After biotin labeling, the reaction solution (20 μL) was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL) on ice, and centrifuged at 15,000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15,000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 20 min at room temperature. The pellet was dissolved with milliQ (20 μL) for HPLC measurement. These samples were analyzed by HPLC as described above.

Reaction monitoring Biotin-SS-DNA1 and GSH by using HPLC (Figure S7)

5 μM Biotin-SS-DNA**1** was reacted with 0 or 50 μM GSH in PB (250 mM, pH 8.6) for 1 h at room

temperature. The reaction mixtures were analyzed by HPLC as described above.

Reaction monitoring of Mal-DNA1 and thiol metabolites (Figure S8)

5 μM Mal-DNA**1** was reacted with 0 or 50 μM GSH, Cys or HCys in PB (250 mM, pH 7.4) for 1 h at room temperature. The reaction mixtures were analyzed by HPLC as described above.

1-3. Detection of target molecules by using qPCR

DNA hybridization

500 nM code DNA and biotin-DNA, Mal-DNA, PB-DNA or NH_2-DNA were hybridized in 5 mM $MgCl_2$. DNA was heated to 95°C for 5 min and then cooled at a rate of 1°C per min to 25°C.

qPCR measurements

qPCR was performed according to the manufacturer's procedure using Luna Universal qPCR Master Mix (NEW ENGLAND Bio Labs): 2 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C.

Simultaneous detection of GSH and H_2O_2 using the DNA probe cocktail (Figure 3b)

Mal-dsDNA1 (1.5 pmol), **PB-dsDNA2** (1.5 pmol) and **Biotin-dsDNA IS** (1.5 pmol) were incubated

with GSH (0 or 1 nmol) and H_2O_2 (0 or 10 nmol) in PB (250 mM, pH7.4) for 1 h at room temperature. After reaction, the reaction solution (20 μL) was mixed with EtOH (60 μL) and AcONa buffer (5 M, pH5.2, 6.5 μL) on ice, and centrifuged at 15,000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15,000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7, 20 μL) and incubated for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation as described above. The pellet was dissolved with binding buffer (10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 1 M NaCl, 0.05% Tween20). The solution was rotating for 10 min with streptavidin coated magnetic beads (50 μg, Magnosphere™ MS300/Streptavidin, JSR corporation) at room temperature. After removal of supernatant, magnetic beads were washed with binding buffer (200 μL) twice and milliQ (100 μL). After wash, milliQ (50 μL) was added and the code DNAs (**1**, **2** and **IS**) were released by heating (95°C for 5 min). After cooling on ice (15 sec), the supernatant was diluted with milliQ water (×1000), code DNAs (**1**, **2** and **IS**) in the sample were quantified by using qPCR as described above.

Quantification of GSH using the Mal-dsDNA1 (Figure 3c)

Mal-dsDNA1 (1.5 pmol) and **Biotin-dsDNA IS** (1.5 pmol) were incubated with GSH (0, 4, 12 or 40

pmol) in PB (250 mM, pH7.4) for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation. The pellet was dissolved with biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7, 20 μL) and incubated for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation. By using streptavidin coated magnetic beads, collected code DNAs (**1** and **IS**) in the sample were quantified as described above.

Detection of biotin-dsDNA1 or NH_2-dsDNA1 using magnetic beads (Figure S4)

Biotin-dsDNA1 (1.5 pmol) or **NH_2-dsDNA1** (1.5 pmol) was incubated with biotin-AC_5 sulfo-OSu (0 or 100 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation. By using streptavidin coated magnetic beads, collected code DNA**1** from **Biotin-dsDNA1** or **NH_2-dsDNA1** was quantified as described above.

Evaluation of an amount of biotin-AC_5 sulfo-OSu and internal standard (Figure S6)

NH_2-dsDNA1 (1.5 pmol) or **dsDNA1** (1.5 pmol) and **biotin-dsDNA IS** (1.5 pmol) were incubated with biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (200 mM, pH8.7) for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation.

By using streptavidin coated magnetic beads, collected code DNAs (**1** and **IS**) from **NH_2-dsDNA1** or **biotin-dsDNA IS** was quantified as described above.

1-4. Detection of GSH in cell extract

Cell culture

Human lung cancer cell line, A549 were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS). The cells were maintained at 37°C in 5% CO_2 / 95% air and were kept in a logarithmic growth phase by routine passages every 2−3 days. Prior to the use of cells, the densities of cells were determined using a hemocytometer.

Detection of GSH derived from cell extract (Figure 3d)

A549 cells (0 or 3.0×10^5 cells per well) were seeded onto 6 well plate in 3 mL DMEM. Plate was maintained at 37°C in 5% CO_2 / 95% air incubator for 24 h. After washing with D-PBS (-) twice, methanol (500 μL) was added into each well and incubated for 15 min. Then, the solution (200 μL) were collected and extracted with milliQ (100 μL) and $CHCl_3$ (200 μL). After centrifugation at 12,000 g for 15 min at 4°C, aqueous layer (250 μL) was lyophilized. Cell extract was dissolved with 0 or 100 mM N-methylmaleimide (NMM) in milliQ (12 μL) and incubated for 30 min. The cell

number used in this assay was determined by using another cell seeded well.

Mal-dsDNA1 (1.5 pmol) and **Biotin-dsDNA IS** (1.5 pmol) was incubated in PB (pH7.4, 250 mM, total 32 μL) containing 31% cell extract solution (NMM +/-) for 1 h at room temperature. After reaction, the reacted solution was mixed with EtOH (96 μL) and AcONa buffer (5 M, pH5.2, 10.5 μL) on ice, and centrifuged at 15,000 g for 10 min at 4°C. After removement of supernatant, 75% EtOH (100 μL) was added to the pellet and centrifuged at 15,000 g for 4 min at 4°C. After removement of supernatant, the pellet was dried in air for 15 min at room temperature. The pellet was dissolved with biotin-AC_5 sulfo-OSu (10 nmol) in carbonate-bicarbonate buffer (pH8.7, 200 mM, 20 μL) and incubated for 1 h at room temperature. After the reaction, the reaction solution was purified by using EtOH precipitation as described above. By using the streptavidin, coated magnetic beads, collected code DNAs (**1** and **IS**) were quantified as described above.

2. Supporting Figures

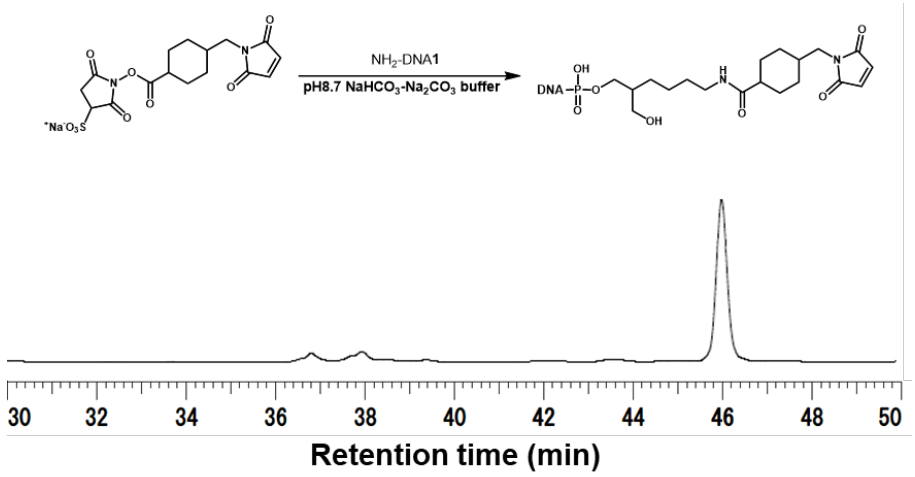

Figure S1. Synthesis of Mal-DNA1. HPLC chart of Mal-DNA**1**.

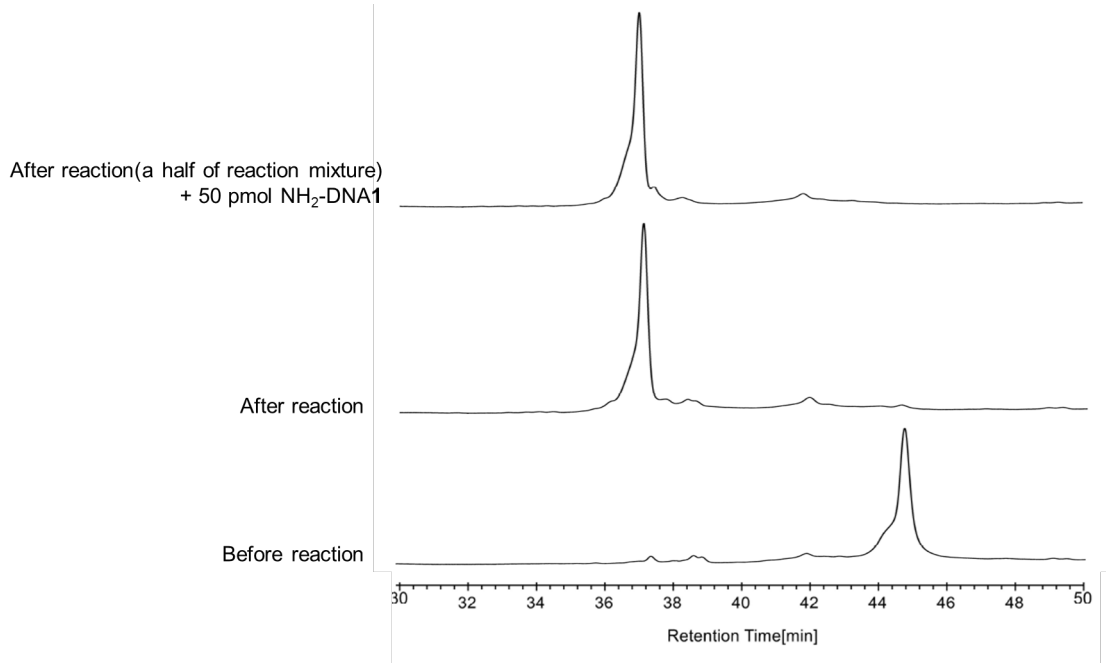

Figure S2. Confirmation of NH_2-DNA1 production using authentic sample. HPLC chart of 5 μM PB-DNA**1** incubated with 1 mM H_2O_2 in 250 mM PB (pH7.4, 20 μL) for 1 h at 37°C and a half of reaction mixture with 50 pmol NH_2-DNA**1**.

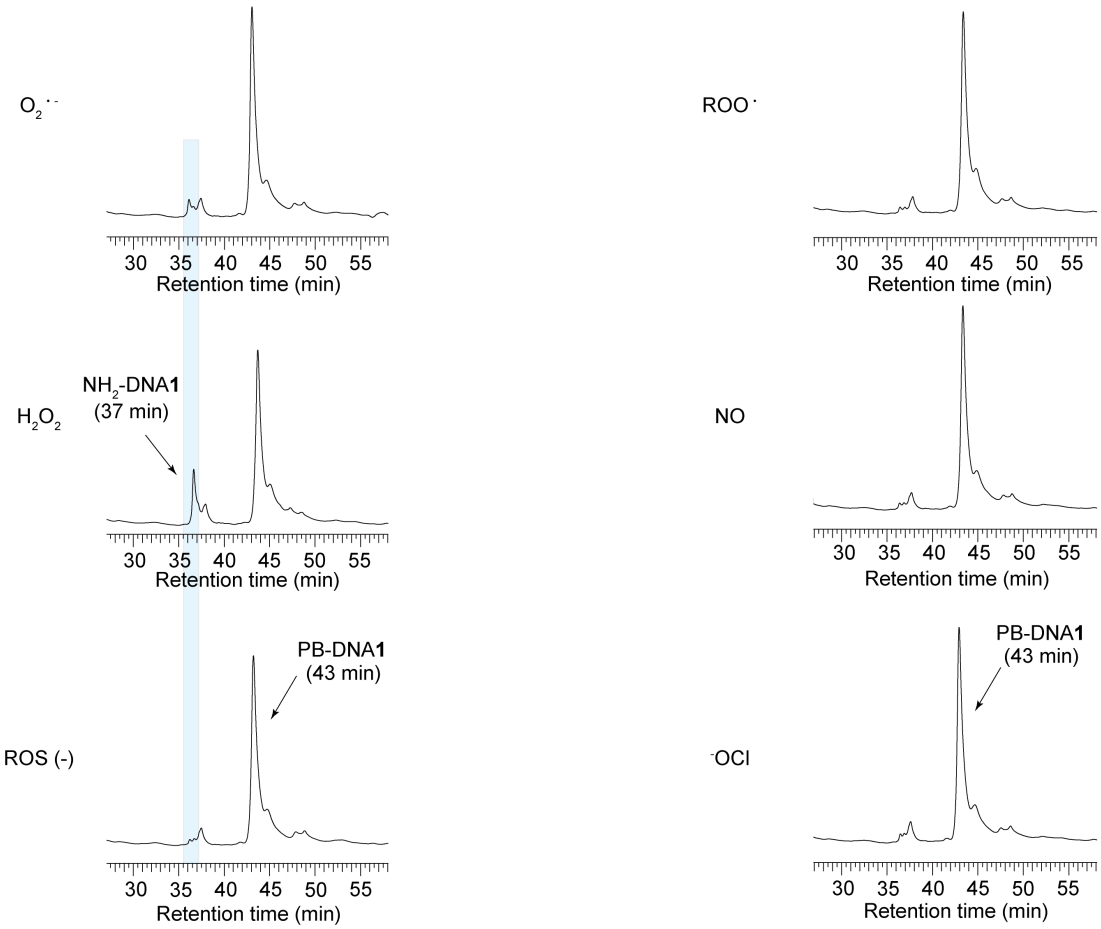

Figure S3. Selectivity of PB-DNA for H_2O_2. 5 μM PB-DNA**1** reacted with 0 or 50 μM H_2O_2, potassium superoxide, NaOCl, NOC7 or AAPH in 250 mM phosphate buffer (pH 7.4) for 1 h at room temperature.

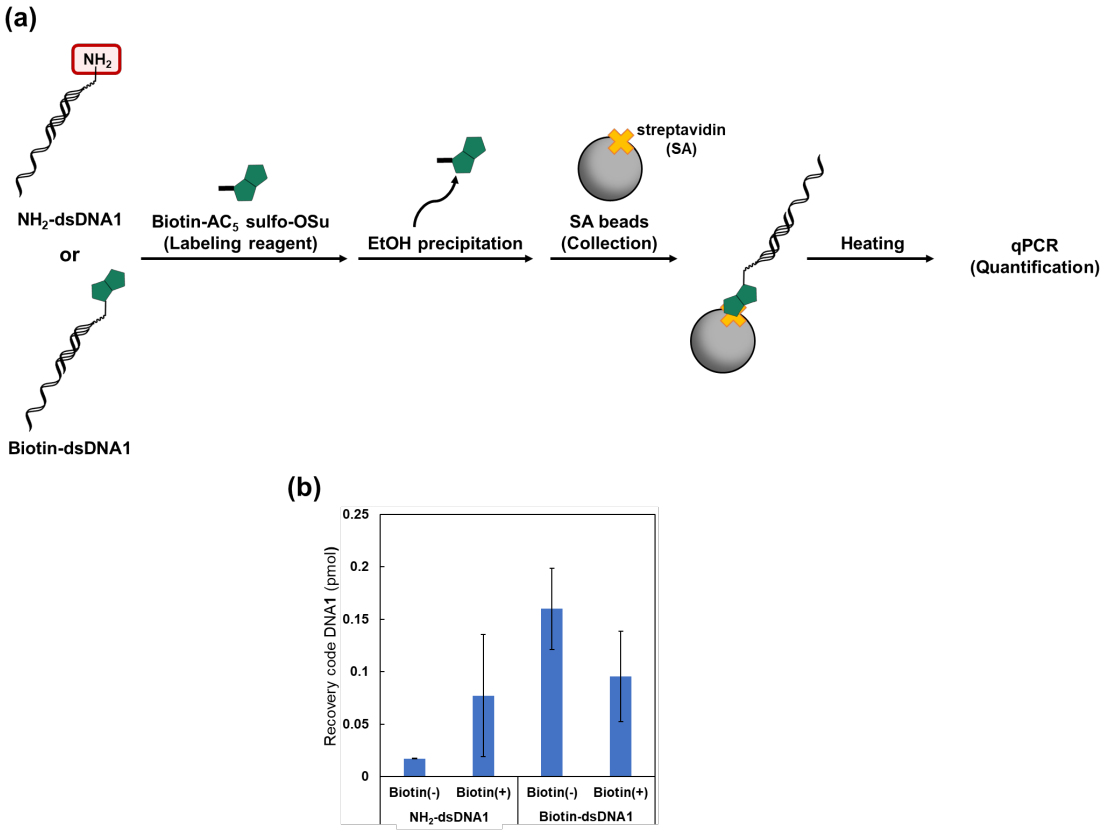

Figure S4. Collection and detection of NH_2-dsDNA1 by using biotin labelling. (a) Schematic illustration of **NH_2-dsDNA1** or **Biotin-dsDNA1** collection by using streptavidin coated magnetic beads. (b) Quantification of collected code DNA**1** from **NH_2-dsDNA1** or **Biotin-dsDNA1**. Data = mean ± SD (n=3).

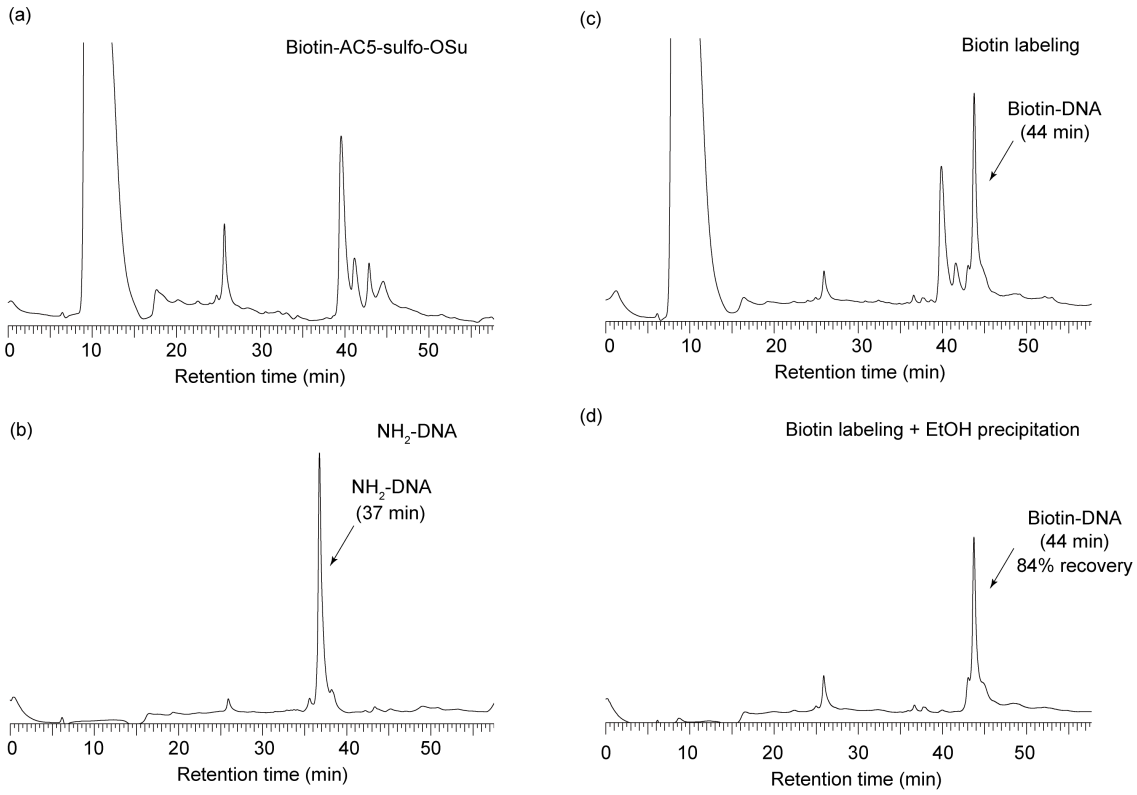

Figure S5. Purification and recovery of biotin-DNA using the EtOH precipitation. (a–c) Reaction monitoring using HPLC; 0 or 150 pmol NH_2-DNA**2** was incubated with 0 or 1 μmol biotin-AC5 sulfo-OSu in 200 mM carbonate-bicarbonate buffer (pH 8.7) for 1 h at room temperature. (d) After biotin labeling, the reaction solution was purified by using EtOH precipitation.

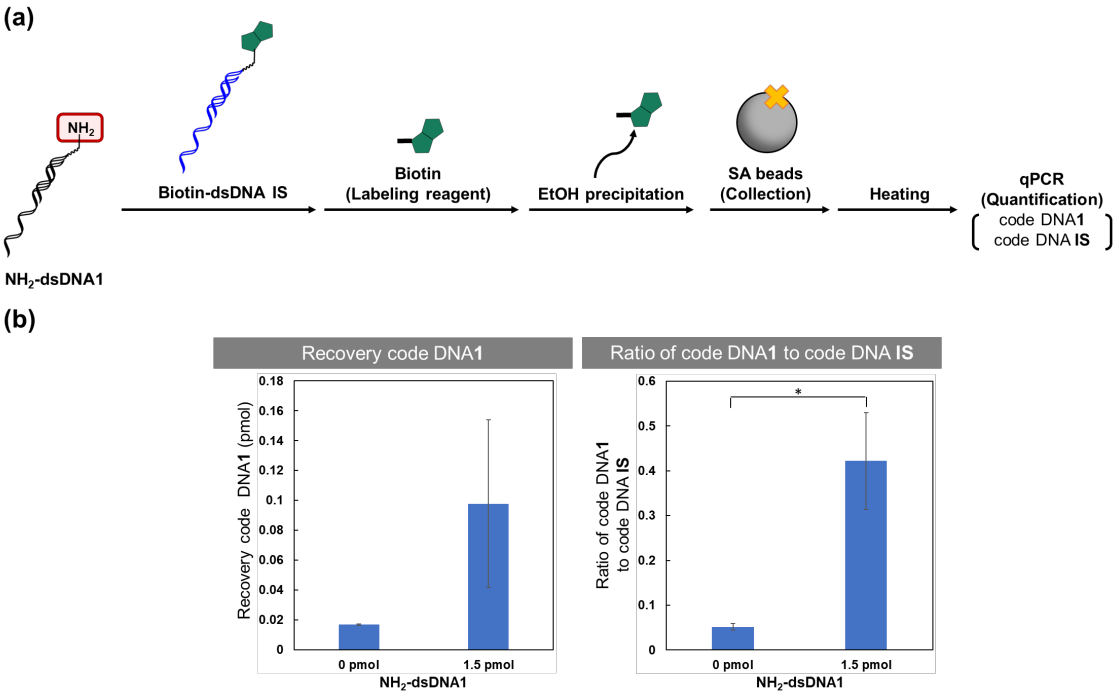

Figure S6. Ratio of code DNA1 to code DNA IS (a) Schematic illustration of code DNA 1 and IS collection by using streptavidin coated magnetic beads. (b) Amount of recovery code DNA **1** and ratio of code DNA**1** to code DNA **IS**. Data = mean ± SD (n=3). * $p < 0.05$ (Student t-test)

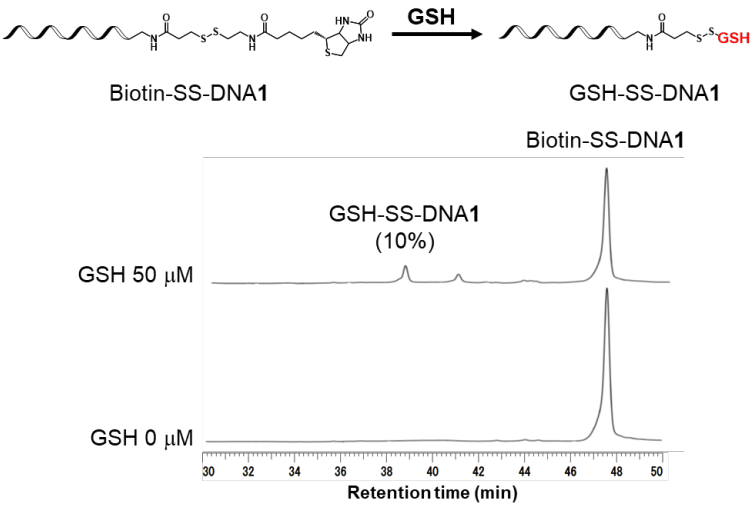

Figure S7. Reaction monitoring of Biotin-SS-DNA1 and GSH (ref 2). 5 μM Bitoin-SS-DNA**1** reacted with 0 or 50 μM GSH in PB (pH8.6, 250 mM) for 1 h at room temperature.

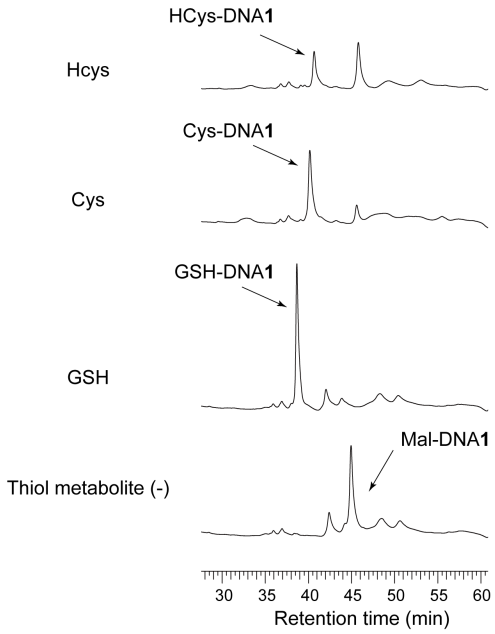

Figure S8. Reaction monitoring of Mal-DNA1 and thiol metabolites Reaction monitoring using HPLC; 5 μM Mal-DNA**1** reacted with 0 or 50 μM GSH, Cys or HCys in 250 mM phosphate buffer (pH 7.4) for 1 h at room temperature.

Table S1. DNA sequences in this study

Strand name	Sequences (5' to 3')
DNA**1**	GGT CCA ATC TAC AGG AAT TC
NH_2-DNA**1**	GGT CCA ATC TAC AGG AAT TC-NH_2
code DNA**1**	GAA TTC CTG TAG ATT GGA CCT TTT TTT TTT TTT TTT TTT CGA CCC TAA GCA TAC ATA CCT
Forward primer (for code DNA**1**)	AGG TAT GTA TGC TTA GGG TCG
Reverse primer (for code DNA**1**)	GAA TTC CTG TAG ATT GGA CC
NH_2-DNA**2**	AAG CTT CGA GTG ACT GCG AT-NH_2
code DNA**2**	ATC GCA GTC ACT CGA AGC TTT TTT TTT TTT TTT TTT TTT CCG TCC TTC ATT ATG GAA GTC
Forward primer (for code DNA**2**)	GAC TTC CAT AAT GAA GGA CG
Reverse primer (for code DNA**2**)	ATC GCA GTC ACT CGA AGC TT
NH_2-DNA **IS**	CTT AAG GAC ATC TAA CCT GG-NH_2
code DNA **IS**	CCA GGT TAG ATG TCC TTA AGT TTT TTT TTT TTT TTT TTT TCC ATA CAT ACG AAT CCC AGC
Forward primer (for code DNA **IS**)	GCT GGG ATT CGT ATG TAT GGA
Reverse primer (for code DNA **IS**)	CCA GGT TAG ATG TCC TTA AG

3. Supporting references

(1) Y. Motohashi, T. Nishihara, and K. Tanabe, *Chem. Lett*. **2023**, 52, 732-735

(2) Y. Motohashi, S. Moritani, T. Nishihara, and K. Tanabe, *ChemistrySelect*, **2023**, 8, e202302662.