Supporting Information

Experimental Methods and Materials

Synthetic RNA Substrates and Probes.

We used the following oligonucleotides, which were synthesized at GeneDesign, Inc. (Osaka, Japan). (m$^6$A) and (*N) indicate N$^6$-methyladenosine and LNA, respectively. We used BNA$^\text{NC}$ [N-H] for C and T (indicated as #C and #T) and BNA$^\text{NC}$ [N-Me] for A (indicated as ##A).$^1$ Donor BNA$^\text{NC}$ probes were designed to be as short as possible to increase $\Delta T_m$ between m$^6$A-containing RNA and unmethylated RNA, and so that the position of the counterpart for m$^6$A was not the 3’ end of the probe, where the fluorescence dye might affect the detection of m$^6$A. In addition, the number of BNA$^\text{NC}$ in donor probes was determined under the assumption that $T_m$ falls within the range of 40°C to 50°C. We did not fix the position of BNA$^\text{NC}$. In contrast, acceptor BNA$^\text{NC}$ probes were designed to be longer to increase sequence specificity and to be thermodynamically more stable than donor probes.

SynDNA: 5’-GCTGTGAAGATGCAGTG-3’

SynRNA: 5’-GCUGUGAAGAUGCAGUG-3’
SynMetRNA: 5’-GCUGUG(m^6A)AGAUGCAGUG-3’

SynRNA2: 5’-UCAGUGAAUAUGAACUCGUGUAGAUGCAG-3’

SynMetRNA2: 5’-UCAGUGAAUAUGAACUCGUGUAG(m^6A)AGAUGCAG-3’

DNA1: 5’-CATCTTCACAGC-(FAM)-3’

LNA1: 5’-(*C)A(*T)C(*T)T(*C)A(*C)AGC-(FAM)-3’

BNA1: 5’-(*C)A(*T)C(*T)T(*C)A(*C)AGC-(FAM)-3’

BNA2: 5’-(*C)TG(*C)A(*T)C(*T)T(*C)AC-(FAM)-3’

BNA3: 5’-(*C)T(*C)T(*C)A(*C)AG(*C)-(FAM)-3’

BNA4: 5’-(*C)A(*T)C(*T)T(*C)A(*C)-(FAM)-3’

BNA5: 5’-CA(*C)C(*T)T(*C)A(*C)AGC-(FAM)-3’

AcDNA1: 5’-(Alexa 594)-CGAGTTCAATTTCAC-3’

AcBNA1: 5’-(Alexa 594)-CGAGT(##T)C(##A)A(##T)T(##C)CA(##C) -3’

AcBNA2: 5’-(Alexa 594)-GT(##T)TGGGG(##T)A(##C)GA(##T)T(##T)G -3’

DoDNA1: 5’-GCATCTTCAC-(Alexa 488)-3’
DoBNA1: 5’-GCATC(T)(#T)(#C)AC-(Alexa 488)-3’

DoBNA2: 5’-GCATC(T)(#T)(#C)A-(Alexa 488)-3’

DoBNA3: 5’-GCATC(#T)(#T)(#C)- (Alexa 488)-3’

DoBNA4: 5’-CAC(#C)(#T)G(#T)GT-(Alexa 488)-3’

E. coli Strains and Culture Conditions.

We obtained E. coli strains BW25113, JW3466 (ArlmJ or AybiR), and JW5107 (ArlmF or AybiN) from the National BioResource Project (Shizuoka, Japan). ArlmJ and ArlmF strains lack rRNA large subunit methyltransferase J (RlmJ) and RlmF, respectively. All E. coli strains were cultured in 2×YT media (1.6% Bacto Tripton, 1% Yeast Extract, 0.5% NaCl; BD Biosciences) with shaking at 37°C. We monitored bacterial growth by an OD-MonitorA&S (Taitec, Saitama, Japan) and collected the cells when the absorbance at 600 nm reached 0.6.

RNA Extraction.

Total RNA was extracted by using TRIZol reagent in accordance with the manufacturer’s instructions (Thermo Fisher Scientific) and dissolved in Nuclease-Free water (Thermo
Fisher Scientific). RNA concentration was measured by using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and adjusted to 3 µg/µl. Total RNAs were stored at -80°C until use.

**Measurement of Melting Temperature.**

Either 50 pmol of synthetic template (DNA and RNA) or 10 µg of total RNA extracted from *E. coli* was mixed with either a single FAM-labeled probe (10 pmol) or dual Alexa 488- and Alexa 594-labeled probes (10 pmol each) in hybridization buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂). The solution was placed in a 96-well plate in a ViiA 7 Real-Time PCR system (Thermo Fisher Scientific) and the melting temperature of each mixture was measured by using the “Melting Curve Experiment” mode. In brief, probes were hybridized to the template by heating to 95°C for 15 sec, followed by cooling from 95°C to 25°C at a rate of 1.6°C/sec. Next, to obtain the melting curve, the fluorescence intensity was serially monitored as RFU (Relative Fluorescence Units) while the temperature was increased from 25°C to 95°C at a rate of 0.1°C/sec. Fluorescence emission from a single FAM-labeled probe is detected at a wavelength of 520 nm after excitation at a wavelength of 495 nm. Given that fluorescence is detected when a single
FAM-labeled probe dissociates from the template, the melting temperature ($T_m$) was defined as the minimum value of $-d(RFU)/dT$ for a single FAM-labeled probe. In the dual probe experiments, by contrast, fluorescence emission from Alexa 594 on the acceptor probe was detected at a wavelength of 619 nm after excitation of Alexa 488 on the donor probe at a wavelength of 495 nm via FRET only when both probes hybridized to the template: therefore, $T_m$ was defined as the maximum value of $-d(RFU)/dT$.

Mass Spectrometric Analysis.

The methylation status at A2030 and A1618 in *E. coli* 23S rRNA was analyzed by using mass spectrometry as previously described with minor modifications. In brief, 60 µg of total RNA derived from *E. coli* was mixed with 200 pmol of DNA oligonucleotide complementary to the sequence around the target m$^6$A site (A2030, 5′-GGTTCAAGGGGCTTTTCCGCCTTGTGACACTGATCTTACAGCG-3′ and A1618, 5′-GCGCTTGGATCTCTCTACCCGCTCGGTTGATGATTTGATT-3′) and 20 µL of 10x Annealing buffer (500 mM Hepes-KOH, 1 M KCl). The total volume was adjusted to 200 µL with Nuclease-free water, and the mixture was heated at 90 °C for 5
min and then gradually cooled to 30°C at a rate of 1.0°C/min for annealing. Next, the mixture was kept at 4°C for 1 h. To digest uncovered regions of 23S rRNA, 500 U of RNase T1, and 0.5 µg of RNase A were added to the mixture and the sample was incubated for 1 h on ice. The resultant heteroduplex was recovered by phenol/chloroform (pH 7.9) extraction and 2-propanol precipitation with glycogen. The precipitate was dissolved in 10 µL of Nuclease-Free water and applied to a 15% denaturing polyacrylamide gel. After electrophoresis at 250 V for 1 h, the gel was stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific). The piece of gel containing the heteroduplex was excised, minced, and mixed with 400 µL of Elution Buffer (1 mM EDTA-NaOH (pH 8.0), 0.1% SDS, 400 mM NaOAc (pH 5.2)). After incubation at 37°C for 3 h with shaking, the heteroduplex was recovered by 2-propanol precipitation. Next, 1 µL of 1000 U of RNase T1 and 4 µL of 50 mM NH₄OAc (pH 5.2) were added to 1/10 volume of the collected sample, and the total volume was adjusted to 10 µL with Nuclease-free water. After incubation at 37°C for 1 h, 10 µL of 100 mM triethylamine-acetate buffer (TEAA) was added to the digested sample, which was analyzed by using an LTQ Orbitrap XL instrument (Thermo Fisher Scientific).
**Statistical Analysis.**

All values are displayed as the mean ± standard error of the mean (SEM). All statistical analyses of differences were carried out by using Student’s t-test. All values are displayed as the mean ± standard error of the mean (SEM). Statistical significance is displayed as *p<0.05, #p<0.05, **p<0.01, or ##p<0.01.*
References

Design, synthesis, and properties of 2′, 4′-BNA(NC): a bridged nucleic acid 

methylation of 23S rRNA triggers late steps of 50S ribosomal subunit assembly. 
Figures and Figure Legends

Figure S1. Structure of N6-methyladenosine (m6A) and bridged nucleic acids (BNAs).

Shown are the structures of adenosine and m6A (A), and those of DNA, 2', 4'-BNA (LNA) and 2', 4'-BNA NC (BNA NC) (B). H, hydrogen group; Me, methyl group.
**Figure S2.** Measurement of melting temperature using single FAM-labeled probes. (A) Fluorescence is detected when a single FAM-labeled probe dissociates from the template. (B) Shown are representative melting curves obtained for the template–probe duplexes formed between SynRNA and DNA1 (red), SynMetRNA and DNA1 (light red), SynRNA and LNA1 (blue), SynMetRNA and LNA1 (light blue), SynRNA and BNA1 (green), and SynMetRNA and BNA1 (light green). The temperature corresponding to the peak with the minimum value of \(-d(RFU)/dT\) was defined as the melting temperature \((T_m)\).
Figure S3. Comparison of melting temperature between a single FAM-labeled BNA<sup>NC</sup> probe with DNA-T and a single FAM-labeled BNA<sup>NC</sup> probe with BNA<sup>NC</sup>-T at the counterpart of m<sup>6</sup>A. (A) Nucleotide sequences of the FAM-labeled BNA<sup>NC</sup> probes BNA1 and BNA5. The positions of BNA<sup>NC</sup> are indicated in green. As compared with BNA1, BNA5 was designed to contain BNA<sup>NC</sup>-T at the counterpart of m<sup>6</sup>A without changing the total number of BNA<sup>NC</sup> bases in the probe. (B) Melting temperature (Tm) of the indicated template–probe duplexes (n.s., not significant; n=3). (C) Comparison of ΔTm (Tm<sub>SynRNA</sub> – Tm<sub>SynMetRNA</sub>) between the BNA1 and BNA5 probes (n.s., not significant; n=3).
Figure S4. Measurement of melting temperature using dual probes. (A) Fluorescence emission from Alexa 594 on the acceptor probe is detected at a wavelength of 619 nm after excitation of Alexa 488 on the donor probe at a wavelength of 495 nm via FRET (fluorescence resonance energy transfer) only when both probes hybridize to the template. (B) Shown are representative melting curves obtained for template–probe duplexes formed between SynRNA2 and AcDNA1-DoDNA1 probes (red), SynMetRNA2 and AcDNA1-DoDNA1 probes (light red), SynRNA2 and AcBNA1-DoBNA1 probes (green) and SynMetRNA2 and AcBNA1-DoBNA1 probes (light green). The temperature corresponding to the peak with the maximum value of -d(RFU)/dT was defined as the melting temperature (Tm).
Figure S5. Detection of m^6A in *E. coli* 23S rRNA by mass spectrometric analysis. Shown are mass chromatograms of m^6AAGp and AAGp, corresponding to A2030 (A), and ACACm^6AGp, corresponding to A1618 (B), derived from wild-type *E. coli* 23S rRNA. Asterisk indicates a non-specific peak.
Figure S6. Application of dual BNA$^{NC}$ probes to the detection of m$^6$A in *E. coli* rRNAs. Comparison of melting temperature (Tm) between wild-type (WT) and the methylation enzyme-deficient strain by using dual BNA$^{NC}$ probes targeting the A2030 (A) and A1618 (B) sites (n=3).