Supplementary Information for

Small Molecule - PNA Oligomer Conjugates for rRNA A-site at neutral pH for FID Assays

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General Information

Abbreviations:

Fmoc = fluorenylmethoxycarbonyl

Bhoc = benzhydryloxycarbonyl

Boc = N-tert-butoxycarbonyl

Alloc = allyloxycarbonyl

TFA = trifluoroacetic acid

SPOC probe = Small molecule – PNA oligomer conjugate probe

Rink Amide Resin was purchased from Merck Millipore (Billerica, MA). Fmoc-protected amino acids were purchased from Sigma-Aldrich (Darmstadt, Germany) and Watanabe Chemical Industries (Hiroshima, Japan). Fmoc-C(Bhoc)-OH PNA monomers were purchased from Panagene (Daejeon, South Korea). Other reagents were commercially available and of analytical grade and were used without further purification. Model hairpin rRNAs were custom synthesized and HPLC purified (99%) by GeneDesign, Inc. (Osaka, Japan). The concentration of the RNAs was determined from the OD_{260} at 80°C and calculated using the OD_{260} = 1 = 40 μg/mL. Ultrapure water (18.2 MΩ-cm specific resistance) was used from an Elix 5 UV water purification system and a MilliQ Synthesis A10 system (Millipore Co., Bedford, MA), followed by filtration through a BioPak filter (Millipore Co.) in order to remove RNase.

Unless otherwise mentioned, all measurements were performed in 10 mM sodium acetate buffer solutions (pH 5.5) or 10 mM sodium phosphate buffer solutions (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Before measurements, annealing of the RNA-containing samples was carried out as follows: heating at 95°C for 10 min, before cooling by 1°C for 1 min all the way down to 25°C, where it is held until the next step.

Synthesis of SPOC Probes and Control Probe

PNA synthesis was done with help from the Biotage® Initiator+ (Biotage, Uppsala, Sweden) inside a corresponding microwave peptide vial. Fmoc-protected PNA monomers were used with orthogonal Alloc and Bhoc/Boc protecting groups where necessary. The progress of the synthesis was monitored by the use of Kaiser tests. The thiazole orange (TO)[1] and ATMND-C_n-NH_2 (n = 2, 3 and 4) [2,3] monomer units was synthesised according to previous literature. TO-C_1-COOH was manually attached to a PNA backbone monomer. ATMND-C_n-NH_2 was coupled to a Glu amino acid unit through an amide bond. We also attempted to couple ATMND-C_n-NH_2 with an Asp amino acid unit, but the synthesis failed due to aspartimide formation. The control probe contains a Gly unit as a spacer instead of the Glu unit. A TFA: m-cresol (85:15) solution was used for probe cleavage from the resin. M-cresol was purchased from Tokyo Chemical Industry Co. Ltd. The solution was added to the resin and the resultant mixture was

shaken for a total of 3 hours, with 2 min of vortexing ever half an hour. The filtrate would be collected in PE centrifuge tubes (GE Healthcare UK Limited, Buckinhamshire, UK) and topped up with cold diethyl ether to precipitate the probes. The tubes would be left in the freezer overnight and the probes would be collected by centrifugation the following day.

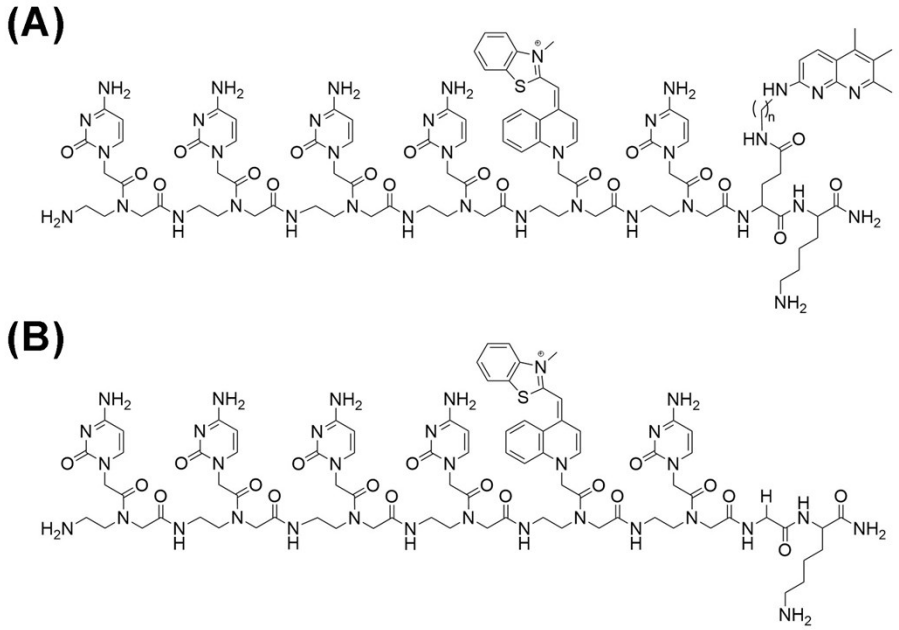

Fig. S1 Chemical structures of the **(A)** SPOC probe (n = 2, 3 and 4) and **(B)** the control probe.

Purification of SPOC Probes and Control Probe

The crude probes were purified via HPLC with an Inertsil ODS-3 5 μm (20 x 250 mm) column (GL Sciences, Torrance, CA), with JASCO UV 2070-Plus spectrometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The wavelengths were set to 510 nm (for TO) and 260 nm (for PNA). The oven temperature was set to 55°C. The solvents used were 0.1% TFA H_2O and 0.1% TFA acetonitrile (Sigma-Aldrich, HPLC grade). Typically, the solvent gradient would be H_2O : Acetonitrile 90:10 → 50: 50 in 50 minutes. The separated fractions would be checked using MALDI-TOF mass spectrometry (matrix: CHCA) (Bruker Daltonics autoflex Speed-S1, Bruker, Billerica, MA) to confirm the presence of the purified probe. The concentration of the control probe was calculated in water via UV-vis spectrophotometer at 25°C using the following molar extinction coefficients at 260 nm: 8800 $M^{-1}cm^{-1}$ for thymine, 7300 $M^{-1}cm^{-1}$ for cytosine, and 9400 M^{-1} cm^{-1} for TO. For the SPOC probes, the following molar coefficients were used: ATMND-C_2-NH_2: 15 187 $M^{-1}cm^{-1}$ at 374.5 nm; ATMND-C_3-NH_2: 14 587 $M^{-1}cm^{-1}$ at 375.5 nm; ATMND-C_4-NH_2: 14 625 $M^{-1}cm^{-1}$ at 375 nm.

UV-Vis Spectroscopic Experiments

UV-Vis experiments were done with a JASCO model V-570 UV-Vis spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan) at 25°C. Two 2 × 10 mm quartz cuvette (optical path length: 10

mm) was used – one for the sample, and the other for the reference (ultrapure water). A baseline correction was first done using only the buffer before UV measurement of the samples.

General Fluorescence Experiments

All fluorescence experiments were carried out using a JASCO FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), with a 3 x 3 mm quartz cuvette at 25°C. Excitation and emission band widths were set to 5 nm, and the sensitivity was set to medium (or low, for the mock FID assays).

For the fluorescence titration experiments, a 1:1 binding isotherm was fitted to it in order to evaluate the association constant (K_a). The dissociation constant, K_d, is the reciprocal of K_a.

Ultimately, the following equation was used to fit the obtained curves for the SPOC and control probes:

$$\Delta F = \frac{F_{\Delta PR}}{2[P]_0}\left([P]_0 + [R]_0 + \frac{1}{K_a} - \sqrt{([R]_0 + [P]_0 + \frac{1}{K_a})^2 + 4[P]_0[R]_0}\right)$$

Where $[P]_0$ and $[R]_0$ are the initial concentrations of the probe and rRNA, respectively. $F_{\Delta PR}$ is the difference in fluorescence intensity between the complex (probe-RNA) and the free probe. The above equation is a derivation from the following two equations:

$$\Delta F = F_{\Delta PR}\left(\frac{[PR]}{[P]_0}\right)$$

$$K_a = \frac{[PR]}{[P][R]}$$, where $[P] = [P]_0 - [PR]$, and $[R] = [R]_0 - [PR]$

For ATMND-C_2-NH_2, the curve-fitting equation used was:

$$\frac{F}{F_0} = \frac{1 + \frac{k_{complex}}{k_{RNA}}K_a[RNA]}{1 + K_a[RNA]}$$

F and F_0 are the observed fluorescence intensities of the ATMND-C_2-NH_2 in the presence and absence of RNA respectively, where $k_{complex}$ and k_{RNA} are the proportionality constants of the complex (ligand-RNA) and the RNA respectively. K_a is the association constant of the system. The free RNA concentration, $[RNA]$ can be related to known total concentrations of the RNA (RNA_0) and the ligand, ATMND-C_2-NH_2, (L_0) by the following equation:

$$RNA_0 = [RNA] + \{L_0K_a[RNA]\}/\{1 + K_a[RNA]\}$$

All data analyses were performed by Kaleidagraph software 4.5 (Synergy Software, Reading, PA).

Fluorescent Indicator Displacement (FID) Assay Experiment

All the conditions used were the same as those of the fluorescence spectroscopic experiments. The test compounds were added in after the RNA had been reannealed and resulting samples were incubated for 30 min. The probe was added in just 5 min before each sample measurement was taken.

Melting Temperature Experiment

The bacterial rRNA (3.0 μM) was first annealed in a pH 7.0 buffer before the conjugate probe (3.0 μM) was added. The experiment was carried out in a 8-microcell quartz cuvette with an optical path of 10 mm and a UV-2450 UV-Visible spectrophotometer from Shimadzu (Kyoto, Japan). The total sample volume was 120 μL and paraffin was added at the top to prevent solvent loss. Absorbance was monitored at 260 nm (data not shown) and 300 nm with a temperature ramp of 0.5°C / min to give the resultant absorption spectra. The melting points were determined using the peaks in the first derivative curves of the absorption spectra.

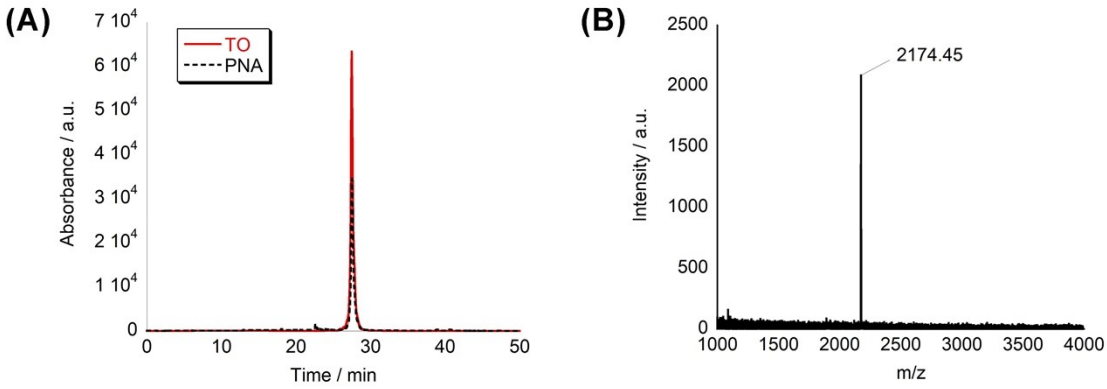

Fig. S2 (A) HPLC chromatogram and **(B)** the corresponding MALDI-TOF-MS spectrum of purified SPOC probe with ATMND-C_2-NH_2.

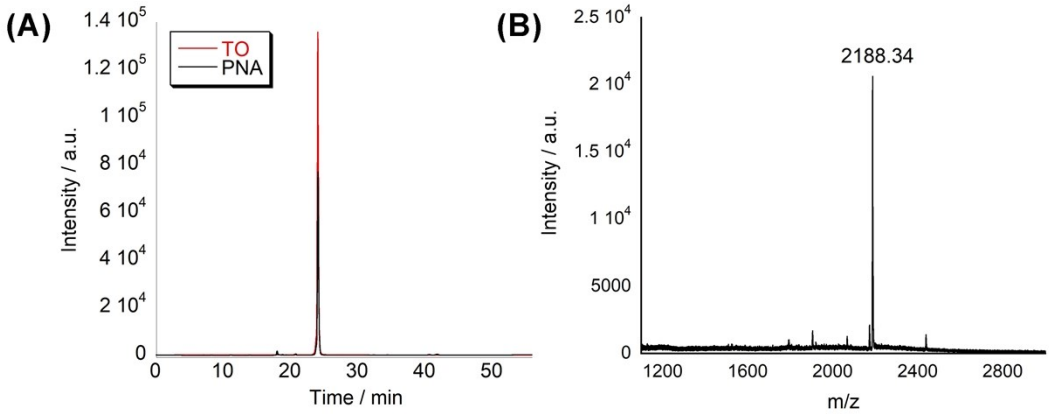

Fig. S3 (A) HPLC chromatogram and **(B)** the corresponding MALDI-TOF-MS spectrum of purified SPOC probe with ATMND-C_3-NH_2.

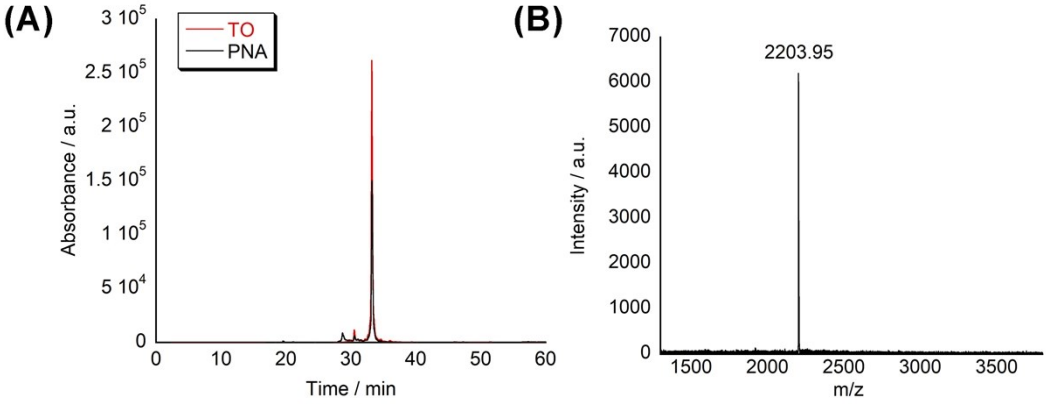

Fig. S4 (A) HPLC chromatogram and **(B)** the corresponding MALDI-TOF-MS spectrum of purified SPOC probe with ATMND-C_4-NH_2.

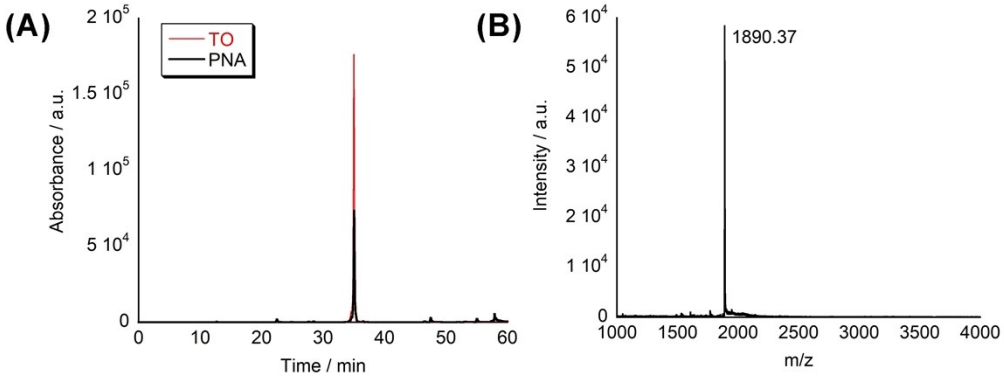

Fig. S5 (A) HPLC chromatogram and **(B)** the corresponding MALDI-TOF-MS spectrum of the purified control probe.

Table S1 MALDI-TOF-MS data for the various probes.

Probe	Chemical Formula	Calculated MW $[M]^+$	Observed m/z
SPOC probe (ATMND-C_2-NH_2)	$C_{98}H_{126}N_{37}O_{20}S$	2174.38	2174.45 $[M]^+$
SPOC probe (ATMND-C_3-NH_2)	$C_{99}H_{128}N_{37}O_{20}S$	2188.41	2188.34 $[M]^+$
SPOC probe (ATMND-C_4-NH_2)	$C_{100}H_{130}N_{37}O_{20}S$	2202.44	2203.95 $[M+H]^+$
Control Probe	$C_{82}H_{106}N_{33}O_{19}S$	1890.02	1890.37 $[M]^+$

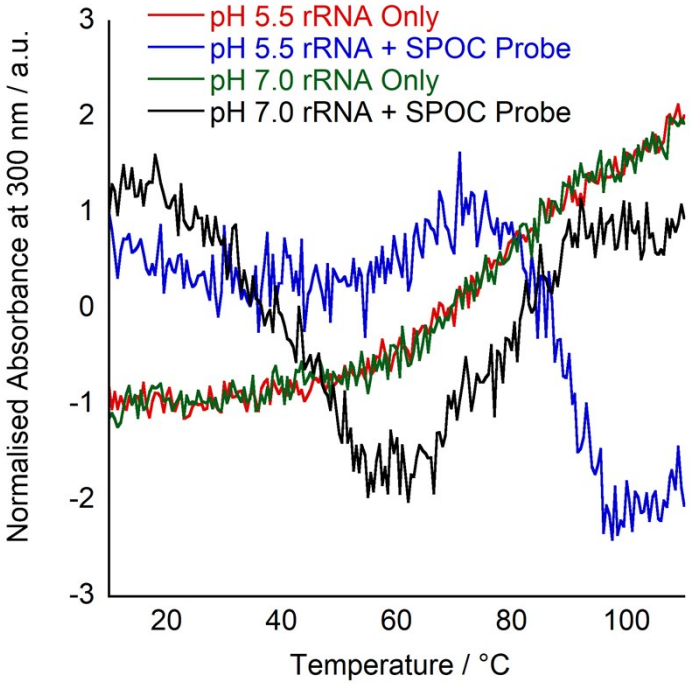

Fig. S6 Melting curves of bacterial rRNA in the presence and absence of the SPOC probe (with ATMND-C_2-NH_2) at pH 5.5 or pH 7.0.

The change in absorbance of the probe was observed at 300 nm, which is the wavelength that has the largest absorption difference between protonated and non-protonated cytosines.[4] From the results of the T_m experiment (Fig. S6), a T_m of 93°C is observed at pH 5.5, suggesting a dissociation of the triplex into its individual strands. Comparatively, the T_m at pH 7.0 is much lower at 49°C, suggesting the dissociation of the SPOC probe from the duplex RNA. The presence of hypochromism at 300 nm at pH 7.0 suggests that even at a neutral pH, the probe contains protonated cytosines, which would indicate that triplex formation is indeed happening to some extent.

Table S2 T_m values from of bacterial rRNA in the presence and absence of the SPOC probe at pH 5.5 or pH 7.0.

	pH 5.5		**pH 7.0**	
Sample	rRNA Only	rRNA + Probe	rRNA Only	rRNA + Probe
T_m / °C	74	93	74	49

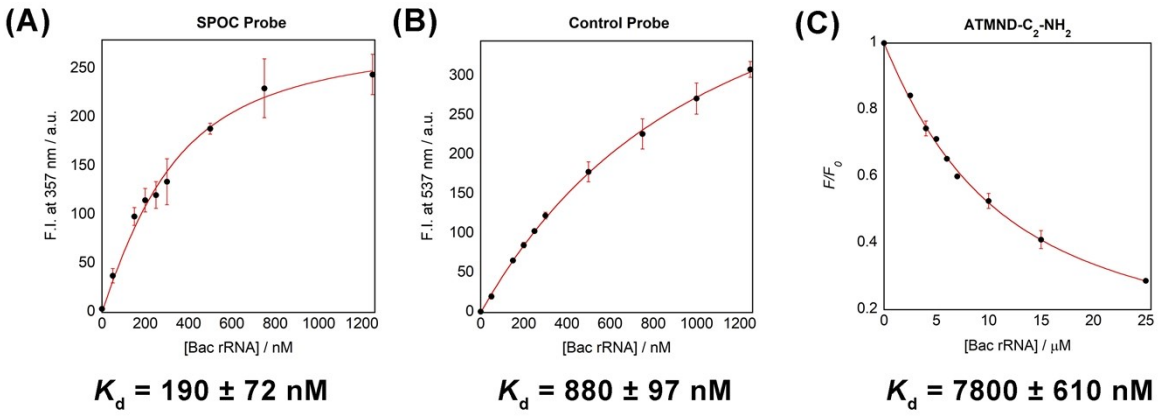

Fig. S7 Concentration dependence curves of **(A)** the SPOC probe, **(B)** the control probe and **(C)** ATMND-C_2-NH_2 with bacterial rRNA A-site. (A) [SPOC probe] = 250 nM, [Bac rRNA] = 0 – 1250 nM, Ex wavelength: 515 nm. (B) [Control Probe] = 250 nM, [Bac rRNA] = 0 – 1250 nM, Ex wavelength: 518 nm. (C) [ATMND-C_2-NH_2] = 5 μM, [Bac rRNA] = 0 – 25 μM, Ex wavelength: 358 nm, F.I. values at 407 nm were used to plot the curve. Error bars were plotted from three independent measurements.

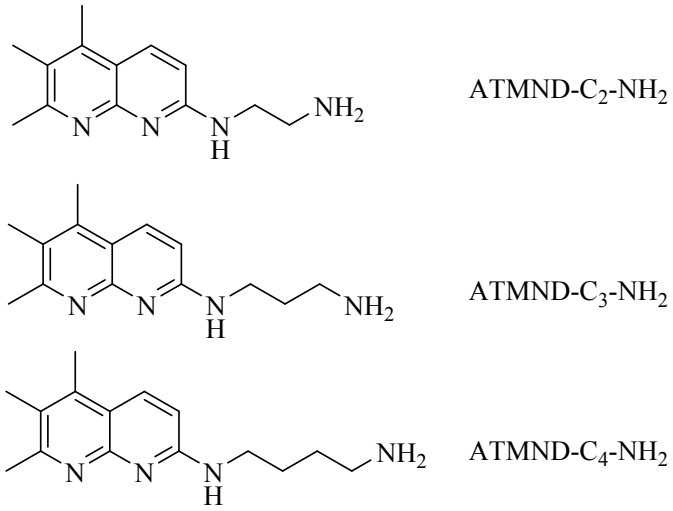

Fig. S8 Chemical structures of ATMND-C_n-NH_2 where n = 2, 3 and 4. These molecules were used to synthesise SPOC probes to optimise the linker length of ATMND-C_n-NH_2.

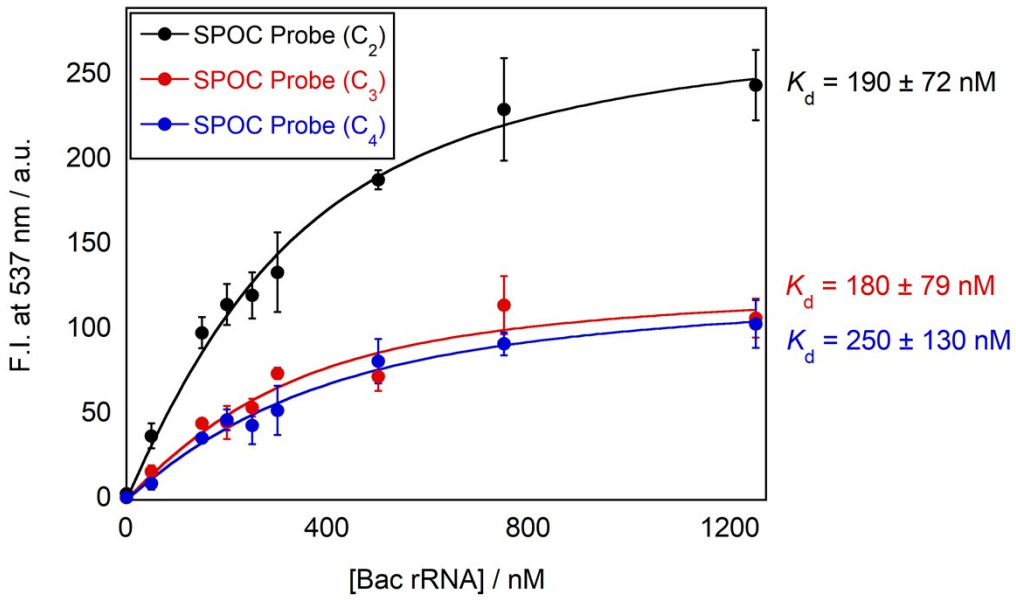

Fig. S9 Concentration dependence curves of the SPOC probe with ATMND-C_n-NH_2, where n=2, 3 and 4 (250 nM) with bacterial rRNA at 25°C, pH 7.0. Excitation wavelength: 515 nm. The error bars were plotted using results from three independent measurements. Calculated K_d values are also shown.

Table S3 Experimental K_d values of the SPOC probe (with ATMND-C_2-NH_2) and ATMND-C_2-NH_2 with bac, cyto and mito rRNAs.

K_d / μM	Bac rRNA	Cyto rRNA	Mito rRNA
SPOC Probe	0.19 ± 0.07	1.40 ± 0.55	1.50 ± 0.16
ATMND-C_2-NH_2	7.8 ± 0.61	32 ± 9.1	48 ± 29

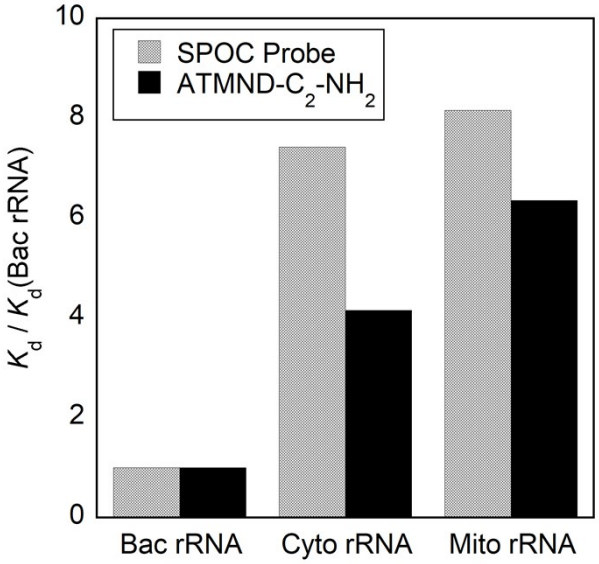

Fig. S10 Plot comparing the selectivity of the SPOC probe and ATMND-C_2-NH_2 with bac, cyto and mito rRNAs. The values were normalised by taking both molecules K_d values with bac rRNA to be 1. The conjugate probe has a K_d value with bac rRNA up to 8 times lower compared to the other two rRNAs, while ATMND-C_2-NH_2 has a K_d value with bac rRNA that is only up to 6 times lower.

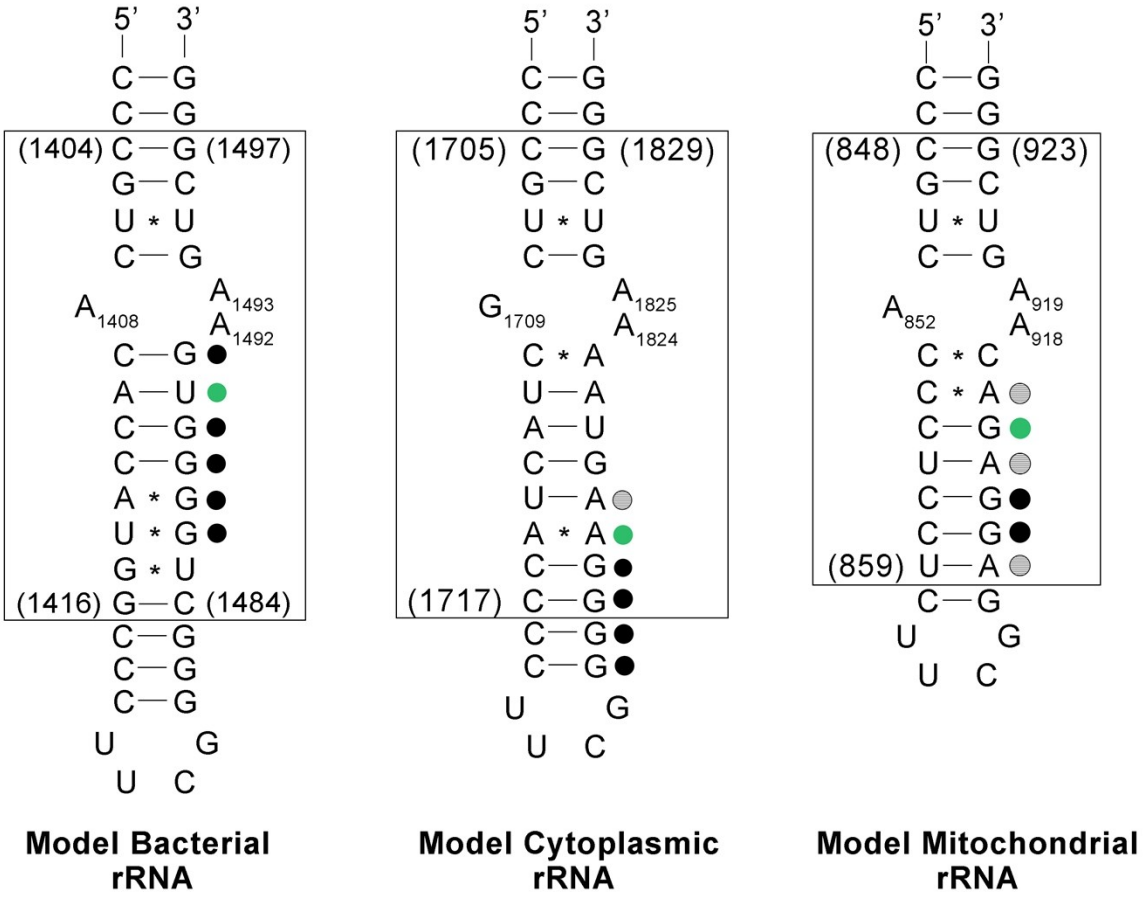

Fig. S11 Model rRNA sequences with proposed binding regions indicated using dots. Filled dots = match. Hashed dots = mismatch. Green dot = Thiazole orange fluorophore. Boxed region indicates the native sequence of the rRNA. Numbering used here follows native sequence numbering.

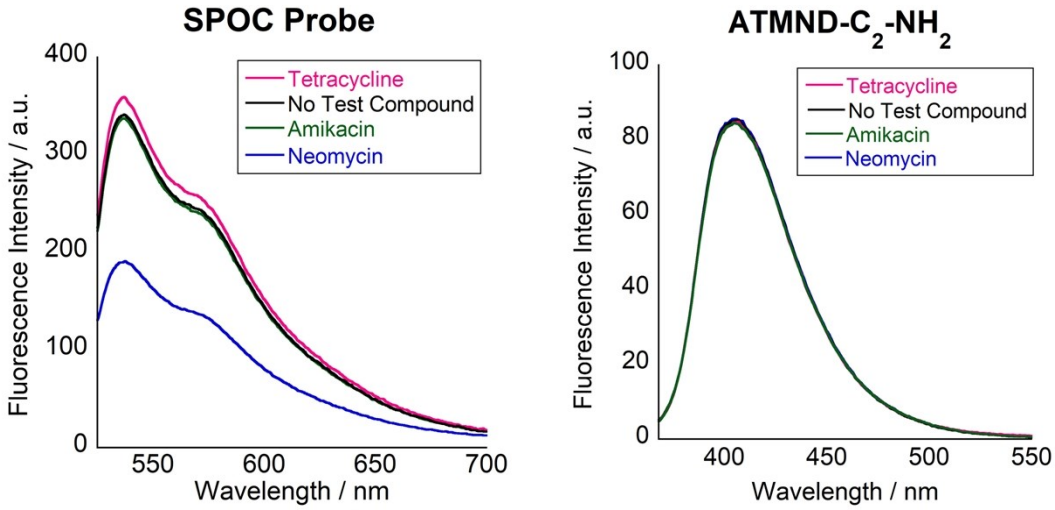

Fig. S12 Fluorescence response of the SPOC probe (100 nM) and ATMND-C_2-NH_2 (100 nM) with Bac rRNA A-site (100 nM) in the presence and absence of test compounds (500 nM) at 25°C, pH 7.0. Ex wavelength: 515 nm (SPOC probe), 358 nm (ATMND-C_2-NH_2).

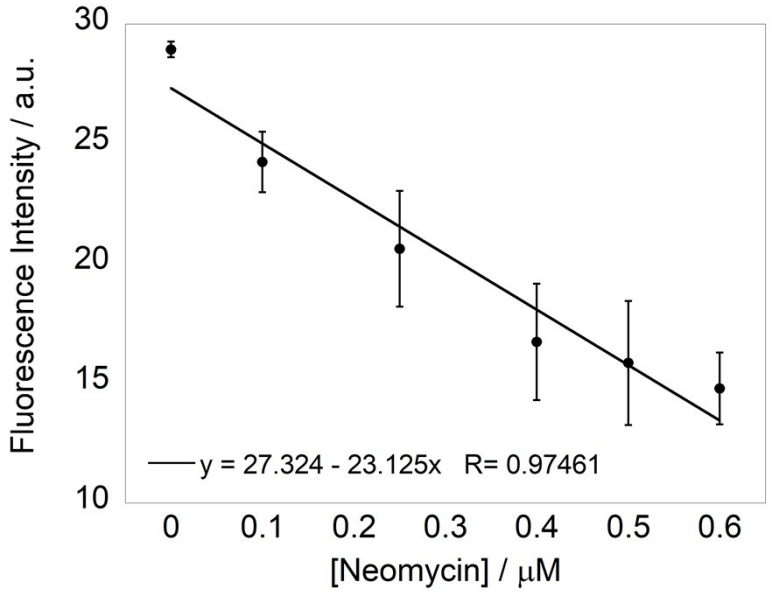

Fig. S13 Calibration curve for calculating the limit of detection (LOD) of the FID assay with neomycin at 25°C, pH 7.0. [SPOC Probe] = [Bac rRNA] = 0.10 μM, [Neomycin] = 0 – 0.60 μM. Error bars were plotted from three independent experiments. Exitation, 515 nm. Analysis, 537 nm.

To calculate the LOD of the FID assay with neomycin, the following equation was used:

$$LOD = 3(\frac{S_{blank}}{Slope})$$

where S_{blank} is the standard deviation of the blank, and the *slope* refers to the slope of the calibration curve.

The absolute value of the calculated slope was 23.1, and the standard deviation of the blank was 0.327.

As such, the calculated LOD is 42 nM (to 2 s.f.).

References

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