Electronic Supplementary Material (ESI) for Analyst.


**Supporting Information for**

# Forced intercalation-induced light-up peptides as fluorogenic indicators for HIV-1 TAR RNA-ligand assay

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*Experimental*

**Reagents:** All of the RNAs were custom-synthesized and HPLC-purified by GeneDesign Inc. (Osaka, Japan). Fmoc-protected amino acids with L-configuration were purchased from Watanabe Chemical Industries (Hiroshima, Japan) or AAPPTec (Luuisville, KY, U.S.A.). Recombinant Tat protein was purchased from ProSpec (Rehovot, Israel). FRET probe (5-FAM-AAARKKRRQRRRAAAK-TAMRA) was purchased from Hipep Laboratories (Kyoto, Japan). Other reagents were commercially available analytical grade and were used without further purification. The concentrations of RNAs were determined according to the litarature.[S1] MALDI-TOF-MS spectra were recorded on a Bruker Daltonics autflex Speed-S1 (Bruker, Germany). Water was deionized (≥18.0 MΩ cm specific resistance) by an Elix 5 UV water purification system and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA, USA), followed by filtration through a BioPak filter (Millipore Corp.) in order to remove RNase.

Unless otherwise mentioned, all measurements were performed at 25°C in 1×PBS buffer (pH 7.4). Before measurements, annealing of RNA-containing samples was conducted as follows: heated at 95°C for 10 min, and gradually cooled to 25°C (1°C/min).

Errors denote the standard deviations obtained from at least three independent experiments.

**Probe synthesis:** All probes were synthesized by Fmoc solid phase peptide synthesis using Biotage Initiator + Alstra microwave-assisted peptide synthesizer (Biotage, Uppsala, Sweden) on a Rink-Amide-ChemMatrix resin (Biotage). Fmoc-Dap (Alloc)-OH was utilized for introducing the TO unit. 1-[(1-(cyano-2-ethoxy-2-oxoethylideneamino-oxy)-dimethylamino-morpholino methylene)]methanaminium hexafluorophosphate (COMU) / diisopropylethylamine (DIEA) system was employed for the coupling reaction. After completion of the elongation of all amino acid residue units, the alloc group of Dab unit was selectively deprotected with 8 equimolar borane dimethylamine complex and an equimolar tetrakis(triphenylphosphine) palladium (0) in dichloromethane. Then, TO-C1-COOH[S1] was coupled with the resulting free amine. The deprotection of the peptide probes and the cleavage from the resin were conducted using trifluoroacetic acid/triisopropylsilane/water (95/2.5/2.5). The solution was dropped into cold diethyl ether in order to precipitate the crude peptide probe. The obtained crude product was purified by a reverse-phase HPLC system (pump, PU-2086 Plus ×2; mixer, MX 2080-32; column oven, CO-1565; detector, UV-2070 plus and UV-1570M (Japan Spectroscopic Co. Ltd., Tokyo, Japan)) equipped with a C18 column (Inertsil ODS3; GL Sciences Inc., Tokyo, Japan) using a gradient of water/acetonitrile containing 0.1% TFA. The probe was verified by MALDI-TOF-MS.

**Fluorescence measurements:** Fluorescence spectra were measured with a JASCO model FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan) equipped with thermoelectrically temperature-controlled cell holders. Excitation wavelength of the probes was set at the maximum absorption wavelength: 524 nm (FiLuP and FiLuP ($R_{52}$ substitution)) and 521 nm (Conventional probe and FiLuP (Lys linkage)). Fluorescence response of the probes was analyzed based on the maximum emission wavelength: 541

nm (FiLuP), 534 nm (Conventional probe and FiLuP (Lys linkage)). Excitation and emission wavelength for FRET probe was set at 497 nm and 583 nm, respectively. Fluorescence titration experiments at different NaCl concentrations were conducted using a microplate reader (Tecan Infinite 200 Pro; Tecan, Switzerland).

Measurements of fluorescence spectra were done using a 3 × 3 mm quartz cell. Dissociation constant ($K_d$) for the binding of the probes for target RNA was determined by fluorescence titration experiments, where the probe concentration was fixed and the concentration of the target RNA was varied. The changes in the fluorescence intensity of the probe at the maximum wavelength were monitored as a function of the concentration of the target RNA. The resulting titration curves were analyzed by nonlinear least-squares regression based on a 1:1 binding fitting model for determination of $K_d$ value according to the literature.[S2] Absolute fluorescence quantum yield ($\Phi$) of FiLuP in the absence and presence of TAR RNA ([probe] = 300 nM, [TAR RNA] = 2250 nM) was determined by Quantaurus-QY C11347-01 (Hamamatsu Photonics, Shizuoka , Japan).

**Salt dependence of the binding affinity:** The 1:1 binding constants ($K_a$) of FiLuP to TAR RNA were determined by fluorescence titration experiments in 10 mM sodium phosphate buffer containing different NaCl concentrations (pH 7.4). The obtained data were analyzed according to the polyelectrolyte theory proposed by Record *et al*. (ref. 27 in the main text). The observed salt dependence of the binding constants was explained by eq. (1).

$$\delta \log K_a / \delta \log [Na^+] = SK \qquad (1)$$

The slope (*SK*) of the plot was associated with the number of counterions released from the nucleic acids upon ligand binding. *SK* value was then used to evaluate the polyelectrostatic contribution ($\Delta G_{pe}$) to the observed binding free energy ($\Delta G_{obs} = -RT \ln K_a$) using the following equation: $\Delta G_{pe} = -(SK) \ln[Na^+]$.

**FID assay:** The FID assay was carried out with FiLuP as a fluorescence indicator. After FiLuP/TAR RNA complex was formed by annealing procedure as shown above, test compounds were added and incubated for 10 min. Fluorescence response of FiLuP/TAR RNA complex to test compounds was then measured with a JASCO model FP-6500 spectrofluorophotometer using a 3 × 3 mm quartz cell.

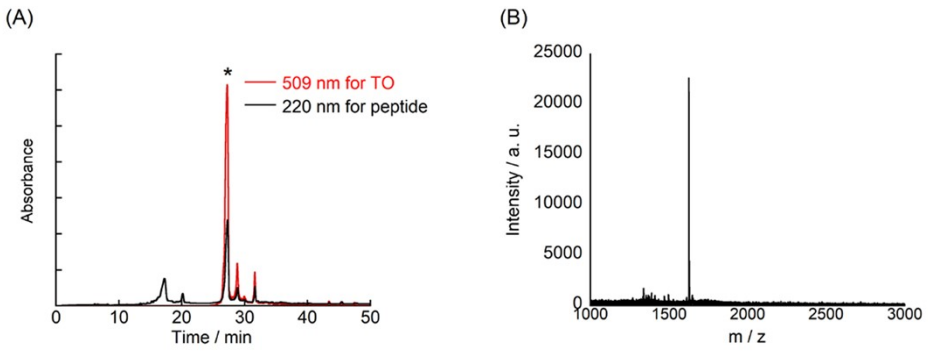


Figure S1. (A) HPLC profile for the purification of FiLuP. Gradient condition: 0-50% $CH_3CN$ (0.1%TFA) in $H_2O$ (0.1% TFA) during 50 min. Absorbance at 509 nm and 220 nm for TO unit and peptide unit, respectively, were monitored. The peak (*) was collected and used for MALDI-TOF-MS. (B) MALDI-TOF-MS spectrum of the purified FiLuP.

Table S1. Probe characterization

| | Sequence | Observed mass (m/z) | Calculated mass $[M+H]^+$ |
|---|---|---|---|
| FiLuP | RKKRR-(Dap-TO)-RRR | 1628.82 | 1627.97 |
| Conventional probe | (TO-Lys)-RKKRRQRRR | 1797.49 | 1798.07 |
| FiLuP ($R_{52}$ substitution) | RKK-(Dap-TO)-RQRRR | 1598.99 | 1599.96 |
| FiLuP (Lys linkage) | RKKRR-(Lys-TO)-RRR | 1670.18 | 1670.02 |

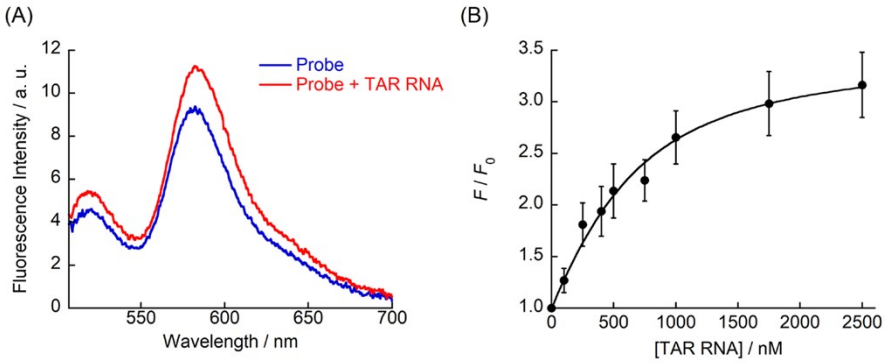


Figure S2. (A) Fluorescence spectra of FRET probe (50 nM) in the (a) absence and (b) presence of 50 nM TAR RNA. (B) Fluorescence titration curve for the binding of FRET probe (500 nM) to TAR RNA (0-2500 nM). Measurements were performed at 25°C in 1×PBS buffer (pH 7.4). Excitation, 497 nm. Analysis, 585 nm.

As observed for the literature (ref.11 in the main text), FRET probe showed the increase in the emission of TAMRA unit ($\lambda_{em}$ = 585 nm) upon binding to TAR RNA whereas the emission of fluorescein also increased. According to the FID assay using this probe (ref.11 in the main text), we analyzed the change in the emission of TAMRA unit-as the fluorescence response of the probe for TAR RNA.

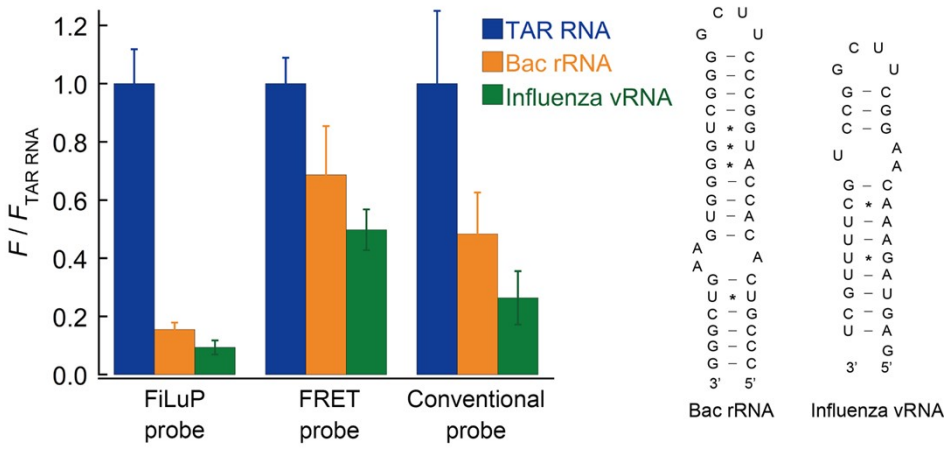


Figure S3. Selectivity of the probes for TAR RNA over bacterial rRNA A-site (Bac rRNA) and influenza virus A promoter RNA (influenza vRNA). $F$ and $F_{\text{TAR RNA}}$ denote the fluorescence intensity of the probe in the presence of target RNA and TAR RNA, respectively. RNA sequences of Bac rRNA and influenza vRNA are also shown. Excitation: 524 nm (FiLuP), 497 nm (FRET probe), 521 nm (Conventional probe). Analysis: 541 nm (FiLuP), 585 nm (FRET probe), 534 nm (Conventional probe).

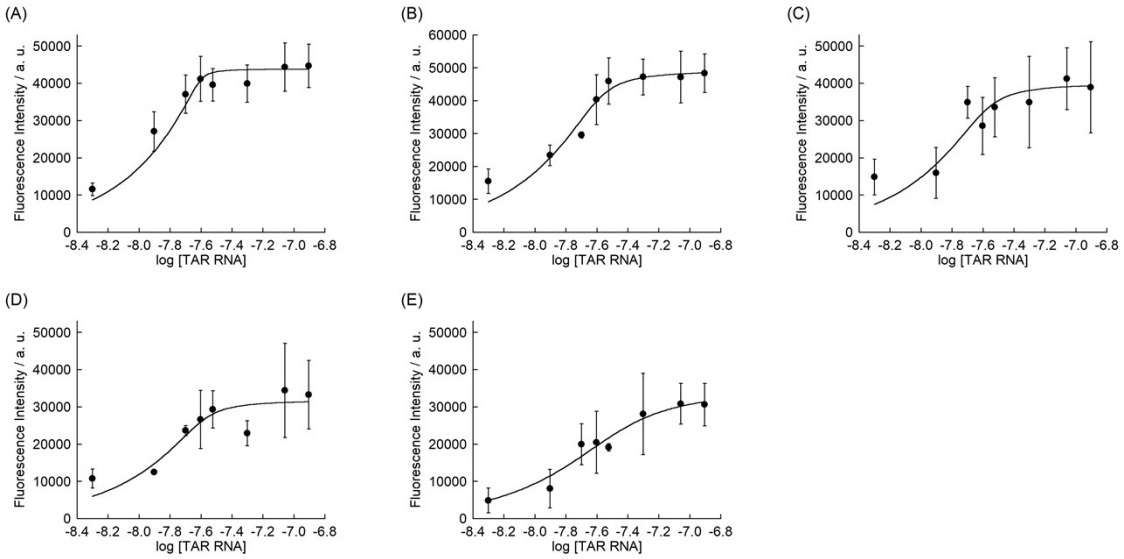


Figure S4. Fluorescence titration curves for the binding of FiLuP (25 nM) to TAR RNA (0-125 nM) in 10 mM phosphate buffer with different NaCl concentration ((A) 100 mM, (B) 200 mM, (C) 300 mM, (D) 500 mM, (E) 700 mM). Excitation, 524 nm. Analysis 541 nm.

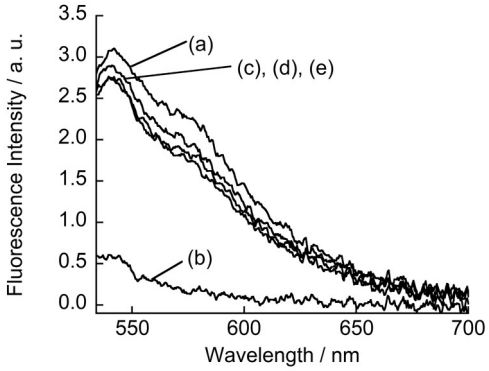


Figure S5. Fluorescence spectra of 2 nM TAR RNA/FiLuP complex in the (a) absence and presence of 120 nM test compounds: (b) Tat protein, (c) mitoxanthrone, (d) ICR191 and (e) neomycin. Excitation, 524 nm.

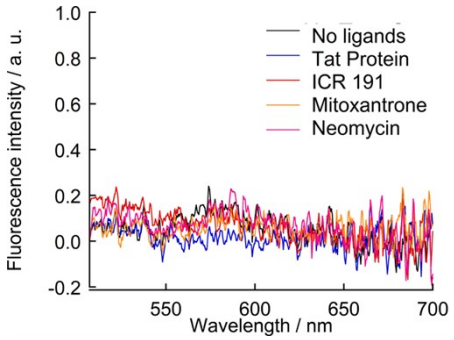


Figure S6. Fluorescence response of FRET probe (2 nM)/TAR RNA (2 nM) complex for test compounds (120 nM). Excitation, 497 nm.

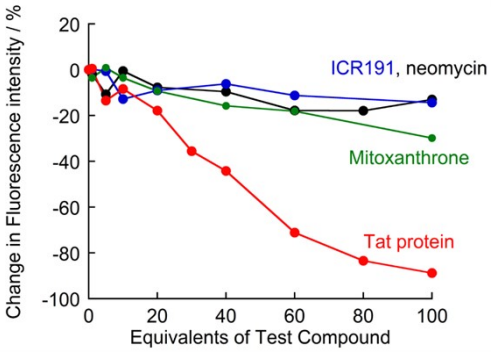


Figure S7. Change in fluorescence intensity at 541 nm against the number of equivalents of the test compounds. For the experiment with Tat protein, [FiLuP] = [TAR RNA] = 2 nM, [Tat Protein] = 0 – 200 nM. For the rest of the test compounds, [FiLuP] = [TAR RNA] = 10 nM, [Test Compound] = 0 – 1000 nM. Excitation, 524 nm.

**References**

S1. Lee, E. T. T.; Sato, Y.; Nishizawa, S. *Chem. Commun.*, **2020**, *56*, 14976-14979.

S2. Sato, T.; Sato, Y.; Nishizawa, S. *J. Am. Chem. Soc.*, **2016**, *138*, 9397-9400.