Supporting Information

Highly selective dual sensing of ATP and ADP by fluorescent ribonucleopeptide sensors

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Experimental Procedures

Material and Methods

Materials

PrimeSTAR HS DNA polymerase for PCR reactions was obtained from TaKaRa Bio Inc. (Shiga, Japan). T7-Scribe Standard RNA IVT Kit was obtained from CELLSCRIPT (Madison, USA). N-a-Fmoc-protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), N, N-diisopropylethyl amine (DIEA), distilled N, N-dimethylformamide (DMF), diisopropylcarbodiimide (DIC), N, N-dimethyl-4-aminopyridine (DMAP), and 4-Hydroxymethylbenzoic acid PEG resin (HMBA-PEG resin) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Dichloromethane (DCM) and HPLC-grade acetonitrile were purchased from Nacalai Tesque (Kyoto, Japan). 6-Carboxyfluorescein N-succinimidyl ester and 1-pyrenesulfonyl chloride were from Life technologies™, Molecular Probes® (Grand Island, NY, USA). A reversed-phase C18 column (4.6 × 150 mm, ULTRON-VX ODS; Shinwa Chemical Industries, Kyoto, Japan) and a COSMOSIL PBr column (4.6 × 150 mm, Nacalai Tesque) were used for purification of peptides for preparative purposes or analysis of nucleotides. Sodium periodate, hydrazine, gel electrophoresis grade acrylamide, bisacrylamide, phenol, thioanisol, and 1,2-ethanditiol were purchased from Wako Pure Chemical industries Ltd (Tokyo, Japan). Adenosine triphosphate (ATP) , adenosine diphosphate (ADP) adenosine monophosphate (AMP), 3', 5'-cyclic AMP (cAMP) and creatine phosphokinase from rabbit and Phosphocreatine disodium salt hydrate were purchased from MilliporeSigma (USA).

Preparation of RNA subunit of RNP receptors

The double-stranded DNA templates for RNA transcription were constructed by PCR amplification to add the promoter for T7 RNA polymerase using PrimeSTAR DNA polymerase (TaKaRa) with 5'-DNA primer and 3'-DNA primer shown in Table S1. RNA transcription was performed using an T7-Scribe Standard RNA IVT Kit (CELLSCRIPT) for 3 h at 37 °C, according to the supplier's recommended protocols. The resulting RNA was purified by denaturing PAGE (8M urea, 12%) and eluted. Concentration of RNA was determined by UV spectroscopy with excitation coefficient at 260 nm (sATP-RRE-I ε = 711,900 M^{-1} cm^{-1}, sATP-RRE-II ε = 696,600 M^{-1} cm^{-1}, sATP-RRE-III ε = 723,300 M^{-1} cm^{-1}, sATP-RRE-VII ε = 820,300 M^{-1} cm^{-1}, sADP-RRE ε = 559,000 M^{-1} cm^{-1}).

Table S1. Nucleotide sequences of RNAs, 5'-DNA primers and 3'-DNA primers

Sequences of RNAs:	
sATP-RRE-I	GGGAGAUCUACGGAUCUCAGGGUCUGGGCGCAUACGUGACGGUACAGGCUACAUGGAAGGAGUCCAUGUGU
sATP-RRE-II	GGGAGAUCUACGGAUCUCAGGUGACGGUACAGGUACGUCUGGGCGCAUACAUGGAAGGAGUCCAUGUGU
sATP-RRE-III	GGGAUGACGGUACAGGCUACGGUCUGGGCGCAUCAGGGCUCUUACGGGAGCUACAUGGAAGGAGUCCAUGUGU
sATP-RRE-VII	GGUCUGGGCGCA GGGAGAUCUACGGAUCUCAG GGCUCU UACG GGAGCU ACAUGGAAGGAGUCCAUGUGU UGACGGUACAGGCC
5'-DNA primers (T7 RNA promoter is underlined)	
For-ATP(P)-RRE	TCTAATACGACTCACTATAGGGAGATCTACGG
For-ATP(P)-RRE-02	TCTAATACGACTCACTATAGGGATGACGGTAC
For-s2	TCTAATACGACTCACTATAGGTCTGGGCGCA
3'-DNA primers	
Rev-ATP(P)-RRE-02	ACACATGGACTCC
Rev-s2	GGCCTGTACCGTC

Construction of Noncovalent Fluorescent RNP Sensors

The nucleotide sequences of RNA subunit of RNP sensors used in this paper were shown in Table S1. For the conversion of synthesized RNAs to noncovalent fluorescent RNP sensors, 1 μM RNA subunit was mixed with 1 μM fluorophore-modified Rev peptide to form a noncovalent complex.

Construction of a Stable Fluorescent RNP Sensor by the Covalent Linking Method

Constructions of covalently-linked RNP sensors were performed as previously described[1] with a few modifications. An RNA subunit (2 nmol) purified by denaturing 12% polyacrylamide gel electrophoresis was treated with freshly prepared 50 eq. of 0.01 M sodium periodate (10 μL; 100 nmol) to convert the cis-diol of the 3-terminal ribose to 3-dialdehyde by periodate oxidation in total 50 μL of 0.03 M sodium phosphate (pH 5.2) solution. The reaction mixture was incubated for 1 hour at 37°C in dark. After the reaction, an excess of sodium periodate was reduced by adding glycerol (final concentration 1M), and the resulting oxidized RNA was purified by ethanol precipitation.

The peptide subunit for the formation of a covalent linkage was synthesized as previously described.[1] A fluorophore was introduced at the N-terminal of Rev peptide and the peptide linker (GS4; GGSGGSGGSG) with a reactive hydrazide group was introduced at the C-terminal (F-Rev-GS4-HZ; Fluorophore-TRQARRNRRRRWRERQR GGSGGSGGSG-hydrazide). The N- and C-terminal modified peptide was purified by reversed-phase HPLC, and characterized by MALDI-TOF mass spectrometry (AXIMA-LNR, Shimadzu). A coupling reaction between the 3'-modified RNA (2 nmol, 40 μM) and fluorophore-modified Rev-GS4-HZ (4.4 nmol, 88 μM) was performed in 0.02 M sodium acetate (pH 5.2) containing 0.01 M NaCl (total 50 μL) at 37°C in dark. After 5 h, the reaction mixture was extracted by phenol-chloroform and then purified by ethanol precipitation. The sample solution was purified by 8 M urea 15% denaturing PAGE and was subsequently quantified by measuring the absorption at 260 nm (sATPRRE-I ε = 711,900 M^{-1} cm^{-1}, sATPRRE-II ε = 696,600 M^{-1} cm^{-1}, sATPRRE-III ε = 723,300 M^{-1} cm^{-1}, sADPRRE ε = 559,000 M^{-1} cm^{-1}). As a result, about 40 μL of 15 μM covalently linked RNP complex (c-sATPRRE-II /6FAM-Rev, c-sATPRRE-III/6FAM-Rev, c-sADPRRE/Pyr-Rev) was recovered (yield, 30%).

Fluorescence Measurements on the Microplate

Fluorescence measurements in 96-well plates were performed on a Tecan Infinite M200 plate reader. A binding solution (30 μL) containing noncovalent or covalent fluorescent RNPs in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20 with an determined amount of substrate was gently swirled for a few seconds and allowed to sit for 30 min at the indicated temperature. Respective excitation and emission wavelengths were Pyr-Rev (350, 390 nm), 6FAM-Rev (485, 535 nm), and 7mC-Rev (350, 390 nm).

Determination of Ligand-Binding Affinity

The ligand-binding affinity of fluorescent RNP was calculated by fitting the ligand titration data using the following equation:

$$F_{obs} = F_{min} + A(([FRNP]_T+[substrate]_T+K_D) - (([FRNP]_T+[substrate]_T+K_D)^2 - 4[FRNP]_T[substrate]_T)^{1/2})/2[FRNP]_T$$

where F_{obs} is the observed fluorescence intensity with each concentration of substrate, A is the increase in fluorescence at saturating substrate concentrations (F_{max} — F_{min}), K_D is the equilibrium dissociation constant, and $[FRNP]_T$ and $[substrate]_T$ are the total concentrations of fluorescent RNP and the substrate, respectively.

Simultaneous Detection of ATP and GTP in the Same Solution

Samples were prepared by mixing c-sATPRRE-II/6FAM-Rev (100 nM), c-sADPRRE/Pyr-Rev (100 nM) and the indicated substrates in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20. The samples were incubated for 30 minutes at 25 °C. Fluorescence intensities were recorded on a Tecan Infinite M200 plate reader. The excitation wavelengths were 485 nm for 6FAM-Rev, and 350 nm for Pyr-Rev. The emission wavelengths were 535 nm for 6FAM-Rev and 390 nm for Pyr-Rev and were used for the determination of relative fluorescence intensity.

Simultaneous Detection of a Substrate ADP and a Product ATP in an Enzymatic Reaction by Covalently Linked Fluorescent RNP Sensor

A sample (30 µL) was prepared by mixing 100 nM c-sATPRRE-II/6FAM-Rev, 100 nM c-sADPRRE/Pyr-Rev, and 1000 µM ADP and 2000 µM phosphocreatine in 50 mM Tris-HCl (pH 7.6) buffer containing 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20. The sample was incubated for 30 min at 25 °C, and then 30 mU of creatine phosphokinase (CPK) was added to start the reaction. For the detection of changes in the concentrations of ATP and ADP, the fluorescence intensities were measured with Tecan Infinite M200 plate reader with an excitation and emission bandwidth of 5 nm (ex.: 485 nm, em.: 535 nm for 6FAM-Rev; ex.: 350 nm, em.: 380 nm for Pyr-Rev) with reaction time. Amounts of the reacted substrate (ADP) and the product (ATP) were converted using the standard curves calculated from the results of fluorescence spectroscopy by using the dual sensors for each substrate with the known concentration. The actual amounts of ATP and ADP with the reaction of CPK were measured by HPLC analysis for comparing to the results traced by the cRNP sensors.

Conditions of HPLC analysis for quantification of ATP and ADP

ATP and ADP were analyzed by HPLC using C18 reverse-phage column (COSMOSIL PBr, 4.6 x 150 mm, particle size 5 µm, Nacalai Tesque). The mobile phase consisted of 20 mM sodium phosphate buffer, pH 7.5 (eluent A) and 20 mM sodium phosphate buffer, pH 7.5 / 50% MeOH (eluent B). Samples were eluted with the gradient of eluent B increased from keeping for 5 min. at 0% then to 10% in 10 min. The flow rate was 1 mL/min.

Supplemental Table and Figures

Table. S2 Equilibrium dissociation constants (K_D) and maximum relative fluorescence intensity (I / I_0) of sATP and sADP RNP sensors.

A

		Non-covalent RNP sensors			
		F-sATPRRE-RNPs			F-sADPRRE-RNPs
	Substrate	sATPRRE-I / 7mC-Rev	sATPRRE-II / 7mC-Rev	sATPRRE-III / 7mC-Rev	sADPRRE / Pyr-Rev
K_D (μM)	ATP	16	61	6.1	> 1000
	ADP	350	520	> 1000	10
I / I_0	ATP	1.7	2.8	1.5	-
	ADP	1.3	1.4	-	2.1

B

		Covalently-linked RNP sensors			
		c-sATP RNP sensors			c-sADP RNP sensors
	Substrate	c-sATPRRE-I / 6FAM-Rev	c-sATPRRE-II / 7mC-Rev	c-sATPRRE-III/7mC-Rev	c-sADPRRE / Pyr-Rev
K_D (μM)	ATP	-	102	19	> 1000
	ADP	-	> 1000	> 1000	30
I / I_0	ATP	-	1.3	1.3	-
	ADP	-	-	-	2.3

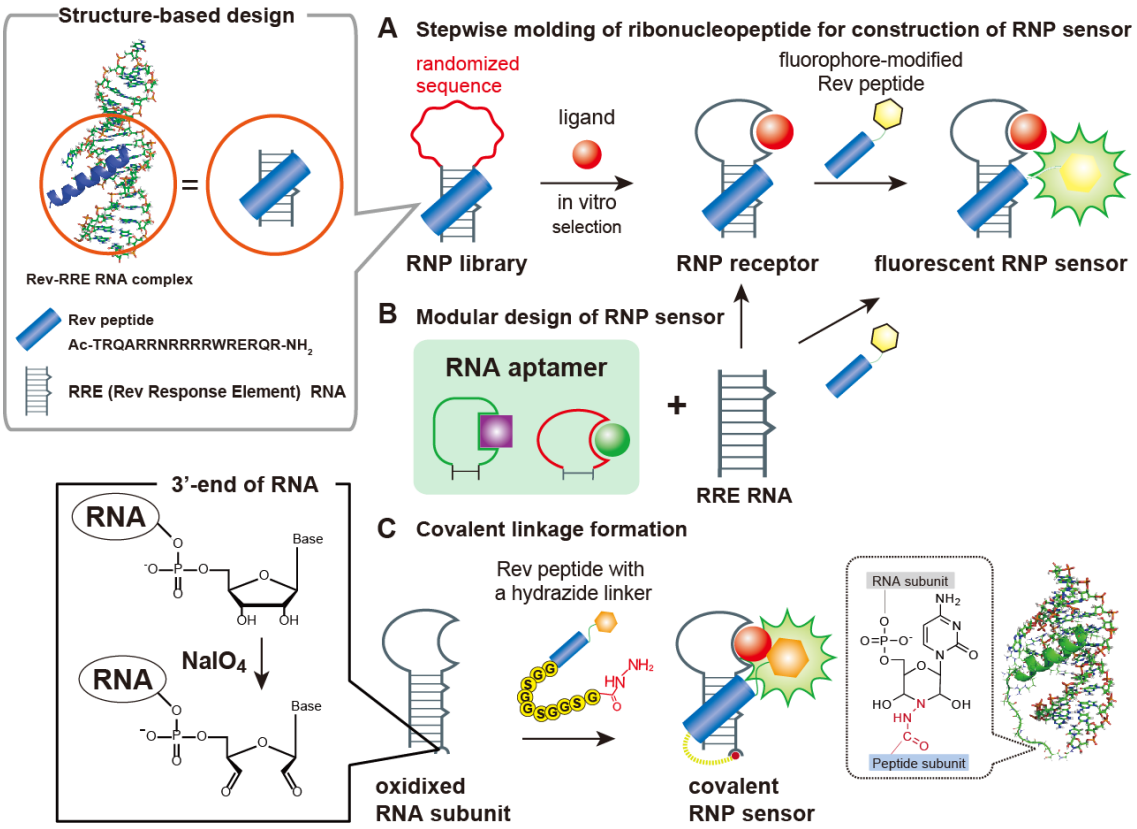

Fig. S1 Schematic illustrates of (A, B) methods for construction of fluorescent ribonucleopeptide (RNP) sensors and (C) a method for formation of a covalent linkage between RNA and peptide subunit. (A) RNP receptors can be selected from RNA-diverse RNP library by applying SELEX method and the receptor can be converted into fluorescent RNP sensor by complexing an RNA subunit of RNP receptor and a fluorophore-modified Rev peptide. (B) RNA aptamer can be utilized as the binding module to construct the RNP receptor and RNP sensor by linking the nucleotide sequence of RRE RNA and the parent RNA aptamer in a plausible stem structure. (C) A covalently-linked RNP can be constructed by mixing an RNA subunit oxidized by sodium periodate to form the di-aldehyde group at the 3'-end and a Rev peptide with a hydrazide group through a flexible peptide linker.

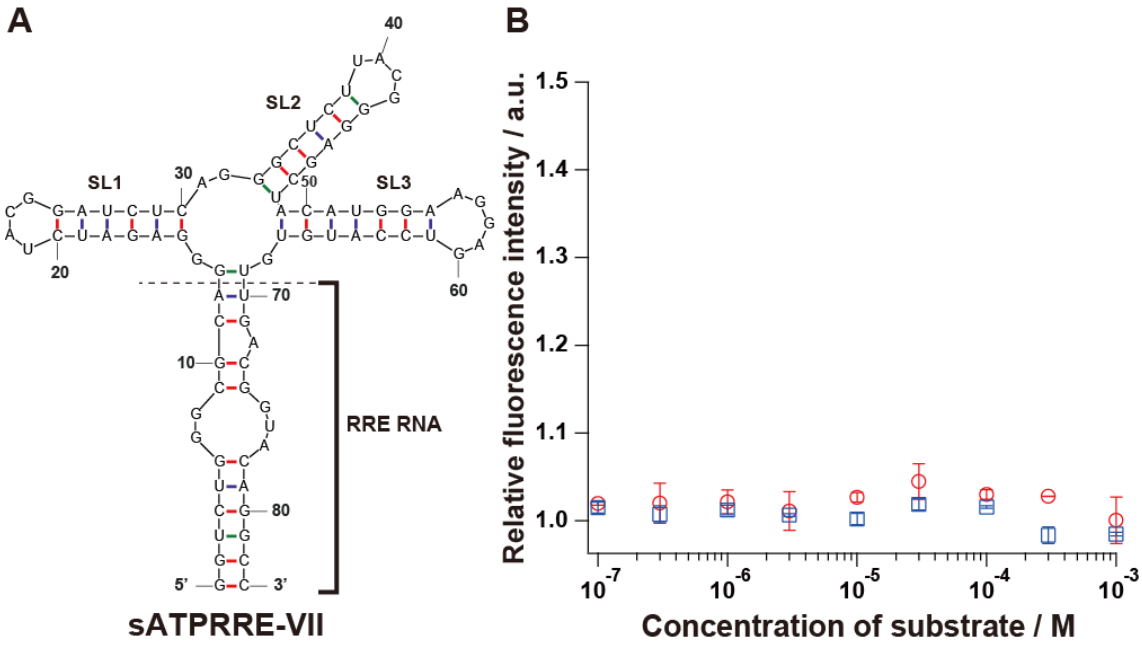

Fig. S2 (A) Estimated secondary structure of RNA subunit of ATP aptamer-conjugated RNP (sATPRRE-VII). The 5'- and 3'-terminal of Sazani's ATP aptamer and the stem-loop region of RRE RNA were directly connected. (B) Titration curves for relative fluorescence intensity change of sATPRRE-VII/7mC-Rev with ATP and ADP. Each experimental data point represents the average of two parallel measurements and the error bar shows standard deviation. The sensor did not show any fluorescence change with changes of ATP or ADP concentrations.

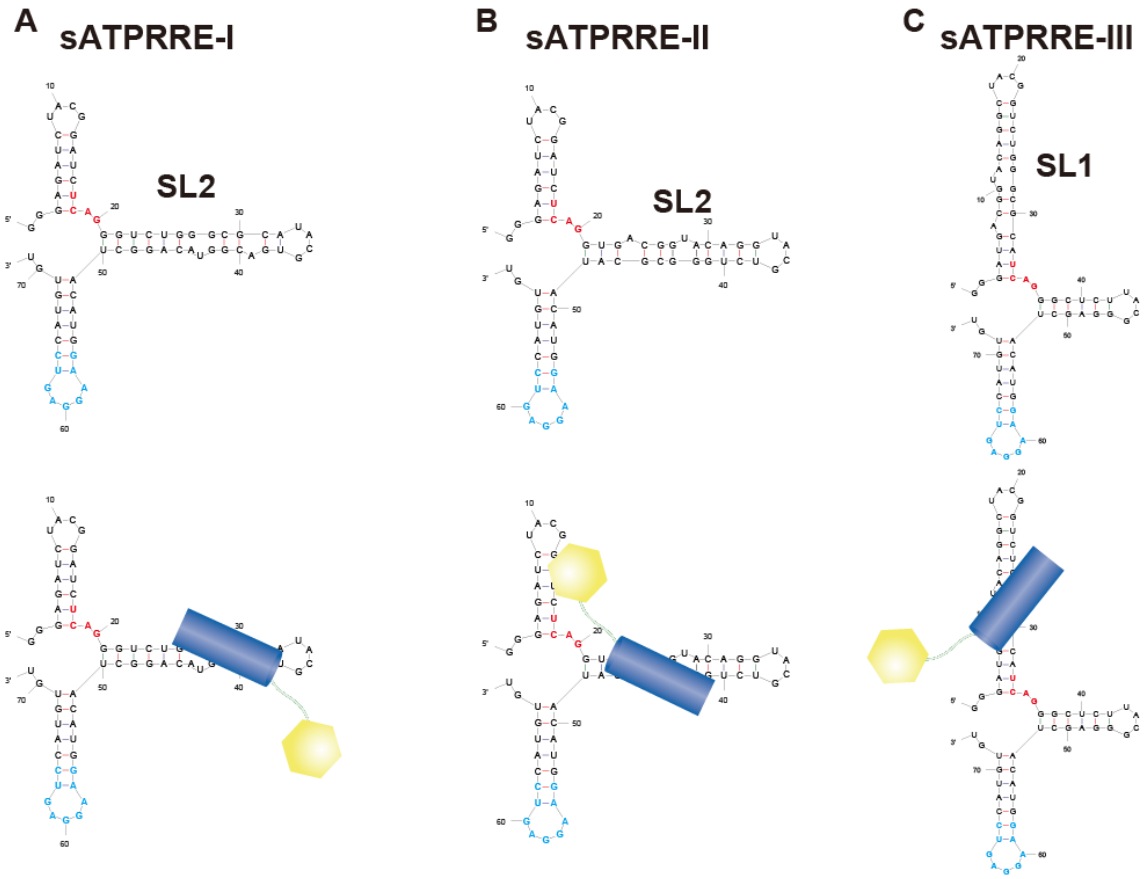

Fig. S3 Plausible secondary structures of RNA subunits of designed sATP RNP sensors (sATPRRE-I, sATPRRE-II and sATPRRE-III) (upper). The consensus sequences of RNA aptamers reported by Sazani *et al.*[2] showed in bold and colored by red and light blue. The lower illustrations showed expected interaction mode of the fluorophore modified-peptide of each sATP RNP sensors. The fluorophores were estimated to be in different positions toward the consensus sequences that should relate to form the binding pocket for the substrate ATP.

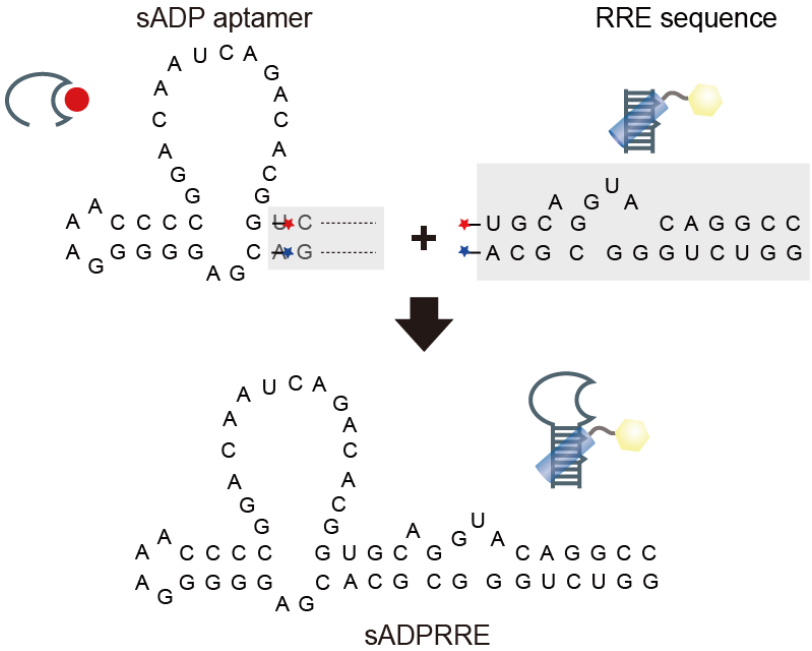

Fig. S4 Modular design of sADPRRE RNA by overlay the stem region of sADP aptamer and RRE RNA.

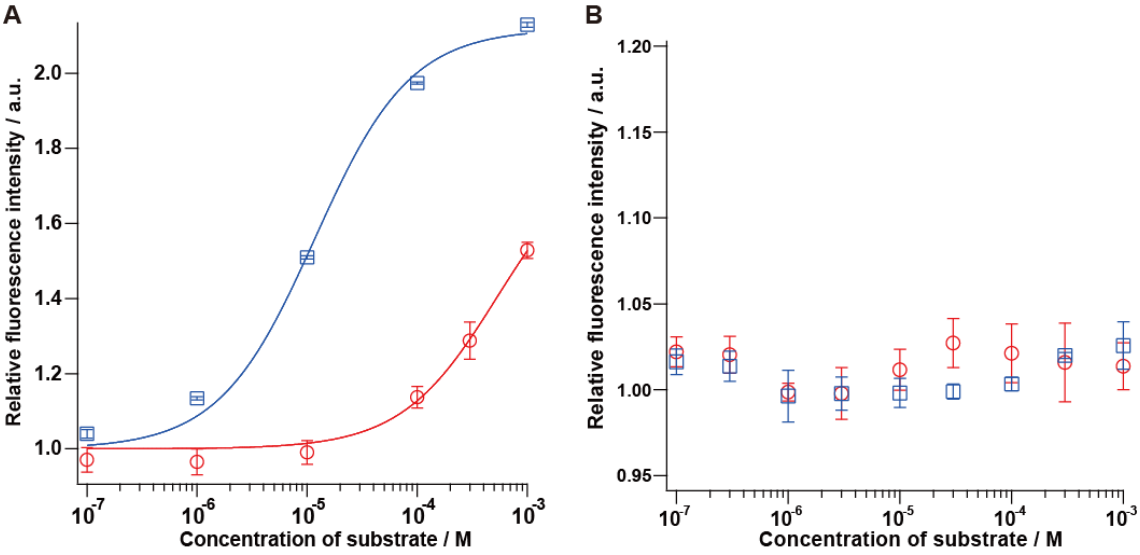

Fig. S5 Saturation curves for the relative fluorescence intensity changes of (A) non-covalent sADPRRE/7mC-Rev sensor (sADPRRE/7mC-Rev) and (B) covalently-linked sATPRRE-I/6FAM-Rev (c-sATPRRE-I/6FAM-Rev) titrated with ADP (blue squares) and ATP (red circles) in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20 at 25 °C. Each experimental data point represents the average of at least three parallel measurements and the error bar shows standard deviation.

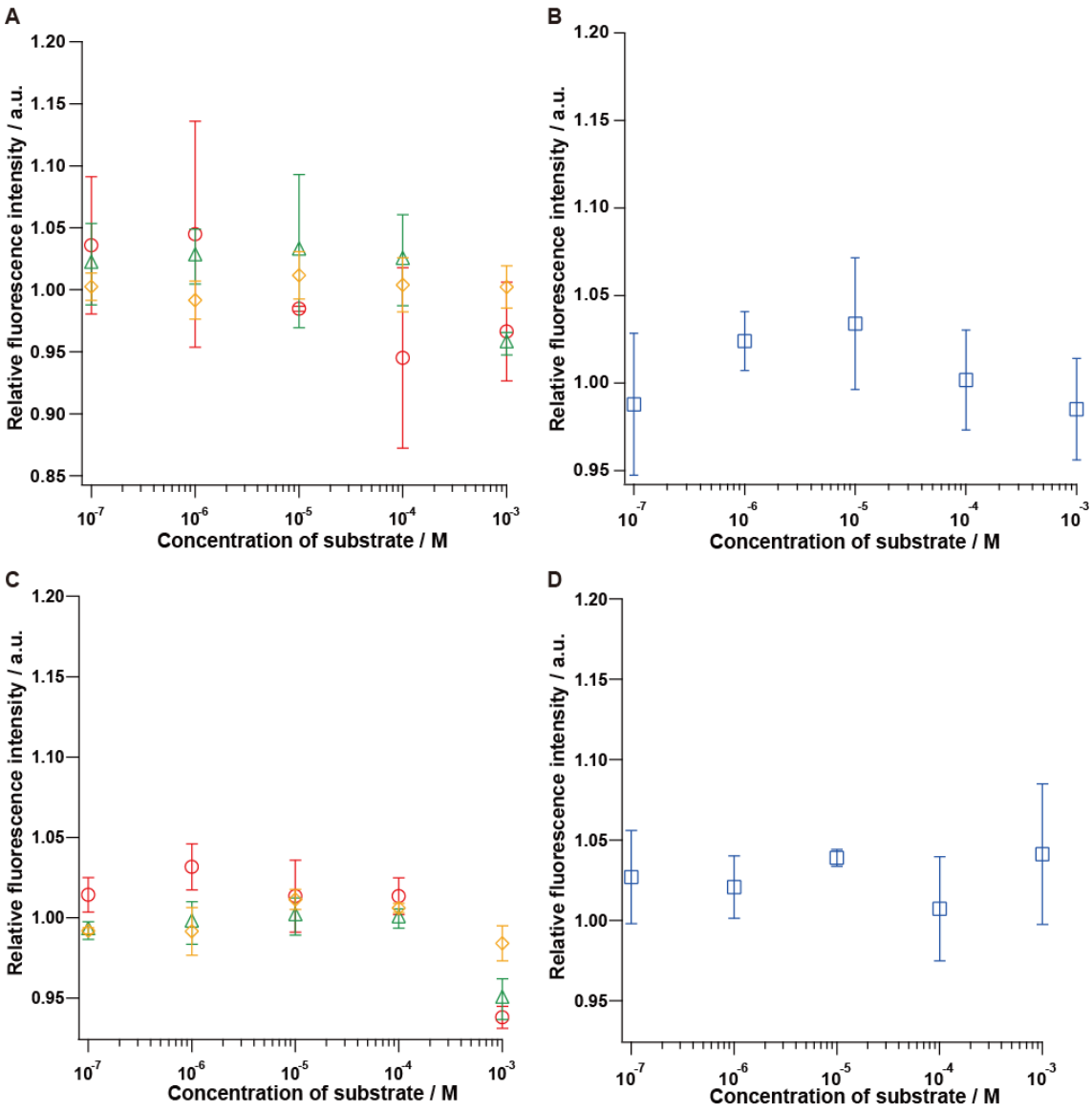

Fig. S6 Relative fluorescence intensity changes of covalently-linked sATPRRE-II/6FAM-Rev sensor (red circles), covalently-linked sATPRRE-III/6FAM-Rev (green triangles), covalently-linked sADPRRE/Pyr-Rev (blue squares), and noncovalent sATPRRE-I/7mC-Rev (orange diamonds) titrated with AMP (A, B) and cAMP (C, D) in a buffer containing 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20 at 25 °C. Each experimental data point represents the average of at least three measurements and the error bar shows standard deviation. All the sensors showed little or no fluorescence change with up to 1mM AMP and 3', 5'-cyclic AMP (cAMP).

A

	Concentration / M	Volume / L	Amount / mol	Peak area / a.u.					
				1	2	3	4	Average	SD
ATP	1.0E−04	5.00E−06	5.0E−10	1686366	1666791	1676704		1676620	9787.768
	5.0E−04	5.00E−06	2.5E−09	8241354	8152966	8130447		8174922	14477.4
	1.0E−03	5.00E−06	5.0E−09	16544290	16550093	17119163	16713573	16731780	292993.1
	3.0E−03	5.00E−06	1.5E−08	49054347	48251341	48739482		48681723	404606.9
ADP	1.0E−04	5.00E−06	5.0E−10	1661837	1690005	2090051		1675921	19917.78
	5.0E−04	5.00E−06	2.5E−09	8092925					
	1.0E−03	5.00E−06	5.0E−09	16130123	16119044			16124584	7834.036
	3.0E−03	5.00E−06	1.5E−08	48039553	50876349	48030354		48982085	1640487

B

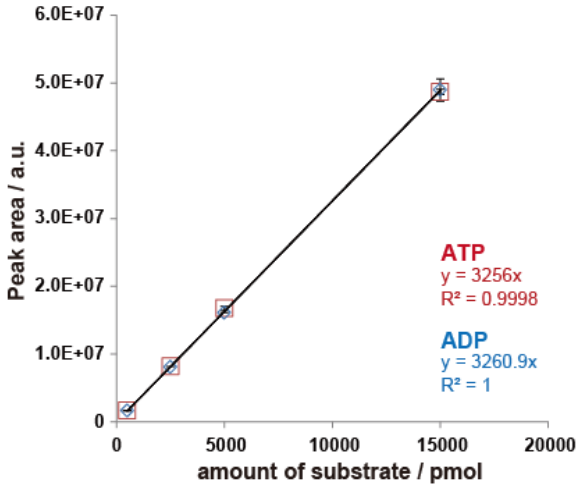

Fig. S7 (A) Quantitation of the peak area for given concentration and injection volume (100, 500, 1000, and 3000 μM, 5 μL) of ATP and ADP measured by HPLC analysis. The values were used for calculating standard curves for the quantitation of the creatine phosphokinase reaction. (B) The quantitative titration curves for conversion from the peak area to the amount of substrate, ATP (pink squares) and ADP (blue diamonds).

A

		Peak area /a.u.		Amount / pmol		Concentration / M (in 2.5 μL)			
	Time/min	1	2	1	2	1	2	Average	SD
ATP	5	2349899	1853693	721.7	569.3	2.9E-04	2.3E-04	2.6E-04	4.3E-05
	10	3728043	3494173	1145.0	1073.1	4.6E-04	4.3E-04	4.4E-04	2.0E-05
	15	3286832	3948526	1009.5	1212.7	4.0E-04	4.9E-04	4.9E-04	1.1E-04
		3619324	5255228	1111.6	1614.0	4.4E-04	6.5E-04		
	30	6353783	5958365	1951.4	1830.0	7.8E-04	7.3E-04	7.6E-04	3.4E-05
	45	7096144	7291064	2179.4	2239.3	8.7E-04	9.0E-04	8.8E-04	1.7E-05
	60	7622334	7373442	2341.0	2264.6	9.4E-04	9.1E-04	9.2E-04	2.2E-05
	90	7880276	7356604	2420.2	2259.4	9.7E-04	9.0E-04	9.4E-04	4.5E-05
ADP	5	6104073	5974839	1871.9	1832.3	7.5E-04	7.3E-04	7.4E-04	1.1E-05
	10	4842189	4846326	1484.9	1486.2	5.9E-04	5.9E-04	5.9E-04	3.6E-07
	15	5209213	4520797	1597.5	1386.4	6.4E-04	5.5E-04	5.0E-04	1.6E-04
		4431468	2281873	1359.0	699.8	5.4E-04	2.8E-04		
	30	2042762	2150017	626.4	659.3	2.5E-04	2.6E-04	2.6E-04	9.3E-06
	45	1415344	862107	434.0	264.4	1.7E-04	1.1E-04	1.4E-04	4.8E-05
	60	632568	367747	194.0	112.8	7.8E-05	4.5E-05	6.1E-05	2.3E-05
	90	620149	277798	190.2	85.2	7.6E-05	3.4E-05	5.5E-05	3.0E-05

B

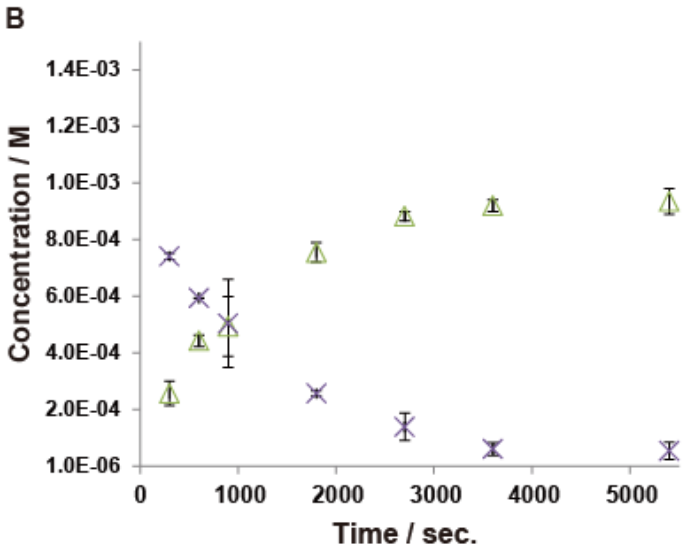

Fig. S8 (A) Peak areas and converted concentrations of the substrate (ADP) and the product (ATP) measured by HPLC analysis in the CPK reaction at a given reaction time (5, 10, 15, 30, 45, 60, and 90 min.). (B) The concentration changes of ATP (light green triangles) and ADP (purple crosses) in the CPK reaction with each determinant time. Each concentration was converted by using the quantitative titration curves (figure S5). A sample was prepared by mixing 1000 μM ADP and 2000 μM phosphocreatine in 50 mM Tris-HCl (pH 7.6) containing 100 mM NaCl, 10 mM $MgCl_2$, and 0.005% Tween 20. The sample was incubated for 15 min at 25°C, and then 30 mU of CPK was added to start the reaction. Each experimental data point represents the average of two or four parallel measurements as shown in (A) and the error bar shows standard deviation.

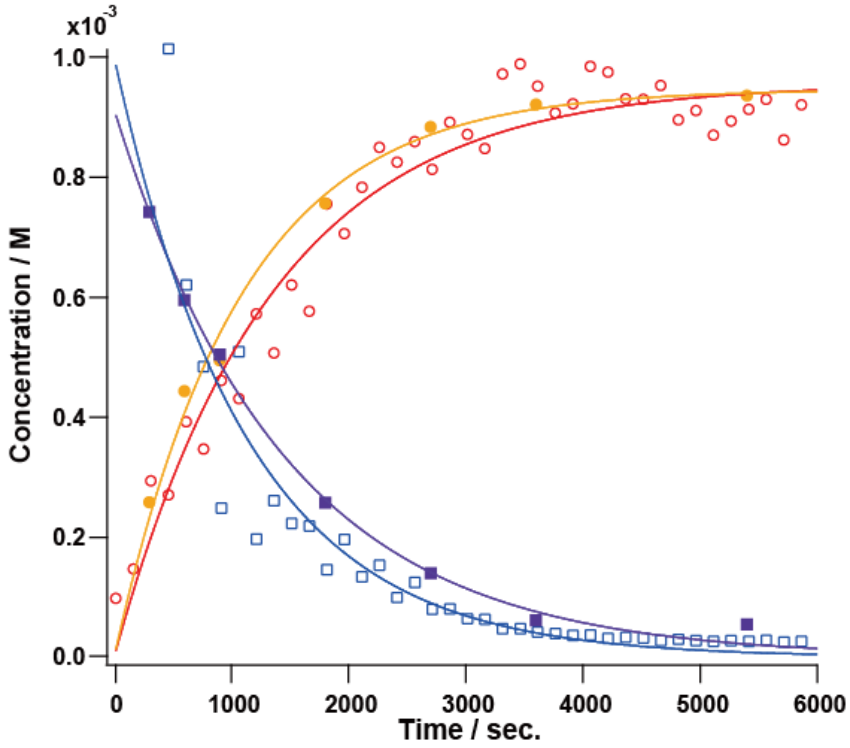

Fig. S9 Changes of the concentrations of ATP and ADP monitored by fluorescent sensors (red open circles for ATP and blue open squares for ADP) and HPLC (orange filled circles for ATP and purple filled squares for ADP) and the fitting curves for the calculation of the reaction rate (k_{obs}) of the reaction with CPK.

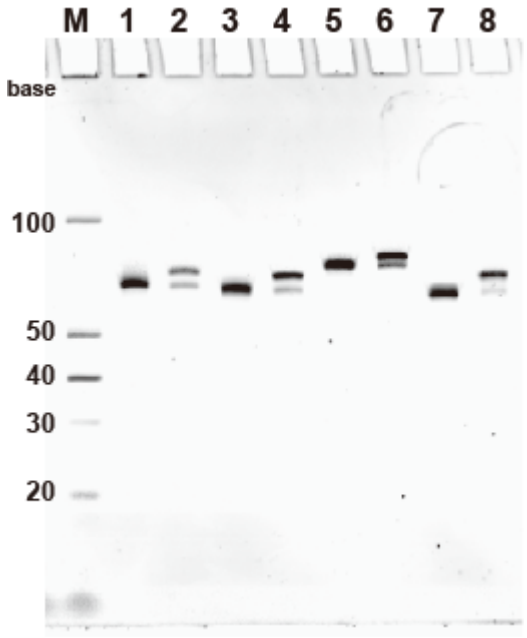

Fig. S10 A PAGE image shows purity of the covalently-linked RNP sensors. The purity of each sample was confirmed by the denaturing gel electrophoresis (8 M Urea, 15% PAGE). Each lane shows sATPRRE-I RNA (Lane 1), c-sATPRRE-I/6FAM-Rev (Lane 2), sATPRRE-II RNA (Lane 3), c-sATPRRE-II/6FAM-Rev (Lane 4), sATPRRE-III RNA (Lane 5), c-sATPRRE-III/6FAM-Rev (Lane 6), sADPRRE RNA (Lane 7), c-sADPRRE/Pyr-Rev (Lane 8), respectively. Band ratio in each even number of lane that shows the purification yields of each c-RNP are 71, 84, 77, and 98% respectively.

References

S1 S. Nakano, M. Fukuda, T. Tamura, R. Sakaguchi, E. Nakata, T. Morii, *J. Am. Chem. Soc.*, 2013, **135**, 3465.
S2 P. L. Sazani, R. Larralde, and J. W. Szostak, *J. Am. Chem. Soc.,* 2004, **126**, 8370.