Supporting Information for

A nucleic acid-based fluorescent sensor for expeditious detection of pyrophosphate anions at nanomolar concentrations

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Experimental

Reagents and Instruments.

The fluorophore labeled single-stranded DNA were synthesized and purified by HPLC (Sangon Co., China). The sequences of all the probes that have been studied in this work are summarized in Table S2. All other reagents are of analytical grade. Assays were carried out in 50 μL sealed tubes on real-time PCR (RotorGene Q, Germany). The thermal program was 180 cycles at room temperature with 10 s per cycle, and fluorescence was measured at the end of each cycle. The excitation and emission wavelengths for FAM and Cy5 were 470/512nm and 625/660 nm, respectively. The fluorescence gain level is 10. All the experiments were performed in triplicates.

Fluorescence spectra of aluminum ions induced fluorescence quenching of F*-ssDNA and pyrophosphate induced fluorescence restoration.

The fluorescence emission spectra were recorded on a Hitachi F-7000 Fluorescence spectrophotometer by exciting fluorescein at 470 nm and Cy5 at 625 nm, respectively. The slit widths for excitation and emission were both 10 nm, and the scan speed was 1200 nm/min. In a typical experiment, 80 nM of P2 or P4 and 8.0 μM of $AlCl_3$ were mixed in 30 mM HEPES buffer in a quartz cell (cross section of 1.0 cm *1.0 cm) firstly and the emission spectra were recorded. Then 40 μM of ppi was added and the emission spectra were recorded per minute.

Optimization of the amounts of F*-ssDNA and Al(III)

The optimum amounts of F*-ssDNA and Al(III) to achieve the lowest background fluorescence were first investigated by titration of F*-ssDNA at different concentrations with $AlCl_3$. Based on the results shown in Figure S2, P2 at different concentrations in the range from 40 to 100 nM was mixed with of $AlCl_3$ with concentration varying from 4.0 μM to 10 μM to achieve the sensitive response to PPi. Totally 16 assays were simultaneously performed to find the optimum conditions of F*-ssDNA and Al(III).

Determination of the calibration curve for the quantification of PPi by the F*-ssDNA/ Al(III) probe

Different amounts of sodium pyrophosphate in the range from 40 nM to 40 μM were mixed with F*-ssDNA/Al(III) by fixing the concentration of P2 at 80 nM and $AlCl_3$ at 8.0 μM. Other conditions are the same as described above.

Investigation on the thermostability of the F*-ssDNA/Al(III)

80 nM of P2 and 8.0 μM of $AlCl_3$ were mixed in HEPES buffer (30 mM, pH 7.4). 60 nM of P2 without Al(III) was used as control. The temperature of the system changed from 298 K to 363 K with an increment of 1K and the fluorescence intensity was measured.

Detection of pyrophosphate in urine and cell lysates

Urine samples of healthy volunteers were collected and diluted 10 times with HEPES buffer (30 mM, pH 7.4). To 49 μL of the probe solution which contains 80 nM of P2 and 8.0 μM of $AlCl_3$, 1.0 μL of the diluted solution of urine sample was added and the fluorescence intensity was measured.

MCF-7 cells (ATCC, Manassas, VA, USA) were cultured in Dulbeco's MEM media supplemented with 1% Penn/Strep, 10% fetal bovine serum and incubated in 5% CO_2 at 37°C. The cells were seeded on a 60 mm petri dish at a confluence of 10–30%. For detection of PPi, MCF7 cells were first washed and suspended with freshly prepared cell culture medium. The cell suspensions were probe-tip sonicated using an ultrasonic homogenizer (Ningbo Scientz Biotechnology Co., Ltd., China). Cell lysis was confirmed by microscopic inspection. The lysate was ultrafiltered at 9000 rpm for 0.5 h with a molecular cutoff of 3 kDa (Microfuge 22R Refrigerated Microcentrifuge, Beckman Coulter, USA). The filtrate was collected and added to the probe solution for PPi detection. The concentrations of P2 and $AlCl_3$ are 80 nM and 8.0 μM, respectively.

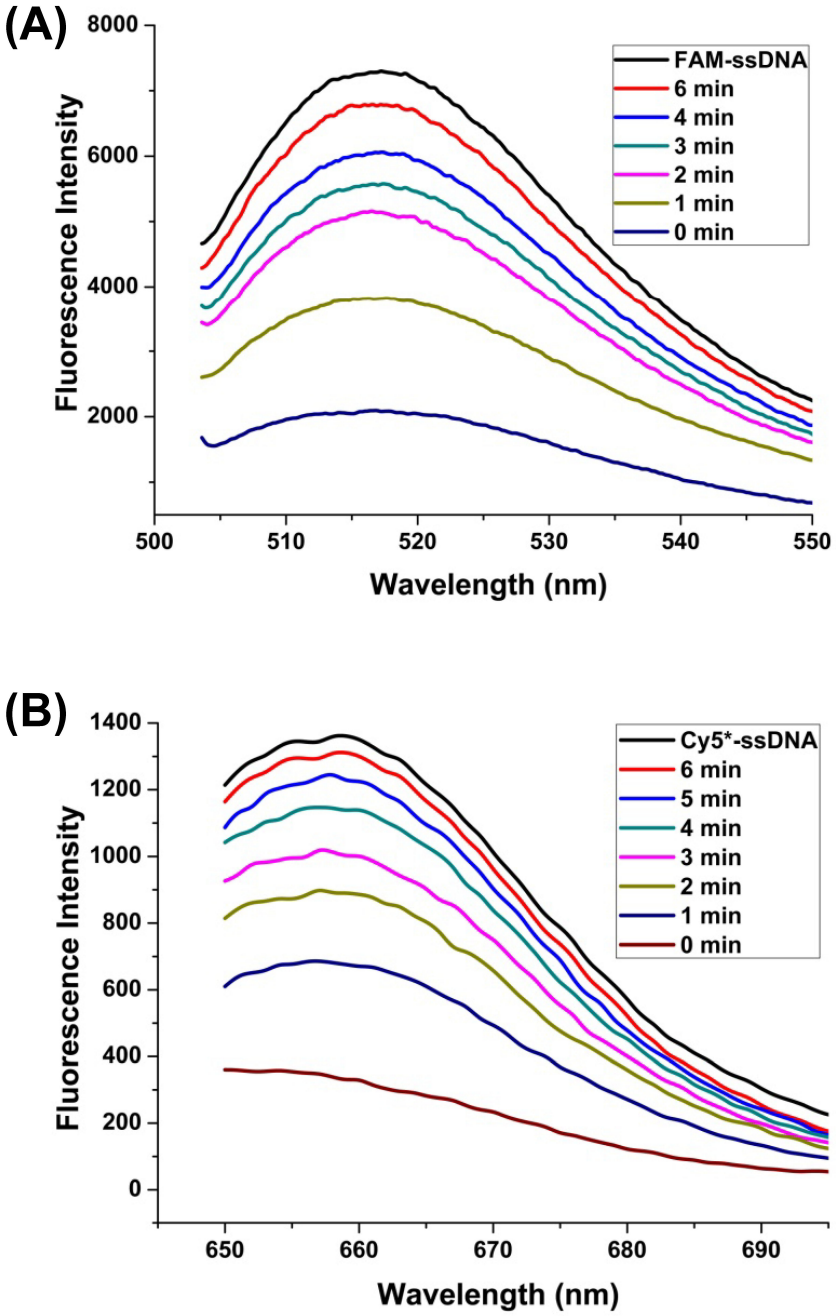

Figure S1. Fluorescence restoration of the F*-ssDNA/Al(III) complex system in the absence (0 min) and presence (1-6 min after starting the reaction) of PPi. (A) F*-ssDNA labelled with FAM; (B) F*-ssDNA labelled with Cy5. Black curves in (A) and (B) show the fluorescence spectra of FAM-ssDNA and Cy5*-ssDNA without the addition of Al(III), respectively. The concentrations of F*-ssDNA, Al(III) and PPi are 80 nM, 8.0 μM and 40 μM, respectively. The sequence of the F*-ssDNA is 5'F*-TTAAACTCACGTATCGGCCT.

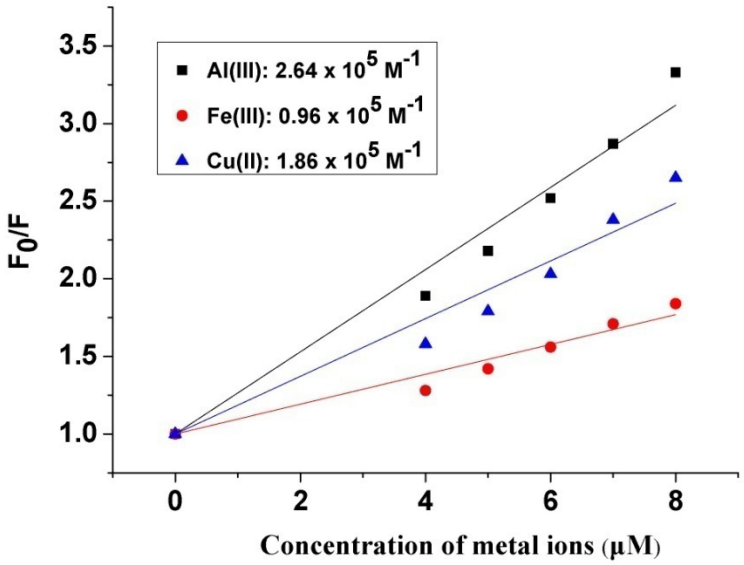

Figure S2. Measurement of the Stern-Volmer quenching constants of the complexes formed between F*-ssDNA and Al(III), Fe(III) and Cu(II). F_0 and F represent the fluorescence intensity of F*-ssDNA in the absence and presence of metal ions, respectively. The sequence of the F*-ssDNA is 5'F*-TTAAACTCACGTATCGGCCT.

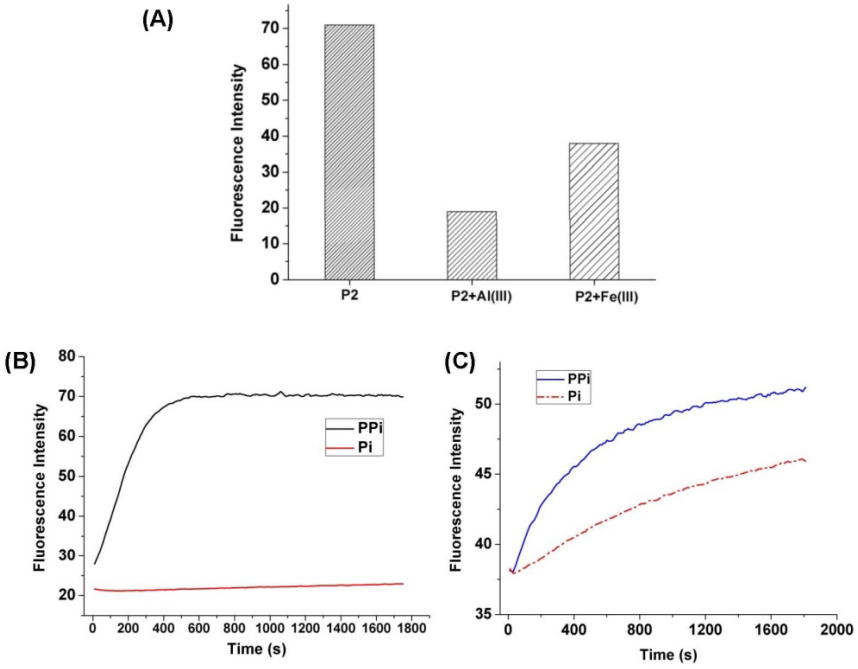

Figure S3. (A) Comparison of the quenching efficiencies of Al(III) and Fe(III) to the fluorescence F*-ssDNA. (B) Comparison of the responses of the F*-ssDNA/Al(III) system to PPi and Pi. (C) Comparison of the responses of the F*-ssDNA/Fe(III) system to PPi and Pi. The sequence of F*-ssDNA (P2) is 5'FAM-TTAAACTCACGTATCGGCCT. The concentrations of F*-ssDNA and Al(III) are 80 nM and 8.0 μM, respectively.

Investigation of the possible influences of native metal ions on the performance of the F*-ssDNA/Al(III) sensor

We further examined whether the presence of Fe(III), Cu(II) and Al(III) in the biological samples had significant influences on the performance of the F*-ssDNA/Al(III) sensor in the detection of PPi. The Fe(III), Cu(II) and Al(III) levels in serum are reported to be about 1.0 μM,[s1] 0.7 μM [s2] and 0.5 μM,[s3] respectively. The urinary concentrations of these three metals are 1.0 μM for Fe(III),[s4] 0.3 μM for Cu(II) [s4] and 0.3 μM for Al(III), [s3] respectively. We compared the fluorescence intensity of F*-ssDNA (80 nM) in the absence and presence of the above three metals at 1.0 μM. The relative decrease of the fluorescence intensity of F*-ssDNA due to the presence of Fe(III), Cu(II) and Al(III) were observed to be 5%, 9% and 12%, respectively. Since the actual concentrations of Cu(II) and Al(III) are lower than 1.0 μM, above results proved that the presence of Fe(III), Cu(II) and Al(III) at their normal concentration levels in the biological samples has no significant influence on the detection of PPi by using the F*-ssDNA/Al(III) sensor.

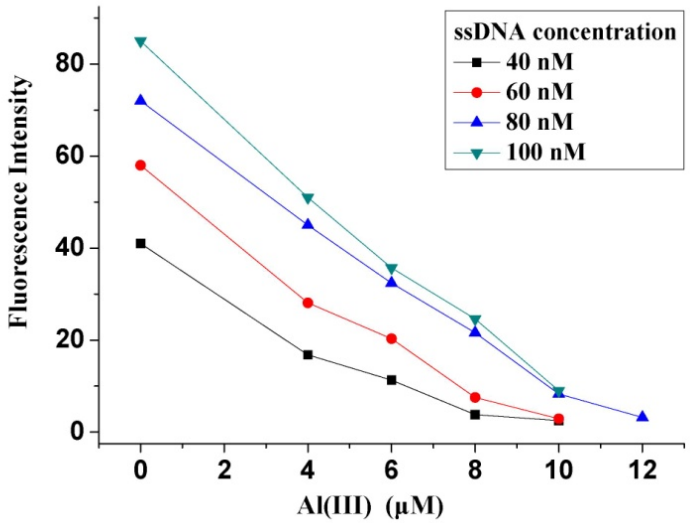

Figure S4. Fluorescence titration curves of the F*-ssDNA/Al(III) system. The sequence of F*-ssDNA is 5'FAM-TTAAACTCACGTATCGGCCT.

Table S1. Fluorescence restoration rates (/s) in the presence of 100 nM PPi at different concentrations of F*-ssDNA and Al(III)

Al (μM) \ P2 (nM)	40	60	80	100
4.0	0.00034	0.00073	0.00074	0.00067
6.0	0.00058	0.00132	0.00095	0.00088
8.0	0.00075	0.00136	0.00138	0.00135
10.0	0.00083	0.00104	0.00129	0.00128

Table S2. Sequences of all the fluorescent oligonucleotide probes studied in this work

Probe Name	Sequence (5'-3')	Number of bases
P1	5'FAM-TTAAACTCAC	10
P2	5'FAM-TTAAACTCACGTATCGGCCT	20
P3	5'FAM-TTAAACTCACGTATCGGCCTCCCGATTAGTATCCA	35
P4	5'Cy5-TTAAACTCACGTATCGGCCT	20
P5	5'Alexa Fluor 488-TTAAACTCACGTATCGGCCT	20
P6	TTAAACTCACGTATCGGCCT	20
P7	TTAAACT(-FAM)CACGTATCGGCCT	20
P8	5'FAM-CCCCGATACGTGAGTTTCAT	20
P9	5'FAM-ATTGAGCCGGCCGTTTGGGA	20

FAM is fluorescein. Cy5 is a type of cyanine dye. Alexa Fluor 488 is disulfonicrhodamine.

Investigation on the influence of the structure design of F*-ssDNA on the performance of the sensor

We examined the influence of the number of nucleotides of the ssDNA on the performance of the F*-ssDNA/Al(III) sensor. In addition to F*-ssDNA/Al(III) with 20 nucleotides in the ssDNA sequence (5'FAM-TTAAACTCACGTATCGGCCT, referred to as P2, see Table S2), two other different singly labelled oligonucleotides with 10 and 35 nucleotides (referred to as P1 and P3, respectively, sequences shown in Table S2) were also tested. It was observed that all the three tested probes showed rapid and sensitive responses to the addition of PPi. However, as shown in Figure S5A, P1 shows an unstable background and high response to Pi, indicating low selectivity for PPi over Pi. By contrast, P2 provided lowest background and best discrimination capability between PPi and Pi. P3 also discern PPi well from Pi, but it requires more Al(III) to obtain a low background. Accordingly, P2 (the 20-nt F*-ssDNA/Al(III)) was selected as the working probe for further investigation.

We also checked the influence of the position of the fluorophore on the DNA strand on the performance of the F*-ssDNA/Al(III) sensor. As shown in Figure S5B, the fluorescence intensity of F*-ssDNA with the fluorophore labelled in the middle of the DNA strand is much lower than that of the same strand with the fluorophore labelled at the end, implying lower sensitivity to the addition of PPi. So the end-labelled F*-ssDNA/Al(III) was chosen for PPi detection.

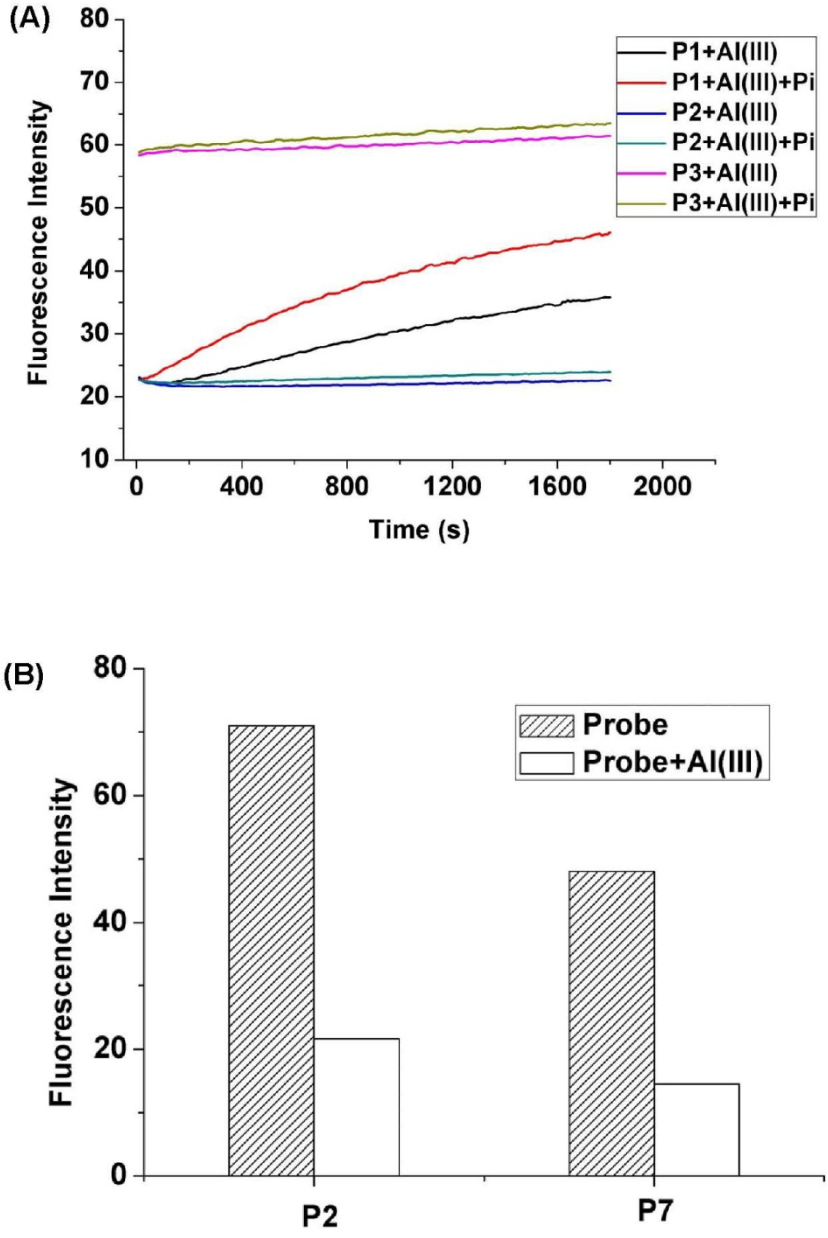

Figure S5. (A) Comparison of the background fluorescence intensity of ssDNA-F*/Al(III) with different number of bases and their responses to Pi. (B) Comparison of the fluorescence intensity of the F*-ssDNA/Al(III) system with the fluorophore labeled at different positions on the DNA strands. The concentrations of F*-ssDNA and Al(III) are 80 nM and 8.0 μM, respectively. The sequences of P1, P2, P3, and P7 are listed in Table S2.

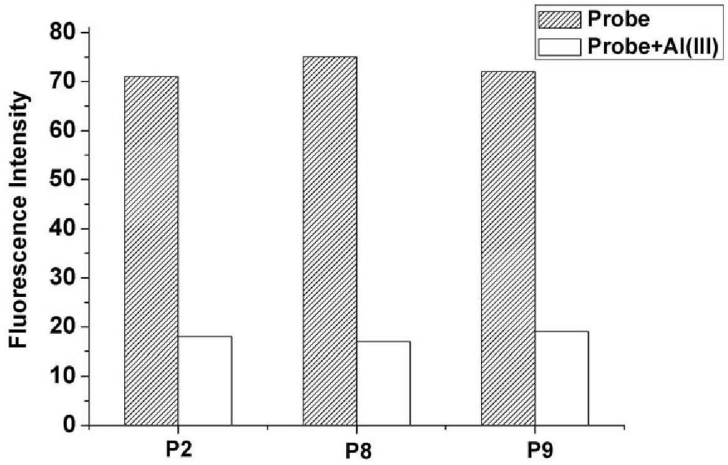

Figure S6. Comparison of the fluorescence intensity of F*-ssDNA with different ssDNA sequences in the absence and presence of Al(III). The concentrations of F*-ssDNA and Al(III) are 80 nM and 8.0 μM, respectively. The sequences of P2, P8 and P9 are listed in Table S2.

Comparison study by doping the F*-ssDNA/Al(III) system with different percentages of non-labelled ssDNA with the same sequence.

First we directly diluted the F*-ssDNA/Al(III) (80 nM/8.0 μM) system to different concentrations (60 nM/6.0 μM, 40 nM/4.0 μM and 20 nM/2.0 μM) with the HEPES buffer (30mM, pH 7.4) and measured the fluorescence intensity of the obtained solutions (Blue bars in Figure 1C). Then we fixed the total amount of ssDNA at 80 nM and Al(III) at 8.0 μM, respectively, but replaced part of the F*-ssDNA with non-labelled ssDNA. The Red bars of 80 nM, 60 nM, 40 nM and 20 nM in Figure 1C represent the concentrations of F*-ssDNA in the complex, which means that 0%, 25%, 50% and 75% of the DNA strands have been replaced by non-labelled ssDNA but the total amount of DNA strands are all 80 nM. It can be seen that the fluorescence signals of red bars were all higher than those of the blue bars. For comparison, the original fluorescence intensities of F*-ssDNA at different concentrations without the addition of Al(III) were also measured and shown in Figure 1C (Black bars), where a linear decrease of the fluorescence of F*-ssDNA with the decrease of concentration can be observed. Since the amounts of fluorophore units in the Red bar system are the same as those of the Blue bar system, the results suggest that the non-labelled DNA strands were involved in the complex formation but made little contribution to the fluorescence quenching.

Investigation of the performance of the F*-ssDNA/Al(III) sensor at different pH and temperature conditions

Figure S7 compared the reaction rates of 20 μM PPi at different pH values. It can be seen that at pH 7.4, the system exhibited the highest sensitivity to PPi. The influences of temperature on the approach were shown in Figure S8. With the increase of temperature, the fluorescence intensity of F*-ssDNA slowly decreased because of collisional quenching of the FAM molecules at high temperatures (Figure S8A). For the F*-ssDNA/Al(III) complex, the fluorescence intensity of the system remained stable in the temperature range from 25°C to 70°C. At higher temperature, the fluorescence intensity gradually increased, indicating dissociation of the complex. Then we further tested the variation of the reaction rates with the changes of pH and temperature. Figure S8B compared the reaction rates of different concentrations of PPi (200 nM, 2.0 μM, and 20 μM) at different temperature (25°C, 37°C and 45°C). As can be seen, the reaction rates slightly increase with the increase of temperature, indicating an even higher sensitivity of the method at an elevated temperature. These results suggest that the F*-ssDNA/Al(III) sensor can be used for PPi detection over a wide temperature range.

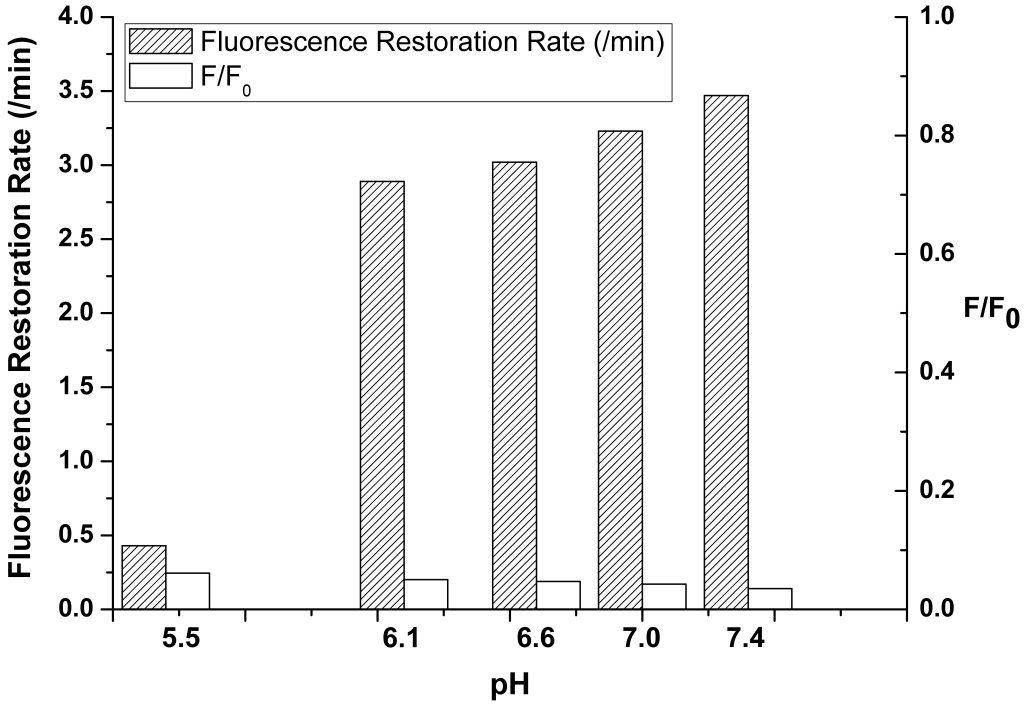

Figure S7. Comparison of the reaction rates of 20 μM PPi at different pH values. Alexa Fluor 488-labeled ssDNA was used for the experiments. The concentrations of F*-ssDNA and Al(III) are 80 nM and 8.0 μM, respectively. The sequence of P2 is listed in Table S2.

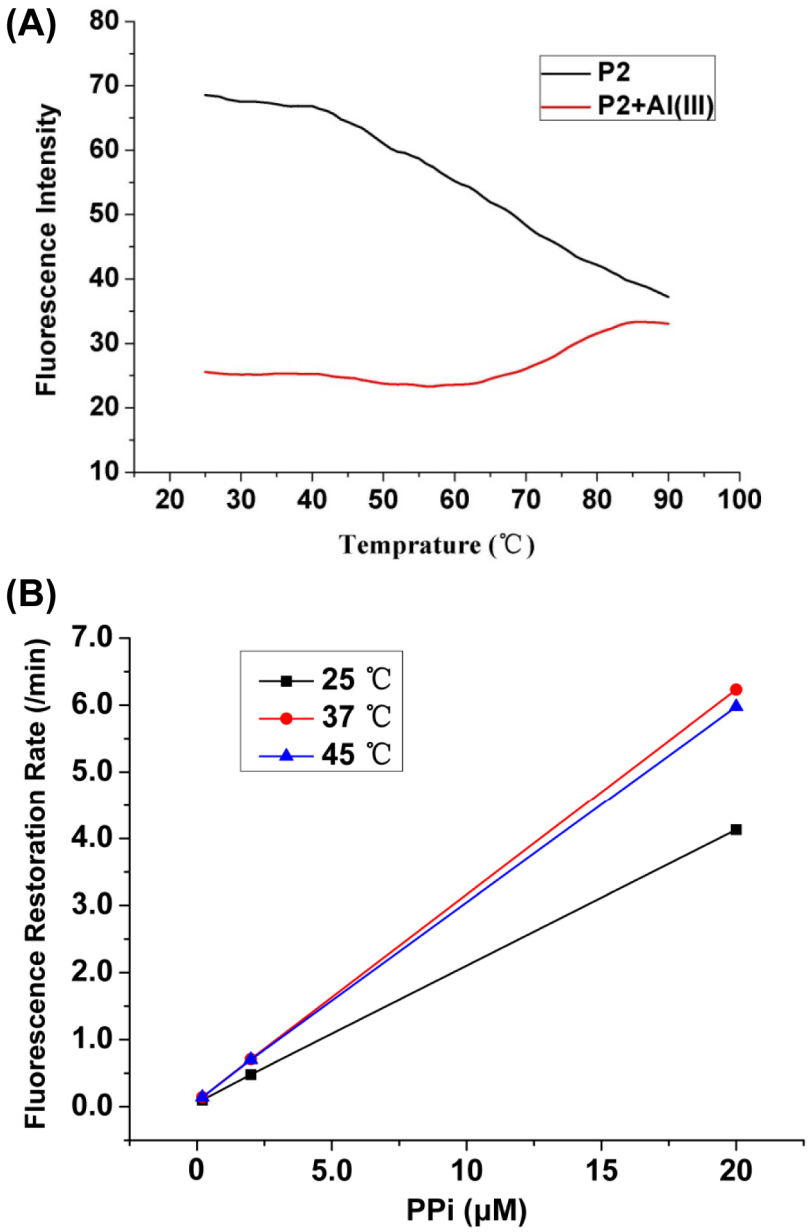

Figure S8. (A) Fluorescence intensity of F*-ssDNA and F*-ssDNA/Al(III) complex at different temperature conditions. (B) Comparison of the reaction rates of different concentrations of PPi at different temperature. The concentrations of F*-ssDNA and Al(III) are 80 nM and 8.0 μM, respectively. The sequence of P2 is listed in Table S2.

Table S3. Urinary PPi detection data

Sample No.	No PPi added	200 nM PPi added		400 nM PPi Added		Original PPi concentration in urine (μM)
	Found (nM)	Found (nM)	Recovery	Found (nM)	Recovery	
1	283	463	85%	623	90%	28.3
2	356	514	88%	708	79%	35.6
3	235	411	76%	539	88%	23.5

Table S4. Detection of PPi in cell lysates

Sample No.	No PPi added	0.5 μM PPi added		1.0 μM PPi added		2.0 μM PPi added	
	Found (μM)	Found (μM)	Recovery	Found (μM)	Recovery	Found (μM)	Recovery
1	0.87	1.38	102%	1.85	98%	2.77	95%
2	0.93	1.42	98%	1.87	94%	2.87	97%
3	0.85	1.90	105%	1.95	110%	2.69	92%

References:

[s1] A. Alexiev, P. R. Bontchev, D. Raykowa, *MikrochimicaActa* [Wien] 1974, 751-758.

[s2] C. J, Gubler, M. E. Lahey, G. E. Cartwright, M. M. Wintrobe, *J. Clin. Invest.*1953, **32,** 405-414.

[s3] P. Allain, Y. Mauras, *Anal. Chem.* 1979, **51,** 2089–2091.

[s4] E. Rodríguez, C. Díaz, *J. Trace Elem. Med. Biol.* 1995, **9,** 200-109.