Electronic Supplementary Information

for manuscript entitled

The Enzyme-free Regioselective Phosphorylation of Ribonucleosides is Promoted by Metal Ions

by

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1. Analytical Data of the Reference Compounds

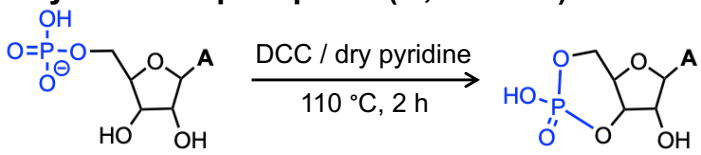
All the phosphorylated regioisomers of adenosine mononucleotides used as references for HPLC-based analysis and NMR spectroscopy, were prepared following the literature procedures.

Protocol for salt exchange of adenosine monophosphate (AMP)

For salt exchange, the resin was carefully prepared. The DOWEX resin was first rinsed with ~30 mL of HPLC-grade water, followed by treatment with 2-3 mL of 1 M HCl until the eluent reached pH 1. It was then washed with water until neutral pH was achieved. A pyridine : water mixture (4:25) was subsequently passed through the column until the pH reached 8-9, followed by further washing with water to restore neutrality.

The commercially available disodium salt of AMP (102 mg) was dissolved in 1 mL of HPLC-grade water and loaded onto the prepared DOWEX resin. The column was eluted with approximately 15 mL of water, and the eluate was collected. Methanol was then added to the collected solution to achieve a final concentration of 25% (*v*/*v*), followed by the addition of 5 equivalents of tributylamine. The mixture was thoroughly mixed prior to lyophilization. After lyophilization, tributylammonium adenosine monophosphate was obtained as a white powder.

Adenosine 3′,5′-cyclic monophosphate (3′,5′-cAMP)[1]



5′-AMP 3′,5′-cAMP

Scheme S1. Synthesis scheme of 3′,5′-cAMP from 5′-AMP

Procedure: The tributyl ammonium salt of adenosine 5′-monophosphate (215 mg, 0.3 mmol) was dissolved in 29 mL of dry pyridine under anhydrous conditions. The solution was added dropwise to the boiling solution of DCC (124 mg, 0.6 mmol) in dry pyridine (29 mL) at 110 °C and refluxed for 2 hr. Subsequently, 100 mL of water was added, and afterwards, the mixture was evaporated to dryness. The resulting semi-solid, off-white substance was partitioned between water and diethyl ether (1:1 *v*/*v*), with insoluble dicyclohexylurea removed by filtration before the aqueous phase was lyophilized.

The desired product was finally purified by RP-HPLC using a gradient of acetonitrile (containing 0.1% TFA) in triethylammonium bicarbonate buffer (50 mM, pH 7). The collected fractions from HPLC were combined together and lyophilized to dryness. Prior to the characterization, the triethylammonium salt form of the product was exchanged with Na^+-form

using 0.3 M $NaClO_4$ solution in acetone/diethyl ether (1:1, *v*/*v*). The product was characterized by proton-decoupled ^{31}P-NMR spectroscopy, and the purity of the product was further confirmed by HPLC (96% purity). The final yield was calculated using UV-vis spectroscopy. Yield: 65%; $^{31}P\{^{1}H\}$-NMR (162 MHz, 10% D_2O in water): δ = -1.8 ppm.

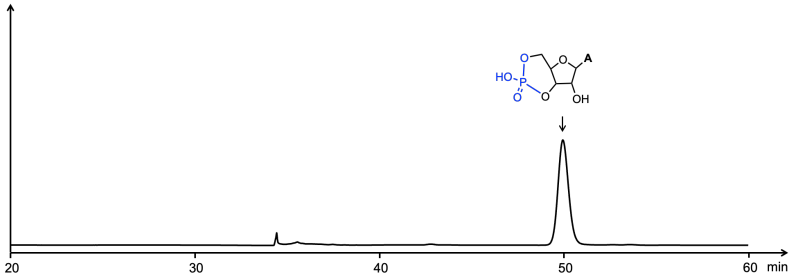


Fig. S1. HPLC purity profile of 3′,5′-cAMP.

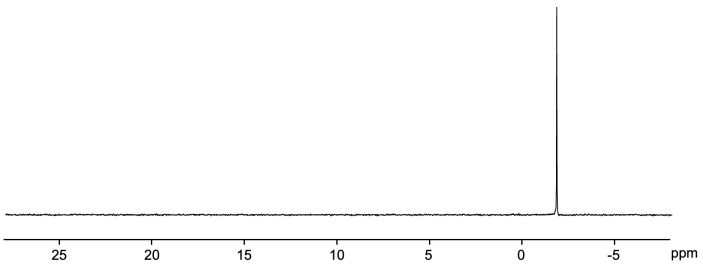
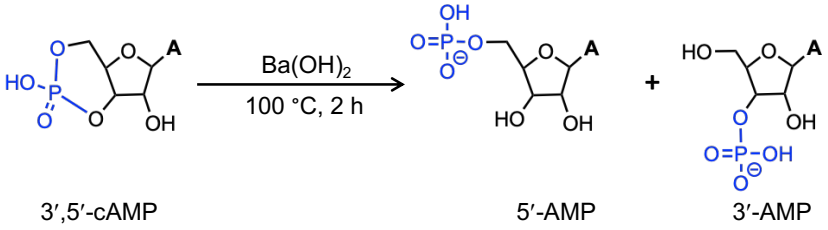


Fig. S2. Proton-decoupled ^{31}P-NMR spectrum of 3′,5′-cAMP.

Adenosine 3′-monophosphate (3′-AMP)



Scheme S2. Synthesis of 3′-AMP from 3′,5′-cAMP

Procedure: Adenosine 3',5'-cyclic monophosphate sodium salt (18 mg, 0.05 mmol) and barium hydroxide octahydrate (158 mg, 0.5 mmol) were mixed together in 10 mL of water and stirred at 100 °C in a 50 mL round-bottom flask. Over the course of the reaction, the clear solution turned cloudy. After 2 hours, 5 mL of 0.5 M sodium sulfate solution (7.1 g per 100 mL of water) was added at room temperature, and the resulting barium sulfate precipitate was filtered out, and the clear solution was concentrated. The desired product was finally purified by HPLC using a gradient of acetonitrile (containing 0.1% TFA) in triethylammonium bicarbonate buffer (50 mM, pH 7). After salt exchange, the product was analyzed by proton-decoupled ^{31}P-NMR

spectroscopy and the purity of the product was further confirmed by HPLC (~ 85% purity). The final yield was calculated using UV-vis spectroscopy. Yield = 38%; $^{31}P\{^{1}H\}$-NMR (162 MHz, 10% D_2O in water): δ = 2.1 ppm.

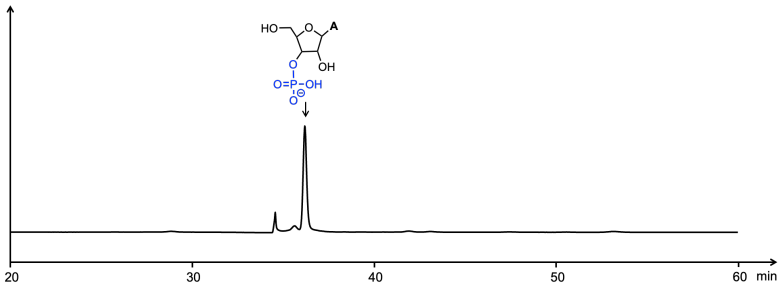


Fig. S3. HPLC purity profile of 3′-AMP.

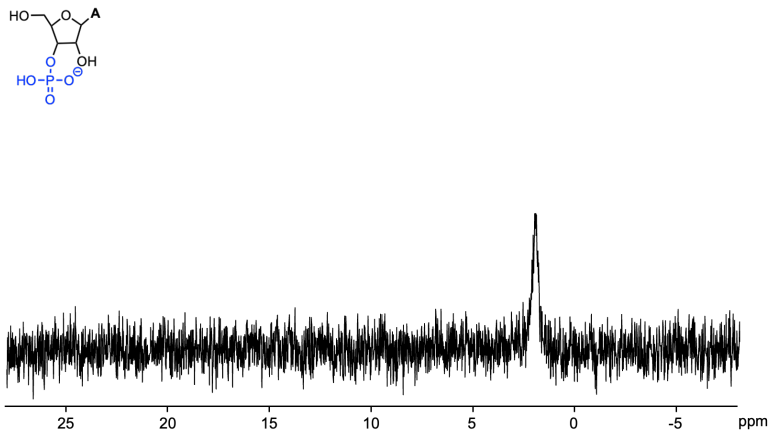
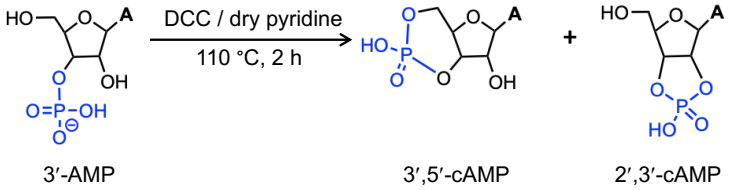


Fig. S4. Proton-decoupled ^{31}P-NMR spectrum of 3′-AMP.

Adenosine 2′,3′-cyclic monophosphate (2′,3′-cAMP)[2]



Scheme S3. Synthesis of 2′,3′-cAMP from 3′-AMP

Procedure: Tributylammonium salt of adenosine 3'-monophosphate (72 mg, 0.1 mmol) was dissolved into 15 mL of dry pyridine under anhydrous conditions. The solution was introduced dropwise into the boiling solution of DCC (41 mg, 0.2 mmol) in dry pyridine (15 mL) at 110°C and subsequently refluxed for 2 hours. Next, 100 mL of water was added, and after stirring at room temperature for 1 hour, the mixture was concentrated to dryness. The crude residue was extracted using a 1:1 (*v*/*v*) mixture of water and diethyl ether. The insoluble dicyclohexylurea was removed by filtration, after which the aqueous layer was isolated and lyophilized. The desired product was finally purified by HPLC using a gradient of acetonitrile (containing 0.1%

TFA) in triethylammonium bicarbonate buffer (50 mM, pH 7). After salt-exchange the product was analyzed by proton-decoupled ^{31}P-NMR and the purity of the nucleotide was further confirmed by HPLC (~ 70% purity). The final yield was calculated using UV-vis spectroscopy. Yield = 45%; $^{31}P\{^{1}H\}$-NMR (162 MHz, 10% D_2O in water): δ = 19.9 ppm.

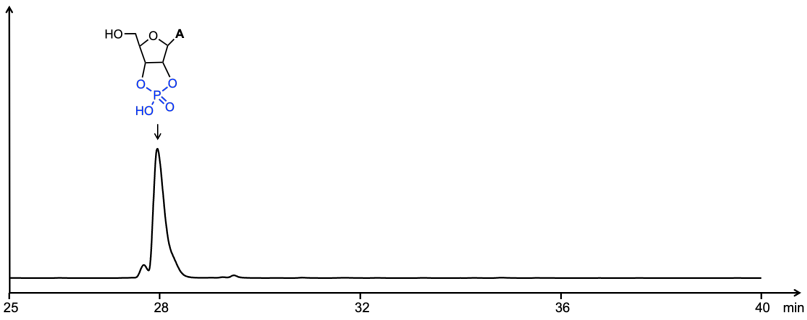


Fig. S5. HPLC purity profile of 2′,3′-cAMP

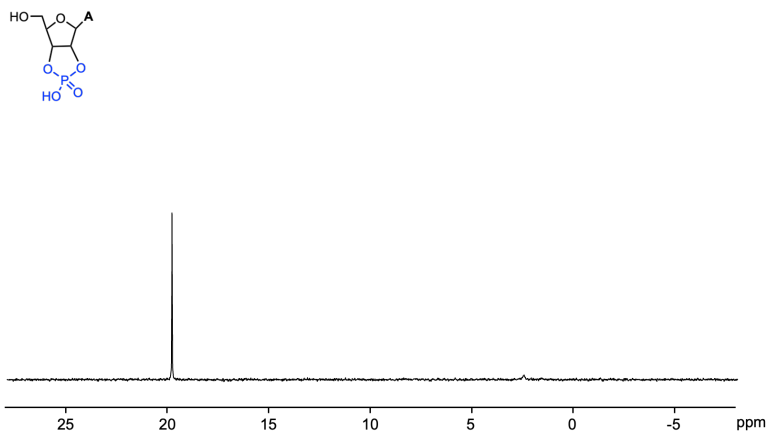
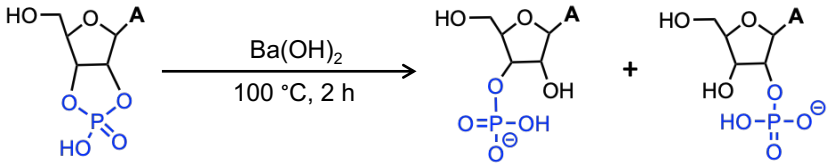


Fig. S6. Proton-decoupled ^{31}P-NMR spectrum of 2′,3′-cAMP.

Adenosine 2′-monophosphate (2′-AMP)



Scheme S4. Synthesis of 2′-AMP from 2′,3′-cAMP

Procedure: Adenosine 2',3'-cyclic monophosphate sodium salt (18 mg, 0.05 mmol) and barium hydroxide octahydrate (158 mg, 0.5 mmol) were dissolved in 10 mL of deionized water. This mixture was stirred in a round-bottom flask at 100 °C, in the course of the reaction, a clear-to-cloudy transition of the solution was observed. Following the reaction period of 2 hours at ambient temperature, 5 mL of 0.5 M sodium sulphate solution (7.1 g per 100 ml of water) was added to promote the precipitation of barium sulphate, which was subsequently isolated via

filtration. The clear solution was then subjected to lyophilization. The desired product was finally purified by HPLC using a gradient of acetonitrile (containing 0.1% TFA) in triethylammonium bicarbonate buffer (50 mM, pH 7). After salt-exchange the product was analyzed by proton-decoupled ^{31}P-NMR spectroscopy and the purity of the nucleotide was further confirmed by HPLC (93% purity). The final yield was calculated using UV-vis spectroscopy. Yield = 32%; $^{31}P\{^{1}H\}$-NMR (162 MHz, 10% D_2O in water) δ = 3.2 ppm.

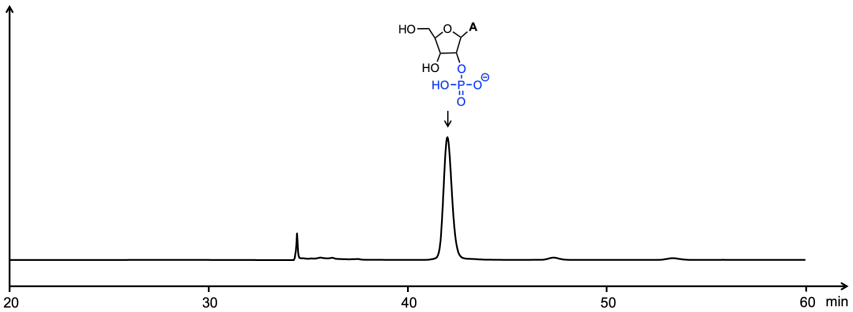


Fig. S7. HPLC purity profile of 2′-AMP.

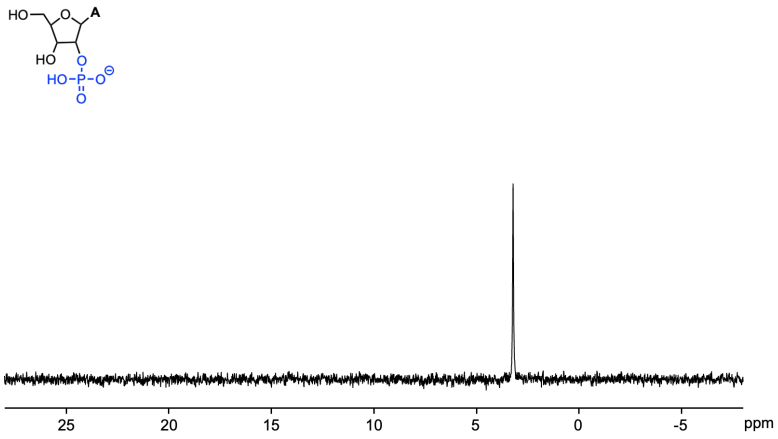


Fig. S8. Proton-decoupled ^{31}P-NMR spectrum of 2′-AMP.

Adenosine 5′-monophosphate (5′-AMP)

The commercially available disodium salt of 5′-AMP was dissolved in water, and subsequently, the purity profile was analyzed by HPLC, and a proton-decoupled ^{31}P-NMR spectrum was recorded.

$^{31}P\{^{1}H\}$-NMR (162 MHz, 10% D_2O in water): δ = 3.7 ppm.

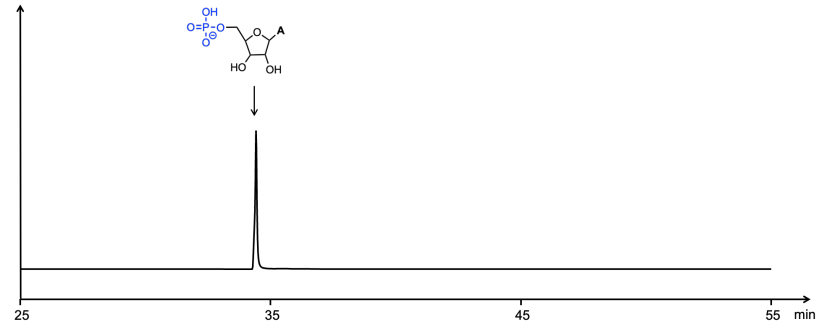


Fig. S9. HPLC purity profile of 5′-AMP.

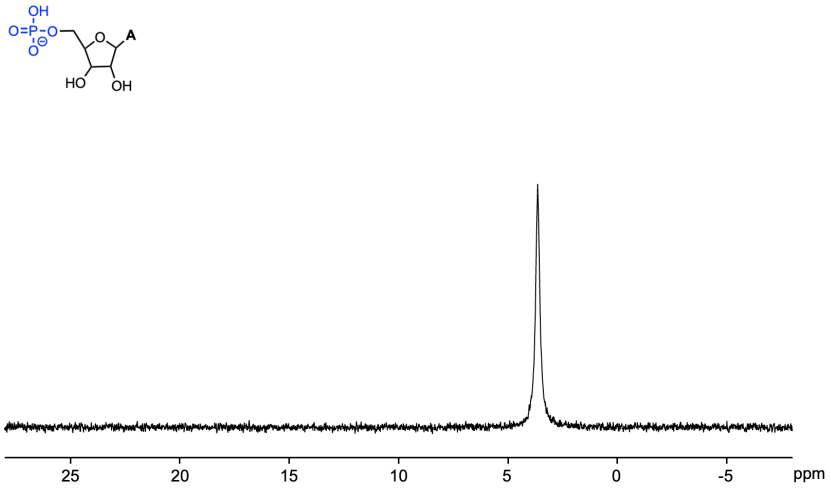


Fig. S10. Proton-decoupled ^{31}P-NMR spectrum of 5′-AMP.

Adenosine (A)

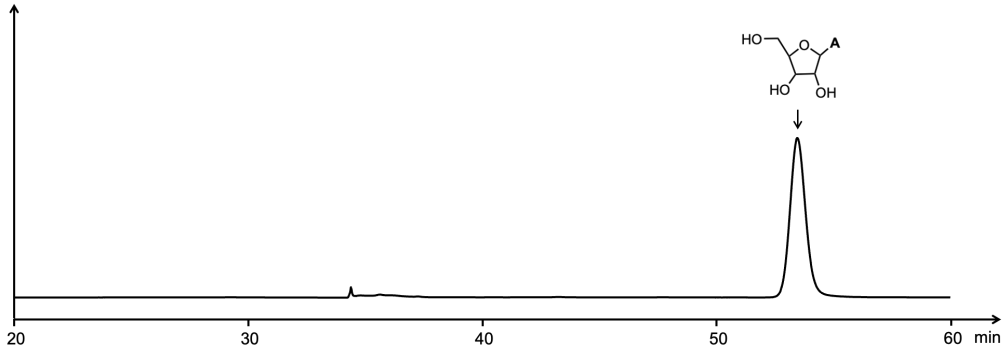


Fig. S11. HPLC purity profile of adenosine.

2. Method optimization for proton-decoupled ^{31}P quantitative NMR

For quantitative nuclear magnetic resonance (qNMR) measurement, a compound with a precisely known concentration is added to the sample as an internal standard. By measuring the ratio of the integrated signal areas between the internal standard and the analyte, the concentration of the analyte can be accurately determined using the following equation[3, 4]:

$$C_a = \frac{I_a}{I_{ref}} \times \frac{P_{ref}}{P_a} \times C_{ref}$$

C_a = concentration of analyte, C_{ref} = concentration of reference molecule, P_a = number of phosphorus in analyte, P_{ref} = number of phosphorus in reference molecule, I_a = signal intensity or integral area under the curve for analyte, I_{ref} = signal intensity or integral area under the curve for reference molecule. This approach is widely used in qNMR due to its precision and reliability. Key factors for selecting an internal reference:

a) No peak overlap between the phosphorus signal of the reference and those of the reagents or products.
b) Chemical stability under the experimental pH and NMR conditions.
c) High water solubility.

In proton-decoupled ^{31}P-qNMR quantitative analysis, potassium dihydrogen phosphate (PDP) serves as a certified reference material (CRM), exhibiting a single peak at δ 0-1 ppm. However, since PDP is inherently present in the reaction mixture, it can't be treated as an internal standard for this study. The present work focuses on the quantitative analysis of 5′-BMP and 2′,3′-cBMP in the assay mixture. For this purpose, diethyl [2-(trihydroxysilyl)ethyl]phosphonate was chosen as the internal reference. This compound offers high water solubility, does not participate in any side reactions with analytes, and displays a distinct single peak at $\delta \sim 37$ ppm (Fig. S12), which is clearly distinguishable from the signals of the reactant and product generated from the assay.

Relaxation delay (d_1) optimization: Selecting an appropriate signal and relaxation delay (d_1) is critical for quantitative NMR measurements. Since different phosphorus-containing molecules possess different relaxation times, it is essential to optimize prior to analyzing the reaction mixture. In our study, the initial optimization was carried out using the reference molecule in the presence of PDP, followed by further refinement of the relaxation delay using the reference molecule with a mononucleotide.

Optimization of d_1 of the reference compound with potassium dihydrogen phosphate (PDP)

In an NMR tube, equimolar amounts of KH_2PO_4 (25 µmol) and the reference compound (25 µmol) were dissolved in 0.5 mL of water (pH 7, containing 10% D_2O). The same sample was analyzed using proton-decoupled ^{31}P-NMR with the zgpg30 pulse sequence at five different relaxation delays (d_1): 2, 5, 10, 20, and 30 s.

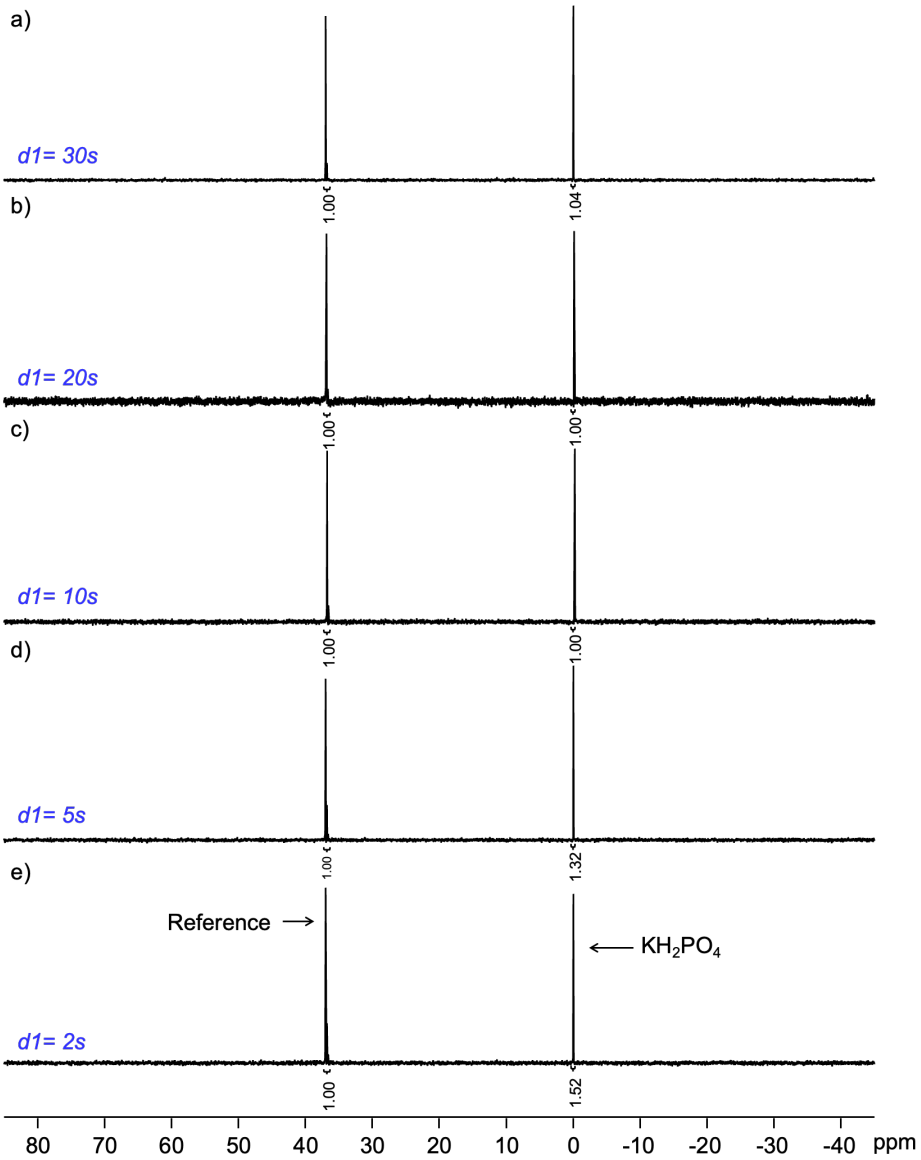


Fig. S12. Proton-decoupled ^{31}P-NMR spectra (qNMR) of relaxation delay (d_1) optimization experiment. The solution comprises of 1:1 molar ratio of KH_2PO_4 and reference molecule, and the NMR spectra recording was conducted at different delay time (d_1) a) 30 sec, b) 20 sec, c) 10 sec, d) 5 sec and e) 2 sec.

The results showed that at shorter relaxation delays (2 s and 5 s), the signal ratio between the reference and PDP did not match with expected values. However, at a relaxation delay of 10 s, the signal ratio was consistent with the molar ratio of the analytes in solution. For further

optimization, measurements were also performed at longer delays (20 s and 30 s), which exhibited minimal deviation from the data obtained at d1 = 10 sec, confirming 10 s as the optimal relaxation delay (Fig. S12).

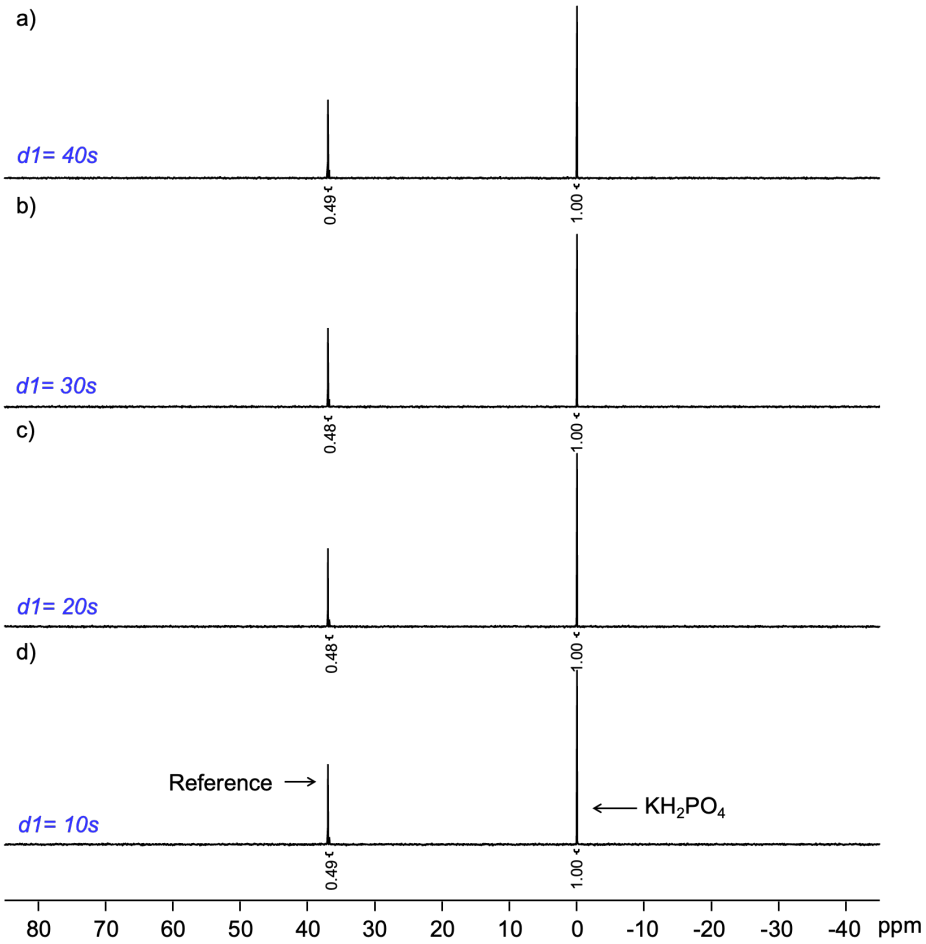


Fig. S13. Proton-decoupled ^{31}P-NMR spectra (qNMR) of relaxation delay (d_1) optimization experiment. The solution comprises of 1:0.5 molar ratio of KH_2PO_4 and reference molecule, and the NMR spectra recording was conducted at different delay time (d_1) a) 40 sec, b) 30 sec, c) 20 sec, and d) 10 sec.

For further validation, a second set of experiments was conducted using 50 µmol of KH_2PO_4 and 25 µmol of the reference (1:0.5 molar ratio) in 0.5 mL of water (pH 7, containing 10% D_2O). Quantitative proton-decoupled ^{31}P NMR measurements were performed with relaxation delays of 10, 20, 30, and 40 s using the zgpg30 pulse sequence. The results consistently confirmed 10 s as the optimal relaxation delay, as no significant change in quantification was observed with increasing d1 (Fig. S13).

Optimization of d_1 for reference compound with 5′-AMP: As our primary objective is nucleotide quantification, differences in relaxation delays between the reference compound and analytes may influence accuracy. To address this, we selected 5′-AMP as a model nucleotide for calibration against the reference molecule. A new set of experiments was performed using a mixture of the reference (25 µmol) and 5′-AMP (17 µmol) in a 1:0.68 molar ratio, dissolved in 0.5 mL of water (pH 7, containing 10% D_2O). This sample was subjected to proton-decoupled ^{31}P-qNMR analysis using the zgpg30 pulse sequence with five different relaxation delays (d1): 2 s, 5 s, 10 s, 20 s, and 30 s (Fig. S14).

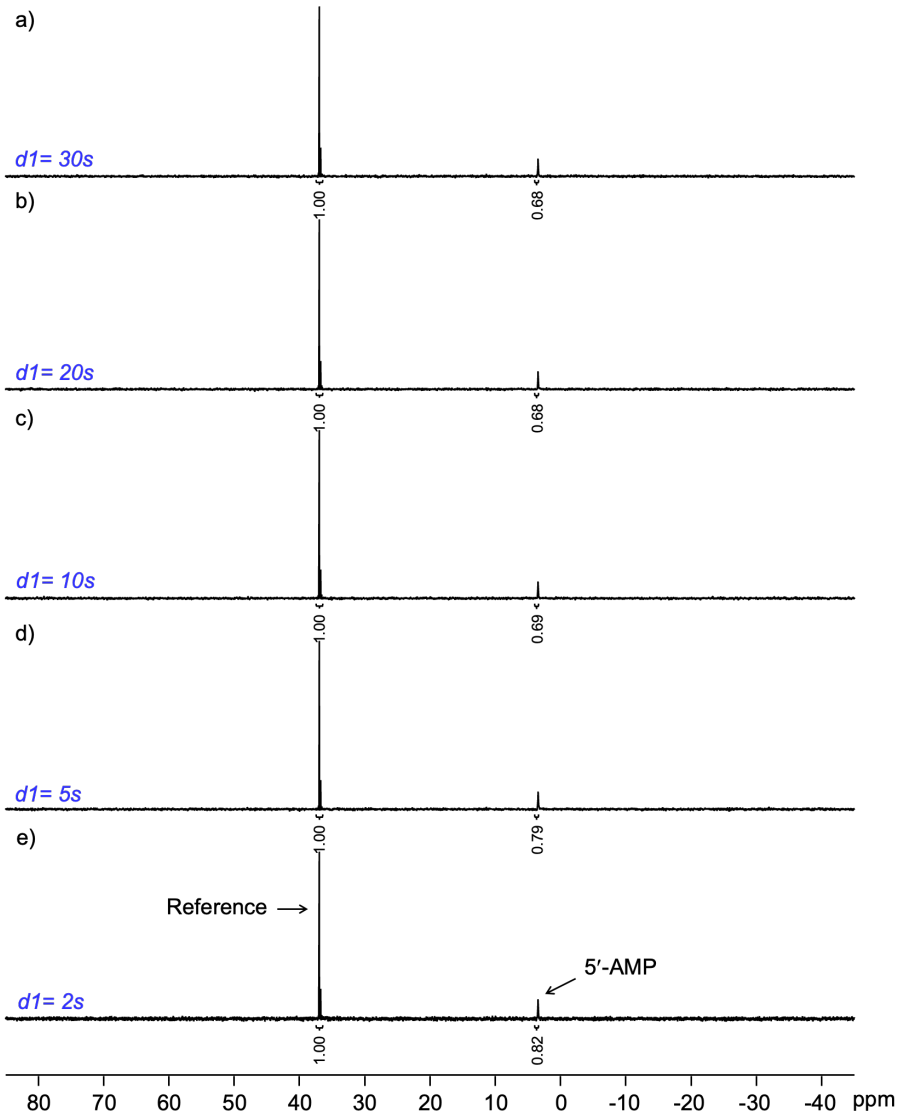


Fig. S14. Proton-decoupled ^{31}P-NMR spectra (qNMR) of relaxation delay (d_1) optimization assay contains 1:0.68 molar ratio of reference molecule and 5′-AMP conducted at different delay time (d_1) a) 30 sec, b) 20 sec, c) 10 sec, d) 5 sec and e) 2 sec.

The results show that at shorter relaxation delays (2 s and 5 s), the integral ratio varies significantly. However, at d1 = 10 s, the integral ratio aligns well with the analytically

determined value. At longer delays (20 s and 30 s), the integral ratio remains consistent with the value obtained at 10 s. These findings validate the accuracy of our quantification method, supporting its application for subsequent analysis of reaction mixtures.

Calibration of different known concentration of 5'-AMP with reference: After optimizing the relaxation delay to 10 s, we next evaluated whether this condition remains valid across a range of analyte concentration (Fig. S15). Four separate samples were prepared in Eppendorf tubes, each containing 2.5 µmol of the reference compound mixed with varying amounts of 5′-AMP (2.5 µmol, 2.0 µmol, 1.5 µmol, and 0.65 µmol) 0.5 mL of water (pH 7, containing 10% D_2O). These solutions correspond to a constant reference concentration of 5 mM, while the 5′-AMP concentrations were 5 mM, 4 mM, 3 mM, and 1.25 mM, respectively.

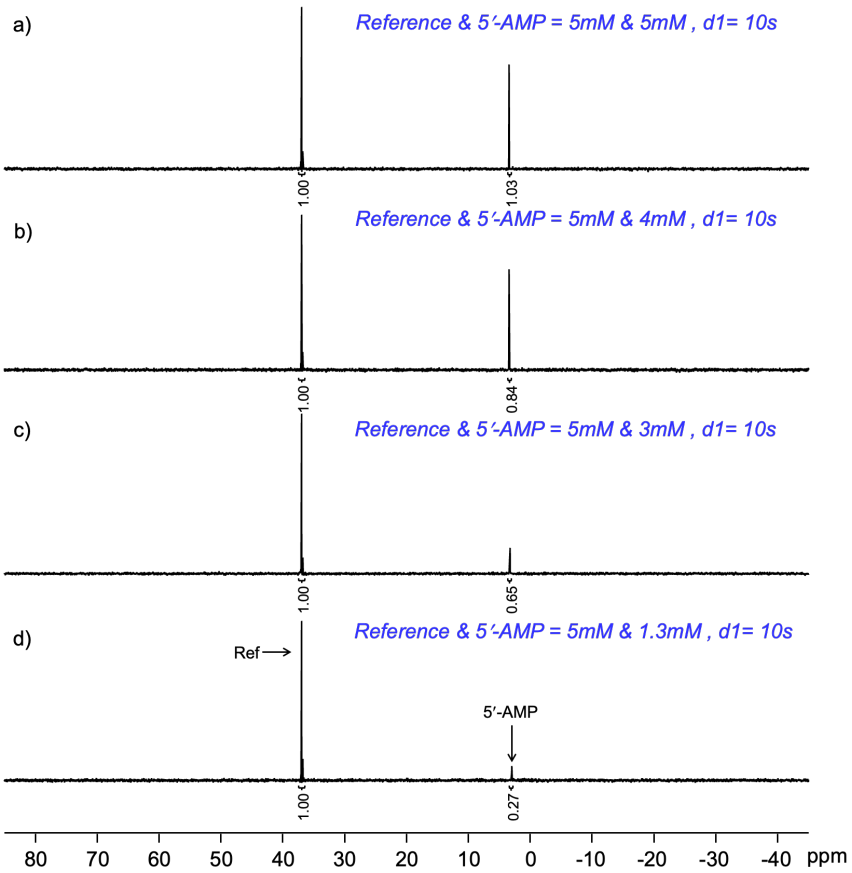


Fig. S15. Proton-decoupled ^{31}P-NMR spectra (qNMR) for concentration calibration of different molar ratio of reference molecule and 5′-AMP at a) 5 mM of 5′-AMP and 5 mM reference, a) 5 mM reference and 5 mM of 5′-AMP, b) 5 mM reference and 4 mM of 5′-AMP, c) 5 mM reference and 3 mM of 5′-AMP and d) 5 mM reference and 1.3 mM of 5′-AMP.

All samples were analyzed using proton-decoupled 31P-qNMR with the zgpg30 pulse sequence and a relaxation delay (d1) of 10 s. The NMR data demonstrate that quantification using the reference molecule remains accurate across this concentration range, confirming the reliability of the method (Fig. S16).

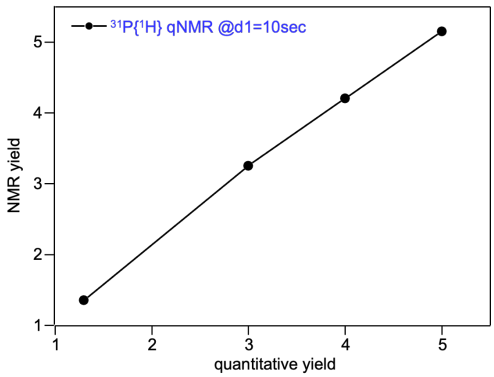


Fig. S16. Calibration plot for proton decoupled ^{31}P quantitative NMR yield vs analytical quantitative yield

3. Phosphorylation Assay

Phosphorylation in formamide medium

Initial screening of metal ions:

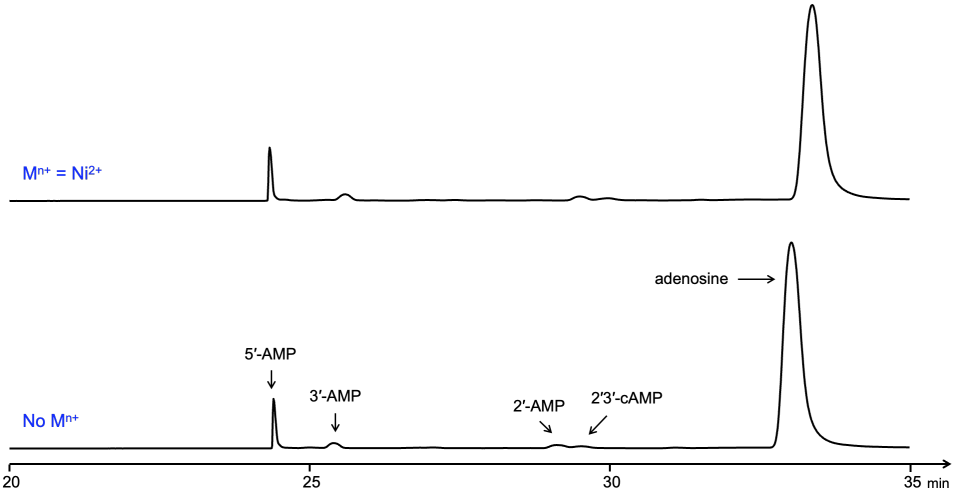


Fig. S17. Stacked representation of HPLC chromatograms from phosphorylation of adenosine assays with formamide in the presence of metal (Ni^{2+}) and absence of metal ions at 70 °C, recorded after 20 days.
Condition: 5 mM adenosine, 50 mM $NH_4H_2PO_4$ and 10 mM of different metal salt in water, containing 13 M formamide.

Table S1. Conversion (%) to different adenosine monophosphate from adenosine and inorganic phosphate at 70 °C for 20 days[a,b,c].

M^{n+}	5′-AMP	3′-AMP	2′-AMP	2′3′-cAMP	3′5′-cAMP
No M^{n+}	4	1	1	1	n.d.
Zn^{2+}	3	1	1	1	n.d.
Fe^{3+}	2	1	1	1	n.d.
Ni^{2+}	4	2	1	1	n.d.

[a] Yields are determined based on the area under the curve of the signals of interest in the HPLC chromatogram at 260 nm.
[b] Condition: 5 mM adenosine, 50 mM $NH_4H_2PO_4$ and 10 mM of different metal salt in water, containing 13 M formamide.
[c] n.d.= not detected

Phosphorylation involving urea and ammonium formate

Adenosine phosphorylation assay

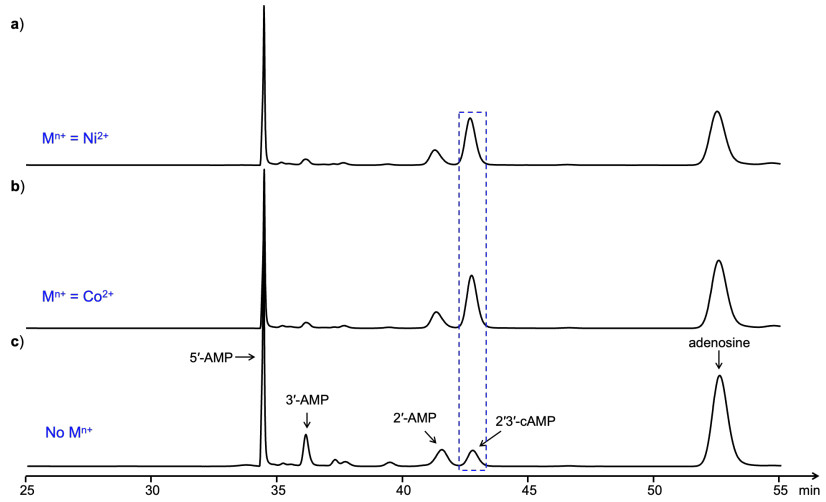


Fig. S18. Stacked representation of HPLC chromatograms from phosphorylation of adenosine assays a) with Ni^{2+}, b) with Co^{2+} and c) in the absence of metal ions, recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

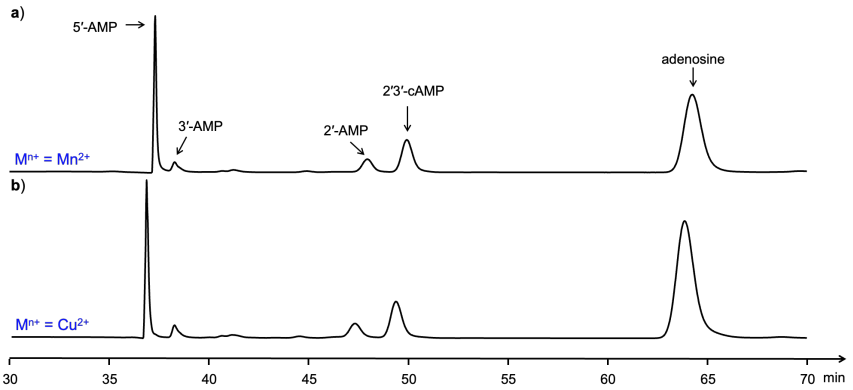


Fig. S19. Stacked representation of HPLC chromatograms from phosphorylation of adenosine assays a) with Mn^{2+}, b) with Cu^{2+}, recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

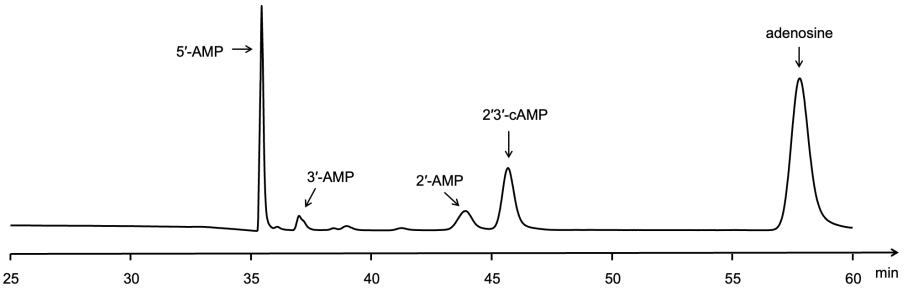


Fig. S20. HPLC chromatograms from phosphorylation of adenosine assays in presence of equimolar mixture of different 3d transition metal salt (Ni^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}: 1 mM each), recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 6 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

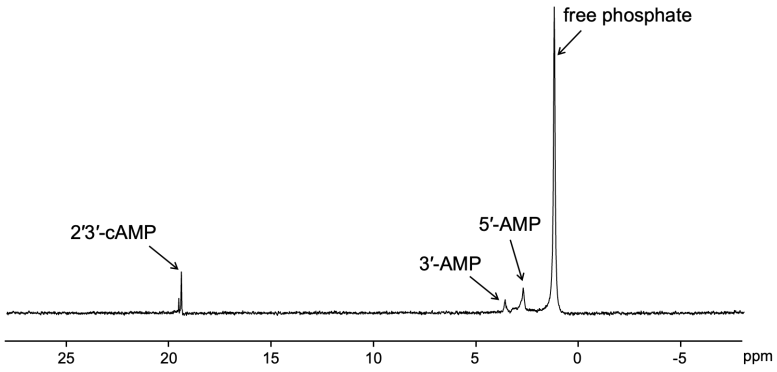


Fig. S21. Proton-decoupled ^{31}P-NMR of the phosphorylation of adenosine assay solution in the absence of metal ions, recorded after 5 wet-dry cycles conducted at 85 °C.
Condition: 5 mM adenosine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

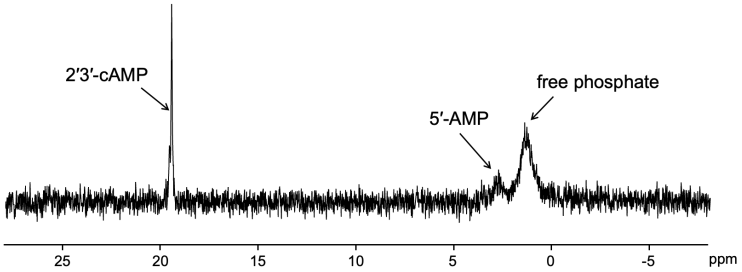


Fig. S22. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of adenosine assay solution in the presence of Ni^{2+}, recorded after 5 wet-dry cycle conducted at 85 °C.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

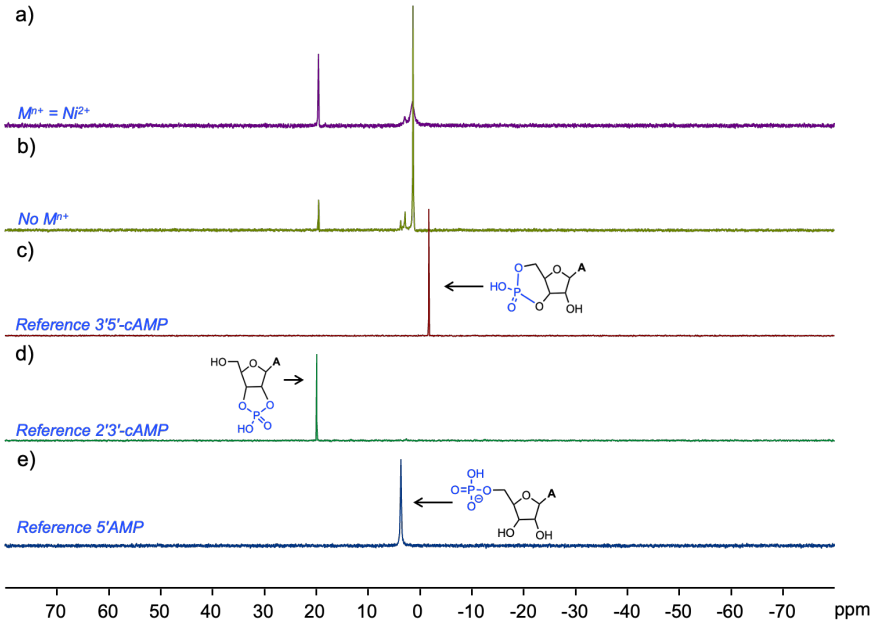


Fig. S23. Stacked representation of proton-decoupled ^{31}P-NMR spectra of a) with Ni^{2+}, b) in the absence of metal ions recorded after 5 wet-dry cycles conducted at 85 °C and synthesized reference standards of c) synthesized and purified 3′5′-cAMP, recorded for reference, d) synthesized and purified 2′3′-cAMP, recorded for reference e) commercially available 5′-AMP, recorded for reference.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

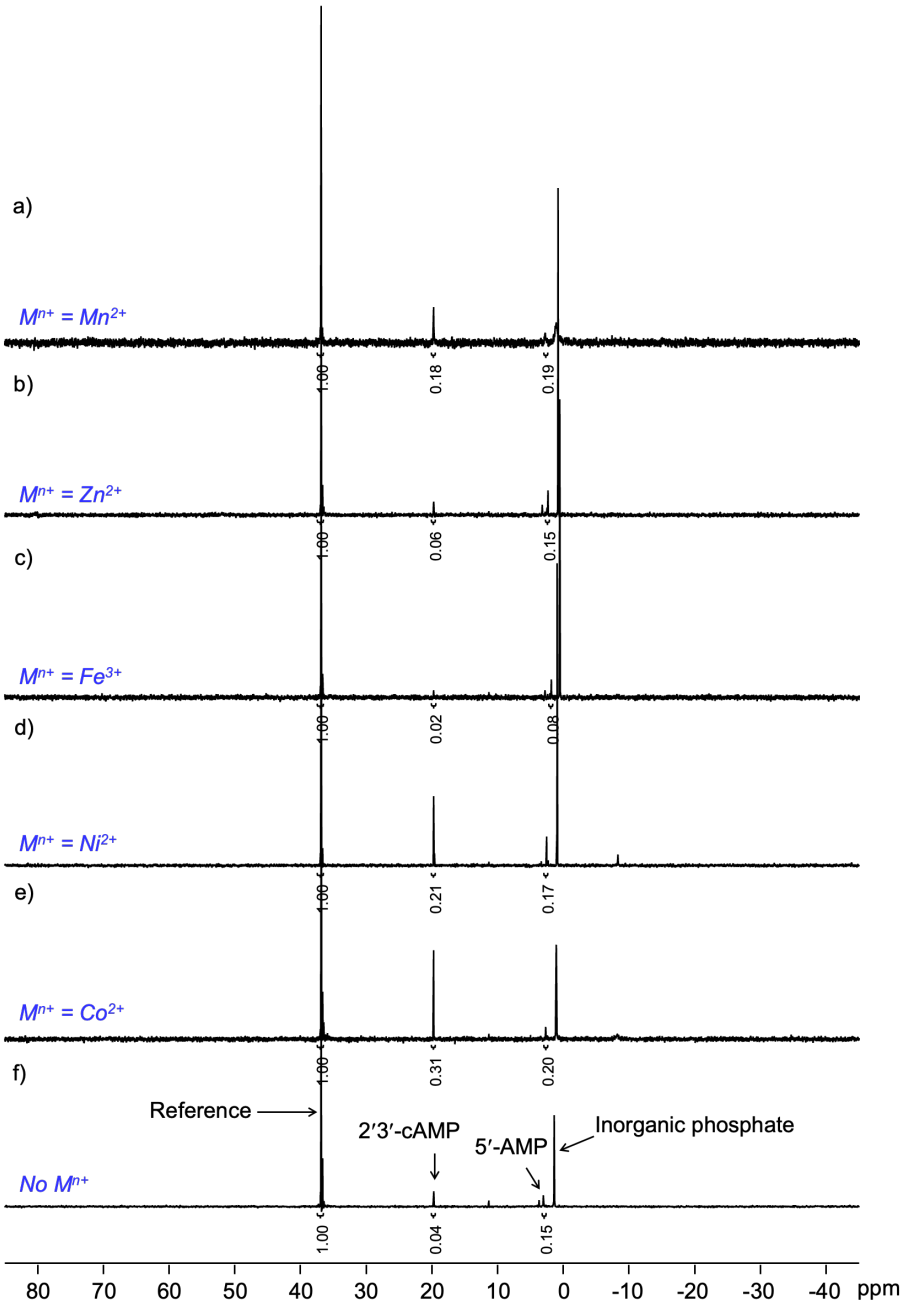


Fig. S24. Stacked representation of proton-decoupled ^{31}P-qNMR spectrum for phosphorylation of the adenosine assay a) with Mn^{2+}, b) with Zn^{2+}, c) with Fe^{3+}, d) with Ni^{2+}, e) with Co^{2+} and f) in the absence of metal ions recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

Table S2. Quantified yield of 5'-AMP and 2'3'-cAMP by proton decoupled ^{31}P-qNMR from phosphorylation assay of adenosine with different transition metal ions, following 5 wet-dry cycle at 85 ºC[a,b].

	Concentration (in mM)		Integral			Yield (%)	
M^{n+}	reference	analyte	reference	5'-AMP	2'3'-cAMP	5'-AMP	2'3'-cAMP
blank[a]	4	2.8	1	0.15	0.04	21.4	5.7
Co^{2+}	2	2.6	1	0.20	0.31	15.4	23.8
Ni^{2+}	2	1.7	1	0.17	0.21	20.0	24.7
Fe^{3+}	2	2.6	1	0.08	0.02	6.2	1.5
Zn^{2+}	2	2.6	1	0.15	0.06	11.5	4.6
Mn^{2+}	2	2.3	1	0.19	0.18	16.5	15.7

[a] Yield represent average of two independent assay
[b] Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

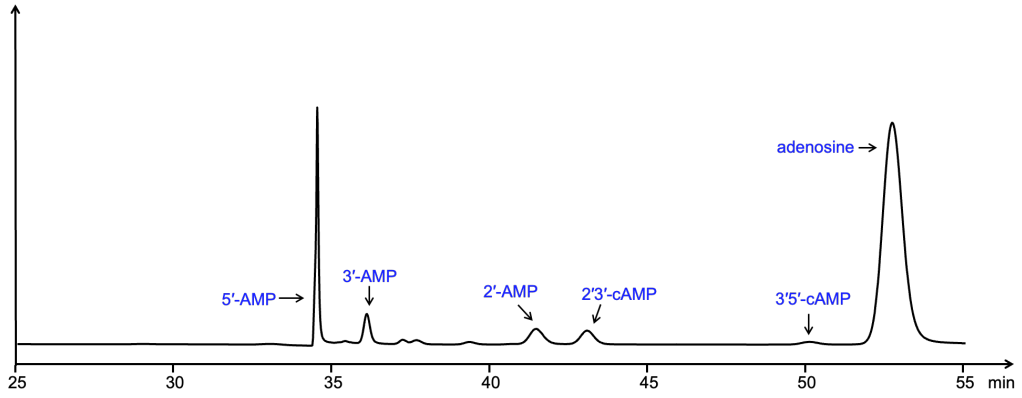


Fig. S25. HPLC chromatograms from phosphorylation of adenosine assays in the absence of metal ions, recorded after 19 wet-dry cycle at 70 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

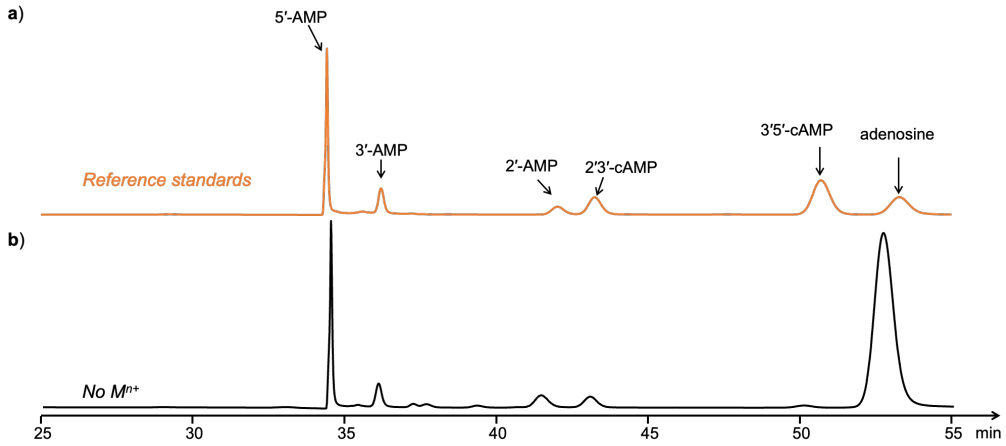


Fig. S26. Stacked representation of HPLC chromatograms from a) mixture of all the synthesised reference standards for finding the optimum run condition to separate the mixture of compounds, b) phosphorylation of the adenosine assay in the absence of metal ions, recorded after 19 wet-dry cycles at 70 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

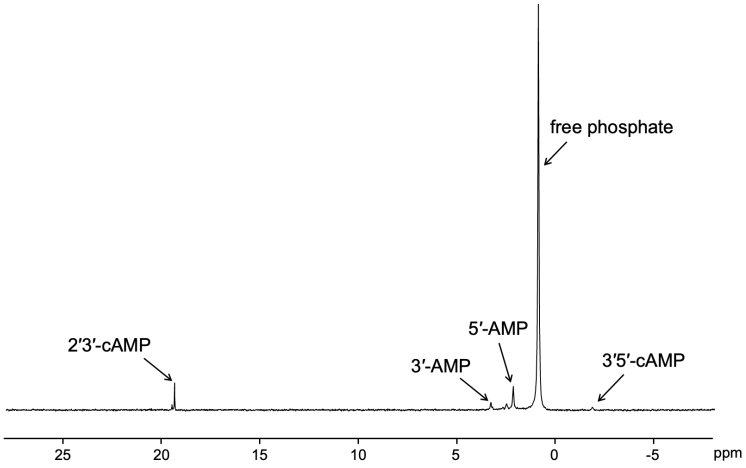


Fig. S27. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of adenosine assay solution in the absence of metal ions, recorded after 19 wet-dry cycle conducted at 70 °C.
Condition: 5 mM adenosine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

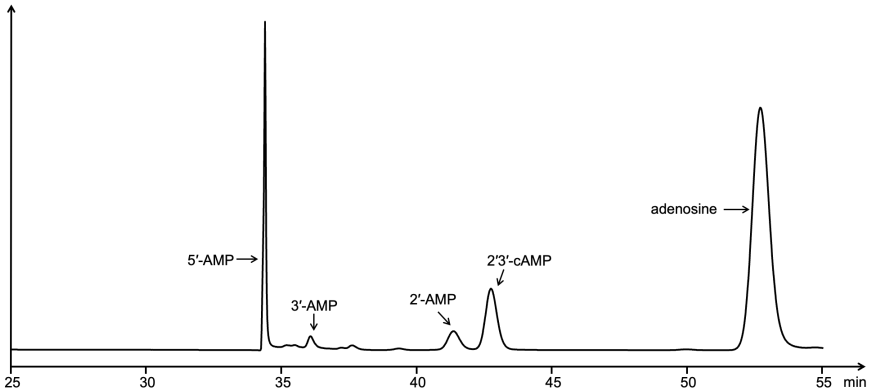


Fig. S28. HPLC chromatogram from phosphorylation assay in presence of Co^{2+}, recorded after 19 wet-dry cycle at 70 °C.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM Co^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

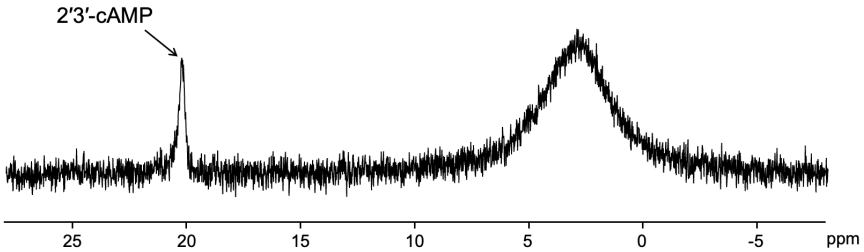


Fig. S29. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of ribonucleoside assay solution in presence of Co^{2+}, recorded after 19 wet-dry cycle conducted at 70 °C.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM Co^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

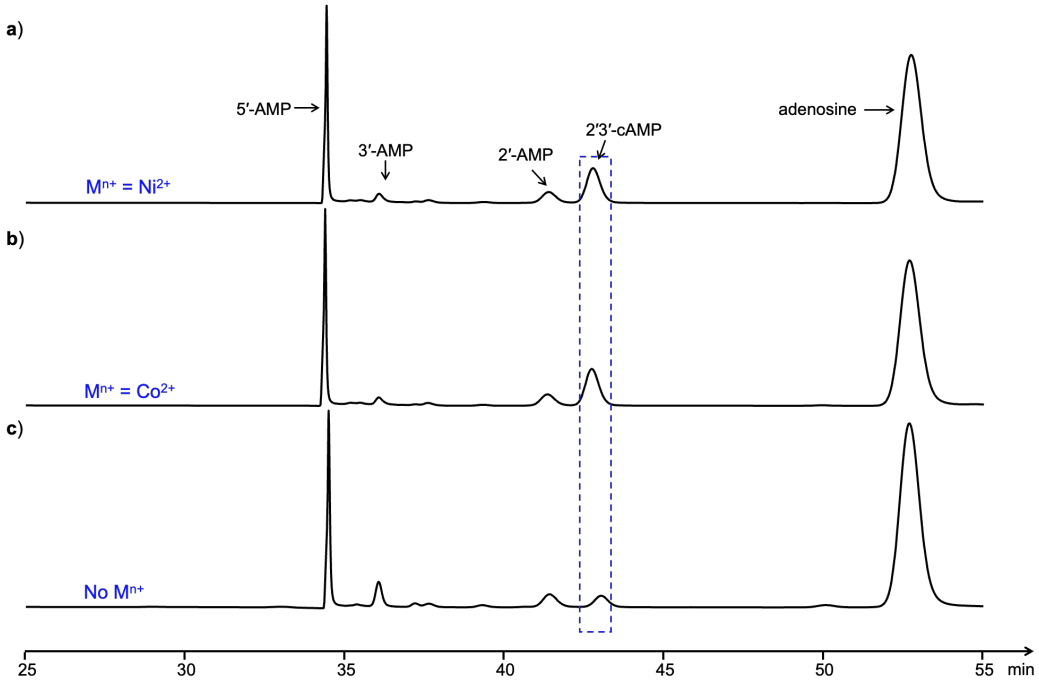


Fig. S30. Stacked representation of HPLC chromatograms from phosphorylation assays a) with Ni^{2+}, b) with Co^{2+} and c) in the absence of metal ions, recorded after 19 wet-dry cycle at 70 ºC.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

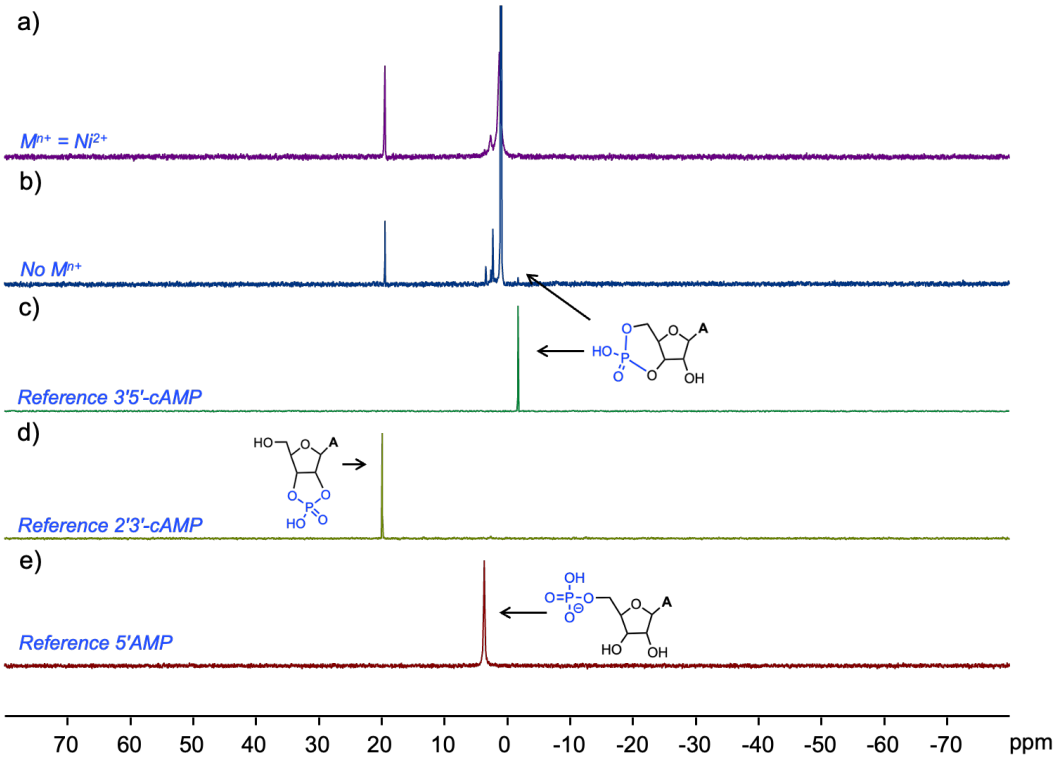


Fig. S31. Stacked representation of proton-decoupled ^{31}P-NMR spectra of a) with Ni^{2+}, b) in the absence of metal ions recorded after 19 wet-dry cycles conducted at 70 °C and synthesized reference standards of c) 3′5′-cAMP, d) 2′3′-cAMP, e) 5′-AMP.
Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

Guanosine phosphorylation assay

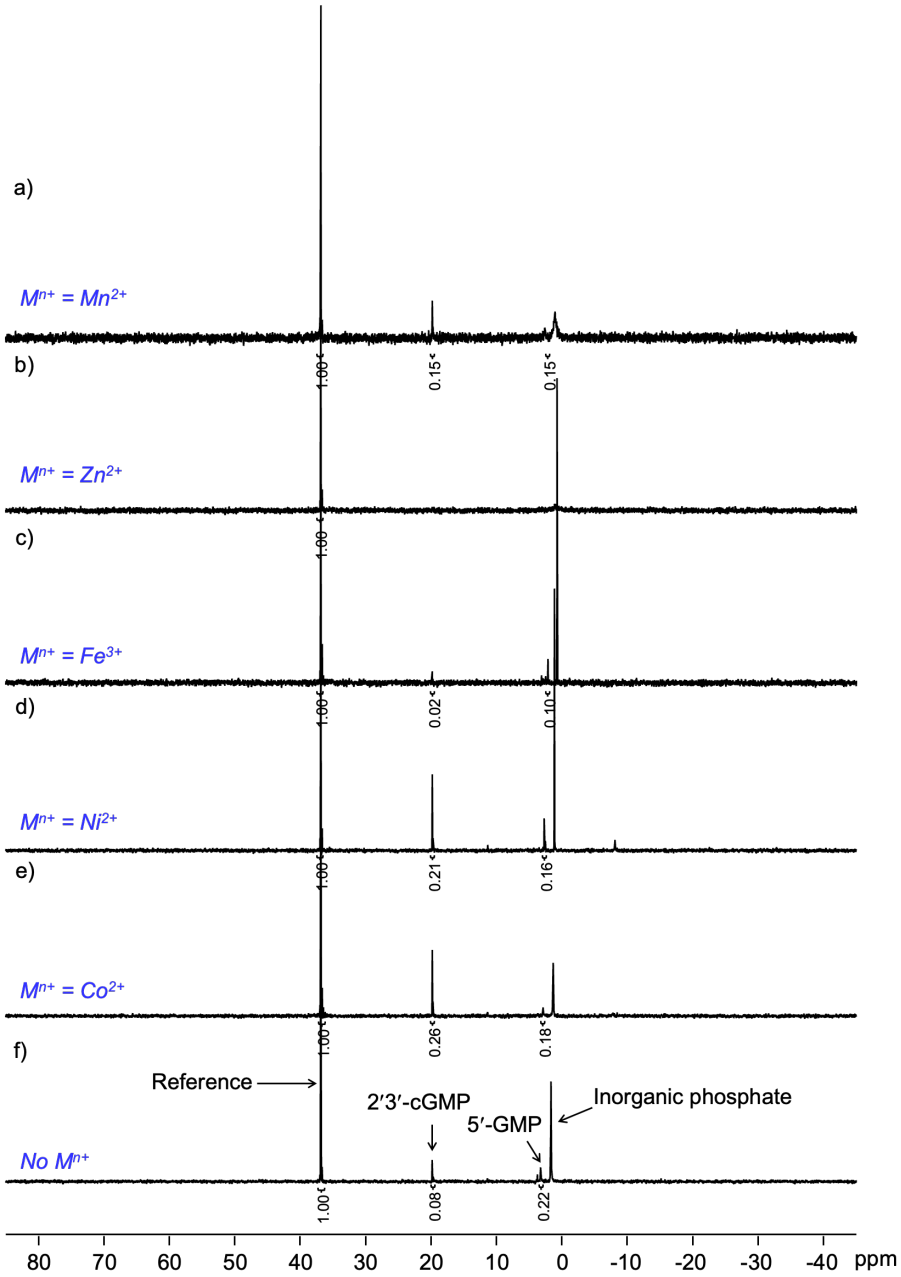


Fig. S32. Stacked representation of proton-decoupled ^{31}P-qNMR spectrum for phosphorylation of the guanosine assay a) with Mn^{2+}, b) with Zn^{2+}, c) with Fe^{3+}, d) with Ni^{2+}, e) with Co^{2+} and f) in the absence of metal ions recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM guanosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

Table S3. Quantified yield of 5'-GMP and 2'3'-cGMP by proton decoupled ^{31}P-qNMR from phosphorylation assay of guanosine with different transition metal ions, following 5 wet-dry cycle at 85 ºC.

	Concentration (in mM)		Integral			Yield (%)	
M^{n+}	reference	analyte	reference	5'-GMP	2'3'-cGMP	5'-GMP	2'3'-cGMP
blank[a]	2	2.3	1	0.22	0.08	19.1	7.0
Co^{2+}	2	2.4	1	0.18	0.26	15.0	21.7
Ni^{2+}	2	2.3	1	0.16	0.21	13.9	18.3
Fe^{3+}	2	2.3	1	0.10	0.02	8.7	1.7
Zn^{2+}	2	2.3	1	ND	ND	0	0
Mn^{2+}	2	2.4	1	0.15	0.15	12.5	12.5

[a] Yield represent average of two independent assay
[b] Condition: 5 mM guanosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

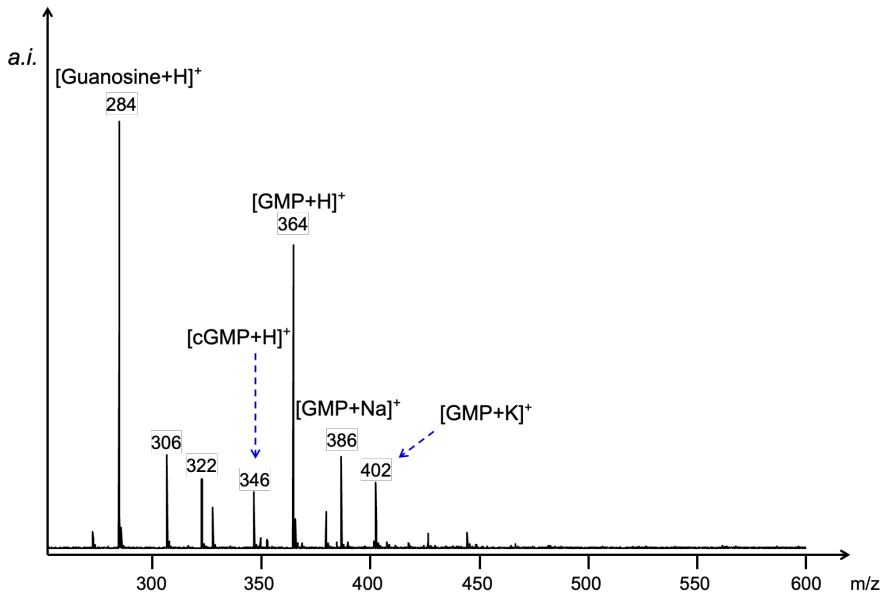


Fig. S33. MALDI-ToF mass spectrum from assay of phosphorylation of guanosine in the presence of Ni^{2+} after the completion of 19 wet-dry cycle at 70 °C.
Condition: 5 mM guanosine, 10 mM KH_2PO_4, 5 mM Ni^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.
The spectrum was recorded in positive mode using CHCA matrix.

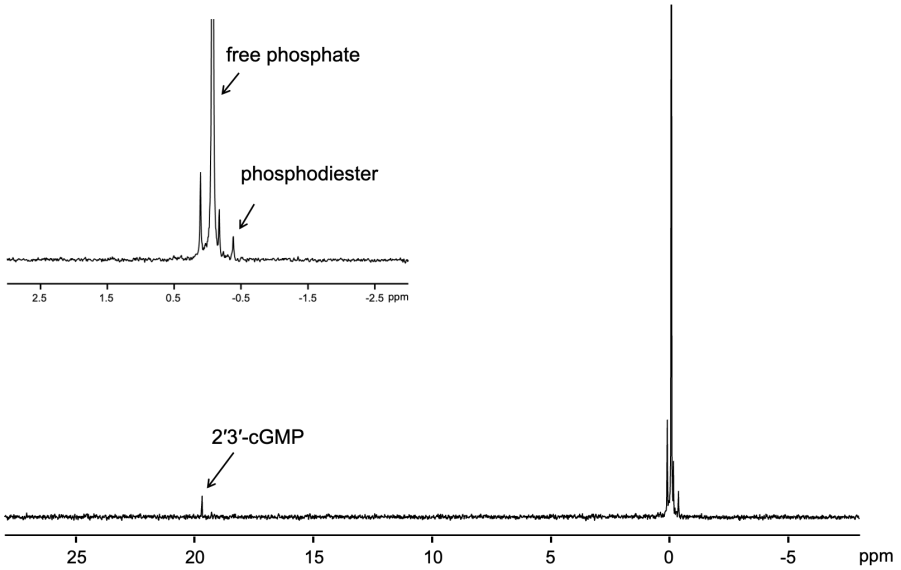


Fig. S34. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of guanosine assay solution in the presence of Ni^{2+} metal ions, recorded after 19 wet-dry cycles conducted at 70°C.
Condition: 5 mM guanosine, 10 mM KH_2PO_4, 5 mM Ni^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

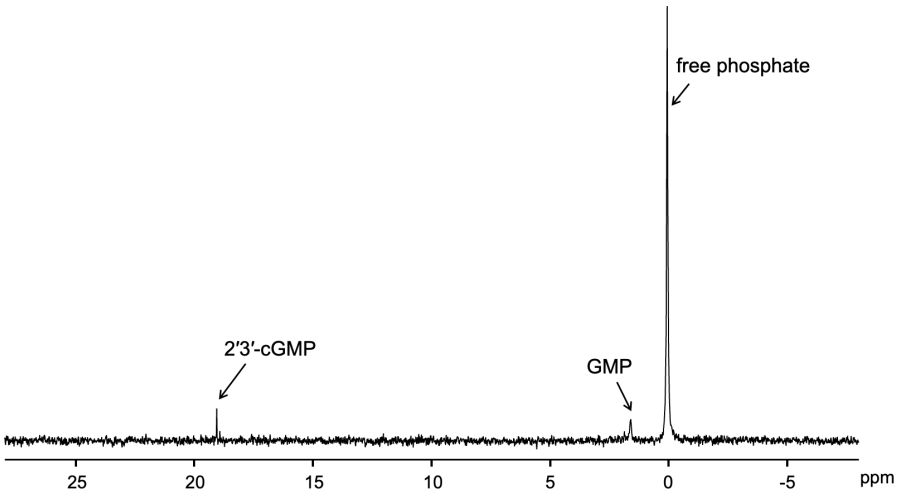


Fig. S35. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of guanosine assay solution in the absence of metal ions, recorded after 19 wet-dry cycles conducted at 70°C.
Condition: 5 mM guanosine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

Note: Interestingly, no phosphodiester bond formation was observed in the assay without metal ions, which can be attributed to the formation of 2′3′-cGMP in low amount. In here, 2′3′-cGMP and GpGp dinucleotide were not synthesized for reference measurement. The assignments in the earlier spectra were made based on the reported chemical shift values of these compounds in the literature[5, 6]

Uridine phosphorylation assay

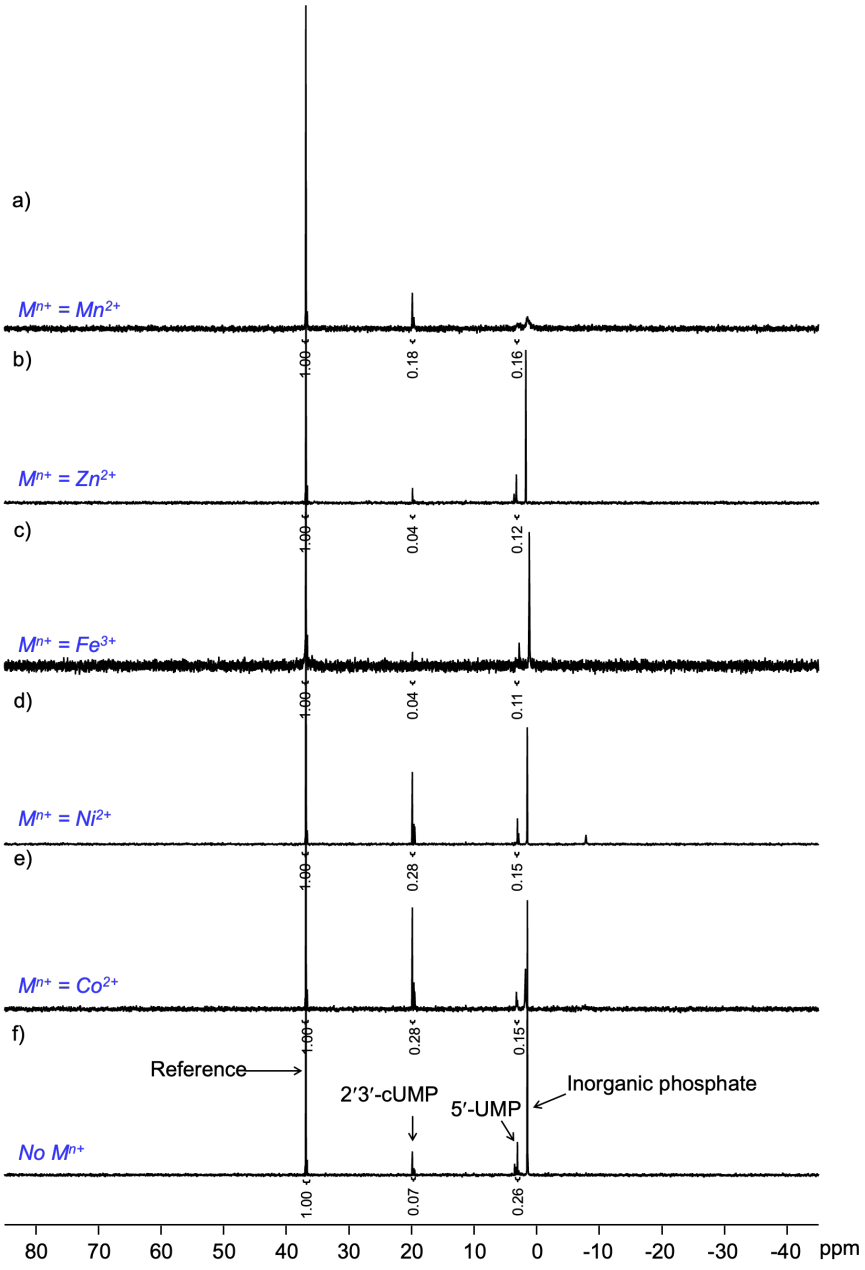


Fig. S36. Stacked representation of proton-decoupled ^{31}P-qNMR spectrum for phosphorylation of the uridine assay a) with Mn^{2+}, b) with Zn^{2+}, c) with Fe^{3+}, d) with Ni^{2+}, e) with Co^{2+} and f) in the absence of metal ions recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM uridine, 10 mM KH_2PO_4, 5 mM metal salt in water in water, containing 3.6 M ammonium formate and 7.2 M urea.

Table S4. Quantified yield of 5'-UMP and 2'3'-cUMP by proton decoupled ^{31}P-qNMR from phosphorylation assay of uridine with different transition metal ions, following 5 wet-dry cycle at 85 ºC.

	Concentration (in mM)		Integral			Yield (%)	
M^{n+}	reference	analyte	reference	5'-UMP	2'3'-cUMP	5'-UMP	2'3'-cUMP
blank[a]	2	2.2	1	0.26	0.07	23.6	6.4
Co^{2+}	2	2.6	1	0.15	0.28	11.5	21.5
Ni^{2+}	2	2.5	1	0.15	0.28	12.0	22.4
Fe^{3+}	2	2.5	1	0.11	0.04	8.8	3.2
Zn^{2+}	2	2.5	1	0.12	0.04	9.6	3.3
Mn^{2+}	2	2.3	1	0.16	0.18	13.9	15.7

[a] Yield represent average of two independent assay
[b] Condition: 5 mM uridine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

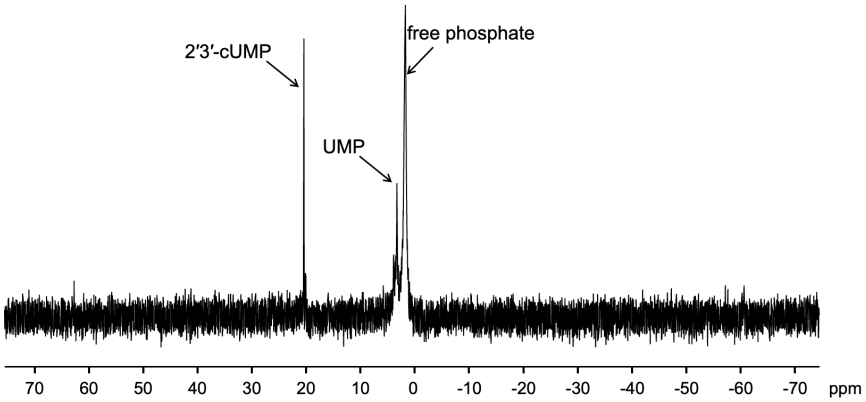


Fig. S37. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of uridine assay solution in the presence of Ni^{2+} metal ions, recorded after 19 wet-dry cycles conducted at 70°C.
Condition: 5 mM uridine, 10 mM KH_2PO_4, 5 mM Ni^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.
The assignments in the spectra was done based on the chemical shift values of these compounds reported in the literature[7]

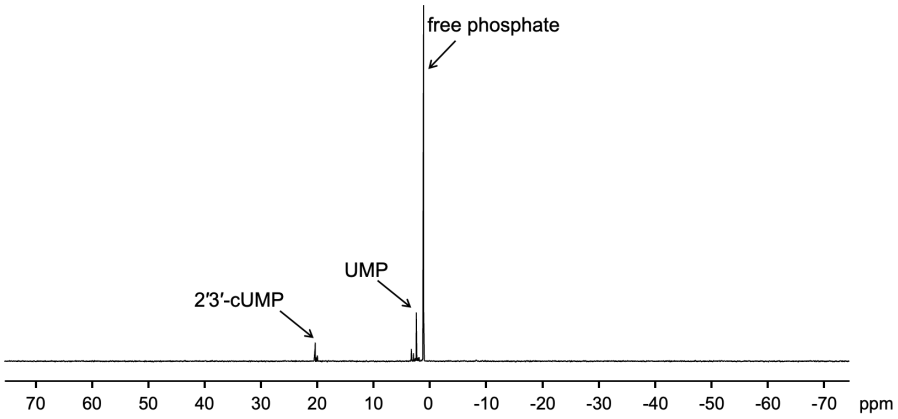


Fig. S38. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of uridine assay solution in the absence of metal ions, recorded after 19 wet-dry cycles conducted at 70°C.
Condition: 5 mM uridine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

Cytidine phosphorylation assay

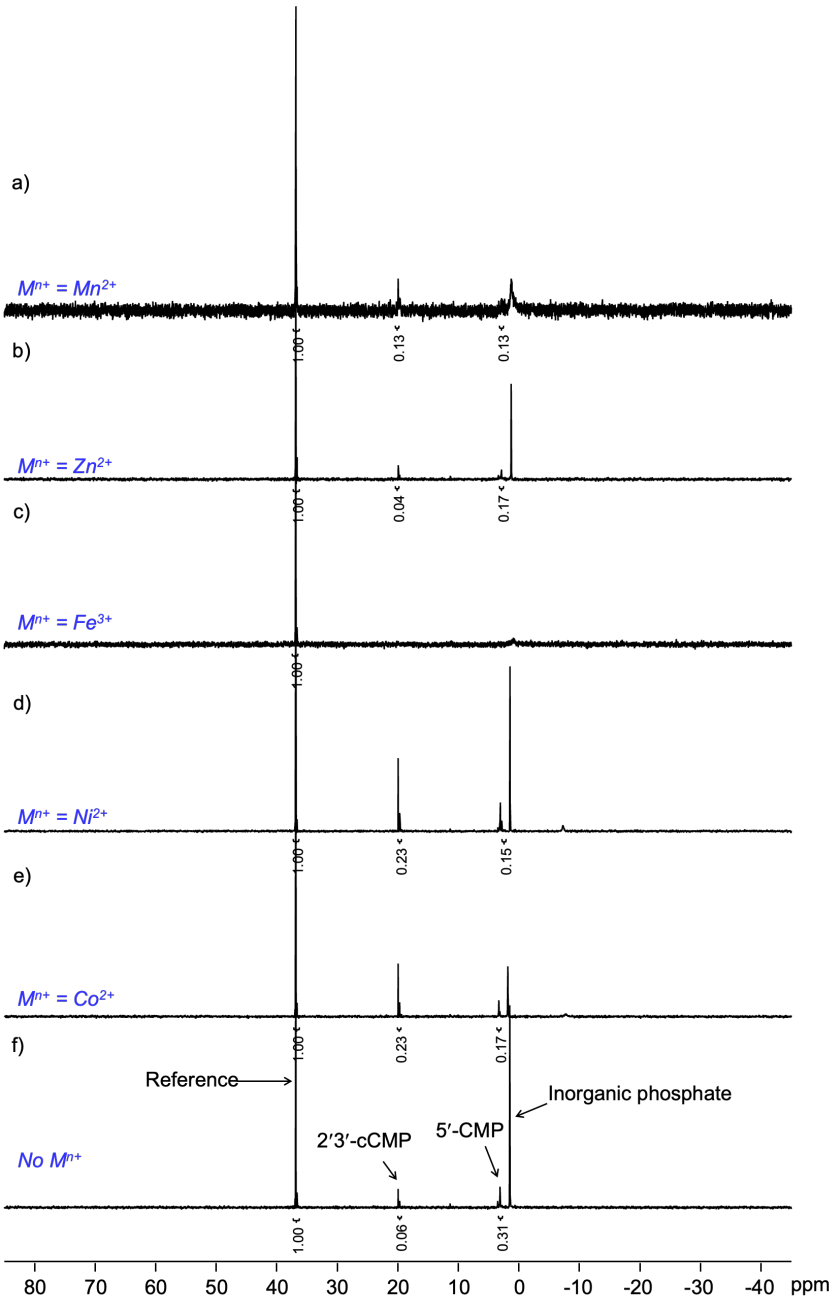


Fig. S39. Stacked representation of the proton-decoupled ^{31}P-qNMR spectrum for phosphorylation of the cytidine assay a) with Mn^{2+}, b) with Zn^{2+}, c) with Fe^{3+}, d) with Ni^{2+}, e) with Co^{2+} and f) in absence of metal ions recorded after 5 wet-dry cycle at 85 ºC.
Condition: 5 mM cytidine, 10 mM KH_2PO_4, 5 mM metal salt in water in water, containing 3.6 M ammonium formate and 7.2 M urea.

Table S5. Quantified yield of 5'-CMP and 2'3'-cCMP by proton-decoupled ^{31}P-qNMR from phosphorylation assay of cytidine with different transition metal ions, following 5 wet-dry cycle at 85 ºC.

	Concentration (in mM)		Integral			Yield (%)	
M^{n+}	reference	analyte	reference	5'-CMP	2'3'-cCMP	5'-CMP	2'3'-cCMP
blank[a]	2	2.2	1	0.31	0.06	28.2	5.5
Co^{2+}	2	2.2	1	0.17	0.23	15.5	20.9
Ni^{2+}	2	2.0	1	0.15	0.23	15	23.0
Fe^{3+}	2	2.1	1	n.d.	n.d.	n.d.	n.d.
Zn^{2+}	2	2.4	1	0.17	0.04	14.2	3.3
Mn^{2+}	2	2.2	1	0.13	0.13	11.8	11.8

[a] Yield represent average of two independent assay
[b] Condition: 5 mM cytidine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea.

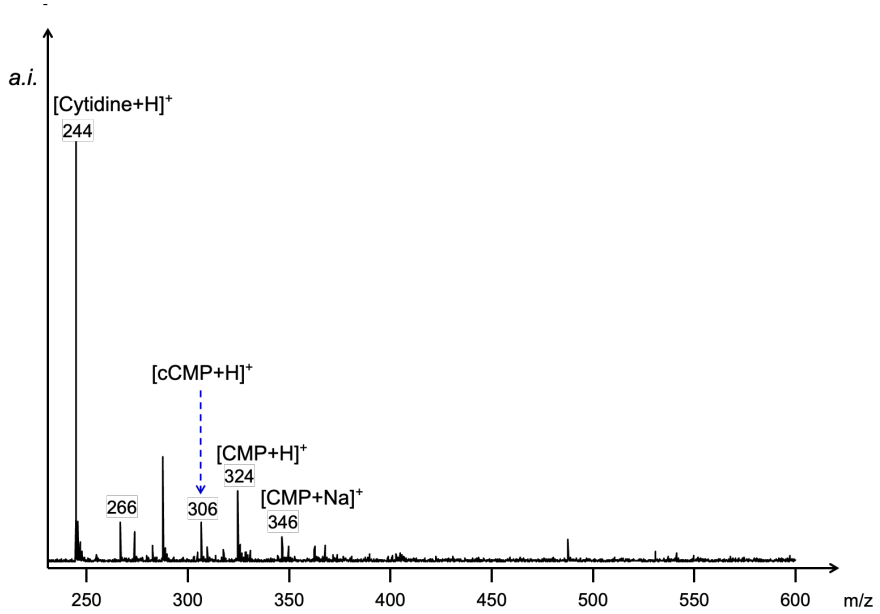


Fig. S40. MALDI-ToF mass spectrum from assay of phosphorylation of cytidine in the presence of Ni^{2+} after the completion of 19 wet-dry cycle at 70 °C.
Condition: 5 mM cytidine, 10 mM KH_2PO_4, 5 mM Ni^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.
The spectrum was recorded in positive mode using CHCA matrix.

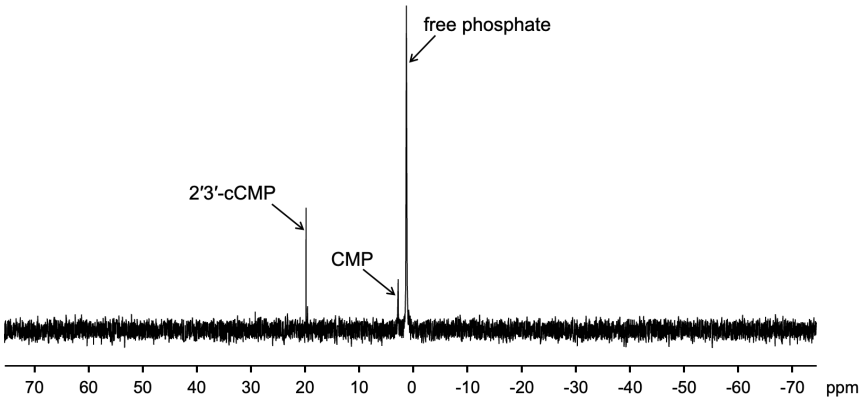


Fig. S41. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of cytidine assay solution in the presence of Ni^{2+} metal ions, recorded after 19 wet-dry cycles conducted at 70 °C.
Condition: 5 mM cytidine, 10 mM KH_2PO_4, 5 mM Ni^{2+} salt in water, containing 3.6 M ammonium formate and 7.2 M urea.
The assignments in the spectra were done based on the chemical shift values of these compounds reported in the literature[7]

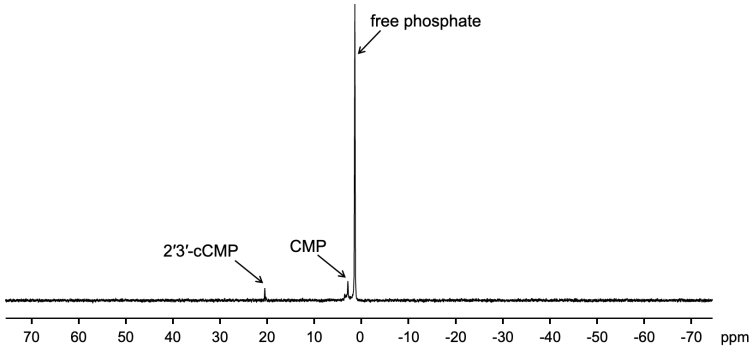


Fig. S42. Proton-decoupled ^{31}P-NMR spectrum of the phosphorylation of cytidine assay solution in the absence of metal ions, recorded after 19 wet-dry cycles conducted at 70 °C.
Condition: 5 mM cytidine, 10 mM KH_2PO_4 in water, containing 3.6 M ammonium formate and 7.2 M urea.

4. Additional Data

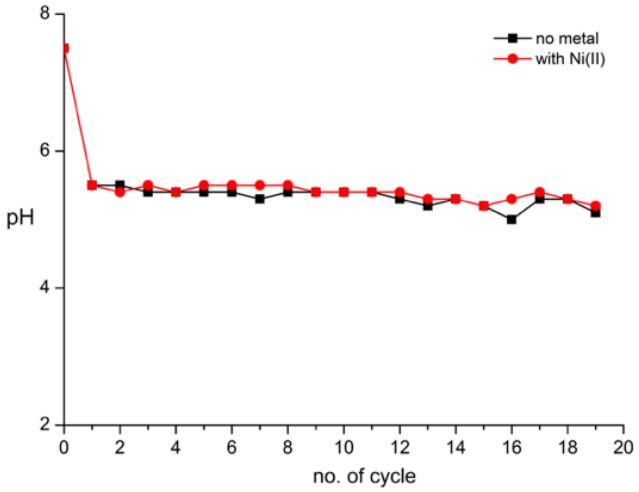


Fig. S43. pH of the assay mixture after each wet-dry cycle conducted in the presence and absence of Ni^{2+}. Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM metal salt in water, containing 3.6 M ammonium formate and 7.2 M urea, 70 °C.

Table S6. Conversion (%) to different regioisomers of adenosine monophosphate over different number of wet-dry cycle at 70 °C.[a,b,]

Cycles	Yield of the phosphorylation of the nucleoside			
	5′-AMP	3′-AMP	2′-AMP	2′,3′-cAMP
5	1	<1	<1	n.d.
10	4	1	1	1
15	11	2	3	7

[a] Yields are determined based on the area under the curve of the signals of interest in the HPLC chromatogram at 260 nm.

[b] Condition: 5 mM adenosine, 10 mM KH_2PO_4, 5 mM Ni^{2+} containing 3.6 M ammonium formate and 7.2 M urea.

Table S7: Conversion (%) to different adenosine monophosphate in presence of inorganic phosphate, as a function of Ni^{2+} concentration in the assay mixture and the number of wet-dry cycles at 70 °C [a].

Cycles	[Ni^{2+}]	5′-AMP	3′-AMP	2′-AMP	2′,3′-cAMP
	2.5 mM	1.5	<1	<1	n.d.
5	5 mM	1.2	<1	<1	n.d.
	10 mM	1.2	<1	<1	n.d.
	2.5 mM	2.5	<1	<1	<1
10	5 mM	4.4	<1	1.3	1.3
	10 mM	4.7	1.0	1.5	1.5
	2.5 mM	5.2	1.2	1.2	1.7
15	5 mM	10.6	1.8	2.8	6.7
	10 mM	10.3	1.6	2.5	7.0

[a] Condition: 5 mM adenosine, 10 mM KH_2PO_4 and varying amount of Ni^{2+} in a mixture containing 3.6 M ammonium formate and 7.2 M urea, 70°C.

Table S8. Conversion (%) of 2′,3′-cAMP into 3′-AMP and 2′-AMP in presence of Ni^{2+} by following 5 wet-dry cycles at 70 °C.[a,b]

	2′,3′-cAMP	3′-AMP	2′-AMP
No metal	91.4	5.5	3.1
Ni^{2+}	97.2	1.2	1.6

[a] Yields are determined based on the area under the curve of the signals of interest in the HPLC chromatogram at 260 nm.
[b] Condition: 2 mM 2′,3′-cAMP, 10 mM KH_2PO_4 and 5 mM Ni^{2+} in a mixture containing 3.6 M ammonium formate and 7.2 M urea.

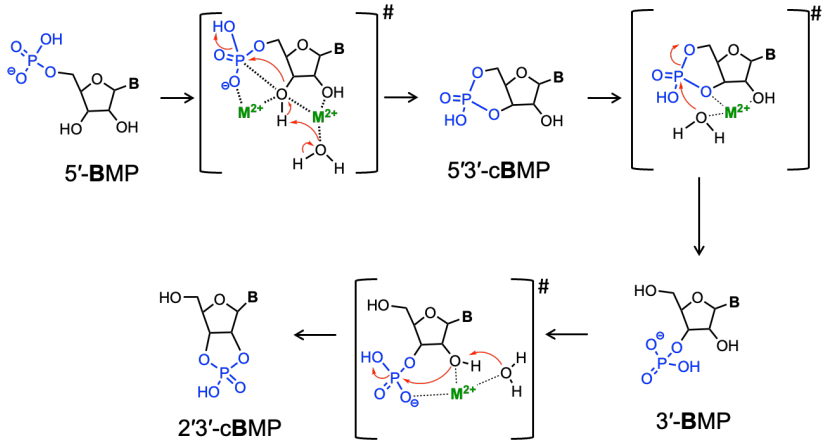


Fig. S44. Plausible mechanism for metal-ion-catalyzed phosphorylation of a ribonucleoside, and its stepwise transformation from 5′-BMP to 2′,3′-cBMP.

5. *References for Supplementary Information*

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