A Universal and Multiplex Kinase Assay Using γ-[$^{18}O_4$]-ATP

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Supporting Information

I. Reagent

cAMP-Dependent Protein Kinase(PKA), cGMP-Dependent Protein Kinase (PKG) and its substrate (Arg-Lys-Ile-**Ser**-Ala-Ser-Glu-Phe), PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay Kit, Trypsin Gold (Mass Spectrometry Grade) are purchased from Promega. Casein Kinase II (CK2) and its peptide substrate (Arg-Arg-Arg-Ala-Asp-Asp-**Ser**-Asp-Asp-Asp-Asp-Asp) are from NEB. PKA substrate kemptide (Leu-Arg-Arg-Ala-**Ser**-Leu-Gly), kinase inhibitors PKI (6-22) amide, H89, KT5823, SB203580, and heparin are from Sigma. $H_2{}^{18}O$ is purchased from Shanghai Research Institute of Chemical Industry (SRICI). Dissembled TiO_2 is a generous gift from Prof. Xiaohong Qian in Beijing Proteome Research Center. Ziptip C18, Ziptip MC, anti-phospho-ATF2 (Thr69/71) (clone AW65) are products of Millipore. MonoTip Trypsin and MonoTip TiO_2 are from GL Sciences. GSTrap column, HisTrap FF column and Mono Q HP column are from GE Healthcare.

II. The synthesis and characterization of γ-[$^{18}O_4$]-ATP

II.A The synthesis of γ-[$^{18}O_4$]-ATP

$KH_2P^{18}O_4$ was prepared using a modified method of Meyerson et al.[1] Phosphorus pentachloride (0.62 g, 3 mmol) was slowly added to $H_2{}^{18}O$ (0.44 ml, 24 mmol) at room temperature in a nitrogen atmosphere. After addition, the solution was heated at 60 ℃ overnight. The excess $H_2{}^{18}O$ and generated HCl were removed by rotary evaporation. The remaining yellow gum was dissolved in 1 ml water and pH was adjusted to 4.5 with 10% KOH solution followed by addition of two volumes of 100% EtOH. Resultant white precipitate was spun down and dried in desiccator.

γ-[$^{18}O_4$]-ATP was synthesized using the procedure described by Webb with a slight modification.[2] Firstly, ADP disodium salt (152 mg, 300 μmol) was converted to its pyridine salt **compound 1** with Dowex 50W×8 (100-200 mesh) resins. Tributylamine (220 μL, 900 μmol) was added and water was removed by rotary evaporation at 30 ℃ and then by freeze-drying. Anhydrous **compound 1** was dissolved in 1 ml of anhydrous dimethylformamide (DMF). Carbonyldiimidazole (243 mg, 1.5 mmol) was added in this solution. After 12 h at room temperature, the mixture was treated with anhydrous methanol (8 equiv, 100 μL) and left for 30 min. The tributylammonium salt of ^{18}O-enriched dihydrogen phosphate **compound 2** (1 mmol), which was prepared and purified as described for **compound 1**, was dissolved in 4 ml anhydrous DMF, and then added with vigorous mixing to a solution of activated ADP **compound 3**. The combined solution was held at room temperature for 24 h, at which time the reaction was about 70% complete. Attempts to allow the reaction to go further to completion unfortunately resulted in lower yields. After the reaction, one volume of 0.1 M triethylamine bicarbonate (TEAB) was added. The γ-[$^{18}O_4$]-ATP was purified on a column of DEAE Sepharose Fast Flow (10 ×1.5 cm diameter) with a linear gradient of TEAB from 0 to 500 mM. Fractions containing ATP were pooled, and the TEAB was removed by freeze-drying and repeating addition and evaporation of methanol. Synthesized TEA salt of γ-[$^{18}O_4$]-ATP was dissolved in methanol to give a solution of 50 mM, followed by addition of 20 mL of a 1 M solution of sodium iodide in acetone. The precipitated sodium salt was collected by centrifugation, repeatedly washed with acetone and desiccated by freeze-drying. The disodium salt of γ-[$^{18}O_4$]-ATP **compound 4** was finally stored frozen at -80 ℃, giving a final yield of 35%.

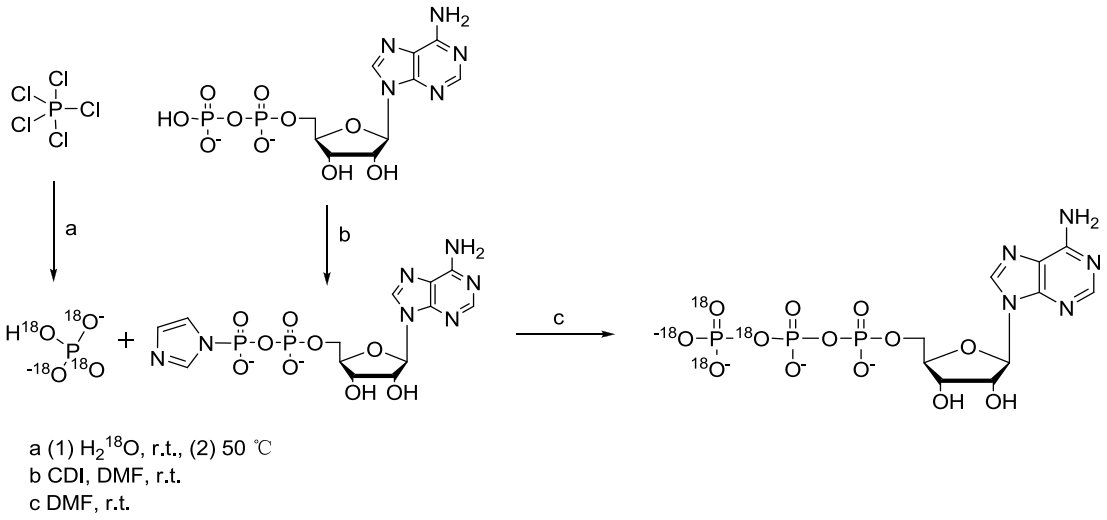
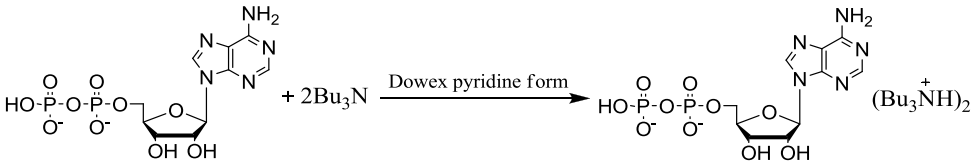


Figure S1. Synthetic route of γ-[$^{18}O_4$]-ATP.

II.B The characterization of intermediates and γ-[$^{18}O_4$]-ATP



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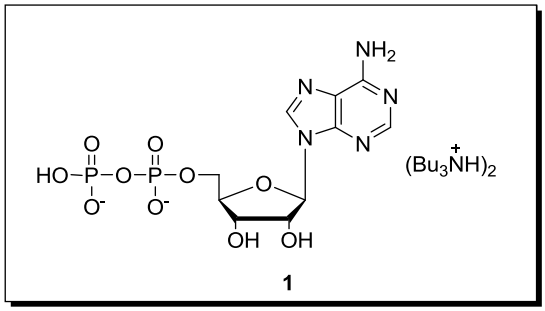
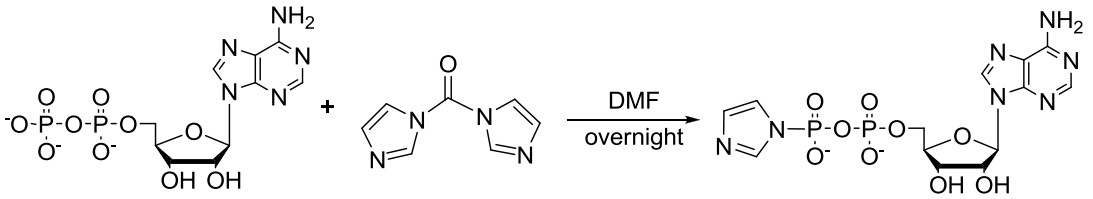


1

Compound 1 Tributylammonium adenosine diphosphate: yellow gum.

^{1}H NMR (400 MHz, D_2O) δ 8.47 (s, 1H), 8.21 (s, 1H), 6.03 (d, *J* = 5.7 Hz, 1H), 4.65 (t, *J* = 5.4 Hz, 1H), 4.49 – 4.41 (m, 1H), 4.35 – 4.26 (m, 1H), 4.14 (d, *J* = 3.7 Hz, 2H), 3.01 (dd, *J* = 9.8, 6.7 Hz, 12H), 1.55 (ddd, *J* = 12.0, 10.1, 6.5 Hz, 12H), 1.37 – 1.15 (m, 12H), 0.82 (t, *J* = 7.4 Hz, 18H).

^{31}P NMR (162 MHz, D_2O) δ -10.76 (d, *J* = 19.6 Hz), -11.30 (d, *J* = 20.8 Hz)



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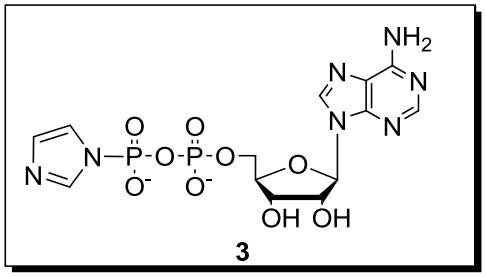
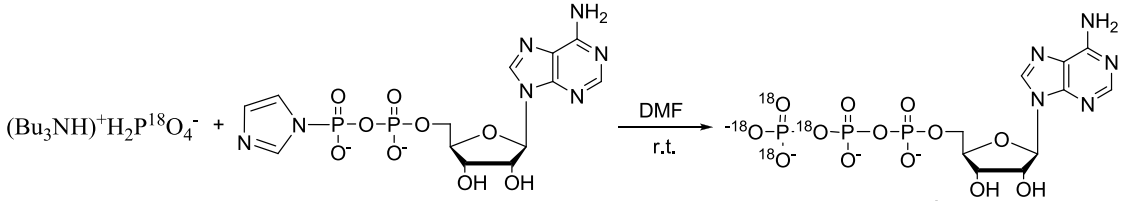


3

Compound 3 imidazole activated ADP

^{31}P NMR (162 MHz, $CDCl_3$) δ -12.02 (d, *J* = 14.0 Hz), -20.38 (d, *J* = 14.4 Hz).



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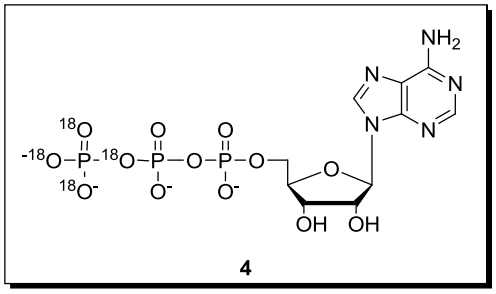

4

Compound 4 Adenosine 5'-triphosphate-P",P",P'-oxy-$^{18}O_4$: white solid.

^{1}H NMR (400 MHz, D_2O) δ 8.33 (s, 1H), 7.95 (s, 1H), 5.96 (d, *J* = 5.4 Hz, 1H), 4.67 – 4.62 (m, 1H), 4.54 – 4.48 (m, 1H), 4.35 – 4.29 (m, 1H), 4.25 – 4.12 (m, 2H).

^{13}C NMR (101 MHz, D_2O) δ 155.07, 152.49, 148.65, 139.75, 118.25, 86.86, 83.72, 74.31, 70.15, 65.12 (d, *J* = 5.3 Hz).

^{31}P NMR (162 MHz, D_2O) δ -7.21 (d, *J* = 17.0 Hz), -10.82 (d, *J* = 18.2 Hz), -21.41 (t, *J* = 16.6 Hz).

MS (ESI): m/z 513.6 ($[M-H]^-$, 20), 427.4 ($[M-P_i-H]^-$, 100).

III. Protein expression and purification:

Plasmid encoded His-p38α, GST-MKK6(E), and GST-ATF2(1-109) are generous gifts from Prof. J. Han at Xiamen University, P. R. China.[3] The above three plasmids were transformed to *E. coli* BL21(DE3) strain, followed by inducing with 1 mM IPTG. Purifications are accordant with reference.[4] Briefly, His-p38α was purified by Ni-NTA resin followed by DEAE resin. GST-MKK6(E) and GST-ATF2(1-109) were purified by GSTrap column according to the standard protocol. Purified p38α (0.5mg/mL) was activated by incubating with GST-MKK6(E) (0.01 mg/mL) in a PBS buffer containing 1 mM ATP, 10 mM $MgCl_2$, 0.62 mM EGTA, and 0.25 mM DTT for 20 hat RT. Activated p38α was separated by another round of His-tag purification. The purity and phosphoryl status of His-p38α were verified by SDS-PAGE (Figure. S2 a) and LC-MS (Figure. S2 b).

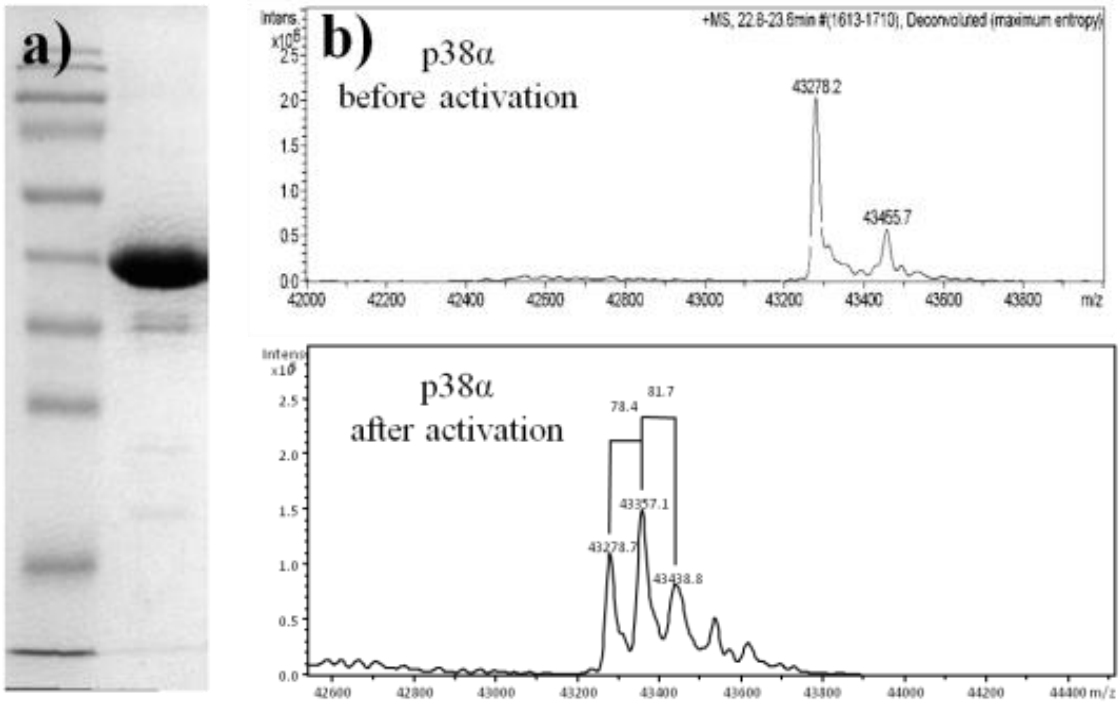

Figure S2. a) SDS-PAGE analysis of purified p38α. b) LC-MS spectra of p38α before and after activation by GST-MKK6(E). Peak with Mw. 43278 indicates purified p38α and peaks with Mw. 43357 and Mw. 43438 represent the active form of p38α.

IV. Cell culture and preparation of crude PKA from cell lysate:

PKA extraction buffer: 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA , 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/mL leupeptin, 1 μg/mL aprotinin, store at -20 ℃. Just before use, add PSMF to a final concentration of 1mM.

HeLa cells were cultured in Eagle's Minimum Essential Medium with 10% (v/v) FBS (fetal bovine serum) at 37 ℃ in 5% CO_2. For preparation of crude PKA, 5×10^6 cells were harvested by trypsin digestion and washed with 5 ml chilled PBS twice. Cell pellet was suspended in 0.5 ml of cold PKA extraction buffer, and homogenized using grinding pestle in 1.5 ml tube. Crude PKA containing supernatant was cleared by centrifugation for 5 mins at 4 ℃ at 14,000 × g and used immediately or aliquoted and stored at -80 ℃.

V. Condition for standard protein kinase assay:

PKA reaction Buffer: 10 mM Tris (pH 7.4), 1 mM $MgCl_2$

p38 reaction Buffer: 50 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.2 mM Na_3VO_4, 1 mM NaF, 0.1% BSA, 2.5 mM DTT.

Universal Kinase reaction Buffer: 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 2.86 uM cGMP.

Standard PKA kinase reaction was carried out in the presence of 50 μM kemptide, 10 μM heavy or light ATP, 0.4 Unit PKA catalytic subunit in 10 μL PKA reaction buffer at 30 ℃ for 10min. The reaction was stopped by heating at 95 ℃ for 2 mins. When using crude PKA, 1 uL lysate instead of purified PKA was added and cAMP was complemented to a final of 1 μM with 1× protease inhibitor (Roche). When performing high throughput screening for PKA inhibitor, $MgCl_2$ is reduced to 0.2 mM.

Standard p38 kinase reaction was carried out in the presence of 100 μM heavy or light ATP, 1 μg GST-ATF2(1-109), 4 ng activated p38α in 10 μL p38 reaction buffer at 30 ℃ for 30min. The reaction was stopped by adding 2.5 μL 1% SDS, 12.5 ul trypsin buffer (Promega), 0.5 μg trypsin (Pormega) and performed digestion at 37 ℃ for 8 hr.

Multiplex kinase reaction was carried out in the presence of 100 μM ATP, 0.4 Unit PKA, 0.4 Unit PKG, 0.4 Unit CKII, and all three peptide substrates for PKA, PKG and CKII,respectively, in the universal kinase reaction buffer at 30 ℃ for 20 min. Reaction was stopped by heating at 95 ℃ for 2min.

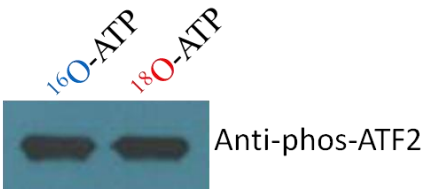

Figure S3. Western blot result of phosphorylated GST-ATF2(1-109) by p38α. Two parallel reactions were carried out under the same conditions with the same amount of all other reagents except using γ-[$^{16}O_4$]-ATP (left lane) or γ-[$^{18}O_4$]-ATP (right lane). The amount of phosphorylated GST-ATF2(1-109) was detected by anti-phos-ATF2 antibody and recorded by exposure to X-ray film according to conventional western blot procedures.

VI. Isotope exchange test:

Standard PKA kinase reaction was set up in $H_2{}^{16}O$ with γ-[$^{18}O_4$]-ATP. Before the addition of protein kinase, the reaction mixture was lyophilized and re-dissolved in the same volume of $H_2{}^{16}O$ or $H_2{}^{18}O$ followed by standard reaction and mass analysis (Figure S4).

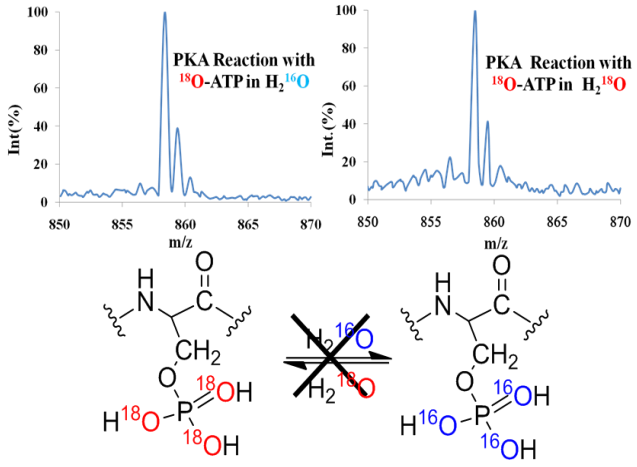

Figure S4. MALDI-TOF-MS spectrum of phosporylated kemptide (theoretical Mw. 858.6) catalyzed by PKA. Upper left panel, *in vitro* kinase reaction using γ-[$^{18}O_4$]-ATP in $H_2{}^{16}O$; Upper right panel, *in vitro* kinase reaction using γ-[$^{18}O_4$]-ATP in $H_2{}^{18}O$. No obvious exchanged peak (theoretical Mw. 852.6) was observed.

VII. Sample preparation and MALDI-TOF parameters:

VII.A Recipes for MALDI-TOF matrix:

CHCA matrix: saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile, 0.1% TFA.

Phospho-peptide matrix: 20 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% acetonitrile, 1% ortho-phosphoric acid

VII.B Experimental Procedures

For PKA reaction, reaction mixture can be equivalently mixed with CHCA matrix and spotted on MALDI plate. In some cases, samples can be further desalted by C18 ziptip (Millipore) according to the manufacturer's instructions for better signal. When performing the high throughput screening for PKA inhibitor, the reaction solution was analyzed without any enrichment or desalting.

For p38 kinase reaction, relevant phosphopeptide was enriched by TiO_2 disassembled from a column chromatography or by MonoTip TiO_2 as described by Tine E Thingholm *et al*.[5] An aliqout of 0.5 uL of the enriched peptides was equivalently mixed with 0.5 uL of phospho-peptide matrix and spotted on MALDI plate.

MALDI-TOF-MS analysis was performed on a Bruker REFLEX III Instrument (SCOUT 384 Source, 1.5/3.0 M TOF tubes, 2 MHz Digitizer). A 337 nm N_2 laser source with 3 ns laser pulse, an accelerating voltage of 20.0 KV and a reflecting voltage of 23.0 KV were applied for the MS experiments. The spectra were acquired in the reflex and positive mode with an accumulation of 300 times (10 times/spot). The external chemicals for the calibration of all the spectra were Angiotensin Ⅱ (1046.5 MW), Bombesin (1619.8 MW), ACTHclip18 ~39 (2465.2 MW) and Somatostatin28 (3147.47 MW). Peak area at appropriate Mw. of interest was chosen for quantitative assay and integrated by Bruker DataAnalysis. The data processing and calculation of IC_{50} were performed by using Graphpad Prism.

VIII. Reference:

(1) Meyerson, S. K., E. S.; Ramirez, F.; Marecek, J. F. *J Am Chem Soc* **1982**, *104*, 7231.
(2) Webb, M. R. *Biochemistry* **1980**, *19*, 4744.
(3) Ge, B.; Gram, H.; Di Padova, F.; Huang, B.; New, L.; Ulevitch, R. J.; Luo, Y.; Han, J. *Science* **2002**, *295*, 1291.
(4) Keesler, G. A.; Bray, J.; Hunt, J.; Johnson, D. A.; Gleason, T.; Yao, Z.; Wang, S. W.; Parker, C.; Yamane, H.; Cole, C.; Lichenstein, H. S. *Protein Expr Purif* **1998**, *14*, 221.
(5) Thingholm, T. E.; Jorgensen, T. J.; Jensen, O. N.; Larsen, M. R. *Nat Protoc* **2006**, *1*, 1929.