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Supplementary Information

Colorimetric and Fluorometric Dual-Readout Protein Kinase Assay by Tuning the Active Surface of Nanoceria

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Contents

1. Experimental section

Materials and reagents

cAMP-dependent protein kinase A (PKA, catalytic subunit) and protein phosphatase 1 (PP1) were purchased from New England Biolabs. H-89 was purchased from EMD Bioscience, and ATP was from Sangon Biotech (Shanghai, China). PKA specific substrate peptides (TAMRA-LRRASLG and LRRASLG) and their phosphorylated counterparts (TAMRA-LRRApSLG and LRRApSLG) were synthesized by GL Biochem (Shanghai, China). CeO_2 nanoparticles (nanoceria) were supplied by Alfa Aesar, and 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from Sigma-Aldrich. All of the other reagents used in this work were analytical grade and used as received without further purification.

Procedures of the nanoceria-based fluorometric assay for the detection of PKA and PP1 activity

Typically, in a 100 μL PKA reaction system, TAMRA-LRRASLG substrate peptide (3 μM) and ATP (24 μM) were incubated with a certain amount of PKA in the reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4) at 37 ºC for 60 min. Then, this reaction solution was directly mixed with an equal volume of colloidal nanoceria (100 μL, 120 μg/mL). Immediately after mixing, the fluorescence spectrum of such mixed solution was recorded on an LS-55 fluorescence spectrophotometer (PerkinElmer) under the excitation of 540 nm.

The procedures for the determination of PP1 activity were similar to the PKA assay stated above. In a typical 100 μL reaction solution, TAMRA-LRRApSLG peptide (3

μM) was reacted with certain dosage of PP1 in HEPES buffer (50 mM HEPES,100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 10 mM $MgCl_2$, pH=7.4) at 37 ºC for 60 min. Then, this reaction solution was directly mixed with an equal volume of colloidal nanoceria (120 μg/mL). The fluorescence spectrum of this mixed solution was measured immediately.

Procedures of the nanoceria-based colorimetric assay for the detection of PKA and PP1 activity

In a 50 μL PKA reaction solution, LRRASLG peptide (20 μM) and ATP (20 μM) were incubated with series dilutions of PKA in Tris-HCl buffer (10 mM, pH 7.4) containing 10 mM $MgCl_2$ at 37 ºC for 60 min. After adjusting the pH of the reaction system to 4.0 by adding acetic acid, colloidal nanoceria was introduced with a final concentration of 30 μg/mL. After mixing these two solutions for about 10 min, 5 μL of TMB (final concentration of 1 mM) was added to make up a final volume of 100 μL. After standing for 1 h at room temperature, the color change of such reaction mixture was recorded by a D90 digital camera (Nikon), and the corresponding visible absorption spectrum was measured on a TU-1901 spectrometer (Pgeneral) by using the two-fold diluted mixture.

Similarly, as for the determination of PP1 activity by the colorimetric strategy, the peptide LRRApSLG (3 μM) was firstly incubated with PP1 at 37 ºC for 40 min in 50 μL of HEPES buffer. After that, acetic acid was used to adjust the pH of the reaction solution. Then the nanoceria (final concentration of 30 μg/mL) and TMB (final concentration of 1 mM) were sequentially introduced to the PP1 reaction system to a

final volume of 100 μL. After the final mixture was incubated for 2 h at room temperature, the photograph was taken by the D90 digital camera, and the absorption spectrum was measured by using the two-fold diluted solution.

PKA inhibition study

For both the nanoceria-based fluorometric and the colorimetric strategies, the experiments for PKA inhibition studies were carried out following the same procedures as those of the corresponding PKA assays stated above, except for the involvement of a fixed PKA concentration and pre-incubating with varies concentrations of H-89.

Cell culture and preparation of cell lysates

MCF-7 cells (6×10^5 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum. The cells were cultured in an incubator at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity.

Cells were starved with serum-free DMEM for 4 h before stimulated with 10 μM forskolin and 20 μM IBMX in dimethylsulfoxide (DMSO), which can effectively activate the cellular cAMP-dependent PKA. After 30 min of stimulation, the cultured cells were washed three times with pre-cold PBS buffer. Then, the cell suspensions were treated by ultrasound with an ultrasonic cell disintegrator of 200 W ultrasonic power, 3 s interval time, 2 s ultrasonic time, and 60 min total working time. After that, the samples were centrifuged at 22000 rpm for 60 min at 4 °C and further ultrafiltered (10 K, Millipore). Finally, the samples collected were proceeded for the proposed kinase activity assay.

2. TEM image of the nanoceria

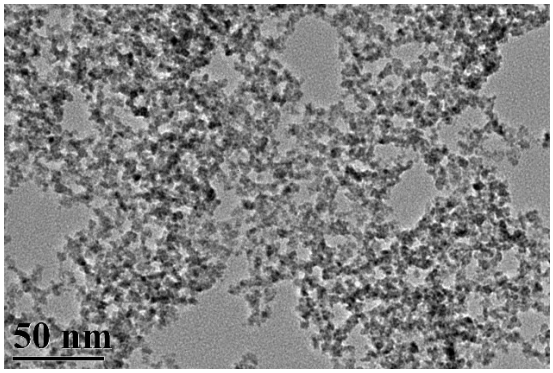

Fig S1. TEM image of the nanoceria used in this study.

3. Optimization of the experimental conditions

3.1. Optimization of nanoceria concentration

A series of 100 μL solutions containing TAMRA-LRRApSLG (TAMRA-p-peptide, 3μM) were prepared. Then the equal volume of nanoceria aqueous suspensions were added into the peptide solutions by controlling the final concentrations of nanoceria at 0, 0.5, 2, 5, 10, 25, 50, 60, and 80 μg/mL, respectively. The fluorescence spectra of each sample were measured immediately after the mixing of the two components under the excitation of 540 nm. The peak values of the fluorescence spectrum at 576 nm were recorded. The TAMRA-LRRASLG (TAMRA-peptide) was used as blank control. One can see from the result that the fluorescence intensity decreases gradually when the concentration of nanoceria ranges from 0 to 5 μg/mL. However, no obvious change in the fluorescence intensity can be found when nanoceria concentration is higher than 5 μg/mL, suggesting that all of TAMRA-LRRApSLG in the reaction solution might be adsorbed by the nanoceria when the concentration

reaches 5 μg/mL. Meanwhile, the fluorescence intensity of the blank control with TAMRA-LRRASLG kept almost unchanged irrespective of the variation of nanoceria concentration. To guarantee the high efficiency of the peptide-nanoceria combination, 60 μg/mL of nanoceria was selected in our experiment.

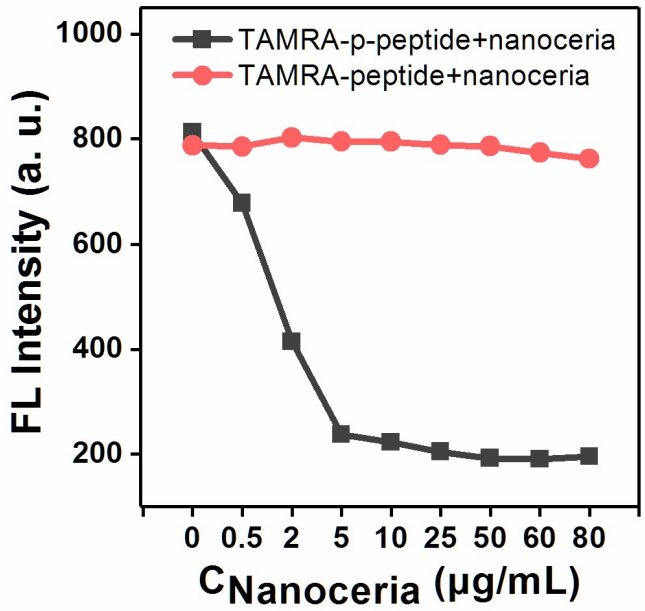

Fig S2. Phosphorylated peptide (black line) and non-phosphorylated peptide (red line) were incubated with different concentrations of nanoceria, respectively.

3.2 Optimization of nanoceria-peptide incubation time

Reaction time is one of the most important parameters for an analysis method. Therefore, the incubation time for phosphorylated peptide-nanoceria has been optimized. In this optimization, the PKA concentration in the phosphorylation reaction system is fixed at 0.01 U μL^{-1}, and 60 μg/mL of nanoceria is used in the final detection system. The fluorescence spectrum was recorded from 0 to 60 min. As shown in Fig. S2, the fluorescence signal decreases immediately when the phosphorylation reaction solution mixed with the colloidal nanoceria, and the fluorescence was almost quenched completely. Moreover, no obvious fluorescence

change was observed even if the reaction time extended, indicating the rapid combination of the phosphorylated peptide and the nanoceria. In contrast, the fluorescence signal of the blank control (without PKA) keeps almost consistent within 60 min. Therefore, this method allows for the mix-and-read fluorescence assay for the determination of protein kinase activity.

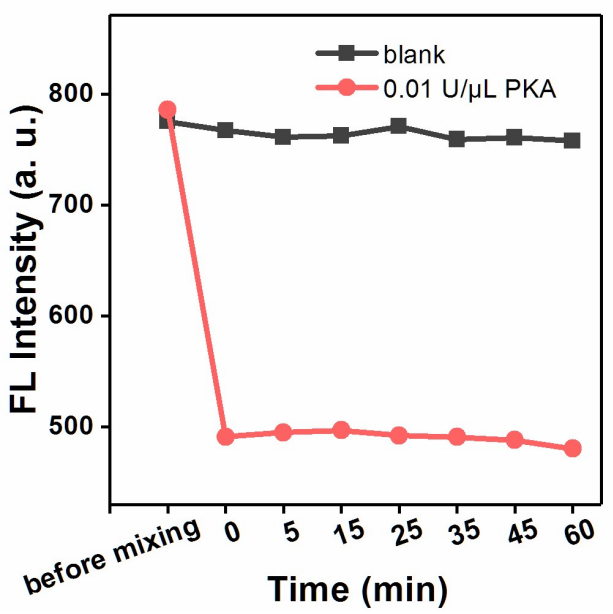

Fig S3. Fluorescence intensity at 576 nm of the reaction system (3 μM substrate peptide, 24 μM ATP) treated with 0.01 U μL^{-1} PKA (red line) and without PKA treatment (blank control, black line). 60 μg/mL nanoceria were used in the final detection mixture.

4. Nanoceria-based PKA activity colorimetric assay

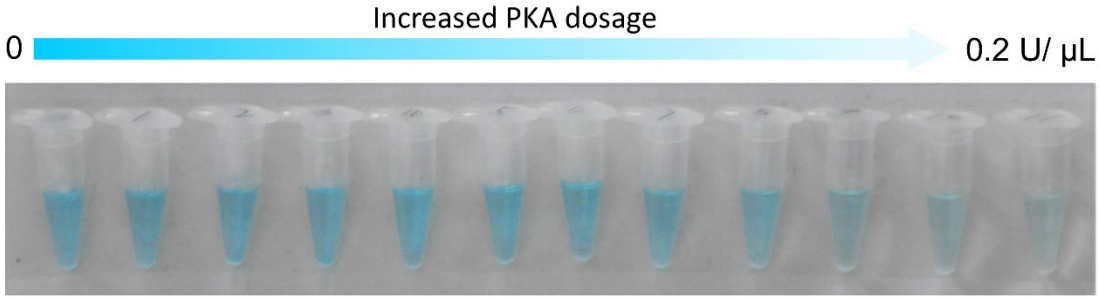

Fig S4. Corresponding photographs of the samples in Fig 3a with different concentrations of PKA.

5. Determination of protein phosphatase activity

The level of phosphorylation is regulated by protein kinases and phosphatases, and the abnormal balance between them will cause many human diseases.[S1] Protein phosphatase (PP) plays an important role in mediating various signal transduction pathways, and it is one of the most important molecular targets in medical research and medical diagnosis.[S2] Therefore, we further analyze the protein phosphatase 1 (PP1, a class of PP) activity by using the proposed colorimetric and fluorometric strategies, which are constructed on the basis of controlling the exposed active surface of the nanoceria.

PP1 catalyzes the hydrolysis of the phosphate monoester to remove the phosphate groups from the phosphorylated serine/threonine.[S3] Hence, the fluorescent-labeled phosphorylated peptide (TAMRA-LRRApSLG) and the phosphorylated kemptide (LRRASLG) were selected for the nanoceria-based fluorometric assay and colorimetric assay respectively. As can be seen from Fig S5 and S6, with the activity of PP1 increasing, both the colorimetric and the fluorescence signals of the dephosphorylation reaction solution gradually enhanced. The reason is that with the increase of PP1 activity the amount of phosphorylated peptide decreases, which leads to the substrate desorbed from the nanoceria surface to restore the oxidization activity/fluorescence quenching capability of the nanoceria. Therefore, the PP1 activity assay further verifies the design principle that tuning the exposed active surface of the nanoceria to achieve the determination of protein kinase activity.

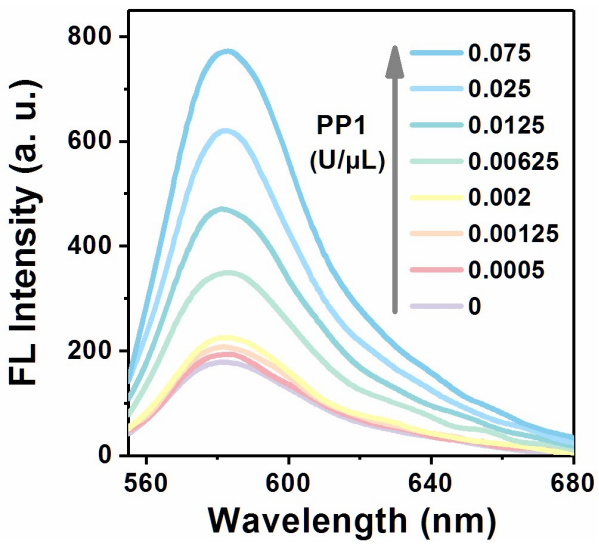

Fig S5. The results of PP1 activity analysis by the proposed nanoceria-based fluorometric assay.

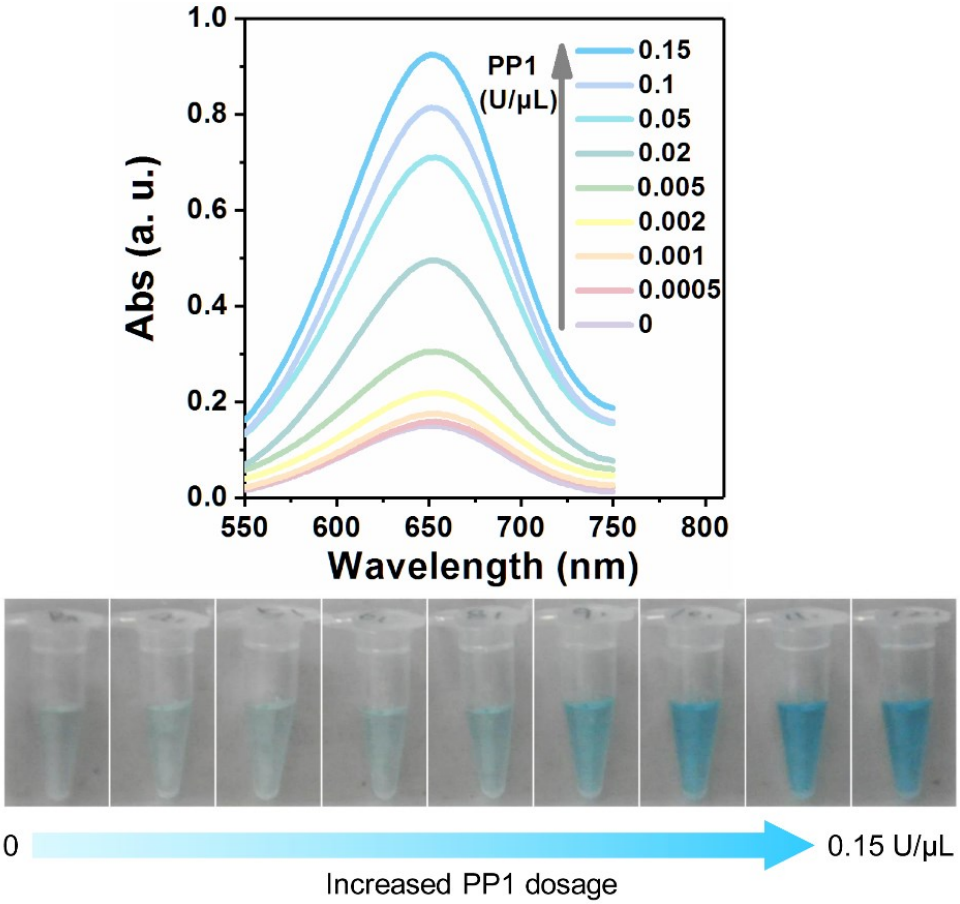

Fig S6. The results of PP1 activity analysis by the proposed nanoceria-based colorimetric assay.

6. Nanoceria-based PKA activity colorimetric assay in cell lysates

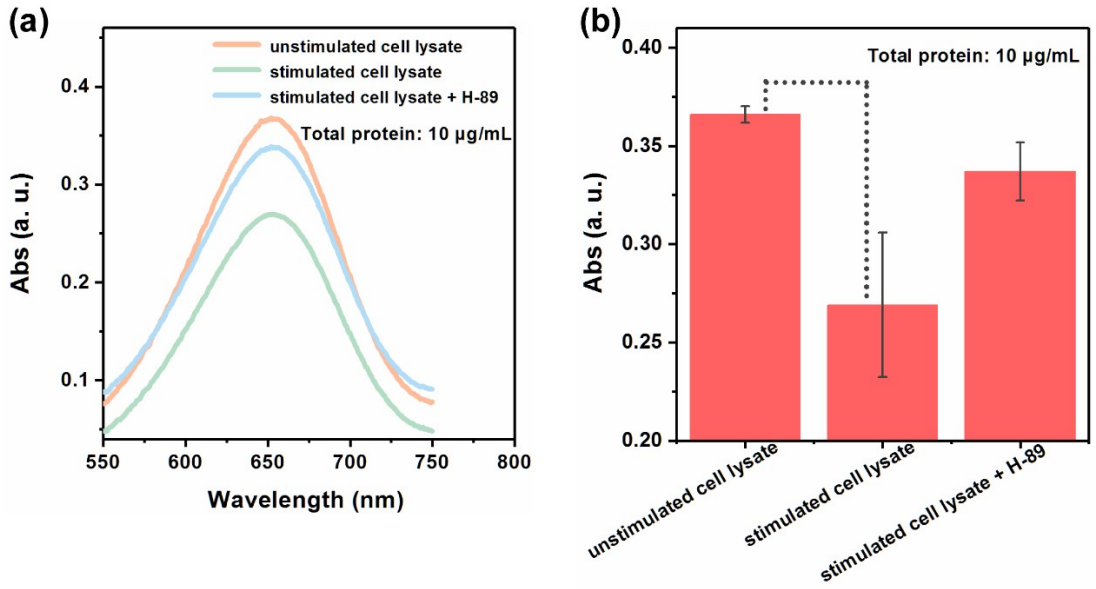

Fig S7. (a) Absorption spectra of the proposed nanoceria-based colorimetric assay for the determination of PKA activities in different types of MCF-7 cell lysates. The total protein concentration is fixed at 10 μg/mL and 20 μM substrate peptide, 20 μM ATP are used in the PKA reaction system. 30 μg/mL nanoceria was used in the final detection solution. (b) Corresponding quantitative analysis result of absorbance intensity at 651 nm.

7. Nanoceria-based fluorometric assay for PKA inhibition studies

As shown in Fig. S8a, the fluorescence signal at 576 nm increases gradually with the increasing concentration of H-89, indicating the effective inhibition of PKA activity. The relationship between the fluorescence intensities at 576nm and H-89 concentrations on the logarithm scale is plotted in Fig. S8b, from which the IC_{50} value of H-89 is estimated to be 134 nM. This result well consistent with the previous literature reports.[S4] This clearly suggests that the nanoceria-based fluorometric assay of kinase activity is feasible for the study of protein kinase inhibition and is of great potential in protein kinases-targeted drug development.

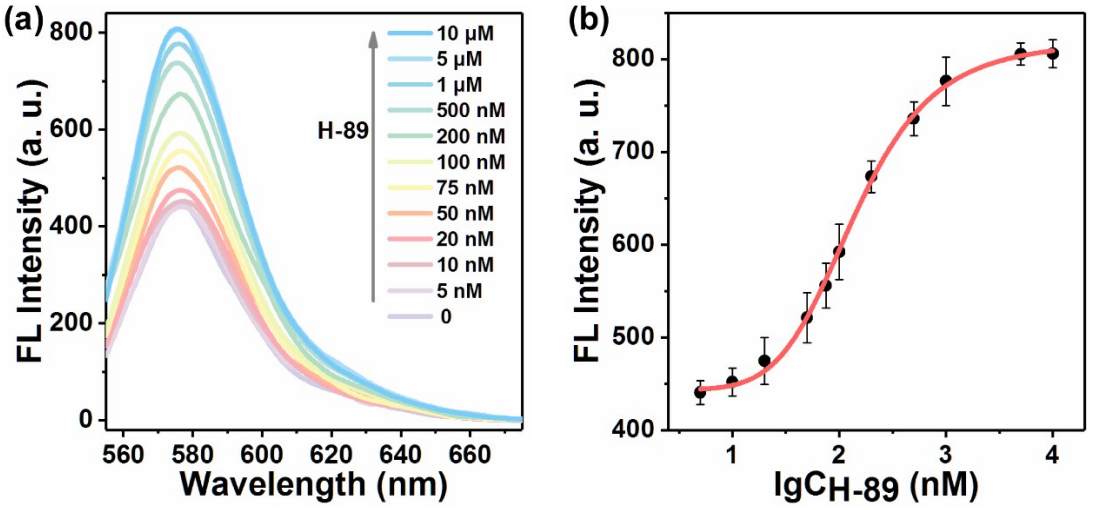

Fig S8. (a) Fluorescence spectra of the proposed nanoceria-based fluorometric system in the presence of different concentrations of H-89 (0-10 μM) by fixing PKA activity at 0.02 U μL^{-1}; (b) The relationship between the fluorescence intensity at 576 nm and H-89 concentration on the logarithm scale. The error bars represent the standard deviation of three replicates for each data point. 1.5 μM peptide, 12 μM ATP, and 60 μg/mL nanoceria were used in the final detection solution.

8. Comparison of different particle-based methods for PKs activity assay.

Table S1. Comparison of different PK activity analysis methods.

Detection methods	Particle species	Sensing mechanism	Detection limit	Reference
Fluorescence method	TiO_2-coated magnetic microbeads	Enriching of the PK-induced phosphorylated peptides labelled with fluorophores on TiO_2-coated magnetic microbeads and measuring the eluted fluorescence signals	0.1 U/mL	S5
Fluorescence method	$NaYF_4$:Yb,Er upconversion nanophosphors	Upconversion nanophosphors capture the PK-induced phosphorylated peptides labelled with fluorophores and transfer energy to light up the captured peptides	0. 05 U/mL	S6
Fluorescence method	AuNPs	AuNPs capture the PK-induced phosphorylated peptides and quench the fluorophores labelled on the peptides.	0.5 U/mL	S 7
Colorimetric method	AuNPs	The PK-induced phosphorylated peptides conjugate to the surface of AuNPs and prevent the aggregation (color change) of AuNP suspension.	0. 5 U/mL	S8
Colorimetric method	Platinum and carbon dot hybrid nanomaterials (Pt/CDs)	The Pk-induced phosphorylated peptides bind to the platinum/carbon dot hybrid nanoparticles to mediate the peroxidase-like activity, which controls the color change of TMB.	0.625 U/mL	S9
Chemiluminescence method	AuNPs	Zr^{4+} ions mediate the conjugation of AuNPs and PK-induced phosphorylated peptides fixed on an electrode to generate luminol signals.	0.09 U/mL	S10
Electrochemical method	AuNPs/multi-walled carbon nanotube nanohybrids	The PK-induced phosphorylated are fixed on an electrode, which will then capture AuNPs/multi-walled carbon nanotube nanohybrids specifically and results in a current change.	0.09 U/mL	S11
Fluorometric method	Zr^{4+}-functionalized magnetic beads (ZrMBs)	Enriching the PK-induced phosphorylated peptides labelled with fluorophores by ZrMBs via Zr-phosphate peptide affinity and measuring the eluted fluorescence signals by glucose meter	0.1 U/mL	S12
Colorimetric and Fluorometric method	Nanoceria	Phosphorylated peptides attach to the surface of nanoceria to mediate the activity and fluorescence quenching property	0.1 U/mL	This work

Reference

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