

## SUPPORTING INFORMATION

### **Multiplex Isotope Dimethyl Labeling of Substrate Peptides for High Throughput Kinase Activity Assay via Quantitative MALDI MS**

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## ***Experimental Procedure***

### *Chemicals and Materials*

Protein kinase A (PKA) was obtained from Promega (Madison, WI, USA); Formaldehyde (CH<sub>2</sub>O), formaldehyde-D<sub>2</sub> (CD<sub>2</sub>O) and formaldehyde-<sup>13</sup>CD<sub>2</sub> (<sup>13</sup>CD<sub>2</sub>O), sodium cyanoborohydride (NaBH<sub>3</sub>CN), sodium cyanoborodeuteride (NaBD<sub>3</sub>CN), adenosine triphosphate (ATP), HEPES were purchased from Sigma (St. Louis, MO, USA); 2, 5-dihydroxybenzoic acid (2, 5-DHB) was purchased from Thermo Electron; Formic acid (FA) was obtained from Fluka (Buches, Germany); 25% ammonia solution (NH<sub>3</sub>·H<sub>2</sub>O), acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany); Magnesium acetate (Mg(CH<sub>3</sub>COO)<sub>2</sub>) were obtained from Tianjin Kermel plant of chemical reagent (Tianjin, China); LRRASLGGK, LRRApSLGGK were synthesized by APeptide (Shanghai, China); HHSPRK was purchased from Enzo Life Sciences (Plymouth meeting, PA, USA); SHKQIYYSDK was synthesized from ChinaPeptides (Shanghai, China); N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) was purchased from Beyotime (Shanghai, China). All the water in the experiments was purified by a Milli-Q system from Millipore Company (Bedford, MA, USA).

### *Standard Procedures for multiplex isotope dimethyl labeling-based PKA Assay*

In a typical 200 μL phosphorylation reaction system of PKA, substrate peptide LRRASLGGK (7.5 μM) was incubated with fixed amount of PKA (10 unit/μL, 10 μL) at 25 °C in 50 mM HEPES-HCl buffer (pH 7.5), containing 20 mM Mg(CH<sub>3</sub>COO)<sub>2</sub> and 0.5 mM ATP. At each time point, an aliquot of 20 μL was taken from the incubation mixture and the PKA catalyzed reaction was quenched by the addition of 2 μL formaldehyde (CH<sub>2</sub>O, CD<sub>2</sub>O or <sup>13</sup>CD<sub>2</sub>O, 4%, vol/vol) and 2 μL cyanoborohydride (NaBH<sub>3</sub>CN or NaBD<sub>3</sub>CN, 0.6 M) immediately. The dimethyl labeling reaction was also initiated by the addition of these reagents. The reaction solution was kept at 25 °C for 1 h for complete dimethyl labeling, and then 3 μL ammonia (10%, vol/vol) was added

and kept for another 20 minutes. Finally the labeling reaction was quenched with the addition of 3  $\mu$ L formic acid.

For multi-substrate PKA assay, the experiments were performed the same with standard PKA assay described above, except for the inclusion of another substrate peptide HHASPRK to the reaction system at the beginning.

For PKA inhibitor assay, different concentrations (5 nM, 15 nM) of H-89 were added in the reaction mixture, respectively. Other experimental conditions were the same as those stated for standard PKA assay.

#### *Sample Preparation for MALDI MS Analysis*

Solutions of Internal standards (IS) for the substrate LRRASLGGK, HHASPRK and the product LRRApSLGGK were prepared and labeled with the heaviest dimethyl group respectively. All the aliquots in the same time course and the corresponding internal standards were mixed together, then the mixture was diluted for 10 times or more to decrease the salt concentration, in order to get more accurate quantification results.

#### *MALDI MS Analysis*

The MALDI-TOF MS analyses were performed on a MALDI-TOF/TOF<sup>TM</sup> 5800 System (AB SCIEX, Foster City, CA) equipped with a 1 kHz OptiBeam<sup>TM</sup> on-axis laser. All mass spectra reported were obtained in the reflector positive ion mode. Typically, 30 laser shots were summed per spectrum. Sample aliquots (0.5  $\mu$ l) and 2, 5-dihydroxybenzoic acid (DHB) matrix (0.5  $\mu$ l, 25 mg mL<sup>-1</sup> in 70% ACN-H<sub>2</sub>O solution containing 1% H<sub>3</sub>PO<sub>4</sub>) were spotted on MALDI plate sequentially and dried at room temperature prior to MALDI-TOF MS analysis.

### ***Supplementary Results and Discussion***

#### *Design of the substrate peptide*

The phosphorylation of substrates by protein kinases always prefer some linear consensus sequence, such as the classical PKA-specific motif [K/R]X<sub>1,2</sub>[S/T]. The substrates will be phosphorylated regardless of the residues out of the preferable

sequence.<sup>1</sup> Consequently, substrate peptides with multiple primary amine groups could be easily designed by adding Lys residues to N or C-terminal of peptides with the motif consensus sequence. Kemptide, the well-known PKA substrate peptide (LRRASLG, 957.1 Da), has only one N-terminal primary amine. To enable the multiplexed assay, we designed a new peptide substrate LRRASLGGK (1037.1 Da) by adding glycine and lysine to the C-terminal. Because the presence of two primary amine groups on this peptides ( $\alpha$ - and  $\gamma$ -amino groups), five-plex isotope dimethyl labeling with mass difference of 4 Da could be achieved (Fig. 1). Since the substrate was modified to get more than one free amino group, it's of interest to know if the recognition of the enzyme to the substrate change significantly after such modifications. The value of Michaelis constant  $K_m$  is a measure of the substrate's affinity to enzyme. If the modification affects the recognition of the enzymes to the substrates, the measured  $K_m$  for the synthesized substrate would display a big difference. The value of  $K_m$  for the original substrate LRRASLG (kemptide) is  $4.4 \pm 0.8 \mu\text{M}$ ,<sup>2</sup> the determined  $K_m$  for the synthesized substrate LRRASLGGK is  $6.16 \pm 0.43 \mu\text{M}$ . Considering that the  $K_m$ s could vary several order of magnitudes, above data indicated that there was no big impact on the substrate property through the modification. In this study, this new substrate peptide was used as the model substrate for the PKA to evaluate the performance of this labeling strategy for the MALDI MS based kinase activity assay.

#### *Quantification performance of the labeling strategy*

Before this labeling strategy was applied to kinase activity assay, we investigated its quantitative performance. Because the enzymatic product was typically measured for enzymatic assay, the phosphorylated product, LRRApSLGGK, was used for this investigation. Solutions of this peptide with different concentrations, 1  $\mu\text{M}$ , 2  $\mu\text{M}$ , 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , 20  $\mu\text{M}$  were prepared. And the same volume of these samples were labeled by using different types of isotopic formaldehyde and cyanoborohydride as shown in Fig. 1 to introduce 5 different isotopic tagged dimethyl groups to the peptides in above five samples. After the differential labeling, the five samples were pooled together and an aliquot of the mixture was transferred onto a spot of a MALDI plate for analysis.

Supplementary Fig. S3a gives the spectra for this analysis. Five clusters of peaks with different intensities were observed. The sample concentration ratios of the five solution was 1:2:5:10:20. It was found that peak intensities have good correlation with their concentration with the  $R^2$  value of 0.99 (see Fig. S3b), indicating excellent performance in quantification. This assay allowed the determination of the relative concentration of five samples by one laser shot which cost only 30 ms (the instrument equipped with a 1 kHz laser and 30 laser shots were summed per spectrum). Clearly this method is well suited for high throughput analysis of kinase activity.

#### *Calculation of kinetic and inhibition constants*

As substrate concentration [S] is a function of time, an integrated Michaelis-Menten equation is obtained.<sup>3</sup>

$$-\Delta S + K_m \ln\left(\frac{[S_0]}{[S_1]}\right) = k_2[E_{tot}]\Delta t \quad (1)$$

where  $[E_{tot}]$  is the initial enzyme concentration,  $[S_x]$  represents the substrate concentration at the quenching time point  $x$ ,  $\Delta t$  is the time interval between the two time-dependent substrate concentration  $S_x$ . Equation (1) can be rearranged as:

$$[S_0] + K_m(\ln[S_0] - \ln[S_1]) - k_2[E_{tot}]\Delta t = [S_1] \quad (2)$$

Equation (2) gives a way to determine  $k_2$  and  $K_m$ , given only change in substrate from the time course results (the time course tendency was drawn by B-Splines<sup>4</sup>).

The  $K_m'$  for the substrate in the system with addition of different concentration of inhibitor was calculated by Equation (2) as well. Inhibition constant  $K_i$ , was obtained on the basis of the Equation (3).

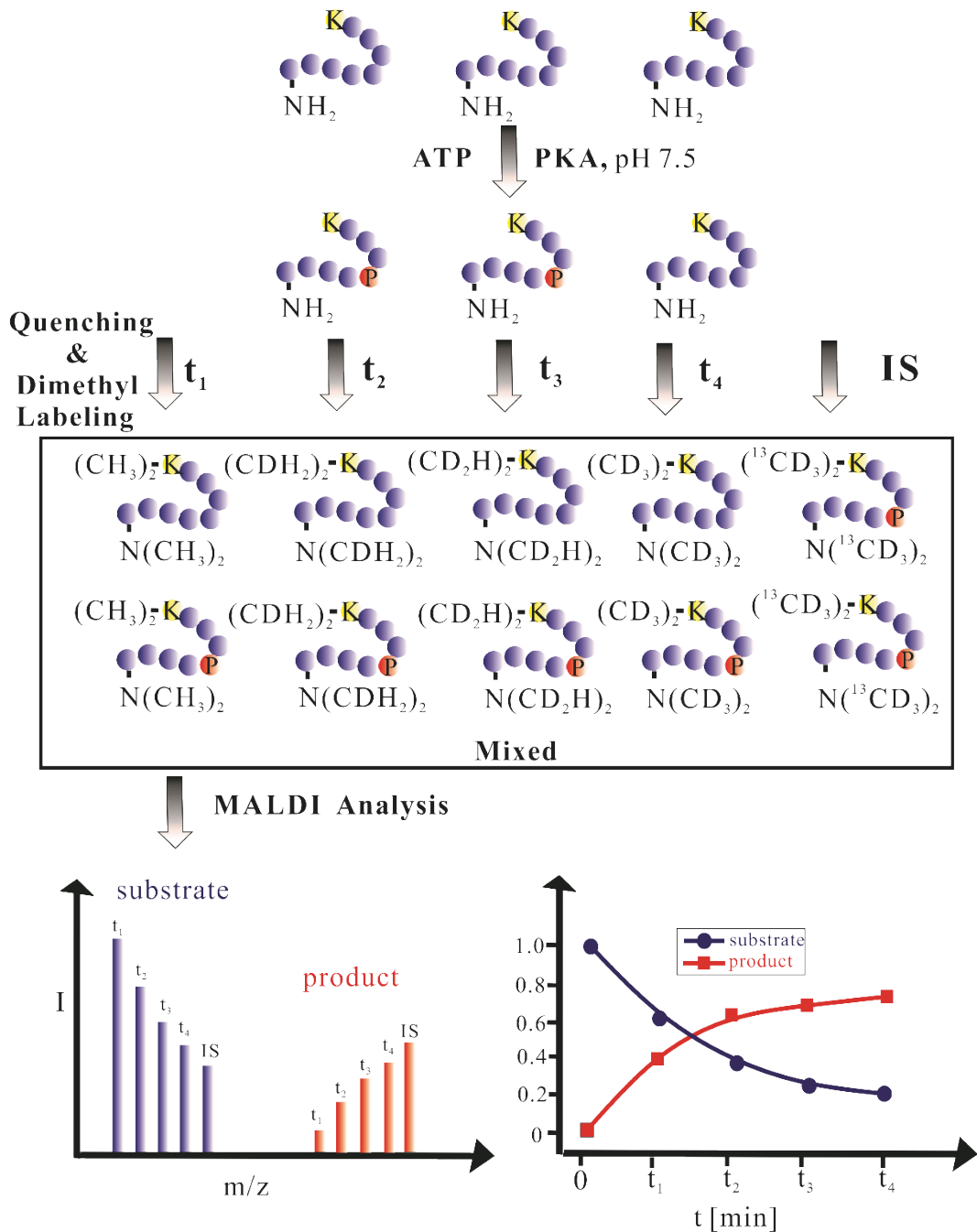
$$K_m' = K_m\left(1 + \frac{[I]}{K_i}\right) \quad (3)$$

where  $[I]$  represents the concentration of the inhibitor.

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4. Carl De Boor, A Practical Guide to Splines, 1978, 113-115.

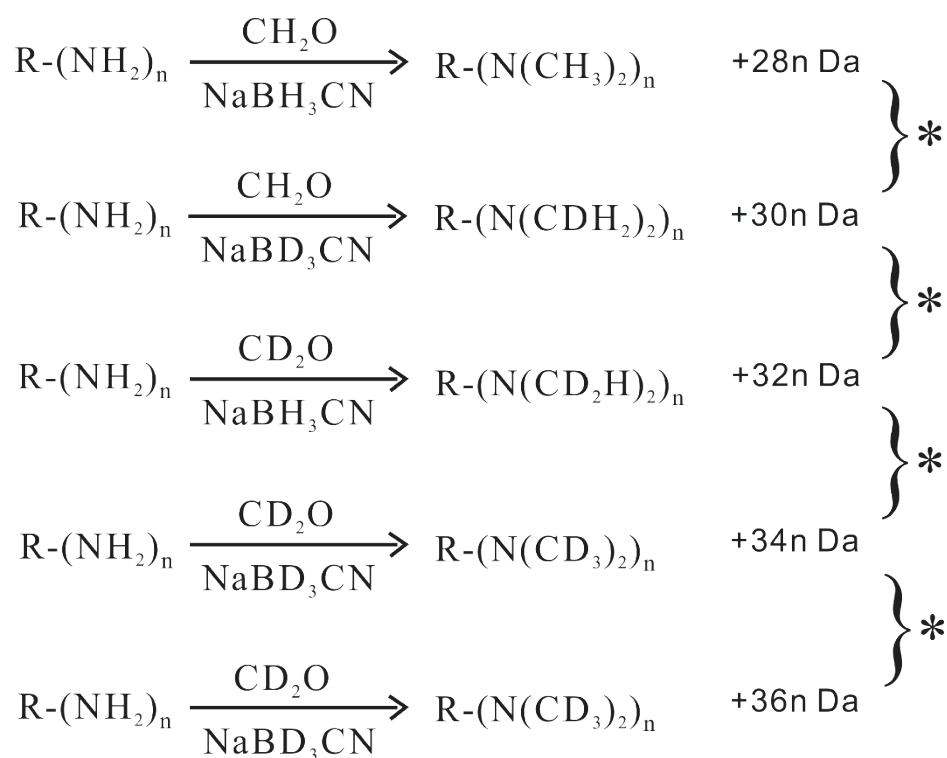
**Supplementary Figures**



**Legends:** Substrate peptide  $\text{NH}_2\text{-K}$ , Product peptide  $\text{NH}_2\text{-P}$

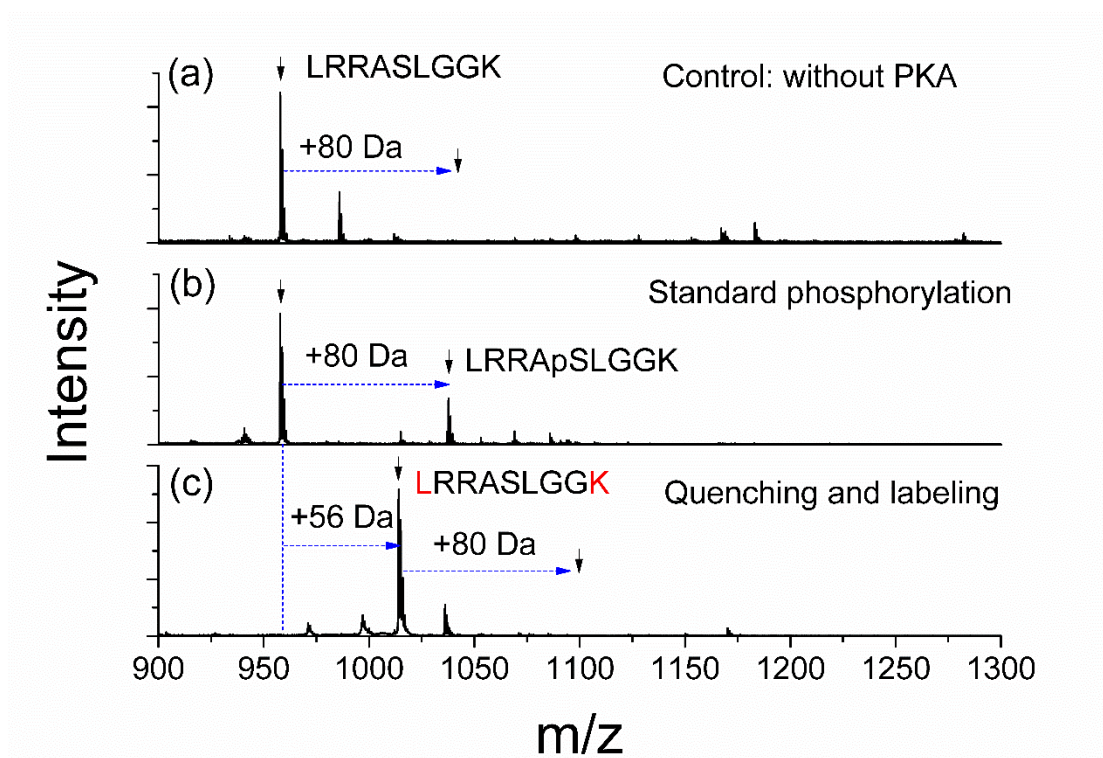
**Supplementary Scheme S1.** Workflow for kinase activity assay by the five-plex

isotope labeling strategy.



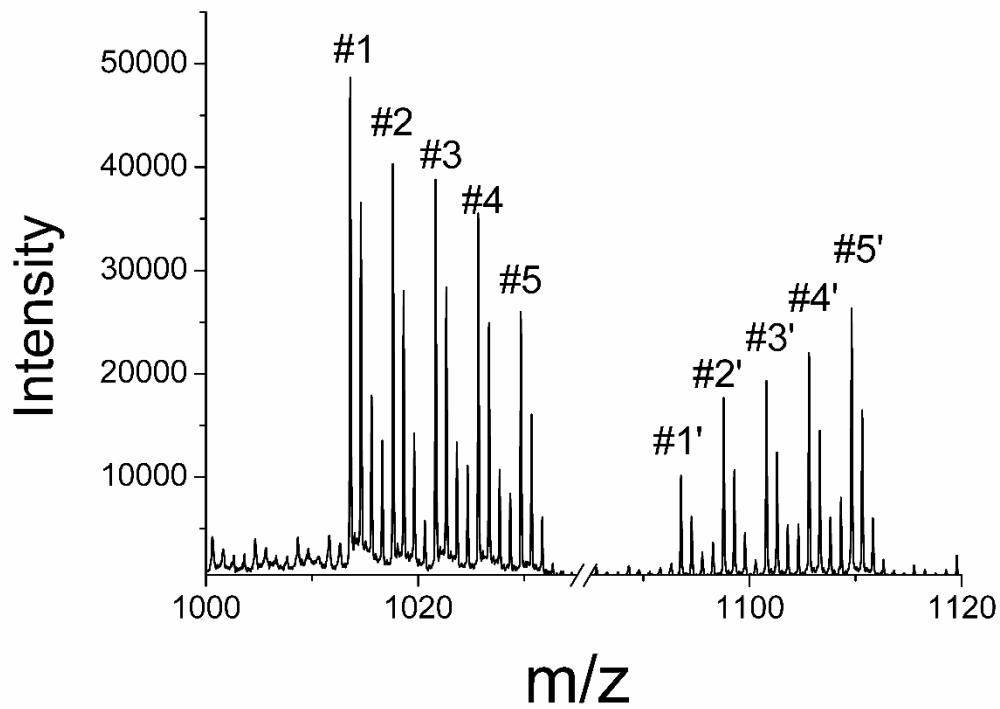
\*  $\Delta\text{mass}=2n \text{ Da}$ ,  $n$ =number of primary amines

**Figure S1** Mechanism of five-plex isotope dimethyl labeling with combination of different isotopic reagents.

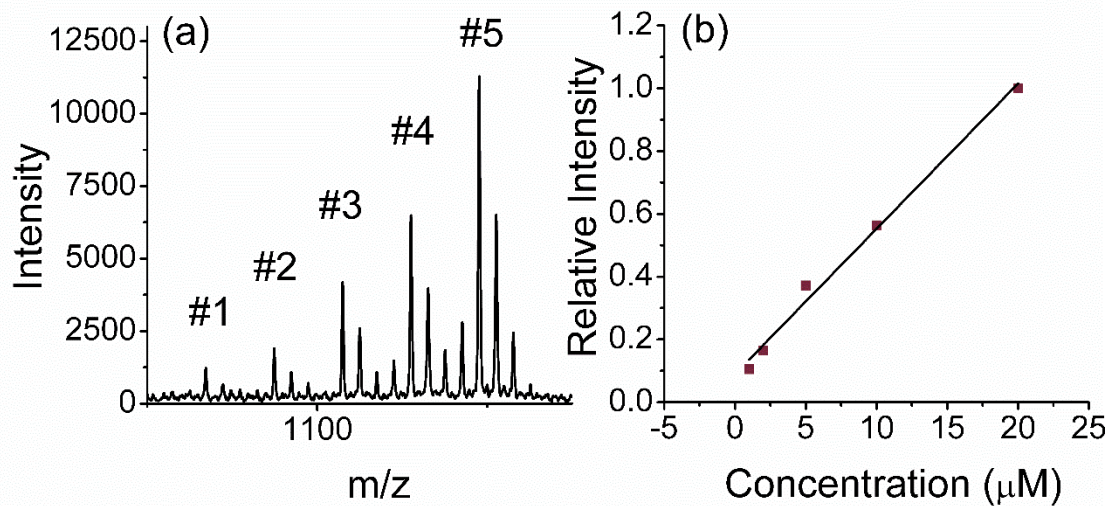


**Figure S2** MALDI MS spectrums for monitoring the PKA catalyzed reactions. (a) Control experiment without addition of PKA. (b) PKA catalyzed reaction. (c) Quenching and dimethyl labeling (Dimethyl labeling reagents were added to the reaction system immediately before the addition of substrate).





**Figure S3** MALDI MS spectrums for time dependent assumption of substrate LRRASLGGK (#1-5) and formation of phosphorylated product LRRApSLGGK (#1'-5') in a time course study.



**Figure S4** Linear dynamic range of a single MALDI MS analysis using standard phosphorylated product LRRApSLGGK. (a) MALDI MS spectrum, the signals marked #1-5 represent dimethyl labeled standard substrates with gradually increasing concentration of 1 μM, 2 μM, 5 μM, 10 μM, 20 μM with the ratio of 1:2:5:10:20. (b) Linear regression with a concentration range of 1:2:5:10:20,  $R^2=0.99$ .