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A fluorometric method for the assay of protein kinase activity

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A B S T R A C T

Protein kinases constitute one of the largest protein families in nature. Current methods to assay their activity involve the use of radioactive ATP or very expensive reagents. In this work, we developed a highly sensitive, cost-effective and straightforward protocol to measure protein kinase activity using a microplate layout. Released ADP is converted into NAD^+, which is quantified by its fluorescent properties after alkaline treatment (linear range 0–10 nmol ADP). To validate our protocol, we characterized a recombinant calcium-dependent protein kinase from potato. Overall, this tool represents a critical step forward in the functional characterization of protein kinases.

Protein phosphorylation is one of the most studied post-translational modifications because it regulates almost every aspect of cellular function [1,2]. Eukaryotic protein kinases (PKs, EC 2.7.11.-) transfer a single phosphoryl group from the γ position of ATP to the hydroxyl groups of serine, threonine and tyrosine residues in protein substrates (Supplemental Fig. S1) [3]. Abnormal protein phosphorylation has been linked to several diseases; thus, PKs are intensively studied in drug-discovery experiments [4]. The main difficulty to assay PKs is their intrinsic, low turnover rates (10^{-2}-10^{-5} s^{-1}) [5]. Several methods have been developed to measure PK activity (Supplemental Fig. S1), but only those based on ADP quantification can be applied to all PK/substrate combinations [6].

The radioactive assay (Supplemental Fig. S1) was the first method used to measure PK activity and is considered the gold standard due to its high sensitivity and robustness [7–10]. As drawbacks, it is time-consuming and generates a large amount of radioactive waste. Non-radioactive methods to assay PK activity are based on peptide or ADP detection (Supplemental Fig. S1). In general, peptide-dependent methods have low sensitivity and can neither be adapted to PKs with unknown motif preference nor used with native proteins as substrates. Among methods to detect ADP, Transcreener ADP^2 Kinase Assay (BellBrook Labs) is the only one that allows direct detection of ADP; it is highly sensitive but very expensive. Coupled methods are based on the use of enzymes to detect the products of the phosphorylation reaction, being ADP-Glo (Promega) the main assay currently available on the market. It measures released ADP using an enzymatic tandem to generate a luminescent signal. This is a highly sensitive method but it is quite expensive and sensitive to compounds that inhibit luciferase [11,12].

In this work, we adapted the enzymatic tandem pyruvate kinase/lactate dehydrogenase to assay PK activity [9]. Released ADP is converted into NAD^+, which can be quantified using a fluorescence detector after the alkaline treatment initially described by Kaplan et al. [13] and further adapted by other authors [14,15] (Supplemental Fig. S2). We characterized a plant PK in parallel with the radioactive method, the ADP-Glo reagent and the fluorometric method. We show this is a cost-effective method, suitable to assay PK activity without radioactive ATP and with any type of substrate.

Solanum tuberosum Calcium Dependent Protein Kinase 1 (*St*CDPK1) was expressed as described by Santin et al. [16], with minor modifications. Transformed cells were grown in LB medium supplemented with $100 \mu g \, ml^{-1}$ ampicillin and induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside for 16 h at 30 °C and 180 rpm. Cells were collected by centrifugation, resuspended in lysis buffer [25 mM Tris-HCl pH 8.0, 300 mM NaCl, 5% (v/v) glycerol and 10 mM imidazole], disrupted by sonication and centrifuged twice at $16000 \times g$ for 20 min at 4 °C. The crude extract was loaded onto a 1-ml HisTrap HP column (GE Healthcare) previously equilibrated with lysis buffer. The enzyme was eluted with a linear imidazole gradient (10–300 mM) in lysis buffer. Fractions containing PK activity were pooled, concentrated and stored at −80 °C until use.

*St*CDPK1 activity was assayed as described by Raíces et al. [17]. Briefly, the enzyme was incubated for 5 min in 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$, 50 μM ATP and 100 μM Syntide-
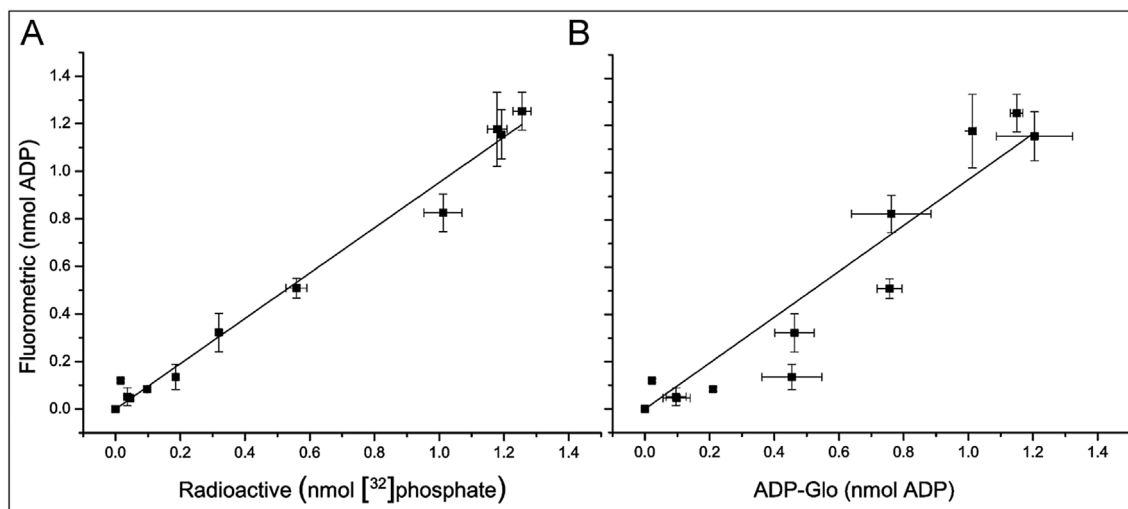
Fig. 1. Validation of the fluorometric method for PK activity assays. Data of released ADP (fluorometric method, y-axis in A and B) were plotted against incorporated [32P]phosphate (radioactive method, x-axis in A) or released ADP (ADP-Glo method, x-axis in B). Data are the mean of two independent experiments ± standard error.

Table 1
Characterization of *St*CDPK1 with three different methods. Kinetic parameters were calculated by fitting activity data to a modified Hill equation, as described in the main text.

Parameter	Radioactive	ADP-Glo	Fluorometric
V_{max} (U mg^{-1})	1.21 ± 0.04	1.2 ± 0.1	1.23 ± 0.06
ATP $S_{0.5}$ (µM)	63 ± 6	54 ± 17	78 ± 11
n_H	1.5 ± 0.2	0.8 ± 0.1	1.3 ± 0.2
Adjusted R^2	0.99	0.98	0.99

2 peptide at 30 °C (a detailed list of all reagents used as well as preparation and storage considerations are available in Supplemental Table S1). Activity measurements with the radioactive method were done in a final volume of 20 µl, following a previously described protocol [10]. Activity measurements with the ADP-Glo reagent (Promega) were done in a final volume of 5 µl, according to the manufacturer's instructions.

Reactions with the fluorometric assay were performed in a final volume of 20 µl in black 96-well microplates (Thermo Scientific) as described above, with the addition of the system necessary to convert released ADP into NAD$^+$ (2 mM phosphoenolpyruvate, 2 mM NADH, 1 U lactate dehydrogenase and 1 U pyruvate kinase) to the PK reaction. Reactions were stopped with 20 µl 0.3 M HCl and incubated for 20 min at room temperature to remove excess NADH (Supplemental Fig. S3). Then, 170 µl 9 M NaOH were added and samples were incubated at room temperature in the dark for 60 min to develop the NAD$^+$ alkaline derivates. Fluorescence was quantified in a fluorometer (Fluoroskan Ascent FL, Thermo Scientific) using excitation and emission filters of 355 and 460 nm, respectively, with an integration time of 20 ms. The amount of generated ADP was calculated using an ADP standard curve constructed using the same conditions. A detailed step by step procedure with key experimental details is presented in Supplemental Fig. S2. Data were analyzed with OriginPro 8 (OriginLab Corporation). Kinetic parameters were obtained by fitting data of enzyme activity versus substrate concentration using a modified Hill equation: $v = V_{max}*S^{nH}/(S_{0.5}^{nH} + S^{nH})$; where v is the initial velocity, $S_{0.5}$ is the concentration of substrate (S) that produces 50% of the maximal velocity (V_{max}), and n_H is the Hill coefficient. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 µmol of ADP per minute under the specified assay conditions.

The radioactive assay detects phosphopeptide formation in the nanomolar range; thus, we optimized conditions to measure ADP in such a range. Standard curves were made in a final volume of 20 µl with increasing amounts of ADP (0–0.5 mM; 0–10 nmol). Curves were linear with a slope of 420 ± 7 RFU mM^{-1} (Adjusted R^2 = 1.00, $P < 0.001$; Supplemental Figs. S4A–B). We obtained identical results when calibration curves were performed with NAD$^+$ (Supplemental Fig. S4 A-B), indicating complete conversion of ADP into NAD$^+$ with the pyruvate kinase/lactate dehydrogenase system. Under our assay conditions, limits of detection and quantification for ADP were 0.05 and 0.15 nmol, respectively (Supplemental Fig. S4C). The fluorometric method showed adequate reproducibility, according to the coefficient of variation (CV) obtained for calibration curves (average < 5%) and *St*CDPK1 activity assays (average < 15%). We also obtained a good signal to background ratio (S:B) and a Z factor > 0.5 above 15 µM ADP (Supplemental Fig. S4D), suggesting potential application in high-throughput screenings. Equations used for calculating LOD, LOQ, S:B and Z factor are presented in Supplemental Information S1.

To validate our method, we performed *St*CDPK1 activity assays with our fluorometric method in parallel with the radioactive and ADP-Glo methods. We observed a linear correlation (in the analyzed range of product formation, 0–1 nmol) between the fluorometric and radioactive methods (Slope = 1.04 ± 0.03, R^2 = 0.99, $P < 0.001$; Fig. 1A). Similar results were obtained between the fluorometric method and the ADP-Glo reagent (Slope = 0.98 ± 0.07, R^2 = 0.95, $P < 0.001$; Fig. 1B).

Kinetic parameters for *St*CDPK1 using Syntide-2 as substrate determined with the fluorometric method were similar to those obtained in parallel with the radioactive method and the ADP-Glo reagent (Table 1 and Supplemental Fig. S4), although slightly different to the ones reported by Grandellis et al. [18]. We also obtained identical results with the fluorometric and radioactive methods when *St*CDPK1 activity was assayed with myelin basic protein as substrate (1.5 ± 0.3 and 1.6 ± 0.3 mU mg^{-1}, respectively).

The fluorometric method described in this work is intended for the functional characterization of PKs. Our method is highly sensitive, which makes it suitable for activity assays in small reaction volumes, either with peptides or proteins as substrates. This is of great interest because PKs phosphorylate proteins more efficiently than artificial peptides [3]. Another advantage of using pyruvate kinase in the PK reaction is that released ADP is recycled back to ATP, thus maintaining a constant substrate concentration [19] (Supplemental Fig. S2). This ensures initial velocity conditions over the course of the enzymatic reaction and avoids PK inhibition by ADP [19,20].

The main drawback of our method is that it cannot be used to

reliably assay PK activity in crude extracts. This is mainly due to the presence of ATPases and non-specific phosphatases, which release ADP and thus increase reaction blanks [12]. Also, the flourometric method could not be employed to assay PKs with ATP $S_{0.5}$ values close to or lower than its detection limit [21]. Besides that, we envisage that our assay will be of great help for the biochemical characterization of PKs. It is worth mentioning that the cost per reaction of the fluorometric method is significantly lower than the radioactive method (5-fold) and the ADP-Glo reagent (19-fold). Kinetic parameters obtained for *St*CDPK1 with all methods used herein (fluorometric, radioactive and ADP-Glo) are in good agreement with those previously reported for other CDPKs (Table 1 and Supplemental Table S2). Therefore, we conclude that the fluorometric method developed in this work represents an important tool better understand the function of PKs.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ab.2018.07.018.

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