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Method Article

Screening of chemical library against whole cell kinome activity via non-radioactive, high throughput kinase assay

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A B S T R A C T

Protein kinases play a crucial role in cellular functions by adding phosphate group to the protein substrates. It is an indispensable post-translational modification that regulates intracellular signaling and key cellular processes. They thus serve as an excellent target for chemotherapeutic interventions. A vast repertoire of protein kinases is present in a cell with diverse substrates as well as phosphorylation sites. To study full kinome for its activity, there is an urgent need of designing a comprehensive, *in vitro* assay which itself is an impractical task. However, in this study, we have attempted to develop a robust assay that not only mimics the *in vivo* nature of the kinases but can also be used in a high throughput drug-screening platform. Herein, the *Leishmania donovani* parasites are lysed and the total protein content is extracted. This extracted proteome is further sub divided into two parts: one active fraction containing cellular kinases and the substrate is heat-denatured fraction that loses all the enzymatic activity but retains the potential phosphorylation sites. These fractions are then co-incubated in the presence of ATP to initiate the kinase reaction and the total kinase activity is measured using ADP-glo kinase assay. Overall, this method

- Presents a simple and robust approach to understand the participation of kinases in signaling networks.
- Presents a high-throughput platform for ex-vivo drug screening.

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A R T I C L E I N F O

Method name: Kinome survey

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Specifications Table

Subject Area	<ul style="list-style-type: none"> • Biochemistry, Genetics and Molecular Biology • Pharmacology, Toxicology and Pharmaceutical Science
More specific subject area:	High throughput kinase assay
Method name:	Kinome Survey
Name and reference of original method	NA
Resource availability	NA

Method details

Materials and methods

Chemicals and assay components

The composition for measuring functional activity of the parasite kinome involves ATP regeneration-based luciferase reaction system resulting from nascent ADP phosphorylation by utilizing ADP-Glo™ kinase assay kit (Promega Corporation, catalog no. V9101). The kit is composed of ADP-Glo™ Reagent, Kinase Detection Reagent (prepared by mixing Kinase Detection Buffer and Kinase Detection Substrate to reconstitute the lyophilized substrate containing Luciferase and D-Luciferin), 10 mM Ultra-Pure ATP and 10 mM Ultra-Pure ADP. The kit is sufficient for 250 assays if performed in 96-well plates using 20 μ l, 20 μ l and 40 μ l of a kinase reaction, ADP-Glo™ Reagent and Kinase Detection Reagent respectively, per sample. The assay is performed in two steps. First, after the kinase reaction is over, an equal volume of ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the residual ATP. Second, the Kinase Detection Reagent is added to simultaneously convert ADP to ATP and allow the newly synthesized ATP to interact with luciferase/luciferin reaction system. The luminescence, thus generated, is proportional to the amount of ADP released in the reaction, thereby representing kinase activity. Luminescence generated in a reaction can also be correlated with amount of ADP released in a reaction by using standard “ATP to ADP conversion curve”.

Unless otherwise stated, all reagents were bought from Sigma-Aldrich. Phosphorylation reactions were performed in 1X kinase assay buffer: 25 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol (DTT) and 10 mM MgCl₂. Detection signal of enzymatic activity of kinases was enhanced by selective inhibition of phosphatases in the cell lysate and this was achieved by incorporating 5 mM β -Glycerophosphate disodium salt hydrate (classical serine-threonine phosphatase inhibitor) and 0.1 mM Sodium-orthovanadate (ATPase, alkaline phosphatase and tyrosine phosphatase inhibitor) in the assay buffer. 5X Stock of the assay buffer was prepared to be subsequently used in the kinase reactions.

Luminometric white-colored 96-well assay plates (Catalog no. 3912) were obtained from Corning Inc. (Corning NY). Luminescence was recorded using Thermo Scientific™ Varioskan™ Flash Multimode Reader. To analyze assay plots and calculate all biochemical parameters related to kinase reactions, both Microsoft Excel and Origin® 2018b Graphing and Analysis Software (OriginLab Corporation) were used.

Standard ATP to ADP conversion curve

To estimate the amount of ADP produced in a given kinase reaction and to access the linearity of the assay, a standard “ATP to ADP conversion curve” was prepared that represents luminescence corresponding to relative amounts of ATP and ADP available in a kinase reaction at a specified conversion percentage. The standard samples used to generate the standard curve were created by combining the appropriate volumes of ATP and ADP stock solutions in the assay buffer. For example, 100 μ M “ATP + ADP” concentration range was created by mixing proportionally amounts of 100 μ M ADP and 100 μ M ATP to always reach a concentration of 100 μ M of total nucleotides. Once samples

(20 μ l of each) for the standard curve were prepared, 20 μ l ADP-Glo™ Reagent (already equilibrated at room temperature) was added to deplete the unconsumed ATP, leaving only a very low background of ATP and incubated at room temperature for 40 min. Then, 40 μ l Kinase Detection Reagent was added to convert ADP to ATP and introduce luciferase and luciferin into the sample mix and incubated at room temperature for 30 min. Each of the points was transferred to the luminometric assay plate and the luminescence was recorded. Linear relationship between the luminescent signal and the amount of ADP in the standard samples was observed at all tested series of “ATP+ADP” concentrations (Fig. 2A).

Note: If desired, plates containing luminescent sample mix(s) can be left longer at room temperature owing to the long half-life of the luminescent signal.

Implementation of the method to detect cellular kinome activity

We developed a novel *in vitro* kinase assay for target-based drug screening against cellular kinome of protozoan parasite, *Leishmania donovani* strain AG83. To simulate the *in vivo* nature of functional activity of the parasite kinome, parasite lysate was taken as source of active kinases and heat-inactivated parasite proteins as source of substrates (Fig. 1). *L. donovani* promastigotes were cultured at 26 °C in Medium 199, Hanks' Balanced Salt (ThermoFisher Scientific, Catalog no. 12350039), supplemented with 10% heat inactivated fetal bovine serum (ThermoFisher Scientific, catalog no. 10437028) and 20 μ g/mL gentamycin (ThermoFisher Scientific, catalog no. 15750060). Sub-culturing was done at regular intervals (72–96 h) after adjusting the inoculum density to 2×10^6 cells/ml. Parasite culture at logarithmic phase of growth was taken and cells were pelleted down by centrifugation at 3600 rpm for 5 min. Pellet thus obtained was washed thrice with 1X PBS to remove out residual “parasite-conditioned medium”, followed by resuspension of the pellet in PBS supplemented with Protease inhibitor cocktail (Sigma, catalog no. S8820), to specifically inhibit proteases including serine, cysteine and metallo-proteases.

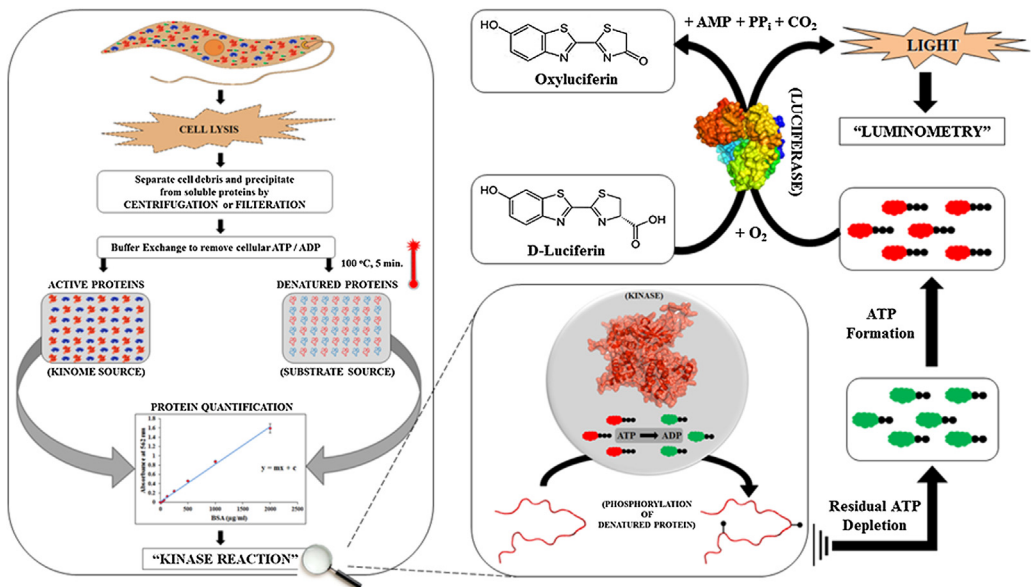


Fig. 1. Pictorial representation of ADP-Glo™ Kinase Assay with *Leishmania donovani* strain AG83 lysate. The assay is broadly composed of two steps. a) Preparation of parasite lysate to generate active kinases (enzymes' source) & heat activated proteins (substrate source), followed by protein quantification. b) Kinase or ATPase reaction. ADP thus formed by active kinases present in the cell lysate is subsequently utilized by ADP-Glo™ Kinase Assay kit to produce light, which is subsequently detected by luminometry.

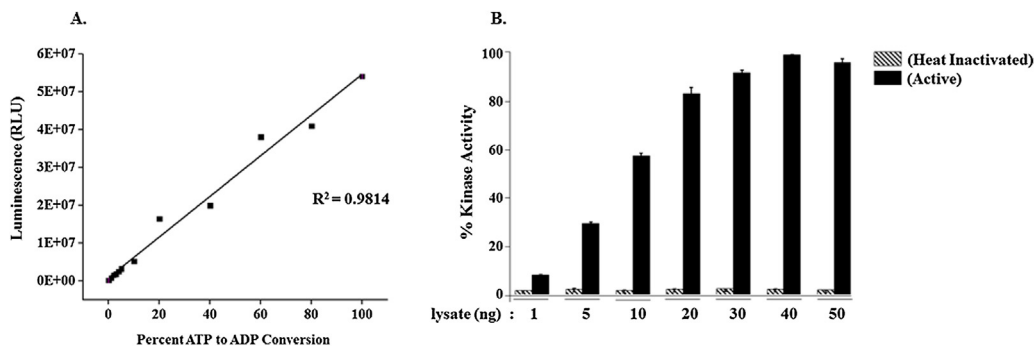


Fig. 2. Evaluation of ADP-Glo™ kinase assay linearity and implementation of the assay for parasite lysate. Panel A. ATP to ADP conversion curves were prepared at the indicated “ATP + ADP” concentration in 20 μ l of 1 \times reaction buffer. Kinase assay was performed as described in methods section. There is a linear relationship observed between the luminescent signal and amount of ADP present in the reaction mix. Panel B. ADP-Glo™ Kinase Assay was utilized to detect functional activity of kinases in the parasite lysate. Reactions were setup by taking varying amounts of the lysate in a total volume of 20 μ l per reaction, as described in methods section. Kinase reactions were initiated by adding 1 μ M ATP and allowed to take place at 30 °C for 1 h, followed by ADP-Glo™ kinase assay. The luminescence signal thus produced increases with the amount of lysate in a kinase reaction. Luminescence values represent the mean of two replicates. Abbreviation: RLU, Relative Lights Unit.

Note: Phosphatase inhibitor cocktails I & II (Sigma, catalog nos. P2850 and P5726 respectively) were also included in the buffer to reduce the background counteracting activities of phosphatases in the cellular lysate.

Cell lysis by repeated freezing and thawing method. Repeated cycles of freezing and thawing is commonly used to physically disrupt cells through a mechanism, wherein cells swell and ultimately break due to formation of ice crystals during the freezing process and then contract during thawing. This technique involves freezing a cell suspension in liquid nitrogen or dry ice and then thawing immediately at room temperature or 37 °C. Multiple cycles are necessary for efficient lysis. Cell lysis by freeze/thaw method has been implemented in the lysis of bacteria, mammalian cells and even in certain cases of protozoan parasites including *Leishmania donovani* and *Plasmodium falciparum* [1–5]. In this regard, cell lysis of *L. donovani* was done by three freeze-thaw cycles (in 1X PBS supplemented with protease and phosphatase inhibitor cocktails as mentioned above) followed by segregation of soluble cytosolic fraction by centrifugation at 13,000 rpm at 4 °C for 30 min. Protein quantification in cell lysate was done using Bicinchoninic Acid (BCA) protein assay kit (GBiosciences, catalog no. 786-570).

Kinase assay conditions. Functional activity of the parasite kinome was measured by the amount of ADP produced in a reaction mix. Phosphorylation experiments were performed by taking varying concentrations of parasite lysate: 1 ng, 5 ng, 10 ng, 20 ng, 30 ng, 40 ng & 50 ng, which served as source of active kinases, in a total volume of 20 μ l in kinase assay buffer. For each of the reactions, equivalent amount of heat inactivated cell lysate was taken as negative control. 200 ng of heat inactivated cell lysate, per reaction, was taken as substrate for the kinases. Kinase reactions were initiated by adding 1 μ M ATP and allowed to take place at 30 °C for 1 h. Once the reactions were complete, the ADP-Glo™ Kinase Assay was performed in two steps. 20 μ l of ADP-Glo™ Reagent was added to stop the kinase reaction and deplete the unconsumed ATP, leaving only ADP and a very low background of ATP, and incubated at room temperature for 40 min. 40 μ l of Kinase Detection Reagent was then added to convert ADP to ATP and introduce luciferase and luciferin into the system to detect ATP, and incubated at room temperature for 30 min, followed by luminometry. The luminescence thus produced increases with the amount of lysate in a kinase reaction (Fig. 2B).

Validation and statistical evaluation of the assay. Two statistical parameters were considered to access quality of the assay, exploited to check activity of kinases in the parasite lysate: Screening window coefficient (Z'-factor) and Signal to Background ratio (S/B).

Z'-factor determination. Screening window coefficient or Z'-factor is a simple and dimensionless parameter which is desirable for use in evaluating the quality of High-Throughput Screening (HTS) assays. This coefficient takes into account the data variations associated with the sample measurements and the reference control measurements. Z-factor greater than 0.5 indicates excellent assay quality. Also, it has been reported that reliable and reproducible data obtains Z-factor values >0.7 [6]. Three replicates, each containing 1 ng, 5 ng, 10 ng, 20 ng, 30 ng, 40 ng and 50 ng of cell lysate (containing active kinases) were compared with 0% ATP to ADP conversion replicates containing equivalent amounts of heat inactivated lysate as control. The Z'-factor values were determined for each reaction with different amounts of active lysate, according to the following formula.

$$Z' = 1 - \frac{3(\text{Standard deviation}_{\text{sample}}) + 3(\text{Standard deviation}_{\text{control}})}{[\text{Mean}_{\text{sample}} - \text{Mean}_{\text{control}}]}$$

Z'-factor for each of the reactions was found to be >0.9, indicating that ADP Glo™ Kinase Assay can be suitably applied to check activity of kinases in cell lysate, *L. donovani* lysate in our case and can subsequently be implemented for hit identification through HTS (Table 1).

Note: Since Z'-factor defines a characteristic parameter of the capability of hit identification for each given assay at the defined screening conditions, it is therefore suitable to use this parameter for evaluating the quality and performance of HTS assays.

Signal to background ratio. ADP-Glo™ Kinase Assay can detect even a very small amount of ADP with a high signal-to-background ratio (S/B). To explore sensitivity of the assay with parasite lysate, S/B ratio was calculated for each of the enzymatic reactions with different amounts of lysate, as mentioned above, using the following formula.

$$S/B = \frac{\text{Mean Signal}_{(\text{Signal due to ADP formed in a reaction})}}{\text{Mean Background}_{(\text{Signal due to residual ATP in a reaction})}}$$

The amount of ADP produced with the varying amounts of parasite lysate results in corresponding increase in S/B ratio, thus validating the sensitivity of the assay (Table 1). A small amount of cellular lysate (containing active kinases) that produces low conversion of ATP to ADP is therefore sufficient for use with the ADP-Glo™ Kinase Assay due to the high S/B ratio generated, thus making the assay ideal for HTS.

Note: The optimal amount of kinase(s) to be used in subsequent compound screens and IC₅₀ determinations is the amount that generates an adequate signal-to-background ratio.

Applications of kinase assay. To explore the possibilities of utilizing ADP-Glo™ Kinase Assay developed with cell lysate, towards useful downstream applications, following two aspects were studied.

Table 1

Validation of the ADP-Glo™ Kinase Assay with parasite lysate. Two statistical parameters were evaluated to check quality and sensitivity of the assay: Signal-to-Background Ratios (S/B) and Z'-Factor. ADP-Glo™ Kinase Assay is highly sensitive as shown by high S/B ratios at all amounts of parasite lysate. Also, very high Z-factor values (>0.9) indicate excellent assay quality.

Signal-to-Background ratios (S/B) and Z' values generated by ADP-Glo™ kinase assay at different amounts of parasite lysate							
	Amount of lysate (ng)						
	1	5	10	20	30	40	50
S/B	5	13.59	33.08	36.61	39.57	44.28	51.14
Z'	0.90	0.90	0.92	0.90	0.95	0.99	0.94

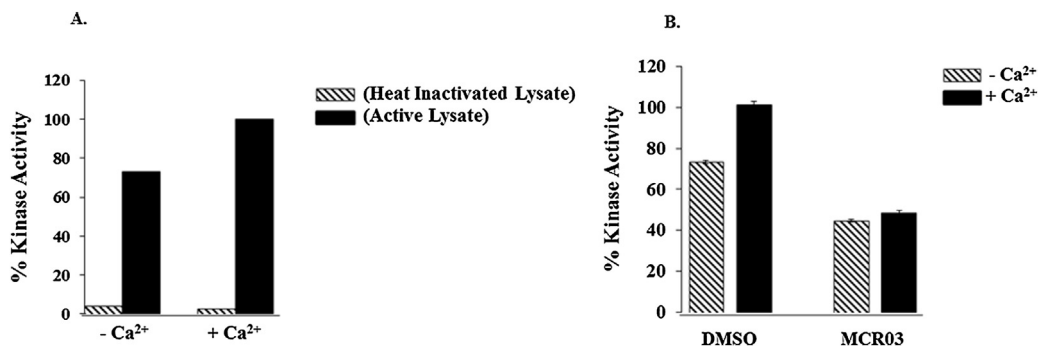


Fig. 3. Applications of ADP-Glo™ kinase assay with parasite lysate. **Panel A.** To Elucidate proportion of calcium-dependent protein kinases in the parasite kinome, kinase reaction was performed using 10 ng of parasite lysate in the presence and absence of 2.5 mM Ca²⁺, as described in methods section. 30% of kinome activity has been reported to impart largely because of calcium-dependent protein kinases. **Panel B.** To check if the assay can be applied for HTS, thio-oxo-imidazolidinone derivative, MCR03 was tested for any effect on the activity of kinases. For this, 10 ng of parasite lysate was allowed to pre-incubate in the reaction buffer with 100 μM of the test compound in the presence and absence of calcium. Once the reactions were complete, the ADP-Glo™ Kinase Assay was performed as described in methods section. MCR 03 significantly exhibited differential inhibition in the presence and absence of calcium. Percent activity represent the mean of two replicates.

Elucidating proportion of calcium-dependent protein kinases in whole kinome. Calcium is a versatile and dynamic cellular messenger that is essential for the survival of all organisms by regulating numerous cellular processes. The pro-apoptotic effects of Ca²⁺ are mediated by a diverse range of Ca²⁺-sensitive factors, including calcium-dependent kinases and phosphatases that are compartmentalized in various intracellular organelles including endoplasmic reticulum, cytosol and mitochondria. At the same time, elevation of Ca²⁺ levels beyond its spatial and temporal boundaries result in cell death mediated through apoptosis or autophagy [7]. Hence, we decided to evaluate the proportion of calcium-dependent protein kinases in the kinome which might be present in parasite intra-cellular milieu. For this, 10 ng of parasite lysate was taken as source of kinases and reaction was setup as described above, in the presence and absence of 2.5 mM Ca²⁺. Using this assay, we found that 30% of kinome activity imparts due to calcium-dependent protein kinases (Fig. 3A).

Validation of the assay for HTS. To further confirm if the proposed kinase assay with parasite lysate can be implemented for HTS, we checked for the effect of imidazolidinone derivatives on the kinome activity. Compounds belonging to this class have been earlier reported to confer anti-leishmanial activities *ex vivo* (Ramu et al., under revision). For this, 10 ng of parasite lysate (containing active kinases) was allowed to pre-incubate in the reaction buffer with 100 μM of the test compound, MCR03 in the presence and absence of calcium, at room temperature for 1 h, followed by initiating the reaction by adding 1 μM ATP. Once the reactions were complete, the ADP-Glo™ Kinase Assay was performed as described above. MCR 03 significantly inhibited phosphorylation activity of parasite kinome, with differential inhibition in the presence and absence of calcium accounting for 38% ± 0.35% and 51.1% ± 0.46% respectively (Fig. 3B).

Significance of the assay

Protein kinases regulate many important cellular processes and represent promising drug targets for a number of human diseases. To determine the global kinome activity, we have developed a novel assay in this study that is robust, high throughput and provides a more physiological condition for kinome activity. Further, the kinase activity can be evaluated under variable conditions such as temperature, pH, calcium, etc. This assay can also provide insights into the various physiological states of the cell and the participation of kinases in the associated signaling events. Further, this method is non-radioactive, very efficient and time saving that can give new directions to the available kinase assays.

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