Cambrex PKLight^{TM} Protein Kinase Assay on the PHERAstar Plate Reader

BMG LABTECH

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Introduction

Protein kinases and their ability to phosphorylate proteins play key roles in the signal transduction pathways of many diseases such as cancer, arthritis and diabetes.[1] The importance of protein kinases makes them common targets for many High Throughput Screening (HTS) departments within the pharmaceutical industry. Current screening technologies employ the use of phospho-state specific antibodies or radioactive beads. The need for new antibodies and beads for each kinase/substrate pair makes these assays expensive and time consuming to develop and run.
Cambrex has developed PKLight^{TM}, a non-radioactive, homogeneous, robust and simple assay suitable for the screening of potentially all protein kinases in 96-, 384- and 1536-well formats. This technology utilises Luciferase bioluminescence to measure ATP consumption as a result of kinase phosphorylation of the target substrate. The assay can be easily optimised for each kinase / substrate pair to produce rapid, quality data suitable for IC_{50} determination of screen compounds.
This technology does not require antibodies, radioactive beads, radio-labelled ATP or specifically modified substrate sequences. The signal is glow luminescence with a half life greater than 2 hours, which is detected using a luminometer, in our case the multifunctional BMG LABTECH PHERAstar plate reader. In this poster we use the Ser/Thr Kinase, cAMP dependant protein kinase (PKA) to demonstrate the assay.

Assay Principle

During a kinase reaction, the level of free ATP in the reaction mixture decreases as the γ-phosphate is transferred from the ATP molecule to the kinase substrate. This drop in free ATP can then be accurately measured using our patented bioluminescent kinase reagents. The bioluminescent reaction is catalysed by the firefly luciferase enzyme and provides speed, sensitivity and convenience.[2] The reagent contains Luciferin and Luciferase, which emits a stable light signal, the intensity of which is proportional to the concentration of ATP (figure 1).

Assay Principle: The amount of free ATP added to the reaction mixture which is consumed during the kinase reaction can be accurately measured using the patented bioluminescent Luciferase kinase reagents from Cambrex. A stable light signal is emitted that has an intensity which is proportional to the concentration of ATP present.

As the kinase reaction progresses the ATP concentration drops and the light emitted becomes less intense.

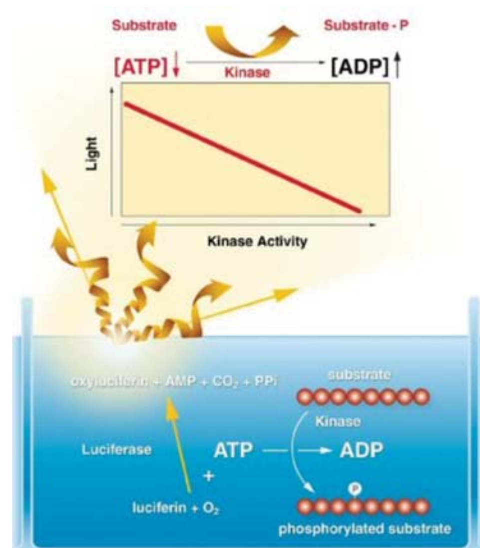

Fig. 1: Schematic assay principle

Results and Discussion

The PKLight^{TM} Protein Kinase Assay uses patented technology to measure ATP consumption and as shown in figure 3 can detect low levels of ATP and has exceptional linearity (R2 > 0.99) over the range used on the PHERAstar. As shown in figures 4 and 5 the PKLight^{TM} assay can accurately determine kinase activity and inhibition, giving reliable IC_{50} data and clean hits.

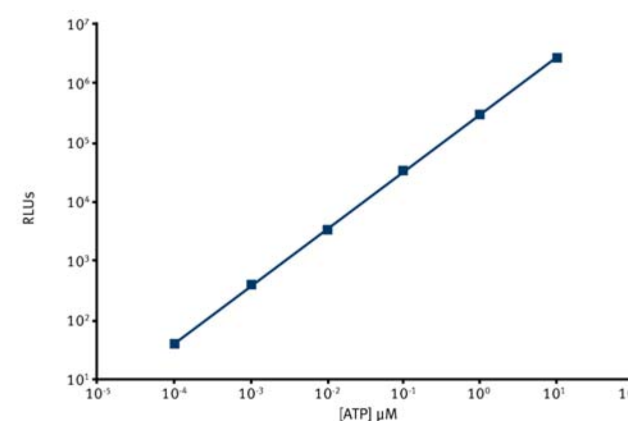

Fig. 3: Measurement of ATP (100 pM to 12.5 µM) using PKLight^{TM} reagents and protocol. The graph demonstrates that the signal is linear over the full ATP concentration range used (R^2 values 0.99)

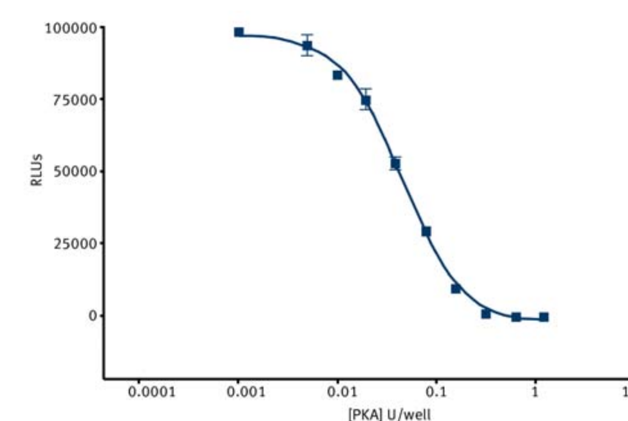

Fig. 4: PKA activity was measured from 0.001 to 1 Units/well using 1 µM ATP and 5 µM Kemptide substrate in a final assay volume of 20 µL per well.

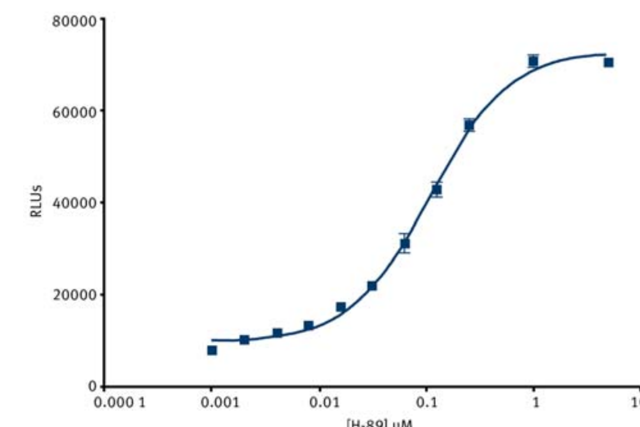

Fig. 5: Inhibition of PKA activity with H-89 dihydrochloride (0.001 - 5 µM) using 0.005 Units/well PKA, 5 µM Kemptide, 1 µM ATP incubated for 20 mins at room temp. Performed in 384 format, 20 µL total reaction volume using PKLight buffer A protocol. IC_{50} = 0.11 µM.

Using the cAMP Dependant Protein Kinase (PKA) and Kemptide substrate as a model we have demonstrated the usefulness of using the patented Cambrex kinase reagents to measure kinase activity and inhibition.

This method is very simple to develop and run and can potentially be applied to any ATP-dependent protein kinase and target substrate. We have proved the assay to be robust with Z' values greater than 0.8 exhibited and reproducible and sensitive enough to determine low potency inhibition.

The PKLight^{TM} assay correlates well with established methods;[3,4] the data obtained for the inhibition of PKA and Kemptide compares well to those obtained by conventional assays used in published data.[5]

The assay platform can be implemented in a variety of ways to suit the requirements of the kinase/substrate pair and the particular demands of the screening group in either 96-, 384- or 1536-well formats. The reagents can be supplied in a custom made manner specific to the size of a particular screen.

Materials and Methods

All materials were obtained through normal distribution channels from the manufacturer stated.
Cambrex PKLight^{TM} kit, Cambrex catalogue, Product Code LT07-500.
PHERAstar, BMG LABTECH, Germany
Microplates, white 384-well, Greiner, Germany
ATP, BSA, Sigma UK cat no. A7699 and A7906
PKA, Kemptide, H-89 dihydrochloride, Calbiochem® cat no. 539486, 05-23-4900 and 371963 respectively.

Fig. 2: PKLight^{TM} protein kinase assay was performed on the PHERAstar multimode microplate reader

In addition, consumables such as pipette tips were used as needed from various manufacturers.

The Cambrex Kinase Assay reagents were used to investigate the inhibition by H-89 dihydrochloride of cAMP dependant protein kinase (PKA) phosphorylation of Kemptide substrate. Enzyme, Substrate, ATP and H-89 dihydrochloride were diluted to working concentrations using the same PKA assay buffer consisting of 40 mM Tris-HCl (pH 7.5); 20 mM $MgCl_2$, and 0.1 mg/mL BSA in purified water.

ATP Detection

ATP was diluted in PKA assay buffer to give a concentration range of 0 – 12.5 µM and added into a Greiner 384-well plate. ATP detection reagent was added and read after 1 minute using a 1 second integration time in luminescence mode on the PHERAstar.

PKA Activity / Inhibition

Using white 384-well plates, 5 µL of PKA, 5 µL Kemptide, 5 µL of ATP and 5 µL of Inhibitor (H-89 dihydrochloride) or PKA assay buffer was added to each well. Giving a total assay volume of 20 µL per well. The reaction mixture was incubated for 20 minutes at room temperature. The remaining amount of ATP was determined by adding 10 µL of the ATP detection reagent to the well and incubating for 10 minutes at room temperature. The 384-well plate was read on the PHERAstar in luminescence mode using 1 second integration.

PKLight is a trademark of CBM Intellectual Properties, Inc.
PKLight and the method for the measurement of kinase activity by ATP consumption are protected by UK patent number GB 2,375,171 B, US patent number 6,599,711 and International Patent Application number PCT/GB01/05506.

References

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