PhosphoWorks™ Fluorimetric ADP Assay Kit

Red Fluorescence

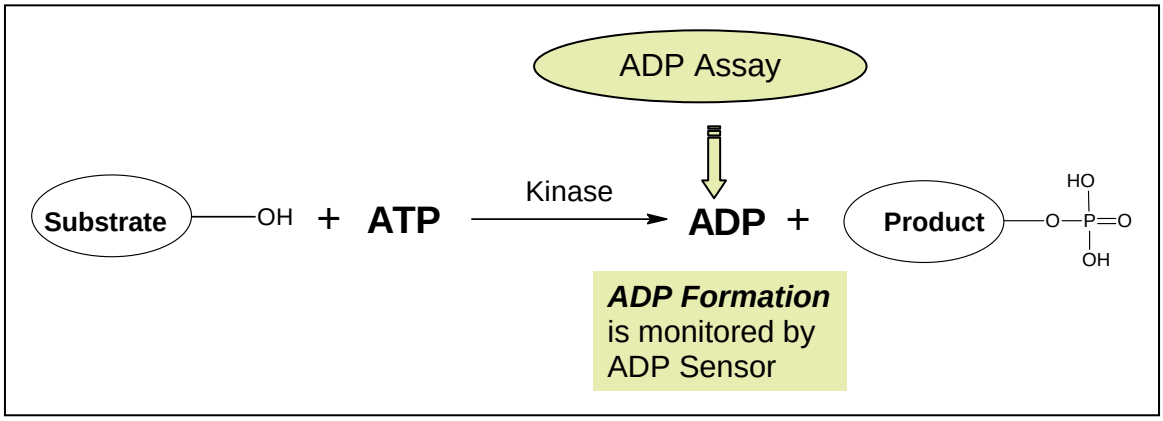
Ordering Information:
Product Number: #21655 (100 assays)

Instrument Platform:
Fluorescence microplate readers

Storage Conditions:
Keep in freezer and avoid light

Introduction

Protein kinases are the enzymes that transfer a phosphate group from a phosphate donor to an acceptor amino acid in a substrate protein. Generally the γ phosphate of ATP, or another nucleoside triphosphate, is the donor, but individual enzymes may have other phosphate donors. The family of protein kinases is large and diverse. The protein kinases serve as molecular switches that can toggle between different conformational states. All signal transduction pathways are regulated, on some level, by phosphorylation, making phosphorylation relevant to most, if not all, areas of cell signaling and neuroscience research. Kinases are of interest to researchers involved in drug discovery due to their broad relevance to diseases. Cancer and other proliferative diseases, inflammatory diseases, metabolic disorders and neurological diseases are among those in which protein kinases play an important role.



Most of commercial protein kinase assay kits are either based on monitoring of phosphopeptide formation or ATP deletion. For the kinase assay kits that are based on detection of phosphopeptides one has to spend time and efforts to identify an optimized peptide substrate while the ATP depletion method suffers various interferences due to the use of luciferase that are inhibited or activated by various biological compounds. The PhosphoWorks™ Fluorimetric ADP Assay Kit is based on the monitoring of ADP formation, which is directly proportional to enzyme phoshphotransferase activity and is measured fluorimetricaly. This kit provides a fast, simple, and homogeneous assay for measure kinases activities. The characteristics of its high sensitivity (<0.2 uM ADP), broad ATP tolerance (1-300 uM), non-antibody based, non-radioactive and no-wash method to detect the amount of ADP produced as a result of enzyme activity make it an ideal kit for determining kinase Michaelis-Menten kinetics and for screening and

identifying kinase inhibitors. The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation with no separation steps required.

Kit Key Features

Universal: Can be used for any kinases that used ATP as phosphate donor.
Continuous: Easily adapted to automation with no mixing or separation protocols.
Convenient: Formulated to have minimal hands-on time.
Non-Radioactive: No special requirements for waste treatment.
Use of Native substrates: Substrates can be proteins, peptides or sugars.
Large Range of ATP Tolerance: ATP can be used from1-300 µM.
Non-Antibody-Based: No antibody is used in the kit.

Kit Components

Materials	#21655 (100 assays)
Component A: ADP Sensor Buffer	1 vial (2 ml)
Component B: ADP Sensor (Light-sensitive)	1 vial (1 ml)
Component C: ADP Standard	1 vial
Component D: ADP Assay Buffer	1 vial (5 ml)

Assay Protocol (for 1 plate)

Brief Summary

Run kinase reaction (20 µL) → Add component A (20 µL) → Add component B (10 µL)→ Incubate at room temperature for 15 min-1 hr → Read fluorescence at Ex 540 nm/Em 590 nm

1. Prepare Samples

1.1 Thaw all the four components at room temperature before use.
1.2 Avoid exposing component B to light directly.
Note: aliquot and store the unused components A and B at -20^{o}C, avoiding freeze/thaw cycles and potential ADP contamination from exogenous biological sources.
1.3 We strongly recommend that black plates are used for achieving the best results.

2. Run Kinase Reaction (Reagents are not provided for this step)

Warning: *The component B is unstable in the presence of thiols such as DTT and β-mercaptoethanol. The final concentration of the thiols higher than 10 uM would significantly decrease the assay dynamic range.*

2.1 Preparation of 20µL kinase reaction as desired. The components of kinase reaction should be optimized as needed (e.g. an optimized buffer system might be required for a specific kinase reaction).
2.2 In most cases, ADP Assay Buffer (Component D) can also be used for running kinase reaction if you do not have your optimized kinase buffer.
2.3 The Phosphoworks™ Fluorimetric ADP Assay Kit is used for determining ADP formation.

3. Run PhosphoWorks™ Fluorimetric ADP Assay

Warning: *The ADP assay should be run at pH from 6.5 to 7.4.*

3.1 Add 20 µL of component A and 10 µL of component B to each well of the 20µL kinase reaction solutions (see Step 2) so that the total ADP assay volume is 50 µL/well.
3.2 Incubate the reaction mixture for 15 min to 1 hour at room temperature.
3.3 Monitor the fluorescence increase with 540 nm excitation and 590 nm emission by using a fluorescence plate reader.

4. Establish ADP Calibration Curve (Not required for screening kinase inhibitors)

ADP standard curve can be generated as described below if required.

3.1 Add 100 µL of H_2O to component C to make 300 µM ADP stock solution. Make a series of dilutions of ADP in the kinase reaction buffer by including a sample without ADP for measuring background fluorescence.
Note: Typically ADP concentrations from 0.05 to 30 uM are appropriate.
3.2 Add the same amount of the serial diluted ADP solutions into an empty plate (20 µL for 384-well plate).
3.3 Add 20 µL of component A and 10 µL of component B to the ADP dilution wells so that total volume per reaction is 50 µL.
3.4 Incubate the reaction mixture for 15 min to 1 hour at room temperature.
3.5 Monitor the fluorescence with 540 nm excitation and 590 nm emission by using a fluorescence plate reader.
3.6 Generate the ADP standard curve.

4. Run Data Analysis

The fluorescence in blank wells (with the kinase buffer only) is used as a control, and is subtracted from the values for those wells with the kinase reactions. The typical data are included in Figures 1 (ADP calibration curve) and 2 (Protein kinase A reaction).
Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

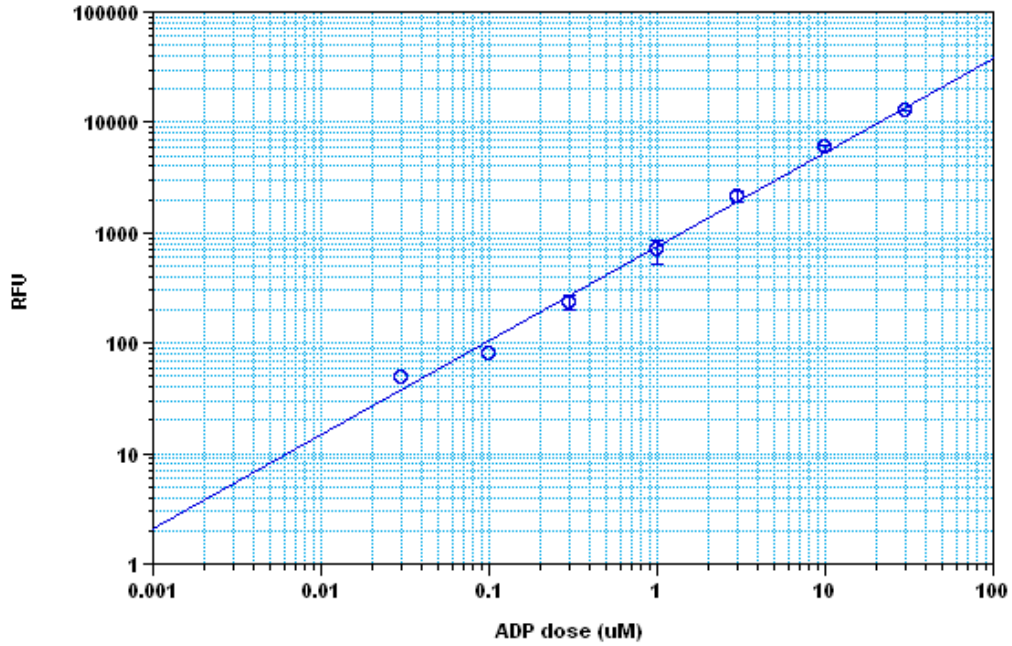


Figure1. ADP dose response on 384-well black plate using a Gemini fluorescence microplate reader (Molecular Devices) measured with the PhosphoWorks™ Fluorimetric ADP Assay Kit. As low as 0.3 µM ADP can be detected with 15, 30 minutes and 1 hour incubation time (Z' factor =0.65).

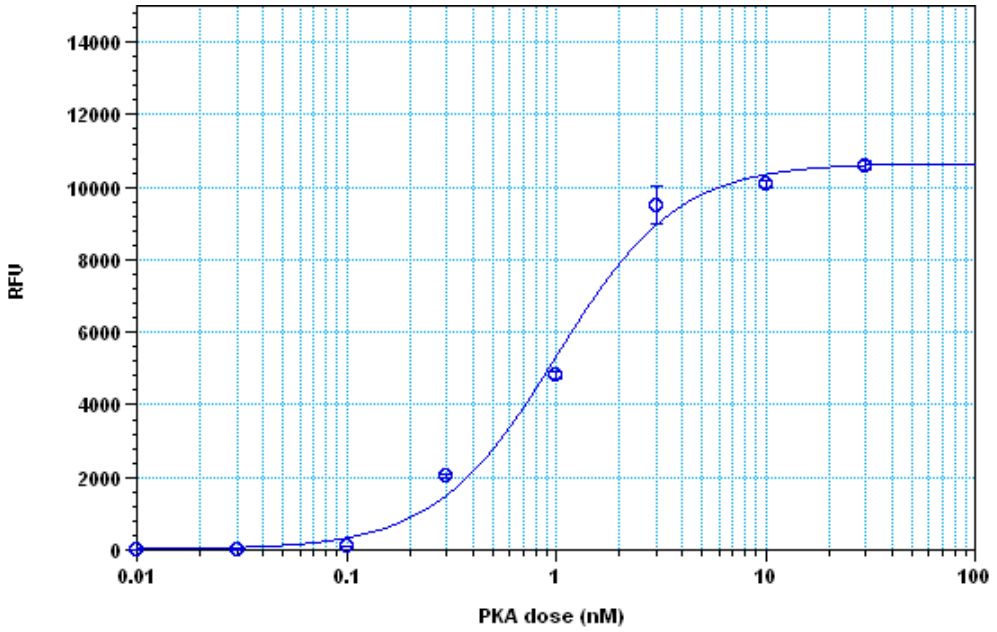


Figure 2. Protein kinase A detection with the PhosphoWorks™ Fluorimetric ADP Assay Kit. The kinase was incubated in the presence of ATP and kemptide peptide substrate for 30 minutes, and ADP generation was detected at 30 min incubation time using the PhosphoWorks™ Fluorimetric ADP Assay Kit.

References:

1. Cohen P (2000). The regulation of protein function by multisite phosphorylation—a 25 year update. *Trends Biochem Sci* **25,** 596-601.
2. Whitmarsh AJ and Davis RJ (2000). Regulation of transcription factor function by phosphorylation. *Cell Mol Life Sci* **57,** 1172-83.
3. Hunter T (1998). The role of tyrosine phosphorylation in cell growth and disease. *Harvey Lect* **94,** 81-119.
4. McCubrey JA, *et al.* (2000). Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* **14,** 9-21.
5. Salli U, *et al.* (2000). Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein is associated with bovine luteal oxytocin exocytosis. *Biol Reprod* **63,** 12-20.
6. Parekh DB, *et al.* (2000). Multiple pathways control protein kinase C phosphorylation. *Embo J* **19,** 496-503.