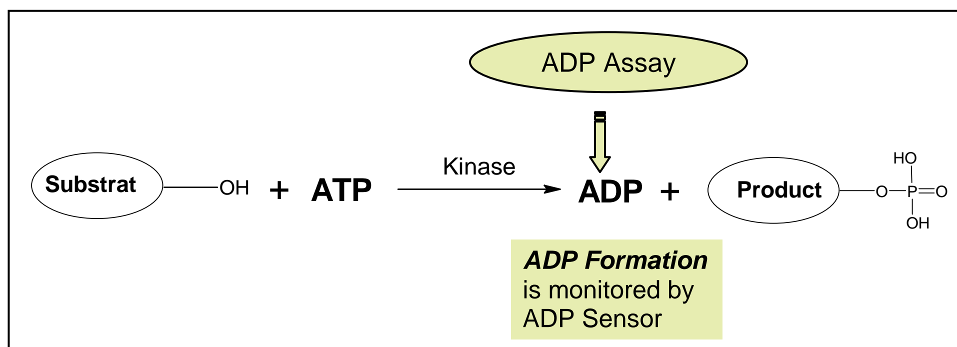
PhosphoWorks™ Fluorimetric ADP Assay Kit
Red Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 21655 (100 assays)	Keep in freezer and protect from light	Fluorescence microplate readers

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 the commercial protein kinase assay kits are either based on monitoring the phosphopeptide formation or ATP depletion. For the kinase assay kits that are based on the 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 the measurement of kinase activities. It is a non-radioactive and no wash method to detect the amount of ADP produced as a result of enzyme activity. Its characteristics of high sensitivity (< 0.3 µM ADP), broad ATP tolerance (1-300 µM) 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 without a separation step.

Kit Key Features

Universal:	Can be used for any kinases that use ATP as a phosphate donor.
Continuous:	Easily adapted to automation without a separation step.
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 from 1 - 300 µM.
Non-Antibody-Based:	No antibody is used in the kit.

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Kit Components

Components	Amount
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 One 96-Well Plate

Brief Summary

Run kinase reaction (20 µL) → Add Component A (20 µL) → Add Component B (10 µL)→ Incubate at room temperature for 15 minutes -1 hour → Monitor fluorescence intensity at Ex/Em = 540/590 nm

1. Prepare samples:

1.1 Thaw all the four components at room temperature before use.

1.2 Avoid direct exposure of component B to light.
Note: Aliquot and store the unused Components A and B at -20 °C. Avoid repeated freeze/thaw cycles and potential ADP contamination from exogenous biological sources.

1.3 Black plates are strongly recommended to achieve 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 µM would significantly decrease the assay dynamic range.*

2.1 Prepare 20 µL of 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 to run kinase reaction if you do not have the optimized kinase buffer.

2.3 The Phosphoworks™ Fluorimetric ADP Assay Kit is used to determine the 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 ADP Sensor Buffer (Component A) and 10 µL of ADP Sensor (Component B) into each well filled with the 20µL of kinase reaction solutions (see Step 2) to make the total ADP assay volume of 50 µL/well.

3.2 Incubate the reaction mixture at room temperature for 15 minutes to 1 hour.

3.3 Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 540/590 nm.

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4. Generate an ADP calibration curve (Not required for the screening of kinase inhibitors):

Note: An ADP standard curve can be generated as described below.

4.1 Add 100 µL of H_2O to the vial of ADP Standard (Component C) to make 300 mM of 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 µM are appropriate.

4.2 Add the same amount of the serial diluted ADP solutions into an empty plate (20 µL/well for a 96-well plate, 10 µL/well for a 384-well plate).

4.3 Add 20 µL of ADP Sensor Buffer (Component A) and 10 µL of ADP Sensor (Component B) into the ADP dilution wells (from Step 4.1) to make the total volume of 50 µL for each reaction.

4.4 Incubate the reaction mixture for 15 min to 1 hour at room temperature.

4.5 Monitor the fluorescence intensity with a fluorescence plate reader at Ex/Em = 540 /590 nm.

4.6 Generate the ADP standard curve.

Data Analysis

The fluorescence in blank wells (with the kinase buffer only) is used as a control, and is subtracted from the values of the wells with the kinase reactions. An ADP calibration curve is shown in Figure 1 and a protein kinase A detection curve is shown in Figure 2.

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.

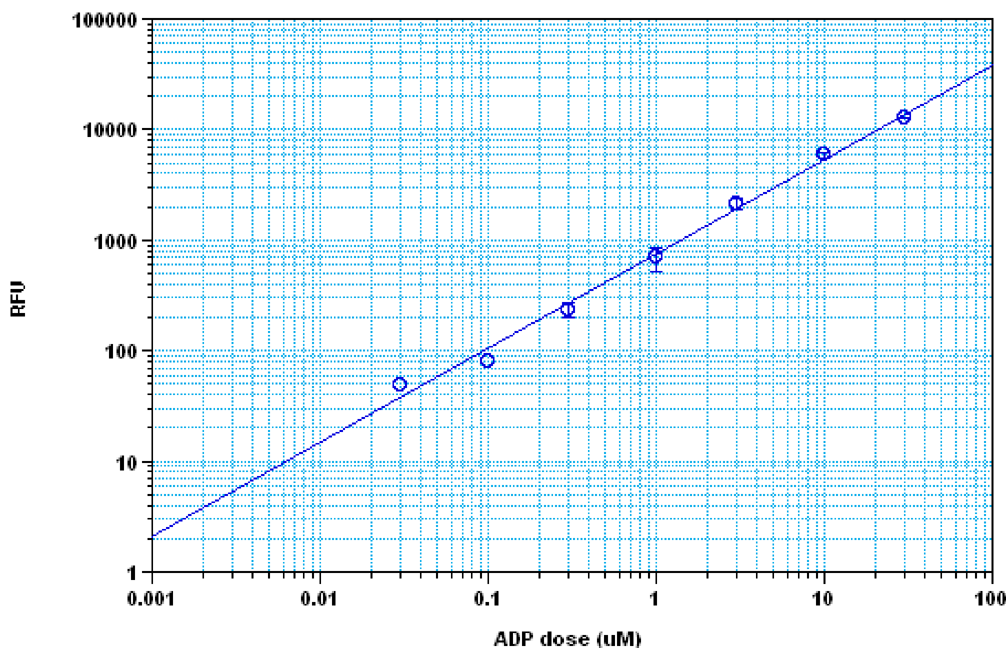

Figure 1. ADP dose response was measured with the PhosphoWorks™ Fluorimetric ADP Assay Kit in a 384-well black plate using a Gemini fluorescence microplate reader (Molecular Devices). As low as 0.3 µM of ADP can be detected with 15, 30 minutes and 1 hour incubation (Z' factor = 0.65).

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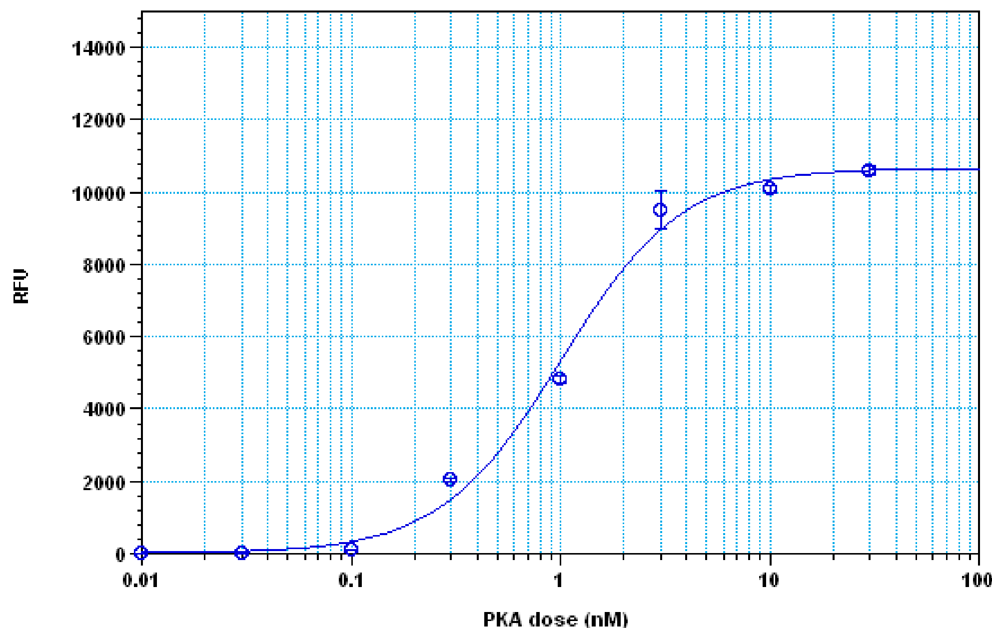

Figure 2. The detection of protein kinase A with the PhosphoWorks™ Fluorimetric ADP Assay Kit. The kinase was incubated in the presence of ATP and kemptide peptide substrate for 30 minutes. ADP generation was detected with the kit after 30 minutes incubation.

References

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