ADP Quest™ Assay

FLUORESCENCE INTENSITY DETECTION

Product Codes: 90-0071 Series

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Read the entire product insert fully before beginning the assay.

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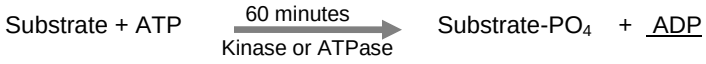
INTENDED USE

The ADP Quest™ Assay is a biochemical assay to measure the accumulation of ADP, a product of kinase and ATPase enzyme activity. The Assay is useful for determining substrate Michaelis-Menten kinetics and for profiling inhibitor potency and mode of action. It can be run in either an endpoint or kinetic mode and is compatible with both peptide substrates and whole proteins. The Assay is for research use only in 96-well or 384-well microplate formats.

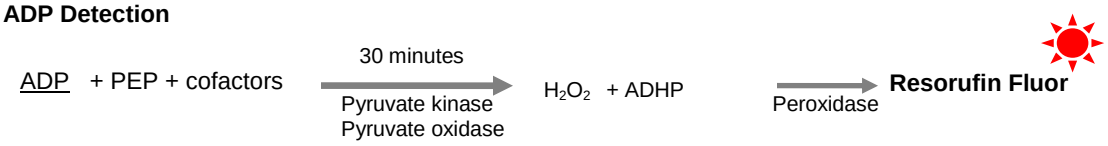
TECHNOLOGY PRINCIPLE

The ADP Quest is a homogeneous assay for the measurement of ADP, which is routinely generated as a product of kinase activity. Unlike alternative generic approaches that monitor the depletion of ATP from a kinase reaction, this method follows the product of the reaction, and offers a convenient gain-of-signal assay format. The assay employs a generic, non-antibody-based, non-radioactive method to detect the amount of ADP produced as a result of enzyme activity. ADP is detected using a coupled enzyme reaction system. The coupled enzyme reaction produces hydrogen peroxide from ADP, which is used to generate the fluorescently active resorufin dye from an

Step 1 Kinase Reaction



Step 2 ADP Detection



STORAGE CONDITIONS

Upon arrival, store reagents at –20°C. Thaw reagents at room temperature before use, and after thawing, store reagents for up to 1 week at 2–8°C. For longer-term storage, aliquots of all the components may be re-frozen at –20°C. Equilibrate reagents to ambient room temperature before use.

MATERIALS PROVIDED

The ADP Quest components are:

Product Code		90-0071	90-0071 M	90-0071 L
Test points in 96-well format		400	1200	5000
Test points in 384-well format		800	2400	10000
Kit Components		Volume in each bottle		
1	ADP Reagent A*	8 mL	3 x 8 mL	100 mL
2	ADP Reagent B*	16 mL	3 x 16 mL	200 mL
3	ADP Standard (225 µM)	1 mL	3 x 1 mL	12 mL
4	ADP Assay Buffer*	20 mL	3 x 20 mL	250 mL

- The ADP Assay Buffer is provided for convenience and may be used as the kinase reaction buffer, to prepare the substrate and/or the kinase, and as the base to add any other kinase reaction cofactors and compounds required. The assay buffer is pH 7.4 containing 15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM $MgCl_2$, and 0.1 mg/mL BGG (bovine-γ-globulins).
- Assay reagents are optimized for consistent performance; however, absolute fluorescence units may vary.
- * Reagents A and B upon thawing may show some particulate matter. This does not affect the performance of the assay. If required, reagents can be centrifuged or filtered through 0.2 micron filters to remove the particulate matter.

MATERIALS NOT PROVIDED

The following additional materials are required:

Equipment	Materials
• 96 or 384-well black microplates • Pipets and pipet tips • Fluorescence intensity reader • Filters and dichroics for the reader for Resorufin Peak Excitation wavelength: 530 nm Peak Emission wavelength: 590 nm	• Kinase enzyme • Kinase substrate • ATP (recommended DiscoveRx ATP Gold, Catalog # 90-0099) • Other cofactors and additives required for kinase reaction components such as phospholipids, and calcium.

GUIDELINES FOR USE

The assay consists of two steps. Step 1 is the kinase reaction and step 2 is the addition of the detection reagents.

Kinase Reaction: The kinase reaction includes a purified or semi-purified active kinase, a substrate (peptide or protein), ATP, other appropriate additives and cofactors, and compounds, in a kinase reaction buffer.

Kinase Reaction Buffer: An assay buffer is provided which may be used as the kinase reaction buffer. If using a different kinase reaction buffer, diluting the ADP standard in your buffer and testing for any interference with the detection reagents is imperative. The assay can tolerate the following concentrations of commonly used components of the kinase reaction. The concentrations here refer to kinase reaction concentrations:

ATP	≤ 200 µM	EDTA	≤ 10 mM	NaCl	≤ 100 mM
BGG	≤ 1 mg/mL	EGTA	≤ 10 mM	$MgCl_2$	≤ 10 mM
DMSO	≤ 10%	Glycerol	≤ 0.5%	Sodium Orthovanadate	≤ 1 mM
DTT	≤ 0.025 mM	HEPES	≤ 15 mM	Optimal pH range	6.0 to 8.0

Kinase reaction optimization:
Components of the kinase reaction need to be optimized for each kinase and may need to be verified with new batches of kinase due to variability in enzyme activity. For optimal performance titrate the following components:

- Substrate
- Kinase
- ATP
- Any cofactors needed for kinase activity

Controls:
A control without kinase should be included in each experiment and signal from this control may be used for correction of background signal, this control should have ATP, substrate, and compounds.

Tips for optimal performance:

- Use the highest quality ATP in performing kinase reactions. Some sources of ATP contain high amounts of contaminating ADP that can significantly increase assay background. We recommend using DiscoveRx ATP Gold (Cat # 90-0099). ATP powder should be stored desiccated at -20 °C. Ideally fresh ATP solutions should be used and made up in 10 mM Tris, pH 7.0. Alternatively stock solution can be aliquoted and stored frozen at -80 °C. Do not freeze-thaw ATP stock solution.
- Avoid using reducing agents such as DTT in the kinase reaction (must be ≤ 0.025 mM) .
- Equilibrate all detection reagents to room temperature for at least 1 hour before use.
- ADP Reagent A and Reagent B must be added separately. **Do not pre-mix**.
- Reagent A must be added before Reagent B for optimal assay performance.
- When using the kinetic mode, first verify that addition of the detection reagents does not stop the kinase activity.
- When using the kinetic mode, compare each kinase concentration with ATP and without ATP.

REAGENT PREPARATION

For ADP Standard Curve: Prepare (7) 3-fold serial dilutions of the ADP standard in the assay buffer provided in polypropylene tubes. The ADP standard concentrations range from 75 to 0.10 µM. Use the assay buffer as the zero ADP control.

For the Kinase Reaction: Add cofactors and/or additives to the assay buffer provided or prepare a kinase reaction buffer. The kinase reaction has a final reaction volume of 20 µL (384-well plate) and 40 µL (96-well plate). The kinase reaction includes a purified or semi purified active kinase, a substrate, ATP, and other appropriate additives/cofactors.

ADP Reagent A: Ready to use, no preparation needed.

ADP Reagent B: Ready to use, no preparation needed.

Assay Procedure (Standard Curve)

The table below outlines the volumes and procedure for the ADP standard curve for a 384-well plate format. For 96-well format, multiply all volumes by a factor of 2.

384-well Plate	ADP Standard
Step 1: Standard Dilutions	20 µL ADP Standard dilutions
Step 2: ADP Detection	10 µL Reagent A
	20 µL Reagent B
Read Fluorescence Intensity signal after 30 minutes at room temperature* Resorufin Excitation wavelength – 530 nm Resorufin Emission wavelength – 590 nm	

* The signal may be measured up to 6 hours after addition of Reagent B; however, since the background continues to increase over time, subtracting the background from the signal is required when comparing different plates.

Assay Procedure (Kinetic mode)

The table below outlines assay volumes and procedure for kinase reactions for kinetic mode. In order to use this mode, first verify that the kinase activity does not stop when detection reagents are added. The volumes in the table below are for 384-well plate format. For 96-well plate, multiply all volumes by a factor of 2.

Kinetic Mode (384-well plate)	Kinase Reaction
Kinase Reaction components	5 µL substrate
	10 µL Kinase
ADP Detection Reagents	10 µL Reagent A
	20 µL Reagent B
Kinase Reaction Start	5 µL ATP
Read Fluorescence Intensity signal every 2-5 minutes* Resorufin Excitation wavelength – 530 nm Resorufin Emission wavelength – 590 nm	

* The amount of time it takes to reach a plateau will depend on kinase activity and kinase reaction concentrations. For each of the ATP levels being tested, a “no kinase” control must be included. If readings are taken more often, some photo bleaching may occur.

Assay Procedure (End-point mode)

The table below outlines assay volumes and procedure for kinase reactions for end-point mode. The volumes in the table are for a 384-well plate format. For 96-well format, multiply all volumes by a factor of 2.

End-Point Mode (384-well Plate)	Kinase Reaction
Step 1: Kinase Reaction	20 µL kinase reaction (kinase, substrate, ATP, compound)
	Kinase reaction incubation*
Step 2: ADP Detection	10 µL Reagent A
	20 µL Reagent B
Read Fluorescence Intensity signal after 30 minutes at room temperature ** Resorufin Excitation wavelength – 530 nm Resorufin Emission wavelength – 590 nm	

* The incubation time and temperature for the kinase reaction should be optimized for each kinase. For some kinases, the addition of ADP Quest™ reagents will stop kinase activity. For those kinases not stopped by the detection reagents, EDTA may be used up to 10 mM.

** The signal may be measured up to 6 hours after addition of Reagent B, however, since the background continues to increase over time, subtracting the background from the signal is required when comparing different plates.

REPRESENTATIVE DATA

The following is representative data for illustrative purposes. Actual results may vary.

Figure 1: The ADP Standard curve is illustrated below. The dynamic assay range is up to 75 µM ADP with a least detectable dose of ≤ 0.6 µM. The data is shown using a second order polynomial curve fit.

Figure 1: ADP Standard Curve

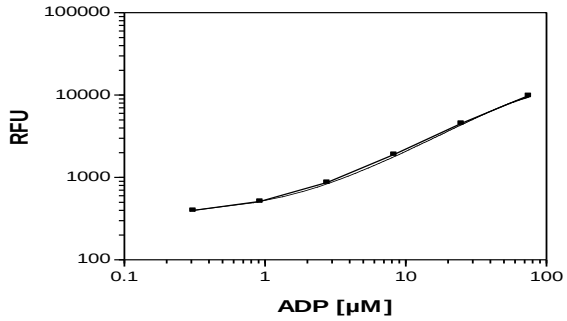


Figure 2: PKA activity was measured at various concentrations with fixed substrate and ATP concentrations in kinetic mode and the data is represented as signal over time. Note that at higher enzyme concentrations the substrate is depleted and a plateau is reached, which is expected.

Figure 2: PKA kinase activity (kinetic mode)

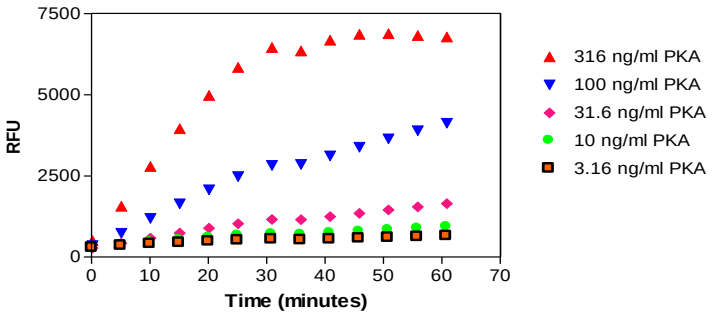


Figure 3: Three kinases (PKAα, JNK2α2, and CK1) with varying activity were tested using the end-point procedure with 100 µM ATP and substrates as indicated (100 µM Kemptide for PKA, 250 µM CK peptide for CK1, and 100 µM ATF2 peptide for JnK2). Kinase reactions were incubated for 1 hour at 30°C, followed by the addition of ADP Reagent A and Reagent B. Figure 3a illustrates rank order of activity of the three kinases PKAα > CK1> JNK2α2. Staurosporine is a potent inhibitor of PKA and a weak inhibitor of both CK1 and JNK2α2. Staurosprine was titrated for all three kinases (PKAα, JNK2α2, and CK1) with peptide substrates and ATP held constant at their respective K_m concentrations using the end-point procedure. Staurosporine inhibition curves are illustrated in figure 3b.

Figure 3a: Kinase Titrations

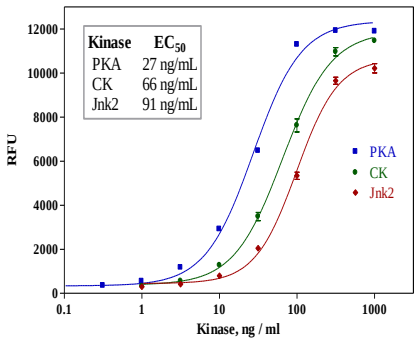
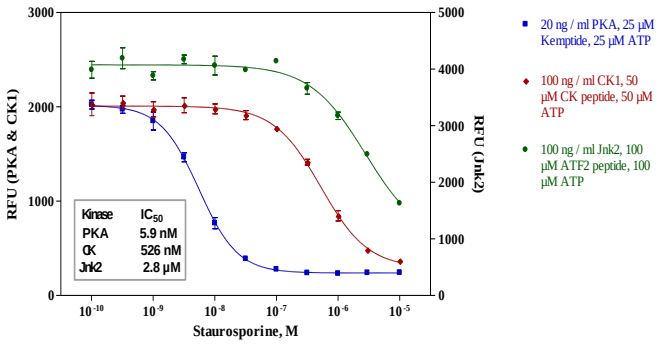


Figure 3b: Staurosporine Dose Response Curve

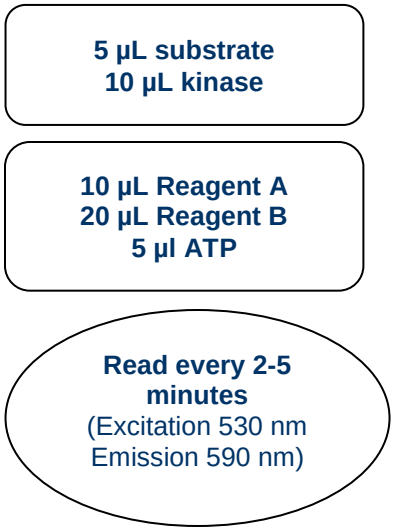


Quick Start Guide

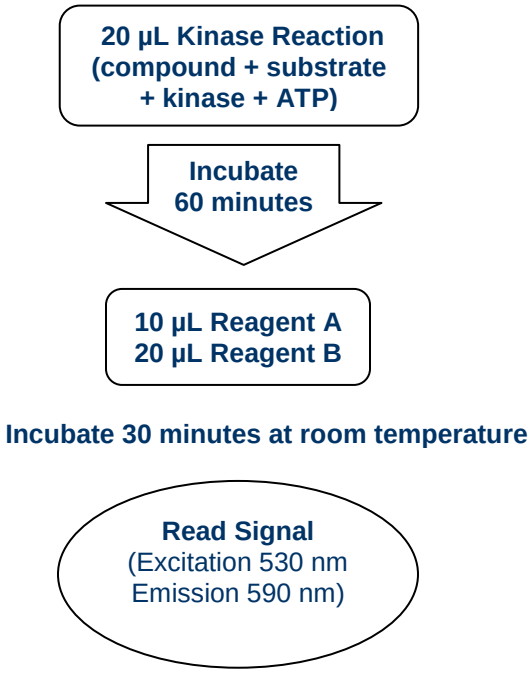
This quick start guide assumes that you have optimized your kinase reaction conditions, for example, ATP concentration, substrate concentration, and kinase amount.

The flowcharts below outline assay volumes and procedure for both kinetic mode and end-point mode. The volumes outlined are for 384-well plate format. For 96-well format, multiply all volumes by a factor of 2. Run appropriate controls with all experiments.

Kinetic Mode *



End-Point Mode *



* For more detailed procedures and guidelines refer to pages 3 and 4 of this product insert.