Product Manual

ADP Assay Kit

Catalog Number

MET-5164 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Adenosine diphosphate (ADP) is an organic molecule that plays a role in energy flow for various cell processes such as enzymatic reactions leading to nerve impulses, muscle contraction, and synthesis of chemicals. ADP is found in all life forms and consists of three components: a nitrogenous base (adenine), the sugar ribose, and the diphosphate group. It is consumed in serial enzymatic reactions that produce ATP such as glycolysis or oxidative phosphorylation. In plants, sunlight is converted from ADP to ATP through a process known as photosynthesis. When used up metabolically, ATP is converted either to adenosine diphosphate (ADP) or to adenosine monophosphate (AMP).

Cell Biolabs' ADP Assay Kit is a simple fluorometric assay that measures the amount of total ADP present in biological samples in a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 100 assays*, including blanks, ADP standards, and unknown samples. Sample ADP concentrations are determined by comparison with a known ADP standard. The kit has a detection sensitivity limit of 3.1 µM ADP.

Note: Each sample replicate requires 2 assays, one treated with pyruvate kinase (+PK) and one without (-PK). ADP is calculated from the difference in RFU readings from the 2 wells.

Assay Principle

Cell Biolabs' ADP Assay Kit measures total ADP within biological samples. ADP and phosphoenolpyruvate (PEP) are converted by pyruvate kinase to ATP and pyruvate. Pyruvate is oxidized by pyruvate oxidase in the presence of phosphate and oxygen into acetyl phosphate, carbon dioxide, and hydrogen peroxide. The resulting hydrogen peroxide is then detected with a highly specific fluorometric probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio. Samples are compared to a known concentration of ATP standard within the 96-well microtiter plate format. Samples and standards are incubated for 30 minutes and then read with a standard 96-well fluorometric plate reader (Figure 1).

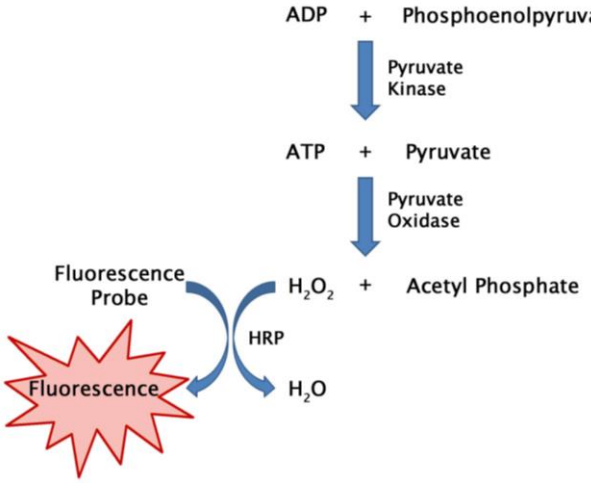

Figure 1. ADP Assay Principle

Related Products

1. MET-5163: ATP Assay Kit
2. MET-5090: Adenosine Assay Kit
3. MET-5158: Methionine Assay Kit

4. MET-5152: s-Adenosylmethionine ELISA Kit
5. MET-5029: Pyruvate Assay Kit (Fluorometric)

Kit Components

Box 1 (shipped on blue ice packs)

1. ADP Standard (Part No. 51641C): One 50 µL tube at 20 mM.
2. $MgCl_2$ (Part No. 51623A): One 200 µL tube of magnesium chloride at 1 M.
3. PEP (Part No. 51585C): One 50 µL tube at 100 mM.
4. Pyruvate Kinase (Part No. 51625D): One 200 µL tube.
5. Pyruvate Oxidase (Part No. 50295C): One 300 µL tube.
6. Fluorometric Probe (Part No. 50231C): One 50 µL tube in DMSO.
7. HRP (Part No. 234402-T): One 10 µL tube of a 100 U/mL solution in glycerol.
8. FAD (Part No. 50293C): One 50 µL tube of 2 mM Flavin Adenine Dinucleotide (FAD).
9. TPP (Part No. 50294C): One 50 µL tube of 2 mM Thiamine Pyrophosphate (TPP).

Box 2 (shipped on blue ice packs)

1. Neutralization Buffer (Part No. 51634A): One 25 mL bottle.
2. 10X Assay Buffer (Part No. 51622A): One 25 mL bottle

Materials Not Supplied

1. Distilled or deionized water
2. 10 kDa molecular weight cutoff (MWCO) centrifuge spin filter (e.g. Amicon Ultra 0.5mL)
3. Standard 96-well fluorescence black microtiter plate and/or black cell culture microplate
4. Concentrated perchloric acid.

Storage

Store the 10X Assay Buffer and Neutralization Buffer at room temperature. Store the Pyruvate Kinase and the Pyruvate Oxidase at -80ºC. Store all other components at -20ºC. The Fluorometric Probe is light sensitive and must be stored accordingly. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 1X Assay Buffer: Dilute the 10X Assay Buffer 1:10 with deionized water for a 1X solution. Stir or vortex to homogeneity. Store at 4°C.
- Reaction Mix and Negative Control Mix: Prepare two separate mixtures according to the table below. The Pyruvate Kinase is omitted from the Negative Control Mix.

Component	Reaction Mix (20 assays)	Negative Control Mix (20 assays)
Pyruvate Kinase	40 µL	------
Pyruvate Oxidase	60 µL	60 µL
PEP	10 µL	10 µL
FAD	10 µL	10 µL
TPP	10 µL	10 µL
$MgCl_2$	40 µL	40 µL
HRP	2 µL	2 µL
Fluorometric Probe	10 µL	10 µL
1X Assay Buffer	818 µL	858 µL
Total	**1000 µL**	**1000 µL**

Note: Prepare only enough for immediate use and scale proportionally as needed.

- 0.5 M Perchloric Acid: Dilute concentrated perchloric acid (not provided in the kit) to 0.5 M with distilled water. Store at room temperature.

Preparation of Samples

- Cell culture supernatants: To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. Collect the supernatant and filter the solution with a 10 kDa spin filter to deproteinate the sample. Collect flow through. The flow through may be assayed directly or diluted as necessary into 1X Assay Buffer.

Note: Maintain pH between 7 and 8 for optimal working conditions as the Fluorometric Probe is unstable at high pH (>8.5).

- Tissue lysates: Sonicate or homogenize 150-300 milligrams of tissue sample in 2 mL of 0.5 M Perchloric Acid at 4°C. Incubate for 5 minutes at 4°C. Centrifuge at 18000 x g for 5 minutes. Collect the supernatant and transfer to a new tube. Add 500 µL of Neutralization Buffer and mix well. Incubate for 5 minutes at 4°C. Centrifuge at 18000 x g for 5 minutes. Collect the supernatant and transfer to a new tube. Store aliquots at -80°C until ready to use. *Upon thawing, if salt crystals are observed, warm the sample to 37°C for 5 minutes and vortex well to resuspend.*
- Cell lysates: Wash cells in PBS and transfer to an Eppendorf tube. Centrifuge 1.5 x 10^7 cells at 1000 x g for 5 minutes. Remove the supernatant and resuspend cell pellet in 200 µL of 0.5 M Perchloric Acid. Incubate for 5 minutes at 4°C. Centrifuge at 18000 x g for 5 minutes. Collect the supernatant and transfer to a new tube. Add 50 µL of Neutralization Buffer and mix well. Incubate for 5 minutes at 4°C. Centrifuge at 18000 x g for 5 minutes. Collect the supernatant and transfer to a new tube. Store aliquots at -80°C until ready to use. *Upon thawing, if salt crystals are observed, warm the sample to 37°C for 5 minutes and vortex well to resuspend.*
- Serum, plasma, saliva, or urine: To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. Collect the supernatant and filter the solution with a 10 kDa spin filter to deproteinate the sample. Collect flow through. The flow through may be assayed directly or diluted as necessary into 1X Assay Buffer.

Notes:

- *All samples should be assayed immediately or stored at -80°C for up to 1-2 months. Run proper controls as necessary. Optimal experimental conditions for samples must be determined by the investigator. Always run a standard curve with samples.*
- *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the Fluorometric Probe and could result in erroneous readings. To minimize this interference, it*

is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL (Votyakova and Reynolds, Ref. 2).

- *Avoid samples containing DTT or β-mercaptoethanol since the Fluorometric Probe is not stable in the presence of thiols (above 10 µM).*

Preparation of Standard Curve

Prepare fresh ADP standards by diluting in distilled water according to Table 1.

Standard Tubes	20 mM ADP Solution (µL)	Water (µL)	ADP(µM)
1	5	495	200
2	250 of Tube #1	250	100
3	250 of Tube #2	250	50
4	250 of Tube #3	250	25
5	250 of Tube #4	250	12.5
6	250 of Tube #5	250	6.25
7	250 of Tube #6	250	3.13
8	0	250	0

Table 1. Preparation of ADP Standards.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.

Note: Each unknown sample replicate requires two paired wells, one to be treated with Pyruvate Kinase (+PK) and one without the enzyme (-PK) to measure endogenous background.

2. Add 50 µL of each ADP standard or unknown sample into wells of a 96-well microtiter plate.
3. Add 50 µL of Reaction Mix to the standards and to one half of the paired sample wells, and mix the well contents thoroughly.
4. Add 50 µL of Negative Control Mix to the other half of the paired sample wells.
5. Mix the well contents thoroughly and incubate for 30 minutes at 37ºC protected from light.

Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the reaction kinetics.

6. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nm range and for emission in the 590-600 nm range.

Example of Results

The following figures demonstrate typical ADP Assay Kit results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

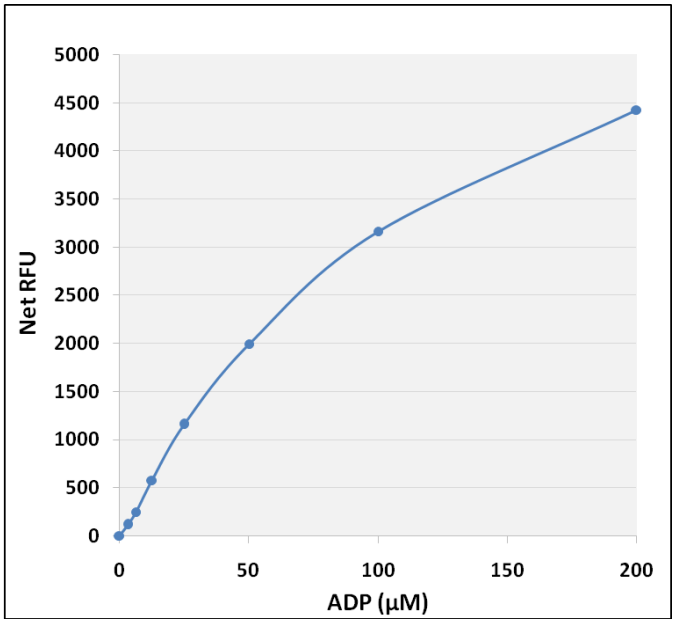

Figure 2: ADP Standard Curve.

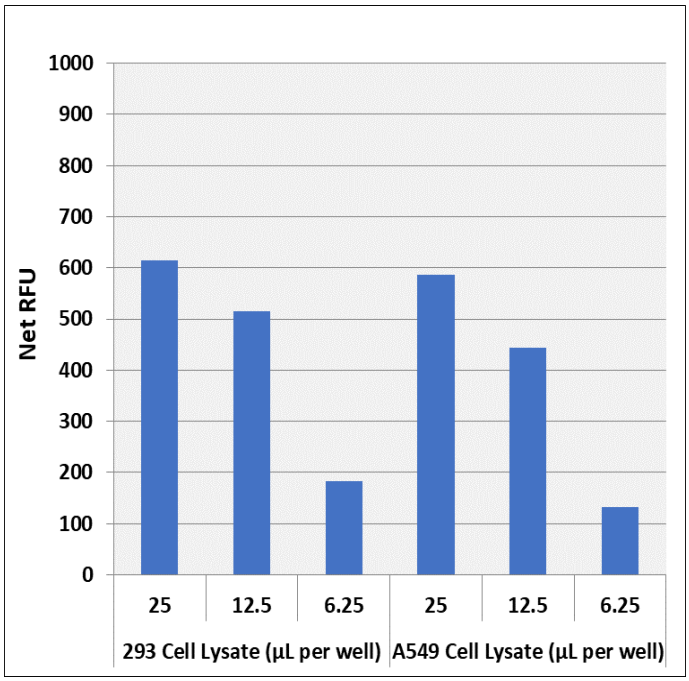

Figure 3: ADP Detection in 293 and A549 cells using the ATP Assay Kit. Cell lysates were prepared according to the preparation of samples section above.

Calculation of Results

1. Determine the average Relative Fluorescence Unit (RFU) values for each sample, control, and standard.
2. Subtract the average zero standard value from itself and all standard values.
3. Graph the standard curve (see Figure 2).

4. Subtract the sample well values without Pyruvate Kinase (-PK) from the sample well values containing Pyruvate Kinase (+PK) to obtain the difference. The fluorescence difference is due to the Pyruvate Kinase activity.

$$\textbf{Net RFU} = (\textbf{RFU}_{+\textbf{PK}}) - (\textbf{RFU}_{-\textbf{PK}})$$

5. Compare the net RFU of each sample to the standard curve to determine and extrapolate the quantity of ADP present in the sample. Only use values within the range of the standard curve.

References

1. Knowles J R (1980) *Annu. Rev. Biochem*. **49**: 877–919.
2. Votyakova TV, and Reynolds IJ (2001) *Neurochem*. **79**:266.
3. Törnroth-Horsefield S and Neutze R. (2008). *Proc Natl Acad Sci USA*. **105**:19565–19566.
4. Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JP, Gonzalez G, Aguilar-Bryan L, Permutt MA, and Bryan J. (1996) *Science*. **272**:1785-1787.
5. Murugappa S, and Kunapuli SP (2006). *Front. Biosci*. **11**: 1977–1986.

Warranty

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