Product Manual

Pyrophosphate Assay Kit

Catalog Number

MET-5159 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Pyrophosphate, also called diphosphate, is any phosphorus oxyanion that contains two phosphorus atoms in a P–O–P linkage. Pyrophosphates are found in important biochemical molecules such as adenosine triphosphate (ATP) and other nucleotide triphosphates. Pyrophosphate is formed when ATP is hydrolyzed to adenosine monophosphate in various biologically important reactions. As an example, when a deoxynucleotide triphosphate (dNTP) or a nucleotide triphosphate (NTP) is used by a DNA polymerase or RNA polymerase to incorporate a nucleotide into a DNA or RNA strand respectively, pyrophosphate is formed. Pyrophosphate complexes readily with transition metals such as calcium, and can be found at sufficiently high enough levels in urine, synovial fluid, and blood plasma to block the accumulation of calcium salts. Non enzymatic membrane pyrophosphate channels such as ankylosis gene product (ANK) may move intracellular pyrophosphate into the extracellular synovial fluid. Over formation of calcium pyrophosphate can crystalize and deposit in joints leading to a form of arthritis in humans.

Cell Biolabs' Pyrophosphate Assay Kit is a simple fluorometric assay that measures the amount of pyrophosphate present in biological samples in a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 100 assays*, including blanks, pyrophosphate standards, and unknown samples. Sample pyrophosphate concentrations are determined by comparison with a known pyrophosphate standard. The kit has a detection sensitivity limit of 4 µM pyrophosphate.

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

Assay Principle

Cell Biolabs' Pyrophosphate Assay Kit measures total pyrophosphate within biological samples. Pyrophosphate (and phosphoenolpyruvate) is converted by PPDK to pyruvate + adenosine triphosphate (ATP) and phosphate. Pyruvate is converted 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 pyrophosphate 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).

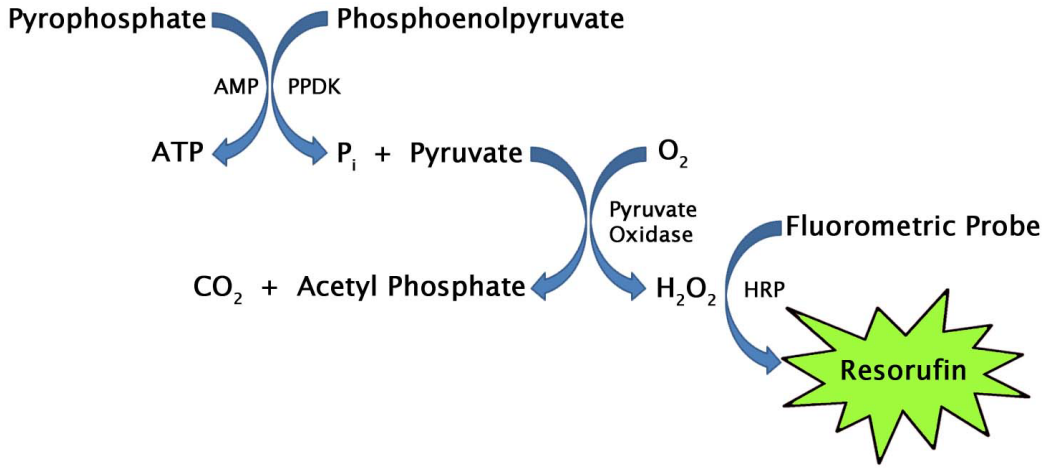

Figure 1. Pyrophosphate Assay Principle

Related Products

1. MET-5029: Pyruvate Assay Kit (Fluorometric)
2. MET-5158: Methionine Assay Kit
3. MET-5013: Lactate Assay Kit (Fluorometric)
4. MET-5023: Glycogen Assay Kit (Fluorometric)
5. STA-681: Glucose Assay Kit (Fluorometric)

Kit Components

Box 1 (shipped on blue ice packs)

1. Pyrophosphate Standard (Part No. 51591C): One 50 µL tube at 25 mM.
2. AMP (Part No. 51584C): One 50 µL tube of adenosine monophosphate (AMP) at 20 mM.
3. PEP (Part No. 51585C): One 50 µL tube of phosphoenolpyruvate (PEP) at 100 mM.
4. Fluorometric Probe (Part No. 50231C): One 50 µL tube in DMSO.
5. HRP (Part No. 234402-T): One 10 µL tube of a 100 U/mL solution in glycerol.
6. FAD (Part No. 50293C): One 50 µL tube of 2 mM Flavin Adenine Dinucleotide (FAD).
7. TPP (Part No. 50294C): One 50 µL tube of 2 mM Thiamine Pyrophosphate (TPP).
8. Pyruvate Oxidase (Part No. 50295C): One 300 µL tube.
9. PPDK (Part No. 51588D): One 200 µL tube of phosphate pyruvate dikinase (PPDK).

Box 2 (shipped on blue ice packs)

1. 10X Assay Buffer (Part No. 51582A): One 25 mL bottle.
2. Na_2HPO4 (Part No. 51586A): One 50 µL tube of sodium phosphate dibasic (Na_2HPO4) at 100 mM.

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. 96-well black or fluorescence microtiter plate

Storage

Upon receipt, store the 10X Assay Buffer and Na_2HPO_4 at room temperature. Store the PPDK 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 PPDK is omitted from the Negative Control Mix.

Component	Reaction Mix (20 assays)	Negative Control Mix (20 assays)
PPDK	40 µL	------
AMP	10 µL	10 µL
PEP	10 µL	10 µL
HRP	2 µL	2 µL
Pyruvate Oxidase	60 µL	60 µL
FAD	10 µL	10 µL
TPP	10 µL	10 µL
Na_2HPO_4	10 µL	10 µL
Fluorometric Probe	10 µL	10 µL
1X Assay Buffer	838 µL	878 µL
Total	**1000 µL**	**1000 µL**

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

Preparation of Samples

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

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 tissue sample in PBS and centrifuge at 10,000 x g for 10 minutes at 4°C. Collect the supernatant and filter the solution with a 10kDa spin filter to deproteinate the sample. Collect flow through. The flow through may be assayed directly or diluted as necessary into PBS.
- Cell lysates: Resuspend cells in PBS. Homogenize or sonicate the cells on ice. Centrifuge to remove debris. Collect the supernatant and filter the solution with a 10kDa spin filter to deproteinate the sample. Collect flow through. The flow through may be assayed undiluted or diluted as necessary into PBS.
- Serum, plasma, saliva, or urine: To remove insoluble particles, centrifuge at 10,000 x g for 5 min. Collect the supernatant and filter the solution with a 10kDa spin filter to deproteinate the sample. Collect flow through. The flow through may be assayed directly or diluted as necessary into PBS.

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 Pyrophosphate standards by diluting into 1X Assay Buffer according to Table 1.

Standard Tubes	25 mM Pyrophosphate Solution (µL)	1X Assay Buffer (µL)	Pyrophosphate (µM)
1	5	995	125
2	250 of Tube #1	250	62.5
3	250 of Tube #2	250	31.25
4	250 of Tube #3	250	15.63
5	250 of Tube #4	250	7.81
6	250 of Tube #5	250	3.91
7	0	250	0

Table 1. Preparation of Pyrophosphate 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 PPDK (+PPDK) and one without the enzyme (-PPDK) to measure endogenous background.

2. Add 50 µL of each pyrophosphate 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 Pyrophosphate 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.

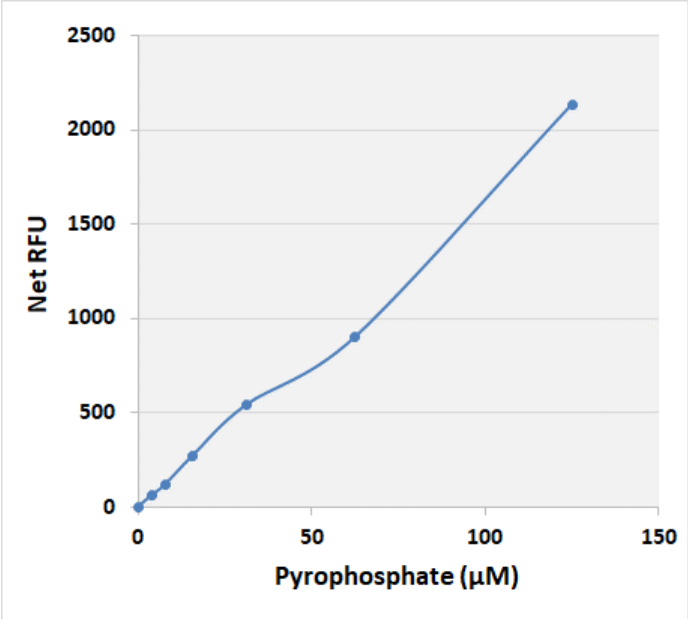

Figure 2: Pyrophosphate Standard Curve.

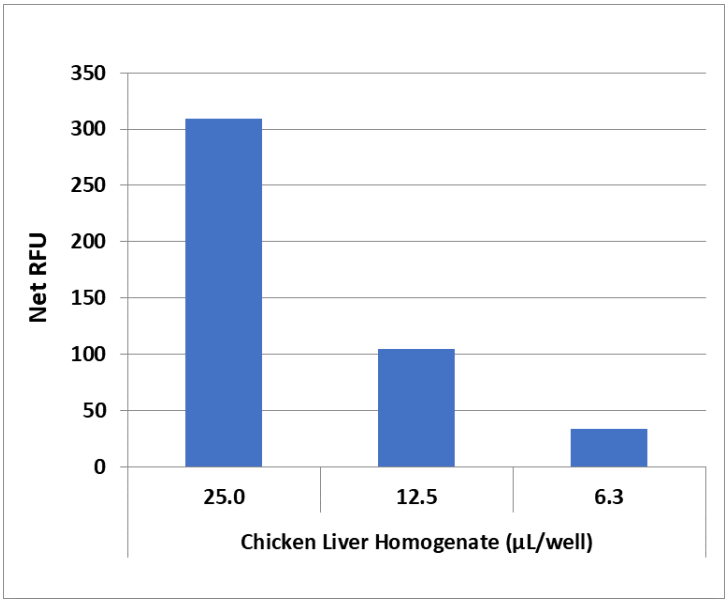

Figure 3: Pyrophosphate Detection in Chicken Liver using the Pyrophosphate Assay Kit. Chicken Liver was homogenized and deproteinated according to the preparation of samples section above.

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 PPDK (-PPDK) from the sample well values containing PPDK (+PPDK) to obtain the difference. The fluorescence difference is due to the PPDK activity.

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

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

References

1. Ho AM, Johnson MD, Kingsley DM (2000). *Science*. **289**: 265–70.
2. Votyakova TV, and Reynolds IJ (2001) *Neurochem*. **79**:266.
3. Rutsch F, Vaingankar S, Johnson K, Goldfine I, Maddux B, Schauerte P, Kalhoff H, Sano K, Boisvert WA, Superti-Furga A, Terkeltaub R (2001). *Am J Pathol.* **158**: 543–54.
4. Ryan LM, Kozin F, McCarty DJ (1979). *Arthritis Rheum*. **22**: 886–91.
5. Rosenthal AK and Lawrence MR (2016). *New England J. Med*. **374**:2575-2584.

Warranty

These products are warranted to perform as described in their labeling and in Cell Biolabs literature when used in accordance with their instructions. THERE ARE NO WARRANTIES THAT EXTEND BEYOND THIS EXPRESSED WARRANTY AND CELL BIOLABS DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR WARRANTY OF FITNESS FOR PARTICULAR PURPOSE. CELL BIOLABS's sole obligation and purchaser's exclusive remedy for breach of this warranty shall be, at the option of CELL BIOLABS, to repair or replace the products. In no event shall CELL BIOLABS be liable for any proximate, incidental or consequential damages in connection with the products.

Contact Information

Cell Biolabs, Inc.
5628 Copley Drive
San Diego, CA 92111
Worldwide: +1 858 271-6500
USA Toll-Free: 1-888-CBL-0505
E-mail: tech@cellbiolabs.com
www.cellbiolabs.com