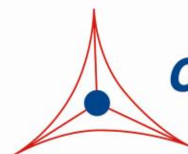
Product Manual

Phosphoenolpyruvate Assay Kit

Catalog Number

MET-5162 100 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



Introduction

Phosphoenolpyruvate (PEP) is an important biochemical intermediate. The phosphate bond of PEP has the highest energy found in living organisms, and the bond plays an important role in metabolic pathways such as glycolysis, where pyruvate kinase generates ATP to form pyruvate. PEP also plays a role in gluconeogenesis, where it is formed from the decarboxylation of oxaloacetate catalyzed by phosphoenolpyruvate carboxykinase. In plants, PEP is used to make various aromatic compounds, and is involved in the process of converting inorganic carbon to organic carbon (carbon fixation). In bacteria, PEP is the source of energy for the phosphotransferase system.

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

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

Assay Principle

Cell Biolabs' Phosphoenolpyruvate Assay Kit measures total PEP within biological samples. PEP in the presence of ADP is converted by Pyruvate Kinase to pyruvate + adenosine triphosphate (ATP). 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 PEP 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).

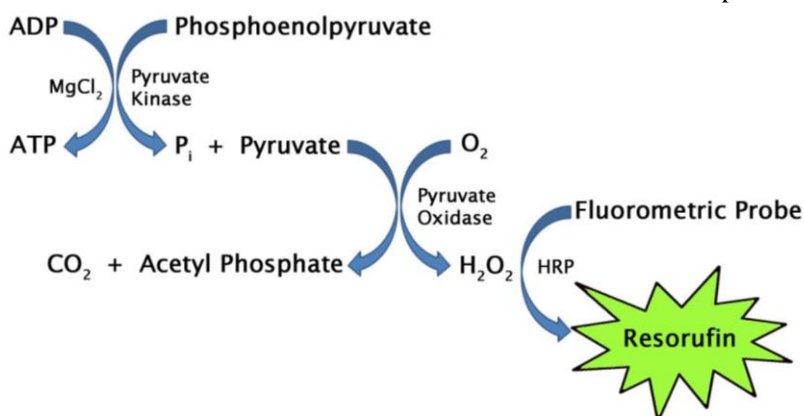


Figure 1. PEP Assay Principle

Related Products

1. MET-5163: ATP Assay Kit (Fluorometric)
2. MET-5164: ADP Assay Kit (Fluorometric)
3. MET-5029: Pyruvate Assay Kit (Fluorometric)

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4. MET-5158: Methionine Assay Kit (Fluorometric)
5. MET-5160: Adenosine Monophosphate Assay Kit (Fluorometric)

Kit Components

Box 1 (shipped on blue ice packs)

1. PEP Standard (Part No. 51621C): One 50 µL tube of Phosphoenolpyruvate at 20 mM.
2. ADP (Part No. 51624C): One 250 µL tube of adenosine diphosphate (ADP) at 200 mM.
3. Fluorometric Probe (Part No. 50231C): One 50 µL tube in DMSO.
4. HRP (Part No. 234402-T): One 10 µL tube of a 100 U/mL solution in glycerol.
5. FAD (Part No. 50293C): One 50 µL tube of 2 mM Flavin Adenine Dinucleotide (FAD).
6. TPP (Part No. 50294C): One 50 µL tube of 2 mM Thiamine Pyrophosphate (TPP).
7. Pyruvate Oxidase (Part No. 50295C): One 300 µL tube.
8. Pyruvate Kinase (Part No. 51625D): One 200 µL tube.

Box 2 (shipped on blue ice packs)

1. 10X Assay Buffer (Part No. 51622A): One 25 mL bottle.
2. $MgCl_2$ (Part No. 51623A): One 200 µL tube of magnesium chloride at 1 M.

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

Storage

Upon receipt, store the 10X Assay Buffer and the $MgCl_2$ at room temperature. Store the Pyruvate Kinase 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.

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Component	Reaction Mix (20 assays)	Negative Control Mix (20 assays)
Pyruvate Kinase	40 µL	------
$MgCl_2$	40 µL	40 µL
ADP	50 µL	50 µL
HRP	2 µL	2 µL
Pyruvate Oxidase	60 µL	60 µL
FAD	10 µL	10 µL
TPP	10 µL	10 µL
Fluorometric Probe	10 µL	10 µL
1X Assay Buffer	778 µL	818 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 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.

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 100-500 milligrams of 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 at 1-2 x 10^6 cells/mL in PBS. Homogenize or sonicate the cells on ice. Centrifuge to remove debris 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 undiluted or diluted as necessary into PBS.
- Serum, plasma, saliva, or urine: To remove insoluble particles, centrifuge at 10,000 x g for 10 minutes. 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 Phosphoenolpyruvate standards by diluting into 1X Assay Buffer according to Table 1.

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Standard Tubes	20 mM Phosphoenolpyruvate Solution (µL)	1X Assay Buffer (µL)	Phosphoenolpyruvate (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 #5	250	3.13
8	0	250	0

Table 1. Preparation of Phosphoenolpyruvate 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 phosphoenolpyruvate 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 Phosphoenolpyruvate 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.

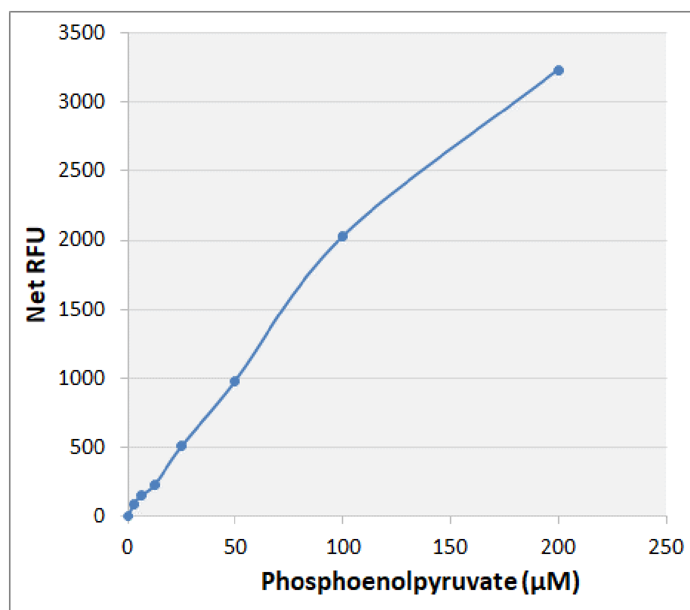

Figure 2: PEP Standard Curve.

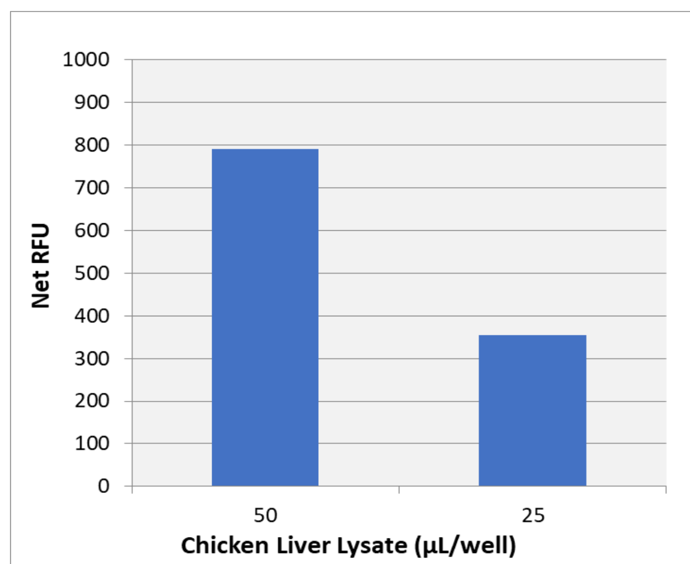

Figure 3: PEP detection in Chicken Liver using the Phosphoenolpyruvate Assay Kit. Chicken liver was homogenized and deproteinated according to the preparation of samples section above.

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

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

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

References

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4. Hopgood M, Ballard FJ, Reshef L and Hanson RW (1973) *Biochem. J*. **134**:445-453.
5. Yu S, Meng S, Xiang M, Ma H (2021) *Mol. Metabol*. **53**:1-12.

Warranty

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