MA-0165

Phosphofructokinase Activity Assay

(100 wells, Colorimetric, OD 450 nm, Store at -20°C)

Introduction:

Phosphofructokinase is a key glycolytic enzyme and plays a major regulatory role in glycolysis. PFK catalyzes the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. It is allosterically regulated by several chemicals including ATP, ADP and citrate. When energy levels are low the ADP to ATP ratio is high which turns on PFK, accelerating glycolysis. Conversely when energy levels are high ATP and citrate work to turn off PFK. Dysregulation of PFK leads to metabolic disorders. PFK has been targeted in cancer therapy since cancer cells display enhanced glycolytic activity. AkrivisBio's Phospho fructokinase Activity Assay is a simple sensitive means of quantifying PFK activity in a variety of biological samples with a sensitivity in the µUnit range.

Assay Principle:

- PFK converts fructose-6-phosphate to dfructose-1,6-bisphosphate with the conversion of ATP to ADP.
- ADP is utilized by an ADP dependent hexokinase to convert glucose to glucose-6-phosphate.
- G-6-P is oxidized by G6PDH converting NAD to NADH in the process.
- NADH is used to reduce the nearly colorless WST-8 tetrazolium to a highly colored formazan measured at 450 nm.

Assay Components:

Assay Buffer	27 ml	WM	MA-0165A
Fructose-6-phosphate	lyoph	Blue	MA-0165B
ATP	lyoph	Orange	MA-0165C
G6PDH/Hexokinase	lyoph	Green	MA-0165D
WST-8 Reagent	lyoph	Red	MA-0165E
NADH Standard	lyoph	Yellow	MA-0165F
PFK Positive Control	lyoph	Purple	MA-0165G

Storage and Handling Considerations:

Store unopened kit at -20°C. Warm all components to room temperature before use. Centrifuge all small vials for a few seconds before opening. Keep all components except the Assay Buffer on ice while in use.
Assay Buffer: Ready to use as supplied. Store at 4°C
Fructose-6-phosphate: Reconstitute with 220 µl Assay Buffer. Store at -20°C.
ATP: Reconstitute with 220 µl DI water. Store at -20°C.
G6PDH/Hexokinase: Reconstitute with 220 µl Assay Buffer. Aliquot to convenient portions and store at -20°C to avoid freeze/thaw cycles.
WST-8 Reagent: Reconstitute with 220 µl DI water. Store at -20°C.
NADH Standard: Reconstitute with 400 µl DI water. Gives a 0.4 mM Standard solution. Ready to use, requires no further dilution. Store at –20°C.
PFK Positive Control: Reconstitute with 100 µl Assay Buffer. Aliquot into 5 portions and store at –20°C.

Phosphofructokinase Activity Assay Protocol:

1. Turn on the plate reader and set the temperature to 37°C.

2. NADH Standard Curve: Transfer 0 – 5 – 10 – 15 – 20 – 25 µl of the 0.4 mM NADH Standard to a series of wells in a 96-well plate, giving 0, 2.5, 5, 7.5, 10, 12.5 nmol of NADH Standard respectively. Adjust all well volumes to 50 µl with Assay Buffer.

3. Sample Preparation: Homogenize tissue (10 mg) or cells (10^6) with 100 µl ice cold Assay Buffer on ice. Centrifuge at 16,000 X g for 5 minutes at 4°C. Transfer the clear supernatant to a fresh tube. Transfer up to 50 µl of each sample to a 96-well plate. Adjust all well volumes to 50 µl with Assay Buffer. Prepare a parallel sample well as background control to avoid interference from ADP and NADH in the sample.
Note: Some samples give a significant matrix effect. If there appears to be very low enzyme activity, 10kDa MWCO centrifugal filters can be used to remove the interference. Pre-wet the column with DI water to remove any glycerol used to store the filters, spin at 10,000 x g for 2 min and discard the water from both upper and lower compartments. Load 100 µl of test sample and centrifuge at 10,000 x g for 10 min at 4°C, discard the filtrate and resuspend the retentate to 100 µl with Assay Buffer. Transfer the sample to the 96-well plate.

4. Positive Control: Transfer 10-20 µl of PFK Positive Control to well(s). Adjust final volume to 50 µl with Assay Buffer.

5. Initiate Reaction: Each Standard**,** Sample and Positive Control well requires 50 µl of Reaction Mix. Each Background Control well requires 50 µl of Background Control Mix. Prepare sufficient material for the total number of wells to be analyzed containing:

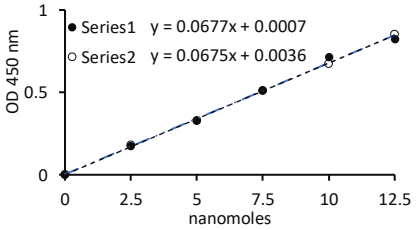
	Reaction Mix	Background Control Mix
Assay Buffer	42 µl	44 µl
G6PDH/Hexokinase	2 µl	2 µl
WST-8 Reagent	2 µl	2 µl
ATP	2 µl	2 µl
Fructose-6-phosphate	2 µl	----

Add 50 µl of the Reaction Mix to each Standard, Sample and Positive Control well and 50 µl of Background Control Mix to each Background Control well.

6. Measurement: Monitor the reaction progress in the plate reader at 37°C for up to 60 minutes at 450 nm.
Note: The Standards will reach its endpoint rather quickly (within a couple minutes). Precise measurements of enzyme activity are best performed below 1 OD. Enzyme activity will go through a lag phase for up to 15-20 minutes then enter a linear phase for a time before bending down due to substrate depletion. The linear portion of the reaction should be used for enzyme activity determination.

7. Typical Results:

nmole Standard	Standard Raw Values OD		Background Corrected Values OD	
0	0.1541	0.1544	0	0
2.5	0.3301	0.3336	0.1760	0.1792
5.0	0.4819	0.4826	0.3278	0.3282
7.5	0.6678	0.6640	0.5137	0.5096
10.0	0.8683	0.8294	0.7142	0.6750
12.5	0.9754	1.0054	0.8213	0.8510



8. Calculation: Subtract the 0 Standard reading from all Standard readings. Plot the NADH Standard Curve. Determine the slope of the Standard Curve. Subtract the Background Control values from the paired Sample readings. Determine the slope of the PFK activity in the linear region. (OD/min) Divide the slope of the PFK activity by the slope of the Standard Curve to get PFK activity in nmoles/min (mU). Divide the PFK activity by the volume of sample added to the well to get PFK/µl of sample. Multiply by 100 to get total PFK activity per sample and divide by mg tissue or # of cells to get PFK activity per mg tissue or per # of cells.

FOR RESEARCH USE ONLY! Not to be used for diagnostic or therapeutic purposes.

48511 Warm Springs Blvd. # 213, Fremont, CA 94539 +1(408)739-9315 contact@akrivisbio.com