MA-0166

Pyruvate Dehydrogenase Activity Assay

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

Introduction:

Pyruvate Dehydrogenase (PDH), a multienzyme complex, catalyzes the oxidative decarboxylation of pyruvate, producing acetyl-CoA, NADH and CO_2. PDH connects the TCA cycle and oxidative phosphorylation to glycolysis, gluconeogenesis, and lipid and amino acid metabolic pathways. The activity of PDH is regulated through reversible phosphorylation of the E1-alpha subunit at specific serine sites. Phosphorylation, catalyzed by PDH kinases 1-4, decreases PDH activity. Dephosphorylation, catalyzed by PDP1 and 2, restores PDH activity. Multiple regulatory signals control PDH activity. Dysregulation is associated with metabolic disorders like diabetes, obesity and cancer. Genetic defects in the PDH complex lead to lactic acidosis and Leigh's disease among others. AkrivisBio's Pyruvate Dehydrogenase Activity Assay is a simple, sensitive way to measure PDH activity with a sensitivity limit below 50 µU.

Assay Principle:

- PDH oxidizes pyruvate with the formation of NADH.
- NADH is used to convert the nearly colorless WST-8 tetrazolium to a highly colored formazan with a λ_{max} of 450 nm.

Assay Components:

Assay Buffer	25 ml	WM	MA-0166A
Sodium Pyruvate	lyoph	Blue	MA-0166B
WST-8 Reagent	lyoph	Red	MA-0166C
NADH Standard	lyoph	Yellow	MA-0166D
PDH Positive Control	100 µl	Orange	MA-0166E

Storage and Handling Considerations:

Store unopened assay at -20°C. Centrifuge all small vials for a few seconds prior to opening. Bring all components except PDH Positive Control to room temperature before using them. Always keep PDH Positive Control on ice.
Assay Buffer: Ready to use as supplied. Store at 4ºC.
Sodium Pyruvate: Reconstitute with 220 µl DI water. Keep solution on ice while in use. Store at -20°C.
WST-8 Reagent: Reconstitute with 220 µl DI water. Let stand for 2-3 minutes then triturate briefly to complete dissolution. Store at -20°C.
NADH Standard: Reconstitute with 400 µl dH_2O giving an 0.5 mM NADH Standard solution. Aliquot and store at –20°C. Keep on ice while in use.
PDH Positive Control: Ready to use as supplied. Aliquot to convenient portions and store at –20°C. Keep on ice at all times.

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 NADH Standard to a series of wells in a 96-well plate, giving 0, 2.5, 5, 7.5, 10, 12.5 nmol NADH Standard, respectively. Adjust all well volumes to 50 µl with Assay Buffer.

3. Sample Preparation: Homogenize tissue (10 mg) or cells (10^6) using 100 µl ice cold Assay Buffer. Let stand on ice for 10 minutes. Centrifuge at 16,000 x g, 5 minutes at 4°C. Transfer the clear supernatant to a fresh tube. Use up to 50 µl of sample per well. Adjust all well volumes to 50 µl with Assay Buffer. Alternatively, isolated mitochondria, prepared using AkrivisBio's PI-0105 Mitochondria/mt-DNA Isolation Kit, may be used as a sample.

4. PDH positive control: Take 1-2 µl of PDH Positive Control and dilute 10X with Assay Buffer. Transfer 2-10 µl of the diluted Positive Control to the a 96-well plate and adjust the well volume to 50 µl with Assay Buffer.

Note:

a. Some samples contain substances which interfere with the assay. An elevated but constant background can be ignored as measuring the rate of change of absorbance is the goal. If an inhibitory or stimulatory effect is suspected, run each sample in duplicate, adding a small amount (2 µl) of Positive Control to one of the pair. The difference in rate between the paired samples should be identical to the same amount of positive control alone. If there is a difference, determine the proportionality factor (PC rate in sample/PC rate alone) to correct the sample rate.

b. Enzymes in samples other than PDH can reduce NAD to NADH, giving a background reaction rate. Run samples in duplicate with one of the pair used as a background control to correct for this.

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

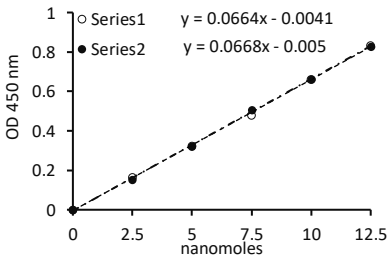
	Reaction Mix	Background Control Mix
Assay Buffer	46 µl	48 µl
WST-8 Reagent	2 µl	2 µl
Sodium Pyruvate	2 µl	----

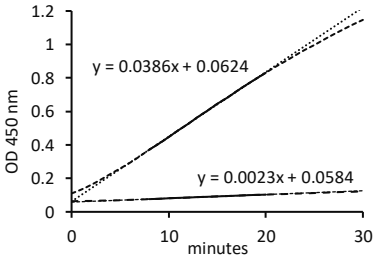
Add 50 µl of Reaction Mix to the Standard, Sample and Positive Control wells. Add 50 µl of Background Control Mix to Background Control wells.

6. Measurement: Monitor absorbance at 450 nm kinetically at 37°C for 0.25 -2 hours (until a sufficient linear reaction rate has been established). The reaction profile is typically sigmoid with an early lag phase lasting ~ 10 minutes and a late slowing down of the reaction rate due to substrate depletion. The middle linear phase is used for activity determinations.

7. Typical Results:

nmole Standard	Standard Raw Values OD		Background Corrected Values OD	
0	0.0879	0.0914	0	0
2.5	0.2534	0.2465	0.1655	0.1551
5	0.4130	0.4143	0.3251	0.3229
7.5	0.5678	0.5980	0.4799	0.5066
10	0.7500	0.7550	0.6621	0.6636
12.5	0.9212	0.9191	0.8333	0.8277





8. Calculations: Subtract the 0 Standard reading from all standard readings. Plot the NADH Standard Curve. Determine the slope of the Standard Curve. Determine the slope of the linear portion of all Samples, Positive Controls and Background Controls. Subtract the slope of any Background Control from its paired Sample slope. Divide the background corrected Sample slope by the slope of the Standard Curve to convert from OD/minute to nanomoles/minute (mU) in the well. To convert back to activity in the original sample:
A – Divide the well activity by the volume of sample (in µl) added to the well = activity (mU) per µl of sample
B – Multiply the activity per µl of sample X the volume of supernatant recovered in step 3 above = total activity per sample
C – Divide the total activity per sample by the mg tissue used (or # of cells, etc.) = mU per mg tissue (or per # of cells, etc.)

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