

Pyruvate Dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit

MSP18

Rev.3.2

DESCRIPTION

Pyruvate Dehydrogenase (PDH) Enzyme Activity Microplate Assay Kit

Kit Contents:

Included in this kit is the necessary buffer and detergent for sample preparation, and reagents for the enzyme reaction. The kit contains a 96-well microplate with an anti-PDH monoclonal antibody pre-bound to the wells of the microplate. This plate can be broken into 12 separate 8-well strips for convenience; therefore the plate can be used for up to 12 separate experiments.

Item	Amount	Storage
20X Buffer	15 mL	4°C
Detergent	2 x 1 mL	4°C
20X Reagent Mix	2 x 0.6 mL	-80°C
5X Stabilizer	13 mL	-20°C
100X Coupler	0.25 mL	-80°C
100X Reagent Dye	0.25 mL	-20°C
96-well microplate (12 strips)	1	4°C

NOTE: Avoid freeze-thaw cycles of frozen components by making appropriate aliquots if the entire plate is not run in a single 96-well experiment.

INTRODUCTION

The Pyruvate Dehydrogenase Enzyme Activity Microplate Assay Kit (MSP18) is used to determine the activity of pyruvate dehydrogenase (PDH) in a sample. The PDH enzyme is immunocaptured within the wells of the microplate and PDH activity is determined by following the reduction of NAD⁺ to NADH, coupled to the reduction of a reporter dye to yield a colored (yellow) reaction product whose concentration can be monitored by measuring the increase in absorbance at 450 nm (Figure 1).

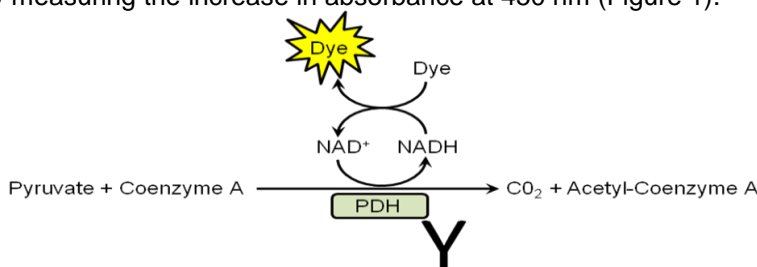


Figure 1. PDH activity assay reaction scheme

As described in the MitoSciences PDH Playbook (see http://www.mitosciences.com/pdh_playbook.pdf), PDH is the key regulatory enzyme of cellular metabolism because it links the TCA cycle and subsequent oxidative phosphorylation with glycolysis and gluconeogenesis as well as with both lipid and amino acid

metabolism. PDH activity is regulated by PDK-dependent phosphorylation and PDP-dependent dephosphorylation of PDH. Phosphorylation inactivates PDH whereas dephosphorylation activates PDH.

The PDH activity assay kit MSP18 can be used to elucidate various aspects of PDH activity, physiologic regulation and phosphorylation status. The MitoSciences PDH Playbook has details regarding these applications, but three are described briefly below:

- A. Specialized sample preparation.** PDH activity in cells and tissues is regulated by reversible phosphorylation (PDH inhibition) and dephosphorylation (activation) caused by endogenous PDH kinases and PDH phosphatases. MSP18 does not include PDH kinase or PDH phosphatase inhibitors. However, these reagents may be incorporated into the sample preparation and/or sample dilution buffers at the researcher's discretion to preserve the ENDOGENOUS phosphorylation and activity status of PDH during cell and tissue solubilization. See the MitoSciences PDH Playbook and MitoSciences PDH Protocols #1 and #2 for details.
- B. Reagents to manipulate the phosphorylation status and activity of PDH AFTER immunocapture.** Exogenous PDH Kinases and PDH phosphatases can be used to manipulate the phosphorylation status and activity of **immunocaptured** PDH. For example, post-capture treatment of immunocaptured PDH with appropriate amounts of PDP1 or PDP2 will fully dephosphorylate the enzyme, resulting in maximum enzyme activity and thus allow measurement of TOTAL PDH activity in a sample. A comparison of TOTAL and ENDOGENOUS PDH activity in a sample allows one to calculate the amount (%) of PDH activity that is inhibited by phosphorylation at the time of immunocapture. Note that PDH activity can also be inhibited by other factors, such as oxidative stress, that are not affected by phosphorylation state. MitoSciences offers a complete selection of recombinant human PDKs and PDPs that can be used in conjunction with MSP18. See the MitoSciences PDH Playbook and MitoSciences PDH Protocols #1 and #2 for details.

*MSP41	PDH Kinase-1 (PDK1), recombinant human
*MSP42	PDH Kinase-1 (PDK2), recombinant human
*MSP43	PDH Kinase-1 (PDK3), recombinant human
*MSP44	PDH Kinase-1 (PDK4), recombinant human
*MSP45	PDH Phosphatase-1 (PDP1), recombinant human
*MSP46	PDH Phosphatase-2 (PDP2), recombinant human

- C. High-Throughput Screening of PDK Kinase inhibitors.** The PDKs regulate PDH activity under normal physiologic conditions, such as in response to exercise and diet, but also play a major role in dysregulation of metabolism in disease such as diabetes and cancer. The MSP18 PDH activity capture assay can be used in conjunction with each of the specific PDKs to aid identification of drugs that regulate PDH kinase activities, namely in development of specific inhibitors of the different PDH kinases as potential treatments for cancer, diabetes, or to identify off-target inhibition of PDKs by kinases directed at other targets. See the MitoSciences PDH Playbook for details.

Additional PDH application resources:

MitoSciences PDH Playbook:

http://www.mitosciences.com/PDF/pyruvate_dehydrogenase_playbook.pdf

MitoSciences PDH Protocols:

http://www.mitosciences.com/pdh_protocols.html

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance at 450 nm
- Deionized water
- Multichannel Pipetting devices
- Protein assay method
- Phosphate buffered saline solution (PBS)
 - 1.4 mM KH_2PO_4
 - 8.0 mM Na_2HPO_4
 - 140 mM NaCl
 - 2.7 mM KCl, pH 7.3

MICROPLATE ASSAY PROTOCOL

The protocol has 4 steps:

- A) Sample preparation
- B) Plate loading
- C) Measurement
- D) Data Analysis

A. Sample Preparation

This PDH enzyme activity assay has been developed for use with human samples. Bovine, mouse, or rat materials are also compatible. Other species have not been tested. Suitable samples include mitochondria, whole tissue extracts and whole cultured cell extracts.

The protein concentration of the sample must be measured prior to sample solubilization. Once diluted to the specified concentration the sample is detergent solubilized and diluted to within the linear range of measurement. A control or normal sample should always be included in the assay as a reference measurement. In addition, a buffer control should be used as a negative control.

1. Mitochondria and whole tissues should be homogenized in PBS, while cultured cell pellets should be suspended in PBS. The protein concentration should then be determined using a standard method such as the BCA method (Pierce). Then, use PBS to adjust the sample concentrations as follows:

5.3 mg/mL for mitochondria

23.7 mg/mL for tissue homogenates

15 mg/mL for cultured cells

(Approximate numbers of cells/mg protein are given in the frequently asked questions section).

2. Solubilize intact, functional PDH by adding Detergent to the sample as described below.

Component	Purified mitochondria at 5.3 mg/mL	Tissue homogenates at 23.7 mg/mL	Cultured cells at 15 mg/mL
Sample	19 volumes	19 volumes	9 volumes
Detergent	1 volume	1 volume	1 volume
Final Protein Concentration (mg/mL)	5.0	22.5	13.5

3. Incubate on ice for 10 minutes.
4. Centrifuge in a tabletop centrifuge for 10 minutes at 4°C as specified below. Carefully collect and save the supernatant. Discard the pellet.

Sample type	RCF (x g)
Purified mitochondria	5,000
Tissue homogenates	1,000
Cultured cells	1,000

5. Add 10 mL of 20X Buffer to 190 mL deionized H₂O. Label this mixture as 1X Buffer.
6. Dilute all samples to the desired concentration in 1X Buffer. The table below shows the linear working range for the assay using various samples. The working range for your sample set should be confirmed by testing a representative reference control sample at a series of dilutions across the expected working range. Results from individual experimental samples can then be compared directly when tested at concentrations within the working range.

Recommended sample dilutions:

Mitochondria extracts	10-100µg / 200 µL/well
Whole tissue extracts	20-100 µg / 200 µL/well
Cultured cell extracts	100-1000 µg / 200 µL/well

Typical intra-assay variations (same day, same sample) <10%

B. Plate Loading

1. Add 200 µL of solubilized, diluted sample prepared in step A6 to each well of the microplate that will be used for this experiment. Be sure to include a normal or control sample as a positive control and a buffer control as a negative control.
2. Incubate microplate for 3 hours at room temperature.

C. Measurement

1. Prepare 1X Stabilizer by mixing 1 volume of thawed 5X Stabilizer with 4 volumes of 1X Buffer.
2. Prepare "Assay Solution" as described in the table below. Warm to room temperature. NOTE: Thaw 20X Reagent Mix immediately before use. If the 20X Reagent Mix is to be used in multiple experiments with fewer than 12 strips/experiment, aliquot and store unused 20X Reagent Mix at -80°C immediately after thawing.

No. of Strips	20X Reagent Mix (μL)	1X Buffer (mL)	100X Coupler (μL)	100X Reagent Dye (μL)
1	88	1.63	18	18
2	175	3.25	35	35
3	263	4.89	53	53
4	350	6.51	70	70
5	438	8.14	87	87
6	525	9.77	105	105
7	612	11.39	123	123
8	700	13.02	140	140
9	788	14.65	158	158
10	875	16.28	175	175
11	963	17.90	192	192
12	1050	19.53	210	210

3. Empty the wells of the microplate and to each well add 300 μL of 1X Stabilizer.
4. Again, empty the wells of the microplate and to each well add 300 μL of 1X Stabilizer.
5. Empty the wells again.
6. Add 200 μL of Assay Solution to each well carefully, to avoid bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.
7. Measure the absorbance of each well at 450 nm at room temperature using a kinetic program for approximately 15 minutes. The interval between readings should be as short as your reader allows but not longer than 1 minute between reads. Incorporate a shake step between reads if possible.

Data Analysis

PDH activity is expressed as the initial rate of reaction, determined from the slopes of the curves generated.

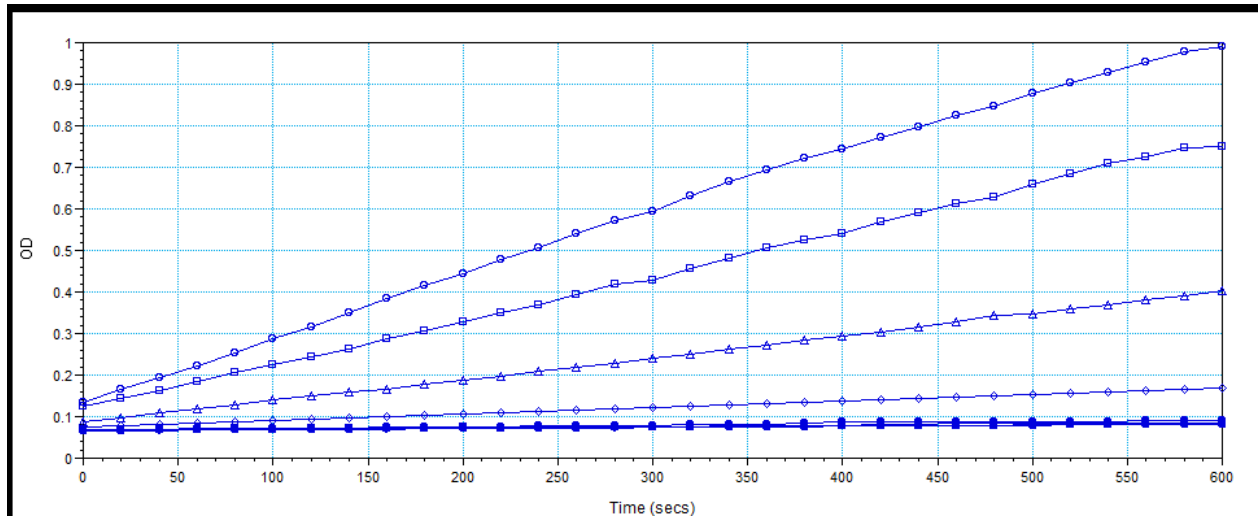


Figure 2. Example of microplate reader recorded data. Bovine heart mitochondria were loaded at 100 $\mu\text{g}/\text{well}$ (top trace) and 2-fold dilutions (stepwise lower traces). Activity should always be related to a control or normal sample to obtain the relative activity of PDH in experimental samples.

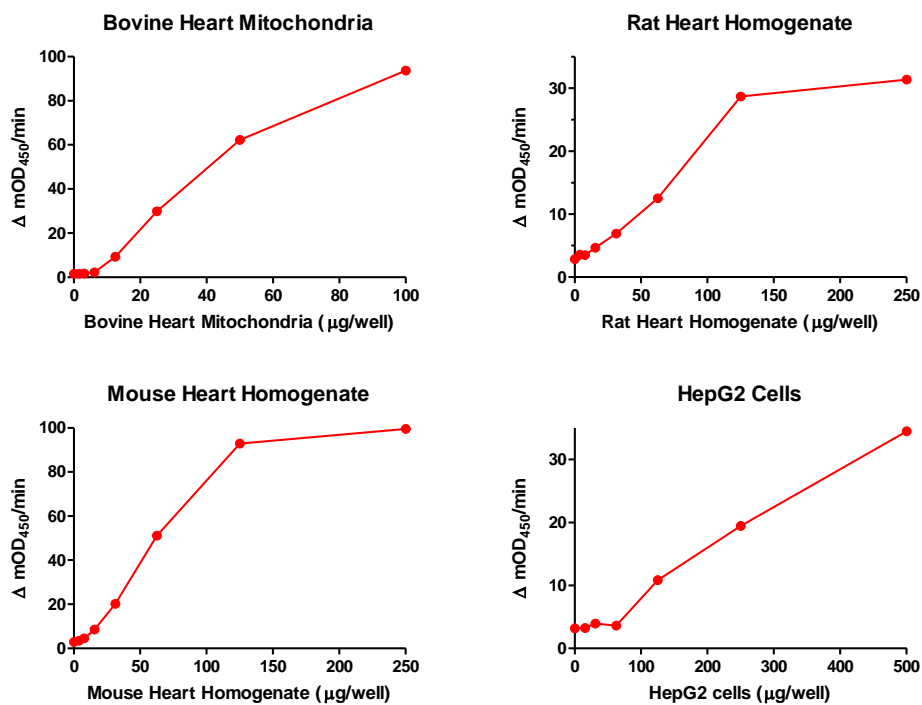
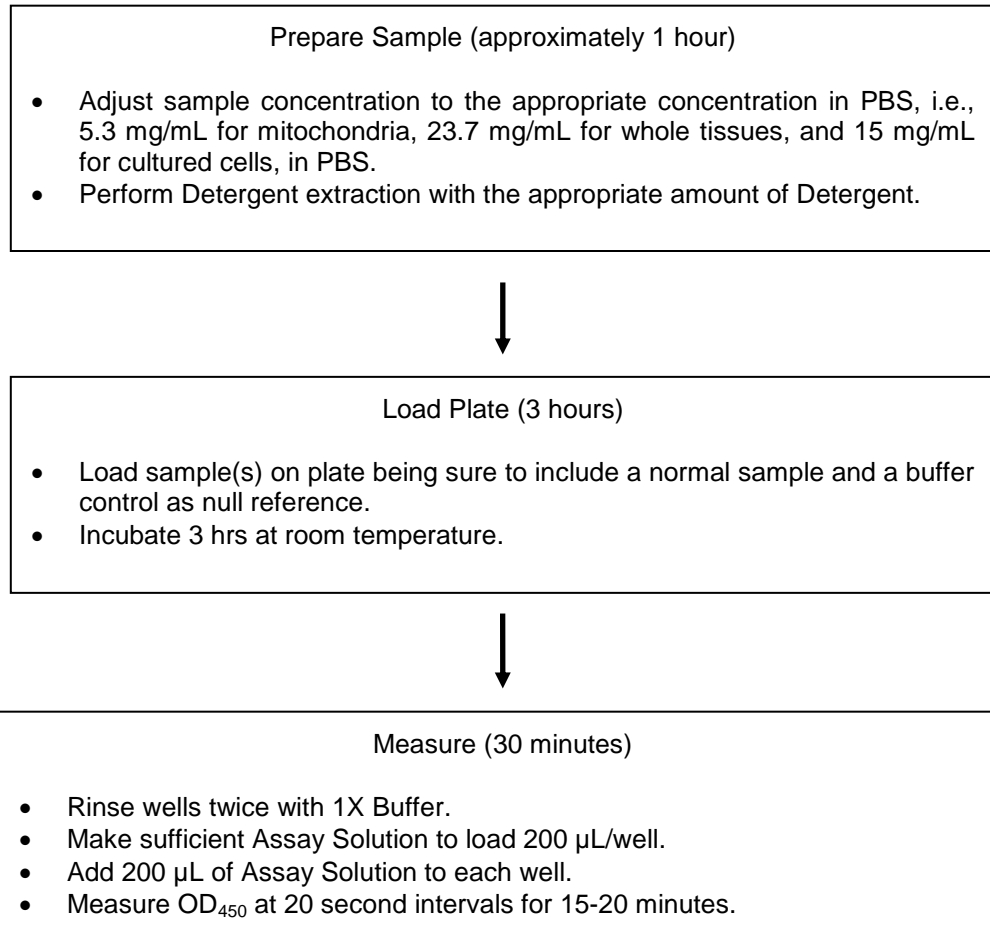


Figure 3. Mitochondria, tissue extracts and whole cultured cell extracts all show linear relationships between signal and sample load at limiting concentrations. The rates shown were determined as change in OD over time, and these are best represented as change in milliOD per minute.

FLOW CHART

(For quick reference only. Be completely familiar with previous details of this document before performing the assay.)



Frequently asked questions

How should I store my reagent mix?

Reagent mix is temperature sensitive. It must be stored at -80°C and is stable for at least six months from date of receipt. If the reagent mix is to be used in multiple experiments with fewer than 12 strips/experiment, aliquot and store unused reagent mix at -80°C immediately after thawing.

How should I aliquot my reagent mix?

Divide the reagent mix into equal aliquots depending on how many experiments you wish to run. The plate comes in 12 strips, therefore it is anticipated that up to 12 experiments on different days could be done. The reagent mix is supplied as 2 x 0.5 mL aliquots, thus for 12 independent experiments each tube of the reagent mix can further be divided into 6 x 83 μL aliquots and immediately frozen at -80°C for storage until use.

How do I optimize sample solubilization?

Mitochondria have two membrane bilayers which must be dissolved by detergent before PDH can be isolated. PDH is a huge, extremely complex, multi-subunit enzyme which is sensitive to high detergent concentrations. Therefore, the correct detergent : protein ratio must be used to efficiently solubilize the mitochondrial outer and inner membranes while maintaining PDH integrity. This kit suggests optimized ratio for specific sample types. However, this ratio may need to be adjusted from sample to sample, particularly when contaminated with excess extracellular proteins such as serum. Therefore, it may be necessary to adjust the concentration of detergent or isolated mitochondria.

How do I isolate mitochondria?

We have found that little or no optimization is necessary if crude mitochondria are isolated from samples. Mitochondria can be prepared by simple differential centrifugation of homogenized samples as described in the protocols accompanying our mitochondria isolation kits MS850, MS851, MS852 and MS853 (http://www.mitosciences.com/mitochondria_isolation_kits.html).

How do I grow and prepare cultured cell samples?

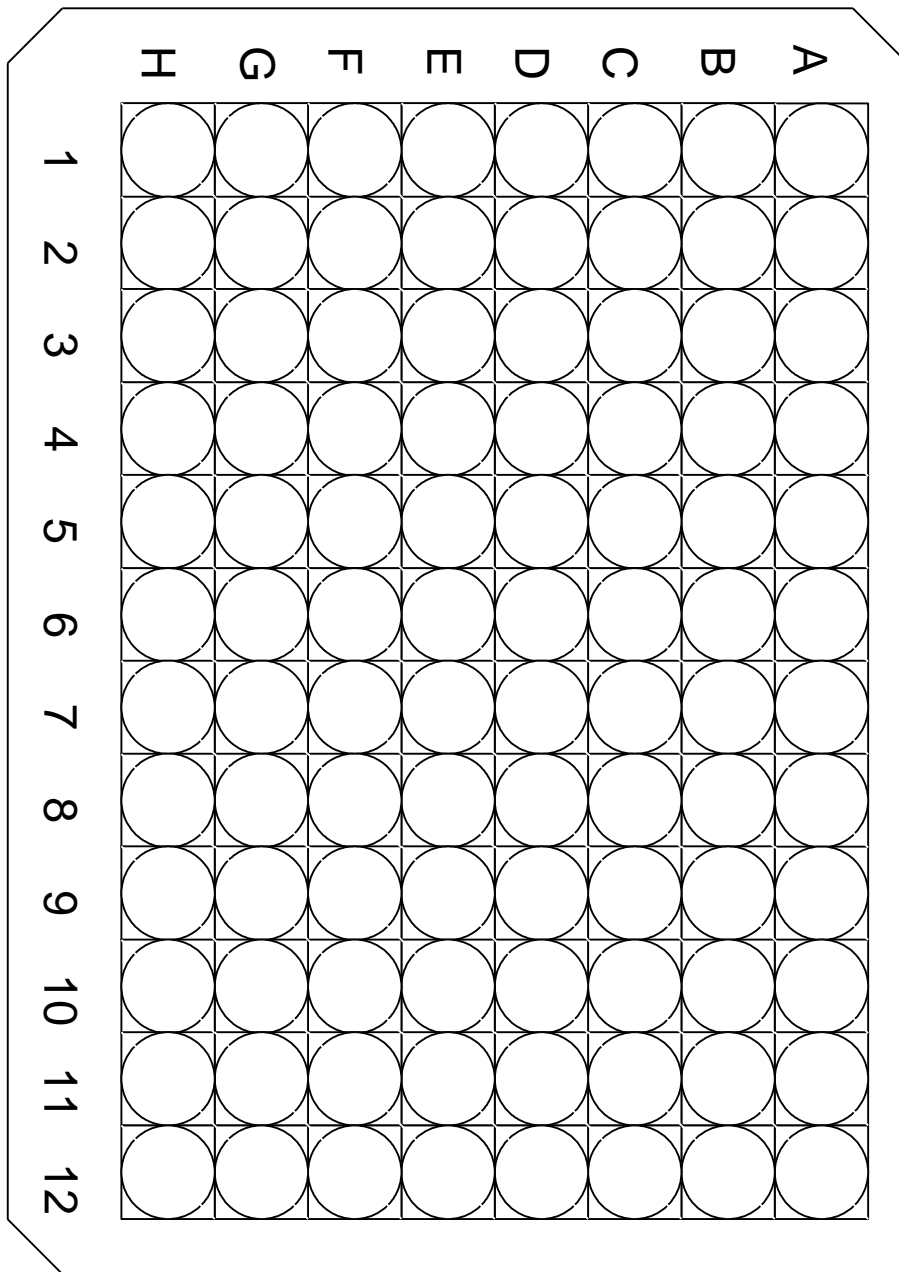
PDH activity in cells from different origins differs greatly. Cells grown in glucose have a lower activity than those grown in galactose/glutamine. Consequently, cell type and growth conditions are a large factor in PDH activity measured.

Approximately how much protein is yielded from my plate of cells?

We find the following typical yield of cells from a single confluent 177 cm^2 plate:

Human fibroblasts	1 x 10 ⁷ cells	1.5 mg total protein
Human HepG2	2 x 10 ⁷ cells	3 mg total protein

It is recommended to accurately determine the number of cells and the total protein yield from an initial confluent plate.



MICROPLATE MS _____ / /