

Pyruvate Dehydrogenase (PDH) Enzyme Activity Dipstick Assay Kit

MSP30

Rev.2

DESCRIPTION

Pyruvate Dehydrogenase (PDH) Enzyme Activity Dipstick Assay Kit

Sufficient materials are provided for 30 (MSP30-30) or 60 (MSP30-90) measurements.

Kit Contents:

Item	MSP30-30	MSP30-90
Dipsticks	30	90
Buffer 1 (Sample buffer)	30 mL	90 mL
Buffer 2 (Blocking buffer)	2 mL	6 mL
Detergent	1 mL	3 mL
Reagent Mix 1 (Enzyme substrate mix)	0.5 mL	1.5 mL
Reagent Mix 2 (Diaphorase)	0.4 mL	1.2 mL
Reagent Mix 3 (NBT)	40 µL	120 µL
96-well microplate	1	3

Storage:

Store dipsticks at room temperature out of direct sunlight in their provided desiccant container. Avoid high humidity conditions. Store Buffer 1 and Buffer 2 at 4°C. Store Reagent Mix 1 and 2 at -80°C, but these may be aliquoted upon receipt to prevent freeze/thaw cycles. Store Reagent Mix 3 in the dark at -80°C.

INTRODUCTION

The PDH (pyruvate dehydrogenase) Enzyme Activity Dipstick Assay Kit is used to quantify the activity of the PDH enzyme complex. This is accomplished by immunocapturing PDH with an anti-PDH antibody immobilized on a defined section of a dipstick. The enzyme complex is immunocaptured in its native form and activity is measured by the reaction scheme as illustrated in Figure 1. PDH activity is visualized by coupling PDH-dependent production of NADH to the reduction of NBT in the presence of excess diaphorase, forming an insoluble intensely colored precipitate at the capture line. The signal intensity is measured by a dipstick reader (MitoSciences' MS1000 Dipstick Reader) or analyzed by other imaging systems such as a flatbed scanner.

Purified mitochondria can be used in this assay. However, homogenized tissue and whole cells can also be used without the need for mitochondrial isolation.

Pyruvate dehydrogenase activity is regulated by PDH kinase and PDH phosphatase. Cells grown in glucose media derive most of their energy from glycolysis; therefore, most of their PDH complex may be in an inactive phosphorylated form at the time of isolation. Growing cells in alternate carbon sources such as glutamine/galactose may up-regulate PDH activity. This kit does not include PDH kinases, PDH phosphatases or their respective inhibitors. These may be incorporated into the assay at the user's discretion.

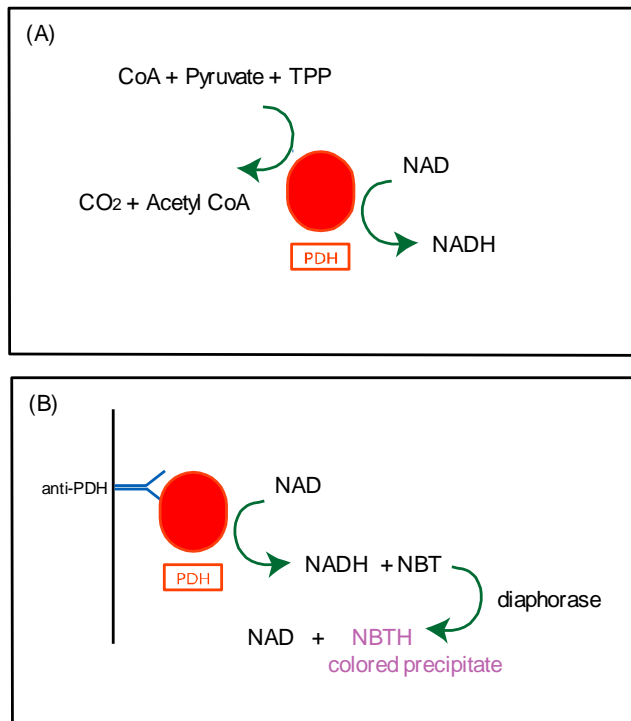


Figure 1: Illustration of the PDH activity reaction. (A) The PDH activity reaction generates NADH. (B) The NADH produced is coupled to the reduction and precipitation of a colored dye.

ADDITIONAL MATERIALS REQUIRED

- Dipstick reader (see MitoSciences' MS1000 Dipstick Reader) or other imaging system
- Method for determining protein concentration
- Pipetting devices
- Protease inhibitors

DIPSTICK ASSAY PROTOCOL

A. Sample Preparation

The preparation of the sample is a critical step of this procedure. The effective solubilization of the mitochondrial membranes in the sample is necessary. However, if the detergent to membrane protein ratio is too high, PDH may become destabilized. Please choose the sample preparation method that best fits your sample type. Follow each of the protocol steps carefully.

Note: *Samples must be kept on ice.*

1. Sample Preparation Method 1: Standard method for purified mitochondria and muscle or brain tissue homogenates.
 - a. Pellet the sample by centrifugation.
 - b. Re-suspend the pellet in Buffer 1 to a protein concentration of 5 mg/mL (measure using a standard protein concentration determination method).
 - c. Add 1/10 volume of supplied detergent.
 - d. Proceed directly to Part B of the Protocol or freeze samples at -80°C.
2. Sample Preparation Method 2: For soft tissue homogenates that contain low levels of PDH (e.g. liver tissue).
 - a. Homogenize tissue thoroughly using a micro-tissue grinder or homogenizer (MS851 Mitochondria Isolation Kit available from MitoSciences).
 - b. Measure the protein concentration.
 - c. Adjust protein concentration of the sample to 25 mg/mL in Buffer 1.
 - d. Add 1/10 volume of the supplied detergent.
 - e. Proceed directly to Part B of the protocol or freeze samples at -80°C.
3. Sample Preparation Method 3: For cultured cell extract containing low levels of PDH.
 - a. Pellet the cells by centrifugation.
 - b. Re-suspend the pellet in 5 volumes of Buffer 1.
 - c. Add 1/10 volume of supplied detergent.
 - d. Keep sample on ice for 10 minutes.
 - e. Centrifuge sample for 10 minutes at 3,000 rpm.
 - f. Transfer extracted supernatant to a new tube and determine the protein concentration by a standard method that is detergent compatible.
 - g. Proceed directly to Part B of the protocol or freeze samples at -80°C.

B. Dipstick Procedure

The assay is most accurate with a user established standard curve for interpolation of signal intensity. Following the protein concentration ranges as defined in Table 1, generate a standard curve using a positive control sample (see example experiment on pg. 7 for reference).

Sample Preparation Method	Sample Type	Working Range/ Sample Volume
1	Tissue mitochondria	1-10 µg/ 25 µL
1	Muscle tissue extract	10-100 µg/ 25 µL
2	Liver tissue extract	25-150 µg/ 25 µL
3	Fibroblast extract	40-250 µg/ 50 µL
3	Hepatocellular carcinoma extract	20-160 µg/ 50 µL

Table 1: Suggested working range for different sample types. Note: Cultured cells may require increased sample volume to accommodate larger sample load.

1. Dilute sample with appropriate volume of Buffer 1 to a protein concentration as per Table 1.
2. Add 25 µL or 50 µL of Buffer 2 to an empty well of the microplate.
3. To the same well, add 25 µL or 50 µL of diluted sample prepared in step 1. Mix by pipetting.
4. Gently add a dipstick to the sample mix in the microplate well. Make sure to place the thin/nitrocellulose end of the dipstick down.
5. Allow the sample to wick up onto the dipstick towards the wicking pad (thick end of dipstick). This takes 12-60 minutes depending on sample volume and viscosity. *The entire sample volume has to be absorbed by the dipstick before proceeding to the next step, but do not allow the dipstick to dry at any time during this procedure.*
6. Now, wash the dipstick by adding 40 µL of Buffer 1 to each microplate well with a dipstick once the sample mix is wicked completely.
7. Allow Buffer #1 to wick for 5-15 minutes. *Do not allow the dipstick to dry out.*
8. Prepare the Activity Buffer by combining Buffer 1, Reagent Mix 1, 2, and 3 as per Table 2.

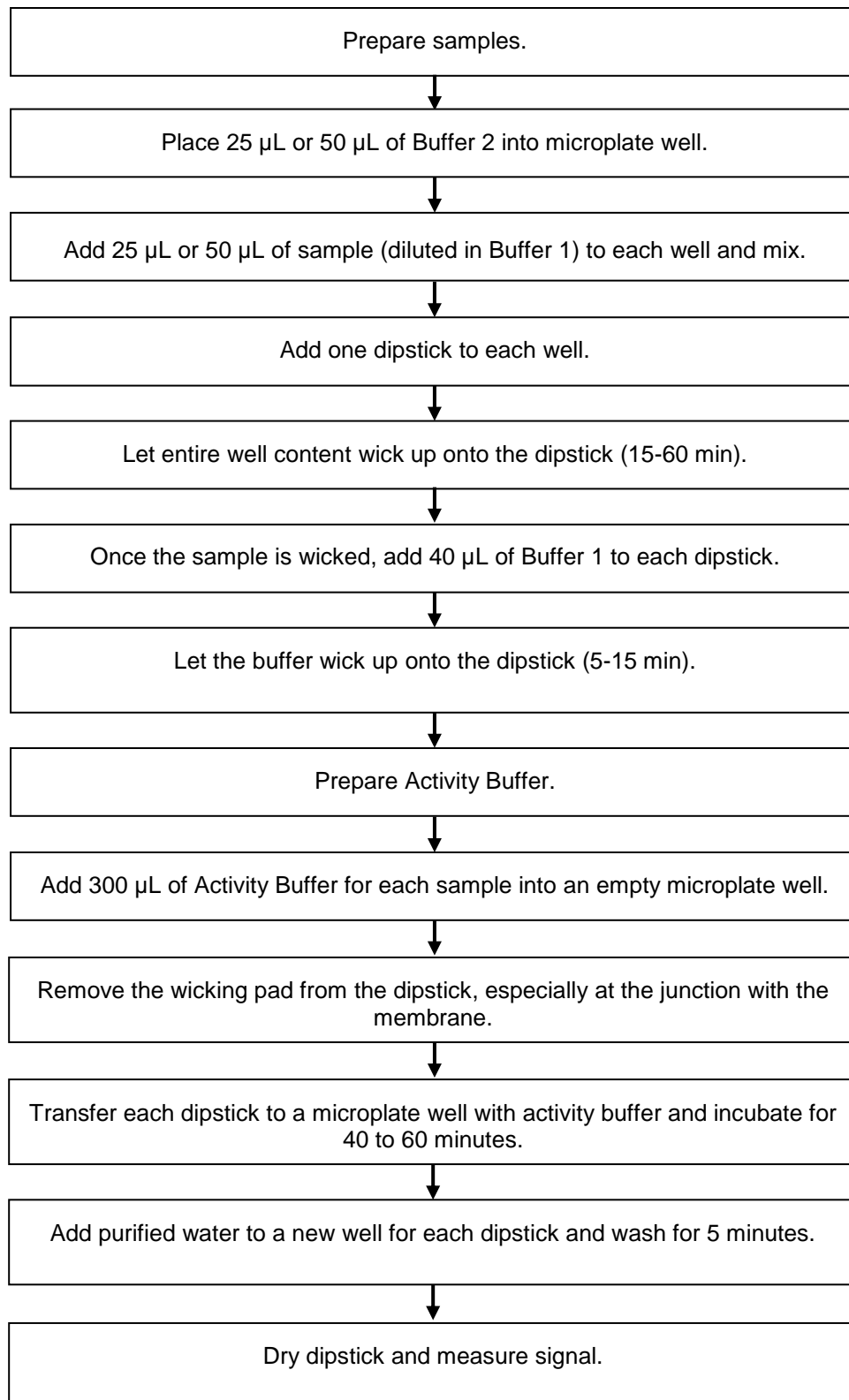
No. of Dipsticks	Buffer 1 (mL)	Reagent Mix 1 (µL)	Reagent Mix 2 (µL)	Reagent Mix 3 (µL)
10	3.3	166	133	7
20	6.6	333	266	14
30	10	500	400	20

Table 2. Preparation of Activity Buffer

9. Add 300 μ L of Activity Buffer to an empty microplate well for each dipstick.
10. Now, remove the wicking pad from the dipstick. Make sure to completely remove the pad at the junction with the membrane.
11. Place the dipstick in a well with Activity Buffer. The signal will appear 5-7 mm from the bottom of the dipstick in approximately 10-20 minutes.
12. Develop for between 40 to 60 minutes. *Since this is an end-point reaction, develop all dipsticks for the same amount of time.*
13. Add 300 μ L deionized water to an empty well of the microplate.
14. After signal development, add the dipstick to the well with deionized water. Wash for 5 minutes.
15. Dry the dipstick and measure the signal intensity with a dipstick reader (MitoSciences' MS1000 Dipstick Reader) or other imaging system, e.g. flat-bed scanner.

FLOW CHART

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

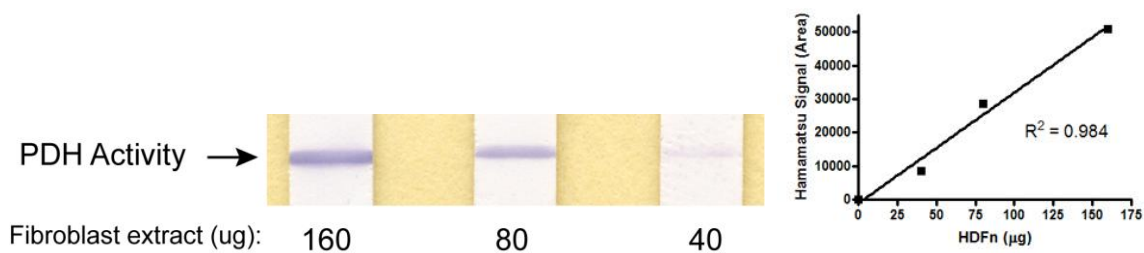


EXAMPLE EXPERIMENT

Below is an example using the MSP30 kit to measure PDH activity in human fibroblast sample. Samples were prepared as described in the Sample Preparation section (Method 3). All data were analyzed using the MS1000 Dipstick Reader and GraphPad software.

Step 1. Generating a standard curve

Shown are developed dipsticks from a 1:2 dilution series using a positive control sample and the associated standard curve. Starting material was 160 μg of fibroblast protein extract in a 100 μL reaction volume (50 μL sample in Buffer #1 plus 50 μL of Buffer 2). The dipsticks were developed for 1 hour in Activity buffer.



Step 2. Analysis of samples

Based on the standard curve, 160 μg of protein extract were loaded onto a dipstick for each sample. The figure below shows two developed dipsticks, a control sample and a sample from a patient with a mutation in the E1 α subunit of PDH. The analysis of the signal intensity and interpolation from the standard curve showed that the patient sample had 8% the PDH activity of the control.



TROUBLESHOOTING GUIDE

Signal is saturated

It is very important that the amount of sample used is within the working range of the assay (use a best fit line for interpolation). For this reason, it is crucial to determine the working range for your sample type and avoid the region of signal saturation.

Signal is too weak

This occurs when the sample lacks measurable amounts of the protein. Increase the signal by adding more sample protein to another dipstick, or leave the dipstick in the activity solution for longer to maximize the signal