

# Pyruvate Dehydrogenase (PDH) Protein Quantity Microplate Assay Kit

**MSP19**

Rev.5

## DESCRIPTION

### Pyruvate Dehydrogenase (PDH) Protein Quantity Microplate Assay Kit

#### Kit Contents:

Detergent and buffer to solubilize PDH from mitochondria, whole tissue homogenates or cultured cells, a microplate with wells pre-coated with a PDH binding antibody, antibodies and enzyme label to detect PDH immunocaptured in the microwells, and a reaction substrate/buffer to quantitate levels of bound antibody (PDH). The kit contains enough material to perform 96 tests. Since the plate is arranged as 12 strips of 8 wells, up to 12 separate experiments can be performed.

Part Number	Item Description	Amount	Storage
8209001	20X Buffer	20 mL	4°C
8201088	Detergent	1 mL	4°C
8209203	10X Blocking Buffer	10 mL	4°C
8209703	5X Stabilizer	13 mL	-20°C
8209003	20X Detector Antibody	1 mL	4°C
8203026	20X HRP Label	1 mL	4°C
8203015	1X HRP Development Solution	20 mL	4°C
8209200	96-well microplate (12 strips)	1	4°C

When stored as recommended the kit is stable for 6 months.

## INTRODUCTION

The Pyruvate Dehydrogenase (PDH) Protein Quantity Microplate Assay Kit (MSP19) can be used to determine the amount of PDH protein in a sample. This assay is a 'sandwich' ELISA, where the PDH enzyme is purified and immobilized by an anti-PDH capture antibody pre-coated in the microplate wells. The amount of captured PDH is determined by adding a second (detector) anti-PDH antibody which binds to the captured PDH, followed by binding of an HRP conjugated goat anti-mouse antibody that binds the detector anti-PDH antibody. The detector-bound HRP then changes the colorless HRP development solution to blue and the color intensity (absorbance) is proportional to the amount of PDH captured.

The Pyruvate Dehydrogenase (PDH) Protein Quantity Microplate Assay Kit (MSP19) can also be used as the basis for additional sandwich assays using alternative detector mAbs specific for certain phosphoserine residues on PDH that are reversibly phosphorylated/dephosphorylated to modify PDH activity in response to metabolic demands. See section on **ALTERNATIVE DETECTOR ANTIBODIES**.

This PDH Protein Quantity Assay has been developed for use with human samples but bovine, mouse, and rat materials are also compatible. Other species have not been tested. Importantly, it is suitable for use with whole tissue or cell lysates without the need for mitochondrial isolation.

Table 1. Typical ranges of measurement.

Tissue extracts	0.5 - 25 µg / 200 µL
Cultured cell extracts <sup>†</sup>	0.5 - 50 µg / 200 µL

Typical intra-assay variation (same day, same sample) <15%

<sup>†</sup> Mitochondrial PDH quantity is controlled by cellular metabolism. Consequently, cells with different metabolic requirements, such as those derived from different tissues, vary widely in their PDH amount. Additionally, cells of the same kind but cultured in different growth conditions show similar effects. For example, cells grown in glucose-rich media derive most of their energy by glycolysis. Cells grown in carbon sources which promote oxidative phosphorylation (such as galactose/glutamine), upregulate mitochondrial enzymes, including PDH. Ultimately, the cell type and growth conditions must be chosen carefully to obtain PDH quantity measurements.

## ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance at 600 nm Deionized water
- Multichannel Pipetting devices
- Protein assay method
- Phosphate buffered saline (PBS) – for recipe see [www.mitosciences.com/PDF/western.pdf](http://www.mitosciences.com/PDF/western.pdf)
- Optional for 450 nm endpoint data measurement – 1 N HCl

## ALTERNATIVE DETECTOR ANTIBODIES

- Phospho-PDH Ser<sup>293</sup> (Site 1) polyclonal antibody (EMD Chemicals catalog #AP1062)
- Phospho-PDH Ser<sup>300</sup> (Site 2) polyclonal antibody (EMD Chemicals catalog #AP1064)
- Phospho-PDH Ser<sup>232</sup> (Site 3) polyclonal antibody (EMD Chemicals catalog #AP1063)

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 primarily by PDK-dependent phosphorylation and PDP-dependent dephosphorylation of PDH. Phosphorylation inactivates PDH whereas dephosphorylation activates PDH. Phosphorylation occurs at Serines 232, 293, and 300 of the human E1 $\alpha$  subunits.

MitoSciences also offers a comprehensive line of PDH-related assays and reagents that can be used in conjunction with MSP19 to elucidate various aspects of PDH activity, physiologic regulation and phosphorylation status. These include all four PDH kinases, both PDH phosphatases, PDH activity microplate assays and PDH protein quantity microplate assays. For convenience, these tools are available combined in several kits and described in additional protocols.

The MitoSciences PDH Playbook (see [http://www.mitosciences.com/pdh\\_playbook.pdf](http://www.mitosciences.com/pdh_playbook.pdf)) has details and protocols regarding these applications. The three alternative phospho-serine detector antibodies listed above can be employed easily with kit MSP19 simply by replacing the “PDH detector mAb” in Step B4 with one of the Phospho-PDH Serine specific antibodies in wells selected for phospho-site detection. Because the phospho-site specific antibodies are of rabbit origin, and not mouse, it is also necessary to replace the HRP-goat-anti-mouse secondary antibody normally employed in Step B6 with an appropriate HRP-goat-anti-rabbit antibody in each well selected for phospho-site detection (we recommend using Southern Biotech cat # 4050-05 at 0.5 µg/mL). As noted in the “Sample Preparation” section, particular

care must be taken to preserve the endogenous phosphorylation state during sample preparation when using the phospho-site-specific alternative detector antibodies.

**Representative data using these alternative detectors is shown in the Appendix to this protocol.**

The MitoSciences PDH Playbook has additional information and protocols regarding applications in which *Pyruvate Dehydrogenase (PDH) Protein Quantity Microplate Assay Kit (MSP19)* can be used with these and other reagents and should be consulted for details.

[http://www.mitosciences.com/pdh\\_playbook.pdf](http://www.mitosciences.com/pdh_playbook.pdf)

## MICROPLATE ASSAY PROTOCOL

### A. Sample Preparation

The protein concentration of the sample should be measured before 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 positive control measurement. In addition, a buffer control should be used as a negative control.

NOTE: If phospho-serine detector antibodies are used in place of the standard PDH detector mAb, it is critical to inhibit the endogenous PDH phosphatases and kinases during sample preparation and immunocapture to ensure the phosphorylation status of the sample does not change during processing. Methods to do so can be found in the detailed protocols appended to the MitoSciences PDH Playbook, and at [http://www.mitosciences.com/pdh\\_protocols.html](http://www.mitosciences.com/pdh_protocols.html).

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 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 samples 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 15 mL of 20X Buffer to 285 mL deionized H<sub>2</sub>O. Label this mixture as **1X Buffer**.
6. Prepare “**Incubation Solution**” by mixing 1 part 10X Blocking Buffer with 9 parts 1X Buffer (the total volume of Incubation Solution needed per experiment depends on the number of wells to be used in the experiment at hand).
7. Dilute all samples to the desired concentration in Incubation Solution. Table 1 (page 2) shows the 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.

## B. Plate Loading and Assay Steps

1. Load wells at 200 µL per well with samples prepared in Section A7. Include a control (normal) sample as a positive control. Also include a buffer control (200 µL Incubation Solution without sample) as a null or background reference.
2. Cover/seal the plate and incubate for 3 hours at room temperature.
  - a. During this time prepare the 1X Detector Antibody by mixing 1 part 20X Detector Antibody with 19 parts Incubation Solution.
  - b. Also prepare 1X Stabilizer by mixing 1 part 5X Stabilizer with 4 parts 1X Buffer.
3. Wash the plate
  - a. Empty the wells by turning the plate over a receptacle and firmly shaking out the well contents in one rapid downward motion.
  - b. Rapidly add 300 µL 1X Stabilizer to each well. The wells must not become dry during any step. **Repeat this wash once more for a total of two washes in 1X Stabilizer.** After the last wash strike the microplate surface onto paper towels to remove excess liquid.
4. Add 200 µL of 1X Detector Antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature.
  - a. During this time prepare 1X HRP label by mixing 1 part 20X HRP Label with 19 parts Incubation Solution
5. Repeat the wash procedure in step B3 except this time use 1X Buffer (without Stabilizer) and do **a total of two washes in 1X Buffer**.
6. Add 200 µL of 1X HRP Label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. Meanwhile prepare the microplate spectrophotometer using the parameters described below.
  - a. During this time, allow the HRP Development solution to warm to room temperature.

7. Repeat the wash procedure in step B5, but perform **a total of three washes with 1X Buffer**.
8. Rapidly add 200  $\mu$ L HRP Development solution to each empty well and record (at room temperature) blue color development in the prepared microplate reader immediately.

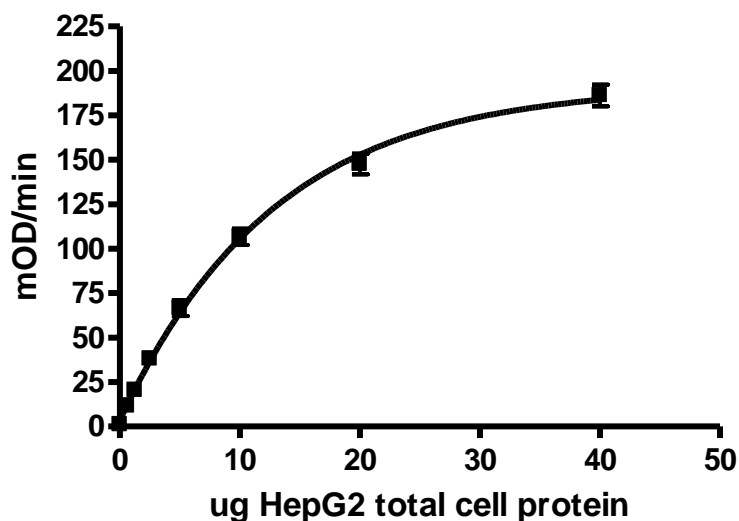
Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 15 min
Interval:	20 sec to 1 min
Shaking:	Shake between readings

Alternative– At a **user defined** color development time, record the endpoint OD data at (i) 600 nm or (ii) stop the reaction by adding 50  $\mu$ L stop solution (1 N HCl) to each well and record OD at 450 nm.

### C. Data Analysis

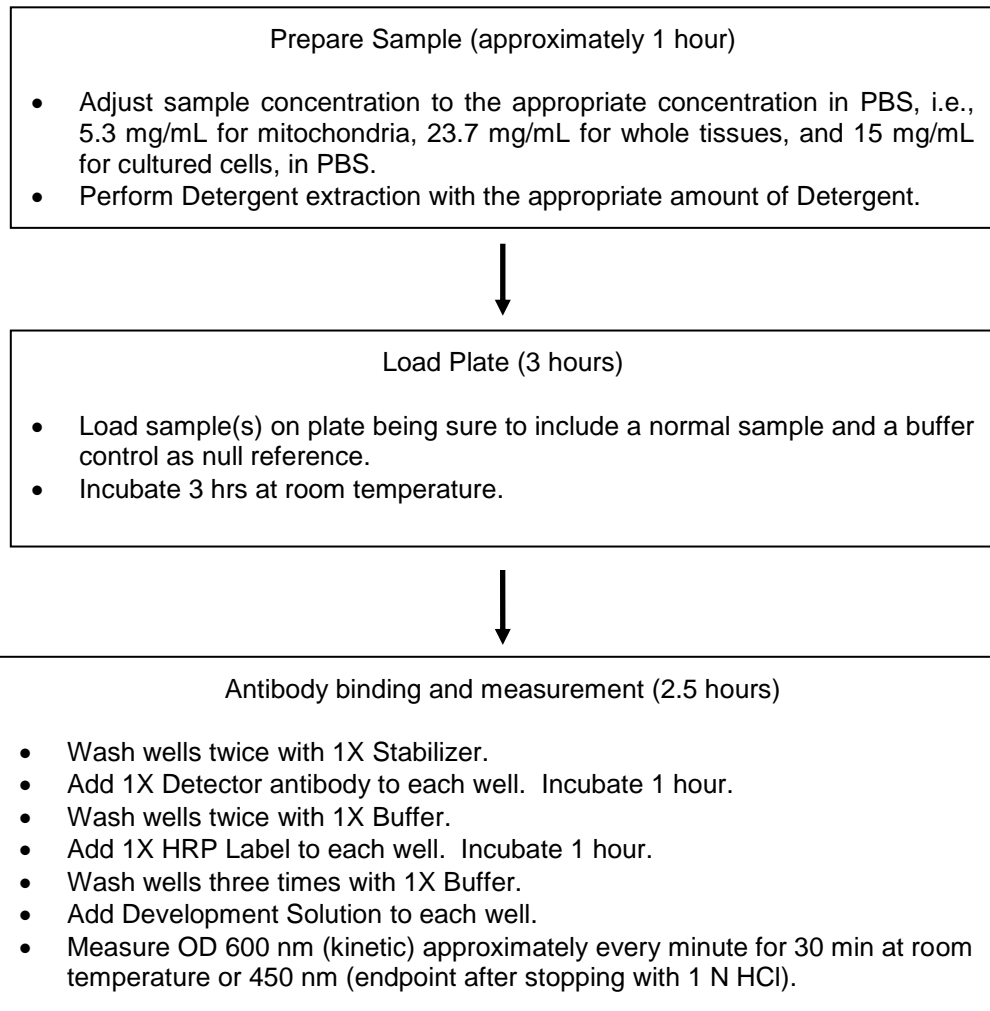
Examine the color development over time in each well. Under the conditions stated above the color development should be linear over the 30 minute time period of measurement. Subtract the initial absorbance reading from the final absorbance reading to determine the quantity of PDH in each well. This quantity should always be related to a control or normal sample to obtain the relative quantity of PDH in experimental samples.

Figure 1 below is an example of the quantity of PDH capture from a HepG2 cultured cell lysate. The sample was diluted to show that over this range of concentrations that can be used. Each sample was measured in 6 replicates. Bars show standard deviations.



**FLOW CHART**

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



## FREQUENTLY ASKED QUESTIONS

*How do I grow and prepare cultured cell samples?*

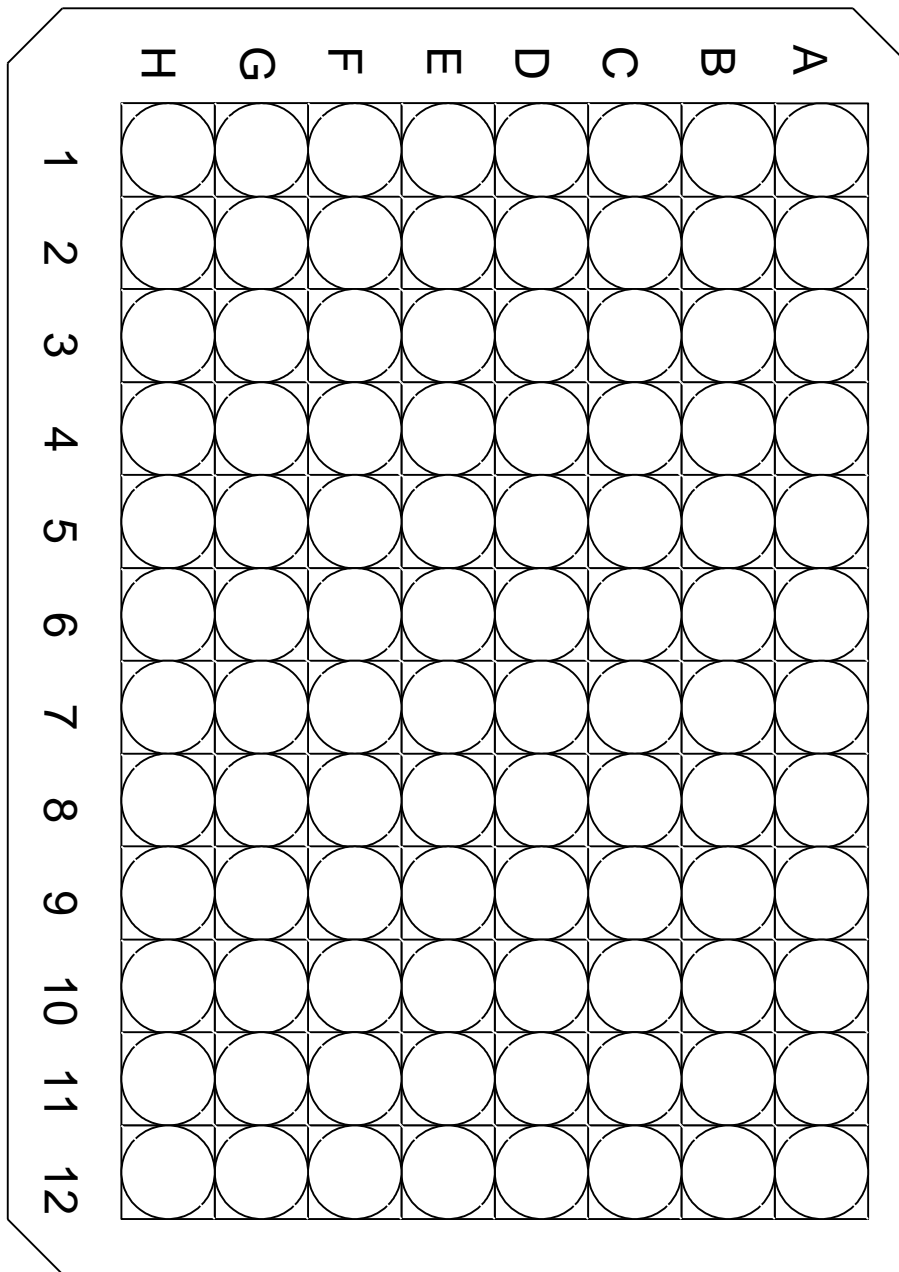
The amount of PDH in cells from different origins differs greatly. Cells grown in glucose have a lower activity than those grown in galactose/glutamine. Consequently, cell type and growth conditions are a large factor in PDH activity measured.

*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<sup>2</sup> plate:

Human fibroblasts	1 x 10 <sup>7</sup> cells	1.5 mg total protein
Human HepG2	2 x 10 <sup>7</sup> cells	3 mg total protein

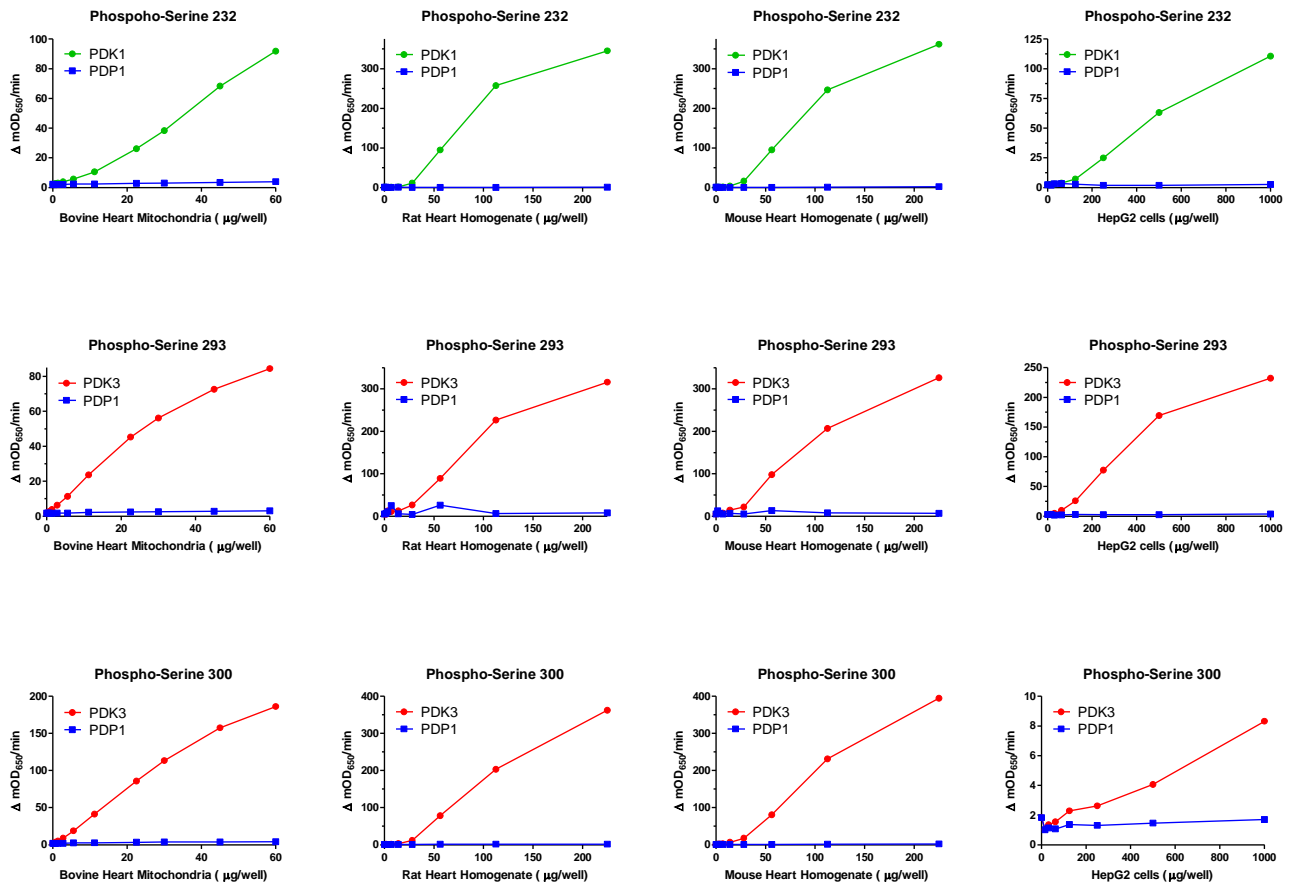
It is recommended that you accurately determine from your first confluent plate the number of cells and the total protein yield.



MICROPLATE MS \_\_\_\_\_ / /

## APPENDIX

## ALTERNATIVE DETECTOR ANTIBODIES



Specificity of Phospho-PDH Serine 232, 293 and 300 antibodies used as detectors in the MSP19 assay. Extracts of bovine heart mitochondria, rat heart homogenate, mouse heart homogenate and human HepG2 cells were treated post-capture with PDK1 (in green) or PDK3 (in red) to fully phosphorylate the captured PDH, or with PDP1 (in blue) to dephosphorylate the captured PDH. Each antibody binds phosphorylated PDH specifically.