

SOD2 Protein Quantity Microplate Assay Kit

MS746

Rev.0

DESCRIPTION

SOD2 Protein Quantity Microplate Assay Kit

Sufficient materials are provided for 96 measurements (one 96-well microplate).

Kit Contents:

Item	MS746
20X Buffer (Tube 1)	20 mL
Detergent	1 mL
20X Detector Antibody (Tube A)	1 mL
20X HRP Label (Tube B)	1 mL
10X Blocking Buffer (Tube 2)	8 mL
Development Solution (Tube 3)	20 mL
96-well microplate (12 strips)	1

The 96-well microplate has a monoclonal antibody pre-bound to the wells. This plate can be separated into twelve 8-well strips and thus can be used for up to 12 individual experiments.

Storage:

Store all components at 4°C. The kit is stable for at least 6 months.

INTRODUCTION

Aerobic organisms have several mechanisms to protect against oxidation, particularly from harmful reactive oxygen species (ROS). Superoxide and its products have been implicated in a wide range of diseases including cancer, inflammation, neurodegenerative diseases, diabetes and aging itself. The principle cellular anti-oxidants are the superoxide dismutase family (SOD, E.C. 1.15.1.1). These enzymes dismutate superoxide into hydrogen peroxide which is further detoxified by other cellular defenses such as glutathione peroxidase and catalase.

The SOD family has 3 members, two of which are Cu-Zn type: the extracellular SOD3 and the cytoplasmic SOD1. The other member is the mitochondrial Mn type SOD2. The mitochondrial Mn-SOD2 is a homotetramer of subunit mass 23 kDa in the mitochondrial matrix with one manganese ion per subunit. SOD2 is vital since SOD1 knockout mice appear to develop normally while SOD2 knockout mice do not and die shortly after birth.

SOD2 can also be the target of oxidative stress Tyr34 near the Mn active site can become nitrated leading to inactivation. It has been postulated that an increased reactive nitrogen species in ALS may contribute to site-specific SOD2 inactivation and exacerbation of ALS pathogenesis.

SOD2 levels may be down-regulated in tumor cells and recent observations show that over expression of SOD2 in tumor cells may suppress cell division and cancer growth (Oberley, Biomedicine & Pharmacotherapy, 2005, 59, p143-8).

Note: This protocol contains detailed steps for measuring SOD2 quantity. Be completely familiar with the protocol and the FAQ section (page 8) before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

The protocol has 4 steps:

- A. Sample preparation
- B. Plate loading
- C. Add detection antibodies
- D. Quantity measurement

This microplate assay (Catalog # MS746) has been developed for use with human samples. Mouse and rat samples may be used in the provided sample range guidelines.

This assay is designed for use with homogenates from cultured cells. Isolated mitochondria and tissue lysates can also be used, but some sample optimization may be necessary. As described below, homogenized samples should be resuspended to 5.5 mg/mL protein. The proteins are detergent extracted and loaded to within the linear range of the assay (see below). A **control** or normal sample should always be included in the assay as a reference. Also, include a **null** or buffer control to act as a background reference measurement.

Typical linear ranges per well (200 μ L) and per milliliter are listed below. The ranges may be extended by using a non-linear fit of the data from a normal sample.

Typical Linear Ranges:		
Human heart mitochondria	1 - 10 μ g/well	5 - 50 μ g/mL
Human liver mitochondria	1 - 10 μ g/well	5 - 50 μ g/well
Whole cultured HepG2 extract	2 - 25 μ g/well	10 - 125 μ g/mL
Whole cultured fibroblast cell extract	12 - 100 μ g/well	5 - 500 μ g/mL
Mouse brain mitochondria	25 - 100 μ g/well	125 - 500 μ g/well

NOTE: SOD2 levels between cell types also vary greatly. Ranges for mitochondria are dependent upon mitochondrial purity and integrity. For sample loading use the recommended amount specified on page 3. Intra assay variation = 12% (n=48), inter assay variation = 10.4 % (n=48)

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer plate reader (Molecular Dynamics SpectraMax recommended) capable of measuring absorbance at 600 nm (or 450 nm after addition of 1N HCl (not supplied)).
- Method for determining protein concentration
- Deionized water
- Multichannel pipette
- PBS (phosphate buffered saline – for recipe see www.mitosciences.com/PDF/western.pdf)

MICROPLATE ASSAY PROTOCOL

A. Buffer and Sample Preparation

1. Prepare the buffer solution by adding 20 mL of 20X Buffer (Tube 1) to 380 mL deionized H₂O. Label this Solution 1.
2. For entire plate (12 strips) add 8 mL of 10X Blocking Buffer (Tube 2) to 72 mL of Solution 1. Label this Incubation Solution.

NOTE: When using fewer strips make proportionately less (i.e. 0.67 mL Tube 2 + 6 mL Solution 1 for each strip used).

3. Determine the sample protein concentration by a standard method and then adjust the protein concentration to 5.5 mg/mL in PBS.

NOTE: If the sample is less than 5.5 mg/mL, centrifuge to pellet again and take up in a smaller volume to concentrate the pellet and repeat protein concentration measurement. The optimal protein concentration for detergent extraction is 5.5 mg/mL.

4. Add 1/10 volume of Detergent to the sample (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent). The final protein concentration is now 5 mg/mL.
5. Mix immediately and then incubate on ice for 30 minutes.
6. Spin in tabletop microfuge at maximum speed (~20,000 g, ~16,000 rpm) for 20 minutes. Carefully collect the supernatant and save as sample. Discard the pellet.
7. The microplate wells are designed for 200 µL sample volume per well, so dilute samples to the following recommended concentrations by adding Incubation Solution :

Sample Type	Recommended amount
Human whole cultured cell extract	20 µg/200 µL (100 µg/mL)
Human Tissue / mitochondria extract	5 µg/200 µL (25 µg/mL)

Note: Do not use more than 40 mL of the Incubation Solution for sample dilution.

8. Keep diluted samples on ice until ready to proceed.

B. Plate Loading

- A. Add 200 µL of each diluted sample into individual wells on the plate. Include a control (normal) sample as a positive control. Also include a buffer control (200 µL Incubation Solution) as a null or background reference.
- B. Incubate for 3 hours at room temperature.

C. Add Detection Antibodies

1. The bound monoclonal antibody has immobilized the protein in the wells. Empty the wells by turning the plate over and shaking out any remaining liquid.
2. Once emptied, add 300 μ L of Solution 1 to each well used.
3. Empty the wells again and add another 300 μ L of Solution 1 to each well used.
4. Separately, add 1 mL 20X Detector Antibody (Tube A) to 20 mL of Incubation Solution. Label this Solution A.

When using fewer strips make proportionately less Solution A.

5. Empty the wells again by turning the plate over and shaking out the remaining liquid.
6. Add 200 μ L of Solution A to each well used.
7. Incubate at room temperature for 1 hour.
8. Separately, add 1 mL 20X HRP Label (Tube B) to 20 mL of Incubation Solution. Label this Solution B.

When using fewer strips make proportionately less Solution B.

9. Empty the wells again quickly by turning the plate over and shaking out the remaining liquid.
10. Add 300 μ L of Solution 1 to each well used. Empty the wells again and add another 300 μ L of Solution 1 to each well used.
11. Empty the wells and add 200 μ L of Solution B to each well used.
12. Incubate at room temperature for 1 hour.

D. Quantity Measurement

1. After 1 hour empty the wells by turning the plate over and shaking out any remaining liquid.
2. Add 300 μ L of Solution 1 to each well used. Repeat this step three more times for a total of 4 rinse steps.
3. Add 200 μ L of Development Solution to each well used. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.
4. Place the plate in the reader and record with the following kinetic program (alternatively an endpoint measurement can be made by stopping the reaction at a user defined time by addition of 100 μ L of 1N HCl per well.

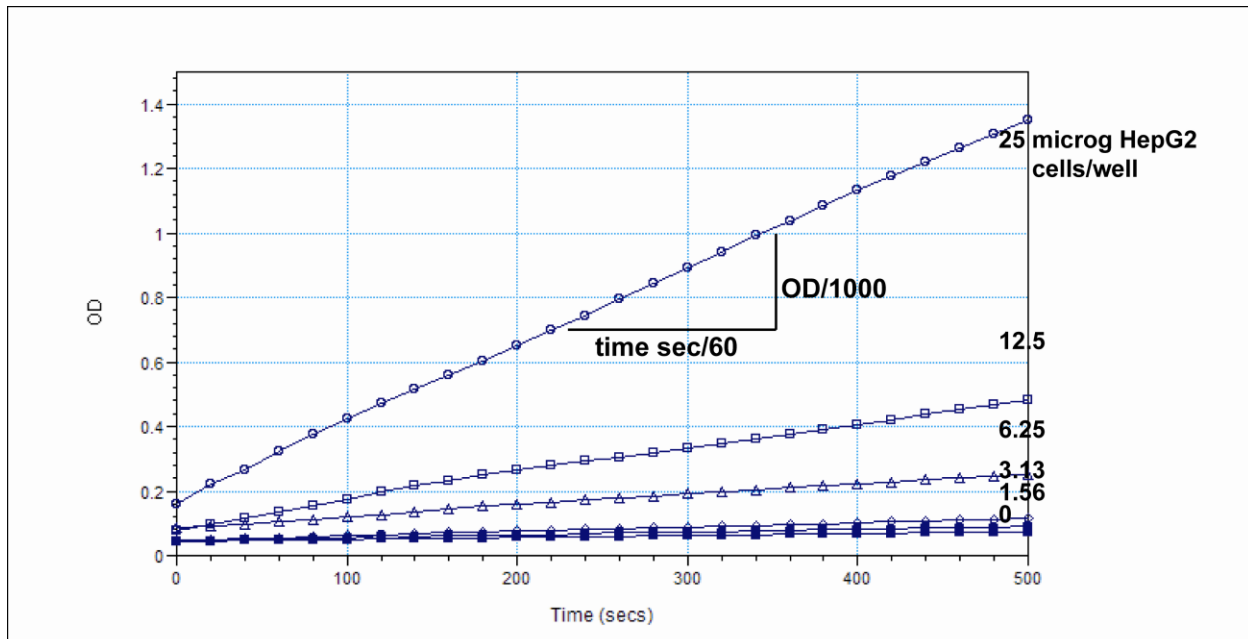
Kinetic measurement	Endpoint Measurement
OD 600 nm	Add 100 μ L 1M HCl/well
Time: 30 mins	Autoshake before reading
Interval: 20 sec - 1 min	Measure OD 450 nm
Autoshake between readings	

5. Save data and analyze as described in the DATA ANALYSIS section.

DATA ANALYSIS

The quantity of SOD2 captured in each well is proportional to the amount of HRP activity within each well. Therefore the relative quantity is the change in absorbance at 600 nm/minute/amount of sample loaded into the well.

Examine the linear rate of increase in absorbance at 600 nm with time. This is shown below where the rate or slope is calculated between two time points. Most microplate software is capable of performing this function. Repeat this for all samples.

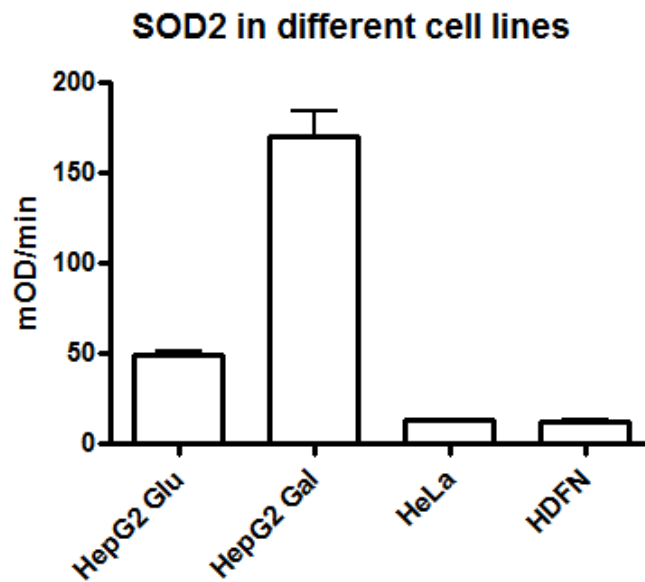


For the control or normal sample, the rate versus amount loaded is plotted as a straight line in the linear region of the assay as shown above.

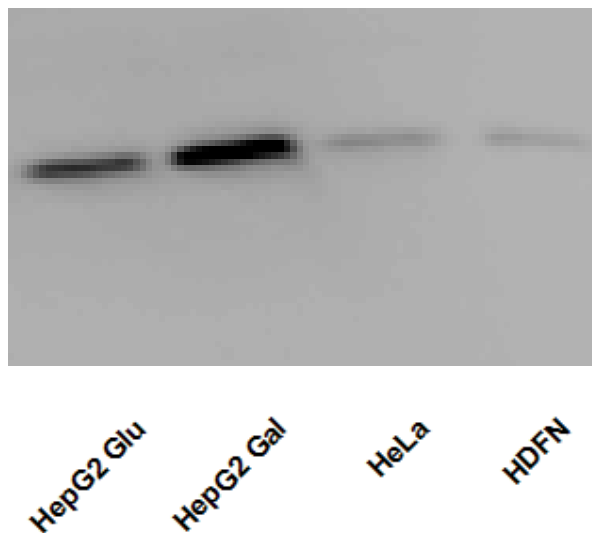
Compare the rates of the **control** (normal) sample and with the rate of the **null** (background) and with your **unknowns**, **experimental** or **treated** samples to get the relative amount of SOD2.

SOD2 levels were quantified in the whole cell extract from three different cell lines; HepG2, HeLa and HDFN cells all grown in glucose as well as HepG2 cells grown in galactose. A striking difference is seen in expression levels, this difference is confirmed in Western blotting. This difference may be due to increased mitochondrial respiratory chain activity in HepG2 galactose grown cells and a resulting need for more antioxidant enzyme activities.

Example: 25 µg whole cell lysate analyzed by MS746 quantity SOD2 microplate.

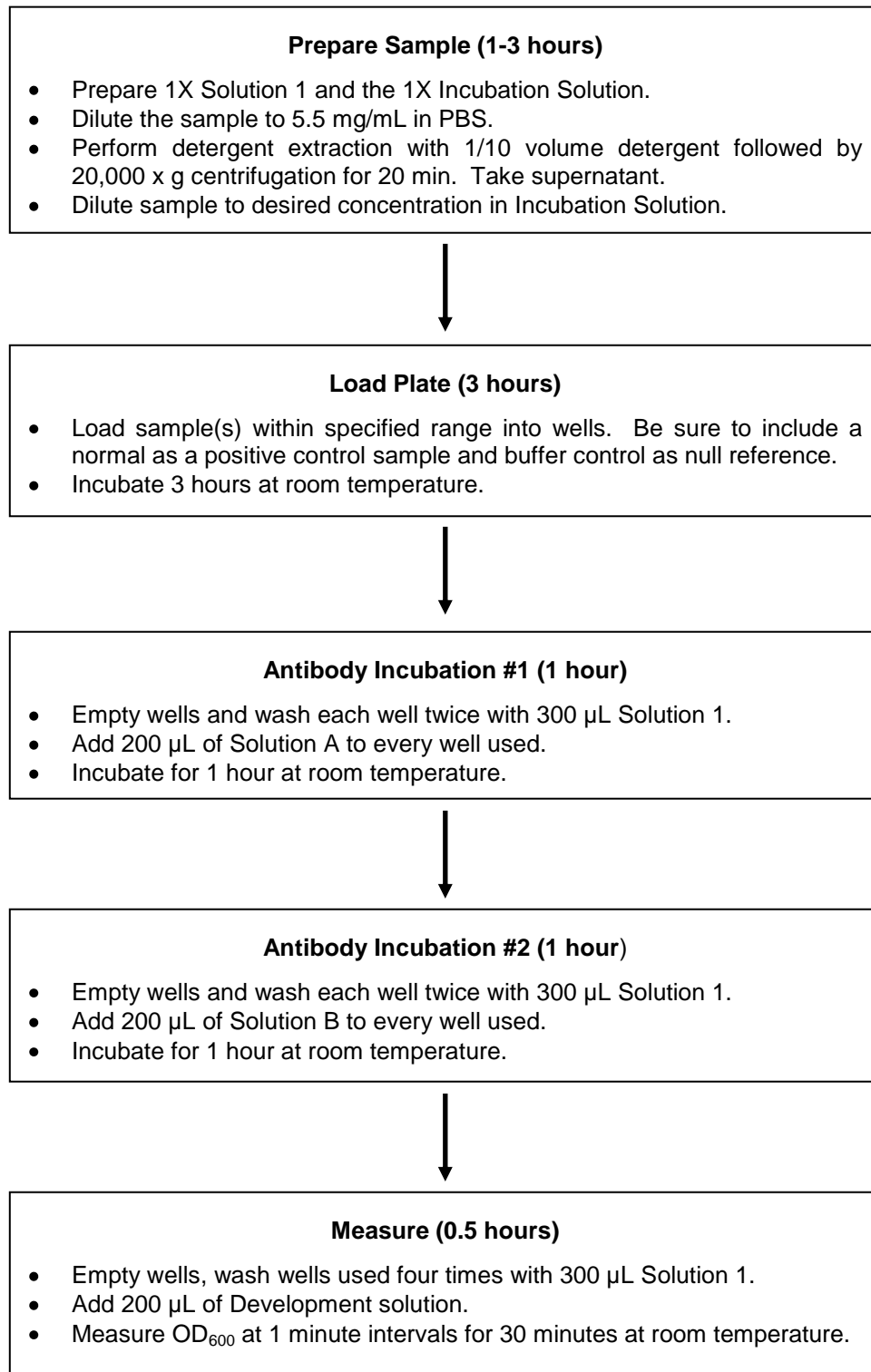


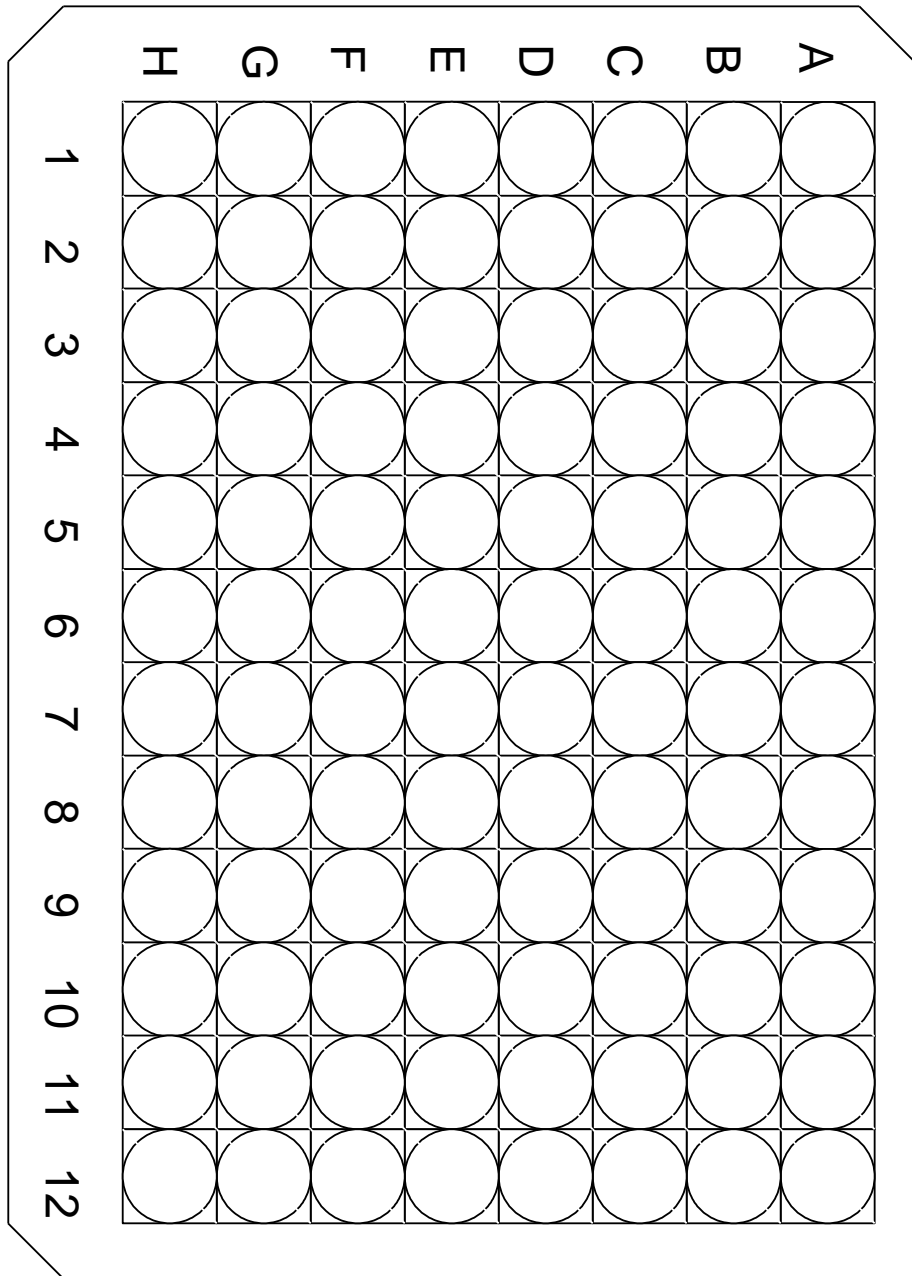
Example – Confirmation by Western blotting. 15 µg whole cell lysate analyzed by an anti-SOD2 Western blot.



FLOW CHART

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





MICROPLATE MS _____ / /

Frequently Asked Questions

1. What is the minimum amount of cells or tissue needed to accurately measure SOD2 quantity?

SOD2 quantity varies from cell line to cell line. A signal of 2x over background was seen with 2 µg and 6 µg for Hepg2 and fibroblast cells respectively. This corresponds to approximately 20,000 -100,000 cells per well.

2. Is it possible to speed up this assay?

Antigen-antibody reactions are dependent on many conditions such as temperature and movement of molecules. The higher the temperature and the faster the movement of molecules and the sooner the saturation of binding sites occur. This assay can be performed in about half the time if sample, detector antibody and HRP label incubation steps are carried out at 37°C on a rotating platform. However, it is crucial to be consistent with all assays for cross-comparisons. Under these specified conditions, samples can be incubated for 1.5 hours and detector and label incubation times can be reduced to 35 minutes each.

3. Can I use this plate to determine SOD2 quantity in tissues from rodents or other animal models?

Yes rat and mouse tissues have been tested but may require more material than is indicated for human samples.

4. Which immunogen was used to develop the antibodies used in this kit?

Purified SOD2.

5. What evidence do you have that the captured protein is in fact pure SOD2?

Immunoprecipitations using these antibodies were performed on a large scale and analyzed by SDS-PAGE purity and Mass Spectrometry for identity.

6. Which is the exact epitope that binds to the capture antibody (attached to the plate) and the detector antibody provided with this kit?

These antibodies bind the native conformation of SOD2, the exact epitopes are unknown.

7. How do I analyze tissue samples?

Tissue samples must be homogenous before detergent extraction. Homogenize tissue samples thoroughly by using a Dounce homogenizer (see MitoSciences' product MS851) or microtissue grinder/Ultraturrax T8. Measure protein concentration by BCA protein assay (ThermoFisher-Pierce). Dilute to 5.5 mg/mL in PBS, then add 1/10 volume of detergent for extraction and proceed from page 3 step A5.