ab119693 –

Mitochondrial Malate Dehydrogenase (MDH2) Activity Assay Kit

Instructions for Use

For the quantitative measurement of mitochondrial malate dehydrogenase (MDH2) activity in multiple species (Human, Mouse, Rat)

View kit datasheet: www.abcam.com/ab119693
(use www.abcam.cn/ab119693 for China, or www.abcam.co.jp/ab119693 for Japan)

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

Principle: ab119693 is used to determine mitochondrial malate dehydrogenase activity (MDH2) in a sample. The enzyme is captured within the wells of the microplate and activity is determined by following the production of NADH in the following MDH2 catalyzed reaction:

malate + NAD+ ⟶ oxaloacetic acid + NADH
(↑ Absorbance at 450 nm)

The generation of NADH is coupled to the 1:1 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 (Dye molar extinction coefficient - 37000 M-1 cm-1). In each well, ab119693 immunocaptures only native MDH2 from the chosen sample; this removes all other enzymes, including MDH1 in cytosol. ab119693 allows researchers to focus on TCA cycle, studying isotype-specific malate dehydrogenase (MDH2) activity assay without the necessity of isolating mitochondria.

Background: Mitochondrial malate dehydrogenase (MDH2, P40926) is a 35.5 kDa enzyme that catalyzes the conversion of malate into oxaloacetate (using NAD+) and vice versa. (EC 1.1.1.37) Several isozymes of malate dehydrogenase exist, depending on where they are localized in the cell and their specific dependence on

NAD+ or NADP+ (only in chloroplasts). There are two main isoforms in eukaryotic cells. One is found in the mitochondrial matrix (MDH2), participating as a key enzyme in the citric acid cycle that catalyzes the oxidation of malate. The other is found in the cytoplasm (MDH1), assisting the malate-aspartate shuttle with exchanging reducing equivalents so that malate can pass through the mitochondrial membrane to be transformed into oxaloacetate for further cellular processes. Because malate dehydrogenase is closely tied to the citric acid cycle, regulation is highly dependent on TCA products. High malate concentrations stimulate MDH activity, and, in a converse manner, high oxaloacetate concentrations inhibit the enzyme. Enzyme activity is enhanced by acetylation.

Limitations:

- FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC PROCEDURES.
- Use this kit before expiration date.
- Do not mix or substitute reagents from other lots or sources.
- If samples generate values outside of the range of the standard curve, further dilute the samples with 1X Incubation buffer and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

Technical Hints:

- To avoid cross contamination, change pipette tips between additions of each standard, sample and between reagent additions. Also use separate clean, dry reservoirs for each reagent.
- Cover plate during incubation steps.
- Thorough and consistent wash technique is essential for proper assay performance. Wash buffer must be forcefully dispensed and completely removed from the wells by aspiration or decanting. Remove remaining wash buffer by inverting the plate and blotting on paper towels.

2. Assay Summary

Prepare samples as instructed.

Determine the protein concentration of extracts.

Bring all reagents to room temperature.

Dilute sample to desired protein concentration in Incubation buffer.

Add 100 μL sample to each well used. Incubate 3 hours at room temperature.

Aspirate and wash each well twice.

Freshly prepare 1X Activity Solution then add 100 μL to each well.

Pop bubbles and immediately record the color development with elapsed time at 450 nm for 15 to 30 minutes.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Item	Quantity
20X Buffer	20 ml
Extraction Buffer	15 ml
10X Blocking Buffer	8 ml
Base Buffer	24 mL
100X Sodium Malate	add 0.25 mL H_2O
100X Reagent Dye	add 0.25 mL H_2O
100X Coupler	add 0.25 mL H_2O
100X NAD+	add 0.25 mL H_2O

MDH2 Microplate (12 x 8 antibody coated well strips)	96 Wells

4. Storage and Handling

All components are shipped cold. Reagent dye, coupler, malate and NAD+ are shipped lyophilized. Before use rehydrate by adding 0.25 mL pure H_2O to each tube and vortex each tube thoroughly to dissolve.

After hydration unused amounts of these four materials should be stored at -80°C for 6 months. Store all other components at 4°C. This kit is stable for 6 months from receipt.

5. Additional Materials Required

- Standard absorbance microplate reader capable of kinetic reading at 450nm
- Multichannel pipette (50 - 300 µL) and tips
- 1.5-mL microtubes
- Paper towels
- Deionized water
- Optional plate shaker for all incubation steps

6. Reagent Preparation

6.1. Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.

6.2. Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer.

6.3. Prepare 100X Sodium Malate, 100X NAD+, 100X Coupler, and 100X reagent dye, as described above (Section 4. Storage and handling).

6.4. Before use (in Step 8.6) prepare 1X Activity Solution. For an entire plate add 0.1 mL 100X Sodium Malate, 0.1 mL 100X NAD+, 0.1 mL 100X Coupler, 0.1 mL 100X Reagent Dye to 9.6 mL Base Buffer provided. *Note – the final concentration of substrate is now 5mM malate, 4mM NAD+.*

7. Test sample Preparation

Note: Extraction buffer <u>DOES NOT CONTAIN PROTEASE INHIBITORS.</u> It can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

7.1 Cell Lysates

7.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.

7.1.2 Rinse cells twice with PBS.

7.1.3 Solubilize cell pellet at $2x10^7$/mL in Extraction Buffer.

7.1.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

7.2 Tissue lysates:

7.2.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

7.2.2 Suspend the homogenate to 25 mg/mL in PBS.

7.2.3 Solubilize the homogenate by adding 4 volumes of Extraction Buffer to a sample protein concentration of 5 mg/mL.

7.2.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

7.3 Sub-cellular organelle lysates e.g. mitochondria:

7.3.1 Prepare the organelle sample by, for example, sub-cellular fractionation.

7.3.2 Pellet the sample.

7.3.3 Solubilize the pellet by adding 9 volumes Extraction Buffer.

7.3.4 Incubate on ice for 20 minutes. Centrifuge at 16,000 x g for 20 at 4°C minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C for 6 months. The sample protein concentration in the extract may be quantified using a protein assay.

7.3.5 These test samples should be diluted to within the working range of the assay in 1X Incubation Buffer.

8. Assay Procedure

Bring all reagents and samples to room temperature before use. It is recommended all samples and standards be assayed in duplicate.

8.1. Prepare all reagents, and samples as directed in the previous sections.

8.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

8.3. Add 100 µL of each diluted sample per well. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a 1X Incubation buffer as a zero standard.

8.4. Cover/seal the plate and incubate for 3 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.

8.5. Aspirate each well and wash, repeat this once more for a total of two washes. Wash by aspirating or decanting from wells then dispensing 300 µL 1X Wash buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash, remove the remaining buffer by

aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

8.6. Gently add 100 μL fresh mixed 1X Activity Solution, described above, to each well minimizing the production of bubbles.

8.7. Pop any bubbles immediately and record absorbance in the microplate reader prepared as follows:

Mode:	**Kinetic**
Wavelength:	450 nm
Time:	15-30 Min.
Interval:	20 Sec.
Shaking:	Shake before and between readings

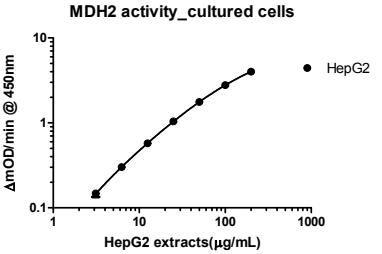
Alternative– In place of a kinetic reading, at a ***user defined***, time record the endpoint OD data at 450 nm in all wells.

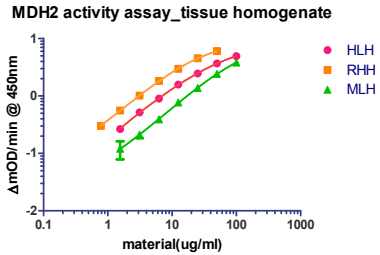
8.8. Analyze the data as described below.

9. Data Analysis

Example data sets are shown below illustrating data analysis of MDH2 activity measurements in HepG2 cells (as an example human cell line derived from liver), and homogenate samples from human, rat and mouse liver tissues.

The starting concentration of malate in the assay is 5 mM and NAD+ is 4 mM. Malate oxidation and production of NADH by MDH2 in each well is 1:1 proportional with dye reduction and increase in absorbance at 450 nm (dye ε450nm = 37 mM-1 cm-1). For simplicity the activity can be expressed as the change in absorbance per minute per amount of sample loaded into the well. Activity was collected as described in this protocol using a Molecular Dynamics microplate reader. Standard curves of reference sample data were exported to graphing software capable of a 4-parameter data analysis (shown below). Activity is clearly measurable in the 6.25-200 μg/ mL range when such a fit is applied for HepG2, and human or mouse liver tissue, and 0.78-50 μg/mL range for rat heart tissue extracts.





Figures 1 and 2. Representative measurements from serially diluted HepG2 extracts and tissue homogenates HLH, RHH, MLH.

Unknown samples should be interpolated from these reference sample graphs. This determined relative activity is the amount of reference sample required to generate the same amount of activity as the unknown sample and usually expressed as a per cent value.

WORKING RANGE

This assay has been demonstrated with human, rat, and mouse liver homogenates, heart tissue homogenate samples as well as HepG2 whole cell lysate, a liver derived human cultured cell line. Typical ranges for several sample types are described below. It is highly recommended to prepare multiple dilutions for each sample to ensure that each is in the working range of the assay (see Data Analysis section).

Sample Type	Range (µg/mL)
Cultured whole cell extracts (type dependant) e.g. HepG2	6.25– 200
Tissue extract e.g. liver extracts	0.78 – 200

REPRODUCIBILITY

Parameter	CV%
Intra (n=3, 3 assays)	1.3 - 6.8
Inter (n=3 days)	13.9

SPECIFICITY

Species– human, rat, mouse, reactive. Others untested.

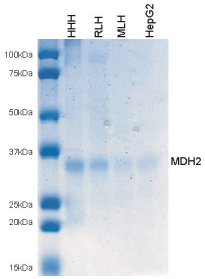


Figure 3. The antibody used to isolate MDH2 in this kit was generated by immunization of purified human MDH2 proteins. The resulting monoclonal mouse antibody isolates, by immunoprecipitation, a single MDH2 band to purity from a number of species.

The immunoprecipitate was confirmed to be MDH2 by mass spectrometry with no other contamination. The specificity of the immunocapture based activity assay is confirmed by replacing the capture antibody with another unrelated capture antibody.

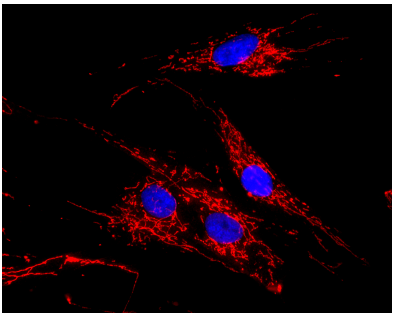


Figure 4. This antibody is cross reactive in immunofluorescence microcopy and labels a mitochondrial intracellular pattern.

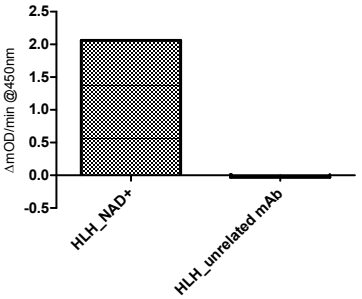


Figure 5. The specificity of the immunocapture based activity assay is confirmed by replacing the capture antibody with another unrelated capture antibody.

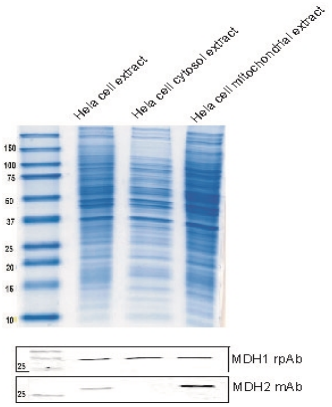


Figure 6.

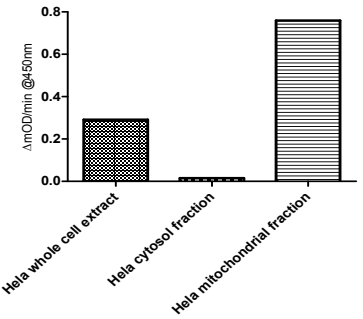


Figure 7.

Figure 6 and 7. The isoform specificity of the malate activity measured by this kit is confirmed by measuring the MDH activity from different cell fractions. Activity was only detected from the mitochondrial fraction (MDH2), not the cytosol fraction (MDH1).

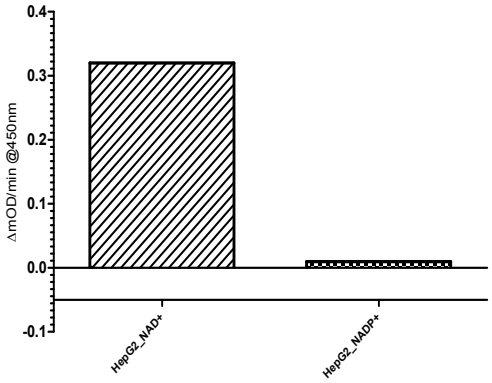


Figure 8. Finally the activity measured by this kit is only compatible with the NAD+ cofactor, there is no activity with NADP+, a feature of mitochondrial malic enzyme, which catalyzes the conversion of malate to pyruvate using NADP+ as cofactor, producing NADPH.

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