ab115348 – Mitochondrial Aldehyde Dehydrogenase (ALDH2) Activity Assay Kit

Instructions for Use

For the measurement of mitochondrial aldehyde dehydrogenase (ALDH2) activity in multiple species (human, mouse, rat)

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

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Table of Contents

1. Introduction

Principle: The microplate assay ab115348 is used to determine mitochondrial aldehyde dehydrogenase activity (ALDH2) 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 ALDH2 catalyzed reaction:

$$\text{acetaldehyde} + NAD^+ \rightarrow \underset{(\uparrow \text{Absorbance at 450 nm})}{\text{acid} + \text{NADH}}$$

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}). ab115348 immunocaptures in each well only native ALDH2 from the chosen sample; this removes all other enzymes, including unrelated aldehyde dehydrogenases.

Background: Mitochondrial aldehyde dehydrogenase (ALDH2, P05091) is a 56 kDa enzyme that catalyzes the hydrolysis of an aldehyde into an acid (EC 1.2.1.3) in the mitochondrial matrix of primarily liver cells but can also be detected in many other tissues. Aldehyde dehydrogenases perform the second step in the major oxidative pathway of alcohol metabolism. ALDH2 is a target for the treatment of alcoholism. Genetic variation in ALDH2 is responsible for individual differences in responses to drinking alcohol

[MIM:610251]. Approximately 50% of Asians have one normal copy of the ALDH2 gene and one mutant copy that encodes an inactive mitochondrial isoenzyme. A remarkably higher frequency of acute alcohol intoxication among Asians than among Caucasians has been repeatedly shown to be related to the very much reduced activity of the mutant ALDH2 (E487K). ALDH2 has also been identified as an important anti-oxidant enzyme and confers protection from ischemic heart damage as well as an important bioactivator of nitroglycerin.

Limitations:

- FOR RESEARCH US 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 1x Incubation buffer.

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

Aspirate and wash each well twice.

Add 200 μL 1X Activity Solution to each well.

Pop bubbles and record immediately the color development with time at 450 nm for 30-120 minutes.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Item	Quantity
20X Buffer	20 ml
1X Extraction Buffer	15 ml
10X Blocking Buffer	6 ml
100X acetaldehyde (2.5 M)	0.25 mL
1X Base Buffer	24 mL
100X Reagent Dye	add 0.25 mL H_2O
100X Coupler	add 0.25 mL H_2O
100X NAD+ (0.1M)	add 0.25 mL H_2O
96 well microplate (12 strips)	1

4. Storage and Handling

All components are shipped cold. Reagent dye, Coupler 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 three materials should be stored at -80°C. Store all other components store at 4°C.

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
- Stop solution (optional) – 1N hydrochloric acid
- Optional plate shaker for all incubation steps

6. Reagent Preparation

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

2. Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Solution to 54 mL 1X Wash Buffer.
3. Before use (in section 8, step 6) prepare 1X Activity Solution. Dissolve the NAD+, Coupler and Reagent dye as described above.
   For an entire plate add 0.25 mL 100X Coupler, 0.25 mL 100X acetaldehyde, 0.25 mL 100X NAD+, 0.25 mL 100X Reagent Dye to 24 mL 1X Base Buffer provided.
   Note – the final concentration of substrate is now 25mM aldehyde, 1mM NAD+.

7. Test sample Preparation

Note: Extraction buffer can be supplemented with phosphatase inhibitors, PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions:

1. Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 g for 10 min at 4^{o}C.

2. Rinse cells twice with PBS.

3. Solubilize cell pellet at 2x10^{7}/mL in Extraction Buffer.

4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

5. Samples should be diluted to within the working range of the assay in 1X Incubation Buffer.

Tissue lysates:

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).

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

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

4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

5. Samples should be diluted to within the working range of the assay in 1X Incubation Buffer.

Sub-cellular organelle lysates e.g. mitochondria:

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

2. Pellet the sample.

3. Solubilize the pellet by adding 9 volumes Extraction Buffer.

4. Incubate on ice for 20 minutes. Centrifuge at 16000 x g 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

5. 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.

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

2. Remove excess microplate strips from the plate frame; return them to the foil pouch containing the desiccant pack, and real.

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.

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.

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.

6. Gently add 200 μL 1X Activity Solution, described above, to each well minimizing the production of bubbles.

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

Mode:	Kinetic
Wavelength:	450 nm
Time:	30-120 min (as desired)
Interval:	20 sec - 1 min
Shaking:	Shake 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.

Analyze the data as described in section 9.

9. Data Analysis

Example data sets are shown below illustrating data analysis of ALDH2 activity measurements in HepG2 cells (as an example human cell line derived from liver), and homogenate samples from whole human, rat and mouse liver tissues.

The starting concentration of aldehyde in the assay is 25 mM and NAD+ is 1mM. Aldehyde oxidation and production of NADH by ALDH2 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 60-500 μg/ mL range when such a fit is applied for HepG2, or rat liver tissue and 60-250 μg/mL range for human or mouse liver tissue extracts.

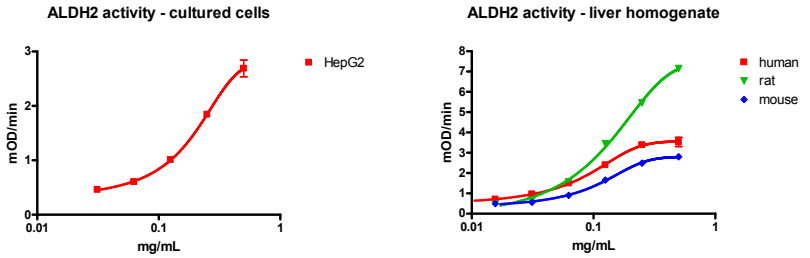

Figure 1. Unknown samples should be interpolated from these reference sample graphs. This determined relative activity is the amount of reference sample required to generate the same amount of activity as the unknown sample and usual expressed as a per cent value.

WORKING RANGE

This assay has been demonstrated with human, rat, and mouse liver and heart tissue homogenate samples as well as HepG2 whole cell lysate, a liver derived human cultured cell line. As an example of a non liver cell line, HeLa cell lysate yielded no ALDH2 activity. 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
Cultured whole cell extracts (type dependant) e.g. HepG2	0.06 – 0.5 mg/mL
Tissue extract	0.03 – 0.25 mg/mL

REPRODUCIBILITY

	CV %
Intra (n= 20)	5
Inter (n=4 days)	12

EXAMPLE DATA - Determination of effect reactive species on ALDH2 activity.

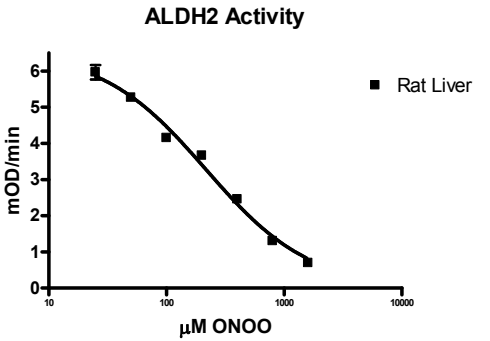

Figure 2. The mitochondrial location of ALDH2 may increase the exposure of this enzyme to high levels of reactive oxygen and nitrogen species generated in mitochondria. ALDH2 has been shown to be specifically inactivated by nitrative stress, particularly by modification or cross-linking of an active site cysteine. To demonstrate this rat liver homogenate was exposed in vitro to a dilution series of peroxynitirite at concentrations - 0, 25, 50, 100, 200, 400, 800, 1600 µM. After exposure the samples were extracted, diluted to 0.25 mg/mL and loaded into microplate wells in duplicate. After following the assay protocol as described above, the activity of each sample was measured and graphed against peroxynitrite exposure concentration. The ALDH2 activity after exposure was reduced with an IC50 approximately 210 µM.

SPECIFICITY

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

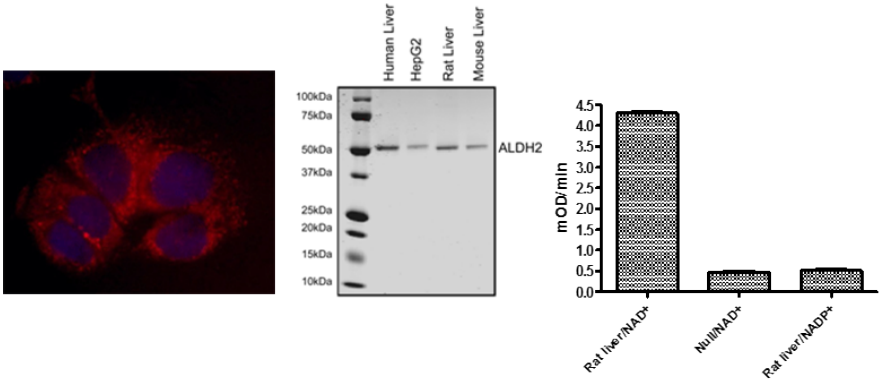

Figure 3. The antibody used to isolate ALDH2 in this kit was generated by immunization of rat mitochondrial proteins. The resulting monoclonal mouse antibody isolates, by immunoprecipitation, a single ALDH2 band to purity from a number of species. The immunoprecipitate was confirmed to be ALDH2 by mass spectrometry with no other contamination aldehyde dehydrogenases. The immunoprecipitate was also confirmed independently by Western blotting. This antibody is cross reactive in immunofluorescence microcopy and labels a mitochondrial intracellular pattern. Finally the activity measured by this kit is only compatible with the NAD+ cofactor; there is no activity with NADP+, a feature of mitochondrial aldehyde dehydrogenase activity over other cytosolic aldehyde dehydrogenases.

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