ab109712 –

Aconitase Assay Kit (Colorimetric)

Instructions for Use

For the quantitative measurement of Aconitase activity in samples from all species

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

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1. Introduction

ab109712 is a simple, reproducible, and sensitive tool for assaying aconitase from tissue homogenates or cell lysates. Unlike other assays this is not a coupled reaction and therefore only aconitase activity is required and measured. Aconitase catalyzes an equilibrium between citrate, cis-aconitate and iso-citrate. These reactions are monitored by measuring the increase in absorbance at 240 nm associated with the formation of the cis-aconitate which has an extinction coefficient of 2.2 OD/mM per well. Therefore the rate of cis-aconitate production is proportional to aconitase activity.

Citrate ↔ cis-aconitate ↔ isocitrate

(↑ Abs. 240nm)

Aconitase preservation solution, assay buffer, reagents and an essential UV microplate are provided for this measurement. The entire assay can be completed within 2 hours.

Note – mitochondrial and cytoplasmic aconitase activities are indistinguishable. Therefore, to measure the mitochondrial activity only, first isolate mitochondria, or for both activities fractionate the cells into cytoplasmic and mitochondrial (see Figures 4-5).

Aconitase (aconitate hydratase; EC 4.2.1.3) is an iron-sulfur protein that catalyzes the reversible inter-conversion of citrate and isocitrate, via a *cis*-aconitate intermediate, in both the TCA and glyoxylate cycles. The enzyme contains a [4Fe-4S] cluster which interacts directly with the substrates. In eukaryotes there are both mitochondrial and cytosolic forms of the enzyme. The mitochondrial form functions not only in the TCA cycle, but also to stabilize mtDNA thereby influencing mitochondrial gene expression. The cytosolic form can function as an aconitase as well as an iron regulatory protein.

The active form of the enzyme is inhibited by citrate analogs, and fluoracetate. Other inhibitors include oxidative stress agents such as peroxynitrite, hydrogen peroxide and superoxide, which inactivate the enzyme by changing the [4Fe-4S] to a [3Fe-4S] cluster. Aconitase is considered a good marker of mitochondrial and cellular oxidative stress. This change in mitochondrial aconitase can lead to a decreased energy production, whereas in cytosolic aconitase it triggers binding of the enzyme to mRNA iron response elements resulting in increased expression of iron uptake proteins and decreased transcription of iron sequestering protein.

A hydroxyl scavenging solution (Aconitase preservation solution) is supplied with this kit to maintain aconitase activity during sample preparation. An inactivated [3Fe-4FS] aconitase may be activated *in vitro* by the addition of iron and cysteine.

2. Assay Summary

Prepare Reagents (10 min)

- Thaw samples, dilute to 5 mg/ml in Aconitase preservation solution, keep on ice.

Prepare Samples and assay buffer (15 min)

- When ready use the supplied Buffer to dilute mitochondria to desired concentration, keep on ice.
- In a separate tube make sufficient Assay buffer by adding Isocitrate and Manganese to the supplied Buffer.

Load and Read Plate (30-45min)

- Add 50 µl of diluted sample to each microplate well. Include buffer control.
- Add 200 µl of Assay buffer to each well of the plate.
- Measure OD_{240} at 20-second intervals for 30 min at room temperature.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Item	Quantity
Aconitase preservation solution	20 ml
Detergent (for cultured cell preparation only)	1 ml
Buffer	50 ml
Isocitrate (25X)	800 µl
Manganese (100X)	200 µl
96 – well UV microplate	1

4. Storage and Handling

Store UV microplate at room temperature.

Store all other components store at 4°C.

5. Additional Materials Required

- Spectrophotometer that measures absorbance at **240nm**
- Multichannel pipette (50 - 300 µl) and tips
- 1.5 ml microtubes
- A fine needle

6. Preparation of Samples

Note: This protocol contains detailed steps for measuring aconitase activity. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

Sample preparation for both mitochondria (section A) and cultured whole cells (section B) are described.

A. Mitochondria Preparation

1. Mitochondria should be made according to a standard protocol. Prepared mitochondria should be stored at -80°C as concentrated as possible, 25-50 mg/ml is recommended. A mitochondrial isolation kit is available from Abcam (ab110168-ab110171/MS850-853).
2. On the day of the assay prepare the mitochondria by thawing and dilute to 5 mg/ml in the supplied, ice-cold, aconitase preservation solution which has been shown to stabilize aconitase activity for >3 hours on ice (see Figure 1). This buffer functions to scavenge hydroxyl radicals that would otherwise inhibit aconitase over time.

B. Cultured Cell Preparation

1. Resuspend the cell pellet to 5 mg/ml in Aconitase preservation solution (approximately 20-50 x106 cells/ml depending on cell type).

2. Add 1/10 volume of supplied detergent.
3. Incubate 30 minutes on ice.
4. Centrifuge 20,000 x g for 10 minutes.
5. Collect the supernatant as sample.

Note - It may be necessary to determine the protein concentration of extracts if extraction efficiency is highly variable between samples being compared

C. Sample Preparation

When ready, dilute the sample in cold Buffer to a concentration that falls within the range as indicated in the table below:

Sample	Sample Protein Range
Tissue Mitochondria	5-100 µg / 50 µl sample
Whole cultured cell extract	25-250 µg / 50 µl sample

Keep samples on ice.

Prepare only sufficient Activity buffer as needed by adding 1/25 volume Isocitrate and 1/100 volume Manganese to the supplied Buffer. Label this Assay buffer.

For example:

No. of wells	Buffer (ml)	Isocitrate (25X) (µl)	Manganese (100X) (µl)
8	1.67	66	17
16	3.33	133	33
24	5.00	200	50
32	6.67	266	67
40	8.33	333	83
48	10.0	400	100
56	11.67	466	117
64	13.33	533	133
72	15.00	600	150
80	16.67	666	167
88	18.33	733	183
96	20.00	800	200

7. Assay Method

A. Plate Loading

1. Add 50 µl of diluted sample to the appropriate wells. Include a buffer control (50 µl Buffer only, no sample) as a null or background reference.

2. Using a multichannel pipette, add 200 µl of Assay buffer made in C2 to each well used. Immediately pop any bubbles with a fine needle.

B. Measurement of Aconitase activity

Place the plate in the reader and record with the following kinetic program. An endpoint measurement can be made if your plate reader is not capable of kinetic measurements.

Kinetic Measurement	Endpoint Measurement
OD 240 nm	OD 240 nm
Duration: 30 mins	Times: 0 minutes and 30 minutes
Interval : 20 – 60 secs (longer if necessary)	Shake plate during incubation
3 sec Auto-shake between readings	Auto-shake before reading final OD
Temperature - room	Temperature - room

8. Data Analysis

A. Calculation of Aconitase activity

The conversion of isocitrate to cis-aconitate is measured as an increase in absorbance at OD 240 nm. The activity could be measured as difference between initial OD and the end OD at 30 mins. However, we recommend a kinetic measurement which is not dependant on the absolute initial OD. Most microplate reader software packages are capable of making kinetic measurements by examining the rate of increase in absorbance at 240 nm over time. To analyze the

data, pick two time points between which the rates are linearly increasing for all samples. We find between 10 and 20 minutes is usually a good window:

$$\text{Rate (OD/min)} = \frac{\text{Absorbance 1} - \text{Absorbance 2}}{\text{Time (min)}}$$

Calculate the average rate and correct for background:

Example: 12.5 µg bovine heart mitochondria

Rates: 37.3 mOD/min
38.5
39.8

Background: 0.4 mOD/min,

Corrected rate: 38.1±1.3 mOD/min
= 17.3 µM/min
0.25 mL/well = 4.3 nmoles/min
12.5 µg /well = 0.35 µmoles/min/mg mitochondria

Extinction coefficient = 2.2 OD mM^{-1}/well*

(* 3.6 OD mM^{-1} cm^{-1})

Examples of data obtained using ab109712:

Aconitase activity is unstable with time. Using the supplied mitochondrial preservation solution, aconitase activity is maintained for longer while samples are stored on ice (Figure 1).

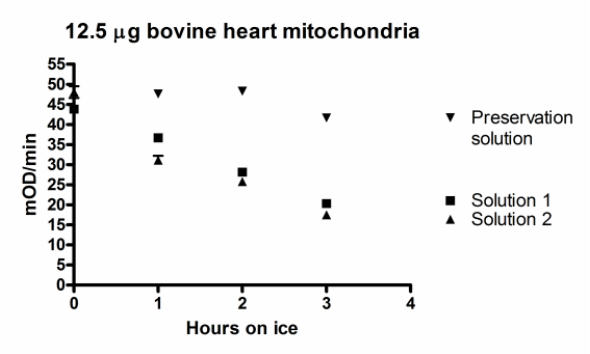

Figure 1. Aconitase preservation solution

Mitochondrial aconitase activity can be measured by first isolating mitochondria from cells or tissues (Figure 2).

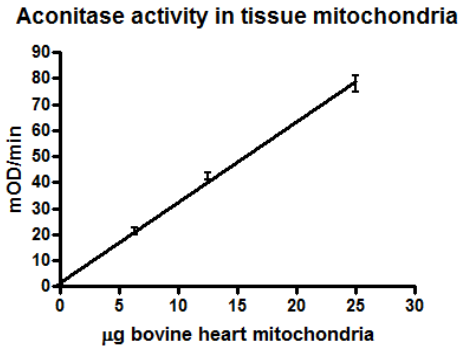

Figure 2. Aconitase activity in tissue mitochondria

Total aconitase activity can also be measured in whole cell lysates, for example, 143B osteosarcoma lysate shown below (Figure 3).

Note – the aconitase activity measured in 143B Rho0 (lacking mitochondrial DNA) is lower. This may be due to lower expression or lower specific activity of the enzyme.

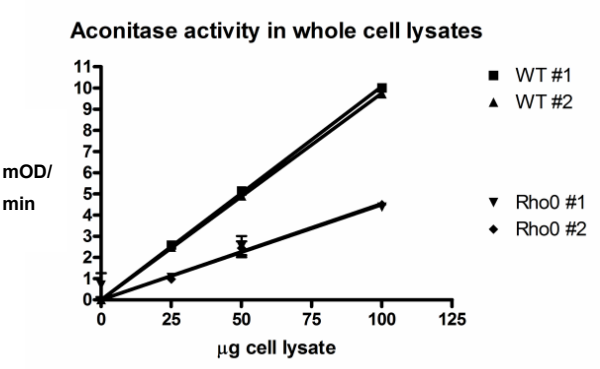

Figure 3. Aconitase activity in whole cell lysate from cultured cells

To determine the activity of mitochondrial aconitase purify mitochondria using Abcam's mitochondrial isolation kit (ab110168/MS850). Alternatively the aconitase activity in both the cytoplasmic and mitochondrial fraction can be determined Abcam's Cell Fractionation Kit (ab109719/ MS861). Typical data shown below for human cell lines HepG2 and Jurkat cells (Figure 4).

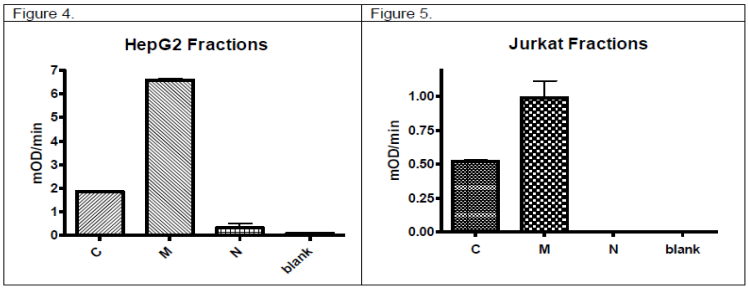

Figure 4. Aconitase activity from cytoplasm (C), mitochondria (M) and nuclear (N) fractions generated using Cell Fractionation Kit (ab109719).

In this example 66-75% of cellular aconitase activity is found in the mitochondrial fraction and attributable to the mitochondrial aconitase isoform (ACONM). The fractionation is confirmed by Western blot of these fractions (Figure 5)

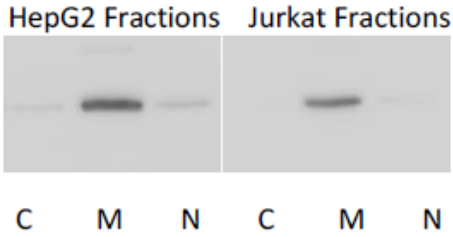

Figure 5. Western blotting of cell fractions with an anti-mitochondrial aconitase antibody indicates mitochondrial aconitase is found specifically in the mitochondria fraction (M) and not in the cytoplasmic (C) or nuclear fraction (N).

Aconitase activity is sensitive to inhibitors including oxidative stress agents such as hydrogen peroxide and peroxynitrite when added to the assay buffer (Figure 6 and 7).

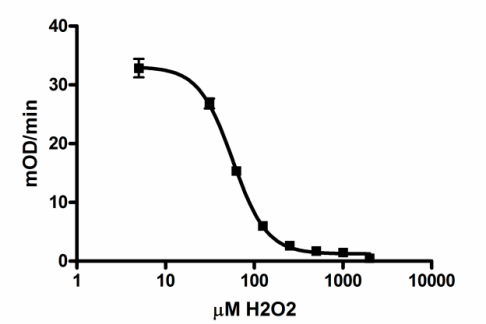

Figure 6. IC_{50} hydrogen peroxide

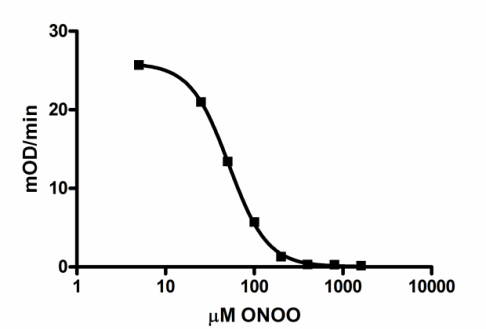

Figure 7. IC_{50} peroxynitrite

B. Reproducibility

Intra-Assay: CV < 10 %
Inter-Assay: CV < 15 %

9. Specificity

Species Reactivity: all species

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

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