Aconitase Assay Kit

Item No. 705502

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
705503	Aconitase Assay Buffer (10X)	1 vial
705511	Aconitase Positive Control	1 vial
705506	Aconitase Substrate Solution (10X)	1 vial
705507	Aconitase Cysteine Hydrochloride	1 vial
705508	Aconitase Ferrous Ammonium Sulfate	1 vial
705509	Aconitase $NADP^+$ Reagent	3 vials
705510	Aconitase Isocitric Dehydrogenase	3 vials
400014	96-Well Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -80°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance at 340 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Aconitase is an iron-sulfur protein containing a $[Fe_4S_4]^{2+}$ cluster that catalyzes the stereo-specific isomerization of citrate to isocitrate *via cis*-aconitate.[1] Aconitase functions in both the tricarboxylic acid (Krebs) and glyoxylate cycles. Unlike the majority of iron-sulfur proteins that function as electron carriers, the $[Fe_4S_4]^{2+}$ cluster of aconitase reacts directly with its substrate. In eukaryotes, there are two forms of the enzyme, a cytosolic (cAcn) and a mitochondrial aconitase (mAcn), which are encoded by two different genes.[2] In bacteria, there are also two forms, aconitase A (AcnA) and B (AcnB).[3] Eukaryotic aconitases contain the $[Fe_4S_4]^{2+}$ cluster which is required for their enzymatic activities. It has been shown that exposure of aconitase to oxidants, particularly superoxide and hydrogen peroxide, renders the enzyme inactive.[4,5] Loss of aconitase activity in cells or in biological samples treated with pro-oxidants has been interpreted as a measure of oxidative damage.[5-10] Oxidative damage during aging targets mitochondrial aconitase.[10] It has been demonstrated *in vitro* that reactivation of aconitase can occur upon removal of oxidants and reinsertion of a ferrous ion into the $[Fe_4S_4]^{2+}$ cluster.[11,12] Aconitase activity is reported to decline during cardiac ischemia/reperfusion events.[8]

About This Assay

Cayman's Aconitase Assay provides a simple, sensitive and reproducible means to assay aconitase activity from tissue homogenate or cell lysates. This assay measures the absorbance of NADPH at 340 nm, which is generated in the coupled reactions of aconitase with isocitric dehydrogenase (Figure 1). The rate at which NADPH is generated is proportional to the activity of aconitase.[13,14]

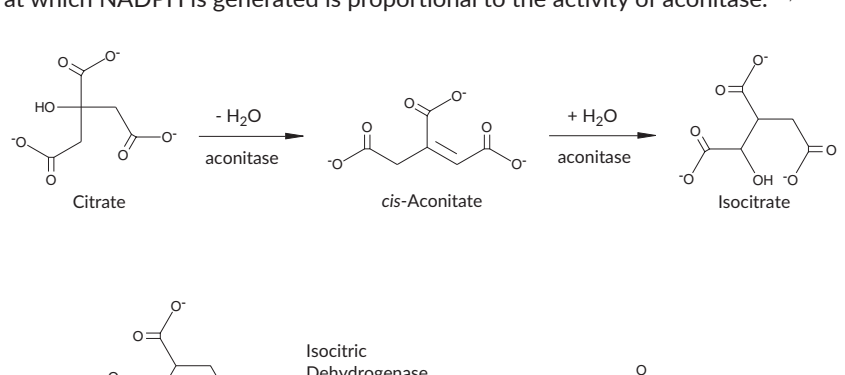

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Aconitase Assay Buffer (10X) - (Item No. 705503)**

   This vial contains 15 ml of concentrated buffer. Mix the 15 ml with 135 ml of HPLC-grade water. When stored at 4°C, this diluted assay buffer is stable for at least six months.

2. **Aconitase Positive Control - (Item No. 705511)**

   This vial contains bovine heart mitochondria. Mix 100 µl of Aconitase Positive Control with 900 µl of cold 1X Aconitase Assay Buffer. Sonicate for 20 seconds. Store the component on ice while preparing the reagents for the assay. Aliquot remaining undiluted Aconitase Positive Control and store it at -80°C to minimize the number of freeze/thaw cycles.

3. **Aconitase $NADP^+$ Reagent - (Item No. 705509)**

   Each vial contains lyophilized $NADP^+$. Immediately before use, reconstitute vial contents with 2 ml of HPLC-grade water. Once reconstituted, this solution is stable for <1 hour. One vial is sufficient to perform 32 reactions. Prepare additional vials as needed.

4. **Aconitase Isocitric Dehydrogenase - (Item No. 705510)**

   Each vial contains lyophilized Isocitric Dehydrogenase. Immediately before use, reconstitute vial contents with 2 ml of HPLC-grade water. Once reconstituted, this solution is stable for <1 hour. One vial is sufficient to perform 32 reactions. Prepare additional vials as needed.

5. **Aconitase Substrate Solution (10X) - (Item No. 705506)**

   This vial contains 2 ml of sodium citrate. Immediately before use, transfer 300 µl of the 10X Substrate Solution to a vial and add 2.7 ml of 1X Assay Buffer. The diluted Substrate Solution is sufficient to perform 32 reactions. Prepare additional substrate as needed.

Optional Reagent Preparation

If your aconitase sample requires activation, please follow the protocol below. The Aconitase Positive Control included in this kit does not require activation.

1. **Aconitase Cysteine Hydrochloride - (Item No. 705507)**

   This vial contains approximately 30 mg of cysteine hydrochloride. Dissolve 9 mg in 1 ml of 1X Assay Buffer. This 50 mM solution will be used to prepare the Activation Solution in #3.

2. **Aconitase Ferrous Ammonium Sulfate - (Item No. 705508)**

   This vial contains approximately 10 mg of ferrous ammonium sulfate. Dissolve 5 mg in 13 ml of 1X Assay Buffer. This 1 mM solution will be used to prepare the Activation Solution in #3.

3. **Aconitase Activation Solution**

   Using Table 1, below, prepare 10 ml of Activation Solution by adding the reagents in the order listed. This solution is used to "activate" some aconitase samples. The Aconitase Positive Control does not require activation. The Activation Solution is stable for two hours.

Reagent	Volume
1X Aconitase Assay Buffer	9.25 ml
50 mM Cysteine Hydrochloride	500 µl
1 mM Ferrous Ammonium Sulfate	250 µl

Table 1. Preparation of Aconitase Activation Solution

4. **"Activate" Aconitase**

   Mix 5 ml of the solution prepared in step 3 with 5 mg of aconitase sample. Incubate the Aconitase Solution for one hour on ice. After incubation, use the activated aconitase within 30 minutes.

Sample Preparation

Tissue Homogenate

Aconitase activity has been detected in the following tissues: heart, liver, and lung[16-19]

1. Weigh tissue and then mince into small pieces.
2. Homogenize in 5-10 ml of cold Assay Buffer per gram of tissue.
3. Centrifuge at 800 x g for 10 minutes at 4°C.
4. Sonicate the supernatant for 20 seconds.
5. If not assaying on the same day, freeze the supernatant at -80°C until use. The sample will be stable for one month.
6. Before performing the assay, further dilute the tissue to 500-1,000 µg/ml total protein with 1X Assay Buffer.

Tissue Mitochondrial Protein

1. Weigh tissue and then mince into small pieces.
2. Homogenize in 5-10 ml of cold Assay Buffer per gram of tissue.
3. Centrifuge the homogenate at 800 x g for 5 minutes at 4°C.
4. Centrifuge the supernatant at 10,000 x g for 10 minutes at 4°C.
5. Discard supernatant.
6. Resuspend the pellet in 0.5-1 ml of cold Assay Buffer and sonicate for 20 seconds.
7. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for one month.
8. Before performing the assay, further dilute the tissue to 500-1,000 µg/ml total protein with 1X Assay Buffer.

Cultured Cells

Aconitase is reported to be found in A549, AT-3, and PC-3 cells.[19-21]

1. Aspirate off media.
2. Add cold PBS and aspirate off to remove any residual medium.
3. Add enough cold PBS to cover cells.
4. Incubate cells at 4°C for 10 minutes.
5. Aspirate off PBS.
6. Add enough cold PBS to cover cells.
7. Scrape off cells with a scraper and add to centrifuge tube.
8. Centrifuge cells at 800 x g for 10 minutes at 4°C.
9. Discard supernatant.
10. Resuspend cell pellet in 1-2 ml of cold Assay Buffer.
11. Sonicate the cell suspension 20X at one second bursts.
12. Centrifuge cell suspension at 20,000 x g for 10 minutes at 4°C.
13. Aliquot supernatant and freeze at -80°C until use.
14. Resuspend pellet in cold Assay Buffer and freeze at -80°C until use.
15. Before assaying, further dilute the samples to 500-1,000 µg/ml total protein with 1X Assay Buffer.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of Aconitase Positive Control, Blank, and Samples to be measured in triplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BL	BL	BL	H	H	H	H	H	H	H	H	H
B	PC	PC	PC	H	H	H	H	H	H	H	H	H
C	S1	S1	S1	H	H	H	H	H	H	H	H	H
D	S1I	S1I	S1I	H	H	H	H	H	H	H	H	H
E	S2	S2	S2	H	H	H	H	H	H	H	H	H
F	S2I	S2I	S2I	H	H	H	H	H	H	H	H	H
G	S3	S3	S3	H	H	H	H	H	H	H	H	H
H	S3I	S3I	S3I	H	H	H	H	H	H	H	H	H

BL - Blank Wells
PC - Aconitase Positive Control
S1-S3 - Samples 1-3
S1I-S3I - Sample Backgrounds 1-3
H - Other Samples

Figure 2. Sample plate format

Pipetting Hints

- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 205 µl in all wells.
- We recommend assaying samples in the presence and absence of substrate.
- Use the diluted Assay Buffer in the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Measure the absorbance at 340 nm.

Performing the Assay

NOTE: If your samples require activation, please follow the protocol outlined on page 8. If you do not know whether your sample requires activation, it is advisable to test your samples with and without activation.

1. **Blank Wells** - Add 55 µl of Assay Buffer, 50 µl of $NADP^+$ Reagent, and 50 µl of Isocitric Dehydrogenase to three wells.
2. **Aconitase Positive Control Wells** - Add 50 µl of Aconitase Positive Control, 5 µl of Assay Buffer, 50 µl of $NADP^+$ Reagent, and 50 µl of Isocitric Dehydrogenase to three wells.
3. **Sample Wells** - Add 50 µl of Sample, 5 µl of Assay Buffer, 50 µl $NADP^+$ Reagent, and 50 µl Isocitric Dehydrogenase to three wells.
4. **Sample Background** - Add 50 µl of Sample, 50 µl of $NADP^+$ Reagent, 50 µl Isocitric Dehydrogenase, and 5 µl of Assay Buffer.
5. Initiate the reactions by adding 50 µl of the diluted Substrate Solution to all control and sample wells, 50 µl of Assay Buffer to Sample Background wells.
6. Measure the absorbance once every minute at 340 nm for 30 minutes at 37°C.

Wells	Blank	Positive Control	Sample	Sample Background
Sample	-	-	50 µl	50 µl
Aconitase Positive Control	-	50 µl	-	-
Aconitase Assay Buffer	55 µl	5 µl	5 µl	55 µl
Aconitase $NADP^+$ Reagent	50 µl	50 µl	50 µl	50 µl
Aconitase Isocitric Dehydrogenase	50 µl	50 µl	50 µl	50 µl
Aconitase Substrate Solution (At initiate step)	50 µl	50 µl	50 µl	-

Table 2. Pipetting summary

ANALYSIS

Calculations

Determination of the Reaction Rate

1. Determine the change in absorbance (ΔA_{340}) per minute:
   a) Plot the absorbance values as a function of time to obtain the slope (rate) of the liner portion of the curve (an example of the Aconitase Positive Control is shown in Figure 3 on page 16)

   OR

   b) Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340} = \left[\frac{A_{340}\,(\text{Time 2}) - A_{340}\,(\text{Time 1})}{\text{Time 2 (min.)} - \text{Time 1 (min.)}} \right]$$

2. Determine the rate ΔA_{340}/min. for the blank and subtract this rate from all the samples, including the Aconitase Positive Control, Sample, and Sample Background.
3. Use the following formula to calculate the aconitase activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00313 μM^{-1}.* One unit of aconitase will convert 1.0 nmol of citrate to isocitrate per minute at 37°C.

Aconitase Activity (nmol/min/ml) =

$$\left[\frac{\Delta A/\text{min. (sample)} - \Delta A/\text{min. (sample background)}}{0.00313\ \mu M^{-1}} \right] \times \frac{0.205\ \text{ml}}{0.05\ \text{ml}} \times \text{Sample dilution}$$

*The actual extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}\ cm^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.503 cm).

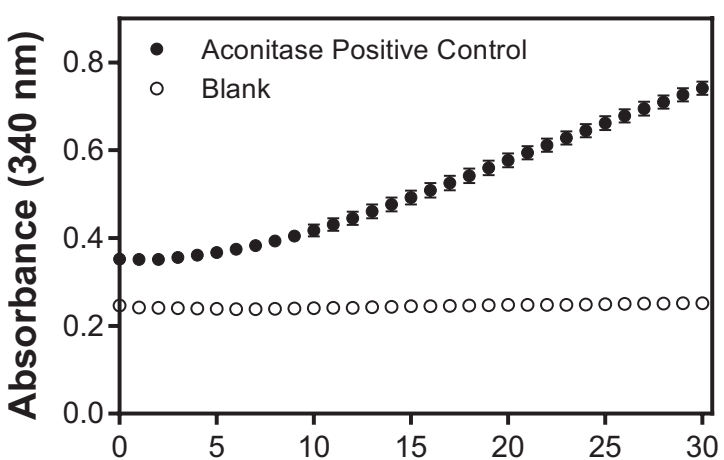

Figure 3. Activity of bovine heart mitochondrial aconitase

Performance Characteristics

Precision:

When a series of 22 aconitase measurements were performed on the same day, the intra-assay coefficient of variation was 9.5%. When a series of 15 aconitase measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 9.4%.

Sensitivity:

Samples containing aconitase activity as low as 1.7 µmol/min/ml can be assayed without further dilution or concentration.

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Borate	Yes
	HEPES	No
	PBS	No
Detergents:	Polysorbate 20 (1%)	No
	Triton X-100 (1%)	No
Chelators:	EDTA (1 mM)	Yes
	EGTA (1 mM)	Yes
Protease Inhibitors/ Enzymes:	Trypsin (10 µg/ml)	Yes
	PMSF (200 µM)	Yes
	Leupeptin (10 µg/ml)	Yes
	Antipain (0.1 mg/ml)	Yes
	Chymostatin (10 µg/ml)	Yes
Solvents:	DMSO (10 µl)	No
	Ethanol (10 µl)	No
	Methanol (10 µl)	No
Others:	Glycerol (10%)	Yes
	Sucrose (250 mM)	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No aconitase activity was detected in the sample	Sample was too dilute	Re-assay the sample using a lower dilution

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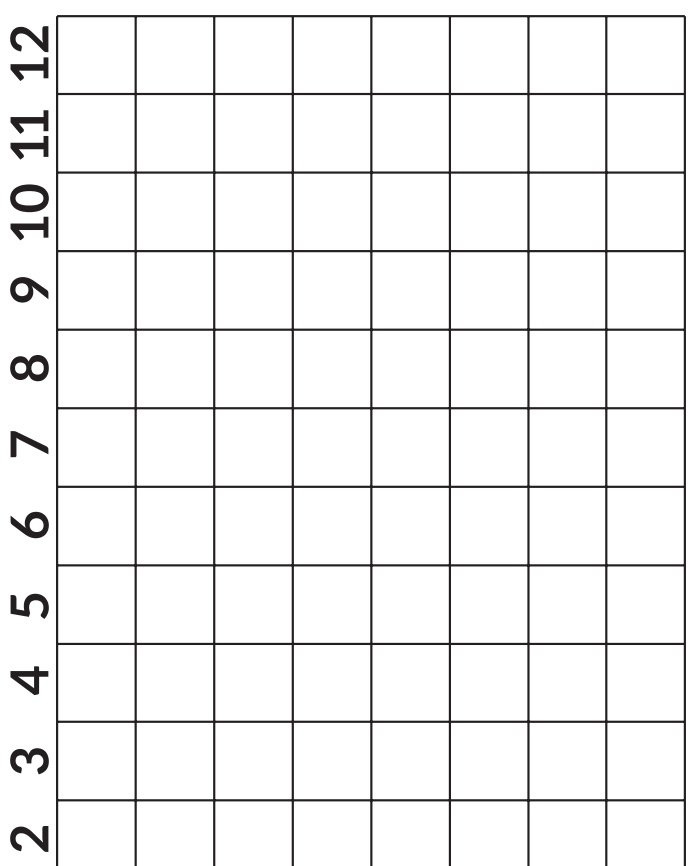

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.