CREATIVE BIOMART®
Assay Kit

Aconitase Assay Kit

Product Information

Common Name

Aconitase

Cat.No.

Kit-0043

Product Overview

Aconitase is an iron-sulfur protein containing a [Fe4S4] 2+ cluster that catalyzes the stereo-specific isomerization of citrate to isocitrate via cis-aconitate. Aconitase functions in both the tricarboxylic acid (Krebs) and glyoxylate cycles. Unlike the majority of iron-sulfur proteins that function as electron carriers, the [Fe4S4] 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. In bacteria, there are also two forms, aconitase A (AcnA) and B (AcnB). Eukaryotic aconitases contain the [Fe4S4] 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. Loss of aconitase activity in cells or in biological samples treated with pro-oxidants has been interpreted as a measure of oxidative damage. Oxidative damage during aging targets mitochondrial aconitase. It has been demonstrated in vitro that reactivation of aconitase can occur upon removal of oxidants and reinsertion of a ferrous ion into the [Fe4S4] 2+ cluster. Aconitase activity is reported to decline during cardiac ischemia/reperfusion events.

Description

Aconitase Assay Kit provides a simple, sensitive and reproducible means to assay aconitase activity from tissue homogenate or cell lysates. This assay measures the absorbance of NAD(P)H at 340 nm, which is generated in the coupled reactions of aconitase with isocitric dehydrogenase. The rate at which NAD(P)H is generated is proportional to the activity of aconitase.

Applications

Functional Studies

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Storage

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Kit Components

Aconitase Assay Buffer (10X) 1 vial
Aconitase Positive Control 1 vial
Aconitase Substrate Solution (10X) 1 vial
Aconitase Cysteine Hydrochloride 1 vial
Aconitase Ferrous Ammonium Sulfate 1 vial
Aconitase NADP+ Reagent 3 vials
Aconitase Isocitric Dehydrogenase 3 vials
96-Well Plate (Colorimetric Assay) 1 plate

Materials Required but Not Supplied

1. A plate reader capable of measuring absorbance at 340 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

Technical Notes

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.
- 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.
- It is recommended replicates be performed triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Measure the absorbance at 340 nm.

Preparation

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Reagent Preparation:
1. Aconitase Assay Buffer (10X)
This vial contains 15 ml of concentrated buffer. Dilute the content of the vial with 135 ml HPLC-grade water. When stored at 4°C, this diluted assay buffer is stable for at least six months.
2. Aconitase Positive Control
The vial contains approximately 10 mg of porcine heart aconitase. In a separate vial, weigh out 5 mg of aconitase and place vial on ice until the Activation Solution is prepared.
3. Aconitase Cysteine Hydrochloride
The 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 #5.
4. Aconitase Ferrous Ammonium Sulfate
The 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 #5.
5. Aconitase Activation Solution Using the table, prepare 10 ml of Activation Solution by adding the reagents in the order listed. This solution is used to "activate" the Aconitase Positive Control. The Activation Solution is stable for two hours.
Reagent Volume
1X 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
6. "Activate" Aconitase Add 5 ml of the solution prepared in step 5 to the tube of aconitase prepared in step 1 and mix until aconitase is dissolved. Incubate the Aconitase Solution for one hour on ice. After incubation, use the activated aconitase within 30 minutes. Aconitase activation is required each day the assay is performed.
7. Aconitase NADP+ Reagent
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.
8. Aconitase Isocitric Dehydrogenase

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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.
9. Aconitase Substrate Solution (10X)
The vial contains 2 ml of sodium citrate in Tris-HCl. 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.

Sample Preparation:
Tissue Homogenate
Aconitase activity has been detected in the following tissues: heart, liver, and lung.
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.

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Cultured Cells
Aconitase is reported to be found in A549, AT-3, and PC-3 cells.
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 to vials 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

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 - After the hour incubation, add 50 µl of "activated" aconitase, 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.

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Analysis

Determination of the Reaction Rate

1. Determine change in absorbance (ΔA340) per minute by:

a) Plot the absorbance values as a function of time to obtain the slope (rate) of the liner portion of the curve

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:

ΔA340 = [A340 (Time 2) - A340 (Time 1)]/[Time 2 (min.) - Time 1 (min.)]

2. Determine the rate ΔA340/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) = [ΔA/min. (sample) - ΔA/min. (sample background)]/0.00313 µM-1 x (0.205 ml/0.05 ml) x Sample dilu□on

*The actual extinction coefficient for NADPH at 340 nm is 0.00622 µM-1 cm-1.

This value has been adjusted for the pathlength of the solution in the well (0.503 cm).

Sensitivity

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

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