OxisResearch™
A Division of AOXRE LLC

BIOXYTECH® Aconitase-340™
Spectrophotometric Assay for Aconitase
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21041

INTRODUCTION

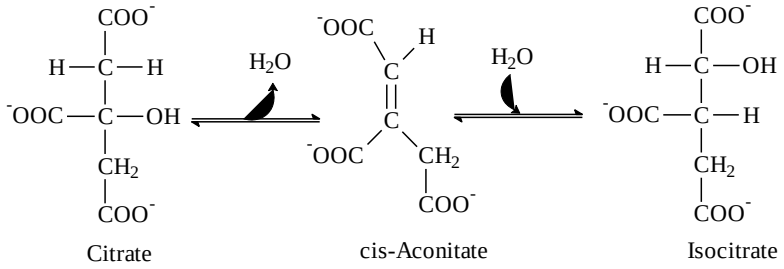
The Analyte

Aconitase is an iron-sulfur enzyme found in both the mitochondria and the cytosol. The catalytically active form of mitochondrial aconitase (citrate-isocitrate hydratase, EC4.2.1.3), which is part of the citric acid cycle, contains a [4Fe-4S] cluster interacting with carboxyl and hydroxyl groups of substrates. In this cluster, three Fe atoms directly bind to the cysteines of the enzyme backbone; the fourth Fe is ligated to sulfur of the inactive [3Fe-4S] cluster. This iron-sulfur ligation provides a free coordination site that is involved in the binding of substrates to the active site of aconitase.[1-2] Cytosolic aconitase is a bifunctional enzyme. The holo [4Fe-4S] form has aconitase activity, while the apo (iron-free) form, known as iron regulatory protein (IRP-1), lacks enzymatic activity but regulates intracellular iron levels.

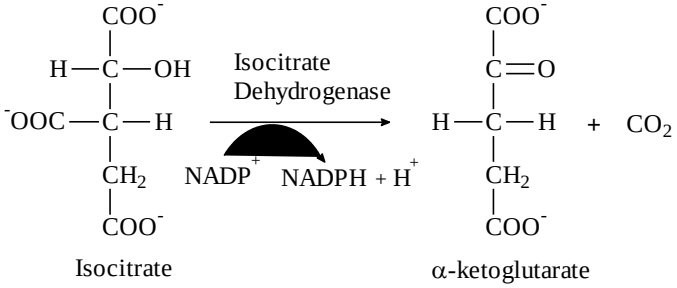
Both mitochondrial and cytosolic aconitases are targets of oxidants in cells due to the oxidant-mediated loss of Fe from the [4Fe-4S] cluster. Various oxidants, including superoxide, hydrogen peroxide, nitric oxide, peroxynitrite, *etc*., can attack the [4Fe-4S] cluster and inactivate aconitase. This can diminish energy production in mitochondria and result in excessive iron uptake by the cell, due to the activation of IRP-1 in the cytosol. Aconitase activity has been measured in various biological tissues such as heart, liver, lung, renal cortical tissue, and prostate epithelial cells. Studies have indicated that aconitase is a sensitive marker of oxidative damage during aging, Parkinson's disease and iron deficiency anemia.[2-4] The Aconitase-340™ assay measures enzymatic activity and not iron-binding capability.[6]

Principle of the Assay

Aconitase belongs to the class of hydratase enzymes, catalyzing the isomerization of citrate to isocitrate via the intermediate, cis-aconitate.



Citrate is isomerized by aconitase into isocitrate, enabling the six-carbon unit to undergo oxidative decarboxylation, catalyzed by isocitrate dehydrogenase, to form α-ketoglutarate. The BIOXYTECH® Aconitase-340™ Assay is based on measurement of the concomitant formation of NADPH from $NADP^+$.



The formation of NADPH is monitored by the increase in absorbance at 340nm. Under appropriate conditions, the rate of NADPH production is proportional to aconitase activity.[3,5] The concentration of aconitase is expressed in units of activity.

One aconitase unit will convert 1.0 µmol of citrate to isocitrate per minute at 25°C and pH 7.4.[2] The increase in isocitrate is equal to the consumption of $NADP^+$, measured as the increase in absorbance at 340 nm. Because the Aconitase-340™ assay is carried out at 37°C, OXIS has determined a temperature coefficient to correct for increased aconitase activity at 37°C. Please note that in the citric acid cycle, NAD^+ is reduced to NADH. However, the use of $NADP^+$ significantly improves assay performance.

REAGENTS

Materials Provided (Sufficient for 100 Tests)

- Assay Buffer — Tris·HCl, pH 7.4, 50 mL.
- Substrate — Trisodium Citrate in Tris·HCl, pH 7.4, 25 mL.
- Enzyme — Isocitrate Dehydrogenase with Tris·HCl, Lyophilized, 3 vials.
- NADP Reagent — $NADP^+$ and Tris·HCl, Lyophilized, 4 vials.

Items Required But NOT Provided

- Spectrophotometer.
- Centrifuge, 20,000 *g* or greater.
- Centrifuge tubes.
- Homogenizer.
- Sonicator.
- Spectrophotometric cuvettes with a 1 cm optical path length.
- Pipettes, preferably adjustable, capable of accurately pipetting 100 to 1000 µL.
- Pipet tips.

Reagent Storage and Handling

Do not allow the reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 2-8°C. Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

- NADP Reagent: Just prior to use, reconstitute the NADP Reagent with 5.5 mL deionized H_2O. Use the same day.
- Enzyme: Reconstitute Enzyme Reagent with 10 mL deionized water. Store at 4°C.

Sample Preparation Guidelines

- Whole Tissue Samples:
    1. Weigh tissue sample.
    2. Mince tissue.
    3. Homogenize at 1%(w/v) in ice-cold 0.2 mM sodium citrate in 50 mM Tris-HCl, pH 7.4, for 15-20 seconds.
    4. Centrifuge at 800 *g* for 10 min at 4°C.
    5. Freeze supernatant at -70°C until use.
    6. Thaw the homogenate.
    7. Sonicate the homogenate for 20 seconds.
    8. Before the assay, further dilute the tissue extract to 50-500 µg/mL with Assay Buffer.

- Mitochondrial Fraction Samples:
    1. Weigh tissue sample.
    2. Mince tissue.
    3. Homogenize at 2-3%(w/v) in ice-cold 0.2 mM sodium citrate in 50 mM Tris-HCl, pH 7.4, for 15-20 seconds.
    4. Centrifuge homogenate at 800 *g* for 10 minutes at 4°C.
    5. Centrifuge supernatant at 20,000 *g* for 10 min at 4°C.
    6. Discard supernatant.
    7. Resuspend pellet in ice-cold 0.2 mM sodium citrate.
    8. Proceed with steps 5-8 as described above for whole tissue samples.

Assay

1. Add 200 µL of Sample or Assay Buffer (Blank) to a cuvette.
2. Add 200 µL of Substrate to the cuvette.
3. Add 200 µL of Enzyme to the cuvette.
4. Add 200 µL of NADP to the cuvette.
5. Mix using pipette.
6. Incubate at 37°C for 15 minutes protected from light.
7. Begin recording absorbance change at 340 nm for 5 minutes at 37°C.

Calculations

1. Calculate the rate of increase in the A_{340} per minute. Note: The ΔA_{340}/minute must be linear over the 5 minute interval.
2. Determine the Net Rate by subtracting the rate of the Blank from the Sample rates.
3. The molar extinction coefficient, ε, for NADPH is 6220 $M^{-1}cm^{-1}$ and the temperature correction coefficient, c, is 2.4435.

Given: ε = 6220 $M^{-1}cm^{-1}$ or if pathlength = 1 cm;
$\varepsilon = 6.220x10^{-3}$ mL/nmol, and;
mU = nmol/min, and;
c = 2.4435, and;
d = Assay dilution = 4

Then:
$$mU = \frac{\text{Net } A_{340}/\text{min}}{c \cdot \varepsilon} \cdot d$$

4. The final concentration of aconitase may be normalized to the protein concentration of the sample.

Example

A sample, containing 8.8 mg/mL protein was diluted 1/10 in Assay Buffer and assayed as described above.

Sample Rate	0.0951 A_{340}/min
Blank Rate	0.0001 A_{340}/min
Net Rate	0.0950 A_{340}/min
Aconitase Activity	$\left(\frac{0.0950}{6.22x10^{-3}\times 2.4435}\right)\times 4 = 25.0$ mU/mL
Sample Dilution Correction	10 x 25.0 mU/mL= 250 mU/mL
Protein Correction	$\frac{250\ \text{mU/mL}}{8.8\ \text{mg/mL}} = 28.4$ mU/mg

PERFORMANCE CHARACTERISTICS

Precision

The precision of the assay was estimated by assaying aconitase controls (1.7 and 6.8 mU/mL aconitase in Assay Buffer) and a rat lung extract sample, all stored at -70°C, in duplicate twice a day for two weeks.

	1.7 mU/mL	6.8 mU/mL	Rat Lung
Mean ($\Delta A_{340/min}$)	0.0052	0.0254	0.0049
Intra-assay precision (%CV)	5.11	1.63	3.64
Inter-assay precision (%CV)	4.82	3.53	8.63
Total precision (%CV)	6.00	3.71	9.01

Sensitivity - Lower Limit of Detection (LLD)

The LLD is a measure of the lowest concentration distinguishable from zero with confidence. The absorbance rate for an Assay Buffer blank was measured 18 times. The concentration equivalent to the rate at 3.29 standard deviations from the mean was determined to give the LLD (~99.5% confidence).

Number of data points	18
Mean ΔA_{340}/min	-0.0005
Standard Deviation	0.0002

LLD in mU/mL (Reaction Mixture) 0.2

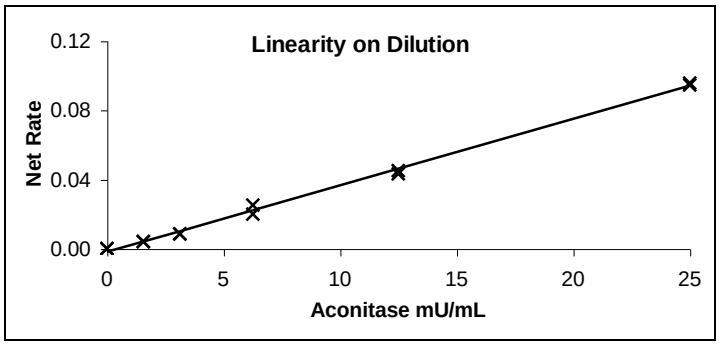
Assay Range

The range of the assay is defined from the LLD to the highest concentration of aconitase that can be added to the reaction mixture and maintain linearity. The range is 0.2 - 25 mU/mL in the reaction mixture.

Accuracy

Linearity on Dilution

Aconitase was diluted in Assay Buffer to several concentrations and the rates determined. The assay is linear (R^2 = 0.9971) up to an aconitase concentration of 25 mU/mL in the original sample.



Addition Recovery

The Aconitase-340™ Assay is linear on dilution; therefore, a single point addition is adequate to demonstrate recovery. The average recovery of aconitase from Assay Buffer spiked with 2.57 mU/mL was determined. Six lung extract samples were measured for aconitase and spiked with 2.57 mU/mL aconitase, then reassayed. The recovery of the added aconitase was determined.

Sample	Aconitase	Net Aconitase	%Recovery
Buffer + 5 mU Aconitase	2.52 ± 0.070		98.0
Lung Alone	3.34 ± 0.055		
Lung + 5 mU Aconitase	5.79 ± 0.128	2.46	95.7

Specificity

Possible interferents were tested in a buffer solution containing 2.57 mU/mL aconitase. The interference is calculated as the percentage difference between the measured concentration and the actual aconitase concentration.

Test Compound	Added Concentration		Measured Aconitase	Percent Interference
Glucose	5	mM	2.58	0.4
Human Albumin	12	mg/mL	2.36	-8.2
Hemoglobin A_0	15	mg/mL	2.37	-7.8
Vitamin C	100	µM	2.50	-2.7
Bilirubin	50	µM	2.36	-8.2
Glutathione	1	mM	2.65	3.1
Homocysteine	20	µM	2.40	-6.6
Cysteine	100	µM	2.63	2.3
Sucrose	100	mM	2.40	-6.6
Dithiothreitol	100	µM	1.50	-41.6
Penicillamine	100	µM	2.47	-3.9
Mercaptopropinylglycine	100	µM	1.78	-30.7
Acetylcysteine	100	µM	2.64	2.7
Captopril	100	µM	2.20	-14.4
Acetaminophen	100	µM	2.31	-10.1
Ibuprofen	100	µM	1.90	-26.1
EDTA	100	µM	1.82	-29.2
DTPA	100	µM	1.97	-22.6
Heparin	14	U/mL	2.32	-9.7
$CuSO_4$	50	µM	0.55	-78.6
$ZnSO_4$	50	µM	3.27	27.2

$MnCl_2$	50 µM	2.77	7.8

REFERENCES

1. Gardner, PR, (1997) Superoxide-Driven Aconitase Fe-S Center Cycling, *Bioscience Reports*, **17**: 33-42.
2. Gardner, PR and Fridovich, I, (1992) Inactivation-Reactivation of Aconitase In *E Coli*: A Sensitive Measure of Superoxide Radical, *J. Biol. Chem.* **267**: 8757-8763.
3. Kennedy, MC, *et. al.*, (1997) An EPR Investigation of the Products of the Reaction of Cytosolic and Mitochondrial Aconitases with Nitric Oxide, *J. Biol. Chem.* **272**: 20340-20347.
4. Yan, LJ, *et. al.*, (1997) Oxidative Damage During Aging Targets Mitochondrial Aconitase, *PNAS*, **94**: 11168-11172.
5. Rose, IA and O'Connell, EL, (1967) Mechanism Of Aconitase Action, *J. Biol. Chem.* **242**: 1870-1879.
6. Hentz, MW and Kühn, LC, (1996) Molecular Control of Vertebrate Iron Metabolism: mRNA based Regulatory Circuits Operated by Iron, Nitric Oxide and Oxidative Stress, PNAS, **93**:8175-8182.

*Oxis*Research™

1499 Rollins Road
Burlingame, CA 94010 U.S.A.
E-mail: info@aoxre.com
Telephone: 650-289-8908
www.aoxre.com

Last revision: May 2019
Made in the U.S.A.