Aconitase Activity Colorimetric Assay Kit (#BN00936)

(Catalog #BN00936; 100 reactions; Store kit at 4° C)

I. Introduction:

Aconitase (aconitate hydratase; EC 4.2.1.3) is an iron-sulfur protein containing an $[Fe_4S_4]^{2+}$ cluster that catalyzes the stereospecific isomerization of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle, a non-redox-active process. Tissue contains two aconitases, a mitochondrial (m-) and a cytosolic (c-) aconitase. They are related, but distinctly different enzymes and are coded for on different chromosomes. Loss of aconitase activity in cells or other biological samples treated with pro-oxidants has been interpreted as a measure of oxidative damage. Assay Genie's Aconitase Assay Kit is a highly sensitive, simple, direct and HTS-ready colorimetric assay for measuring Aconitase activity in biological samples. In the assay, citrate is converted by aconitase into isocitrate, which is further processed resulting in a product that converts a nearly colorless probe into an intensely colored form (λ = 450nm).

II. Kit Contents:

Component	BN00936	Cap Color	Part Number
Assay Buffer	25 ml	WM	BN00936-1
Substrate (lyophilized)	1 vial	Blue	BN00936-2
Developer (lyophilized)	1 vial	Purple	BN00936-3
Enzyme Mix	200 µl	Green	BN00936-4
Cysteine-HCl (lyophilized)	1 vial	Red	BN00936-5
$(NH_4)_2Fe(SO_4)_2$ (lyophilized)	1 vial	Brown	BN00936-6
Isocitrate Standard (100 mM)	100 µl	Yellow	BN00936-7

III. Storage and Handling:

Store the kit at 4° C, protected from light. Warm the assay buffer to room temperature before use. Briefly centrifuge vials before opening. Read the entire protocol before performing the assay.

IV. Reagent Reconstitution and General Consideration:

Substrate: Dissolve with 220 µl ddH_2O; sufficient for 100 assays. Store at 4° C.

Developer: Dissolve with 1.1 ml Assay Buffer before use; sufficient for 100 assays. Store at 4° C.

Aconitase Activation Solution: Dissolve cysteine-HCl and $(NH_4)_2Fe(SO_4)_2$ with 0.5 ml Assay Buffer separately, and store at -20° C. Use within one month. Take out 0.1 ml cysteine-HCl and $(NH_4)_2Fe(SO_4)_2$ solutions and mix together to prepare fresh activation solution.
Ensure that the Assay Buffer is at room temperature before use. Keep samples, Enzyme Mix and Aconitase solution on ice during the assay.

V. Aconitase Activity Assay:

1. Sample Preparations:

Homogenize 20 - 40 mg fresh tissue or 10^6 recently harvested cells on ice in 0.1 ml cold Assay Buffer; Centrifuge at 800 x g for 10 min at 4°C; Collect the supernatant for c-aconitase assay. For m-aconitase assay, centrifuge the supernatant at 20,000 x g for 15 min at 4°C and collect the pellet, dissolve into 0.1 ml cold Assay Buffer, sonicate for 20 sec. Keep samples at -80° C for storage.
Add 10 µl activation solutions to 100 µl sample; incubate on ice for 1 hr to activate aconitase in the sample.
Add 2 – 50 µl activated samples into each well, and adjust volume to 50 µl. We suggest using a background control group as well as several doses of your sample to ensure the readings are within the linear range.

2. Isocitrate Standard Curve:

Dilute 10 µl with 490 µl assay buffer to prepare 2 mM isocitrate standard solution. Add 0, 2, 4, 6, 8, 10µl 2 mM Isocitrate Standard solution into 96-well plate in duplicate to generate 0, 4, 8, 12, 16, 20 nmol/well Isocitrate standard. Bring the final volume to 50 µl with Assay Buffer.

3. Reaction Mix:

Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50 µl Reaction Mix:

Sample Reaction Mix	Background Mix
46 µl Assay Buffer	48 µl Assay Buffer
2 µl Enzyme Mix	2 µl Enzyme Mix
2 µl Substrate	

Add 50 µl of the Sample Reaction Mix to each test samples, background control and Isocitrate standards. Mix well and incubate at 25° C for 30-60 min. Add 10µl Developer to each well, mix and incubate at 25° C for 10 min. Measure OD 450nm.

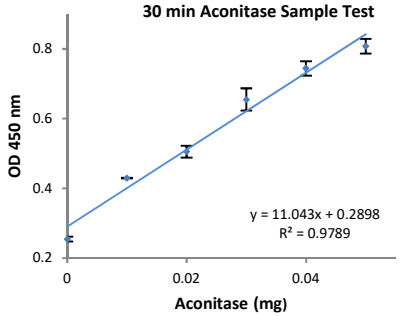
4. Calculation:

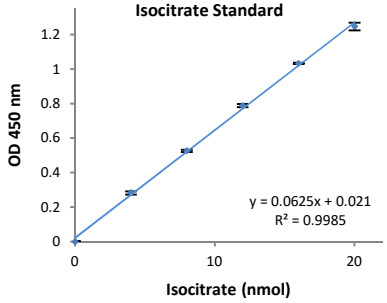
Plot the Isocitrate standard curve. $\Delta OD = OD_{sample} - OD_{background}$, apply the ΔOD to the Isocitrate standard curve to get B nmol of isocitrate generated by aconitase in 30 – 60 min.

$$\text{Aconitase Activity} = \frac{B}{T \times V} \times \text{Sample Dilution Factor} = \text{nmol/min/ml} = \text{mU/ml}$$

Where: **B** is the isocitrate amount from Standard Curve (in nmol)
T is the time incubated (in min)
V is the pretreated sample volume added into the reaction well (in ml)

Unit definition: One unit of Aconitase is the amount of enzyme that will isomerize 1.0 µmol of Citrate to Isocitrate per min at pH 7.4 at 25° C.





FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		