ab83459

Aconitase Assay Kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of aconitase activity in various samples.

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

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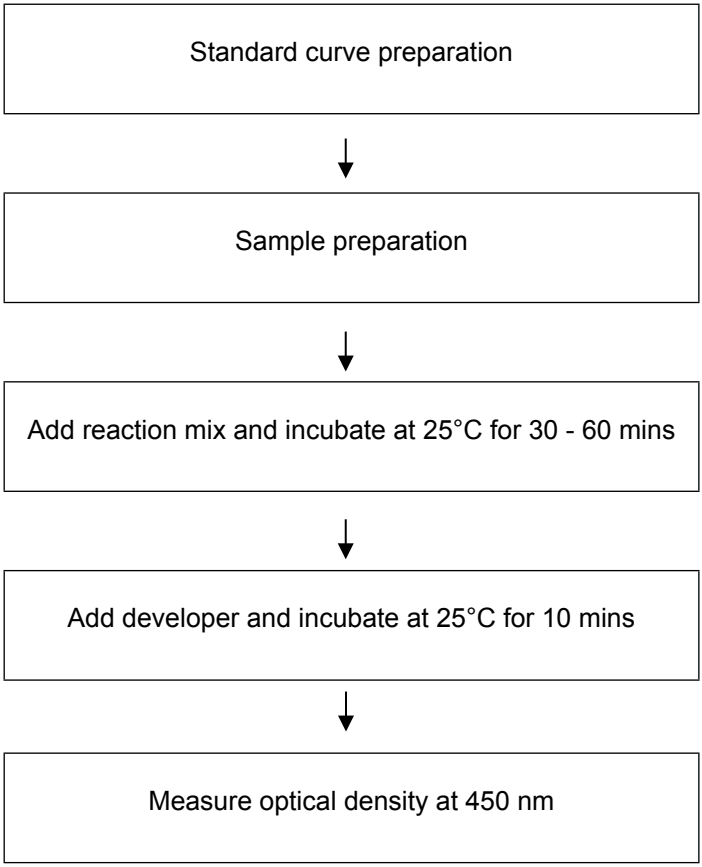
1. BACKGROUND

Aconitase Assay Kit (colorimetric) (ab83459) 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 with a λ_{max} at 450nm.

Aconitase (aconitate hydratase) 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.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 4ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 1 month.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer	25 mL	4°C	4°C
Substrate (Lyophilized)	1 vial	4°C	4°C
Developer (Lyophilized)	1 vial	4°C	4°C
$(NH_4)_2Fe(SO_4)_2$ (Lyophilized)	1 vial	4°C	-20°C
Isocitrate Standard (100 mM)	100 µL	4°C	4°C
Cysteine-HCl (Lyophilized)	1 vial	4°C	-20°C
Enzyme Mix	600 µL	4°C	4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Colorimetric microplate reader – equipped with filter for OD450 nm
- 96 well plate (clear plates)
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.2 **Substrate:**

Reconstitute with 220 µL of ddH_2O. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at 4ºC.

9.3 **Developer:**

Reconstitute with 1.1 mL of Assay Buffer. Aliquot so that you have enough volume to perform the desired number of assays. Store at 4ºC.

9.4 **$(NH_4)_2Fe(SO_4)_2$:**

Reconstitute with 500 µL of Assay Buffer. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20ºC. Use within 1 month.

9.5 **Isocitrate Standard:**

Ready to use as supplied. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at 4°C. Keep on ice during use.

9.6 **Cysteine-HCL:**

Reconstitute with 500 µL of Assay Buffer. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20ºC. Use within 1 month.

9.7 **Enzyme Mix:**

Ready to use as supplied. Aliquot enzyme mix so that you have enough volume to perform the desired number of assays. Store at 4°C. Keep on ice during use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 Prepare a 2 mM Isocitrate Standard by adding 10 µL Isocitrate Standard into 490 µL Assay Buffer.

10.2 Using 2 mM standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End [Isocitrate] in well
1	0	150	50	0 nmol/well
2	6	144	50	4 nmol/well
3	12	138	50	8 nmol/well
4	18	132	50	12 nmol/well
5	24	126	50	16 nmol/well
6	30	120	50	20 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples – for c-aconitase:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of cold Assay Buffer.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge sample for 10 minutes at 4°C at 800 *x g* using a microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.2 Cell (adherent or suspension) samples – for m-aconitase:

11.2.1 Follow steps 11.1.1 to 11.1.4.

11.2.2 Centrifuge sample for 15 minutes at 4°C at 20,000 *x g* using a microcentrifuge and collect the pellet.

11.2.3 Resuspend pellet in 100µL cold Assay buffer, and sonicate for 20 seconds.

11.2.4 Keep on ice or store samples at -80°C.

11.3 **Tissue samples – for c-aconitase:**

11.3.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 20 – 40 mg).

11.3.2 Wash tissue in cold PBS

11.3.3 Resuspend tissue in 100 µL of ice cold Assay Buffer.

11.3.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.3.5 Centrifuge sample for 10 minutes at 4°C at 800 *x g* using a microcentrifuge to remove any insoluble material.

11.3.6 Collect supernatant and transfer to a clean tube.

11.3.7 Keep on ice.

11.4 **Tissue samples - for m-aconitase:**

11.4.1 Follow steps 11.3.1 to 11.3.4.

11.4.2 Centrifuge sample for 15 minutes at 4°C at 20,000 *x g* using a microcentrifuge and collect the pellet.

11.4.3 Resuspend pellet in 100 µL cold Assay buffer, and sonicate for 20 seconds.

11.4.4 Keep samples at -80°C for storage.

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12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 **Aconitase Activation Solution:**

- Add 100 µL of cysteine HCL to 100 µL of $(NH_4)_2Fe(SO_4)_2$ and mix together to prepare fresh activation solution.

12.2 Add 10 µL activation solution to 100 µL sample.

12.3 Incubate on ice for 1 hour to activate aconitase in the sample. Use this activated sample in the following steps.

12.4 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 2 – 50 µL activated samples (adjust volume to 50 µL/well with Assay Buffer).
- Sample Background wells = 2 – 50 µL activated samples (adjust volume to 50 µL/well with Assay Buffer).
- Background wells (blank control) = 50 µL Assay Buffer.

12.5 **Reaction Mix:**

Prepare Reaction Mix for each reaction:

Component	Colorimetric Reaction Mix (µL)	Background Reaction Mix (µL)
Assay Buffer	**42**	**44**
Enzyme Mix	**6**	**6**
Substrate	**2**	**0**

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + Standards +1)

12.6 Add 50 µL of appropriate Reaction Mix to each well except sample background wells. In sample background wells add 50µl background reaction mix.

12.7 Incubate at 25ºC for 30 - 60 minutes.

12.8 Add 10 µL Developer to each well.

12.9 Mix and incubate at 25ºC for 10 minutes.

12.10 Measure output on a microplate reader.

- Colorimetric assay: measure OD450 nm.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard, sample readings and sample background control. This is the corrected absorbance.

13.3 Subtract the sample background from sample readings if applicable.

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of Isocitrate.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 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.

13.7 Activity of aconitase in the test samples is calculated as:

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

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 1.0µmol of Citrate to Isocitrate per min at pH 7.4 at 25 °C.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

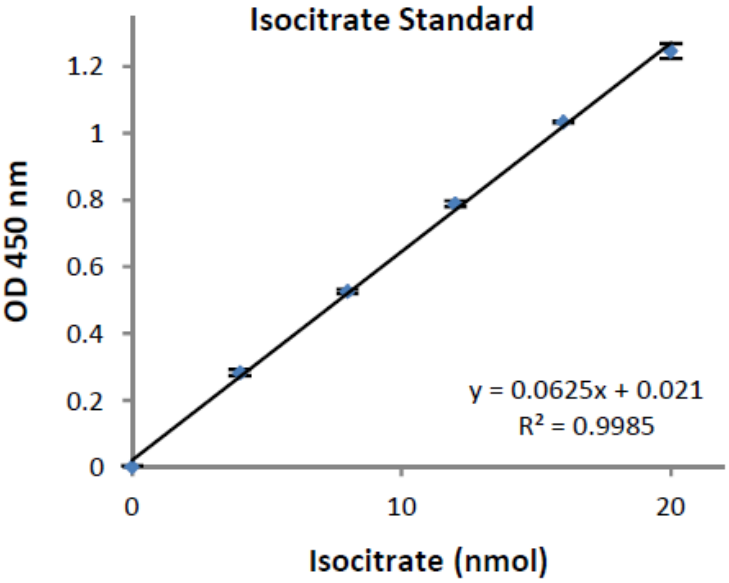

Figure 1: Typical isocitrate standard calibration curve using colorimetric reading.

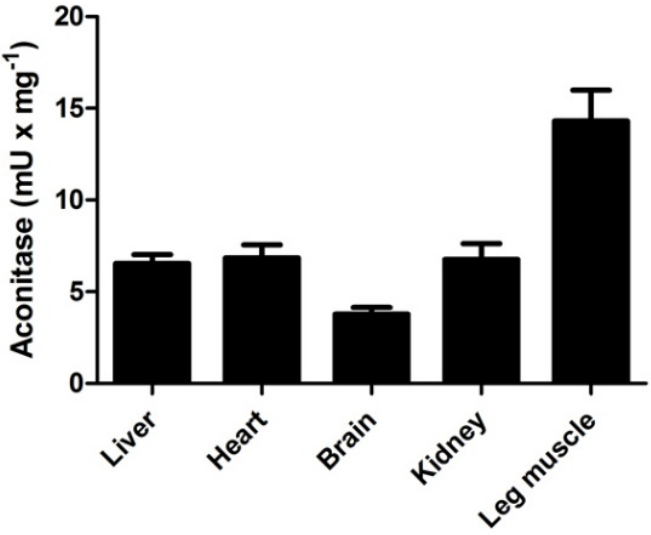

Figure 2: Aconitase measured in mouse tissue lysates showing quantity (mU) per mg of tested sample.

15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare substrate, developer and activation solutions (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Add 10 µL activation solution to 100 µL sample.
- Incubate on ice for 1 hr to activate aconitase in the sample.
- Set up plate for standard (50 µL), activated samples (50 µL) and activated sample background wells (50 µL).
- Prepare Reaction Mix (Number samples + standards + background control samples +1)

Component	Colorimetric Reaction Mix (µL)	Background Reaction Mix (µL)
Assay Buffer	**46**	**48**
Enzyme Mix	**2**	**2**
Substrate	**2**	**0**

- Add 50 µL of Reaction Mix to each well.
- Incubate 25ºC 30 - 60 mins.
- Add 10 µL Developer to each well.
- Incubate plate 25ºC 10 mins.
- Measure plate at OD450 nm for colorimetric assay.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. **FAQ**

18. **INTERFERENCES**

19. **NOTES**

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