MitoCheck® Complex II/III Activity Assay Kit

Item No. 700950

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Customer Service 800.364.9897
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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
700951	Mitochondrial Complex III Activity Assay Buffer	2 vials/10 ml	-20°C
700952	Cytochrome *c* Assay Reagent	1 vial/3 mg	-20°C
700019	Bovine Heart Mitochondria Assay Reagent	1 vial/100 µl	-80°C
700021	Succinate Assay Reagent	1 vial/100 µl	-20°C
700020	Half Volume 96-Well Clear Plate	1 plate	RT

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.

NOTE: It is recommended that gloves be worn at all time when working with isolated mitochondria and mitochondrial inhibitors.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

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 as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance a 550 nm at 30 second intervals
2. Adjustable and multichannel pipettes
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Mitochondrial Inhibitors - Rotenone, TTFA, Potassium Cyanide, or Antimycin A
5. 0.1 M NaOH

INTRODUCTION

Background

Complex III (CoQ cytochrome *c* oxidoreductase) is an essential protein for mitochondrial oxidative phosphorylation. Complex III functions as both a gatekeeper for mitochondrial respiration and as a major source of reactive oxygen species III.[1,2] Complex III accepts electrons from complexes I and II in the form of QH_2, the reduced form of the electron carrier ubiquinone. Once bound to complex III, QH_2 undergoes a series of redox reactions, known as the Q-cycle. During the Q-cycle, electrons are passed from QH_2 onto cytochrome *c* *via* the Rieske iron-sulfur protein and cytochrome c_1 resulting in the translocation of $4H^+$ and the generation of $O_2^{\bullet-}$.[3] This assay is designed to measure the complex III-dependent reduction of cytochrome *c*. Because the mechanism is coupled to complex II, it is also sensitive to complex II inhibitors. It is therefore recommended that all potential complex III inhibitors be pre-screened against complex II. (See Cayman's MitoCheck® Complex II Activity Assay Kit (Item No. 700940) for more details.)

About This Assay

Cayman's MitoCheck® Complex II/III Activity Assay measures the reduction of excess cytochrome *c* (550 nm absorbance) as catalyzed by complex III. This assay is coupled to succinate co-enzyme Q oxidoreductase (complex II) for the generation of QH_2. Due to the dependence on complex II activity, a counter-screen for complex II activity (Item No. 700940) should be performed in order to truly measure complex III activity. Cayman's Mitochondrial Complex II/III Activity Assay allows for the activity of complexes II/III to be determined without the need to isolate mitochondria or pre-incubate with antibodies. Since electrons can flow backwards through complex I and cytochrome *c* is readily reduced by complex IV, it is recommended that rotenone and potassium cyanide (not supplied) are present throughout the assay.[2,4,5] (Rotenone and potassium cyanide are not supplied.) For the use of this kit with other types of tissue mitochondria, please see Cayman's MitoCheck® Mitochondrial (Tissue) Isolation Kit (Item No. 701010).

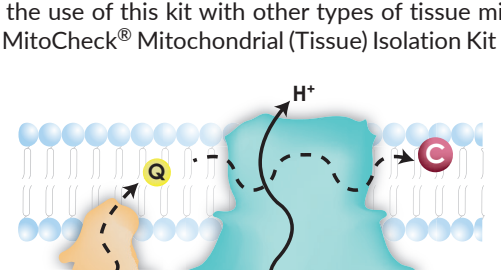


Figure 1. The coupled reaction of complex II and III as measured by this assay kit.

PRE-ASSAY PREPARATION

Reagent Preparation

All assay reagents, unless listed below, are ready to use as supplied.

1. **Mitochondrial Complex III Activity Assay Buffer - (Item No. 700951)**

   This buffer is ready to use as supplied. It is important that the buffer is warmed to room temperature prior to use. Additionally, vortex well to be sure that any crystals that may have precipitated have dissolved.

2. **Mitochondrial Inhibitors - *(Not Supplied)***

   1. **Potassium Cyanide (KCN)** - KCN should be present to inhibit the ETC (complex IV) and prevent the oxidation of Q. It is important **that extreme care is taken when preparing and using KCN**. Protocol: In a **ventilated hood**, weigh out 6.5 mg of KCN and dissolve in 1 ml of 0.1 M NaOH to yield a 100 mM stock solution of KCN. **Do not use water or any acidic solvents to make up KCN.** Store stock solution on ice and make fresh less than three hours prior to running this assay. Use appropriate personal protective equipment (PPE).
   2. **Rotenone (Item No. 13995)** - to ensure inhibition of complex I, use concentrations ≥1 µM. Can be made up in DMSO or ethanol. If making up in DMSO, avoid freeze/thaws. Use appropriate PPE.
   3. **Antimycin A** - to ensure inhibition of complex III, use concentrations ≥10 µM. Can be made up in DMSO or ethanol. Use appropriate PPE.
   4. **2-Thenoyltrifluoroacetone (TTFA)** - to ensure inhibition of complex II, use concentrations ≥1 mM. Can be made up in DMSO or ethanol. Use appropriate PPE.

ASSAY PROTOCOL

Pipetting Hints

- Use different tips to pipette each reagent.
- Avoid introducing bubbles into the well.
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicates preferred).
- The assay is performed in the kinetic read mode at 25°C.
- Monitor the absorbance at 550 nm every 30 seconds for 15 minutes.

Performing the Assay

Label two polystyrene tubes as A and B and add the following reagents. *Isolated mitochondria can settle over time, so make sure contents of each tube are well mixed. Store tubes on ice until ready to use. Volumes indicated below are suitable for 20 reactions (or wells). Customer may scale volumes as needed.*

Tube A (1 ml)	Tube B (675 µl)
958 µl of Complex III Activity Assay Buffer	607 µl of Complex III Activity Assay Buffer
20 µl Bovine Heart Mitochondria Assay Reagent	8 µl of Succinate Assay Reagent
2 µl of 1 mM Rotenone *not supplied*	60 µl Cytochrome *c* Assay Reagent
20 µl of 100 mM KCN (1 mM) *not supplied*	

Table 1. Assay preparation

All assays are carried out at 25°C.

1. Add 50 µl of the contents of tube A to each well.
2. Add 20 µl of compound, positive control, or vehicle to each well. Allow for pre-incubation if required.
3. Add 30 µl of the contents of tube B to each well. This should be done quickly as the reaction will start immediately.
4. Immediately place plate in plate reader and measure absorbance at 550 nm (30 second intervals for 15 minutes at 25°C).

ANALYSIS

Calculations

1. Plot data as absorbance (y-axis) *versus* time (x-axis).
2. To determine the reaction rate, calculate the slope for the linear portion of the curve.
3. Determine % activity relative to the vehicle control using the equation indicated below.
4. To determine an IC_{50} value for each compound, plot the slope as a function of test compound concentration.

$$\text{Complex II/III Activity (\%)} = \left[\frac{\text{Rate of Sample wells}}{\text{Rate of Vehicle Control}}\right] \times 100$$

Performance Characteristics

The data shown below are an example of data obtained with this kit. Your results will not be identical to these. Do not use these data to directly compare your samples as your results may vary substantially.

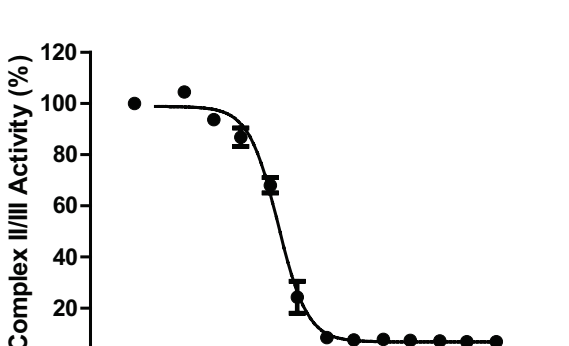


Figure 3. A typical concentration response curve for inhibition of complex III activity by antimycin A (IC_{50} = 136 nM). "Veh." represents compound vehicle control.

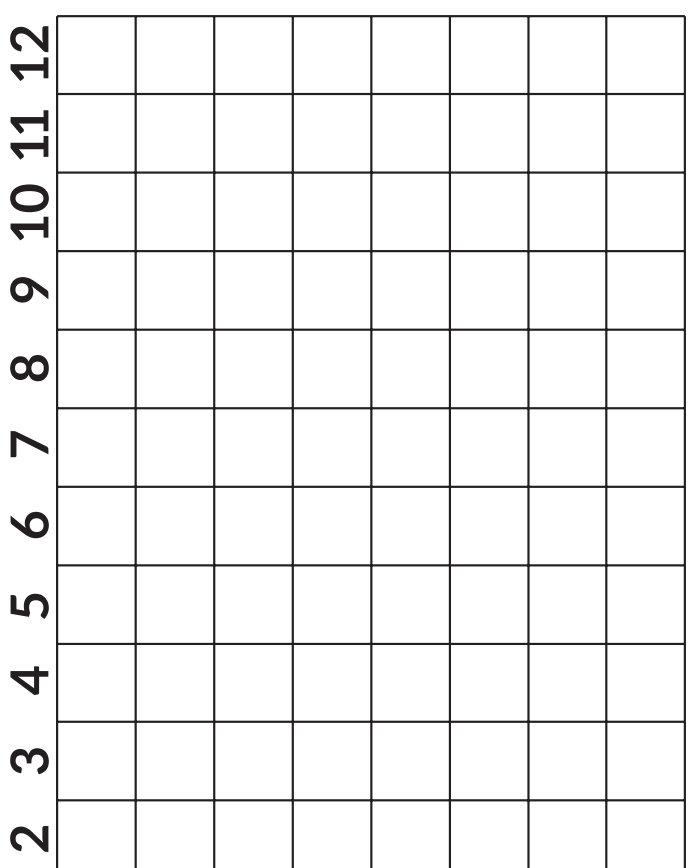
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s) C. Poor test compound solubility	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 C. Test solubility with assay buffer
No activity was detected in test compound wells	Test compound is a potent inhibitor	Check vehicle controls to be sure complex III is active
Test compound absorbance is above saturating positive control (*i.e.*, antimycin A) absorbance	Test compound absorbs at 550 nm	Determine absorbance of compounds in the absence of Cytochrome *c*; subtract this value from all wells containing test compound
Positive control doesn't inhibit	A. Inhibitor has gone off B. Excess superoxide generated at Complex I	Make sure positive controls and rotenone are fresh; avoid freeze thaw cycles

References

1. Muller, F., Crofts, A.R., and Kramer, D.M. Multiple Q-cycle bypass reactions at the Qo site of the cytochrome bc1 complex. *Biochemistry* **41(25)**, 7866-7874 (2002).
2. Hoffman, D.L. and Brookes, P.S. Oxygen sensitivity of mitochondrial reactive oxygen species generation depends on metabolic conditions. *J. Biol. Chem.* **284(24)**, 16236-16245 (2009).
3. Crofts, A.R., Holland, J.T., Victoria, D., *et al.* The Q-cycle reviewed: How well does a monomeric mechanism of the bc1 complex account for the function of a dimeric complex. *Biochim. Biophys. Acta* **1777(7-8)**, 1001-1019 (2008).
4. St-Pierre, J., Buckingham, J.A., Roebuck, S.J., *et al.* Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* **277(47)**, 44784-44790 (2002).
5. Lambert, A.J. and Brand, M.D. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J. Biol. Chem.* **279(38)**, 39414-39420 (2004).



NOTES

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