MitoCheck® Complex IV Activity Assay Kit

Item No. 700990

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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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
700991	Mitochondrial Complex IV Activity Assay Buffer	2 vials/10 ml	-20°C
700992	Reduced Cytochrome *c* Assay Reagent	1 vial/6 mg	-80°C
700019	Bovine Heart Mitochondria Assay Reagent	1 vial/100 µl	-80°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 at 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 Inhibitor - Potassium Cyanide
5. 0.1 M NaOH

INTRODUCTION

Background

Complex IV (cytochrome *c* oxidase) is the terminal electron acceptor in the mitochondrial electron transport chain. Complex IV functions by oxidizing cytochrome *c* and completing a four-electron reduction of oxygen (O_2) to form H_2O. During this process, two protons are translocated from the mitochondrial matrix to the intermembrane space, contributing the mitochondrial membrane potential required for ATP synthesis.[1] This assay is designed to measure the direct oxidation of cytochrome *c* by complex IV in an isolated bovine heart mitochondrial system. Common inhibitors of complex IV include potassium cyanide, azide, O_2 limitation, nitric oxide, and carbon monoxide.[2,3]

About This Assay

Cayman's MitoCheck® Complex IV Activity Assay allows for the quick and easy measurement of complex IV activity. This assay measures the oxidation rate of reduced cytochrome *c* using isolated bovine heart mitochondria supplied within the kit. For the use of this kit with other types of tissue mitochondria, please see Cayman's MitoCheck® Mitochondrial (Tissue) Isolation Kit (Item No. 701010).

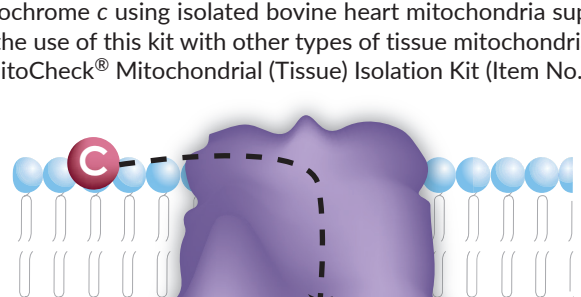


Figure 1. Oxidation of cytochrome *c* by Complex IV.

PRE-ASSAY PREPARATION

Reagent Preparation

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

1. **Mitochondrial Complex IV Activity Assay Buffer - (Item No. 700991)**

   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 are dissolved.

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

   Potassium Cyanide (KCN) is a prototypical inhibitor of complex IV. Because of this, it is important **that extreme care is taken when preparing and using KCN**. In a **ventilated hood**, weigh out 32.5 mg of KCN and dissolve in 1 ml of 0.1 M NaOH; **do not use water or any acidic solvents.** This will provide you with a 500 mM stock of KCN. Store on ice and make fresh less than three hours prior to running this assay.

3. **Reduced Cytochrome c Assay Reagent - (Item No. 700992)**

   Thaw reagent prior to use. Once thawed, the reagent should be kept on ice unopened until use. Once opened, the concentration of reduced cytochrome *c* will diminish slowly due to oxidation. Significant oxidation of cytochrome *c* will impact the performance of the kit. Therefore, after use, component should be frozen immediately and discarded after two weeks.

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.
- The kinetics of this assay are first order with respect to cytochrome *c*. When analyzing data, be sure to use the linear portion of the curve. This portion is typically observed between 2-8 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)
995 µl of Complex IV Activity Assay Buffer	615 µl of Complex IV Activity Assay Buffer
5 µl Bovine Heart Mitochondria Assay Reagent	60 µl Reduced Cytochrome *c* Assay Reagent

Table 1. Assay preparation

Preparation of positive control inhibitor

NOTE: KCN and 0.1 M NaOH are not provided in this assay kit and must be supplied by the user.

An example for preparing a positive control is given below; customer may scale volumes as needed. *NOTE: KCN is not stable at neutral pH, therefore preparation of KCN is carried out in 0.1M NaOH.*

1. In a separate plate or Eppendorf tube, take 14.6 µl of 500 mM KCN stock solution and dilute into 131.4 µl of 0.1M NaOH to give a 50 mM solution.
2. Using the 50 mM KCN, perform log or half log serial dilutions into 0.1 M NaOH. Serial dilutions should be carried out in a separate 96-well plate, or in Eppendorf tubes, and will be used to generate an inhibitor concentration response. When 20 µl of the 50 mM solution is added to the well this will provide a 10 mM concentration of KCN. Subsequent concentrations of KCN will depend on dilutions. Ensure that 0.1 M NaOH is used as a vehicle control.

For each assay condition:

1. Add 50 µl of the contents of tube A to each well.
2. Add 20 µl of test compounds diluted in 0.1 M NaOH, vehicle, or positive control to each well. If test compounds are not stable in 0.1 M NaOH, UltraPure water can be substituted. Depending on the inhibition characteristics of the test compound, a preincubation may be required. KCN does not require preincubation.
3. Add 30 µl of the contents of tube B to each well to start the reaction. This should 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 time-dependent reaction 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 Complex IV Activity (%) as a function of test compound concentration.

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

Performance Characteristics

The data shown below are an example of data obtained with this kit. Your results will not be identical to these.

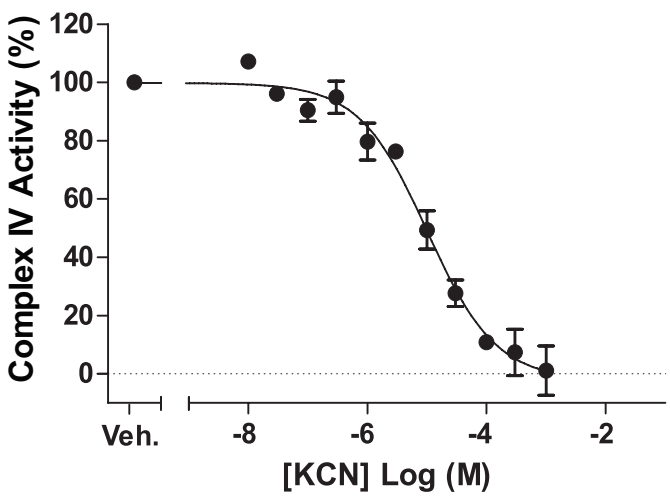


Figure 3. A typical concentration response curve for inhibition of complex IV activity by KCN. "Veh." represents compound vehicle control.

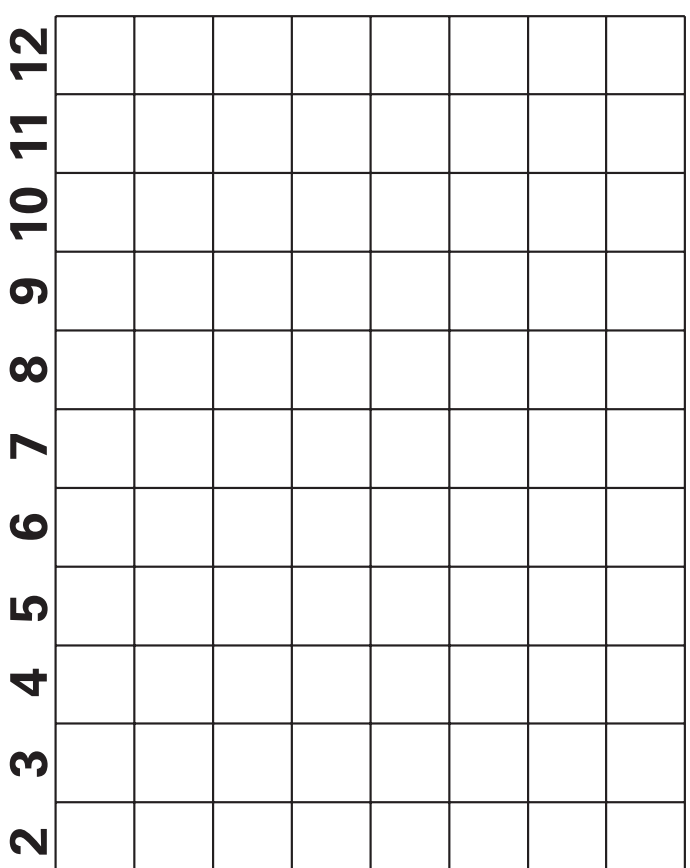
RESOURCES

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	A. Test compound is a potent inhibitor B. Cytochrome *c* is oxidized	A. Check vehicle controls to be sure complex IV is active B. Dilute Reduced Cytochrome *c* Assay Reagent (1:100) in ultrapure H_2O. Perform a wavelength scan (450-600 nm) to ensure an absorbance peak at 550 nm
Test compound absorbance is above saturating positive control absorbance	Sample compound absorbs at 550 nm	Determine absorbance of compounds in the absence of cytochrome *c*; subtract this value from all wells containing sample compound
No inhibition seen with positive control	Positive control has degraded	A. Make sure positive controls are fresh; avoid Freeze thaw cycles B. If using KCN, make sure all dilutions and vehicle controls are prepared in 0.1 M NaOH

References

1. Capaldi, R.A. Structure and function of cytochrome c oxidase. *Annu. Rev. Biochem.* **59**, 569-596 (1990).
2. Cooper, C.E. and Brown, G.C. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: Chemical mechanism and physiological significance. *J. Bioenerg. Biomembr.* **40(5)**, 533-539 (2008).
3. Petersen, L.C. The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. *Biochim. Biophys. Acta.* **460(2)**, 299-307 (1977).



NOTES

Warranty and Limitation of Remedy

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