MitoCheck® Complex V Activity Assay Kit

Item No. 701000

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
701001	Mitochondrial Complex V Activity Assay Buffer	10 ml/2 per kit	-20°C
701002	Mitochondrial Complex V Enzyme Mix	4 ml	-20°C
700019	Bovine Heart Mitochondria Assay Reagent	1 vial/100 µl	-80°C
701003	Mitochondrial Complex V NADH Reagent	3 mg	-20°C
701004	Mitochondrial Complex V ATP Reagent	4 mg	-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 at 340 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 - Oligomycin (Item No.11341) (stock at 1 mg/ml); Rotenone (Item No. 13995) (stock at 1 mM)

INTRODUCTION

Background

The mitochondrial F_1F_O ATP synthase; while not a complex of the mitochondrial electron transport chain (ETC), is commonly referred to as complex V. Under normal physiological conditions, this multi-subunit protein utilizes energy in the form of proton gradient, generated by the ETC, to phosphorylate ADP to form ATP in the presence of P_i. This coupled reaction, commonly known as oxidative phosphorylation is responsible for ~90% of ATP generation in mammalian cells. Should this proton gradient collapse, complex V can also run in reverse, as an F_1F_O ATPase, dephosphorylating ATP to yield ADP and P_i. Protypical inhibitors for complex V include oligomycin and aurovertin. For more information on complex V, please see references 1-3.

About This Assay

Cayman's MitoCheck® Complex V Activity Assay measures the activity of Complex V as an ATPase, since the ATP synthase reaction typically requires freshly isolated, coupled mitochondria. In this assay, ATP is converted to ADP by complex V. The ADP is then utilized by pyruvate kinase to convert phosphoenolpyruvate into pyruvate with the concomitant generation of ATP. Pyruvate, in the presence of NADH and lactate dehydrogenase, is then reduced to lactate and NAD^+. The rate of NADH oxidation can be monitored at 340 nm. Isolated bovine heart mitochondria are provided in this kit, however fresh mitochondria can be isolated from tissue (Item No. 701010) and used with this kit. The reaction scheme for for this assay is shown below.

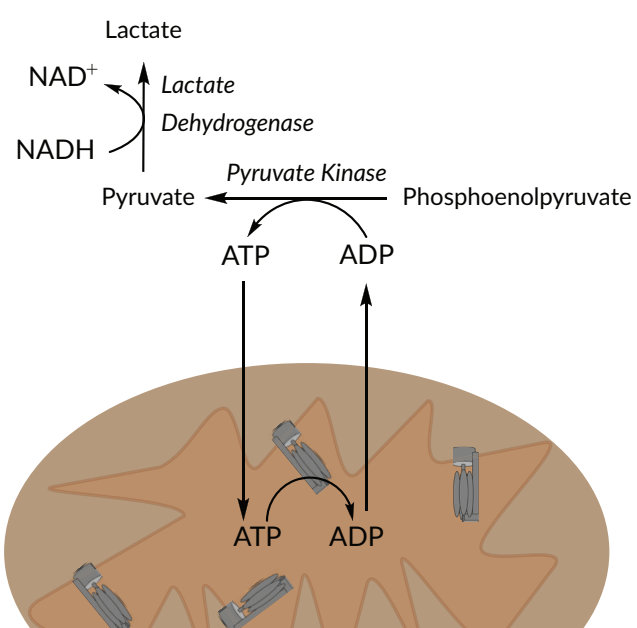


Figure 1. Reaction scheme of Cayman's Mitochondrial Complex V Activity Assay

PRE-ASSAY PREPARATION

Reagent Preparation

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

1. **Mitochondrial Complex V Activity Assay Buffer - (Item No. 701001)**

   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 ensure that any crystals that may have precipitated are dissolved. Any unused portion can be stored at -20°C.

2. **Mitochondrial Complex V Enzyme Mix - (701002)**

   This item is ready to use as supplied. It is recommended that the Mitochondrial Complex V Enzyme Mix is thawed on ice before use and that any unused portion be aliquoted and stored at -20°C.

3. **Bovine Heart Mitochondria Assay Reagent - (Item No. 700019)**

   This item is ready to use as supplied. It is recommended that the Bovine Heart Mitochondria Assay Reagent is thawed on ice before use. It is recommend that, for any unused portion, aliquots be taken and stored at -80°C.

4. **Mitochondrial Complex V NADH Reagent - (Item No. 701003)**

   This vial contains 3 mg of lyophilized Mitochondrial Complex V NADH Reagent. Reconstitute vial contents by adding 120 µl of UltraPure water. Once reconstituted, this reagent is stable for two weeks when stored at -20°C.

5. **Mitochondrial Complex V ATP Reagent - (Item No. 701004)**

   This vial contains 4 mg of lyophilized Mitochondrial Complex V ATP Reagent. Reconstitute vial contents by adding 120 µl of UltraPure water. Once reconstituted, this reagent is stable for one month when stored the indicated temperature.

ASSAY PROTOCOL

Pipetting Hints

- Use different tips to pipette each reagent.
- Avoid introducing bubbles into the well(s).
- 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.

Performing the Assay

Label two microfuge tubes as A and B and add the following reagents. *Isolated mitochondria can settle over time, ensure that the 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). Scale volumes as needed.*

Tube A (1 ml)	Tube B (675 µl)
978 µl of Complex V Activity Assay Buffer	635 µl of Complex V Assay Enzyme Mix
20 µl Bovine Heart Mitochondria Assay Reagent	20 µl of Complex V ATP Reagent
2 µl of 1 mM Rotenone *not supplied*	20 µl of Complex V NADH Reagent

***NOTE: Rotenone stock should be prepared in ethanol, but can be prepared in DMSO*

Table 1. Assay preparation

Preparation of positive control inhibitor

Oligomycin is soluble in ethanol (200 proof) and DMSO to ~20 mg/ml (*NOTE: oligomycin is not provided in this assay kit and must be supplied by the user.*) It is recommended control compounds be dissolved in same solvent as test compounds. The concentration of this solvent in the final reaction should not exceed 1%.

Example: From a 1 mg/ml solution of oligomycin, take 50 µl and dilute into 950 µl of Complex V Activity Assay Buffer. This will result in a 50 µg/ml solution of oligomycin to be used as a positive control. This stock solution, when used in accordance with the reaction setup below, results in a 10 µg/ml final concentration. It is recommended that a concentration response curve (log or ½ log dilutions) be generated for all positive controls and test compounds.

NOTE: Heart tissue contains oligomycin insensitive ATPases.[4,5] While isolated mitochondria are provided, some carryover of these ATPases from the sarcoplasmic reticulum, and the dissociated F_1 subunit of complex V is unavoidable. Because of this, it is necessary to always counterscreen against a saturating concentration of oligomycin (e.g., 10 µg/ml). This ensures that test compounds are specific to complex V, and not inhibiting non-specific ATPases. Failure to fully inhibit with oligomycin with any mitochondrial sample may result in a false positive.

Isolated bovine heart mitochondria, at a concentration of 5 mg/ml, are supplied with this kit. However, users can also isolate mitochondria for use with this assay (Item No. 701010). Following mitochondrial isolation it is important that:

1. Protein concentration is accurately determined (Lowry or BCA, NOT Bradford).
2. Isolated mitochondria are diluted to working concentration of 5 mg/ml in Complex V Activity Assay Buffer.
3. Once diluted, working stock of isolated mitochondria should undergo a minimum of two freeze-thaw cycles (dry ice ethanol, or liquid nitrogen) prior to performing this assay.

NOTE: Amounts of complex V/mg protein can vary greatly between tissue types. Because of this, the activity of user isolated samples should not be compared to that of supplied bovine heart mitochondria. Complex V activity should only be compared to isolated mitochondria from the same tissue and species type.

Reaction Set Up

For each assay condition:

1. Add 50 µl of the contents of tube A to each well.
2. Add 20 µl of positive control or test compounds.
3. Add 30 µl of the contents of tube B to each well to start the reaction.

Immediately measure absorbance at 340 nm (30 second intervals for 30 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 (15-30 minutes).
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 V Activity (%) as a function of test compound concentration.

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

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.

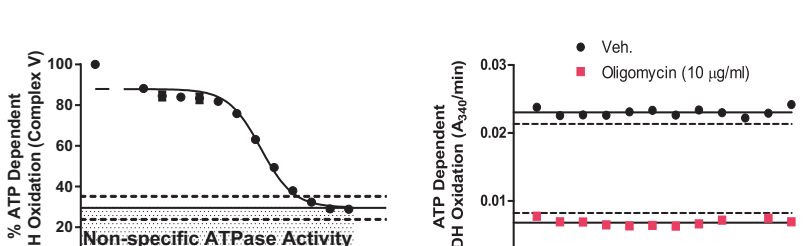


Figure 2. A typical concentration response curve for oligomycin inhibition of complex V ATPase activity. Note that ~25% of ADP Dependent NADH Oxidation is oligomycin insensitive. Because of this, it is important to test experimental compounds in the presence of saturating concentrations of oligomycin (10 µg/ml). "Veh." represents compound vehicle control.

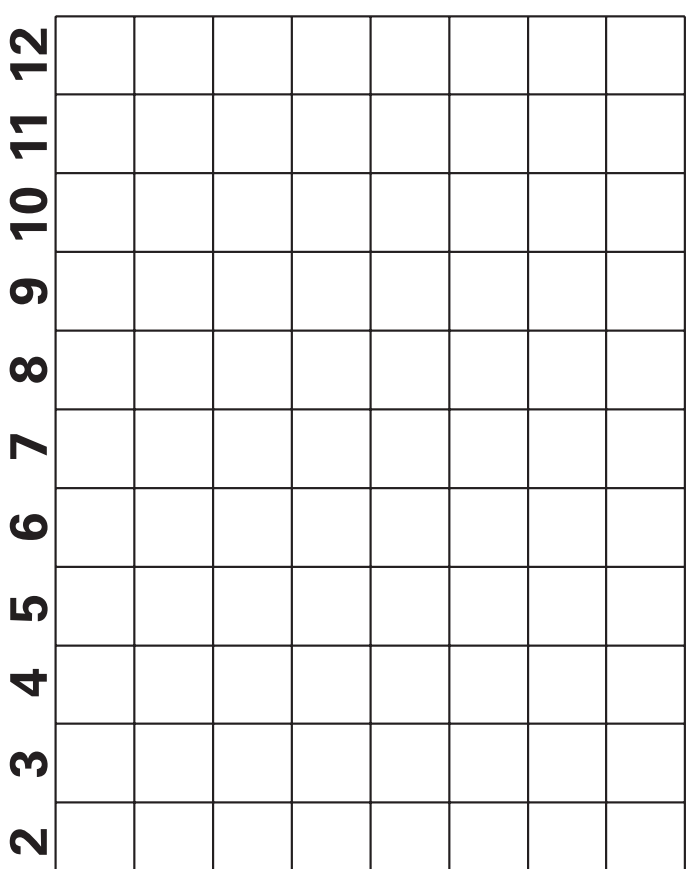
RESOURCES

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 well(s) 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 well(s)	A. Test compound is a potent inhibitor B. NADH is oxidized	A. Check vehicle controls to be sure complex V is active B. Measure absorbance at 340 nm to ensure >1
Sample absorbance is higher than saturating inhibitor absorbance	Sample compound absorbs at 340 nm	Determine absorbance of compounds in Assay Buffer with mitochondria; subtract this value from all wells containing sample compound
No inhibition seen with positive control	A. Rotenone/Oligomycin needs to be fresh - complex I activity can result in oxidation of NADH B. Non-specific ATPase activity - this is normal providing that vehicle control has a greater rate than positive control	A. Make sure positive controls are fresh; avoid freeze thaw cycles B. Ensure that vehicle control has a greater rate of NADH oxidation than positive control

References

1. Hederstedt, L. and Rutberg, L. *Microbiol. Rev.* **45(4)**, 542-555 (1981).
2. Selak, M.A., Armour, S.M., MacKenzie, E.D., *et al. Cancer Cell.* **7(1)**, 77-85 (2005).
3. Pollard, P.J., Wortham, N.C., and Tomlinson, I.P. *Ann. Med.* **35(8)**, 632-639 (2003).
4. King, A., Selak, M.A., and Gottlieb, E. *Oncogene* **25(34)**, 4675-4682 (2006).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.