ab109907 – MitoTox™ Complex V OXPHOS Activity Microplate Assay

Instructions for Use

For the quantitative measurement of MitoTox™ Complex V OXPHOS activity in Human, mouse, rat and bovine samples

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

Complex V, also known as the ATP synthase complex (EC 3.6.3.14) or F1F0 ATPase, is one of the five complexes involved in oxidative phosphorylation in mitochondria. This enzyme complex makes 95% of the cell’s ATP using energy generated by the proton-motive force and can also function in the reverse direction in the absence of a proton-motive force, hydrolyzing ATP to generate ADP and inorganic phosphate.

The production of ADP by ATP synthase can be coupled to the oxidation of NADH to NAD+, and the progress of the coupled reaction monitored as a decrease in absorbance at 340 nm, as shown in figure 1.

![Figure 1. Schematic representation of monitored reaction. PK- Pyruvate kinase, LDH-Lactate dehydrogenase, PEP-phosphoenolpyruvate](image)

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ab109907 (MTOX5) is designed for testing the direct inhibitory effect of compounds on Complex V activity by using enzyme immunocaptured from bovine heart mitochondria. Bovine heart mitochondria are provided in this kit as they are a rich source of Complex V. The phospholipids provided in this kit are essential for Complex V activity.

The 96-well plate in the kit is coated with an anti-Complex V monoclonal antibody (mAb) in rows B to G, enabling Complex V to be captured in a functionally active form in these rows. Rows A and H are coated with a null capture mAb and, hence, can be used as blanks. There are two ways in which this kit can be used to test the effect of compounds on the activity of Complex V. The first is by screening up to 23 compounds at a single concentration in triplicate (for example, in wells B1, C1, D1) along with the appropriate blank which has all the components of the assay except for the immunocaptured enzyme (for example, in well A1). The second approach is by generating a dose response of two compounds known to affect the activity of the enzyme. In this scenario, each compound will have 11 data points, in triplicate (for example rows B to D for one compound, with row A used as blank for that compound). Two 12-well troughs are included with the kit to facilitate assay set up, so that compounds can be mixed with the activity buffer prior to addition to the plate. The first trough will have compounds 1 to 12 at a single concentration (for the screening assay) or the dilution series of compound 1 (for the dose response
assay), whereas the second trough will have compounds 12 to 23 or the dilution series of compound 2. Oligomycin, an inhibitor of Complex V activity, may be used as a positive control. 50% inhibition of Complex V activity is obtained with 10 ± 2 nM oligomycin for the assay conditions described in this protocol. The entire assay can be completed within 4 hours.

Figure 2. Schematic representation of assay set up format. Panel A shows assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) as provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel B shows assay set up for the dose response format with the plate and two 12-well troughs (depicted above and below the plate) as provided in the kit. Each color gradient represents a compound titration.
2. Assay Summary

Add detergent-solubilized bovine heart mitochondria to 96-well plate (2 hours)

- Dilute 320 μl of detergent-solubilized bovine heart mitochondria with 5 ml of Mito Buffer.
- Add 50 μl of the diluted mitochondria to each well in the 96-well plate.
- Incubate for 2 hours at room temperature.

Add Phospholipids (45 min)

- Thaw the phospholipids.
- Prepare the “Wash Solution” by adding the contents of Wash Buffer to 95 ml water
- Rinse wells twice with the Wash Solution.
- Add 40μl of phospholipids to each well.
- Incubate for 45 minutes at room temperature.
- Thaw the Complex V Activity solution.

Add Complex V Activity solution and compounds to be screened and measure activity (1 hour)

- Add the compounds to the Complex V Activity solution in both 12-channel reagent reservoirs.
- Add 200 μl of Complex V Activity solution and compounds to each well of the plate.
- Measure OD$_{340}$ at 1 minute intervals for 1 hour at 30°C.
3. Kit Contents

Sufficient materials are provided for one 96-well microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Mito Buffer</td>
<td>5 ml</td>
</tr>
<tr>
<td>Bovine Heart Mitochondria</td>
<td>360 µl</td>
</tr>
<tr>
<td>Detergent</td>
<td>100 µl</td>
</tr>
<tr>
<td>20X Wash Buffer</td>
<td>5 ml</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>6 ml</td>
</tr>
<tr>
<td>Complex V Activity Buffer</td>
<td>24 ml</td>
</tr>
<tr>
<td>Pre-coated 96-well microplate</td>
<td>1</td>
</tr>
<tr>
<td>12-channel reagent reservoirs</td>
<td>2</td>
</tr>
</tbody>
</table>

4. Storage and Handling

Pre-coated 96-well microplate, Mito buffer, Wash Buffer and detergent should be stored at 4°C. Phospholipids should be stored at -20°C. Bovine heart mitochondria and Activity buffer should be
stored at -80°C. Reagent reservoirs should be stored at room temperature.

5. Additional Materials Required

- Spectrophotometer that measures absorbance at 340nm
- Deionized water
- Multichannel pipette (50 - 300 μl) and tips
- Reagent reservoirs
- A syringe needle
- Oligomycin if desired by the user
- Paper towels

6. Assay Method

Note: This protocol contains detailed steps for measuring Complex V activity. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained. Ensure all reagents and compounds are thawed before the start of the experiment. DO NOT use compounds that have been diluted for more than 3 to 6 months.
A. Solubilization and addition of bovine heart mitochondria to plate

1. Add 40 µl of detergent to the bovine heart mitochondria aliquot given with the kit (360 µl at 5.5 mg/ml).

2. Vortex well.

3. Incubate on ice for 30 minutes.

4. Centrifuge at 25,000 x g for 20 minutes at 4°C.

5. **Save the Supernatant** (solubilized BHM) as sample and discard the pellet.

6. Mix: 5ml of 1X Mito-Buffer + 320 µl of solubilized BHM.

7. Add 50 µl (15 µg) of mitochondria to each well of pre-coated 96-well microplate.

8. Cover plate and incubate for 2 hours at room temperature.
B. Addition of the phospholipids

1. Add 5ml of 20X Wash Buffer to 95 ml deionized H$_2$O. Label this solution as “Wash solution”.

2. Empty the wells of the 96-well plate.

3. Wash the plate TWICE by adding 300 μl of the Wash solution to each well.

4. Empty the wells of the plate and tap the plate upside down a couple of times on l paper towels to ensure all wells are empty.

5. Add 40 μl of phospholipids to each well.

6. Cover the plate and incubate for 45 minutes at room temperature.
C. Addition of the Complex V Activity solution and the compounds to be tested

1. When the incubation period with the phospholipids is almost complete, add 900 μl of Complex V Activity solution to each channel of two 12-channel reagent reservoirs. Add the compounds, to be tested, to the channels of the reservoirs, leaving at least one channel for addition of DMSO (or other solvent the compounds are dissolved in). The volume of the compound should not exceed 1.8% of that of the Complex V Activity solution in each channel of the reservoir. Mix the contents of each channel using a multichannel pipette. To determine the volume of compound to add to each channel, see “calculation of compound concentration” in the Data Analysis section below.

2. When the 45 minute incubation period with the phospholipids is complete, do NOT empty the wells of the 96-well plate since the phospholipids are necessary for the assay.

3. Transfer 200 μl of Complex V Activity solution containing the compounds from each channel of ONE of the reservoirs and add it to each well in row A. Repeat this step for rows B, C and D.
4. Transfer 200 μl of Complex V Activity buffer containing compounds from each channel of the second 12-channel reagent reservoir and add it to each well in row E. Repeat this step for rows F, G and H.

5. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.

D. Measurement of ATPase activity

- Set the 96-well plate in the reader. Measure the absorbance at 340 nm at 30°C in kinetic mode taking absorbance measurements every minute for 60 minutes. Ensure that the limit of Maximum OD is set to read at 1 and Kinetic reduction reads as $V_{\text{max}}$ (mOD per minute). In SoftMax Pro these settings can be adjusted from the reduction tab located on the main window. If the activity buffer is cool when is transferred onto the plate, this assay may have a flat kinetic reading during the first 5 to 15 minutes of the assay. NADH only becomes oxidized once the activity buffer inside the plate reaches 30°C. This can be overcome once the assay has been completed by changing the default lag time settings in the reader. To guarantee that $V_{\text{max}}$ is
calculated in the linear range, confirm that the $R^2$ is close to 0.99 for every measurement in the raw graph window.

- With the settings specified above, calculate the CV activity rate automatically. However, it will not calculate blank subtractions for each individual compound (see Data analysis section below to determine final activity of CV minus background). If analysis software is not available, the rate can be calculated manually (see Data analysis section below).

7. Data Analysis

A. Calculation of the activity of Complex V

The activity of the ATP synthase enzyme is coupled to the molar conversion of NADH to NAD⁺ measured as a decrease in absorbance at OD 340 nm. Examine the rate of decrease in absorbance at 340 nm over time. The assay starts slowly and takes time to stabilize. The fastest, most linear rate of activity is usually seen between 12 and 50 minutes. This is shown below where the rate is calculated between these two time points. Most microplate analysis software is capable of
performing this function. Repeat this calculation for all samples measured.

\[ \text{Rate (mOD/min)} = \frac{\text{Absorbance 1} - \text{Absorbance 2}}{\text{Time (min)}} \]

The activity of immunocaptured Complex V is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex V are 10.5, 10.3 and 10.7 mOD/min and the background rate is 0.15 mOD/min, the activity of Complex V is \((10.5 + 10.3 + 10.7) / 3 - 0.15\) which is 10.35 mOD/min. Once the background rate has been subtracted, the activity of immunocaptured Complex V in the presence and absence of compound can be compared.
B. Calculation of compound concentration in each well

If 16.2 μl of “A” mM compound is added to 900 μl of Complex V Activity solution in each channel of the 12-channel reagent reservoir, the final concentration of the compound in each well in the 96-well plate will be

\[
\frac{16.2 \text{ μl}}{900 \text{ μl}} \times \text{ (“A” mM)} \times \frac{200 \text{ μl}}{240 \text{ μl}}.
\]

For example, if 16.2 μl of 10 mM compound is added to each channel of the 12-channel reagent reservoir containing 900 μl of Complex V Activity solution, the final concentration of the compound in the 96-well plate is 150 μM.

C. Reproducibility

The intra-assay variation and interassay variation with this assay are each less than 10%.

8. Example of a dose response assay

*Note – A dose response assay should be run in the same way as the screening assay (shown in the protocol above) except for step C (Addition of Complex V activity solution and the compounds to be screened).*

Dose response assay for Oligomycin (1:10 dilution series) and test compound (1:3 dilution series)
1. When the incubation period with the **phospholipids** is almost complete, take ONE 12-channel reservoir and add 900 μl of complex V activity solution to each channel.

2. Add 5.5 μl of **Oligomycin**, at 10 mM stock, to channel 1 (50 μM final concentration in the well in a total volume of 240 μl = 200 μl of activity solution + 40 μl of phospholipids).

3. Generate 1:10 serial dilutions by taking 90 μl of channel 1 into channel 2. Repeat this until 11 serial dilutions have been generated for oligomycin (ensure that the contents of each channel are well mixed before transferring compound from one channel to the next).

4. Add 11 μl of **DMSO** to channel 12 of the FIRST 12-channel reservoir (1% DMSO in the well in a total volume of 240 μl = 200 μl of activity solution + 40 μl of phospholipids).

5. Take the SECOND 12-channel reservoir.

6. Add 1.5 ml of **complex V activity solution** to channel 1.
7. Add 1 ml of complex V activity solution to channels 2 to 12.

8. Add 18 μl of Test compound, at 10 mM stock, to channel 1 (100 μM final concentration in the well in total a volume of 240 μl = 200 μl of activity solution + 40 μl of phospholipids).

9. Generate 1:3 serial dilutions by taking 500 μl of channel 1 into channel 2. Repeat this until 12 serial dilutions have been generated for the test compound (ensure that the contents in each channel are well mixed before transferring compound from one channel to the next).

10. Do NOT empty the wells of the 96-well plate after the 45 minute incubation period with the phospholipids, since the phospholipids are necessary for the activity assay.

11. Transfer 200 μl of Complex V Activity solution containing oligomycin serial dilution and DMSO control from each channel of the FIRST 12-channel reagent reservoir and add it to each well in row A. Repeat this step for rows B, C and D. Minimize addition of bubbles during pipetting. Final volume in the well will be 240 μl.
12. Transfer 200 μl of Complex V Activity solution containing test compound serial dilution from each channel of the SECOND 12-channel reagent reservoir and add it to each well in row E. Repeat this step for rows F, G and H. Minimize addition of bubbles during pipetting. Final volume in the well will be 240 μl.

13. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.

14. Continue on to step C of the main protocol.

9. Specificity

Species Reactivity: Bovine
UK, EU and ROW
Email: technical@abcam.com
Tel: +44 (0)1223 696000
www.abcam.com

US, Canada and Latin America
Email: us.technical@abcam.com
Tel: 888-77-ABCAM (22226)
www.abcam.com

China and Asia Pacific
Email: hk.technical@abcam.com
Tel: 108008523689 (中國聯通)
www.abcam.cn

Japan
Email: technical@abcam.co.jp
Tel: +81-(0)3-6231-0940
www.abcam.co.jp

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