MitoTox™ Complete
OXPHOS Activity Assay Kit
(5 Assays)

Instructions for Use

Assays measure the direct effects of drugs on the 5 OXPHOS complexes

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

The assays measure the direct effects of drugs on the 5 OXPHOS complexes. Complexes I, II, IV and V are each immunocaptured from bovine heart mitochondria (included) in 96-well plates, and their activities are measured by simple spectrophotometric assays. Complex III is measured in an in-solution colorimetric assay.

ab110419 consists of the following assays:

- MitoTox™ Complex I OXPHOS Activity Microplate Assay (ab109903)
- MitoTox™ Complex II OXPHOS Activity Microplate Assay (ab109904)
- MitoTox™ Complex III OXPHOS Activity Microplate Assay (ab109905)
- MitoTox™ Complex IV OXPHOS Activity Microplate Assay (ab109906)
- MitoTox™ Complex V OXPHOS Activity Microplate Assay (ab109907)
MitoTox™ Complex I
OXPHOS Activity
Microplate Assay
(ab109903)

Instructions for Use

For the quantitative measurement of Complex I activity in Bovine samples.

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

Complex I, also known as NADH ubiquinone oxidoreductase (EC 1.6.5.3) is one of the five complexes involved in oxidative phosphorylation in mitochondria. The enzyme complex catalyzes electron transfer from NADH to the electron carrier, ubiquinone, concomitantly pumping protons across the inner mitochondrial membrane.

\[
\text{NADH} + H^+\text{ubiquinone} \rightarrow \text{NAD}^+ + \text{ubiquinol}
\]

The progression of the above reaction can be monitored by following the oxidation of NADH as a decrease in absorbance at 340 nm.

ab109903 is designed for testing the direct inhibitory effect of compounds on Complex I 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 I. The phospholipids provided in the kit are essential for rotenone-sensitive Complex I activity, rotenone being a well-known inhibitor of Complex I.

The 96-well plate in the kit has been coated with an anti-Complex I monoclonal antibody (mAb) in rows B to G, enabling Complex I to be captured in a functionally active form in these rows. Rows A and H have been 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 I. 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 on 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 (see Fig.1). The user may wish to use rotenone, an inhibitor of Complex I activity, in the screening procedure as a positive control. Rotenone (10 mM), an inhibitor of Complex I activity, may be used as a positive control. 50% inhibition of Complex I activity is obtained with 13 ± 5 nM rotenone for assay conditions described in this protocol. The entire assay can be completed within 5 hours.
Figure 1. 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 I Activity solution.

Prepare Complex I Activity solution by adding Ubiquinone 1 to the contents of Complex I Activity Buffer

Add Complex I Activity solution and compounds to be screened and measure activity (1 hour)
- Add the compounds to the Complex I Activity solution in both 12-channel reagent reservoirs.
- Add 200 µl of Complex I Activity solution and compounds to each well of the plate.
- Measure OD$_{340}$ at 1 minute intervals for 2 hours at 30°C.
3. Kit Contents

Sufficient materials for 90 measurements.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>1X Mito Buffer</td>
<td>5 ml</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 I Activity Buffer</td>
<td>24 ml</td>
</tr>
<tr>
<td>Detergent</td>
<td>100 μl</td>
</tr>
<tr>
<td>Bovine heart mitochondria</td>
<td>360 μl</td>
</tr>
<tr>
<td>Ubiquinone 1</td>
<td>60 μl</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

Store Microplate, Wash buffer, Mito Buffer, and detergent at 4°C. Reagent reservoirs may be stored at RT. Store Phospholipids at -20°C. Store Bovine heart mitochondria, Ubiquinone and Activity Buffer at -80°C.
5. Additional Materials Required

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

6. Assay Method

*Note – This protocol contains detailed steps for measuring Complex I 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.*

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: 5 ml of 1X Mito Buffer + 320 µl of solubilized BHM (supernatant).

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 5 ml of 20X Wash Buffer to 95 ml deionized H₂O. 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 add 40 µl of Phospholipids to each well.

5. Cover the plate and incubate for 45 minutes at room temperature.

6. Meanwhile, thaw Complex I Activity Buffer and the compounds to be tested.
C. Preparation of Complex I Activity solution

Add all of Ubiquinone 1 to Complex I Activity Buffer and mix well. Label this solution “Complex I Activity Solution”.

D. Addition of the Complex I Activity solution and the compounds to be tested

1. When the incubation period with the phospholipids is almost complete, add 900 µl of Complex I Activity solution to each channel of both 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 I Activity solution in each channel of the reservoirs. Mix the contents of each channel using a multichannel pipette.

2. 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. Transfer 200 µl of Complex I Activity solution containing the compounds 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.

3. Transfer 200 µl of Complex I Activity solution 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.

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

E. **Measurement of Complex I 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 2 hours. Ensure that the limit of Maximum OD is set to read at 1.5 and Kinetic reduction reads as $V_{\text{max}}$ (mOD-units per minute). If the activity solution is cool when is transferred onto the plate, this assay may have a flat kinetic reading during the first 10 to 15 minutes of the assay. NADH only becomes oxidized once the activity solution in the plate wells reaches 30°C. This can be overcome once the assay has been completed by changing the default lag time settings in the reader to 600 or 900 seconds. To guarantee that $V_{\text{max}}$ is calculated in the linear range, confirm that the
R² is close to 0.99 for every measurement in the raw graph window.

7. Data Analysis

A. Calculation of the activity of Complex I

The oxidation of NADH to NAD⁺ by Complex I is measured as a decrease in absorbance at OD 340 nm. Examine the rate of decrease in absorbance at 340 nm over time. Calculate the rate between two time points for all the samples where the decrease in absorbance is most linear.

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

The activity of immunocaptured Complex I is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex I are 4.2, 4.1 and 4.7 mOD/min and the background rate is 0.4 mOD/min, the activity of Complex I is (4.2 + 4.1 + 4.7) / 3 - 0.4 which is 3.93 mOD/min. Once the background rate has been subtracted, the activity of immunocaptured Complex I 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 I 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 \((\frac{16.2 \, \mu L}{900 \, \mu L}) \times \text{“A”} \times (\frac{200 \, \mu L}{240 \, \mu L})\). 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 I Activity solution, the final concentration of the compound in the 96-well plate is 150 µM.

C. Reproducibility

Intra-Assay: CV < 10 %

Inter-Assay: CV < 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 4 (Addition of Complex I activity solution and the compounds to be screened).

Dose response assay for Rotenone (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 **Rotenone**, 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 Rotenone (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 I activity solution to channel 1.

7. Add 1 ml of complex I 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).

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 I Activity solution** containing rotenone 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 I 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 5 of the main protocol.

Figure 2: Typical Dose Response Assay using Rotenone

9. Specificity

Species Reactivity: Bovine
MitoTox™ OXPHOS
Complex II Activity Kit
(ab109904)

Instructions for Use

For testing the direct inhibitory effect of compounds on Complex II activity

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

Complex II, also known as succinate-coenzyme Q reductase (SDH, EC 1.3.5.1), is one of the five complexes involved in oxidative phosphorylation in the inner mitochondrial membrane. It catalyzes electron transfer from succinate to the electron carrier, ubiquinone, but unlike the other four complexes it is not a proton pump. The product ubiquinol is utilized by complex III in the respiratory chain and fumarate is necessary to maintain the tricarboxylic acid (TCA) cycle.

\[
\text{Succinate + ubiquinone (Q) \rightarrow Fumarate + ubiquinol (QH2)}
\]

The production of ubiquinol can be monitored by the addition of DCPIP (2,6-dichlorophenolindophenol) which when reduced by ubiquinol decreases in absorbance at 600 nm and recycles the substrate ubiquinone.

\[
\text{ubiquinol (QH2) + DCPIP (blue) \rightarrow ubiquinone (Q) + DCPIPH2 (colorless)}
\]

ab109904 MitoTox™ OXPHOS Complex II Activity Kit (MTOX2) is designed for screening the direct inhibitory effect of compounds on Complex II activity and does this using enzyme immunocaptured from Bovine heart mitochondria. Bovine heart mitochondria are provided in this kit as they are a rich source of Complex II.
The 96-well plate in the kit has been coated with an anti-Complex II monoclonal antibody (mAb) in rows B to G, enabling Complex II to be captured in a functionally active form in these rows. Rows A and H have been coated with a null capture mAb and thus 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 II. 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 (See Figure 1). TTFA (2-Thenoyltrifluoroacetone), an inhibitor of Complex II activity, may be included as a positive control. 50% inhibition of Complex II activity is obtained with 20 ± 2 μM TTFA under the assay conditions described in this protocol. The entire assay can be
completed within 4 hours. The intra-assay variation and inter-assay variation with this assay are each less than 15%.

Figure 1. 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

For quick reference only. Be completely familiar with details of this document before performing the assay.

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

- Dilute 513 μl detergent-solubilized Bovine heart mitochondria with 20 ml of Mito Buffer.
- Add 200 μl of the diluted mitochondria to each well in the 96-well plate.
- Incubate for 2 hours at room temperature.

↓

Wash plate (5 minutes)

- Prepare the “Buffer Solution” by adding 5 ml of 20X Buffer to 95 ml water. Rinse wells twice with the Buffer Solution.
- Add 300 μl Buffer Solution.
- Ensure Complex II Activity components are thawed.
Prepare Complex II Activity solution (5 minutes)

- Add Ubiquinone 2, DCPIP and Succinate to the contents of Complex II Activity Buffer.

Add Complex II Activity Solution and compounds to be screened, measure activity (0.5 hours)

- Add the compounds to the Complex II Activity Solution in two 12-channel reagent reservoirs.
- Empty the wells of the 96-well plate
- Add 200 μl of Complex II Activity Solution and compounds to each well of the plate.
- Measure OD600 at 1 minute intervals for 1 hour at room temperature.
3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x Buffer</td>
<td>5 ml</td>
</tr>
<tr>
<td>Bovine heart mitochondria</td>
<td>2 x 360 µL</td>
</tr>
<tr>
<td>10 X Detergent</td>
<td>1 ml</td>
</tr>
<tr>
<td>Complex II Activity Buffer</td>
<td>24 ml</td>
</tr>
<tr>
<td>DCPIP</td>
<td>250 µl</td>
</tr>
<tr>
<td>Succinate</td>
<td>250 µl</td>
</tr>
<tr>
<td>Ubiquinone 2</td>
<td>60 µl</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

Succinate, Ubiquinone 2, BHM, DCPIP should be stored at -80°C. Reagent reservoirs may be stored at RT. All other components should be stored at -4°C.

5. Additional Materials Required

- Spectrophotometer that measures absorbance at 600nm
- Deionized water
- Multichannel pipette (50 - 300 μl) and tips
- Reagent reservoirs
- A fine needle
- Optional: TTFA (2-thenoyltrifluoroacetone), an inhibitor of Complex II activity
- Paper towels

6. Assay Method

*Note: This protocol contains detailed steps for measuring Complex II 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.*

*Note – Ensure all reagents and compounds are thawed before the start of the experiment.*
Note – 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 each of the Bovine heart mitochondria aliquots given with the kit (2 x 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. Collect the supernatant from each and pool. The pooled supernatant (solubilized BHM) will be the sample to use in step A7 below. Discard the pellets.

6. Add 5 ml of 20X Buffer to 95 ml deionized H₂O. Label this solution as “Buffer Solution”.

7. Mix 20 ml of Buffer Solution + 513 μl of solubilized BHM.
8. Add 200 µl (25 µg) of mitochondria to each well of pre-coated 96-well microplate.

9. Cover plate and incubate for 2 hours at room temperature.

B. Plate washing

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

2. Add 300 µl of the Buffer Solution to each well.

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

4. Add 300 µl of the Buffer Solution to each well.

5. Leave on the bench until ready to add activity solution with compounds.

C. Preparation of Complex II Activity Solution

Add all of the Ubiquinone 2, DCPIP and Succinate to Complex II Activity BUFFER. Mix well. Label this solution “Complex II Activity SOLUTION”.

D. Addition of the Complex II Activity Solution and the compounds to be tested
1. Add 900 μl of Complex II Activity SOLUTION to each channel of both 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 II Activity SOLUTION in each channel of the reservoir. Mix the contents in each channel using a multi-channel pipette. To determine the volume of compound to add to each channel, see “calculation of compound concentration” in the Data Analysis section below.

2. Empty the wells of the 96-well plate and tap the plate a couple of times upside down on paper towels to ensure all wells are empty.

3. Using the multi-channel pipette, transfer 200 μl of Complex II Activity Solution containing the compounds 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.

4. Transfer 200 μl of Complex II Activity Solution 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.

E. Measurement of Complex II activity

Set the 96-well plate in the reader. At room temperature, measure the absorbance at 600 nm in kinetic mode taking absorbance measurements every minute for 1 hour. Ensure that the limit of Maximum OD is set to read at 1.0 and Kinetic reduction reads as Vmax (mOD per minute). Vmax must be calculated in the linear range. To guarantee this, it may be necessary to adjust the default lag and end time settings in the reader so that the calculated R2 is close to 0.99 for every measurement in the raw graph window to calculate the CII activity rate automatically. 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 II

The initial OD should be approximately 0.2 OD units at 600 nm. The reduction of ubiquinone and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD 600 nm. Examine the rate of decrease in absorbance at 600 nm over time. Calculate the rate between two time points for all the samples where the decrease in absorbance is most linear, this linear region is typically between 5 mins and 10 mins. After 15 minutes the rate of reduction in absorbance is declining for the most active samples due lack of substrate so do not calculate the rate after this point.

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

The activity of immunocaptured Complex II is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex II are 2.3, 2.6 and 2.7 mOD/min and the background rate is 0.07 mOD/min, the activity of Complex I is \((2.3 + 2.6 + 2.7) / 3 - 0.07\) which is 2.46 mOD/min. Once the background rate has been subtracted, the activity of immunocaptured Complex II
in the presence and absence of compound can be compared.

B. Calculation of compound concentration in each well

If 13.5 μl of “A“ mM compound is added to 900 μl of Complex IV Activity solution in each division of the 12-channel reagent reservoir, the final concentration of the compound in each well in the 96-well plate will be (13.5 μl / 900 μl) x “A“. For example, if the stock concentration of the compound is 10 mM, the final concentration of the compound in the 96-well plate is 150 μM.

8. Example of a Dose Response Assay

Note – A dose response assay should be performed in the same way as the screening assay (shown in the protocol above) except for step 4 (Addition of Complex II Activity Solution and test compounds).

Dose response assay for TTFA (1:2 dilution series) and test compound (1:2 dilution series)

1. Take ONE 12-channel reservoir and add 1.8 ml of Complex II Activity Solution to channel 1.
2. Add 900 µl of Complex II Activity Solution to channels 2 to 12.

3. Add 9 µl of TTFA (100 mM stock) to channel 1 (500 µM final concentration in the well).

4. Generate 1:2 serial dilutions by taking 900 µl from channel 1 and adding it to channel 2. Repeat this until 11 serial dilutions have been generated for TTFA (ensure that the contents of each channel are well mixed before transferring compound from one channel to the next).

5. Add 4.5 µl of DMSO to channel 12 of the FIRST 12-channel reservoir (0.5% DMSO in the well).

6. Take the SECOND 12-channel reservoir.

7. Add 1.8 ml of Complex II Activity Solution to channel 1.

8. Add 900 µl of Complex II Activity Solution to channels 2 to 12.

9. Add 9 µl of Test compound (20 mM stock) to channel 1 (100 µM final concentration in the well).

10. Generate 1:2 serial dilutions by taking 900 µl from channel 1 and adding it to 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).

11. Empty the wells of the 96-well plate after the washing steps.

12. Using a multi-channel pipette transfer 200 μl of Complex II Activity Solution containing TTFA 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.

13. Transfer 200 μl of Complex II 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.

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

15. Continue with step 5 of the main protocol.
9. Reproducibility

Reproducibility

Intra-Assay: CV < 15 %

Inter-Assay: CV < 15 %
MitoTox™ OXPHOS Complex III Activity Kit (ab109905)

Instructions for Use

For screening the direct inhibitory effects of compounds on Complex II and III activity

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

Complex II (succinate-ubiquinone oxidoreductase), one of the mitochondrial respiratory chain complexes, transfers electrons from succinate generated during the citric acid cycle to Complex III (ubiquinolcytochrome c oxidoreductase), via a mobile electron shuttle, ubiquinone. Complex III transfers electrons to Complex IV (cytochrome c oxidase) via another mobile electron shuttle, cytochrome c, as shown in Figure 1.

ab109905 MitoTox™ OXPHOS Complex III Activity Kit (MTOX3) is designed for screening the direct inhibitory effects of compounds on Complex II and III activity. Bovine heart mitochondria are provided in this kit as they are a rich source of these two enzymes. The kit contains succinate (Solution 1) which is the electron donor of Complex II, as well as cytochrome c (oxidized), the electron acceptor of Complex III. Ubiquinone, the electron acceptor of Complex II, is present in the mitochondria. The rate of the coupled Complex III reaction is measured in whole mitochondria by monitoring the conversion of cytochrome c inits oxidized form to its reduced form, as a linear increase in absorbance at 550 nm. The kit requires rotenone (not supplied) and KCN (not supplied). Rotenone inhibits Complex I and its addition as described in this protocol blocks electron transfer from NADH, via Complex I, to ubiquinone, thereby assuring that all reduction of cytochrome c is via Complex II. KCN
inhibits Complex IV, ensuring that there is no re-oxidation of cytochrome c by this enzyme.

**Figure 1:** Pathway of electron transfer in mitochondria during oxidative phosphorylation

Since the assay is performed in whole mitochondria, the 96-well plate in the kit is not coated with monoclonal antibody and therefore the user has the freedom to set up the assay layout. However, to optimize the number of data points, it is suggested to use rows B to G for measurements of functionally active Complex II+III and use rows A and H as blanks. With this arrangement, the kit can be used to screen the effect of various compounds on the activity of Complex II+III or to test the IC50 of two compounds in a dose-response format. The screening format could test up to 23 compounds at a single concentration in triplicate (for example, in wells B1, C1, D1) along with the appropriate blank which would have all the components of the assay except for whole mitochondria (for example, in well A1). The dose–response format could test up to two compounds known to affect the activity of the enzyme. In this last 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 and added to the plate in rows A or H, prior to addition of whole mitochondria into the troughs so that enzyme activity can be monitored in rows B to G. 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 (see Fig.2). The user may add antimycin, a strong inhibitor of Complex III activity, as a positive control. 50% inhibition of Complex III activity is obtained with 22 ± 4 nM antimycin under the assay conditions described in this protocol. The entire assay can be completed within 30 minutes. The intra-assay variation and inter-assay variation with this assay are each less than 10%.
**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. Blanks are dispensed in rows A and H soon after diluting the compound in activity buffer in each of the troughs. Once blanks are dispensed, whole mitochondria is added to each of the channels of the two-12 well troughs soon after dispensation in rows B to G so that activity of Complex II+III can be monitored.
2. Assay Summary

For quick reference only. Be completely familiar with details of this document before performing the assay.

Prepare Complex III activity solution (10 minutes)

- Prepare the Complex III activity solution by adding 120 μl of 0.2 M KCN, 15.2 μl rotenone and 500 μl cytochrome c (Oxidized) to 12 ml of Succinate solution.
- Mix well.
- Add the Complex III activity solution to a single-channel reagent reservoir.

Add compounds to be screened to the Complex III activity solution (10 minutes)

- Transfer 460 μl of the Complex III activity solution to each channel of both 12-channel reagent reservoirs.
- Add the compounds to be screened to the 12-channel reagent reservoirs and mix well.
• Transfer 100 μl from each channel of the reagent reservoirs to a row of the 96-well plate. The wells in these rows will be the blanks.

Add mitochondria to the Complex III activity solution containing the compounds. Measure activity (10 minutes)

• Dilute 120 μl of mitochondria with 880 μl of Complex III Mito Buffer; add the diluted mitochondria to a single channel reagent reservoir.
• Using a multi-channel pipette, RAPIDLY transfer 20 μl of the diluted mitochondria to each channel of the 12-channel reagent reservoirs containing the compounds and the Complex III activity solution.
• Mix well.
• Rapidly transfer 100 μl of from each channel of the reagent reservoirs to THREE rows of the 96 well plate.
• Measure OD$_{550}$ at 20 second intervals for 5 minutes at room temperature.
3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Succinate Solution</td>
<td>12 ml</td>
</tr>
<tr>
<td>Bovine heart mitochondria (5 mg/ml)</td>
<td>300 µl</td>
</tr>
<tr>
<td>1X Complex III Mito Buffer</td>
<td>1 ml</td>
</tr>
<tr>
<td>Cytochrome c (Oxidized)</td>
<td>550 µl</td>
</tr>
<tr>
<td>96-well microplate</td>
<td>1</td>
</tr>
<tr>
<td>Single-channel reagent reservoirs</td>
<td>2</td>
</tr>
<tr>
<td>12-channel reagent reservoirs</td>
<td>2</td>
</tr>
</tbody>
</table>

4. Storage and Handling

Store at -80°C: Bovine Heart Mitochondria, Cytochrome c (oxidized).
Store at 4°C: Succinate Solution, Microplate, Mito Buffer and Reagent Reservoirs: Reagent Reservoirs may be stored at RT.
5. Additional Materials Required

- KCN, diluted to 0.2 M stock in deionized water or in 0.1 M Sodium Hydroxide.
- Rotenone, diluted to 10 mM in ethanol or DMSO.
- Spectrophotometer measuring absorbance at 550 nm.
- Deionized water
- Multichannel pipettes (5-50 μl and 50 - 300 μl) and tips
- Optional: Antimycin A

6. Assay Method

*Note: This protocol contains detailed steps for measuring Complex III 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.*

*Note – Ensure all reagents and compounds are thawed before the start of the experiment.*

*Note – DO NOT use compounds that have been diluted for more than 3 to 6 months.*
A. Preparation of Complex III Activity solution

1. Succinate is an electron donor of Complex II. Prepare the **Complex III activity solution** by adding to the following to 12 ml of **Succinate**:

   - 120 μl of 0.2 M KCN (not provided in the kit). KCN should be prepared fresh, on the day of the experiment if diluted in deionized water. KCN may be prepared in advanced if prepared in 0.1 M Sodium Hydroxide and stored at -80°C.

   - 15.2 μl of 10 mM rotenone (not provided in the kit). Rotenone may be prepared in advanced and stored at -20°C or -80°C.

   - 500 μl of cytochrome c (Oxidized)

2. Add the **Complex III activity solution** to a single-channel reagent reservoir.

B. Addition of the compounds to be screened to the Complex III activity solution

1. Using a multichannel pipette, transfer 460 μl of the **Complex III activity solution** to each channel of both 12-channel reagent reservoirs.
2. Add the compounds to be screened to the channels of the reservoirs. Mix well. To determine the volume of compound to add to each channel, see “calculation of compound concentration” in the Data Analysis section below.

3. Transfer 100 μl of the Complex III activity solution and compounds from each channel of the FIRST 12-channel reagent reservoir to row A in the 96-well microplate.

4. Transfer 100 μl of the Complex III activity solution and compounds from each channel of the SECOND 12-channel reagent reservoir to row H.

5. Rows A and H will be the blanks.

6. Spectrophotometer MUST be set up at this time.

C. Addition of whole mitochondria to start the Complex III reaction

1. Dilute 120 μl of 5 mg/ml of Bovine heart mitochondria using 880 μl of CIII Mito Buffer. The diluted mitochondria are now at 0.6 mg/ml. Add the diluted mitochondria to a single-channel reagent reservoir.
2. Using a multichannel pipette, RAPIDLY transfer 20 µl of the diluted mitochondria to every channel of both 12-channel reagent reservoirs containing the Complex III activity solution and compounds. Mix well.

3. RAPIDLY mix and transfer 100 µl from each channel of the FIRST 12-channel reagent reservoir and add it to each well in row B. Repeat this step for rows C and D.

4. RAPIDLY mix and transfer 100 µl 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 and G.

D. Measurement of Complex III activity

Set the 96-well plate in the reader. At room temperature, measure the absorbance at 550 nm in kinetic mode taking absorbance measurements every 20 seconds for 5 minutes. Ensure that the reader is set for Kinetic measurement with Vmax in mOD per minute. If the user takes more than 2 minutes transferring the mitochondria into the 12-channel reservoirs and subsequently onto the plate, it is possible that substrate may be consumed before the reaction ends. In this scenario, the rate of reduced...
Cytochrome c accumulation will plateau (as shown below from 180 to 300 seconds). To avoid including plateau measurement points in the overall Vmax calculation, change the default end time settings in the software. In the example below, end time was set to 120 seconds.

With the settings specified above, the software will automatically calculate the CIII activity rate. However, it will not calculate blank subtractions for each individual compound (see Data analysis section below to determine final activity of III 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 relative activity of Complex III

Examine the rate of increase in absorbance at 550 nm over time. Calculate the rate between two time points for all the samples where the increase in absorbance is most linear.

The relative activity of Complex III is the mean of measurements obtained with mitochondria minus the rate obtained without mitochondria. For example, if the rates of Complex III are 20.1, 22.3 and 21.5 mOD/min and the background rate is 0.3 mOD/min, the activity of Complex III is \( \frac{20.1 + 22.3 + 21.5}{3} - 0.3 \) which is 21 mOD/min. Once the background rate has been subtracted, the activity of Complex III in the presence and absence of compound can be compared.

B. Calculation of compound concentration in each well

If 6.9 \( \mu l \) of “A” mM compound is added to 460 \( \mu l \) of Complex III Activity solution in each division of the 12-channel reagent reservoir, the final concentration of the compound in each well in the 96-well plate will be \( \frac{6.9 \mu l}{460 \mu l} \times A \). For example, if the stock concentration of the compound is 10 mM, the final concentration of the compound in the 96-well plate is 150 \( \mu M \).
8. Example of a Dose Response Assay

Note – A dose response assay should be performed in the same way as the screening assay (shown in the protocol above) except for step 2 (Addition of Complex III Activity Solution).

Dose response assay for Antimycin (1:4 dilution series) and test compound (1:2 dilution series)

1. Take ONE 12-channel reservoir.

2. Add 600 μl of Complex III Activity Solution to channel 1.

3. Add 450 μl of Complex III activity solution to channels 2 to 12.

4. Add 3 μl of Antimycin, at 10 mM stock, to channel 1 (50 μM final concentration in the well).

5. Generate 1:4 serial dilutions by taking 150 μl of division 1 into division 2. Repeat this until 11 serial dilutions have been generated for antimycin (ensure that each division is well mixed before transferring compound from one channel to the next).

6. Add 4.5 μl of DMSO to division 12 of the FIRST 12-channel reservoir (1% DMSO in the well).
7. Mix well with a multichannel pipettor.

8. Transfer 100 μl of **Complex III Activity Solution** with antimycin serial dilution and DMSO to row A (these wells will be the blanks for antimycin and DMSO).

9. Take the SECOND 12-channel reservoir.

10. Add 900 μl of complex III activity solution to channel 1.

11. Add 450 μl of complex III activity solution to channels 2 to 12.

12. Add 9 μl of **Test compound**, at 10 mM stock, to channel 1 (100 μM final concentration in the well).

13. Generate 1:2 serial dilutions by taking 450 μL of division 1 into division 2. Repeat this until 12 serial dilutions have been generated for the test compound (ensure that each division is well mixed before transferring compound from one channel to the next).

14. Mix well with a multichannel pipette.

15. Transfer 100 μl of **Complex III activity solution** with the test compound serial dilution to row H (these wells will be the blanks for the test compound).
16. Spectrophotometer MUST be set up at this time.

17. Continue with step 3 of the main protocol (section 6).

9. Reproducibility

Reproducibility

Intra-Assay: CV < 10 %

Inter-Assay: CV < 10 %
MitoTox™ OXPHOS
Complex IV Activity Kit
(ab109906)

Instructions for Use

For screening the direct inhibitory effects of compounds on Complex IV activity

This product is for research use only and is not intended for diagnostic use.
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9. Reproducibility 72
1. Introduction

Complex IV, also known as cytochrome c oxidase (EC 1.9.3.1), is one of the five complexes involved in oxidative phosphorylation in mitochondria. It transfers electrons from reduced cytochrome c (cytochrome c2+) to molecular oxygen and concomitantly pumps protons across the inner mitochondrial membrane.

\[
4 \text{cytochrome } c^{2+} + 4H^+ + O_2 \rightarrow 4 \text{cytochrome } c^{3+} + 2H_2O
\]

The progression of the above reaction can be monitored by following the oxidation of reduced cytochrome c as a decrease in absorbance at 550 nm.

ab109906 MitoTox™ OXPHOS Complex IV Activity Kit (MTOX4) is designed for screening the direct inhibitory effect of compounds on Complex IV activity and does this by using enzyme immunocaptured from Bovine heart mitochondria. Bovine heart mitochondria are provided in this kit as they are a rich source of Complex IV.

The 96-well plate in the kit has been coated with an anti-Complex IV monoclonal antibody (mAb) in rows B to G, enabling Complex IV to be captured in a functionally active form in these rows. Rows A and H have been coated with a null capture mAb and thus, 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 IV. 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 (see Fig.1). KCN may be used in the screening procedure as a positive control (KCN is not provided in this kit). 50% inhibition of Complex IV activity is obtained with 1 - 4 μM KCN under the assay conditions described in this protocol. The entire assay can be completed within 5 hours. The intra-assay variation and inter-assay variation with this assay are each less than 10%.
**Figure 1: 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

For quick reference only. Be completely familiar with details of this document before performing the assay.

Add blocking solution to 96-well plate (1 hour)

- Prepare the Blocking Solution by dissolving 1.5 g of the Blocking Powder in 30 ml of Blocking Buffer.
- Add 300 μl of Blocking Solution to each well of the 96-well plate.
- Incubate for 1 hour at room temperature.

Add detergent-solubilized Bovine heart mitochondria to plate (3 hours)

- Transfer Rinse wells ONCE with blocking buffer. Empty the wells. Discard Blocking buffer.
- Dilute the detergent-solubilized Bovine heart mitochondria (4.4 μl) with 22 ml of Mito Buffer.
- Add 200 μl of diluted mitochondria to each well of the 96-well plate.
- Incubate for 3 hours at room temperature.
Prepare Complex IV Activity Solution
2 ml of Reagent C + 22 ml of Mito Buffer

Add Add Complex IV Activity Solution and compounds to be tested and measure activity (1 hour)

- Rinse wells twice with Mito Buffer.
- Add compounds to Complex IV Activity Solution in both 12-channel reagent reservoirs.
- Add 200 μl of Complex IV Activity Solution with compounds to each well of the 96-well plate.
- Measure OD$_{550}$ at 1 minute intervals for 1 hour at room temperature.
### 3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking powder</td>
<td>1.8 g</td>
</tr>
<tr>
<td>1X Blocking buffer (Solution 1)</td>
<td>70 ml</td>
</tr>
<tr>
<td>1X Mito Buffer (Solution 2)</td>
<td>110 ml</td>
</tr>
<tr>
<td>Bovine Heart Mitochondria</td>
<td>90 µl</td>
</tr>
<tr>
<td>Detergent</td>
<td>50 µl</td>
</tr>
<tr>
<td>Reagent C (reduced cytochrome c)</td>
<td>2 x 1 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

Store at -80°C: Bovine Heart Mitochondria.
Store at -20°C: Reagent C (store at -80°C for long term storage)
Store at 4°C: Pre-coated 96-well plate, Blocking Buffer, Buffer and Detergent
Store at RT or 4°C: Blocking powder and reagent reservoirs.

5. Additional Materials Required

- Spectrophotometer measuring absorbance at 550 nm
- Deionized water
- Multichannel pipettes (50 - 300 μl) and tips
- A syringe needle
- Optional: KCN. Dilute in deionized water or in 0.1 M Sodium Hydroxide. When diluting in deionized water, prepared fresh on the day of the experiment. When diluting in sodium hydroxide, it may be prepared in advanced and stored at -80°C.
- Paper towels
6. Assay Method

Note: This protocol contains detailed steps for measuring Complex IV 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.

Note – ***Ensure all reagents and compounds are thawed before the start of the experiment.***

Note – ***DO NOT use compounds that have been diluted for more than 3 to 6 months.***

A. Addition of Blocking solution to plate

1. Mix 1.5 g of **Blocking Powder** + 30 ml of **Blocking Buffer**. Label as Blocking Solution.

2. Add 300 μl of **Blocking Solution** to each well of the 96-well plate.

3. Cover the plate and incubate at room temperature for 1 hour.

B. Solubilization and addition of Bovine heart mitochondria to plate

1. Add 10 μl of **Detergent** to the **Bovine Heart Mitochondria** aliquot given with the kit (90 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 (detergent-solubilized Bovine heart mitochondria) as sample and leave on ice until used in step 10. Discard the pellet.

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

7. Wash the plate ONCE by adding 300 μl of Blocking Buffer to each well.

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

9. DISCARD Blocking Buffer as it is not needed for subsequent steps in this protocol. Inadvertent use of Blocking Buffer in subsequent steps will result in loss of Complex IV activity in the assay.

11. Add 200 μl of the diluted mitochondria into each well of the 96-well plate. The amount of mitochondria in each well is 200 ng.

12. Cover the plate and incubate for 3 hours at room temperature.

C. Preparation of Complex IV Activity solution

When the 3-hour incubation period is almost complete, mix the following: 2 ml of Reagent C (reduced cytochrome c) + 22 ml of Mito Buffer. Label as Complex IV Activity Solution.

D. Addition of the Complex IV Activity Solution and the compounds to be tested

1. When the 3-hour incubation period is almost complete, mix the following: 2 ml of Re Add 900 μl of the Complex IV Activity solution to each channel of both 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 compound solvent). The volume of the compound solution should not exceed 1.5% of the Complex IV Activity Solution in each channel of the reservoir. Mix the contents in each channel
using a multi-channel pipette. To determine the volume of compound to add to each channel, see “calculation of compound concentration” in the Data Analysis section below.

2. After the 3-hour incubation period, rinse the wells of the plate by adding 300 μl Mito Buffer to each well.

3. Empty the wells of the plate and tap the plate a couple of times upside down on paper towels to ensure all wells are empty. Repeat steps 2 and 3.

4. Using a multi-channel pipette, transfer 200 μl of Complex IV Activity Solution containing the compounds 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.

5. Using a multi-channel pipette, transfer 200 μl of Complex IV Activity Solution 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.

6. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.
E. Measurement of Complex IV activity

Set the 96-well plate in the reader. At room temperature, measure the absorbance at 550 nm 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 $V_{\text{max}}$ (milli-units per minute). 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.

7. Data Analysis

Calculation of the relative activity of Complex IV

Since the Complex IV reaction is product inhibited, the rate of activity should be expressed as the initial rate of oxidation of cytochrome c. Examine the rate of decrease in absorbance at 550 nm over time. Calculate the rate between two time points for all the samples where the decrease in absorbance is most linear. The activity of immunocaptured Complex IV is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex IV are 5.5, 5.2 and 5.1 mOD/min and the background rate is 0.5 mOD/min, the activity of Complex IV is $(5.5 + 5.2 + 5.1) / 3 - 0.5$ which is 4.76 mOD/min. Once the background
rate has been subtracted, the activity of immunocaptured Complex IV in the presence and absence of compound can be compared.

**Calculation of compound concentration in each well**

If 13.5 μl of "X" mM compound is added to 900 μL of Complex IV Activity Solution in each division of the 12-channel reagent reservoir, the final concentration of the compound in each well in the 96-well plate will be (13.5 μl / 900 μl) x “X”. For example, if the stock concentration of the compound is 10 mM, the final concentration of the compound in the 96-well plate is 150 μM.

**8. Example of a Dose Response Assay**

Note – A dose response assay should be performed in the same way as the screening assay (shown in the protocol above) except for step 4 (Addition of Complex IV Activity Solution and the compounds to be screened).

Dose response assay for KCN (1:10 dilution series) and test compound (1:2 dilution series)

1. Take ONE 12-channel reservoir and add 900 μl of complex IV activity solution to all divisions.

2. Add 1 μl of KCN (100 mM stock) in 0.1 M NaOH, to division 1 (100 μM final concentration in the well).
3. Generate 1:10 serial dilutions by taking 90 μl from channel 1 to channel 2. Repeat this until 11 serial dilutions have been generated for KCN (ensure that each division is well mixed before transferring compound from one division to the next).

4. Add 9 μl of 0.1 M NaOH to division 12 of the FIRST 12-channel reservoir (1 mM NaOH in the well).

5. Take the SECOND 12-channel reservoir.

6. Add 1.8 ml of complex IV activity solution to division 1.

7. Add 900 μl of complex IV activity solution to divisions 2 to 12.

8. Add 18 μl of Test compound, at 10 mM stock, to division 1 (100 μM final concentration in the well).

9. Generate 1:2 serial dilutions by taking 900 μl of division 1 into division 2. Repeat this until 11 serial dilutions have been generated for the test compound (ensure that each division is well mixed before transferring compound from one division to the next).

10. Add 9 μl of DMSO to division 12 of the SECOND 12-channel reservoir (1% DMSO in the well).
11. When the 3 hour incubation period with the mitochondria is complete, rinse the wells of the plate TWICE by adding 300 μl of Buffer to each well.

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

13. Using a multichannel pipette transfer 200 μl of Complex IV Activity Solution containing KCN serial dilution and NaOH control from each division 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.

14. Transfer 200 μl of Complex IV Activity Solution containing test compound serial dilution and DMSO control from each division 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.

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

16. Continue on to step 5 of the main protocol (section 6).
9. Reproducibility

Reproducibility

Intra-Assay: CV < 10 %

Inter-Assay: CV < 10 %
MitoTox™ Complex V OXPHOS Activity Microplate Assay (ab109907)

Instructions for Use

For the quantitative measurement of MitoTox™ Complex V OXPHOS activity in Bovine samples

This product is for research use only and is not intended for diagnostic use.
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1. Introduction  
2. Assay Summary  
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8. Example of a Dose Response assay  
9. Specificity
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.png)
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 H₂O. 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 filter 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.

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

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 \( \frac{(10.5 + 10.3 + 10.7)}{3} - 0.15 \) 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 

\[
\frac{16.2 \mu l}{900 \mu l} \times ( "A" \text{ mM}) \times \frac{200 \mu l}{240 \mu l}.
\]

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 3 (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 4 of the main protocol (section 6).
9. Specificity

Species Reactivity: Bovine
UK, EU and ROW
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