Version 5c Last updated 4 January 2021

ab109903 MitoTox™ Complex I OXPHOS Activity Assay Kit

For the rapid, sensitive and accurate screening of potential inhibitors of Complex I activity *in vitro*.

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

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1. Overview

MitoTox™ Complex I OXPHOS Activity Assay Kit (ab109903) is designed for testing the direct inhibitory effect of compounds on Complex I activity in only 5 hours. Complex I extracted from the provided bovine heart mitochondria (a rich source of Complex I) is immunocaptured by specific antibodies on the plate. Complex I activity can be observed as decrease in absorbance at OD 340 nm.

The intra-assay and inter-assay variation of this assay are both < 15%.

The inhibitory effects of compounds on Complex I activity can be tested in two different ways:

- Screening format (Figure 1, panel A): in this scenario, a maximum of up to 23 compounds can be tested at a single concentration, in triplicate, along with the appropriate blank.
- Dose response (IC_{50}) format (Figure 1, panel B): In this scenario, two compounds known to affect Complex I activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

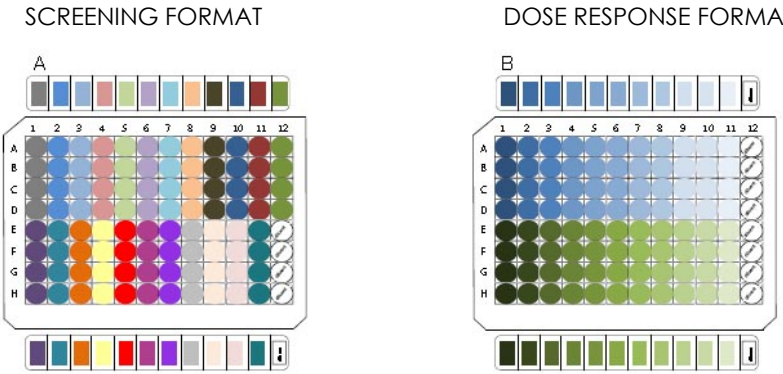


Figure 1. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel B: assay set up for the dose response format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color gradient represents a compound titration.

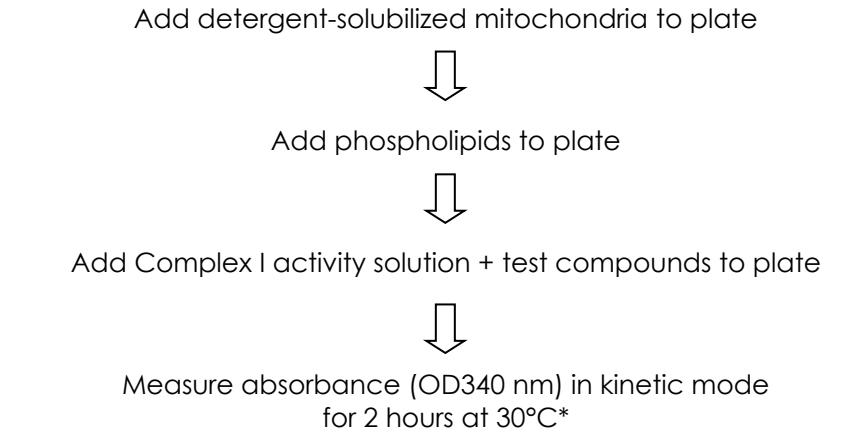
Testing for mitochondrial function has become a key aspect of drug discovery. Mitochondria can be affected by drug treatment, resulting into cardio- and hepatotoxic side effects that can lead to drug withdrawal from the market. Therefore, there is increasing emphasis on testing the impact on mitochondria early on in the drug development process to reduce failure rates during preclinical and clinical phases.

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

$NADH + H^+$ ubiquinone $\rightarrow NAD^+ +$ ubiquinol

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

2. Protocol Summary



**For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at 4°C (store Bovine Heart Mitochondria, Complex I activity Buffer, Phospholipid and Ubiquinone 1 at -80°C) in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
1X Mito Buffer	5 mL	4°C	4°C
20X Wash Buffer	5 mL	4°C	4°C
Phospholipids	6 mL	-80°C	-80°C
Complex I Activity Buffer	24 mL	-80°C	-80°C
Detergent	100 µL	4°C	4°C
Bovine Heart Mitochondria	360 µL	-80°C	-80°C
Ubiquinone 1	60 µL	-80°C	-80°C
96-well microplate	1 unit	4°C	4°C
12-channel reagent reservoirs	2 units	4°C/RT	N/A

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 340 nm
- Double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Disposable single channel reservoirs
- Syringe needle
- (Optional) Rotenone – Complex I inhibitor: we recommend Rotenone (ab143415) at 10 mM stock solution (in DMSO)

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 1X Mito Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

9.2 20X Wash Buffer:

Prepare 1X Wash Buffer by diluting 20X Wash Buffer in ddH_2O: make 100 mL 1X Wash Buffer by combining 5 mL 20X Wash Buffer with 95 mL ddH_2O and mix thoroughly and gently. Label this mixture as **Wash Solution**. Equilibrate to room temperature before use. Store 1X Wash Solution at 4°C.

9.3 Phospholipids:

Ready to use as supplied. Keep on ice while in use. Avoid multiple freeze/thaw cycles. Store at -80°C.

9.4 Complex I Activity Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -80°C.

9.5 Detergent:

Ready to use as supplied. Thaw on ice. Keep on ice while in use. Store at 4°C.

9.6 Bovine Heart Mitochondria (5.5 mg/mL):

Ready to use as supplied. Thaw on ice. Avoid multiple freeze/thaw cycles. Store at -80°C.

9.7 Ubiquinone 1:

Ready to use as supplied. Thaw on ice. Avoid multiple freeze/thaw cycles. Store at -80°C.

9.8 96-well microplate:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

Δ Note: We do not recommend using the plate more than once as there is risk of contaminating unused wells.

9.9 12-channel reagent reservoirs:

Ready to use as supplied. Store at room temperature. Do not reuse and discard after using.

10. Sample preparation

General sample information:

- Always prepare a fresh set of dilutions for every use.
- Do not use compounds that have been diluted in solvent for more than 3-6 months.

Test Compounds:

Dissolve test compounds into appropriate solvent.

The volume of the compound should not exceed 1.8% of the total volume of the activity solution in which they are diluted for the assay.

Use the following formula to calculate how much you need to add the activity solution to achieve the desired final concentration on the reaction well:

$$Vtest = 900\ \mu L\ x\ \frac{[Compound]}{[Stock]}\ x\ \frac{240\ \mu L}{200\ \mu L}$$

Where:

[Compound] = desired concentration of test compound in well.

[Stock] = stock concentration of test compound.

900 µL = total volume of Complex I Solution/Compound.

200 µL = volume of Complex I Solution/Compound added to reaction well.

240 µL = reaction well volume (phospholipids + Complex I Solution/Compound).

Δ Note: See Assay Procedure section for more details

11. Assay Procedure – SCREENING ASSAY

- The 96-well microplate provided has been coated with anti-Complex I monoclonal antibody in rows B-G. Rows A and H have been coated with a null capture monoclonal antibody to be used as background controls.
- We recommend using Rotenone in the screening procedure as a positive control. Rotenone is a well-known inhibitor of Complex I activity. Following the assay procedure, 50% inhibition of Complex I activity is obtained with 13 ± 5 nM rotenone.
- Do not use compounds that have been diluted in solvent for more than 3-6 months.
- The 12-well troughs included in the kit will 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 1-12, whereas the second trough will have compounds 12-23 (see Figure 1).

11.1 Bovine Heart Mitochondria (BHM) solubilization and plating:

11.1.1 Add 40 µL of Detergent to the provided tube containing bovine heart mitochondria (BHM) (360 µL at 5.5 mg/mL).
11.1.2 Mix well by vortexing.
11.1.3 Incubate tube on ice for 30 minutes.
11.1.4 Centrifuge at 25,000 x *g* for 20 minutes at 4°C in a cold microcentrifuge.
11.1.5 Collect sample supernatant containing solubilized BHM (approx. 320 µL) and discard pellet.
11.1.6 Add 5 mL 1X Mito Buffer to the solubilized BHM (~ 320 µL). Mix well by pipetting up and down.
11.1.7 Add 50 µL of BHM (~ 15 µg mitochondria) to each well of the precoated 96-well microplate.
11.1.8 Cover plate and incubate for 2 hours at room temperature.

11.2 Phospholipid addition:

11.2.1 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.

11.2.2 Add 300 µL of 1X Wash Solution to each well.

11.2.3 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.

11.2.4 Add 300 µL of 1X Wash Solution to each well.

11.2.5 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.

11.2.6 Add 40 µL Phospholipids to each well.

11.2.7 Cover the plate and incubate for 45 minutes.

11.3 Test Compound preparation:

11.3.1 Prepare Complex I Activity Solution immediately prior to use by adding all the content of the Ubiquinone 1 vial (~ 60 µL) to the bottle of Complex I Activity Buffer, Mix well. Label this solution "**Complex I Activity Solution**".

11.3.2 Add each compound (up to 23 different compounds) to be tested to each channel of both 12-channel reagent reservoirs. Leave 1 channel (preferably, last channel) for the addition of a solvent only control.

11.3.3 When phospholipid incubation time is almost complete (Step 11.2.7), add Complex I Activity Solution to each channel of both 12-channel reagent reservoirs to a final volume of 900 µL.

11.3.4 Mix contents of each channel by pipetting up and down with a multichannel pipette.

11.4 Test Compound addition to plate:

Δ Note: 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.

11.4.1 Once phospholipid incubation step (Step 11.2.7) is finished, using a multi-channel pipette, add 200 µL of Complex I Activity Solution/Compound from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.

11.4.2 Using a multi-channel pipette, add 200 µL of Complex I Activity Solution/Compound from each channel of the second 12-channel reagent reservoir to each well in row E, F, G, H.

Δ Note: Any bubbles in the wells should be popped with a fine needle as quickly as possible.

11.5 Measurement:

11.5.1 Measure output immediately at OD 340 nm on a microplate reader in kinetic mode, every minute, for at least 2 hours at 30°C protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1.5 and Kinetic reading reads as Vmax (mOD-units per minute).

If activity solution is cool when transferred onto the plate, the assay may have a flat kinetic reading during the first 10-15 min. This is because NADH is only oxidized once the activity solution in the wells reaches 30°C. This can be overcome once the assay has been completed by changing the default lag time settings in the reading to 600 or 900 seconds.

12. Assay Procedure – DOSE RESPONSE ASSAY

- The 96-well microplate provided has been coated with anti-Complex I monoclonal antibody in rows B-G. Rows A and H have been coated with a null capture monoclonal antibody to be used as background controls.
- We recommend using Rotenone in the screening procedure as a positive control. Rotenone is a well-known inhibitor of Complex I activity. Following the assay procedure, 50% inhibition of Complex I activity is obtained with 13 ± 5 nM rotenone.
- Do not use compounds that have been diluted in solvent for more than 3-6 months.
- The 12-well troughs included in the kit will 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 dilution series of compound 1, whereas the second trough will have dilution series of compound 2 (see Figure 1).

12.1 Bovine Heart Mitochondria (BHM) solubilization and plating:

12.1.1 Add 40 µL of Detergent to the provided tube containing bovine heart mitochondria (BHM) (360 µL at 5.5 mg/mL).
12.1.2 Mix well by vortexing.
12.1.3 Incubate tube on ice for 30 minutes.
12.1.4 Centrifuge at 25,000 x *g* for 20 minutes at 4°C in a cold microcentrifuge.
12.1.5 Collect sample supernatant containing solubilized BHM (approx. 320 µL) and discard pellet.
12.1.6 Add 5 mL 1X Mito Buffer to the solubilized BHM (~ 320 µL). Mix well by pipetting up and down.
12.1.7 Add 50 µL of BHM (~ 15 µg mitochondria) to each well of the precoated 96-well microplate.
12.1.8 Cover plate and incubate for 2 hours at room temperature.

12.2 Phospholipid addition:

12.2.1 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
12.2.2 Add 300 µL of 1X Wash Solution to each well.
12.2.3 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
12.2.4 Add 300 µL of 1X Wash Solution to each well.
12.2.5 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
12.2.6 Add 40 µL Phospholipids to each well.
12.2.7 Cover the plate and incubate for 45 minutes.

12.3 Test Compound preparation:

12.3.1 Prepare Complex I Activity Solution immediately prior to use by adding all the content of the Ubiquinone 1 vial (~ 60 µL) to the bottle of Complex I Activity Buffer, Mix well. Label this solution "**Complex I Activity Solution**".
12.3.2 Compound 1 dose response:
12.3.2.1 When phospholipid incubation time is almost complete (Step 12.2.7), add 900 µL of Complex I Activity Solution to channels 2-12 of the first reagent reservoirs.
12.3.2.2 Add Compound 1 to channel 1 of the first reservoir. Add Complex I Activity Solution to a final volume of 900 µL.

Δ Note: The volume on channel 1 might vary depending on the serial dilutions you are performed. Please take that in consideration when calculating the volume of compound and/or buffer that you need to add to the channel.

12.3.2.3 Starting with channel 1, generate serial dilutions from channel 2 till channel 11.
12.3.2.4 Add only solvent to channel 12 of the first reservoir as a control.
12.3.3 Repeat same procedure described in Step 12.3.2 for compound 2.
12.3.4 Mix contents of each channel by pipetting up and down with a multichannel pipette.

12.4 Test Compound addition to plate:

Δ Note: 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.

12.4.1 Once phospholipid incubation step (Step 12.2.7) is finished, using a multi-channel pipette, add 200 µL of Complex I Activity Solution/Compound 1 Serial Dilution from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.

12.4.2 Using a multi-channel pipette, add 200 µL of Complex I Activity Solution/Compound 2 Serial Dilution from each channel of the first 12-channel reagent reservoir to each well in row E, F, G, H.

Δ Note: Any bubbles in the wells should be popped with a fine needle as quickly as possible.

12.5 Measurement:

12.5.1 Measure output immediately at OD 340 nm on a microplate reader in kinetic mode, every minute, for at least 2 hours at 30°C protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1.5 and Kinetic reading reads as Vmax (mOD-units per minute).

If activity solution is cool when transferred onto the plate, the assay may have a flat kinetic reading during the first 10-15 min. This is because NADH is only oxidized once the activity solution in the wells reaches 30°C. This can be overcome once the assay has been completed by changing the default lag time settings in the reading to 600 or 900 seconds.

13. Data Analysis

- Use only the linear rate for calculation. To guarantee that Vmax (mOD-units per minute) is calculated in the linear range, confirm that the R^2 is close to 0.99 for every measurement in the raw graph window.
- Complex I activity is proportional to the decrease in absorbance at OD 340 nm.

13.1 Calculation of activity of Complex I:

13.1.1 Examine the linear rate of decrease in absorbance at OD 340 nm over time.

13.1.2 For all reaction wells, choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding OD values at those points (OD1 and OD2).

13.1.3 Calculate reaction rate (mOD/min). Most microplate analysis software can perform this function. Alternative, use the following formula:

Reaction Rate (mOD/min) = (OD1 – OD2)/ (T1 – T2)

13.1.4 Average the triplicate reading for each sample.

13.1.5 Calculate activity of Complex I as follows:

C I activity = Rate sample – Rate background (row A/H)

14. Typical Data

Data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

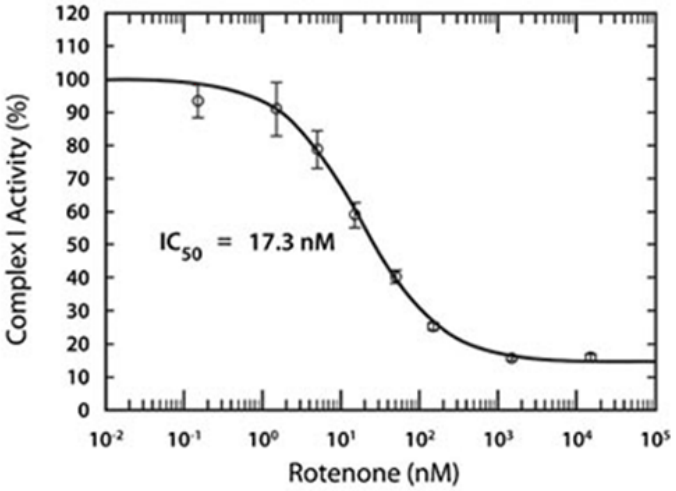


Figure 2. Typical dose response curve for rotenone. Assay was performed following the Dose Response Assay Procedure using rotenone, a well known Complex I inhibitor. Rotenone was prepared in DMSO to generate a 10 mM stock. Starting with a 50 µM final concentration in well (5.5 µL in channel 1), 1:10 serial dilutions of rotenone were generated.

15. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Assay with erratic readings	Pipetting errors	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
No signal above background in inhibitor wells	Inhibitor concentration too high	Reduce concentration of inhibitor
No inhibition seen in test compound wells	Compound is not an inhibitor	Use another compound for your test. Use a known inhibitor as positive control (rotenone)

16. FAQs

Q. Why do I need to use phospholipids?

A. The phospholipids provided in the kit are essential for rotenone-sensitive Complex I activity, rotenone being a well-known inhibitor of Complex I.

Q. I want to treat my cells with OXPHOS inhibitors and then look at the effect they have in the OXPHOS complexes activity. Can I use this kit?

A. No, we do not recommend this product.

If you treat the cells, let's say, with rotenone, and then isolate the mitochondria from rotenone-treated cells, all the rotenone present in the cells will wash off during the sample preparation procedure and there will no inhibitor present when the assay is performed.

The MitoTox™ range has been specifically designed to test normal mitochondria with inhibitor compounds *in vitro*.

Q. Can I use my own isolated mitochondria?

A. Yes, you can use mitochondria isolated from human, mouse, or rat cells for this assay. This is because the antibody used to capture Complex I in the plate recognizes Complex I from these species. We would still recommend to run a control reaction with the provided mitochondria from cow to ensure the assay is working.

17. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)