Version 8a Last updated 16 June 2022

ab110419 MitoTox™ Complete OXPHOS Activity Assay Kit (5 assays)

For the rapid, sensitive and accurate screening of potential inhibitors of the 5 OXPHOS complexes activity *in vitro*.

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

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1. Overview

MitoTox™ Complete OXPHOS Activity Assay Kit (5 assays) (ab110419) is designed for testing the direct inhibitory effect of compounds on the 5 OXPHOS complexes.

The inhibitory effects of compounds on OXPHOS complexes activity can be tested in two different ways:

- Screening format: 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: In this scenario, two compounds known to affect specific Complex activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

Complexes I, II, IV and V activity are each immunocaptured from bovine heart mitochondria by specific antibodies on 96-well microplates, and their activities measured by simple spectrophotometric assays. Complex III activity is measured by an in-solution colorimetric assay.

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.

This product consists of the following assays:

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

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Version 5c Last updated 16 June 2022

ab109903 MitoTox™ Complex I OXPHOS Activity Assay Kit

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

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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.

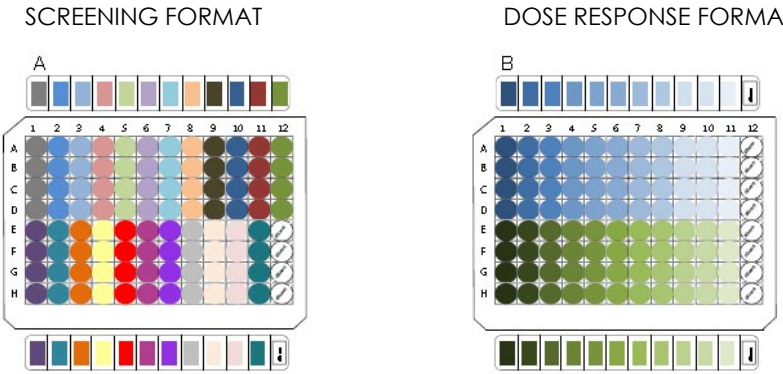


Figure 1. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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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.

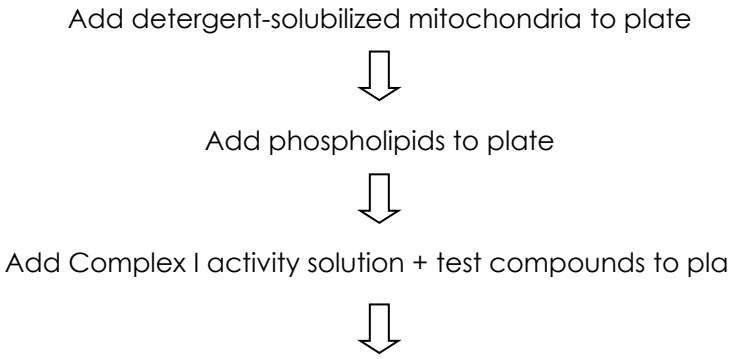
OXPHOS Complex I (NADH ubiquinone oxidoreductase, EC 1.6.5.3) is one of the five complexes involved in oxidative phosphorylation (OXPHOS) in mitochondria. The enzyme complex catalyzes electron transfer from NADH to the electron carrier, ubiquinone, concomitantly pumping protons across the inner mitochondrial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

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2. Protocol Summary



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

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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.

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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

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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)

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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.

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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.

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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

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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.

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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.

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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.

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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.

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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.

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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.

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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)

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14. Typical Data

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

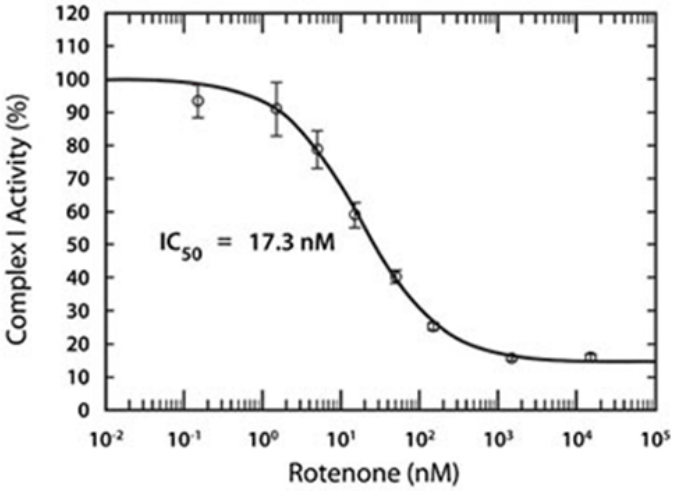


Figure 2. Typical dose response curve for rotenone. Assay was performed following the Dose Response Assay Procedure using rotenone, a well known Complex I inhibitor. Rotenone was prepared in DMSO to generate a 10 mM stock. Starting with a 50 µM final concentration in well (5.5 µL in channel 1), 1:10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

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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)

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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.

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ab109904 MitoTox™ Complex II OXPHOS Activity Assay Kit

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

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1. Overview

MitoTox™ Complex II OXPHOS Activity Assay Kit (ab109904) is designed for testing the direct inhibitory effect of compounds on Complex II activity in only 4 hours. Complex II extracted from the provided bovine heart mitochondria (a rich source of Complex II) is immunocaptured by specific antibodies on the plate. Complex II activity can be observed as decrease in absorbance at OD 600 nm.

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

The inhibitory effects of compounds on Complex II 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 II activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

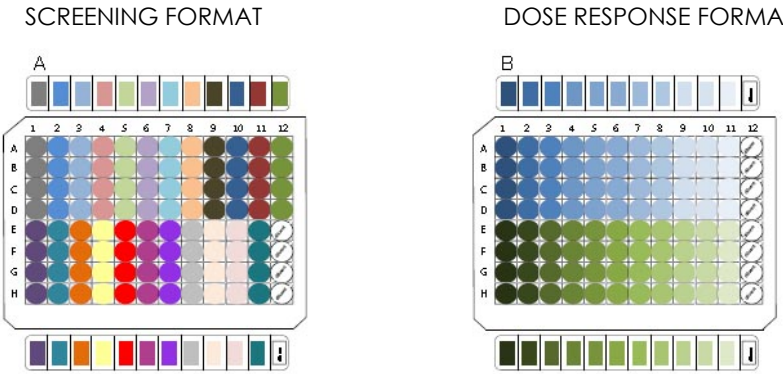


Figure 1. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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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 II (Succinate-coenzyme Q Reductase, SDH, EC 1.3.5.1) is one of the five complexes involved in oxidative phosphorylation (OXPHOS) in mitochondria. The enzyme complex 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 used by Complex III in the respiratory chain and fumarate is necessary to maintain the Kreb's/TCA cycle.

Succinate + ubiquinone (Q) → Fumarate + ubiquinol (QH2)

The production of ubiquinol can be monitored by the addition of DCPIP (2,6-dichlorophenolindophenol), which when reduced by ubiquinol recycles the substrate ubiquinone and is transformed from a blue product to a colorless product, leading a decrease in absorbance at OD 600 nm.

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2. Protocol Summary

Add detergent-solubilized mitochondria to plate



Add Complex II activity solution + test compounds to plate



Measure absorbance (OD600 nm) in kinetic mode
for 30 minutes at RT*

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

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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, Succinate, DCPIP and Ubiquinone 2 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.

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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)
20X Buffer	5 mL	4°C	4°C
Complex II Activity Buffer	25 mL	4°C	4°C
Detergent	1 mL	4°C	4°C
Bovine Heart Mitochondria	2 x 360 µL	-80°C	-80°C
DCPIP/DCIP	250 µL	-80°C	-80°C
Succinate	500 µL	-80°C	-80°C
Ubiquinone 2	60 µL	-80°C	-80°C
Pre-coated 96-well microplate	1 unit	4°C	4°C
12-channel reagent reservoirs	2 units	4°C/RT	N/A

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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 600 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) TTFA (2-thenoyltrifluoroacetone) – Complex II inhibitor: use at 100 mM stock solution (in DMSO)

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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.

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9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 20X Buffer:

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

9.2 Complex II Activity Buffer:

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

9.3 Detergent:

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

9.4 Bovine Heart Mitochondria (5.5 mg/mL):

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

9.5 DCPIP:

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

9.6 Succinate:

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

9.7 Ubiquinone 2:

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

9.8 Pre-coated 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.

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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:

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

Where:

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

[Stock] = stock concentration of test compound.

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

Δ Note: See Assay Procedure section for more details

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11. Assay Procedure – SCREENING ASSAY

- The 96-well microplate provided has been coated with anti-Complex II 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 TTFA in the screening procedure as a positive control. TTFA is a well-known inhibitor of Complex II activity. Following the assay procedure, 50% inhibition of Complex II activity is obtained with 20 ± 2 µM TTFA (see example in section 14. Typical data).
- 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 36 µL of Detergent to each of the provided tubes containing bovine heart mitochondria (BHM) (2 x 360 µL at 5.5 mg/mL each).
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 from each tube and pool together (approx. 513 µL). Discard pellets.
11.1.6 Add 20 mL Buffer Solution to the solubilized BHM (~ 513 µL). Mix well by pipetting up and down.
11.1.7 Add 200 µL of BHM (~ 25 µg mitochondria) to each well of the precoated 96-well microplate.
11.1.8 Cover plate and incubate for 2 hours at room temperature.

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11.2 Plate washing:

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 Buffer 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 Buffer Solution to each well.
11.2.5 Cover the plate and leave at room temperature (on the bench) until ready to add compounds.

11.3 Test Compound preparation:

11.3.1 Prepare Complex II Activity Solution immediately prior to use by adding all the content of the Ubiquinone 2 vial (~ 60 µL) + 250 µL DCPIP and 500 µL succinate to 24 mL of Complex II Activity Buffer, Mix well. Label this solution "**Complex II 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 Add Complex II 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:

11.4.1 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
11.4.2 Using a multi-channel pipette, add 200 µL of Complex II Activity Solution/Compound from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.
11.4.3 Using a multi-channel pipette, add 200 µL of Complex II 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.

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11.5 Measurement:

11.5.1 Measure output immediately at OD 600 nm on a microplate reader in kinetic mode, every minute, for at least 1 hour at room temperature protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1.0 and Kinetic reading reads as Vmax (mOD-units per minute).

The initial OD should be approximately 0.2 OD-units at 600 nm. The reduction of ubiquininone and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD 600 nm.

The linear rate is usually seen between 5-10 minutes. After 15 minutes, the rate of reduction in absorbance is declining for the most active samples due lack of substrate – do not calculate the rate after this point.

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12. Assay Procedure – DOSE RESPONSE ASSAY

- The 96-well microplate provided has been coated with anti-Complex II 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 TTFA in the screening procedure as a positive control. TTFA is a well-known inhibitor of Complex II activity. Following the assay procedure, 50% inhibition of Complex II activity is obtained with 20 ± 2µM TTFA.
- 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 36 µL of Detergent to each of the provided tubes containing bovine heart mitochondria (BHM) (2 x 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 from each tube and pool together (approx. 513 µL). Discard pellets.
12.1.6 Add 20 mL Buffer Solution to the solubilized BHM (~ 513 µL). Mix well by pipetting up and down.
12.1.7 Add 200 µL of BHM (~ 25 µg mitochondria) to each well of the precoated 96-well microplate.
12.1.8 Cover plate and incubate for 2 hours at room temperature.

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12.2 Plate washing:

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 Buffer 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 Buffer Solution to each well.
12.2.5 Cover the plate and leave at room temperature (on the bench) until ready to add compounds.

12.3 Test Compound preparation:

12.3.1 Prepare Complex II Activity Solution immediately prior to use by adding all the content of the Ubiquinone 2 vial (~ 60 µL) + 250 µL DCPIP and 500 µL succinate to 24 mL of Complex II Activity Buffer, Mix well. Label this solution "**Complex II Activity Solution**".
12.3.2 Compound 1 dose response:
12.3.2.5 Add 900 µL of Complex II Activity Solution to channels 2-12 of the first reagent reservoirs.
12.3.2.6 Add Compound 1 to channel 1 of the first reservoir. Add Complex II 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.

12.3.2.7 Starting with channel 1, generate serial dilutions from channel 2 till channel 11.
12.3.2.8 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.

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12.4 Test Compound addition to plate:

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

12.4.2 Using a multi-channel pipette, add 200 µL of Complex II Activity Solution/Compound 1 from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.

12.4.3 Using a multi-channel pipette, add 200 µL of Complex II Activity Solution/Compound 2 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.

12.5 Measurement:

12.5.1 Measure output immediately at OD 600 nm on a microplate reader in kinetic mode, every minute, for at least 1 hour at room temperature protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1.0 and Kinetic reading reads as Vmax (mOD-units per minute).

The initial OD should be approximately 0.2 OD-units at 600 nm. The reduction of ubiquininone and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD 600 nm.

The linear rate is usually seen between 5-10 minutes. After 15 minutes, the rate of reduction in absorbance is declining for the most active samples due lack of substrate – do not calculate the rate after this point.

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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 II activity is proportional to the decrease in absorbance at OD 600 nm.

13.1 Calculation of activity of Complex II:

13.1.1 Examine the linear rate of decrease in absorbance at OD 600 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 II as follows:

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

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14. Typical Data

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

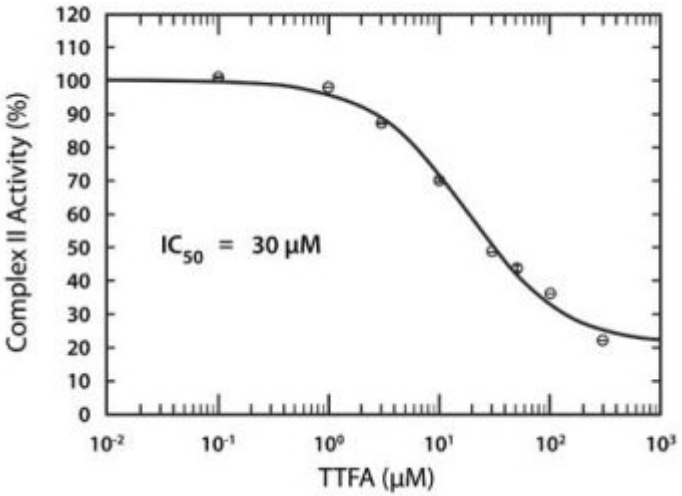


Figure 2. Typical dose response curve for TTFA (2-thenoyltrifluoracetone). Assay was performed following the Dose Response Assay Procedure using TTFA, a well known Complex II inhibitor. TTFA was prepared in DMSO to generate a 100 mM stock. Starting with a 500 µM final concentration in well (9 µL in channel 1), 1:2 serial dilutions of TTFA were generated.

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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 (TTFA)

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16. FAQs

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. Is there a specific pH that the reaction solution should be in order to measure activity? The inhibitor I'm studying is a strong acid with a pH around 2.

A. The assay will not work on an acidic pH; it needs to be at a neutral pH (7-7.4).

You could add your compound and bring the pH back to neutral, but keep in mind that then you will be testing the salt version and the not acid directly in the assay. For example, malonic acid is a known inhibitor of Complex II, as shown in literature: we have tested the effect of potassium and sodium malonate (which is malonic acid after pH has been adjusted with KOH or NaOH, respectively), and have found that malonate (salt form) is able to inhibit activity of Complex II when tested with our assay.

Q. Can I use my own isolated mitochondria?

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

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Version 5e Last updated 16 June 2022

ab109905 MitoTox™ Complex II + III OXPHOS Activity Assay Kit

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

View kit datasheet: www.abcam.com/ab109905
(use www.abcam.cn/ab109905 for China, or www.abcam.co.jp/ab109905 for Japan)

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

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1. Overview

MitoTox™ Complex II+ III OXPHOS Activity Assay Kit (ab109905) is designed for testing the direct inhibitory effect of compounds on Complex III activity in only 30 minutes. The assay is performed using whole bovine heart mitochondria, a rich source of OXPHOS complexes. Succinate (electron donor of Complex II) and oxidized cytochrome c (electron acceptor of Complex III) are added to the mitochondria to start the electron transfer reaction that takes place during oxidative phosphorylation (Figure 1). The rate of coupled Complex II + III reaction is measured by monitoring the conversion of oxidized cytochrome c into reduced form, which can be observed as increase in absorbance at OD 550 nm.

The assay requires rotenone and KCN (not supplied in the kit): rotenone (Complex I inhibitor) blocks electron transfer to ubiquinone, ensuring that all reduction of cytochrome c happens via Complex II. The addition of KCN (Complex IV inhibitor) ensures that there is no re-oxidation of cytochrome c.

The intra-assay and inter-assay variation of this assay are both < 10%.

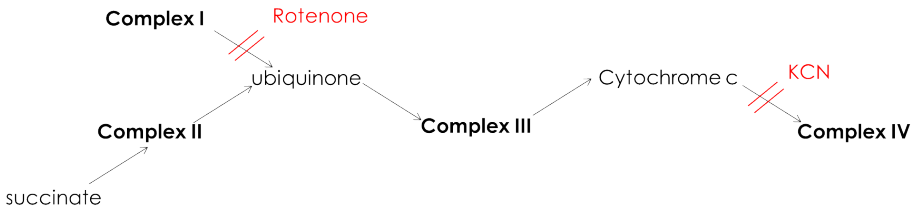


Figure 1. Pathway of electron transfer in mitochondria during oxidative phosphorylation.

The inhibitory effects of compounds on Complex III 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 III activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

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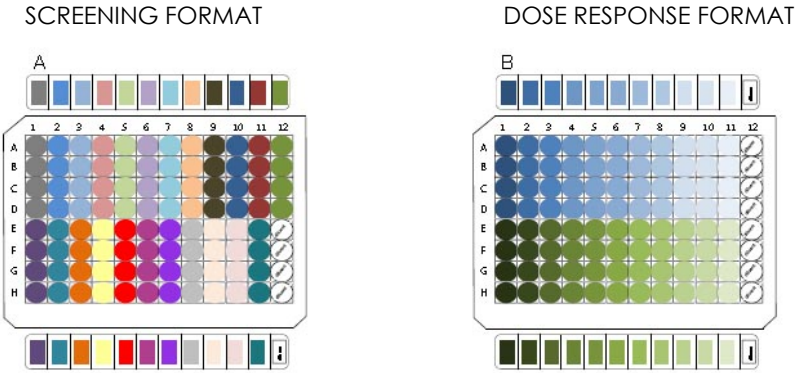


Figure 2. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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2. Protocol Summary

Add Complex III activity solution + test compounds to plate



Add mitochondria to plate



Measure absorbance (OD550 nm) in kinetic mode
for 5 minutes at RT*

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

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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 and Cytochrome c 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.

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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 Complex III Mito Buffer	1 mL	4°C	4°C
1X Succinate Solution	12 mL	4°C	4°C
Bovine Heart Mitochondria	300 µL	-80°C	-80°C
Cytochrome c (III)	550 µL	-80°C	-80°C
96-well microplate	1 unit	4°C/RT	N/A
12-channel reagent reservoirs	2 units	4°C/RT	N/A
Disposable single channel reservoirs	2 units	4°C/RT	N/A

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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 550 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
- Rotenone – we recommend Rotenone (ab143145): prepare a 10 mM stock solution in DMSO or ethanol. Store at -20°C or -80°C.
- KCN: on the day of the assay, prepare a 0.2 M stock in ddH_2O. KCN can be prepared in advanced in 0.1M NaOH solution and stored at -20°C or -80°C. Please be aware that KCN is extremely unstable in water – do not use a KCN made in the morning in ddH_2O if running the assay in the afternoon.
- (Optional) Antimycin A (Complex III inhibitor) – we recommend Antimycin A (ab141904) at a 10 mM stock in DMSO

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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.

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9. 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:

$$Vtest = 460\ \mu L\ x\ \frac{[Compound]}{[Stock]}$$

Where:

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

[Stock] = stock concentration of test compound.

460 µL = total volume of Complex III Solution/Compound.

Δ Note: See Assay Procedure section for more details

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10. Assay Procedure – SCREENING ASSAY

- We recommend using Antimycin A in the screening procedure as a positive control. Antimycin A is a well-known inhibitor of Complex III activity. Following the assay procedure, 50% inhibition of Complex III activity is obtained with 22 ± 4 nM Antimycin A.
- 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 2).

10.1 Test Compound preparation and addition to plate:

10.1.1 Prepare **Complex III Activity Solution** immediately prior to use by adding the following to the 12 mL of succinate: 500 µL Cytochrome c (III) + 120 µL of 0.2 M KCN (not provided) + 15.2 µL 10 mM rotenone (not provided). Mix well.

10.1.2 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.

10.1.3 Add Complex III Activity Solution to each channel of both 12-channel reagent reservoirs to a final volume of 460 µL.

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

10.1.5 Using a multi-channel pipette, add 100 µL of Complex III Activity Solution/Compound from each channel of the first 12-channel reagent reservoir to each well in row A.

10.1.6 Using a multi-channel pipette, add 100 µL of Complex III Activity Solution/Compound from each channel of the second 12-channel reagent reservoir to each well in row H.

Δ Note: rows A and H are set as reaction blanks.

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

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10.2 Bovine Heart Mitochondria (BHM) and plating:

10.2.1 Dilute 120 µL of bovine heart mitochondria (BHM) (5 mg/mL) into 880 µL 1X Complex III Mito Buffer to get 1 mL of BHM at 0.6 mg/mL.

10.2.2 Add diluted BHM to a single-channel reagent reservoir.

10.2.3 Using a multi-channel pipette, rapidly transfer 20 µL of diluted BHM (0.6 mg/mL) to every channel of both 12-channel reagent reservoirs containing Complex III Activity Solution/Compounds (Step 11.1.2 + 11.1.3).

10.2.4 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, C, D.

10.2.5 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, F, G.

10.3 Measurement:

10.3.1 Measure output immediately at OD 550 nm on a microplate reader in kinetic mode, every 20 seconds, for 5-10 minutes at room temperature protected from light.

Δ Note: Ensure the Kinetic reading reads as Vmax (mOD-units per minute).

Δ Note: If it takes you more than 2 minutes to transfer the mitochondria into the 12-channel reservoirs and into the plate, it is possible that the substrate is consumed before the reactions ends. In this scenario, the rate of reduced cytochrome c accumulation will plateau (as seen in Figure 2, Section 13, 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 (for example, to 120 seconds as seen in Figure 3).

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11. Assay Procedure – DOSE RESPONSE ASSAY

- We recommend using Antimycin A in the screening procedure as a positive control. Antimycin A is a well-known inhibitor of Complex III activity. Following the assay procedure, 50% inhibition of Complex III activity is obtained with 22 ± 4 nM Antimycin A.
- 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 2).

11.1 Test Compound preparation and addition to plate:

11.1.1 Prepare **Complex III Activity Solution** immediately prior to use by adding the following to the 12 mL of succinate: 500 µL Cytochrome c (III) + 120 µL of 0.2 M KCN (not provided) + 15.2 µL 10 mM rotenone (not provided). Mix well.

11.1.2 Compound 1 dose response:

11.1.2.9 Add 460 µL of Complex III Activity Solution to channels 2-12 of the first reagent reservoirs.

11.1.2.10 Add Compound 1 to channel 1 of the first reservoir. Add Complex III Activity Solution to a final volume of 460 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.

11.1.2.11 Starting with channel 1, generate serial dilutions from channel 2 till channel 11.

11.1.2.12 Add only solvent to channel 12 of the first reservoir as a control.

11.1.3 Repeat same procedure described in Step 12.1.2 for compound 2.

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

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11.1.5 Using a multi-channel pipette, add 100 µL of Complex III Activity Solution/Compound from each channel of the first 12-channel reagent reservoir to each well in row A.

11.1.6 Using a multi-channel pipette, add 100 µL of Complex III Activity Solution/Compound from each channel of the second 12-channel reagent reservoir to each well in row H.

Δ Note: rows A and H are set as reaction blanks.

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

11.2 Bovine Heart Mitochondria (BHM) and plating:

11.2.1 Dilute 120 µL of bovine heart mitochondria (BHM) (5 mg/mL) into 880 µL 1X Complex III Mito Buffer to get 1 mL of BHM at 0.6 mg/mL.

11.2.2 Add diluted BHM to a single-channel reagent reservoir.

11.2.3 Using a multi-channel pipette, rapidly transfer 20 µL of diluted BHM (0.6 mg/mL) to every channel of both 12-channel reagent reservoirs containing Complex III Activity Solution/Compounds (Step 12.1.2 + 12.1.3).

11.2.4 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, C, D.

11.2.5 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, F, G.

11.3 Measurement:

11.3.1 Measure output immediately at OD 550 nm on a microplate reader in kinetic mode, every 20 seconds, for 5-10 minutes at room temperature protected from light.

Δ Note: Ensure the Kinetic reading reads as Vmax (mOD-units per minute).

Δ Note: If it takes you more than 2 minutes to transfer the mitochondria into the 12-channel reservoirs and into the plate, it is possible that the substrate is consumed before the reactions ends. In this scenario, the rate of reduced cytochrome c accumulation will plateau (as seen in Figure 2, Section 13, 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 (for example, to 120 seconds as seen in Figure 3).

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12. 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 III activity is proportional to the increase in absorbance at OD 550 nm.

12.1 Calculation of activity of Complex III:

12.1.1 Examine the linear rate of increase in absorbance at OD 550 nm over time.

12.1.2 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).

12.1.3 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)

12.1.4 Average the triplicate reading for each sample.

12.1.5 Calculate activity of Complex II as follows:

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

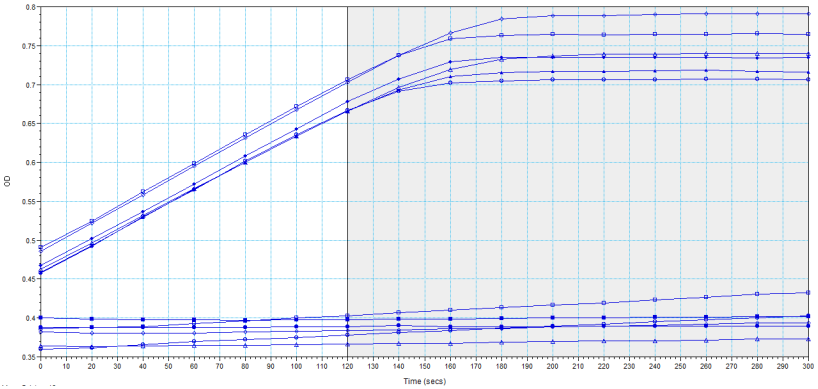


Figure 3. Reduced cytochrome c accumulation kinetic curve.

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13. Typical Data

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

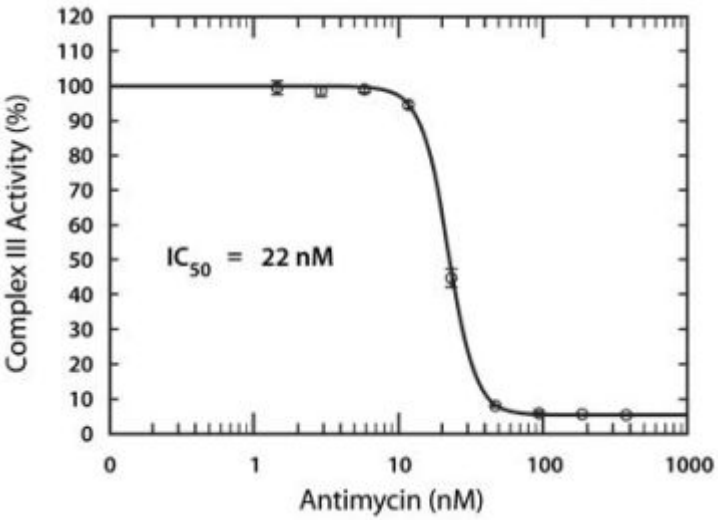


Figure 4. Typical dose response curve for antimycin A. Assay was performed following the Dose Response Assay Procedure using antimycin A, a well known Complex III inhibitor. Antimycin A was prepared in DMSO to generate a 10 mM stock. Starting with a 150 µM final concentration in well (3 µL in channel 1), 1:4 serial dilutions of antimycin A were generated.

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14. 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 (antimycin A)

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15. FAQs

Q. Why is the product measuring also Complex II activity?

A. Measuring Complex III activity poses a technical challenge due to the instability of ubiquinol (Complex III substrate). Therefore, Complex III activity must be measured in a coupled reaction. Complex III liability or inhibition can be inferred from using MitoTox™ Complex II OXPHOS Activity Assay (ab109903) alongside this product (ie, there is inhibition of Complex III when there is reduced activity seen using ab109905 but not when using ab109904).

Q. Can I use another wavelength to measure the activity?

A. No, you should only read the assay at OD 550nm. This is because the peak spectra of oxidized cytochrome c is at OD 542 nm and the peak spectra of reduced cytochrome c is at OD 550 nm. Measuring at any OD other than 550 nm will likely result in misrepresentation of the enzyme activity.

Q. Can I use cells or tissue extracts for this assay?

A. No, you need to use whole mitochondria. Contrary to the other MitoTox™ kits, this assay uses a coupled reaction for which intact mitochondria are needed.

Q. What can affect readout of the assay?

A. KCN and rotenone must be fresh in order to have good activity. Rotenone may be kept at -20°C in DMSO for no longer than 3 months. KCN must be prepared immediately before running the assay if prepared in ddH_2O, or it can be stored at 4°C for 3 months if diluted in 100 mM NaOH. Please be aware that KCN is extremely unstable in water – do not use a KCN made in the morning in ddH_2O if running the assay in the afternoon.

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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, mitochondria isolated from other species can be used for the assay. We would still recommend to run a control reaction with the provided mitochondria to ensure the assay is working.

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Version 5b Last updated 16 June 2022

ab109906 MitoTox™ Complex IV OXPHOS Activity Assay Kit

For the rapid, sensitive and accurate screening of potential inhibitors of Complex IV 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 IV OXPHOS Activity Assay Kit (ab109906) is designed for testing the direct inhibitory effect of compounds on Complex IV activity in only 5 hours. Complex IV extracted from the provided bovine heart mitochondria (a rich source of Complex IV) is immunocaptured by specific antibodies on the plate. Complex IV activity can be observed as decrease in absorbance at OD 550 nm.

The intra-assay and inter-assay variation of this assay are both < 10%.

The inhibitory effects of compounds on Complex IV 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 IV activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

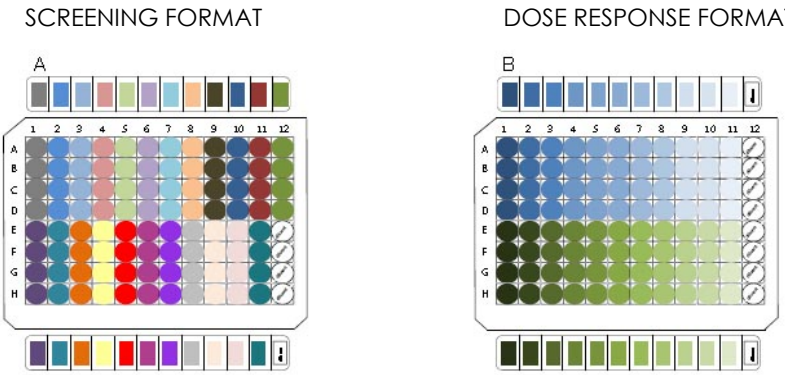


Figure 1. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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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 IV (Cytochrome c oxidase, EC 1.9.3.1) is one of the five complexes involved in oxidative phosphorylation (OXPHOS) in mitochondria. The enzyme complex transfers electrons from reduced cytochrome c (cytochrome c^{2+}) to molecular oxygen, and concomitantly pumps protons across the inner mitochondrial membrane.

$$4 \quad \text{cytochrome } c^{2+} + 4H^{+} + O_2 \rightarrow 4 \text{ cytochrome}^{3+} + 2H_2O$$

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2. Protocol Summary

Add Blocking Solution to plate



Add detergent-solubilized mitochondria to plate



Add Complex IV activity solution + test compounds to plate



Measure absorbance (OD550 nm) in kinetic mode
for 60 minutes at RT*

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

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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 and Reagent C 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.

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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 Blocking Buffer	70 mL	4°C	4°C
1X Complex IV Mito Buffer	110 mL	4°C	4°C
Blocking powder (1.8 g)	1 vial	4°C	N/A
Detergent	20 µL	4°C	4°C
Bovine Heart Mitochondria	90 µL	-80°C	-80°C
Reagent C	2 x 1 mL	-80°C	-80°C
Pre-coated 96-well microplate	1 unit	4°C	4°C
12-channel reagent reservoirs	2 units	4°C/RT	N/A

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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 550 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) KCN: on the day of the assay, prepare a 0.2 M stock in ddH_2O. KCN can be prepared in advanced in 0.1M NaOH solution and stored at -20°C or -80°C. Please be aware that KCN is extremely unstable in water – do not use a KCN made in the morning in ddH_2O if running the assay in the afternoon.

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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.

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9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Blocking powder (1.8 g):

Dissolve 1.5 g of Blocking powder in 30 mL of 1X Blocking Buffer (Step 9.1). Mix well by pipetting up and down. Label this mixture as **Blocking Solution**. Equilibrate to room temperature before use. Store at 4°C.

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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:

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

Where:

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

[Stock] = stock concentration of test compound.

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

Δ Note: See Assay Procedure section for more details

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11. Assay Procedure – SCREENING ASSAY

- The 96-well microplate provided has been coated with anti-Complex IV 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 KCN in the screening procedure as a positive control. KCN is a well-known inhibitor of Complex IV activity. Following the assay procedure, 50% inhibition of Complex IV activity is obtained with 3.2 ± 0.5 µM KCN.
- 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 Blocking Solution addition:

11.1.1 Add 300 µL Blocking Solution (Step 9.1) to each well of the 96-well plate.
11.1.2 Cover plate and incubate at room temperature for 1 hour.

11.2 Bovine Heart Mitochondria (BHM) solubilization and plating:

11.2.1 Add 10 µL of Detergent to the provided tube containing bovine heart mitochondria (BHM) (90 µL at 5.5 mg/mL).
11.2.2 Mix well by vortexing.
11.2.3 Incubate tube on ice for 30 minutes.
11.2.4 Centrifuge at 25,000 x *g* for 20 minutes at 4°C in a cold microcentrifuge.
11.2.5 Collect sample supernatant containing solubilized BHM and discard pellet. Keep on ice until use.
11.2.6 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
11.2.7 Add 300 µL of Blocking Solution to each well.
11.2.8 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.

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Δ Note: Discard Blocking Solution as it is not needed for subsequent steps in the protocol. Inadvertent use of Blocking Solution in subsequent steps will result in loss of Complex IV activity in the assay.

11.2.9 Dilute 20 µL of solubilized BHM (Step 11.2.5) into 22 mL of 1X Mito Buffer. Mix well by pipetting up and down.

11.2.10 Add 200 µL of diluted BHM (~ 909 ng mitochondria) to each well of the precoated 96-well microplate.

11.2.11 Cover plate and incubate for 3 hours at room temperature.

11.3 Test Compound preparation:

11.3.1 Prepare Complex IV Activity Solution immediately prior to use by gently mixing 2 mL of Reagent C with 22 mL of 1X Mito Buffer. (to prevent bubble formation) Label this solution "Complex IV 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 mitochondria incubation time is almost complete (Step 11.2.11), add Complex IV 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:

11.4.1 Once mitochondria incubation step (Step 11.2.11) is finished, rinse the wells by adding 300 µL 1X Mito Buffer to each well.

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

11.4.3 Add 300 µL of 1X Mito Buffer to each well.

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

11.4.5 Using a multi-channel pipette, gently add 200 µL of Complex IV Activity Solution/Compound from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.

11.4.6 Using a multi-channel pipette, add 200 µL of Complex IV Activity Solution/Compound from each channel of the

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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 550 nm on a microplate reader in kinetic mode, every minute, for at least 60 minutes at room temperature protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1 and Kinetic reading reads as Vmax (mOD-units per minute).

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12. Assay Procedure – DOSE RESPONSE ASSAY

- The 96-well microplate provided has been coated with anti-Complex IV 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 KCN in the screening procedure as a positive control. KCN is a well-known of Complex IV activity. Following the assay procedure, 50% inhibition of Complex IV activity is obtained with 3.2 ± 0.5 µM KCN.
- 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 Blocking Solution addition:

12.1.1 Add 300 µL Blocking Solution (Step 9.1) to each well of the 96-well plate.
12.1.2 Cover plate and incubate at room temperature for 1 hour.

12.2 Bovine Heart Mitochondria (BHM) solubilization and plating:

12.2.1 Add 10 µL of Detergent to the provided tube containing bovine heart mitochondria (BHM) (90 µL at 5.5 mg/mL).
12.2.2 Mix well by vortexing.
12.2.3 Incubate tube on ice for 30 minutes.
12.2.4 Centrifuge at 25,000 x *g* for 20 minutes at 4°C in a cold microcentrifuge.
12.2.5 Collect sample supernatant containing solubilized BHM and discard pellet. Keep on ice until use.
12.2.6 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
12.2.7 Add 300 µL of 1X Blocking Buffer to each well.
12.2.8 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.

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Δ Note: Discard Blocking Solution as it is not needed for subsequent steps in the protocol. Inadvertent use of Blocking Solution in subsequent steps will result in loss of Complex IV activity in the assay.

12.2.9 Dilute 200 µL of solubilized BHM (Step 12.2.5) into 22 mL of 1X Mito Buffer. Mix well by pipetting up and down.

12.2.10 Add 20 µL of diluted BHM (~ 200 ng mitochondria) to each well of the precoated 96-well microplate.

12.2.11 Cover plate and incubate for 3 hours at room temperature.

12.3 Test Compound preparation:

12.3.1 Prepare Complex IV Activity Solution immediately prior to use by gently mixing 2 mL of Reagent C with 22 mL of 1X Mito Buffer. (to prevent bubble formation) Label this solution "Complex IV Activity Solution".

12.3.2 Compound 1 dose response:

12.3.2.13 When mitochondria incubation time is almost complete (Step 12.2.11), add 900 µL of Complex IV Activity Solution to channels 2-12 of the first reagent reservoirs.

12.3.2.14 Add Compound 1 to channel 1 of the first reservoir. Add Complex IV 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.

12.3.2.15 Starting with channel 1, generate serial dilutions from channel 2 till channel 11.

12.3.2.16 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:

12.4.1 Once mitochondria incubation step (Step 12.2.11) is finished, rinse the wells by adding 300 µL 1X Mito Buffer to each well.

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

12.4.3 Add 300 µL of 1X Mito Buffer to each well.

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

12.4.5 Using a multi-channel pipette, gently add 200 µL of Complex IV Activity Solution/Compound 1 from each channel of the first 12-channel reagent reservoir to each well in row A, B, C, D.

12.4.6 Using a multi-channel pipette, add 200 µL of Complex IV Activity Solution/Compound 2 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.

12.5 Measurement:

12.5.1 Measure output immediately at OD 550 nm on a microplate reader in kinetic mode, every minute, for at least 60 minutes at room temperature protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1 and Kinetic reading reads as Vmax (mOD-units per minute).

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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 IV activity is proportional to the decrease in absorbance at OD 550 nm.

13.1 Calculation of activity of Complex IV:

13.1.1 Examine the linear rate of decrease in absorbance at OD 550 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 IV as follows:

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

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14. Typical Data

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

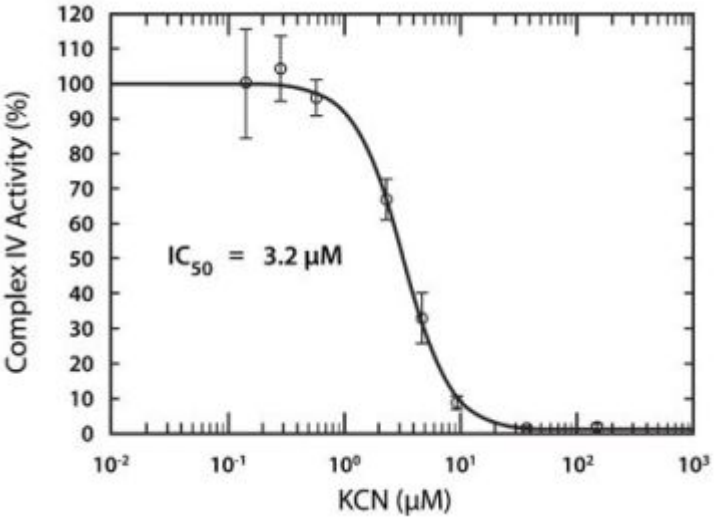


Figure 2. Typical dose response curve for KCN. Assay was performed following the Dose Response Assay Procedure using KCN, a well known Complex IV inhibitor. KCN was prepared in 0.1 M NaOH to generate a 100 mM stock. Starting with a 100 µM final concentration in well (1 µL in channel 1), 1:10 serial dilutions of KCN were generated.

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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 (KCN)

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16. FAQs

Q. Why does this assay require a blocking step? None of the other MitoTox™ OXPHOS kits requires this blocking step.

A. This is because the plate requires fresh blocking to prevent non-specific binding of the enzyme to the plate. We believe this occurs because Complex IV is a highly hydrophobic enzymatic complex.

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 cells for this assay. This is because the antibody used to capture Complex IV in the plate recognizes Complex IV from these species. We would still recommend to run a control reaction with the provided mitochondria from cow to ensure the assay is working.

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Version 4 Last updated 16 June 2022

ab109907 MitoTox™ Complex V OXPHOS Activity Assay Kit

For the rapid, sensitive and accurate screening of potential inhibitors of Complex V (ATP synthase) 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 V OXPHOS Activity Assay Kit (ab109907) is designed for testing the direct inhibitory effect of compounds on Complex V activity in only 4 hours. Complex V extracted from the provided bovine heart mitochondria (a rich source of Complex V) is immunocaptured by specific antibodies on the plate. Complex V activity can be observed as decrease in absorbance at OD 340 nm.

The intra-assay and inter-assay variation of this assay are both < 10%.

The inhibitory effects of compounds on Complex V 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 V activity can be tested at 11 different data points in triplicate, along the appropriate blanks.

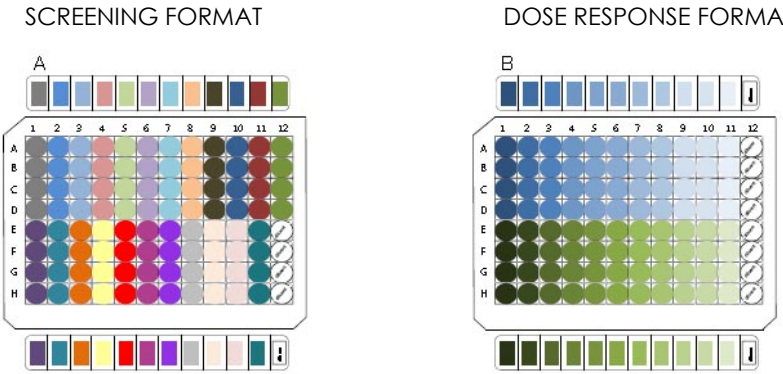


Figure 1. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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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 V (ATP synthase, F1F0 ATPase, EC 3.6.3.14) is one of the five complexes involved in oxidative phosphorylation (OXPHOS) in mitochondria. This enzyme complex makes about 95% of 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 OD 340 nm, as shown in Figure 2.

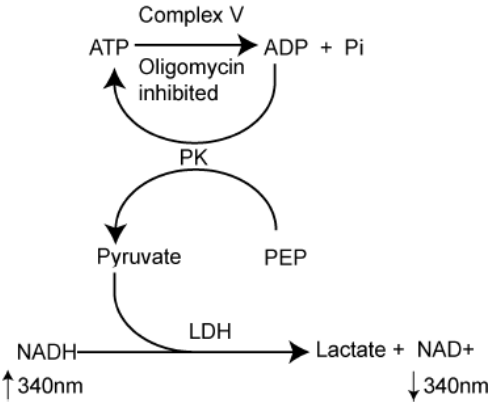


Figure 2. Schematic representation of the reaction set up. PK: pyruvate kinase; PEP: phosphoenolpyruvate; LDH: lactate dehydrogenase.

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2. Protocol Summary

Add detergent-solubilized mitochondria to plate



Add phospholipids to plate



Add Complex V activity solution + test compounds to plate



Measure absorbance (OD340 nm) in kinetic mode
for 60 minutes at 30°C*

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

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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 V Activity Buffer and Phospholipid at - 80°C) in the dark immediately upon receipt. Unopened Kit has a storage time of 1 year from receipt.

Refer to list of materials supplied for storage conditions of individual components.

Aliquot components in working volumes before storing at the recommended temperature. Please avoid freeze/thaw. Discard all the unused components once the assay is done.

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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
1X Mito Buffer	5 mL	4°C
20X Wash Buffer	5 mL	4°C
Phospholipids	6 mL	-80°C
Complex V Activity Buffer	24 mL	-80°C
Detergent	100 µL	4°C
Bovine Heart Mitochondria	360 µL	-80°C
Pre-coated 96-well microplate	1 unit	4°C
12-channel reagent reservoirs	2 units	4°C/RT

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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) Oligomycin – Complex V inhibitor: we recommend Oligomycin (ab141829) at 10 mM stock solution (in DMSO)

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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.

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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 V 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 Pre-coated 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.

9.8 12-channel reagent reservoirs:

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

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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 V Solution/Compound.

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

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

Δ Note: See Assay Procedure section for more details

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11. Assay Procedure – SCREENING ASSAY

- The 96-well microplate provided has been coated with anti-Complex V 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 Oligomycin in the screening procedure as a positive control. Oligomycin is a well-known inhibitor of Complex V activity. Following the assay procedure, 50% inhibition of Complex V activity is obtained with 10 ± 2 nM oligomycin.
- 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.

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

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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 60 minutes at 30°C protected from light.

Δ Note: Ensure the limit of maximum OD is set read at 1 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 5-15 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.

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12. Assay Procedure – DOSE RESPONSE ASSAY

- The 96-well microplate provided has been coated with anti-Complex V 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 Oligomycin in the screening procedure as a positive control. Oligomycin is a well-known inhibitor of Complex V activity. Following the assay procedure, 50% inhibition of Complex V activity is obtained with 10 ± 2 nM oligomycin.
- 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.

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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 Compound 1 dose response:

12.3.1.17 When phospholipid incubation time is almost complete (Step 12.2.7), add 900 µL of Complex V Activity Buffer to channels 2-12 of the first reagent reservoirs.

12.3.1.18 Add Compound 1 to channel 1 of the first reservoir. Add Complex V Activity Buffer 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.

12.3.1.19 Starting with channel 1, generate serial dilutions from channel 2 till channel 11.

12.3.1.20 Add only solvent to channel 12 of the first reservoir as a control.

12.3.2 Repeat same procedure described in Step 12.3.2 for compound 2.
12.3.3 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 V 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.

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12.4.2 Using a multi-channel pipette, add 200 µL of Complex V 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.

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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 V activity is proportional to the decrease in absorbance at OD 340 nm.

13.1 Calculation of activity of Complex V:

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 V as follows:

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

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14. Typical Data

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

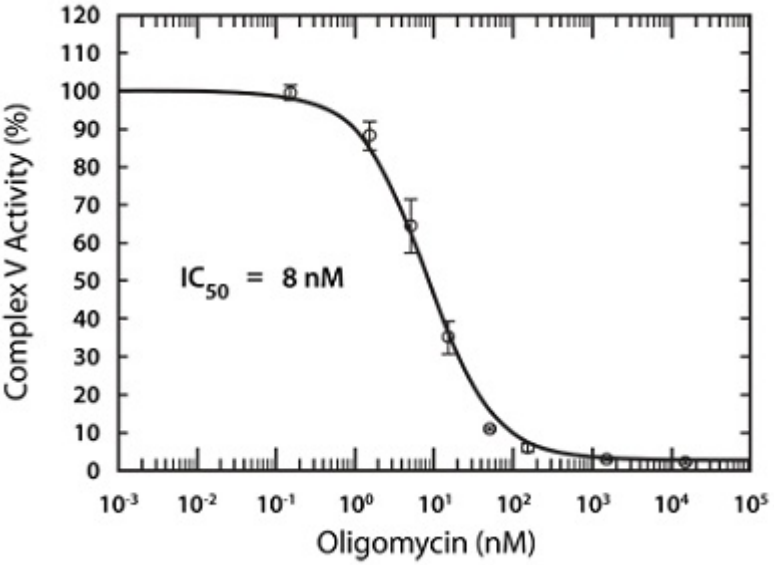


Figure 3. Typical dose response curve for oligomycin. Assay was performed following the Dose Response Assay Procedure using oligomycin, a well known Complex V inhibitor. Oligomycin was prepared in DMSO to generate a 10 mM stock. Starting with a 50 µM final concentration in well (5.5 µL in channel 1), 1:10 serial dilutions of oligomycin were generated.

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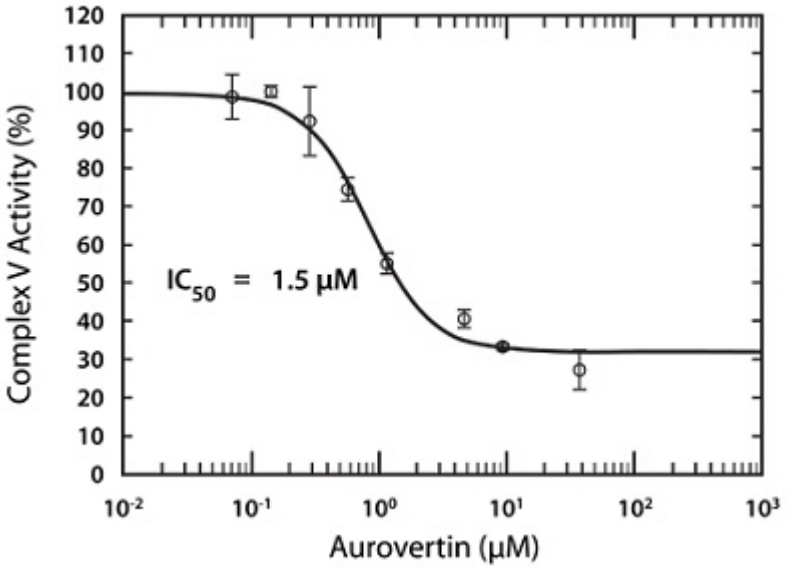


Figure 4. Dose response curve for aurovertin, a non-competitive inhibitor of Complex V.

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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 (oligomycin)

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16. FAQs

Q. Why do I need to use phospholipids?

A. The phospholipids provided in the kit are for the coupled NADH oxidation reaction.

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 or rat cells for this assay. This is because the antibody used to capture Complex V in the plate recognizes Complex V from these species. We would still recommend to run a control reaction with the provided mitochondria from cow to ensure the assay is working.

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Notes

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