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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%.

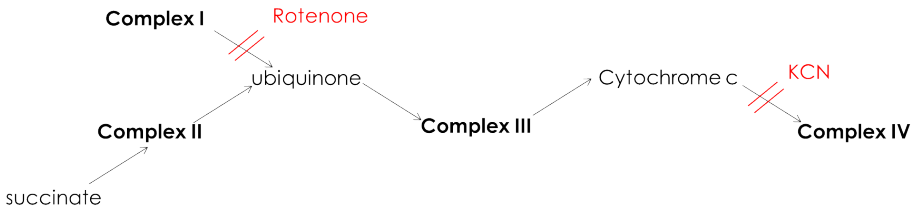

Figure 1. Pathway of electron transfer in mitochondria during oxidative phosphorylation.

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.

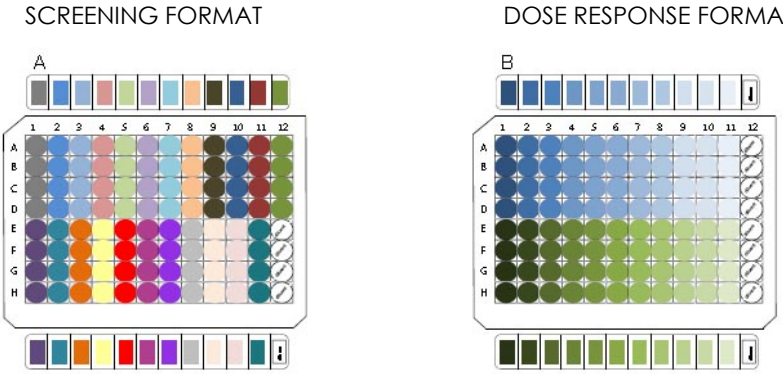

Figure 2. Schematic representation of assay set up format. Panel A: assay set up for the screening format with the plate and two 12-well troughs (depicted above and below the plate) provided in the kit. Each color represents a different compound diluted at a single concentration in activity buffer. Panel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

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*

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.

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

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

8. Technical Hints

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

9. 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

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.

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).

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:

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

11.1.2.2 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.

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

11.1.2.4 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.

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).

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)

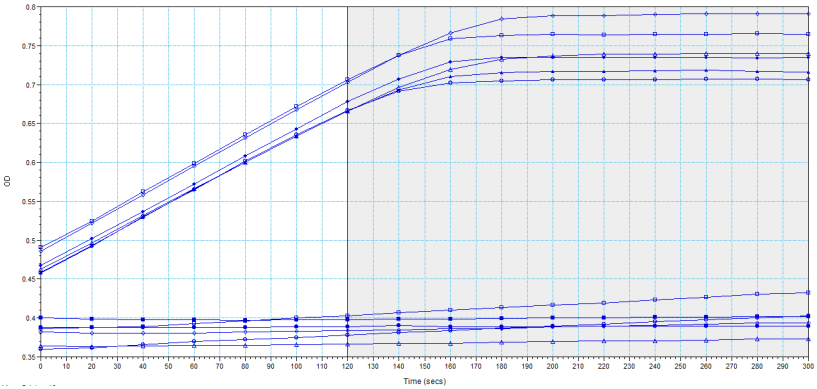

Figure 3. Reduced cytochrome c accumulation kinetic curve.

13. Typical Data

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

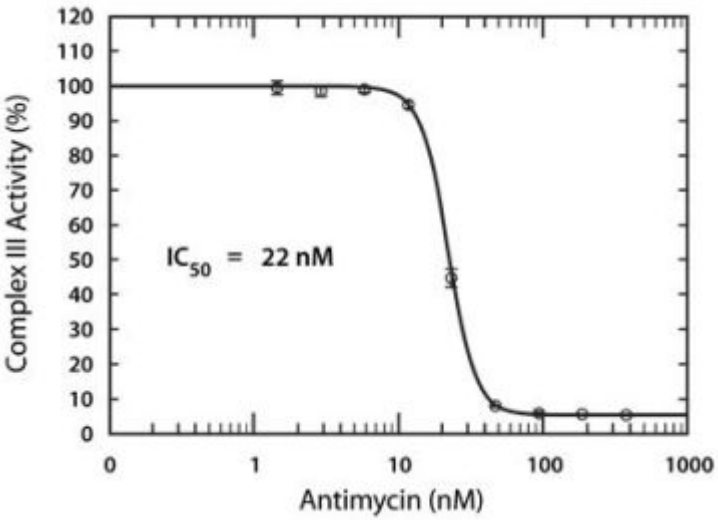

Figure 4. Typical dose response curve for antimycin A. Assay was performed following the Dose Response Assay Procedure using antimycin A, a well known Complex III inhibitor. Antimycin A was prepared in DMSO to generate a 10 mM stock. Starting with a 150 µM final concentration in well (3 µL in channel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

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)

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 (ab109904) 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.

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.

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)