Version 4a Last updated 7 June 2023

ab109908 Complex II Enzyme Activity Microplate Assay Kit (Colorimetric)

View Complex II Enzyme Activity Microplate Assay Kit datasheet:

www.abcam.com/ab109908

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For the measurement of Complex II enzyme activity in human, rat, mouse and bovine cell and tissue extracts.

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1. Overview

Complex II Enzyme Activity Microplate Assay Kit (ab109908) is designed for the analysis of mitochondrial OXPHOS Complex II enzyme activity from human, rat, mouse and bovine cell and tissue extracts.

This kit recognizes Complex II in human, rat, mouse and bovine cell extracts and isolated mitochondria – tissue lysates can also be used but some sample optimization may be necessary. Each of the 96 wells in the kit has been coated with an anti-Complex II monoclonal antibody (mAb) which purifies the enzyme from a complex. After this in-well purification, the production of ubiquinol by the enzyme is coupled to the reduction of the dye DCPIP (2,6-diclorophenolindophenol) and a decreases in its absorbance at OD600 nm which in turn recycles the substrate ubiquinone, as shown below.

ubiquinol (QH2) + DCPIP (blue)

ubiquinone (Q) + DCPIPH2 (colorless)

By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables, an accurate measurement of the enzyme's functional state can be understood.

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

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

Sample preparation (5.5 mg/mL)

- Bring sample to 5.5 mg/mL in PBS.
- Perform detergent extraction with 1/10 volume Detergent
- Incubate on ice for 30 minutes
- Centrifuge at 12,000 x g for 20 minutes at 4°C and then collect supernatant.
- Adjust concentration to recommended dilution for plate loading in Incubation buffer.

Load sample(s) on plate

- Load sample(s) on plate being sure to include positive control sample and buffer control as a null reference.

Incubate for 2 hours at RT

Add 200 µL of Activity Solution to each well

- Prepare sufficient Activity Solution
- Rinse wells twice with 1X Buffer.
- Add 40 µL of Lipid mix to wells.
- Add 200 µL Activity solution into the lipid mix in each well.

Measure Optical Density (OD600 nm) in a kinetic mode at RT for 60 minutes*

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

2. Materials Supplied and Storage

Store kit immediately on receipt. **check below for storage for individual components.** Kit can be stored for 1 year from receipt, if components have not been reconstituted.

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

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temp (before prep)	Storage temp (after prep)
20X Buffer	15 mL	4°C	4°C
10X Blocking Solution	5 mL	4°C	4°C
Detergent	2 x 1 mL	4°C	4°C
Complex II Activity Buffer	25 mL	4°C	4°C
DCPIP/DCIP	250 µL	-80°C	-80°C
Succinate	500 µL	-80°C	-80°C
Ubiquinone 2	60 µL	-80°C	-80°C
Phospholipids	6 mL	-80°C	-80°C
96-well microplate (12 x 8 well strips)	1	4°C	4°C

3. 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.
- MilliQ water or other type of double distilled water (ddH2O)
- 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
- Dounce homogenizer (if using tissue)
- Method for determining protein concentration: we recommend BCA Protein Quantification Kit (ab102536)
- For mitochondria isolation:
- Mitochondria Isolation Kit for Cultured Cells (ab110170)
- Mitochondria Isolation Kit for Tissue (ab110168) or Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer) (ab110169)

4. General guidelines, precautions, and troubleshooting

4.1 Interferences:

RIPA buffer will cause interferences in this assay causing compromised results or complete failure. It contained SDS, which can destroy or decrease the activity of the enzyme.

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

5.1 20X Buffer:

Prepare 1X Buffer by diluting 20X Buffer in ddH2O: to make 300 mL 1X Buffer, combine 15 mL 20X Buffer with 285 mL ddH2O. Mix thoroughly and gently. Label this mixture as "1X Buffer". 1X Buffer can be stored at 4°C. Equilibrate to room temperature before use.

5.2 10 Blocking Solution:

Dilute 10X Blocking Solution in 1X Buffer (Step 5.1) to create Incubation Solution: to make 50 mL Incubation Buffer, combine 5 mL of 10X Blocking Solution with 45 mL of 1X Buffer. Mix thoroughly and gently. Label this mixture as "Incubation Solution". Incubation Solution can be stored at 4°C. Equilibrate to room temperature before use.

5.3 Detergent:

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

5.4 Complex II Activity Buffer:

Ready to use as supplied. Store at 4°C.

5.5 DCPIP:

Ready to use as supplied. Aliquot DCPIP so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at - 80°C. Keep on ice while in use.

5.6 Succinate:

Ready to use as supplied. Aliquot succinate so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -80°C. Keep on ice while in use.

5.7 Ubiquinone 2:

Ready to use as supplied. Aliquot Ubiquinone 2 so that you have enough volume to performed the desired number of assays. Avoid repeated freeze/thaw, Store at -80°C. Keep on ice while in use.

5.8 Phospholipids:

Ready to use as supplied. Keep on ice while in use. Store at 80°C.

5.9 96-well microplate (12 x 8-well strips):

Ready to use as supplied. This plate can be broken into 12 separate 8-well strips for convenience. Equilibrate to room temperature before use. Store at 4°C.

6. Sample Preparation

General sample information:

We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction (once protein concentration has been determined) and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice and continue with the detergent extraction procedure. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Complex II activity in cells or tissue from different origins differs greatly. Cell type and growth conditions are a major factor in Complex II activity measurement.

Treat cells with Complex II activators/inhibitors as per your experimental requirements. The enzyme is sensitive to 2 thenoyltrifluoroacetone (TTFA) a specific inhibitor of Complex II with IC50 of 30 µM.

6.1 Preparation of extracts from cells (adherent or suspension):

6.1.1 Harvest suspension cells by centrifugation or scrape to collect adherent cells from a confluent culture flask (initial recommendation = 1 – 2 x 10^7 cells).

6.1.2 Wash cells twice with PBS

6.1.3 Resuspend and dilute the cell pellet with 9 volumes of PBS (e.g. 50 µL pellet + 450 µL PBS to a total volume of 500 µL).

6.1.4 Determine the sample protein concentration by extracting a portion of your sample (using a standard method such as BCA). Adjust concentration of the sample with PBS so that the final sample protein concentration is **5.5 mg/mL**.

6.1.5 Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/10 (e.g. if the total sample volume is 500 µL add 50 µL of Detergent solution). Mix well.

6.1.6 Incubate the tube on ice for 30 minutes to allow solubilization.
6.1.7 Centrifuge the sample for 20 minutes at 4°C at 12,000 x *g* in a cold centrifuge.
6.1.8 Collect supernatant and transfer to a clean tube. Keep samples on ice. Please note the sample concentration now is **5 mg/mL**. This is the optimal concentration for intact Complex II solubilization.
6.1.9 Dilute your samples to the desired concentration in Incubation Solution (from step 5.2). Table 1 indicates a typical linear range for the assay.

6.2 Preparation of extracts from tissue:

6.2.1 Harvest tissue for the assay (initial recommendation = 100 – 200 mg).
6.2.2 Wash tissue thoroughly in cold PBS to remove blood.
6.2.3 Resuspend tissue in 500 µL – 1 mL of ice cold PBS. It is most convenient to resuspend sample to approximately 10 mg/mL.
6.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 20 – 40 passes, or until sample is fully homogenized and is completely smooth. ***NOTE:*** *it is very important to achieve a thorough homogenization as sample must be completely homogenous.*
6.2.5 Collect homogenate and transfer to a clean tube.
6.2.6 Determine the sample protein concentration by extracting a portion of your sample (using a standard method such as BCA). Adjust concentration of the sample with PBS so that the final sample protein concentration is **5.5 mg/mL**.
6.2.7 Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/10 (e.g. if the total sample volume is 500 µL add 50 µL of Detergent solution). Mix well.
6.2.8 Incubate the tube on ice for 30 minutes to allow solubilization.
6.2.9 Centrifuge the sample for 20 minutes at 4°C at 12,000 x *g* in a cold centrifuge.
6.2.10 Collect supernatant and transfer to a clean tube. Keep samples on ice. Please note the sample concentration now is **5 mg/mL**.

Dilute your samples to the desired concentration in Incubation Solution (from step 5.2). Table 1 indicates a typical range for the assay. However, optimal concentrations for each sample type should be determined by the user:

Sample Type	Recommended Conc. (µg/50 µL volume)	Tested Linear range
Whole cell culture and whole tissue extracts	60 µg/50 µL (1.2 mg/mL)	4-250 µg/well [80 – 5000 µg/mL]
Tissue extracts (mitochondria) – bovine heart	10 µg/50 µL (0.2 mg/mL)	1 – 25 µg/well [20 – 500 µg/mL]

Table 1. Typical range of measurement per assay.

6.3 Preparation of isolated mitochondria:

You can isolate mitochondria using mitochondrial isolation kits such as Mitochondria Isolation Kit for Cultured Cells (ab110170) or Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer) (ab110169).
Alternatively, mitochondria can be prepared by simple differential centrifugation of homogenized tissue samples.
Sample must be completely homogenous, so pipet sample up and down to distribute the mitochondria evenly in the solution.

6.3.1 Determine the sample protein concentration by extracting a portion of your sample (using a standard methods such as BCA). Adjust concentration of the sample with PBS so that the final sample protein concentration is 5.5 mg/mL.

6.3.2 Extract the proteins from the sample by adding Detergent solution to sample to a final dilution of 1/10 (e.g. if the total sample volume is 500 µL add 50 µL of Detergent solution). Mix well.

6.3.3 Incubate the tube on ice for 30 minutes to allow solubilization.

6.3.4 Centrifuge the sample for 20 minutes at 4°C at 12,000 x g in a cold centrifuge.

6.3.5 Collect supernatant and transfer to a clean tube. Please note the sample concentration now is approximately 5 mg/mL. Dilute your samples to the desired concentration in Solution 1. Table 1 indicates a typical linear range for the assay.

7. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

7.1 Plate Loading:

Sample wells: Add 50 µL of sample prepared as described in the Sample Preparation section to each well of the microplate that will be used for this experiment.

Background/Buffer only control wells: Add 50 µL 1X Incubation Solution to buffer only wells.
Positive control sample wells: Add 50 µL of positive sample to appropriate wells.

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

7.2 Measurement:

7.2.1 Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel.
7.2.2 Add 300 µL of 1X Buffer to each well used.
7.2.3 Empty the wells of the microplate by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel. Rinse all wells once more with 300 µL 1X Buffer.
7.2.4 Empty the wells again.
7.2.5 Add 40 µL of Phospholipids to each well used. Incubate for 30 minutes.

7.3 Prepare Activity Solution:

In a sealable tube, prepare only enough solution proportional to the number of microplate strips used according to the following table. Mix gently by inversion.

Number of strips	Ubiquinone (µL)	Succinate (µL)	DCPIP (µL)	Complex II Activity Buffer (mL)
1	5	42	21	2.1
2	10	83	42	4.2
3	15	125	63	6.3
4	20	167	83	8.3
5	25	208	104	10.4
6	30	250	125	12.5
7	35	292	146	14.6
8	40	333	167	16.7
9	45	375	188	18.8
10	50	417	208	20.8
11	55	458	229	22.9
12	60	500	250	25

7.3.1 Do not empty the wells; instead add 200 µL of Activity solution to each well (already containing 40 µL of Phospholipids) for a total of 240 µL. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.

7.3.2 Place the plate in the reader and record with the following kinetic program

Mode	Kinetic
Wavelength	600 nm
Time	60 minutes
Interval	20 seconds
Shaking	No
Temperature	Room Temperature

NOTE: Sample incubation time can vary depending on enzyme activity in the samples. It is possible to make an endpoint measurement in place of kinetic measurement, but be sure to measure the endpoint before the most active sample has begun to slow down (see example in Calculations section for BHM sample >1600 s).

7.3.3 Save data and analyze as described in the "Data Analysis" section.

8. Data Analysis

- The initial solution for the activity measurement should be blue in appearance with an OD of approximately 0.2 OD units at OD = 600 nm.
- The reduction of ubiquinone and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD = 600 nm (see Figure 1). Monitor the rate of decrease in absorbance at 600 nm over time. Calculate the rate between two time points for all the samples where the decrease in absorbance is the most linear (typically between 15 minutes and 25 minutes – shown below). After 30 minutes the rate of reduction in absorbance may decline for the most active samples due lack of substrate so do not calculate the rate after this point.

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

- The activity of immunocaptured Complex II is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex II are 3.2, 3.1 and 3.7 mOD/min and the background rate (null sample) is 0.1 mOD/min, the activity of Complex II is (3.2+3.1+3.7)/3 - 0.1 which is 3.23 mOD/min. Now the activity of immunocaptured Complex II in between samples can be compared.
- This assay is compatible with different sample types such as mitochondria, tissue or cell lysates and in multiple species including human and rodent samples. Typical linear range data are shown below in Figures 1 and 2.

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

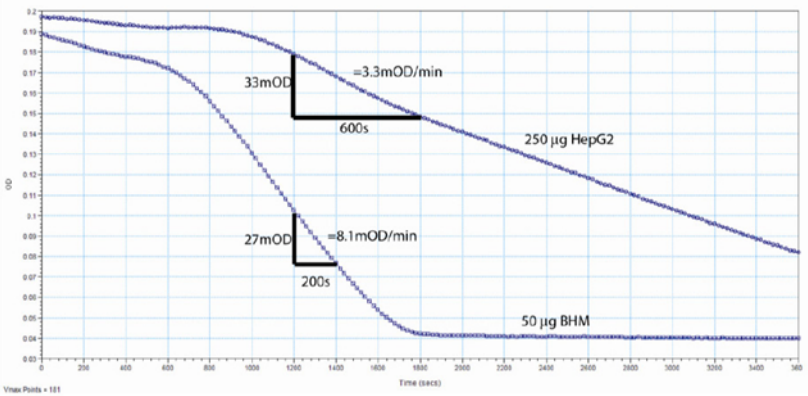

Figure 1. Example of raw data. Note the lag period before activity. Also note the activity of mitochondria (BHM, bovine heart mitochondria) is higher than whole cell lysate (HepG2, human hepatoblastoma) and the reaction ends at >1600 seconds because the substrates are used up.

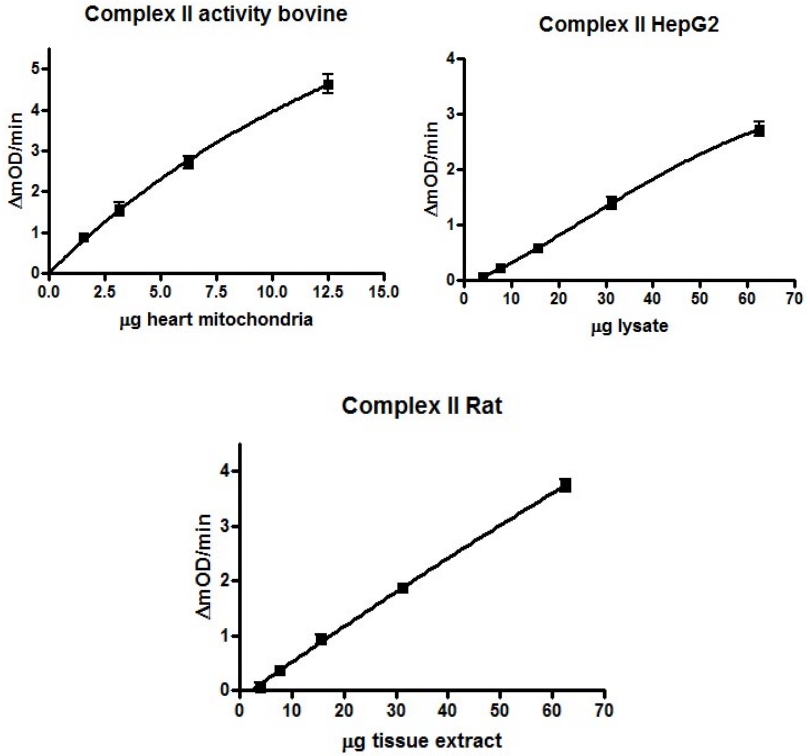

Figure 2. Data are most easily interpreted by working in the linear range of the assay as shown here; however, the range can also be extended by non-linear curve fitting.

Typical Sample Values

Precision:

	Intra Assay
n =	60
CV (%)	<15

9. FAQs / Troubleshooting

How do I prepare my mitochondria samples?

We have found that little or no optimization is necessary if crude mitochondria are made from samples. Mitochondria can be prepared by simple differential centrifugation of homogenized samples.

Can you recommend any positive controls?

Any of the lysates mentioned below can be used as positive control in this assay:

ab110338 – Bovine Heart Mitochondrial lysate
ab110346 – Rat Liver Mitochondrial lysate
ab110347 – Rat Heart Mitochondrial lysate
ab113048 – Rat Brain Mitochondrial lysate
ab110349 – Mouse Liver Mitochondrial lysate
ab110350 – Mouse Heart Mitochondrial lysate
ab110351 – Mouse Brain Mitochondrial lysate

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use deproteinization protocol provided
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till

Problem	Cause	Solution
		use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	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
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

10. Notes

Technical Support

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