

ab109909

Complex IV Human Enzyme Activity Microplate Assay Kit

Instructions for Use

For the quantitative measurement of Complex IV activity in Human and Bovine samples.

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

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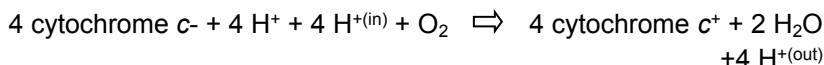
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1. BACKGROUND

Abcam's Complex IV Human Enzyme Activity Microplate Assay Kit (ab109909) is designed for the analysis of mitochondrial OXPHOS Complex IV enzymatic activity from human and bovine cell and tissue extracts.

This kit recognizes Complex IV in human and bovine cell extracts and isolated mitochondria – tissue lysates can also be used but some sample optimization may be necessary. Each of the 12 8-well strips in the kit has been coated with an anti-Complex IV monoclonal antibody (mAb) which isolates the target in the well. After the target has been immobilized in the well, substrate is added, and enzyme activity is analysed by measuring the change in absorbance of either the substrate or the product of the reaction (depending upon which enzyme is being analyzed). Activity is determined colorimetrically by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. The overall reaction is as follows:



Reduced

↑Abs 550 nm

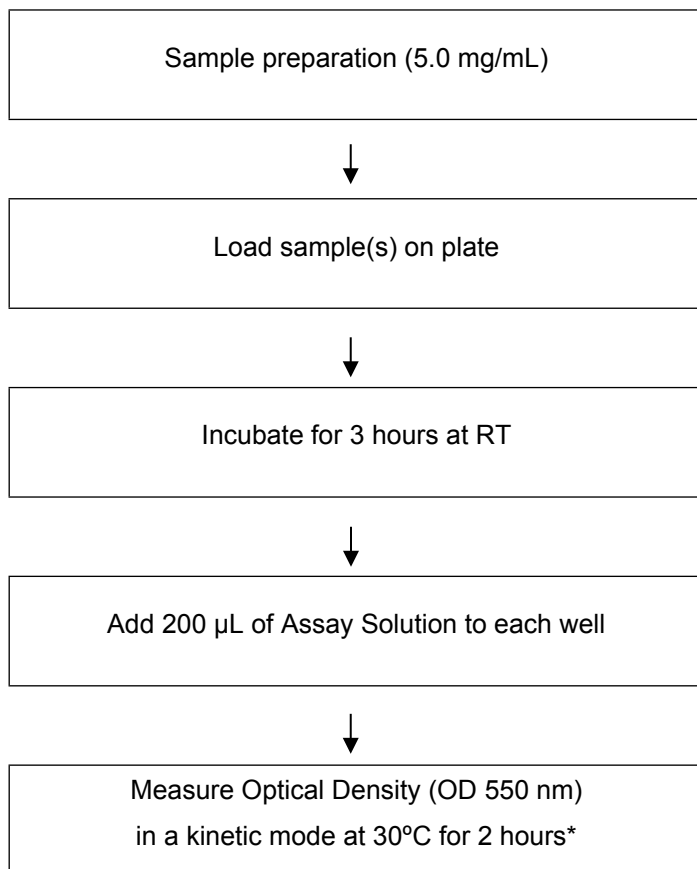
Oxidised

↓Abs 550 nm

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 IV, also known as cytochrome c oxidoreductase or cytochrome c oxidase (EC 1.9.3.1), is a complex of 13 different subunits, three of which (I, II and III) are encoded on mitochondrial DNA and the remainder in the nuclear DNA. The complex contains two heme groups (a and a3) and two copper atoms as prosthetic groups. Genetic alterations of this enzyme complex are a common cause of OXPHOS diseases and the enzyme is altered in patients with Alzheimer's disease. Also, there are reports of reduced amounts of this complex in hypoxic cancer cells.

2. ASSAY SUMMARY



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

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit components at +4°C except Reagent C which should be -80°C, in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Material 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	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Tube 1 (Buffer)	10mL	+4°C	+4°C
Detergent	1 mL	+4°C	+4°C
Reagent C (cytochrome c)	1 mL	-80°C	-80°C
96-well microplate (12 strips)	1 vial	+4°C	+4°C

7. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD 550 nm
- Dounce homogenizer or pestle (if using tissue)
- Optional: Protease inhibitors
- 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)

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.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure the heat block/water bath and microplate reader are switched on.
- Avoid multiple freeze/thaw of samples.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Reagent C:**

Ready to use as supplied. Aliquot Reagent C 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.

9.2 **Tube 1 (Buffer):**

Prepare the buffer solution by adding Tube 1 (10 ml) to 190 ml ddH₂O. Label this solution as Solution 1. Store at 4°C.

9.3 **10X Detergent:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C. Do not freeze.

9.4 **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, which allows performing up to 12 separate experiments. Equilibrate to room temperature before use. Store at 4°C.

10. 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, tissue or mitochondria in liquid nitrogen (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 IV activity in cells or tissue from different origins differs greatly. Cell type and growth conditions are a major factor in Complex IV activity measurement.
- Treat cells with Complex IV activators/inhibitors as per your experimental requirements.
- Add protease inhibitor cocktail to Solution 1 prior use.

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

- 10.1.1 Harvest suspension cells by centrifugation or scrape to collect adherent cells from a confluent culture flask (initial recommendation = $1 - 2 \times 10^7$ cells).
- 10.1.2 Wash cells twice with PBS.
- 10.1.3 Resuspend and dilute the cell pellet with 5 volumes of cold Solution 1 (see Step 9.2) (e.g. 100 μL pellet + 400 μL Solution 1 to a total volume of 500 μL).
- 10.1.4 Determine the sample protein concentration (using a standard methods such as BCA), by extracting a portion of your sample. Adjust concentration of the sample with Solution 1 so that the final sample protein concentration is **5 mg/mL**.
- 10.1.5 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 10X Detergent solution). Mix well.

- 10.1.6 Incubate the tube on ice for 30 minutes to allow solubilization.
- 10.1.7 Centrifuge the sample for 20 minutes at 4°C at 12,000 x g in a cold centrifuge.
- 10.1.8 Collect supernatant and transfer to a clean tube. Please note the sample concentration now is approximately **4.5 mg/mL**.
- 10.1.9 Dilute your samples to the desired concentration in Solution 1. Table 1 indicates a typical linear range for the assay.

10.2 Preparation of extracts from tissue:

- 10.2.1 Harvest tissue for the assay (initial recommendation = 100 – 200 mg).
- 10.2.2 Wash tissue thoroughly in cold PBS to remove blood.
- 10.2.3 Resuspend tissue in 5 volumes of cold Solution 1 (see Step 9.2).
- 10.2.4 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.*
- 10.2.5 Collect supernatant and transfer to a clean tube.
- 10.2.6 Determine the sample protein concentration (using a standard methods such as BCA) by extracting a portion of your sample. Adjust concentration of the sample with PBS so that the final sample protein concentration is **5.0 mg/mL**.
- 10.2.7 Extract the proteins from the sample by adding 10X 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 10X Detergent solution). Mix well.

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

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

10.2.10 Collect supernatant and transfer to a clean tube. Please note the sample concentration now is approximately **4.5 mg/mL**.

Dilute your samples to the desired concentration in Solution 1. Table 1 indicates a typical linear range for the assay.

Sample Type	Recommended Concentration (µg/200 µL volume)
Cell culture extracts	1 - 25 µg / 200 µL
Tissue extracts	0.1 - 10 µg / 200 µL
Tissue mitochondria	0.2 - 1 µg / 200 µL

Table 1. Typical ranges of measurement per 200 µL well volume.

10.3 Preparation of isolated mitochondria:

Mitochondria can be prepared by simple differential centrifugation of homogenized tissue samples – please see Section 17 for a general mitochondrial purification protocol.

Alternatively, you can isolate mitochondria using mitochondrial isolation kits such Mitochondria Isolation Kit for Cultured Cells (ab110170) or Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer) (ab110169).

Sample must be completely homogenous, so pipet sample up and down to distribute the mitochondria evenly in the solution.

10.3.1 Determine the sample protein concentration (using a standard methods such as BCA) by extracting a portion of your sample. Adjust concentration of the sample with PBS

so that the final sample protein concentration is **5.0 mg/mL**.

- 10.3.2 Extract the proteins from the sample by adding 10X 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 10X Detergent solution). Mix well.
- 10.3.3 Incubate the tube on ice for 30 minutes to allow solubilization.
- 10.3.4 Centrifuge the sample for 20 minutes at 4°C at 12,000 x g in a cold centrifuge.
- 10.3.5 Collect supernatant and transfer to a clean tube. Please note the sample concentration now is approximately **4.5 mg/mL**.

Dilute your samples to the desired concentration in Solution 1. Table 1 indicates a typical linear range for the assay.

11. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all controls and samples in duplicate. For recommended positive controls please check the FAQ section.

11.1. Plate Loading:

- Sample wells = 200 μ L of sample prepared in Sample Preparation section to each well of the microplate that will be used for this experiment.
- Background wells = 200 μ L Solution 1.
- Background control sample wells = **200 μ L of sample. Do not add assay solution.**

11.1.1. Incubate microplate for 3 hours at room temperature.

11.2. Measurement:

11.2.1. The bound monoclonal antibody has immobilized the enzyme in the wells. 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 μ L of Solution 1 to each well used.

11.2.3. 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.

11.2.4. Rinse all wells once more with 300 μ L Solution 1.

11.2.5. Add 300 μ L of Solution 1 to each well

11.3. Prepare Assay Solution:

Prepare appropriate amount of Assay Solution according to the following table. Mix gently by inversion.

Number of strips	Solution 1 (mL)	Reagent C (μL)
1	1.67	84
2	3.33	167
3	5	250
4	6.67	333
5	8.33	417
6	10	500
7	11.67	583
8	13.33	667
9	15	750
10	16.67	833
11	18.33	917
12	20	1,000

11.3.1. Empty the wells again.

11.3.2. Add 200 μL of Assay Solution (from step 11.3) to each well carefully to avoid bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.

- 11.3.3. Place the plate in the reader and record with the following kinetic program.

Mode:	Kinetic
Wavelength:	550 nm
Time:	120 minutes
Interval:	1-5 minutes
Shaking:	No Shaking
Temperature	30°C

NOTE: Sample incubation time can vary depending on enzyme activity in the samples.

- 11.3.4. Save data and analyze as described in the “Data Analysis” section.

12. CALCULATIONS

- Calculation of Complex IV activity. Since the Complex IV reaction is product inhibited, the rate of activity is always expressed as the initial rate of oxidation of cytochrome c. This oxidation is seen as a decrease in absorbance at OD = 550 nm. The initial rate should be a linear decrease. At lower activity levels the linear range is extended.
- To determine the activity in samples, calculate the slope by using microplate software or by manual calculations using one of the two methods shown below in Section 13.

Samples with enzymatic activity should have rates higher than the background rate.

Compare the experimental sample rates with those of the control (normal) samples.

$$\text{Rate} = \frac{\text{Absorbance at time t1} - \text{Absorbance at time t2}}{\text{Time (min)}}$$

13. TYPICAL DATA

TYPICAL SAMPLE VALUES

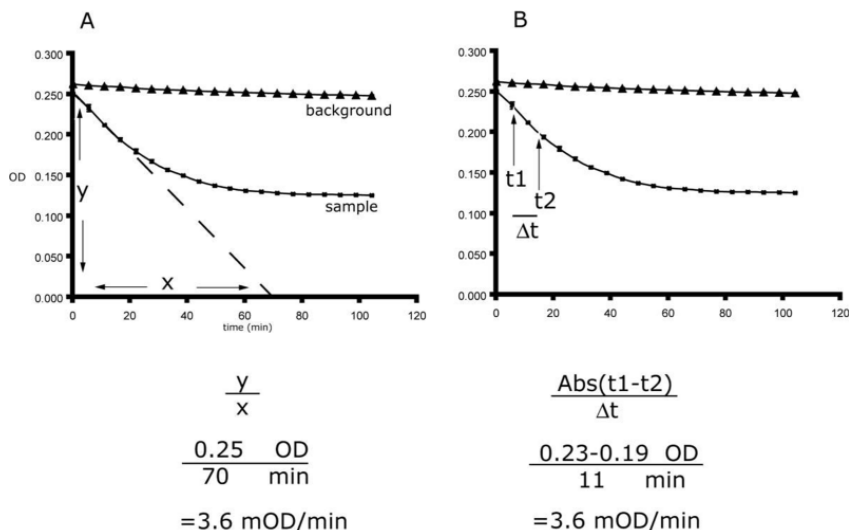


Figure 1. Example of raw data. In graph A, the rate is determined by calculating the gradient of the initial slope over the linear region. In graph B, the rate is determined by calculating the slope between two points within the linear range.

Note: 1 OD = 1000 mOD

PRECISION –

	Intra-Assay	Inter-Assay
CV (%)	<7%	<10%

SPECIES REACTIVITY

Human, Bovine.

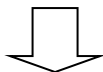
Not suitable for mouse or rat.

14. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

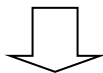
Prepare Sample (1 – 2 hours)

- Adjust sample concentration to 5.0 mg/mL in Solution 1.
- Perform Detergent extraction with 1/10 volume 10X Detergent.
- Incubate on ice for 30 minutes.
- Centrifuge at 16,000 x g for 20 minutes and then collect supernatant.
- Adjust concentration to recommended dilution for plate loading in incubation buffer.



Load Plate (3 hours)

- Load sample(s) on plate being sure to include a positive control sample and a buffer control as null reference.
- Incubate 3 hours at room temperature.



Measure (2 hours)

- Prepare sufficient Assay Solution.
- Rinse wells twice with Solution 1.
- Add 200 μ L of Assay Solution to each well.
- Measure OD₅₅₀ at approximately 1-5 minute intervals for 120 minutes.

15. TROUBLESHOOTING

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 PCA precipitation protocol for deproteinization
	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 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

RESOURCES

Problem	Cause	Solution
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

16. FAQ

Are there any chemicals or biological materials that can interfere with this assay?

It is not recommended to use RIPA buffer as it contains SDS, which can destroy or decrease the activity of the enzyme.

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

17. MITOCHONDRIAL PURIFICATION PROTOCOL

Mitochondrial Purification Protocol

Reagents needed:

NKM buffer	1 mM Tris HCl, pH 7.4 0.13 M NaCl 5 mM KCl 7.5 mM MgCl ₂
Homogenization buffer	10 mM Tris-HCl 10 mM KCl 0.15 mM MgCl ₂ 1 mM PMSF 1 mM DTT

Always add PMSF and DTT immediately before use to NKM and homogenization buffer.

Mitochondrial suspension buffer	10 mM Tris HCl, pH 6.7 0.15 mM MgCl ₂ 0.25 mM sucrose 1 mM PMSF
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Procedure

1. Collect cells by centrifugation at approximately 370 g for 10 min. Decant supernatant and resuspend cells in 10 packed cell volumes of NKM buffer.
2. Pellet cells and decant supernatant, repeat this washing step 2 times. Resuspend cells in 6 packed cell volumes of homogenization buffer.
3. Transfer cells to a glass homogenizer and incubate for 10 min on ice. Using a tight pestle homogenize the cells. Check under the microscope for cell breakage, the optimum is around 60%. This may require 30 strokes or so of the pestle.
4. Pour homogenate into a conical centrifuge tube containing 1 packed cell volume of 2 M sucrose solution and mix gently.

5. Pellet unbroken cells, nuclei and large debris at 1,200 x *g* for 5 min and transfer the supernatant to another tube. This treatment is repeated twice, transferring the supernatant to a new tube each time, discarding the pellet.
6. Pellet the mitochondria by centrifuging at 7,000 *g* for 10 min. Resuspend the mitochondrial pellet in 3 packed cell volumes of mitochondrial suspension buffer. Mitochondria are ready to use.

Technical Support

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