Catalog #KHM2241

MitoProfile® Human Complex IV Activity and Quantity

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INTENDED USE

The Human Complex IV (cytochrome *c* oxidase) Activity and Quantity kit is a microplate assay used to determine the activity and quantity of the enzyme in a human sample. This activity multiplexing plate has been developed for use with human samples. Bovine material is compatible, however mouse and rat samples are not. Other species have not been tested.

The activity microplate and quantity microplate are also available as individual microplate kits (Invitrogen Cat. no. KHM2041 and KHM2141, respectively).

INTRODUCTION

Complex IV, also called cytochrome *c* oxidoreductase or cytochrome *c* oxidase (COX), 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 a_3) 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.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

Complex IV is immunocaptured within the wells and activity is determined colorimetrical by following the oxidation of reduced cytochrome *c* as an absorbance decrease at 550 nm. The overall reaction is as follows.

$$4 \text{ cytochrome } c^{+} + 4\,H^{+} + 4\,H^{+(in)} + O_2 \rightarrow 4 \text{ cytochrome } c^{2+} + 2\,H_2O + 4\,H^{+(out)}$$

reduced — oxidized

↑Abs 550 nm — ↓Abs 550 nm

Subsequently *in the same well/s* the quantity of enzyme is measured by adding a Complex IV specific antibody conjugated with alkaline phosphatase. This phosphatase changes a substrate from colorless to yellow at 405 nm. This reaction takes place in a time dependent manner proportional to the amount of protein captured in the wells.

This assay is designed for use with purified mitochondria. However, homogenized tissue and whole cells can also be used. Samples should be solubilized, the protein extracted and measured within the linear range as described below. A control or normal sample should always be included in the assay as a reference. Also include a null or buffer control to act as a background reference measurement. Intra-assay and inter-assay precision is <10% CV.

Typical linear ranges:

Cultured cell extracts	1-20 µg/200 µL
Tissue extracts	0.1-10 µg/200 µL
Tissue mitochondria	0.01-1 µg/200 µL

REAGENTS PROVIDED

The kit contains a 96-well microplate with a monoclonal antibody pre-bound to the wells of the microplate. This plate can be broken into 12 separate 8-well strips for convenience; therefore the plate can be used for up to 12 separate experiments.

Item	Quantity
Buffer	15 mL
Wash Buffer	2 mL
Development Buffer	10 mL
AP Development Solution	0.4 mL
Detergent	1 mL
Detector Antibody (contains 0.1% sodium azide)	1 mL
AP Label	1 mL
96-Well Plate (12 strips)	1
Cytochrome *c*	1 mL

STORAGE

Store Buffer, Wash Buffer, Development Buffer, Detergent, Detector Antibody, AP Label and plate at 4°C – DO NOT FREEZE. Store AP Development Solution and Cytochrome *c* at -20°C or at -80°C for longer term storage.

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Spectrophotometer measuring absorbance of 550 ± 1 nm and 405 ± 1 nm.
2. Deionized water.
3. Multichannel pipetting devices.
4. Protein assay method.

PROTOCOL AND RECOMMENDED PROCEDURES

Note: This protocol contains detailed steps for measuring Complex IV activity and quantity. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

A. Sample Preparation

1. Prepare the buffer solution by adding *Buffer* (15 mL) to 285 mL deionized H_2O. Label this solution as Solution 1.
2. Pellet the sample by centrifugation.

3. Resuspend the sample by adding 5 volumes of Solution 1. The sample must be homogenous before detergent extraction. Therefore, resuspend the sample thoroughly by pipetting (cultured cells), or homogenize with a microtissue grinder/ultra turrax T8 (tissue). Determine the protein concentration by a standard method and then adjust the concentration to 5 mg/mL.

   Note: The optimal protein concentration for detergent extraction is 5 mg/mL.

4. Add 1/10 volume of *Detergent* to the sample, (e.g. if the total sample volume is 500 µL, add 50 µL of *Detergent*). Mix immediately and then incubate the sample on ice for 30 minutes.
5. Spin in tabletop microfuge at maximum speed (~16,000 rpm) for 20 minutes.
6. Carefully collect the supernatant and save as sample. Discard the pellet.
7. The microplate wells are optimized for 200 µL sample volume, so dilute samples to the following recommended concentrations by adding Solution 1.

Cultured cell extracts	5-20 µg/200 µL
Tissue extracts	1-10 µg/200 µL
Tissue mitochondria	0.01-1 µg/200 µL

8. Keep diluted samples on ice until ready to proceed to Section B (Plate Loading).

B. Plate Loading

1. Add 200 µL of each diluted sample prepared in Section A into individual wells on the plate. Include a normal sample as a positive control. Include a buffer control (200 µL Solution 1) as a null or background reference.
2. Incubate the plate for 3 hours at room temperature.

C. Measurement

1. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining liquid.
2. Add 300 µL of Solution 1 to each well.
3. In a sealable tube prepare an appropriate amount of Assay Solution using *Cytochrome c* and Solution 1. Mix gently by inversion. See table below for amounts required. Set Assay Solution aside.

No. of Strips	Cytochrome *c* (µL)	Solution 1 (mL)
1	84	1.67
2	167	3.33
3	250	5.00
4	333	6.67
5	417	8.33
6	500	10.0
7	583	11.67
8	667	13.33
9	750	15.00
10	833	16.67
11	917	18.33
12	1000	20.00

4. Set up the plate reader to a kinetic program to measure absorbance at 550 nm at 30°C for 120 minutes, with measurement interval of approximately 1 minute (however a longer measurement interval may be used if necessary).
5. Empty wells and add 300 µL Solution 1 to each well used. Repeat this rinse.
6. Empty the wells again and now add 200 µL of Assay Solution to each well used, be careful to avoid the formation of bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.

7. Set plate in plate reader and begin recording immediately. For Activity Assay Data Analysis, see Section F.
8. After data recording proceed to section D, Addition of Detection Antibodies, for enzyme quantitation. Alternatively the plate can be covered and stored overnight at 4°C before proceeding.

D. Addition of Detection Antibodies

Note: The volume of Solution 2, 3 and Development Solution prepared below is for the analysis of all 96 wells. For fewer wells reduce the volume of solution prepared proportionally.

1. Add entire contents of *Detector Antibody* to 20 mL of Solution 1. Label this solution as Solution 2.
2. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining activity assay solution.
3. Add 200 µL of Solution 2 to each well used.
4. Incubate the plate for 1 hour at room temperature.
5. Empty the wells and add 300 µL of Solution 1 to each well.
6. Add entire contents of *AP Label* to 20 mL of Solution 1. Label this as Solution 3.
7. Empty the wells and add 200 µL of Solution 3 to each well used.
8. Incubate the plate for 1 hour at room temperature.

E. Quantity Measurement

1. Empty the wells and add 300 µL of Solution 1 to each well used. Repeat this step.
2. Add 2 mL of *Wash Buffer* to 40 mL deionized H_2O. Label this as Solution 4.

3. Empty the wells and add 300 µL of Solution 4 to each well used.
4. Add 0.4 mL of *AP Development Solution* and 10 mL of *Development Buffer* to 10 mL of deionized H_2O. Label this as Development Solution.
5. Empty the wells and add 200 µL of Development Solution to each well used. Rapidly pop any bubbles that form with a needle.
6. Measure the absorbance of each well at 405 nm at room temperature. Take a measurement every 1.5 minutes for 20 measurements for a total time of 30 minutes.
7. Analyze data as described in Section G, the Quantity Assay Data Analysis.

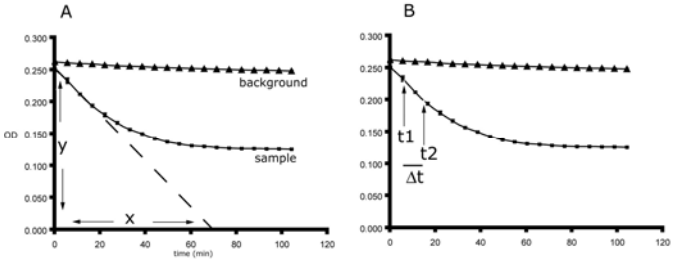
F. Activity Assay Data Analysis

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 550 nm. The initial rate should be a linear decrease. At lower activity levels the linear range is extended.

To determine the activity in the sample, calculate the slope by using microplate software or by manual calculations using one of the two methods shown below. Compare the sample rate with the rate of the control (normal) sample and with the rate of the null (background) to get the relative Complex IV activity.

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

Example:



$$\frac{y}{x} \qquad \frac{Abs(t1-t2)}{\Delta t}$$

$$\frac{0.25 \quad OD}{70 \quad min} \qquad \frac{0.23-0.19 \quad OD}{11 \quad min}$$

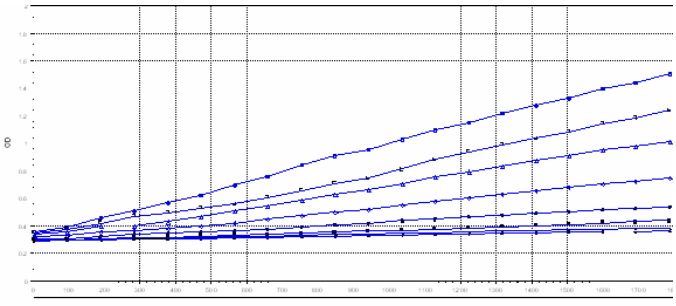
=3.6 mOD/min =3.6 mOD/min

a. The rate is determined by calculating the gradient of the initial slope over the linear region.

b. The rate is determined by calculating the slope between two points within the linear region.

G. Quantity Assay Data Analysis

The quantity of Complex IV is expressed as the amount relative to a normal or control sample. Examine the color development and ensure that the rates are linear as shown below. Subtract the initial absorbance reading from the final absorbance reading to determine the relative quantity of Complex IV captured in each well.



Time (sec)

SPECIFIC ACTIVITY

By measuring both quantity and activity of COX in a sample, the ratio of the two parameters, i.e. a COX activity/quantity ratio, can be calculated. This is the relative specific activity and should be compared to the normal or control, an extremely useful value since three possibilities exist.

1. The relative activity is the same in a sample as in normal (i.e., the amount of assembled COX has a proportional amount of activity.
2. The relative activity is lower than normal as in the case of a catalytic point mutation, chemical inhibition, or an inhibitory modification.
3. The relative activity is higher than normal which might occur if the activity of COX has been up-regulated perhaps by modification such as phosphorylation/dephosphorylation.

ASSAY SUMMARY

(For quick reference only. Be completely familiar with previous details of this document before performing the assay).

Prepare Sample (1-3 hours).

- Homogenize sample, pellet, and adjust sample to 5 mg/mL in Solution 1.
- Perform detergent extraction with 1/10 volume Detergent followed by 16,000 rpm centrifugation for 20 minutes.
- Adjust concentration to recommended dilution for plate loading.

Load Plate (3 hours).

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

Measure (2 hours).

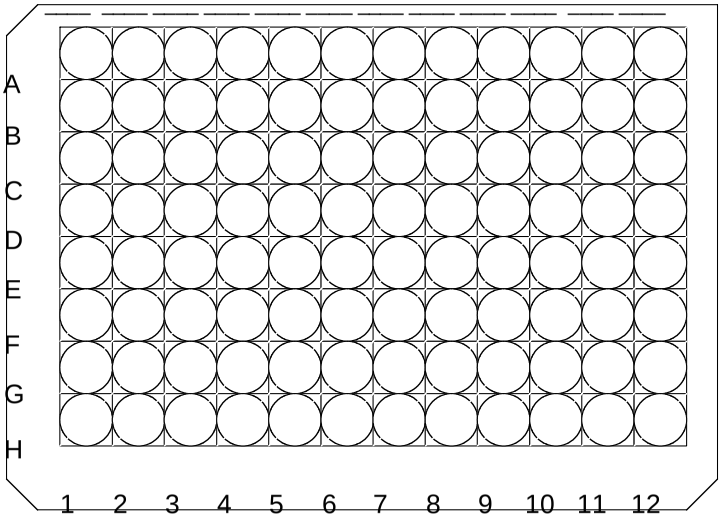
- Rinse wells three times with Solution 1.
- Prepare appropriate volume of Assay Solution and add to wells. Measure OD_{550} at 1-5 minute intervals for 2 hours at 30°C.
- Proceed to Detector Antibody binding immediately or store plate covered overnight at 4°C.

Detector Antibody Binding (2.5 hours).

- Empty wells.
- Add Solution 2 to each well and incubate 1 hour at room temperature
- Rinse wells with Solution 1.
- Add Solution 3 to each well and incubate 1 hour at room temperature.

Measure (1 hour).

- Rinse wells twice with Solution 1.
- Rinse wells with Solution 4.
- Add Development Solution to each well.
- Measure OD_{405} at 1.5 minute intervals for 30 minutes at room temperature.


A
B
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1 2 3 4 5 6 7 8 9 10 11 12

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