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User's Manual and Instructions

Mitochondria Activity Assay (Cytochrome C Oxidase Activity Assay) Kit

Catalog Number: KC310100

Introduction

Mitochondria are the eukaryotic subcellular organelles that contain the enzymes of the citric acid cycle, the electron transport chain, and oxidative phosphorylation. The organelle is composed of an inner and outer membrane. The outer membrane contains a mixture of enzymes involved in such diverse activities as the oxidation of epinephrine, the degradation of tryptophan, and the elongation of fatty acids. The enzymes for the electron transport chain and oxidative phosphorylation are embedded in the matrix side of the inner membrane. And majority of the enzymes of the citric acid cycle are located within the matrix. Mitochondria can be prepared from a variety of mammalian cultured cells and tissues by differential centrifugation. For optimal preparation of mitochondria, it is recommended to use the BioChain's Mitochondria Isolation Kit (Catalog # KC010100). The mitochondria activity in an isolated subcellular fraction can be measured by assaying for mitochondrial-specific enzymes. This mitochondria activity assay kit is designed for measuring the mitochondria-specific cytochrome c oxidase activity in soluble and membrane bound mitochondria samples. The enzyme cytochrome c oxidase (EC 1.9.3.1) is a large transmembrane protein located in the inner membrane of the mitochondria and is the terminal electron acceptor in the electron transfer chain, taking 4 reducing equivalents from cytochrome c and converting molecular oxygen to water. In the process, it translocates protons, helping to establish a chemisomotic potential that the ATP synthase then uses to synthesize ATP:

4 Fe^{+2}-cytochrome c (ferrocytochrome c) + $4H^+$ + O_2 → 4 Fe^{+3}-cytochrome c (ferricytochrome c) + H_2O.

Cytochrome c has a sharp absorption band at 550 nm in the reduced state. Upon oxidation, this band becomes weaker and broader (Figure 1). This colorimetric assay is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. It is typical to determine the extent of reduction of cytochrome *c* by measuring the difference in absorbances at 550 nm and 565 nm. In this assay, cytochrome c is reduced with dithiothreitol (DTT) and reoxidized by the active cytochrome c oxidase. At 550 nm reduced cytochrome c (ferrocytochrome c) has a different extinction coefficient than oxidized cytochrome c (ferricytochrome c). The difference ($\triangle \varepsilon^{mM}$) is 21.84. The cytochrome c oxidase reaction is a first-order rate reaction with respect to the cytochrome concentration, showing an exponential decay over time. The reaction equation is:

$$[C] = [C]_o \exp(-k\,t)$$

where $[C]_o$ is the concentration of cytochrome c at $t = 0$, and [C] is the concentration at time t. k is the first-order rate constant.

It can be rewritten as :

$$k = \frac{\ln [C]_o - \ln [C]}{t}$$

In this assay, we measure the first-order rate constant in the first 55 seconds of reaction. And the activity of the cytochrome c oxidase is expressed in units of μmol of cytochrome c reduced per ml of mitochondria sample per min.

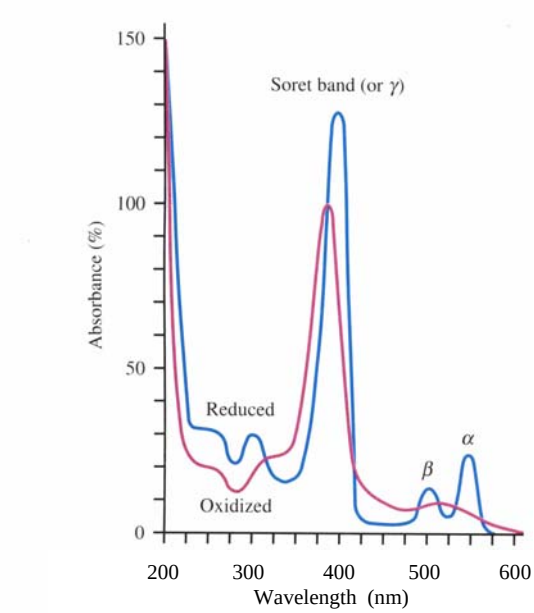

Figure 1. Absorption spectra of oxidized and reduced horse cytochrome c.
Units are absorbance in percent, normalized to the Soret band of the oxidized form.

Features

- **Sensitive** – detect cytochrome c oxidase activity as low as 0.35 milliunit.
- **Simple** – Colorimetric based assay facilitates the easy measurement of mitochondria integrity and activity.

Applications

- Detects the presence of mitochondria in subcellular fractions
- Measure the activity of mitochondria in subcellular fractions
- Measure the integrity of mitochondria outer membrane.

Description

Components in this kit are prepared with pure chemicals according to our proprietary technology. BioChain's Mitochondria Activity Assay (Cytochrome C Oxidase Activity Assay) Kits are designed to measure the cytochrome c oxidase activity in mitochondria sample, as well as the integrity of mitochondria. Different amounts of cytochrome c oxidase positive control were assayed using this kit to define the linear range of enzyme activity for the assay: 0.35 milliunit to 3.5 milliunit (Figure 2). One kit is consisted of reagents enough for performing 100 assays.

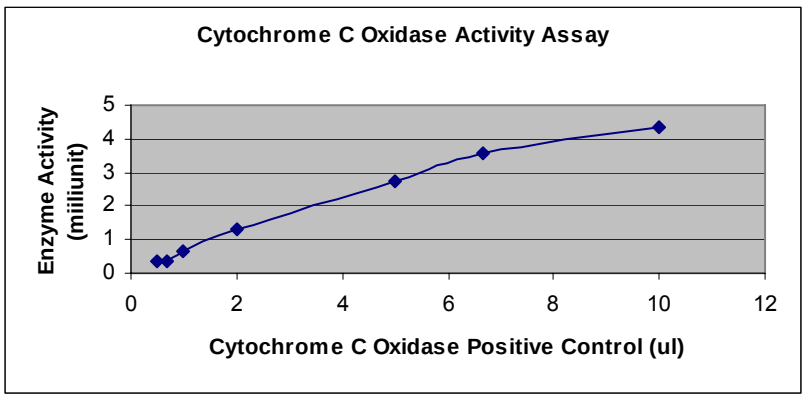

Figure 2. The activity assay of cytochrome c oxidase positive control at various amounts using BioChain's Mitochondria Activity Assay (Cytochrome C Oxidase Activity Assay) Kit. The linear range is achieved when the activity is between 0.35 milliunit and 3.5

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Quality Control
1 kit of this lot has been tested to go through the complete assay procedure. Results from this lot of kits are comparable to those obtained with control (previous) lot.

Components
Mitochondria Activity Kit: Reagents are sufficient for 100 assays.
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Item	Amount	Part No.
1. Enzyme Assay Buffer (5x)	25 ml	KC310100-1
2. Enzyme Dilution Buffer (2x)	20 ml	KC310100-2
3. Cytochrome C	1 bottle	KC310100-3
4. Cytochrome C Reducing (DTT) solution (10x)	400 µl	KC310100-4
5. n-Dodecyl β-D-Maltoside solution (100x)	200 µl	KC310100-5
6. Cytochrome C Oxidase Positive Control	200 µl	KC310100-6

Reagents and Equipments Required but not Supplied in this Kit:
1. Spectrophotometer
2. 1ml Cuvettes
3. ultrapure water

Storage and Stability
Store at –20 ºC. The kit is stable for one year when handled properly.

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Protocol

I. Preparation of Reagents

Use ultrapure water for the preparation of water.

Solution	Preparation	Storage	Notes
1x Enzyme Assay Buffer	Dilute an aliquot of 5x enzyme assay buffer 5-fold with water.	2-8°C	
1x Enzyme Dilution Buffer	Dilute an aliquot of 2x enzyme dilution buffer 2-fold with water.	2-8°C	
Enzyme Dilution Buffer with 1x n-Dodecyl β-D-Maltoside	Add 20 µl of 100x n-Dodecyl β-D-Maltoside solution to 2 ml of 1x Enzyme Dilution Buffer.	-20°C	
1x Cytochrome C Reducing Solution	Dilute an aliquot of the 10x cytochrome c reducing solution 10-fold with water.	-20°C	Aliquot 10x cytochrome c reducing solution into undiluted working aliquots and stored at -20 ºC.
Cytochrome C Oxidase Positive Control	For the positive control assay, dilute an aliquot of control sample 10-fold with enzyme dilution buffer and use 20-50 µl for each control assay.	-20°C	Undiluted and diluted positive control samples should be stored at -20°C.
Cytochrome C Solution	Add 6 ml of water to the cytochrome c bottle and mix well to make sure all the cytochrome c powder goes into the solution. Divide cytochrome C solution into working aliquot.	-20°C	
Ferrocytochrome C Substrate Solution	To reduce the cytochrome c, add 5 µl of 1x cytochrome c reducing solution, to 1 ml of cytochrome c solution, mix gently, and wait for 10-15 min. The color of the solution will change from dark red to orange red. Take 50 µl of cytochrome c and dilute 20-fold with 1x enzyme assay buffer. Measure the A_{550}/A_{565} ratio of this solution. The A_{550}/A_{565} should be between 10 to 20.	-20°C	If the A_{550}/A_{565} ratio remains less than 10, the substrate has not been sufficiently reduced and the enzyme activity will not be valid. In this case prepare a fresh 1x cytochrome c reducing solution.

II. Measurement of Cytochrome C Oxidase Activity

Reaction Setup (the total volume of the reaction is 1ml)

Sample	Enzyme Assay Buffer (µl)	Sample (µl)	Enzyme Dilution Buffer (µl)	Ferrocytochrome C Substrate Solution (µl)
Blank	850	--	100	50
Positive control	850	20-50	50-80	50
Unknown sample	850	X*	100-X	50

*The linear range is achieved when the enzyme activity in the reaction is between 0.35 and 3.5 milliunit. Assay various amount of samples to find a linear range for the sample.

1. Bring the 1x enzyme assay buffer and 1x enzyme dilution buffer to room temperature before use. Set up the spectrophotometer at absorption 550 nm before starting any reaction (the wavelength set up is very critical and can deviate by no more than 2 nm).
2. Add 850 µl of 1x enzyme assay buffer to the cuvette and zero the spectrophotometer.
3. Bring the enzyme or sample to 100 µl with 1x enzyme dilution buffer and add to cuvette. Mix by inversion.
4. Start the reaction by adding 50 µl of ferrocytochrome c substrate solution and mix by inversion. Read the absorption at 550 nm **immediately** following a kinetic program: 5 second delay; 10 second interval; 5 readings (A_{5s}, A_{15s}, A_{25s}, A_{35s}, A_{45s}, A_{55s}).
5. Determine the first-order rate constant *k*:
   Plot -ln [A] vs. time (s) and find a line to best fit the data. (This can be done using Microsoft Excel or other spreadsheet programs). Calculate the slope of the line. The

slope of the line is the first-order rate constant *k* (s^{-1}).

6. Calculate the activity of the sample.

$$\text{Unit/ml} = \frac{\triangle k \times 60 \times \text{dilution} \times 1}{\text{Vol}_{(\text{sample})} \times \ln 21.84}$$

$\triangle k = k_{(\text{sample})} - k_{(\text{blank})}$ ($k_{(\text{blank})}$ is usually between 0.001 and 0.003).
60 = 60 sec in 1 minute
dilution = dilution factor of sample
1 = total assay volume in ml
$\text{Vol}_{(\text{sample})}$ = volume of sample in ml
21.84 = $\triangle \varepsilon^{mM}$ between ferroctyochrome c and ferricytochrome c at 550 nm.

Unit Definition: One unit oxidizes one micromole of ferrocytochrome c per minute at 25°C, pH 7.0.

III. Measurement of the outer membrane integrity of mitochondria

The outer membrane of the mitochondria may be damaged during mitochondria preparation procedure. The integrity of the outer membrane is assessed by measuring cytochrome c oxidase activity in mitochondria membrane in the presence and absence of the detergent, n-Dodecyl β-D-maltoside. Cytochrome c oxidase locates in the inner membrane of the mitochondria. Cytochorme c can not access the cytochrome c oxidase when the outer membrane is intact. So the ratio between activity without and with n-Dodecyl β-D-maltoside presence is a measure of the integrity of the mitochondria outer membrane. N-Dodecyl β-D-maltoside is one of the few detergents that maintain the cytochrome c oxidase dimer in solution at low detergent concentration, therefore maintaining the enzyme activity.

1. Dilute two parallel samples of the mitochondria suspension to 0.1 to 0.2 mg protein/ml with either 1x enzyme dilution buffer (measuring cytochrome c oxidase activity in intact mitochondria) or with the enzyme dilution buffer containing 1x n-Dodecyl β-D-maltoside (measuring the total cytochrome c oxidase activity).
2. Incubate the diluted samples on ice for 10 – 15 min.
3. Take 1 – 2 μg of mitochondrial protein and measure the cytochrome c oxidase activity (Section II, step 1 – 6).
4. Calculate the first-order rate constant *k* for each sample (Section II, step 4&5)
5. Calculate the degree of mitochondrial integrity.
   % mitochondria with intact mitochondria outer membrane:

$$\% = \frac{(\triangle k_{(\text{w/ detergent})} - \triangle k_{(\text{w/o detergent})})}{\triangle k_{(\text{w/ detergent})}}$$

Related Products
Mitochondria Isolation Kit, Compartment Protein Isolation Kit

References
1. Berry E.A. and Trumpower, B.L. Anal. Biochem., 1987.161:1-15.
2. Birch-Machin and TrunBull. Methods Cell Biol. 1991. 65:97-117.
3. Lemberg, M.R. Physiol. Rev., 1969. 49:48-121.
Suarez, M.D., et al. J. Biol. Chem. 1984. 259:13791-13799.