The Cytochrome C Oxidase Assay Kit

Cat. #: CTG-BK012
Size: 100 Tests, 96-well Plate Format

Description
The Cytochrome c Oxidase Assay Kit is designed for the determination of cytochrome c oxidase activity in soluble and membrane bound mitochondrial samples. Cytochrome c oxidase is the last enzyme in the respiratory electron transport chain of mitochondria. Its main function is to convert molecular oxygen to water and aid in establishing mitochondrial membrane potential. Cytochrome c oxidase locates to the inner membrane which separates the mitochondrial matrix from the intermembrane space. 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. This kit is suitable for detection of mitochondrial outer membrane integrity and for detection of mitochondria in subcellular fractions.

Components
This kit is sufficient for 100 tests (96 well plate format).
5X Assay Buffer: 10 ml
2X Enzyme Dilution Buffer: 10 ml
Cytochrome c: 0.5ml (5 mg)
0.5 M Dithiothreitol (DTT): Solution 0.1 ml,
Cytochrome c Oxidase(positive control): 0.5 ml (1 vial)
10% Permeable reagent: 1 ml

1X Assay Buffer: 10 mM Tris-HCl, pH 7.0, containing 120 mM KCl – Dilute an aliquot of Assay Buffer 5-fold with water. Keep at room temperature (~25 °C).
1X Enzyme Dilution Buffer: 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose. Keep at 2–8 °C.
Enzyme Dilution Buffer with 0.1% Permeable reagent (for measurement of mitochondrial integrity): 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and 0.1% Permeable reagent.
50mM M Dithiothreitol (DTT) Solution: Dilute an aliquot of the 0.5 M DTT Solution 10-fold with ultrapure water to a concentration of 50 mM.

Ferrocytochrome c Substrate Solution (0.22 mM): In order to reduce the protein, add the 0.1 M DTT Solution to Cytochrome C solution to a final concentration of 0.5 mM, mix gently, and wait for 15 minutes. The color of the solution should go from dark orange-red to pale purple-red. Measure the A550/A565 ratio of an aliquot diluted 20-fold with 1X Assay Buffer (50 ml in 950 ml of 1X Assay Buffer). Use the 1X Assay Buffer to zero the spectrophotometer. The A550/A565 ratio should be between 10 and 20.

Cytochrome c Oxidase Positive Control and samples: For the enzyme assay, further dilute the sample 10-fold with 1X Enzyme Dilution Buffer. The sample may be stored at 2–8 °C for at least 3 weeks or frozen in aliquots at –20 °C. Enzyme Sample: The best results are achieved when the enzyme activity is between 0.4–4.0 milliunits of cytochrome c oxidase per reaction. For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the assay.

Reagents and Equipment Required but Not Provided.
- Spectrophotometer
- 1 ml Cuvettes
- Analytical balance
- Ultrapure water

Procedure
A. Measurement of cytochrome c oxidase activity

The absorption of cytochrome c at 550 nm changes with its oxidation state. This property is the basis for the assay.3 Cytochrome c is reduced with dithiothreitol and then reoxidized by the cytochrome c oxidase. The difference in extinction coefficients ($\Delta\varepsilon^{mM}$) between reduced and oxidized cytochrome c is 21.84 at 550 nm. The oxidation of cytochrome c by cytochrome c oxidase is a biphasic reaction with a fast initial burst of activity followed by a slower reaction rate (Figure 1).

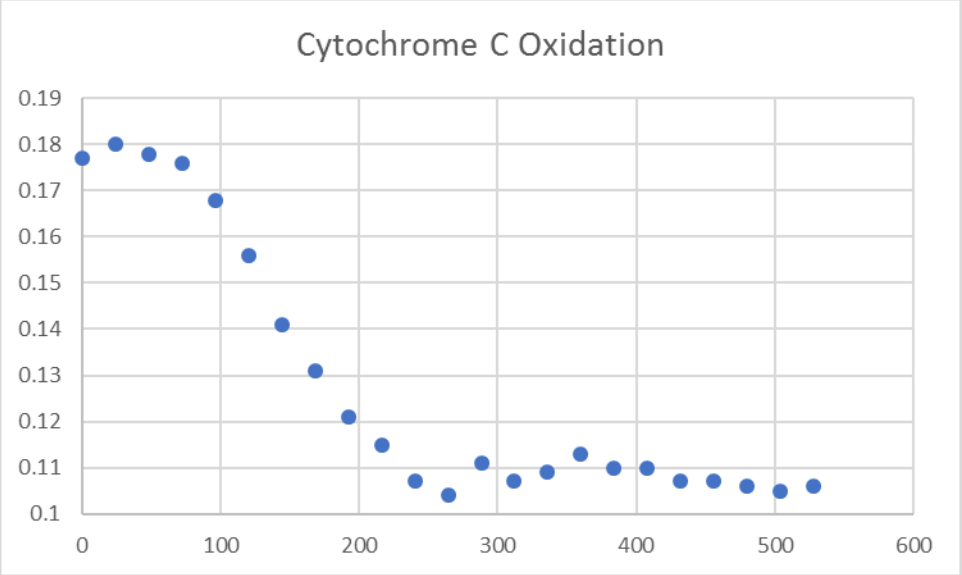

Figure 1. Cytochrome Oxidation Diagram

Total volume of the reaction is 110 µl (see Table 1).

Spectrophotometer settings:

Follow the decrease in absorption at 550 nm at room temperature (25 °C) using a kinetic program: 5 second delay; 10 second interval; 6 readings. Set up the instrument prior to starting any reaction. The wavelength setting is very critical and can deviate by no more than 2 nm. No signal is observed with a deviation of 10 nm.

Table 1. Reaction Scheme

Sample	Assay Buffer (µl)	Enzyme Dilution Buffer (µl)	Sample (µl)	Ferrocytochrome c Substrate Solution(µl)
Blank	95	10	–	5
Unknown sample	95	(10–x)	x	5
Positive control	95	6-8	2-4	5

1. Add 95 µl of 1X Assay Buffer to a well and zero the spectrophotometer.
2. Add a suitable volume of enzyme solution or mitochondrial suspension to the wells, and bring the reaction volume to 105 µl with 1X Enzyme Dilution Buffer. Mix by inversion.
3. Start the reaction by the addition of 5 µl of Ferrocytochrome c Substrate Solution and mix by inversion.
4. Read the A550/minute immediately due to the rapid reaction rate of this enzyme.
5. Background values are expected between 0.001 and 0.005 A550/minute.
6. Calculate the activity of the sample.

Calculation:

$$\text{Units/ml} = \frac{\Delta A/\text{min X dil X 1.1}}{\text{(vol of enzyme) X 21.84}}$$

$\Delta A/min = A/minute_{(sample)} - A/minute_{(blank)}$
dil = dilution factor of enzyme or sample
1.1 = reaction volume in ml
vol of enzyme = volume of enzyme or sample in ml
21.84 = $\Delta\varepsilon^{mM}$ between ferrocytochrome c and ferricytochrome c at 550 nm
Unit definition: One unit will oxidize 1.0 mmole of ferrocytochrome c per minute at pH 7.0 at 25 °C.

B. Measurement of the outer membrane integrity of mitochondria
The integrity of the outer membrane is assessed by measuring cytochrome c oxidase activity in mitochondrial membranes in the presence and absence of the detergent, Permeable reagent, which is one of the few detergents that allows the maintenance of the cytochrome c oxidase dimer in solution at low detergent concentrations. The ratio between activity with and without Permeable reagent present is a measure of the integrity of the mitochondrial outer membrane, since the membrane is a barrier for the entrance of cytochrome c into the organelle. Membrane integrity of mitochondria from various organs is dependent on the mode of preparation. Some tissues are much more difficult to homogenize and the shearing forces involved may cause considerable damage to the mitochondrial outer membrane.

Use of frozen tissues may cause rupture of the subcellular organelles and therefore, it is recommended to use freshly prepared tissues.

1. Dilute two parallel samples of the mitochondrial suspension to 0.1–0.2 mg protein/ml with either 1X Enzyme Dilution Buffer (cytochrome c oxidase activity in intact mitochondria) or with the Enzyme Dilution Buffer containing 0.1% Permeable reagent (total cytochrome c oxidase activity).
2. Incubate the samples at 2–8 °C for at least 10 minutes before assaying.
3. Take 1–2 mg of mitochondrial protein and assay for cytochrome c oxidase activity (Section A, steps 1-6).
4. Determine the DA550/minute for each sample:
$\Delta A_{(intact)} = \Delta A_{(intact\ sample)} - \Delta A_{(blank)}$
$\Delta A_{(total)} = \Delta A_{(total\ sample)} - \Delta A_{(blank)}$
5. Calculate the degree of mitochondrial integrity:

% mitochondria with undamaged outer membranes

$$\% = \frac{(\Delta A_{(total)} - \Delta A_{(intact)}) \text{ X } 100}{\Delta A_{(total)}}$$

Storage/Stability
The kit ships on wet ice and storage at –20 °C is recommended. When stored unopened, the components in this kit are stable for 24 months. After initial thawing of the 0.5 M Dithiothreitol Solution, divide the solution into undiluted working aliquots (still at 0.5 M concentration) and store at –20 °C.