Cytochrome c Oxidase Assay Kit

Cat. No. COA-320K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The Cytochrome c Oxidase Assay Kit uses an optimized colorimetric assay based on observation of the decrease in absorbance of ferrocytochrome c measured at 550 nm, which is caused by its oxidation to ferricytochrome c by cytochrome c oxidase.
Applications	This kit is suitable for the detection of mitochondrial outer membrane integrity/mitochondrial stress and for the detection of mitochondria in subcellular fractions.
Usage	1 Kit is sufficient for 100 tests
Storage	The kit ships on wet ice and storage at -20 centigrade is recommended. When stored unopened, the components in this kit are stable for 24 months. After initial thawing of the 1 M Dithiothreitol Solution, divide the solution into undiluted working aliquots (still at 1 M concentration) and store at -20 centigrade.
Kit Components	Assay Buffer 5 (50 mM Tris-HCl, pH 7.0, containing 600 mM KCl) 25 ml Enzyme Dilution Buffer 2 (50 mM Tris-HCl, pH 7.0, containing 600 mM KCl) 20 ml Cytochrome c 50 mg 1 M Dithiothreitol (DTT) Solution (1 M DTT in deionized water) 0.4 ml Cytochrome c Oxidase (positive control) 1 vial n-Dodecyl b-D-maltoside 10 mg
Materials Required but Not Supplied	· Spectrophotometer · 1 ml Cuvettes

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	· Analytical balance · Ultrapure water
Preparation	Use ultrapure water for the preparation of reagents. 1×Assay Buffer: 10 mM Tris-HCl, pH 7.0, containing 120 mM KCl-Dilute an aliquot of Assay Buffer 5 5-fold with water. Keep at room temperature (~25 centigrade). 1×Enzyme Dilution Buffer: 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose – Dilute an aliquot of Enzyme Dilution Buffer 2´ 2-fold with water. Keep at 2-8 centigrade. Enzyme Dilution Buffer with 1 mM n-Dodecyl b-D-maltoside (for measurement of mitochondrial integrity): 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and 1 mM n-dodecyl-b-D-maltoside – Dissolve 1.02 mg of n-Dodecyl-b-D-maltoside (MW 510.6 Da) in 2 ml of 1´ Enzyme Dilution Buffer. 0.1 M Dithiothreitol (DTT) Solution: Dilute an aliquot of the 1 M DTT Solution 10-fold with ultrapure water to a concentration of 0.1 M. Ferrocytochrome c Substrate Solution (0.22 mM): Dissolve 2.7 mg of cytochrome c (MW 12,384 Da) in 1 ml of water. In order to reduce the protein, add 5 ml of the 0.1 M DTT 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 1´ Assay Buffer (50 ml in 950 ml of 1×Assay Buffer). Use the 1×Assay Buffer to zero the spectrophotometer. The A550/A565 ratio should be between 10 and 20. Note: If the A550/A565 ratio remains less than 10, the substrate has not been sufficiently reduced and the enzyme activity will not be valid. Cytochrome c Oxidase Positive Control: Dissolve the vial in the volume of water specified in the instructions on the label/CofA. For the enzyme assay, further dilute the sample 10-fold with 1´ Enzyme Dilution Buffer and use 20–40 ml for each control reaction mixture. The sample may be stored at 2-8 centigrade for at least 3 weeks or frozen in aliquots at –20 centigrade. 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.
Assay Protocol	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. Cytochrome c is reduced with dithiothreitol and then re oxidized by the cytochrome c oxidase. The difference in extinction coefficients 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. In this assay the initial reaction rate is measured during the first 45 seconds of the reaction. Total volume of the reaction is 1.1 ml. Spectrophotometer settings: Follow the decrease in absorption at 550 nm at room temperature (25 centigrade) 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. Calculation: Units/ml = (DA/min×dil×1.1) /(vol of enzyme)×21.84 DA/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 = De 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 centigrade. 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, n-dodecyl b-D-maltoside, which is one of the few detergents that allows the maintenanc

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e of the cytochrome c oxidase dimer in solution at low detergent concentrations. The r atio between activity with and without n-dodecyl b-D-maltoside 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 fo rces 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/m l with either 1×Enzyme Dilution Buffer (cytochrome c oxidase activity in intact mitocho ndria) or with the Enzyme Dilution Buffer containing 1 mM n-dodecyl b-D-maltoside (t otal cytochrome c oxidase activity).
2. Incubate the samples at 2-8 centigrade 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:

DA(intact) = DA(intact sample)-DA(blank)

DA(total) = DA(total sample) – DA(blank)

5. Calculate the degree of mitochondrial integrity:

% mitochondria with undamaged outer membranes % = ((DA(total)-DA(intact)) ×100)/ DA(total)

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