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Product Information

Cytochrome c Oxidase Assay Kit

Catalog Number **CYTOCOX1**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product 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 [EC 1.9.3.1.] is the principle terminal oxidase of high affinity oxygen in the aerobic metabolism of all animals, plants, yeasts, and some bacteria.[1] The enzyme is present in mitochondria of the more highly developed cells and in the cytoplasmic membrane of bacteria. This enzyme is probably unique in providing the energy for the cell by coupling of the electron transport through the cytochrome chain with the process of oxidative phosphorylation. Cytochrome c oxidase is located on the inner mitochondrial membrane that divides the mitochondrial matrix from the intermembrane space and it has been used for many years as a marker for this membrane.[2-6]

The colorimetric assay in this kit 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[7] and for detection of mitochondria in subcellular fractions. For optimal preparation of mitochondria it is recommended to use the MITOISO1 kit, which enables the fast and easy isolation of an enriched mitochondria fraction from animal tissues. The MITOISO2 and MITOISO3 kits are available for preparation of mitochondria from cultured cells and yeast, respectively.

Components

This kit is sufficient for 100 tests.

Component	Amount
Assay Buffer 5× (Catalog Number A0599) 50 mM Tris-HCl, pH 7.0, containing 600 mM KCl	25 ml
Enzyme Dilution Buffer 2× (Catalog Number E2903) 20 mM Tris-HCl, pH 7.0, containing 500 mM sucrose	20 ml
Cytochrome c	50 mg
1 M Dithiothreitol (DTT) Solution (Catalog Number D7059) 1 M DTT in deionized water	0.4 ml
Cytochrome c Oxidase (positive control) (Catalog Number C8109)	1 vial
n-Dodecyl β-D-maltoside (Catalog Number D4641)	10 mg

Reagents and Equipment Required but Not Provided.

- Spectrophotometer
- 1 ml Cuvettes
- Analytical balance
- Ultrapure water (≥18 MΩ×cm resistivity at 25 °C)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Use ultrapure water (≥18 MΩ×cm resistivity at 25 °C) 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× (A0599) 5-fold with water. Keep at room temperature (~25 °C).

1× Enzyme Dilution Buffer: 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose – Dilute an aliquot of Enzyme Dilution Buffer 2× (E2903) 2-fold with water. Keep at 2–8 °C.

Enzyme Dilution Buffer with 1 mM *n*-Dodecyl β-D-maltoside (for measurement of mitochondrial integrity): 10 mM Tris-HCl, pH 7.0, containing 250 mM sucrose and 1 mM *n*-dodecyl-β-D-maltoside – Dissolve 1.02 mg of *n*-Dodecyl-β-D-maltoside (D4641; 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 (D7059) 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 μl 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 A_{550}/A_{565} ratio of an aliquot diluted 20-fold with 1× Assay Buffer (50 μl in 950 μl of 1× Assay Buffer). Use the 1× Assay Buffer to zero the spectrophotometer. The A_{550}/A_{565} ratio should be between 10 and 20.
Note: 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 refer to the Troubleshooting Guide (see Appendix).

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 μl for each control reaction mixture. 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.

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 1 M Dithiothreitol Solution, divide the solution into undiluted working aliquots (still at 1 M concentration) and store at –20 °C.

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.[9]

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.[10,11] 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 (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	950	100	–	50
Unknown sample	950	(100–x)	x	50
Positive control	950	60-80	20-40	50

1. Add 0.95 ml of 1× Assay Buffer to a cuvette and zero the spectrophotometer.
2. Add a suitable volume of enzyme solution or mitochondrial suspension to the cuvette, and bring the reaction volume to 1.05 ml with 1× Enzyme Dilution Buffer. Mix by inversion.
3. Start the reaction by the addition of 50 μl of Ferrocytochrome c Substrate Solution and mix by inversion.
4. Read the A_{550}/minute **immediately** due to the rapid reaction rate of this enzyme.
5. Background values are expected between 0.001 and 0.005 A_{550}/minute.
6. Calculate the activity of the sample.

Calculation:

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

$\Delta A/\text{min} = A/\text{minute}_{(\text{sample})} - A/\text{minute}_{(\text{blank})}$
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 μmole 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, *n*-dodecyl β-D-maltoside, which is one of the few detergents that allows the maintenance of the cytochrome c oxidase dimer in solution at low detergent concentrations.[6] The ratio between activity with and without *n*-dodecyl β-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.[8]

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. Outer membrane damage in various tissues is shown in Table 2.

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

Table 2.
Percent damage to outer mitochondrial membranes from various tissues[8]

Organ	% Damage of outer membrane
Rat liver	5–10%
Rat heart	20–44%
Rat brain	8–30%
Rat kidney	22%
Rabbit heart	16%
Beef heart	16%

Note: The described procedure is for a mitochondrial suspension and not for the purified enzyme.

1. Dilute two parallel samples of the mitochondrial suspension to 0.1–0.2 mg protein/ml with either 1× Enzyme Dilution Buffer (cytochrome c oxidase activity in **intact** mitochondria) or with the Enzyme Dilution Buffer containing 1 mM *n*-dodecyl β-D-maltoside (**total** cytochrome c oxidase activity).
2. Incubate the samples at 2–8 °C for at least 10 minutes before assaying.
3. Take 1–2 μg of mitochondrial protein and assay for cytochrome c oxidase activity (Section A, steps 1-6).
4. Determine the ΔA_{550}/minute for each sample:

   $\Delta A_{(\text{intact})} = \Delta A_{(\text{intact sample})} - \Delta A_{(\text{blank})}$

   $\Delta A_{(\text{total})} = \Delta A_{(\text{total sample})} - \Delta A_{(\text{blank})}$

5. Calculate the degree of mitochondrial integrity:

   % mitochondria with undamaged outer membranes

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

References

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SBC,EM,ESS,NDH,TA,PY,ALC,MAM 09/19-1

Appendix

After reducing the cytochrome c solution with 0.5 mM DTT, the expected absorbance of a 20-fold diluted sample in a quartz cuvette is as follows: A_{550} = 0.210–0.280 and A_{565} = 0.009–0.020.

Troubleshooting Guide

Problem	Possible Cause	Solution
The color of the cytochrome c solution did not change.	The DTT solution is oxidized.	Prepare a fresh 1 M DTT solution from powder and dilute to 0.1 M.
	The incubation time was too short.	Wait 15–20 minutes before reading absorbance.
		It is possible to add up to 30 μl of 0.1 M DTT in order to reduce the cytochrome c.
The color of the cytochrome c solution did change, but the A_{565} readings are too high.	The spectrophotometer is not suited to distinguish between readings at 550 and 565 nm, due to a bandwidth larger than 4–5 nm.	Measure absorbances at 550 and 575 nm, instead of 550 and 565 nm.
	The cuvette is not suitable.	Read absorbances using a quartz cuvette.
The color of the cytochrome c solution did change, but the A_{550} readings are too low.	The concentration of cytochrome c is too low.	Dilute the sample for the absorbance measurement 10-fold instead of 20-fold.

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