Cytochrome Oxidase Activity Colorimetric Assay Kit

(Catalog #BN00545; 100 assays; Store at -20°C)

I. Introduction:

Cytochrome c Oxidase (EC 1.9.3.1) or Complex IV is the fourth complex of the Electron Transport Chain located in the mitochondrial (or bacterial) membrane. It provides energy to the cell by coupling electron transport through the Cytochrome c chain with the process of oxidative phosphorylation. Complex IV contains 13 different subunits encoded by both mitochondrial DNA and nuclear DNA. It receives an electron from each of the four Cytochrome c molecules, and transfers it to one oxygen molecule, converting it into two molecules of water. In this process, it also binds to four proton molecules and translocates them across the membrane to establish electrochemical gradient, which is utilized for the synthesis of ATP.

Cytochrome Oxidase Activity Assay Kit is simple, fast and high-throughput adaptable. This assay kit can be used for purified mitochondria or tissue extracts containing mitochondria. The activity of the enzyme is determined colorimetrically by following the oxidation of reduced Cytochrome c as an absorbance decrease at 550 nm. The overall reaction is as follows:

$$4\ \text{Cytochrome c}^{-} + 4H^{+} + 4H^{+(in)} + O_2 \longrightarrow 4\ \text{Cytochrome c}^{+} + 2H_2O + 4H^{+(out)}$$

II. Application:

- Fast and simple measurement of Cytochrome Oxidase enzymatic activity in 96-well plate format.
- Mitochondrial respiration studies, assembly of the complexes, Mitochondria outer membrane integrity.

III. Sample Type:

- Purified mitochondria
- Cells/Tissue extracts

IV. Kit Contents:

Components	BN00545	Cap Code	Part Number
Cytochrome Oxidase Assay Buffer	25 ml	WM	BN00545-1
Enzyme Dilution Buffer	8 ml	NM	BN00545-2
1 M DTT	1 ml	Green	BN00545-3
Cytochrome c	2 vials	Red	BN00545-4
96-well Plate	1 Plate	-	BN00545-5

V. User Supplied Reagents and Equipment:

- Multi-well spectrophotometer capable of reading absorbance.
- Multi-channel pipet

VI. Storage and Handling:

Store kit at -20°C, protected from light. Warm Assay Buffer to room temperature before use. Keep Enzyme Dilution Buffer on ice. Read the entire protocol before performing the assay.

VII. Reagent Preparation and Storage Conditions:

- **DTT:** Aliquot and store at -20°C. Thaw just before use.
- **Cytochrome c:** Reconstitute each vial with 1 ml of Cytochrome Oxidase Assay Buffer. Mix by vortexing to dissolve completely. Add 5 µl of DTT solution. Mix well and wait for 15 min. at room temperature. Keep this working solution at room temperature. After assay is completed, aliquot and save rest of the Cytochrome c solution at -20°C. This is now reduced form of Cytochrome c.

VIII. Complex IV Activity Assay Protocol:

1. **Efficiency of Reduction of Cytochrome c:** In a 96-well plate**,** mix 20 µl of reduced Cytochrome c with 100 µl of Cytochrome Oxidase Assay Buffer. Prepare a parallel well as blank with only Assay Buffer. Read OD at 550 nm. The OD at 550 nm of reduced Cytochrome c is between 0.2-0.6. If not, add 5 µl of DTT/ml of reconstituted Cytochrome c and wait for 15 min. to read again the OD.
2. **Sample Preparation:** Isolate mitochondria from cultured cells, yeast or tissues by using Mitochondria/Cytosol Fractionation Kit (Assay Genie cat. # BN00523) or Yeast Mitochondria Isolation Kit (# BN00527) or use cell or tissue lysate. The recommended range of purified mitochondria is 0.5- 5 µg and tissue extract is 1-60 µg per reaction. Dilute the test samples, if needed by Enzyme Dilution Buffer.
3. **Cytochrome c preparation:** Prepare 1:6 dilution of Cytochrome c by using pre-warmed Cytochrome Oxidase Assay Buffer (one part of Cytochrome c to 5 parts of buffer) in a separate tube depending on the number of assay samples and controls. Prepare 120 µl of diluted Cytochrome c per reaction.
4. **Complex IV Activity Assay:** Before the reaction, set the spectrophotometer at 550 nm on kinetic program for 30-45 minutes at 30 sec interval. Add the test samples (approx. volume 5-10 µl) to each well of a 96-well plate. For negative control (Blank), add equal volume of Enzyme Dilution Buffer. Add 120 µl of the diluted Cytochrome c from Step 3 to each sample and control using a multichannel pipette. Shake and immediately read and record decrease in OD over a period of 30-45 min.

   Note: The rate of the reaction is relative to a control or normal sample. The rate is calculated in linear range.
5. **Calculations:** Calculate rate of the reaction by calculating change in OD: ΔOD/min by using the maximum linear rate. The oxidation of

Cytochrome c by complex IV is biphasic reaction with an initial fast burst followed by slower activity. The rate of the reaction will be calculated in the linear range.

$$\textbf{Cytochrome Oxidase Activity (Units/mg)} = \frac{\Delta OD/Time\ (\Delta t)}{\varepsilon \times protein\ (mg)}$$

Where: **ΔOD** is the difference in OD at time (t1) and time (t2)

Δt is the difference in time (min); t1 - t2.

Ɛ is the molar extinction coefficient of reduced Cytochrome c at 550 nm in the given 96-well plate; 7.04 $mM^{-1}cm^{-1}$

Protein is the conc. of sample (mg) used per reaction.

Unit definition: One unit would oxidize 1 µmole reduced Cytochrome c per minute at pH 7.2 at 25°C.

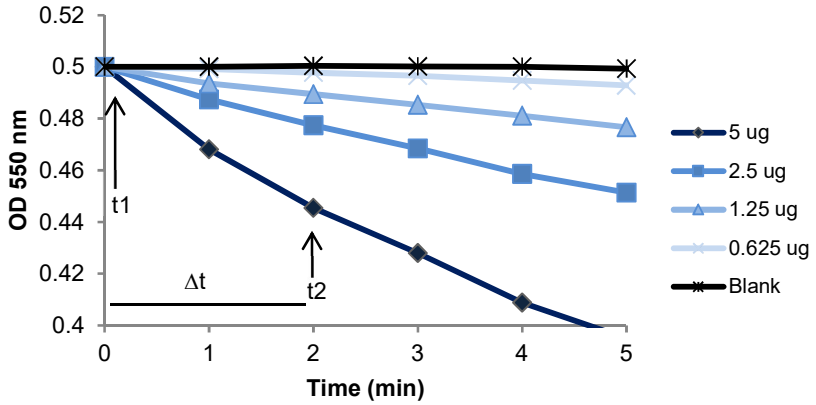

Figure: Cytochrome Oxidase Activity: Purified mitochondria (Samples) were used to calculate a decrease in OD at 550 nm (Conc. 0.625-5 µg/reaction). In Blank, no change in the OD was observed. Rate is calculated by subtracting the initial OD reading from the final OD, t1 and t2 represents linear rate of reaction.

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