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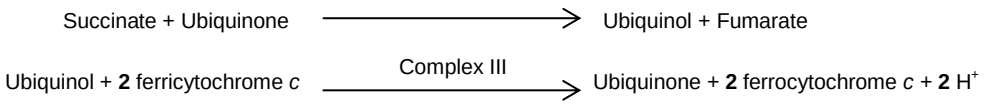
Mitochondrial Complex III Activity Assay Kit

(Catalog # K520-100; 100 assays; Store kit at -20°C)

I. Introduction:

Mitochondrial Complex III or Ubiquinol-Cytochrome *c* reductase (EC 1.10.2.2) is the third complex of the Electron Transport Chain located in the mitochondrial membrane. It is present in the mitochondria of all aerobic eukaryotes and also in the inner membranes of most bacteria. It transfers electrons from CoQH (reduced coenzyme Q or ubiquinol) to cytochrome *c*, resulting in the reduction of cytochrome *c*. This reduced cytochrome *c* is a substrate for complex IV of the electron transport chain. BioVision's Mitochondrial Complex III Activity Assay is based on this reduction of cytochrome *c* through the activity of Complex III. The absorbance of reduced cytochrome *c* can be measured at 550 nm. It is a fast and reliable method to determine the activity of complex III in isolated mitochondria. The kit can detect as low as 10 mU/ml and is linear up to 30 mU/ml.



II. Application:An

- Fast and simple measurement of Mitochondrial Complex III enzymatic activity in 96 well plate format

III. Sample Type:

- Isolated Mitochondria

IV. Kit Contents:

Components	K520-100	Cap Code	Part Number
Complex III Assay Buffer	25 ml	WM	K520-100-1
Cytochrome c	4 X 1 vials	Blue	K520-100-2
Complex III Inhibitor Antimycin A	220 µl	Orange	K520-100-3
1 M DTT	1 ml	Green	K520-100-4
Clear 96-well half area plate	1 plate	-	K520-100-5

V. User Supplied Reagents and Equipment:

- Multiwell spectrophotometer capable of reading absorbance in kinetic mode
- DMSO (anhydrous)
- Deionized water

VI. Storage Conditions and Reagent Preparation:

Store kit components at -20ºC. Briefly centrifuge small vials before opening. Read entire protocol before performing the assay.

- **Complex III Assay Buffer:** Store at 4°C. Bring to RT before use.
- **Cytochrome *c*:** Reconstitute one vial at a time with 175 µl Complex III Assay Buffer to obtain 2 mM solution. One reconstituted vial is enough for 25 reactions. Centrifuge briefly after mixing. Store at -20ºC. The reconstituted vial stored at -20°C should be stable for at least one month.
- **Complex III Inhibitor Antimycin A:** Aliquot and store at -20ºC.
- **DTT:** Thaw before use**.**

  Note: Antimycin A and DTT are stable for at least 3 months at -20°C.

VII. Mitochondrial Complex III Assay Protocol:

1. **Sample Preparation:** Isolate mitochondria from cultured cells or tissue using preferred procedure. We recommend Mitochondria Isolation Kit for Tissue & Cultured Cells (BV cat # K288-50) and Yeast Mitochondria Isolation Kit (BV cat # K259-50) for maximum yield and results consistency. Estimate the protein concentration of isolated mitochondrial samples using Bradford assay.

   Notes:

   a. Isolated mitochondria should be stored at -80ºC unless being used for the assay immediately. Avoid freeze-thaw.
   b. Mitochondria should be placed on ice during the assay.
   c. Different dilutions of the mitochondrial sample should be tested to make sure that the activity falls in the linear range of the assay.
   d. Dilutions should be prepared in Complex III Assay Buffer immediately before performing the assay.

2. **Reduced cytochrome c Standard Curve:** Prepare 750 mM DTT solution by mixing 1 M DTT solution and Assay buffer in a ratio 3:1. Add 0, 2, 4, 6, 8 and 10 µl of 2.0 mM cytochrome *c* standard into a series of wells in the provided 96 well plate to generate 0,4, 8, 12, 16, 20 nmol/well of cytochrome *c*. Adjust the volume to 23 µl/well with Complex III Assay Buffer. Add 2 µl of the prepared 750 mM DTT solution in each well in order to completely reduce cytochrome *c*. Mix well and incubate at RT for 5 min. Measure the absorbance at 550 nm (end point).

3. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare reaction mix containing:

	Background Control	Sample Mix	Sample Mix with Complex III Inhibitor
Assay Buffer	17 µl	15 µl	15 µl
Inhibitor Antimycin A	-	-	2 µl
DMSO	2 µl	2 µl	-

Add the reaction mix to wells of the clear bottom 96-well half area plate which is provided with the kit.

4. **Mitochondrial Sample**: Add 1 to 2 µl mitochondrial samples (1.5 to 10 µg protein) to wells containing "Sample Mix" and "Sample Mix with Complex III Inhibitor". Mix well. Add 6 µl cytochrome c (substrate) and read immediately.
   Note: Have the plate reader ready at 550 nm on kinetic mode.
5. **Measurement:** Immediately start recording absorbance at 550 nm, at 30 seconds intervals for up to 10 minutes at RT.
6. **Calculation:** Complex III specific activity may be calculated by comparing sample OD values (after subtraction of background control) to the reduced cytochrome c standard curve. Calculate the concentration of reduced cytochrome c, at time t1 and t2 by reading off the standard curve. Calculate Δ [cytochrome c] between time t1 and t2. Apply the following equation to obtain activity of complex III.

Complex III specific activity = ΔC /(Δt x p) x D (Units / µg)

Where: **ΔC =** change in reduced cytochrome c concentration during Δt
Δt = t2 – t1 (min)
p = mitochondrial protein sample (µg)
D = dilution factor

Net Complex III Activity in sample = Activity in reaction without Antimycin A – Activity in reaction with Antimycin A

Unit Definition: One unit of Complex III is the amount of enzyme that will cause reduction of 1.0 µmol of cytochrome c per min at pH 7.4 at room temperature.

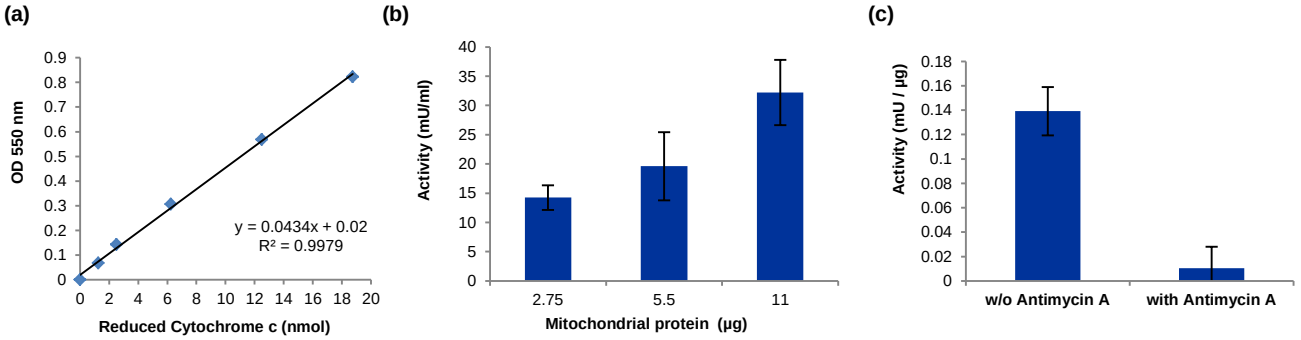


Figure: **(a)** Standard curve for reduced cytochrome c when reduced with DTT. **(b)** Complex III activity in increasing amounts of bovine heart mitochondria. **(c)** Activity of Complex III in isolated bovine heart mitochondria in absence and presence of Antimycin A (units/µg protein). Assays were performed following the kit protocol.

VIII. Related Products

Mitochondria Isolation Kit for Tissue & Cultured Cells (K288-50)
Yeast Mitochondria Isolation Kit (K259)
BCA Protein Assay Kit (K813-2500)
Cytochrome Oxidase Activity Colorimetric Assay Kit (K287)
Dounce Tissue Homogenizer (1998)

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