A convenient, easy-to-follow shortened protocol is provided with this assay.
For a detailed protocol go to www.caymanchem.com/pdfs/700990.pdf

MitoCheck® Complex IV Activity Assay Kit Short Protocol

Item No. 700990

REAGENT PREPARATION

All reagents unless listed below are ready to use as supplied.

1. **Mitochondrial Complex IV Activity Assay Buffer - (Item No. 700991)** - This buffer is ready to use as supplied. It is important that the buffer is warmed to room temperature prior to use. Additionally, vortex well to ensure that any crystals that may have precipitated out have dissolved.

2. **Mitochondrial Inhibitor - (Not Supplied)**
   Potassium Cyanide (KCN) is a prototypical inhibitor of complex IV. Because of this, it is important that extreme care is taken when preparing and using KCN. In a ventilated hood, weigh out 32.5 mg of KCN and dissolve in 1 mL of 0.1M NaOH; do not use water or any acidic solvents. This will provide you with a 500 mM stock of KCN. Store on ice, and make fresh less than three hours prior to running this assay.

BUFFER PREPARATION

Label 2 polystyrene tubes as A and B and add the following reagents. Isolated mitochondria can settle over time, so make the sure contents of each tube are well mixed. Store tubes on ice until ready to use. Volumes indicated below are sufficient for 20 experiments (20 wells). Customer may scale volumes as needed.

Tube A (1 mL)	Tube B (675 mL)
995 µl of Complex IV Activity Assay Buffer	615 µl of Complex IV Activity Assay Buffer
5 µl Bovine Heart Mitochondria Assay Reagent	60 µl Reduced Cytochrome *c* Assay Reagent

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PERFORMING THE ASSAY

1. **Positive Control** - See full version of assay protocol for detailed information on the preparation of KCN.

2. **For each assay condition:**
   - Add 50 µl of the contents of tube A to each well.
   - Add 20 µl of test compounds, diluted in 0.1 M NaOH, vehicle, or positive control to the appropriate wells. Allow for pre-incubation if required.
   - Add 30 µl of the contents of tube B to each well to start the reaction.

   Immediately place plate on plate reader and measure absorbance at 550 nm (30 second intervals for 15 min @ 25°C).

CALCULATIONS

1. Plot time-dependent reaction data as absorbance (y-axis) versus time (x-axis).
2. To determine the reaction rate, calculate the slope for the linear portion of the curve.
3. Determine % activity using the equation below.
4. To generate a concentration response curve, plot the % activity as a function of test compound concentration.

$$\text{Complex IV Activity (\%)} = \left[\frac{\text{Rate of Sample wells}}{\text{Rate of Vehicle Control}}\right] \times 100$$