CREATIVE BIOMART®
Assay Kit

Mit Complex IV Activity Assay Kit

Product Information

Cat.No. Kit-0248

Product Overview

Complex IV Activity Assay is designed to measure the direct oxidation of cytochrome c by complex IV, in an isolated bovine heart mitochondrial system that is supplied within the kit. Complex IV Activity Assay allows for the quick and easy measurement of complex IV activity.

Storage

at -80°C

Shipping

Dry ice

Size

96 wells

Kit Components

Mitochondrial Complex IV Activity Assay Buffer: 2 vials/10 ml, - 20°C;
Reduced Cytochrome c Assay Reagent: 1 vial/6 mg, -80°C;
Bovine Heart Mitochondria Assay Reagent: 1 vial/100 µl, -80°C;
Half Volume 96-Well Clear Plate: 1 plate, Room temperature

Materials Required but Not Supplied

1. A plate reader capable of measuring absorbance at 550 nm at 30 second intervals
2. Adjustable and multichannel pipettes
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Mitochondrial Inhibitor - Potassium Cyanide
5. 0.1 M NaOH

Technical Notes

- Use different tips to pipette each reagent.
- Avoid introducing bubbles into the well.

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Mit Complex IV Activity Assay Kit

• Do not expose the pipette tip to the reagent(s) already in the well.
• The final volume of the assay is 100 µl in all wells.
• It is not necessary to use all the wells on the plate at one time.
• It is recommended that the samples be assayed at least in duplicate (triplicates preferred).
• The assay is performed in the kinetic read mode at 25°C.
• Monitor the absorbance at 550 nm every 30 seconds for 15 minutes.
• The kinetics of this assay are first order with respect to cytochrome c. When analyzing data, be sure to use the linear portion of the curve. This portion is typically observed between 2-8 minutes.

Preparation

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

1. Mitochondrial Complex IV Activity Assay Buffer

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 be sure that any crystals that may have precipitated are 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 0.1 M 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.

3. Reduced Cytochrome c Assay Reagent

Thaw reagent prior to use. Once thawed, the reagent should be kept on ice unopened until use. Once opened, the concentration of reduced cytochrome c will diminish slowly due to oxidation. Significant oxidation of cytochrome c will impact the performance of the kit. Therefore, after use, component should be frozen immediately and discarded after two weeks.

Assay Protocol

Label two polystyrene tubes as A and B and add the following reagents. Isolated mitochondria can settle over time, so make sure contents of each tube are well mixed. Store tubes on ice until ready

Mit Complex IV Activity Assay Kit

to use. Volumes indicated below are suitable for 20 reactions (or wells). Customer may scale volumes as needed.
Tube A (1 ml) Tube B (675 µl)
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
Table 1. Assay preparation

Preparation of positive control inhibitor
NOTE: KCN and 0.1 M NaOH are not provided in this assay kit and must be supplied by the user.

An example for preparing a positive control is given below; customer may scale volumes as needed. NOTE: KCN is not stable at neutral pH, therefore preparation of KCN is carried out in 0.1M NaOH.
1. In a separate plate or Eppendorf tube, take 14.6 µl of 500 mM KCN stock solution and dilute into 131.4 µl of 0.1M NaOH to give a 50 mM solution.
2. Using the 50 mM KCN, perform log or half log serial dilutions into 0.1 M NaOH. Serial dilutions should be carried out in a separate 96-well plate, or in Eppendorf tubes, and will be used to generate an inhibitor concentration response. When 20 µl of the 50 mM solution is added to the well this will provide a 10 mM concentration of KCN. Subsequent concentrations of KCN will depend on dilutions. Ensure that 0.1 M NaOH is used as a vehicle control.

For each assay condition:
1. Add 50 µl of the contents of tube A to each well.
2. Add 20 µl of test compounds diluted in 0.1 M NaOH, vehicle, or positive control to each well. If test compounds are not stable in 0.1 M NaOH, UltraPure water can be substituted. Depending on the inhibition characteristics of the test compound, a preincubation may be required. KCN does not require preincubation.
3. Add 30 µl of the contents of tube B to each well to start the reaction. This should done quickly as the reaction will start immediately.
4. Immediately place plate in plate reader and measure absorbance at 550 nm (30 second

Mit Complex IV Activity Assay Kit

intervals for 15 minutes at 25°C).

Analysis

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 relative to the vehicle control using the equation indicated below.
4. To determine an IC50 value for each compound, plot the Complex IV Activity (%) as a function of test compound concentration.

Complex IV Activity (%) = (Rate of Sample wells/Rate of Vehicle Control) x 100

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