Protocol for 96-well plate based Oxidative Phosphorylation Inhibitors

Complex I (CI, NADH:Ubiquinone oxidoreductase)

1) Add 2 ug mitochondria to x wells of a 96 well plate and dilute to 140 uL. Include atleast three positive control wells and three negative control wells which include rotenone.
2) To three of the six wells add 20 uL of potassium phosphate buffer (0.5 M, pH 7.5), and 12 uL of fatty acid-free BSA (50 mg/ml), and 6 uL of KCN (10 mM) and 2 uL of NADH (10 mM)
3) Adjust the volume to 198.8 uL with distilled water
4) In three control wells add in parallel the same reagents from steps 2-3 but with 2 uL, 1 mM rotenenone.
5) In parallel add the same reagents from steps 2-3 but include 2 uL of test compounds at 10-three-fold dilutions.
    a. Concentrations should be calculated using 200 uL final volume. I.e. to get 10 uM final concentration, make a 1 mM stock solution.
6) Mix plate on shaker and read baseline at 340 nm for 2 min. Specific complex I activity is the rotenone-sensitive activity.

Complex II (CII, succinate dehydrogenase).

1) Add 120 µl ddH2O, 10 µL potassium phosphate buffer (0.5 M, pH 7.5), 4 µL of fatty acid-free BSA (50 mg/ml), 6 µL KCN (10 mM), 5 µL of succinate (400 mM), and 0.5 µg of mitochondria, and 29 µL of DCPIP.
2) Adjust the volume to 198.8 µL with ddH2O.
3) Mix plate in instrument using shaker for 8 minutes and read for 2 min at 600 nm.
4) Start the reaction by adding 0.8 µL of 12.5 mM DUB, mix and follow the decrease of absorbance at 600 nm for 3 min.
5) Check the specificity of complex II activity by running the assay after the addition of 10 µL of either 1 M malonate or 50 mMTTFA before starting the reaction. The degree of inhibition usually exceeds 85% with TTFA and 95% with malonate, indicating a high specificity of reaction.

Complex III (decylubiquinol cytochrome c oxidoreductase)

1) Add 146 uL of ddh20, 10 uL of potassium phosphate buffer (0.5 M, pH 7.5), 15 uL of oxidized cytochrome c, 10 uL of KCN (10 mM), 4 uL of EDTA (5 mM, pH 7.5), 2 µL of Tween-20 (2.5%) vol/vol)) and 0.1 µg mitochondria to x wells of a 96 well plate and dilute to 140 uL.
2) Prepare, in paraellel, atleast three control wells containing the same reagents as above, but with the addition of 2 µL of a 1 mg/ml antimycin A stock.
3) Adjust the volume to 198 µL using ddH2O and mix by shaking.
4) Read baseline at 550 nm for 2 min.
5) Start the reaction by adding 2 µL of a 10 mM decylubiquinol solution, mix rapidly by and immediately read the increase in absobance at 550 nm for 1-2 minutes. Specfici complex II activity is the antimycin A-sensitive activity.

Complex IV (CIV, cytochrome c oxidase)

1) Add 80 µL of ddH20, 100 µL of phosphate buffer (0.1 M, pH 7.0), 12 µL of reduced cytochrome C (1 mM) and read the baseline activity at 550 nm for 2 minutes.

2) Adjust the volume to 199 µL
    a. 7 µL ddH20
3) Start the reaction by adding 1 µL of sample (0.1 µg of mitochondria), mix and monotir the decrease of absorbance at 550 nm. Should take 3 minutes.
4) To check the specificity of complex IV, ad 30 µL of 10 mM KCN in a spate reaction

↳ 1.5 mM

For 3.489 mg/mL

↳ need 15 mM

↳ 0.1 mg x 15 → 1.5 mg / 3.489 mg/mL

= 0.44 mL into

14.56 mL TRIS