

Complex II Enzyme Activity Microplate Assay Kit

MS241

Rev.0

DESCRIPTION

Complex II Enzyme Activity Microplate Assay Kit

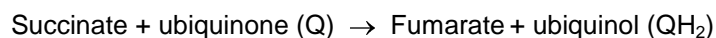
Sufficient materials are provided for one 96-well microplate.

Kit Contents:

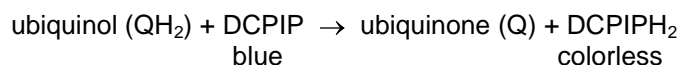
Item	MS241
20X Buffer	15 mL
10X Detergent	2 x 1 mL
10X Blocking Solution	5 mL
Complex II Activity Buffer	25 mL
DCPIP	250 µL
Succinate	500 µL
Ubiquinone 2	60 µL
Lipid Mix	6 mL
Pre-coated 96-well microplate (12 strips)	1

INTRODUCTION

Complex II, also known as succinate-coenzyme Q reductase (SDH, EC 1.3.5.1), is one of the five complexes involved in oxidative phosphorylation in the inner mitochondrial membrane and also a member of the tricarboxylic acid cycle (TCA). It catalyzes electron transfer from succinate to the electron carrier, ubiquinone, but unlike the other four complexes it is not a proton pump. The product ubiquinol is utilized by complex III in the respiratory chain and the product fumarate is necessary to maintain the TCA cycle.



The Complex II Enzyme Activity Microplate Assay (MS241) is designed for determining the Complex II activity in a sample. Each of the 96 wells in the kit has been coated with an anti-Complex II monoclonal antibody (mAb) which purifies the enzyme from a complex sample such as mitochondria, tissue homogenate or cell lysate. After this in-well purification the production of ubiquinol by the enzyme is coupled to the reduction of the dye DCPIP (2,6-dichlorophenolindophenol) and a decrease in its absorbance at 600 nm which in turn recycles the substrate ubiquinone, as shown below.



Note: This protocol contains detailed steps for measuring Complex II activity. Be completely familiar with the protocol and the Notes section (page 9) before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

The protocol has 3 steps:

- A. Sample preparation
- B. Plate loading
- C. Measurement

This microplate assay (Catalog # MS241) has been developed for use with human samples. However, the kit also works with bovine, mouse and rat samples. Other species are untested at this time.

This assay is designed for use with homogenates from cultured cells, and isolated mitochondria - tissue lysates can also be used but some sample optimization may be necessary. As described below, homogenized samples should be resuspended to 5.5 mg/mL protein. The proteins are detergent extracted and loaded to within the linear range of the assay (see below). A **control** or normal sample should always be included in the assay as a reference. Also, include a **null** or buffer control to act as a background reference measurement.

Typical linear ranges per well (50 μ L) and per milliliter are listed below. The ranges may be extended by using a non-linear fit of the data from a normal sample.

Example Ranges:		
Bovine heart mitochondria	1 -25 μ g/well	20 - 500 μ g/mL
Whole cultured cell extract	4 - 250 μ g/well	80 - 5000 μ g/mL

NOTE: Ranges for tissue extract may vary slightly. The lowest amount indicated is the lowest amount tested (>2x background). For sample loading use the recommended amount specified on page 3. Intra assay variation = <15 %

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer plate reader (Molecular Dynamics SpectraMax recommended) capable of measuring absorbance at 600 nm.
- Method for determining protein concentration
- Deionized water
- Multichannel pipette
- PBS (phosphate buffered saline) – for recipe see www.mitosciences.com/PDF/western.pdf

COMPLEX II ACTIVITY ASSAY

Note: *This protocol contains detailed steps for measuring Complex II activity. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained. When doing multiple experiments it is recommended to make only proportionally enough working solutions and reagents from the supplied concentrated stocks.*

A. Sample preparation

1. Prepare the buffer solution by adding 15 mL of 20X buffer to 285 mL deionized H₂O. Label this 1X Buffer solution. Mix, store in the refrigerator.
2. Prepare the Incubation solution by adding 5 mL 10X Blocking Solution to 45 mL 1X Buffer. Label this 1X Incubation solution. Mix, store in the refrigerator.
3. Resuspend sample – tissue homogenate, cell culture pellet, or mitochondria to 5.5 mg/mL in PBS.
4. Add 1/10 volume of Detergent to the sample (e.g. if the total sample volume is 500 μ L, add 50 μ L of Detergent). Therefore the final protein concentration is 5 mg/mL.
5. Mix immediately and incubate for 30 minutes on ice.
6. Centrifuge at 25,000 g for 20 minutes at 4°C. Collect the supernatant discard the pellet.

B. Plate Loading

7. Dilute the sample in 1X Incubation solution, to the desired concentration per mL shown in the table below.

Sample Type	Recommended amount
Whole cultured cell extract	60 μ g/50 μ L (1.2 mg/mL)
Heart mitochondria	10 μ g/50 μ L (0.2 mg/mL)

8. Keep the diluted samples on ice until ready to begin.
9. Add 50 μ L of diluted sample per well. Be sure to include a normal or control sample and also a buffer only sample (1X Incubation solution). A dilution series of a normal control sample is also recommended.
10. Cover the plate and incubate for 2 hours at room temperature.

C. Measurement

11. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by turning the plate over and shaking out any remaining liquid.
12. Once emptied, add 300 μ L of 1X Buffer solution to each well used.

13. Empty the wells again and add another 300 μL of 1X Buffer solution to each well used.
14. Empty the wells and add 40 μL of lipid solution to each well used. Incubate 30 minutes.
15. Prepare the Activity solution. Make only enough solution proportional to the number of microplate strips used.

Use the following table to calculate how much solution to make for 1-12 microplate strips used.

No. of microplate strips	Ubiquinone 2 (μL)	Succinate (μL)	DCPIP (μL)	Activity buffer (mL)
1	5	42	21	2.1
2	10	83	42	4.2
3	15	125	63	6.3
4	20	167	83	8.3
5	25	208	104	10.4
6	30	250	125	12.5
7	35	292	146	14.6
8	40	333	167	16.7
9	45	375	188	18.8
10	50	417	208	20.8
11	55	458	229	22.9
12	60	500	250	25

16. Do not empty the wells; instead add 200 μL of Activity solution (A15) to each well already containing 40 μL lipids for a total of 240 μL . Any bubbles in the wells should be popped with a fine needle as rapidly as possible, begin microplate reading using the following parameters:

Activity - Kinetic measurement

OD 600 nm

Room temperature

Time: 60 mins

Interval: 20 sec - 1 min

NO shake before or between readings

Save data and analyze as described in the DATA ANALYSIS section.

Note – it is possible to make an endpoint measurement in place of kinetic measurement, but be sure to measure the endpoint before the most active sample has begun to slow down (see example below BHM sample >1600s).

DATA ANALYSIS

COMPLEX II ACTIVITY

The initial solution for the activity measurement should be blue in appearance with an OD of approximately 0.2 mOD units at 600 nm. The reduction of ubiquinone and subsequent reduction of DCPIP is measured as a decrease in absorbance at OD 600 nm (see Figure 1). Monitor the rate of decrease in absorbance at 600 nm over time. Calculate the rate between two time points for all the samples where the decrease in absorbance is the most linear (typically between 15 mins and 25 mins – shown below). After 30 minutes the rate of reduction in absorbance may decline for the most active samples due lack of substrate so do not calculate the rate after this point.

$$\text{Rate (mOD/min)} = \frac{\text{Absorbance 1} - \text{Absorbance 2}}{\text{Time (min)}}$$

The activity of immunocaptured Complex II is the mean of measurements obtained with immunocaptured enzyme minus the rate obtained without immunocaptured enzyme. For example, if the rates of immunocaptured Complex II are 3.2, 3.1 and 3.7 mOD/min and the background rate (null sample) is 0.1 mOD/min, the activity of Complex II is $(3.2+3.1+3.7)/3 - 0.1$ which is 3.23 mOD/min. Now the activity of immunocaptured Complex II in between samples can be compared.

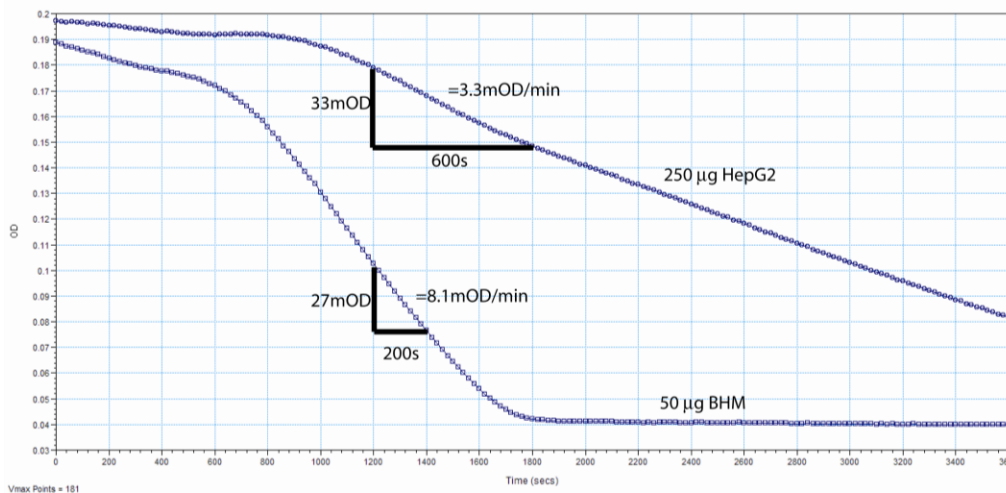
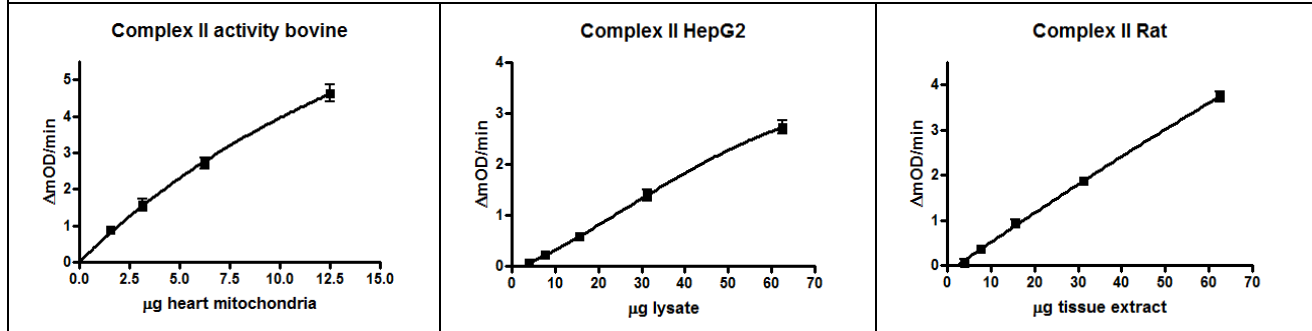


Figure 1. Example of raw data. Note the lag period before activity. Also note the activity of mitochondria (BHM, bovine heart mitochondria) is higher than whole cell lysate (HepG2, human hepatoblastoma) and the reaction ends at >1600 seconds because the substrates are used up.

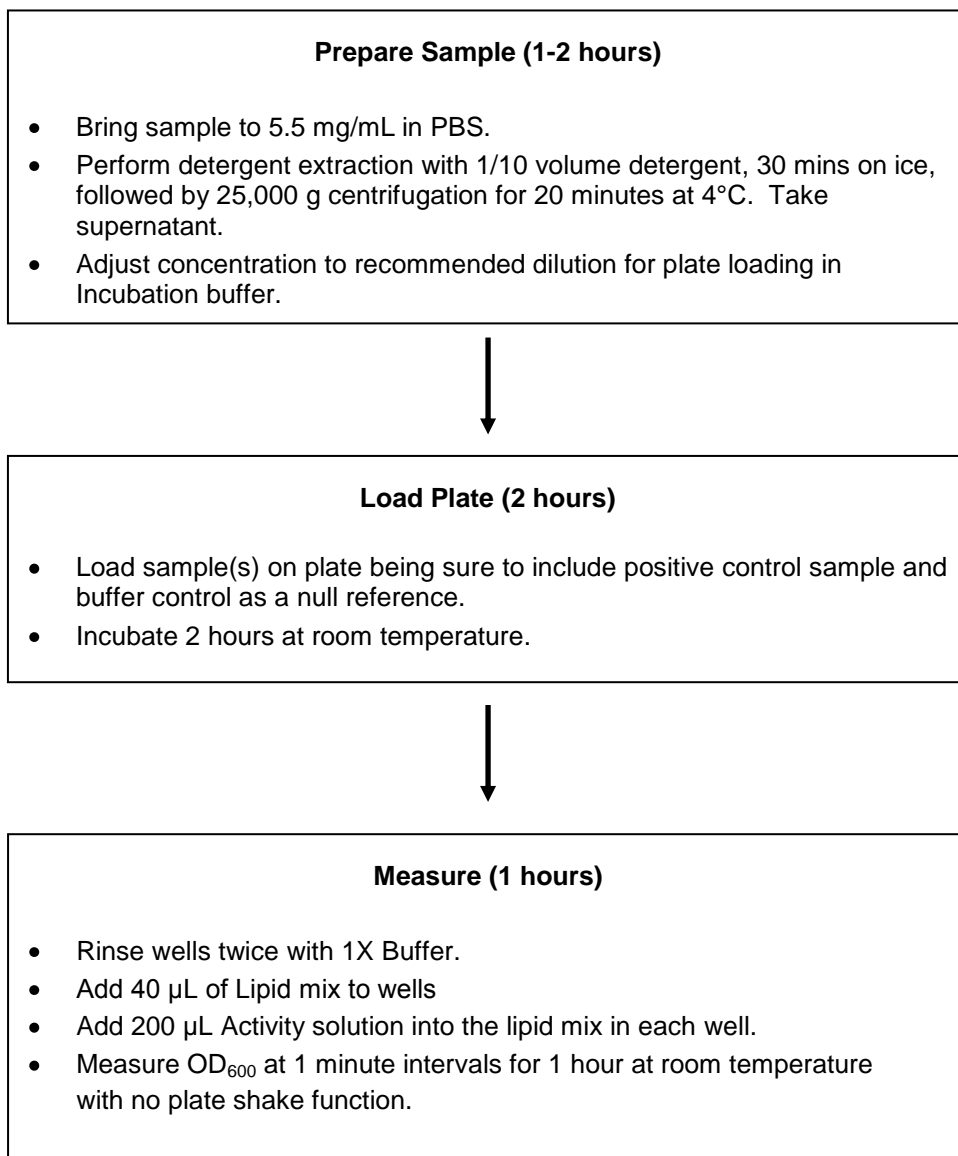
This assay is compatible with different sample types such as mitochondria, tissue or cell lysates and in multiple species including human and rodent samples. Typical linear range data are shown below in Figure 2.

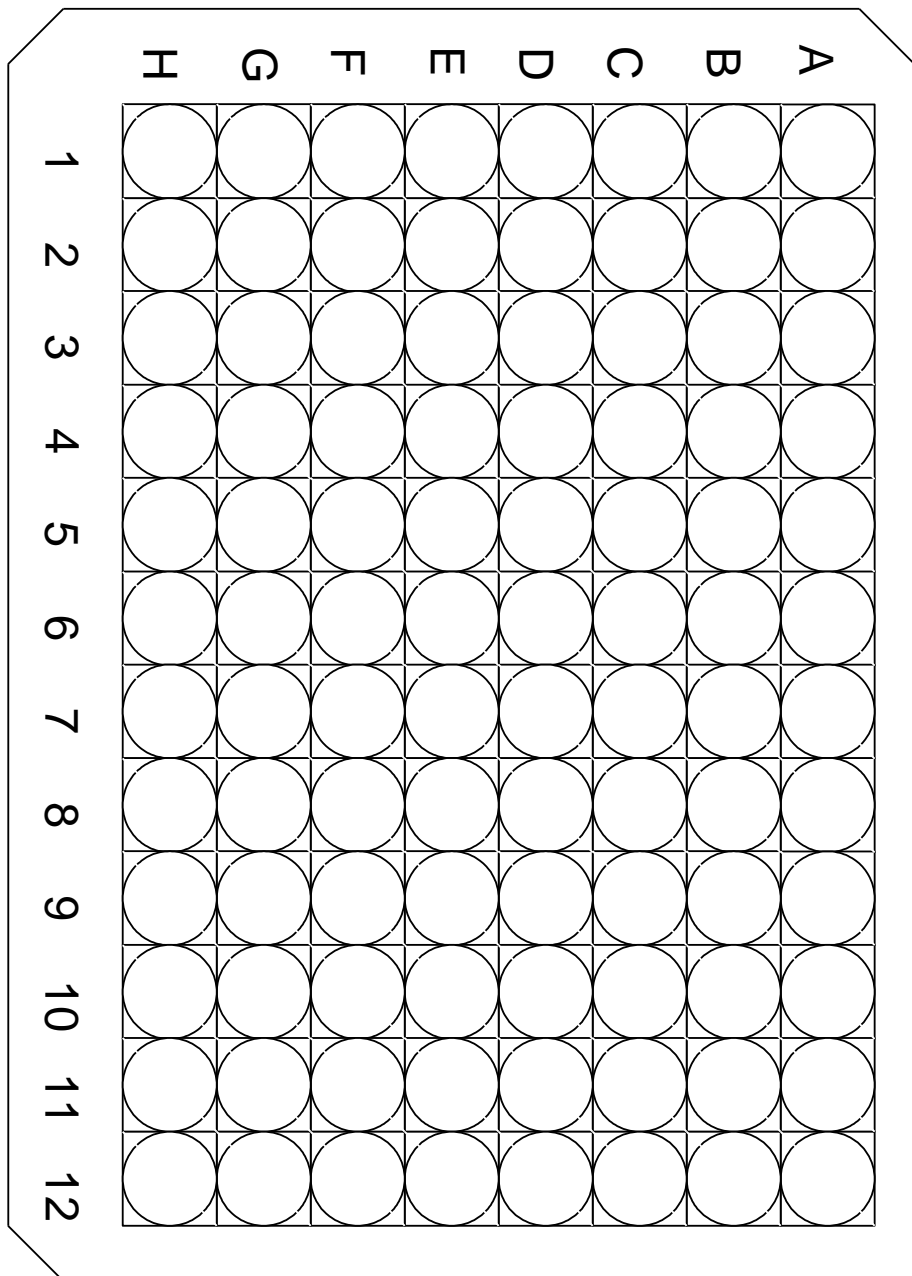
Figure 2. Data are most easily interpreted by working in the linear range of the assay as shown here, however the range can also be extended by non-linear curve fitting.



FLOW CHART

For quick reference only. Be completely familiar with previous details of this document before performing the assay.





MICROPLATE MS _____

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NOTES

Sample preparation is crucial to a successful analysis. Key parameters:

Homogenization

Samples must be completely homogenous. For cultured cells this should only require pipetting up and down to break apart clumps of cells. Similarly for mitochondrial preparations, pipetting is enough to distribute the mitochondria evenly in solution. For soft tissue, and especially for hard tissues such as muscle, thorough homogenization must occur. This is best accomplished with a hand held tissue grinder such as an electric Ultra Turrax T8 tissue grinder or a Dounce glass tissue grinder available from MitoSciences. It is recommended to use one of MitoSciences Mitochondrial Isolation Kits (MS850-MS853).

Sample solubilization

It is most convenient to resuspend to approximately 10 mg/mL. Then determine the exact protein concentration by BCA method (Pierce). Then add solution to a protein concentration of 5.5 mg/mL in PBS. The sample can now be extracted by adding 1/10 volume of the supplied detergent. The final protein concentration is now 5 mg/mL, which is the optimal concentration for intact Complex II solubilization by the supplied detergent. The sample is incubated, centrifuged and supernatant (detergent extract) is collected.

Inhibitor sensitivity

The enzyme is sensitive to 2-thenoyltrifluoroacetone (TTFA) a specific inhibitor of Complex II with IC_{50} of 30 μ M.