Novagen®

Mitochondrial Complex II Activity Assay Kit

Table of Contents

About the Kits 2
- Description 2
- Components 2
- Storage 2
- Materials Required But Not Supplied 2

Assay Preparation 3
- Considerations Before You Begin 3
- Buffer and Sample Preparation 3

Assay Protocol 4
- Plate Loading 4
- Measurement 4

Performance Characteristics 5

Additional Notes 6
- Key parameters 6

Appendix A: Assay Flow Chart 7

Appendix B: Assay Plate Design 8

USA and Canada
Tel (800) 628-8470
bioscienceshelp@
emdchemicals.com

Europe

France	**Germany**	**Ireland**	**United Kingdom**	**All other European Countries**
Freephone	Freecall	Toll Free	Freephone	+44 115 943 0840
0800 126 461	0800 100 3496	1800 409 445	0800 622 935	

All Other Countries
Contact Your Local Distributor
www.merck4biosciences.com
bioscienceshelp@
emdchemicals.com

techservice@merckbio.eu

www.merck4biosciences.com

About the Kits

Mitochondrial Complex II Activity Assay Kit AAMT002-1KIT

Description

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.

$$\text{Succinate + ubiquinone (Q)} \rightarrow \text{Fumarate + ubiquinol } (QH_2)$$

The Mitochondrial Complex II Activity Assay 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-diclorophenolindophenol) and a decreases in its absorbance at 600 nm which in turn recycles the substrate ubiquinone, as shown below.

$$\underset{\text{blue}}{\text{ubiquinol } (QH_2) + \text{DCPIP}} \rightarrow \underset{\text{colorless}}{\text{ubiquinone (Q)} + DCPIPH_2}$$

Components

15 ml	20X Buffer, (72581-15ML)
25 ml	Complex II Activity Buffer, (72582-25ML)
2 x 1 ml	10X Detergent, (72583-1ML)
500 µl	50X Succinate Solution, (72584-500UL)
250 µl	100X DCPIP, (72585-250UL)
60 µl	Ubiquinone 2, (72586-60UL)
5 ml	10X Blocking Solution, (72587-5ML)
6 ml	Phospholipids, (72588-6ML)
1 ea	Microplate, (72589-1EA)

* This kit contains sufficient materials for 96 measurements (one 96-well microplate).

Note: The 96-well microplate has a monoclonal antibody pre-bound to the wells.

Storage

Store the 20X Buffer, Complex II Activity Buffer, 10X Detergent, 10X Blocking Solution, and Microplate at 4°C. Store the 50X Succinate Solution, 100X DCPIP, Ubiquinone 2, and Phospholipids at –80°C.

Materials Required But Not Supplied

- Spectrophotometer plate reader (Molecular Dynamics SpectraMax® recommended) capable of measuring absorbance at 600 nm.
- Method for determining protein concentration
- Deionized water
- Multichannel pipette
- PBS (1.4 mM KH_2P0_4, 8 mM Na_2HP0_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3)

Assay Preparation

Considerations Before You Begin

- 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.
- **The Mitochondrial Complex II Activity Assay has been developed for use with human samples. However, the kit also works with bovine, mouse and rat samples.** Other species have not been tested 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). If performing mitochondrial toxicity testing, an untreated 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 µl) and per milliliter are listed below. The ranges may be extended by using a non-linear fit of the data from a normal sample.

Sample Type	Typical Linear Ranges	
Bovine heart mitochondria	1–25 µg/well	20–500 µg/ml
Whole cultured cell extract	4–250 µg/well	80–5000 µg/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 in the next section. Intra-assay variation ≤ 15 %

Buffer and Sample Preparation

1. Prepare the buffer solution by adding 15 ml of 20X buffer to 285 ml deionized H_2O. Label this solution as 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 solution as 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 µl, add 50 µl 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 x g for 20 minutes at 4°C. Collect the supernatant discard the pellet.
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 µg/50 µl (1.2 mg/ml)
Heart mitochondria	10 µg/50 µl (0.2 mg/ml)

8. Keep the diluted samples on ice until ready to begin.

Assay Protocol

Plate Loading

1. Add 50 µl of diluted sample (from the previous section) per well. If performing mitochondrial toxicity testing, an untreated control or normal sample and also a buffer only sample (1X Incubation solution) should always be included in the assay as a reference. A dilution series of a normal control sample is also recommended.
2. Cover the plate and incubate for 2 hours at room temperature.

Measurement

1. 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.
2. Once emptied, add 300 µl of 1X Buffer solution to each well used.
3. Empty the wells again and add another 300 µl of 1X Buffer solution to each well used.
4. Empty the wells and add 40 µl of Phospholipids solution to each well used. Incubate 30 minutes.
5. Prepare the Activity Solution. Make only enough solution proportional to the number of microplate strips used. (Use the following table for your reference.)

No. of 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	25.0

6. Do not empty the wells; instead add 200 µl of Activity Solution to each well already containing 40 µl Phospholipids for a total of 240 µl. Any bubbles in the wells should be popped with a fine needle as soon as possible, begin microplate reading using the following parameters:

Kinetic Measurement
$OD_{600\ nm}$
Time: 60 minutes
Interval: 20–60 seconds
Temperature: Room Temperature
Do **NOT** shake before or between readings

7. Save data and analyze as described in the Performance Characteristics 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).

Performance Characteristics

Note: All data and results shown below are representative and not lot specific. Please refer to certificate of analysis for lot specific testing data.

- 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 minutes and 25 minutes – 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.

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

- 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.

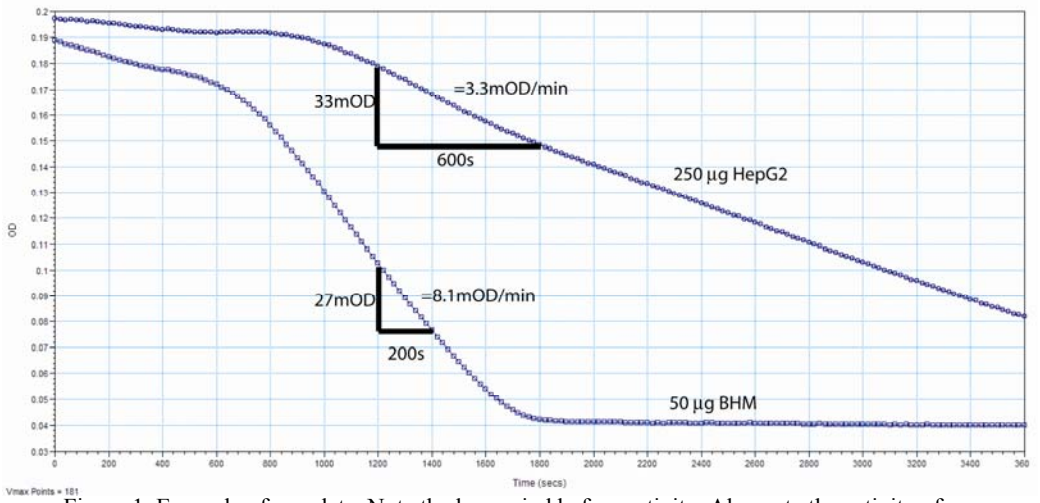

Figure 1. Example of raw data. Note the lag period before activity. Also note the activity of mitochondria (BHM, bovine heart mitochondria) is higher than whole cell lysate (HepG2, human hepatoblastoma) and the reaction ends at > 1600 seconds because the substrates are used up.

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 in the Figure below.

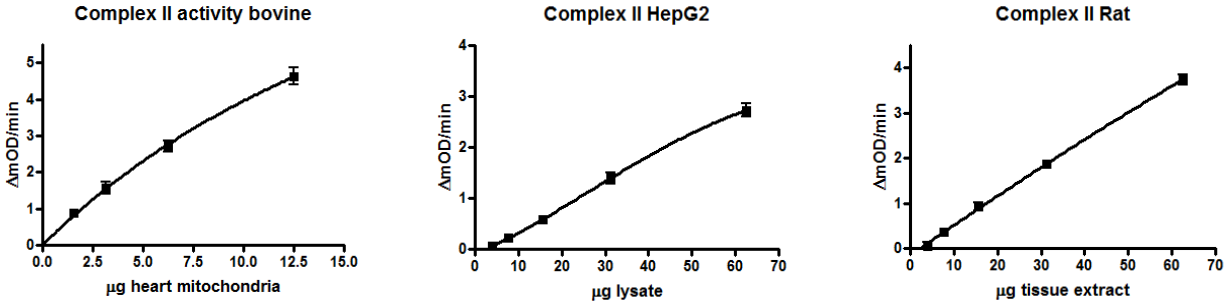

Figure 2. Data are most easily interpred by working in the linear range of the assay as shown here; however, the range can also be extended by non-linear curve fitting.

Additional Notes

Key parameters

Note: Sample preparation is crucial to a successful analysis.

A. 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. Also, search literature for a general protocol on mitochondrial isolation.

B. 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.

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

Appendix A: Assay Flow Chart

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

Prepare Sample (1-2 hours)

- Adjust sample concentration to 5.5 mg/ml in PBS.
- Perform Detergent extraction with 1/10 volume Detergent.
- Incubate on ice for 30 minutes.
- Centrifuge at 12,000 x g for 20 minutes at 4°C and then collect supernatant.
- Adjust concentration to recommend dilution for plate loading in incubation buffer.

Load Plate (2 hours)

- Load sample(s) on plate being sure to include a normal sample and a buffer control as null reference.
- Incubate 3 hours at room temperature.

Measure (1 hour)

- Rinse wells twice with Buffer.
- Add 40 µl of Lipid mix to each well.
- Add 200 µl Acitivity solution into the lipid mix in each well
- Measure OD_{600} at approximately 1 minute intervals for 1 hour at room temperature with no plate shake function.

Appendix B: Assay Plate Design