ab109908 –
Complex II Enzyme Activity Microplate Assay Kit

Instructions for Use

For the quantitative measurement of Complex II activity in samples from Human, Rat, Mouse and Cow

This product is for research use only and is not intended for diagnostic use.
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1. Introduction

Abcam’s enzyme activity assays apply a novel approach, whereby target enzymes are first immunocaptured from tissue or cell samples before subsequent functional analysis. All of our ELISA kits utilize highly validated monoclonal antibodies and proprietary buffers, which are able to capture even very large enzyme complexes in their fully-intact, functionally-active states.

Capture antibodies are pre-coated in the wells of premium Nunc MaxiSorp™ modular microplates, which can be broken into 8-well strips. After the target has been immobilized in the well, substrate is added, and enzyme activity is analyzed by measuring the change in absorbance of either the substrate or the product of the reaction (depending upon which enzyme is being analyzed). By analyzing the enzyme's activity in an isolated context, outside of the cell and free from any other variables, an accurate measurement of the enzyme's functional state can be understood.

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.
ab109908 (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-diclorophenolindophenol) and a decreases in its absorbance at 600 nm which in turn recycles the substrate ubiquinone, as shown below.

\[
\text{ubiquinol (QH}_2\text{)} + \text{DCPIP (blue)} \rightleftharpoons \text{ubiquinone (Q) + DCPIPH}_2\text{ (colorless)}
\]

This microplate assay 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 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 table below:

<table>
<thead>
<tr>
<th>Example Ranges</th>
<th>1 - 25 μg / well</th>
<th>20 – 500 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Heart Mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cultured cell extract</td>
<td>4 - 250 μg / well</td>
<td>80 – 5000 μg/ml</td>
</tr>
</tbody>
</table>

**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. Intra assay variation = <15 %.
2. Assay Summary

Prepare Sample (1-2 hours)

- Bring sample to 5.5 mg/ml in PBS.
- Perform detergent extraction with 1/10 volume detergent, 30 mins on ice, followed by 25,000 g centrifugation for 20 minutes at 4°C. Take supernatant.
- Adjust concentration to recommended dilution for plate loading in Incubation buffer.

Load Plate (2 hours)

- Load sample(s) on plate being sure to include positive control sample and buffer control as a null reference.
- Incubate 2 hours at room temperature.

Measure (1 hour)

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

Sufficient materials are provided for one 96-well microplate.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20x Buffer</td>
<td>15 ml</td>
</tr>
<tr>
<td>10X Detergent</td>
<td>2 x1 ml</td>
</tr>
<tr>
<td>10X Blocking Solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Complex II Activity Buffer</td>
<td>25 ml</td>
</tr>
<tr>
<td>DCPIP</td>
<td>250 µl</td>
</tr>
<tr>
<td>Succinate</td>
<td>500 µl</td>
</tr>
<tr>
<td>Ubiquinone 2</td>
<td>60 µl</td>
</tr>
<tr>
<td>Lipid Mix</td>
<td>6 ml</td>
</tr>
<tr>
<td>Pre-coated 96-well microplate (12 strips)</td>
<td>1</td>
</tr>
</tbody>
</table>
4. Storage and Handling

Succinate, Ubiquinone 2 and DCPIP should be stored at -80°C. Lipid Mix should be stored at -20°C. All other components should be stored at 4°C.

5. Additional Materials Required

- Spectrophotometer capable of measuring absorbance at 600nm
- Method for determining protein concentration.
- Deionized water
- Multichannel pipette (50 - 300 μl) and tips
- PBS (phosphate buffered saline)
6. Preparation of Samples

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.

1. Prepare the buffer solution by adding 15 ml of 20X buffer to 285 ml deionized H$_2$O. 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 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 g for 20 minutes at 4°C. Collect the supernatant discard the pellet.
7. Assay Method

A. Plate Loading

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Protein Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cultured cell extract</td>
<td>60 μg / 50 μl (1.2mg/ml)</td>
</tr>
<tr>
<td>Heart mitochondria</td>
<td>10 μg / 50 μl (0.2mg/ml)</td>
</tr>
</tbody>
</table>

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

3. Add 50 μl 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.

4. Cover the plate and incubate for 2 hours at room temperature.
B. 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 lipid 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 to calculate how much solution to make for 1-12 microplate strips used.
<table>
<thead>
<tr>
<th>No. of microplate strips</th>
<th>Ubiquinone 2 (μl)</th>
<th>Succinate (μl)</th>
<th>DCPIP (μl)</th>
<th>Activity buffer (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>42</td>
<td>21</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>83</td>
<td>42</td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>125</td>
<td>63</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>167</td>
<td>83</td>
<td>8.3</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>208</td>
<td>104</td>
<td>10.4</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>250</td>
<td>125</td>
<td>12.5</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>292</td>
<td>146</td>
<td>14.6</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>333</td>
<td>167</td>
<td>16.7</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>375</td>
<td>188</td>
<td>18.8</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>417</td>
<td>208</td>
<td>20.8</td>
</tr>
<tr>
<td>11</td>
<td>55</td>
<td>458</td>
<td>229</td>
<td>22.9</td>
</tr>
<tr>
<td>12</td>
<td>60</td>
<td>500</td>
<td>250</td>
<td>25</td>
</tr>
</tbody>
</table>
6. 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

**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).
8. Data Analysis

A. Calculation of 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.

**Examples of data obtained using ab109908:**

![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.](image)

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.
B. Reproducibility

Intra-Assay: CV < 15 %

9. Specificity

Species Reactivity: Human, Rat, Mouse, Bovine

10. 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 tissue grinder or a Dounce glass tissue. It is recommended to use one of Abcam’s Mitochondrial Isolation Kits (ab110168-ab110171/MS850-MS853).
**Sample solubilization**

It is most convenient to resuspend to approximately 10 mg/ml. Then determine the exact protein concentration by BCA method. 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 μM.
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