

Complex IV Human Enzyme Activity Microplate Assay Kit

MS441

Rev.1

DESCRIPTION

Complex IV Human Enzyme Activity Microplate Assay Kit

Kit Contents:

Included in this kit is the necessary buffer (Tube 1), detergent for sample preparation, and substrate for the reaction (Reagent C). The kit contains a 96-well microplate with a monoclonal antibody pre-bound to the wells of the microplate. This plate can be broken into 12 separate 8-well strips for convenience; therefore the plate can be used for up to 12 separate experiments.

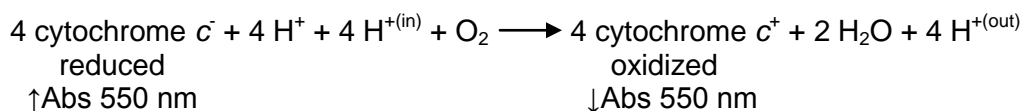
Item	MS441
TUBE 1 (Buffer)	10 mL
DETERGENT	1 mL
REAGENT C (Cytochrome c)	1 mL
96-well microplate (12 strips)	1

Storage:

For multiple experiments Reagent C may be aliquoted and stored at -20°C or preferably -80°C. Tube 1, Detergent and the covered microplate should be stored at 4°C.

INTRODUCTION

The Complex IV Human Enzyme Activity Microplate Assay Kit (MS441) is used to determine the activity of the cytochrome c oxidase enzyme (EC 1.9.3.1) in a human sample. Complex IV is immunocaptured within the wells and activity is determined colorimetrically by following the oxidation of reduced cytochrome c as an absorbance decrease at 550 nm. The overall reaction is as follows:



This activity microplate (MS441) has been developed for use with human samples. Bovine material is compatible, however mouse and rat are not, and separate microplate assay kits are available for these species. Other species have not been tested.

This assay is designed for use with purified mitochondria. However, homogenized tissue and whole cells can also be used. Samples should be solubilized, the protein extracted and measured within the linear range as described 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:

Cultured cell extracts	1-20 μg / 200 μL
Tissue extracts	0.1-10 μg / 200 μL
Tissue mitochondria	0.01-1 μg / 200 μL

Typical intra-assay variations (same day, same sample) <7%
Typical inter-assay variation (day to day, same sample) <10%

The protocol has 4 steps:

- A) Sample preparation
- B) Plate loading
- C) Measurement
- D) Data Analysis

This activity microplate can be performed in conjunction with the Complex IV Human Protein Quantity Microplate Assay Kit (MS442) to establish specific activity. A multiplexing microplate (MS443) is also available as a separate kit.

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance of $550\pm 1\text{nm}$
- Deionized water
- Multichannel Pipetting devices
- Protein assay method

MICROPLATE ASSAY PROTOCOL

Note: *This protocol contains detailed steps for measuring Complex IV activity and quantity. 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.*

A. Sample Preparation

1. Prepare the buffer solution by adding Tube 1 (10 mL) to 190 mL deionized H₂O. Label this solution as Solution 1.
2. Pellet the sample by centrifugation.
3. Resuspend the sample by adding 5 volumes of Solution 1. The sample must be homogenous before detergent extraction. Therefore, resuspend the sample thoroughly by pipetting (cultured cells), or homogenize with a microtissue grinder/ultra turrax T8 (tissue). Determine the protein concentration by a standard method and then adjust the concentration to 5 mg/mL.

Note: *The optimal protein concentration for detergent extraction is 5 mg/mL.*

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). Mix immediately and then incubate the sample on ice for 30 minutes.
5. Spin in tabletop microfuge at maximum speed (~16,000 rpm) for 20 minutes.
6. Carefully collect the supernatant and save as sample. Discard the pellet.
7. The microplate wells are optimized for 200 μ L sample volume, so dilute samples to the following recommended concentrations by adding Solution 1:

Cultured cell extracts	5-20 μ g / 200 μ L
Tissue extracts	1-10 μ g / 200 μ L
Tissue mitochondria	0.01-1 μ g / 200 μ L

8. Keep diluted samples on ice until ready to proceed to Section B.

B. Plate Loading

1. Add 200 μ L of each diluted sample prepared in Section A into individual wells on the plate. Include a normal sample as a positive control. Include a buffer control (200 μ L SOLUTION 1) as a null or background reference.
2. Incubate the microplate for 3 hours at room temperature.

C. Measurement

1. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by quickly turning the plate upside down and shaking out any remaining liquid.
2. Add 300 μ L of Solution 1 to each well.

3. In a sealable tube prepare an appropriate amount of Assay Solution using Reagent C and Solution 1. Mix gently by inversion. See table on the next page for amounts required. Set Assay Solution aside.

No. of Strips	REAGENT C (μ L)	SOLUTION 1 (mL)
1	84	1.67
2	167	3.33
3	250	5.00
4	333	6.67
5	417	8.33
6	500	10.0
7	583	11.67
8	667	13.33
9	750	15.00
10	833	16.67
11	917	18.33
12	1000	20.00

4. Set up the plate reader to a kinetic program to measure absorbance at 550 nm at 30°C for 120 minutes, with measurement interval of approximately 1 minute (however a longer measurement interval may be used if necessary).
5. Empty wells and add 300 μ L Solution 1 to each well used. Repeat this rinse.
6. Empty the wells again and then add 200 μ L of Assay Solution to each well used, be careful to avoid the creation of bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.
7. Set plate in plate reader and begin recording immediately.
8. Analyze data as described below in Section D.

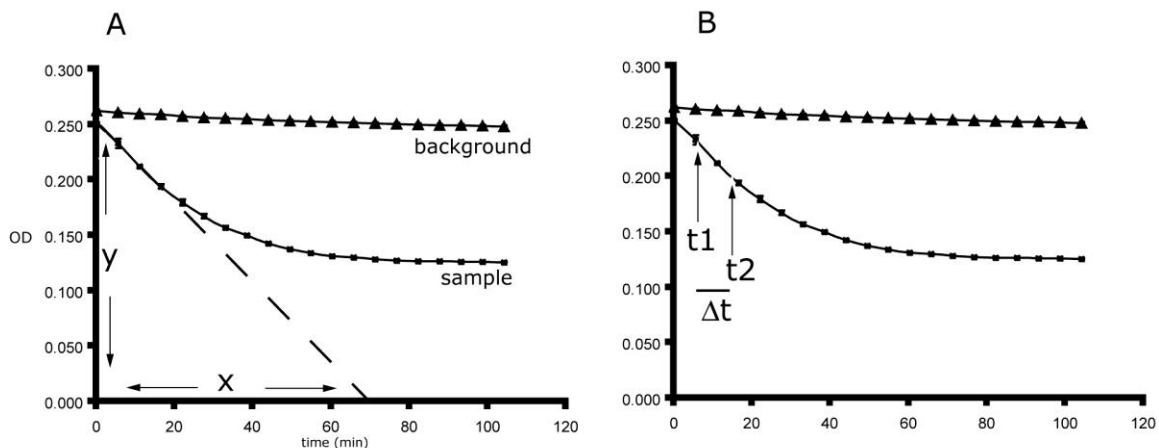
D. Data analysis

Since the Complex IV reaction is product inhibited, the rate of activity is always expressed as the initial rate of oxidation of cytochrome *c*. This oxidation is seen as a decrease in absorbance at 550 nm. The initial rate should be a linear decrease. At lower activity levels the linear range is extended.

To determine the activity in the sample, calculate the slope by using microplate software or by manual calculations using one of the two methods shown below. Compare the sample rate with the rate of the control (normal) sample and with the rate of the null (background) to get the relative Complex IV activity.

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

Example:



$$\frac{y}{x}$$

$$\frac{0.25 \text{ OD}}{70 \text{ min}}$$

$$= 3.6 \text{ mOD/min}$$

$$\frac{\text{Abs}(t1-t2)}{\Delta t}$$

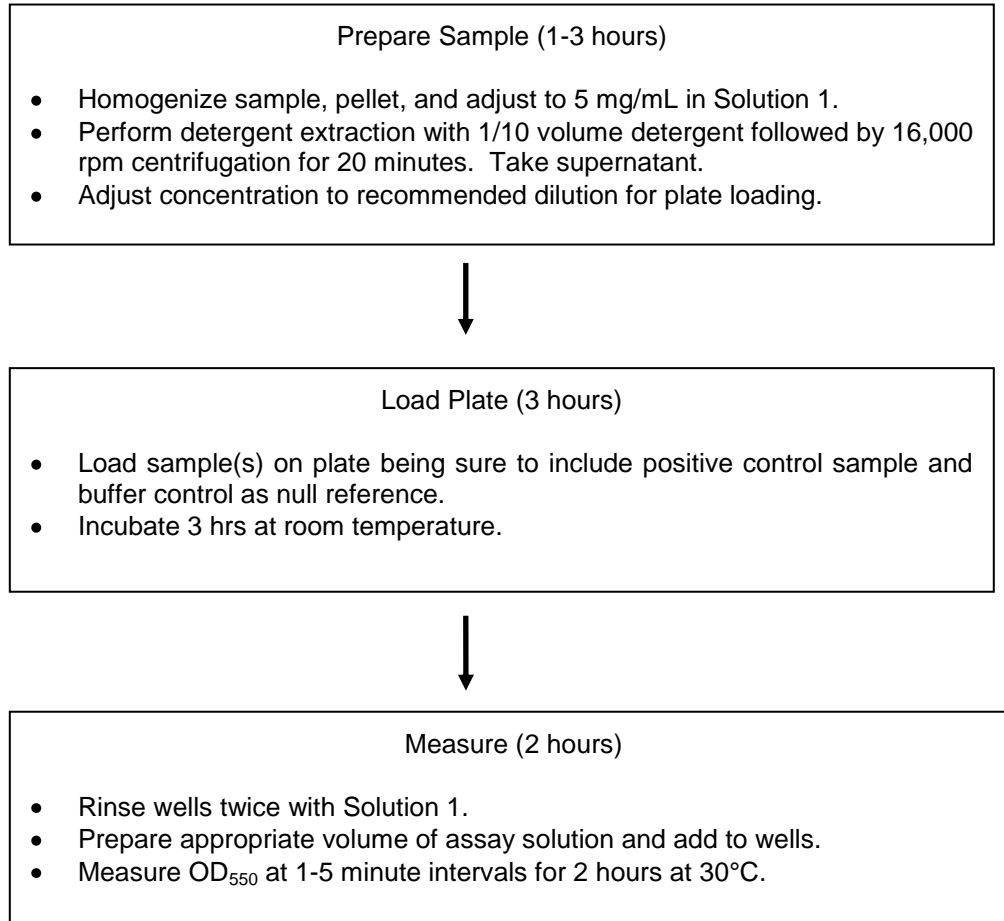
$$\frac{0.23-0.19 \text{ OD}}{11 \text{ min}}$$

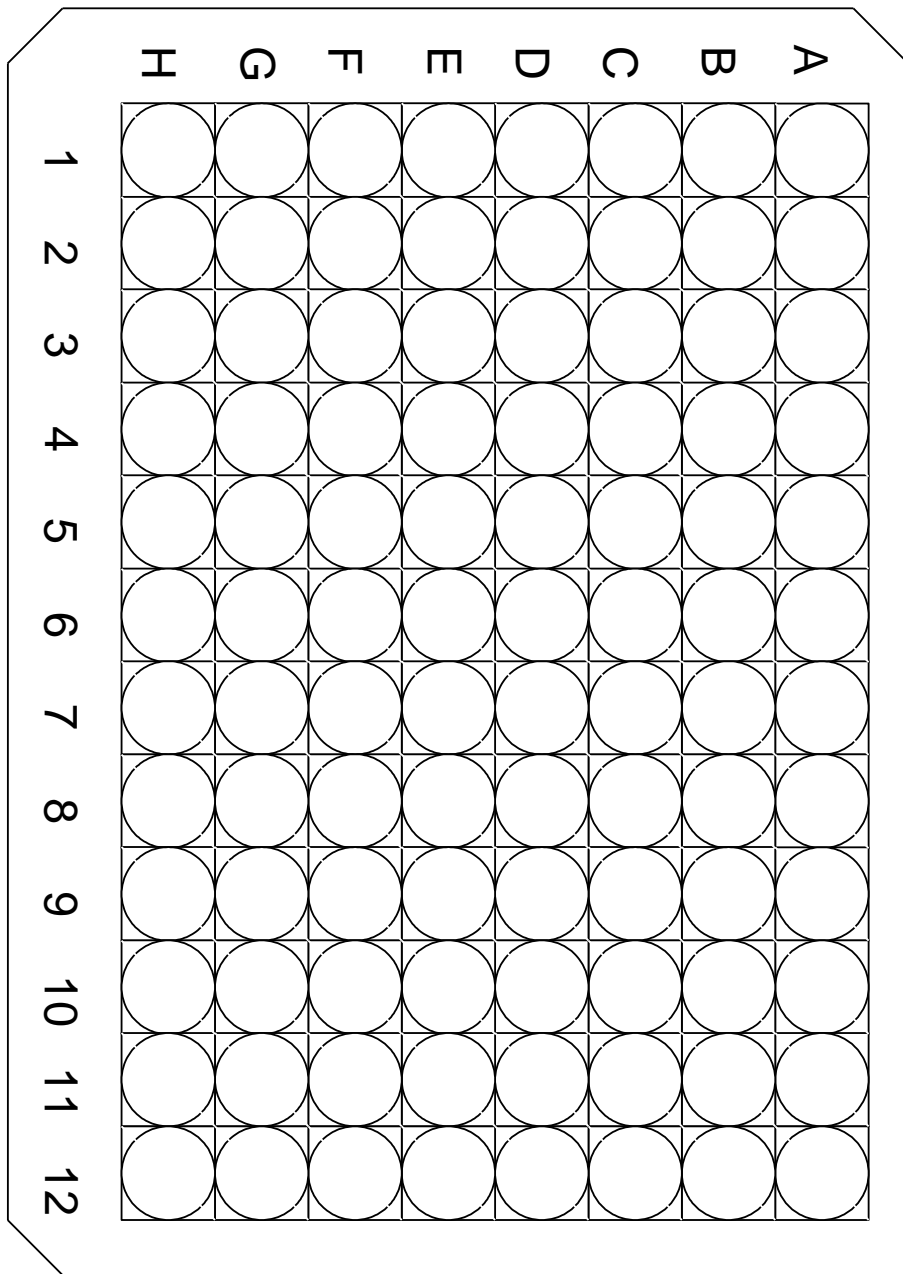
$$= 3.6 \text{ mOD/min}$$

- The rate is determined by calculating the gradient of the initial slope over the linear region.
- The rate is determined by calculating the slope between two points within the linear region.

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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