PROTOCOL

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Complex I Enzyme Activity Microplate Assay Kit

MS141

Rev.1

DESCRIPTION

Complex I Enzyme Activity Microplate Assay Kit

Included in this kit is the necessary buffer, detergent and block for sample preparation, Reagents 1 and 2 for the activity measurement. 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.

Kit Contents:

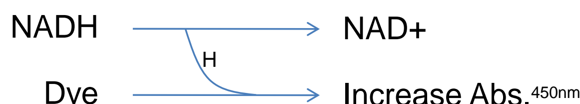
Item	MS141
Buffer (20X)	25 mL
Block (10X)	10 mL
Detergent (10X)	1 mL
NADH Reagent 1 (20X)	1 mL
Reagent 2 (100X)	0.2 mL
96-well microplate (12 strips)	1

Storage:

Buffer, Block, Detergent, and the covered microplate should be stored at 4°C. Reagents 1 and 2 should be stored at -80°C and for multiple experiments must be further aliquoted. For more details on aliquoting and storage see frequently asked questions.

INTRODUCTION

The Microplate Assay for Human Complex I Activity (MS141) is used to determine the activity of mitochondrial OXPHOS Complex I (NADH dehydrogenase, E.C. 1.6.5.3). The enzyme is immunocaptured within the wells of the microplate and activity is determined by following the oxidation of NADH to NAD+ and the simultaneous reduction of a dye which leads to increased absorbance at 450 nm.



Complex I activity reaction scheme. The oxidation of NADH is linked to the reduction of a dye which has increased absorbance at 450 nm and so appears yellow in color.

This rapid Complex I microplate has been developed for use with human, rat and mouse samples (bovine samples are also compatible). Samples may be prepared as mitochondria, crude mitochondria and in most cases whole tissue or cell lysates are suitable but may require some sample optimization.

The protein concentration of the sample should be measured. Yields of protein / cell numbers from cell culture pellets are suggested in frequently asked questions. Once diluted to the specified concentration

the sample is detergent solubilized and diluted to within the linear range of measurement. A control or normal sample should always be included in the assay as a reference measurement. In addition, a buffer control should be used as a negative control.

Table 1. Typical ranges of measurement per 200 µL well volume

Tissue mitochondria extract	5-100 µg
Cultured cell extracts†	25-250 µg

† Complex I activity is controlled by enzyme amount and by post-translational phosphorylation at key specific regulatory residues. Cellular metabolism governs these two factors. Ultimately the cell type and growth conditions will affect Complex I activity measurements.

Note that this activity assay measures the diaphorase-type activity of Complex I. This activity is not dependent on the presence of ubiquinone and therefore inhibitors, such as rotenone, which bind at or near the ubiquinone binding site do not inhibit this assay. However the activity assay is affected by assembly deficiencies.

Typical intra-assay variations (same day, same sample) <10% (n=60), inter-assay variation <15% (n=60)

The protocol has 4 steps:

A) Sample preparation
B) Plate loading
C) Activity Measurement.
D) Data analysis

ADDITIONAL MATERIALS REQUIRED

- Spectrophotometer measuring absorbance at 450 nm
- Deionized Water
- Multichannel Pipetting devices
- Protein assay method
- Phosphate buffered saline solution (PBS)

PBS
1.4 mM KH_2PO_4
8 mM Na_2HPO_4
140 mM NaCl
2.7 mM KCl, pH 7.3

MICROPLATE ASSAY PROTOCOL

A. Sample Preparation

1. Add contents of 20X Buffer (25 mL) to 475 mL deionized H_2O. Label this mixture as “Buffer”. Mix well.

2. Pour off 90 mL of Buffer and add to this amount 10 mL of Blocking Solution. Label this “Incubation Solution”.

3. Determine the sample protein concentration using a standard method such as BCA method (Pierce). Adjust the concentration of the sample to 5.5 mg/mL using PBS.
For details of sample solubilization conditions see frequently asked questions section.

4. Extract the proteins by adding Detergent. To do this add 1/10 of a volume of Detergent (e.g. if the total sample volume is 500 µL, add 50 µL of Detergent). Mix well. Place the tube on ice for 30 minutes to allow solubilization. Note the sample concentration is now 5 mg/mL.

5. Centrifuge the sample for 20 minutes at approximately 12,000 g for 20 minutes. Collect the supernatant.

6. Dilute all samples to the desired concentration in Incubation Solution. Table 1 above shows a good linear range for the assay, Table 2 below shows a recommended mid-range value at which to load an experimental sample.

Table 2. Recommended sample dilutions per well (200 µL):

Tissue mitochondria extract	
Heart	20 µg
Liver	50 µg
Cultured cell extracts	
Fibroblast (MRC5)	100 µg
Hepatoblastoma (HepG2)	200 µg

B. Plate Loading

1. Add 200 µL of sample prepared in Section A6 to each well of the microplate that will be used for this experiment. Be sure to include a normal or control sample in addition to a buffer control as described in the introduction.

2. Incubate microplate for 3 hours at room temperature.

C. Measurement

For multiple experiments Reagents 1 and 2 should be aliquoted and stored at -80°C according to frequently asked questions.

1. Thaw Reagents 1 and 2.

2. Empty the wells by turning the plate over and shaking out any remaining liquid. Blot the plate face down on paper towel. Add 300 µL of Buffer.

3. Make Assay Solution (1.75 mL/strip) according to the following table:

No. of Strips	Buffer (mL)	20X Reagent 1 (µL)	100X Reagent 2 (µL)
1	1.67	84	17
2	3.33	167	33
3	5.00	250	50
4	6.67	333	67
5	8.33	417	83
6	10.0	500	100
7	11.67	583	117
8	13.33	667	133
9	15.00	750	150
10	16.67	833	167
11	18.33	917	183
12	20.00	1000	200

4. Empty the wells of the microplate as above.

5. Rinse all wells once more with 300 µL Buffer.

6. Empty the wells again.

7. Add 200 µL of Assay Solution to each well carefully to avoid bubbles. Any bubbles should be popped with a fine needle as rapidly as possible.

8. Place the plate in the reader and record with the following kinetic program.

Kinetic measurement
OD 450 nm
Time: 30 min
Interval: 20 sec - 1 min
Temperature : room temp.
Autoshake between readings

9. Save data and analyze as described in the “Data Analysis” section.

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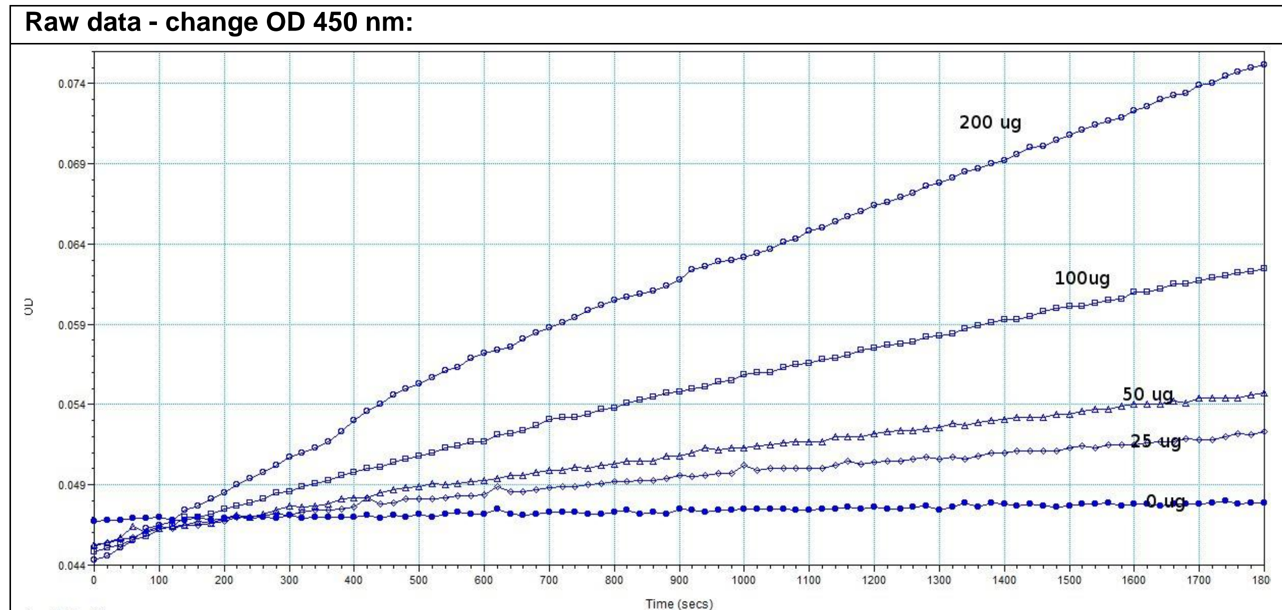
D. Data Analysis

Complex I activity in each well is proportional to the increase in absorbance at 450 nm within each well. The activity is expressed as the change in absorbance per minute per amount of sample loaded into the well.

Examine the linear rate of increase in absorbance at 450 nm over time. An example is shown below where the rate/slope is calculated between these time points. Most microplate software is capable of performing this function. Repeat this for all samples.

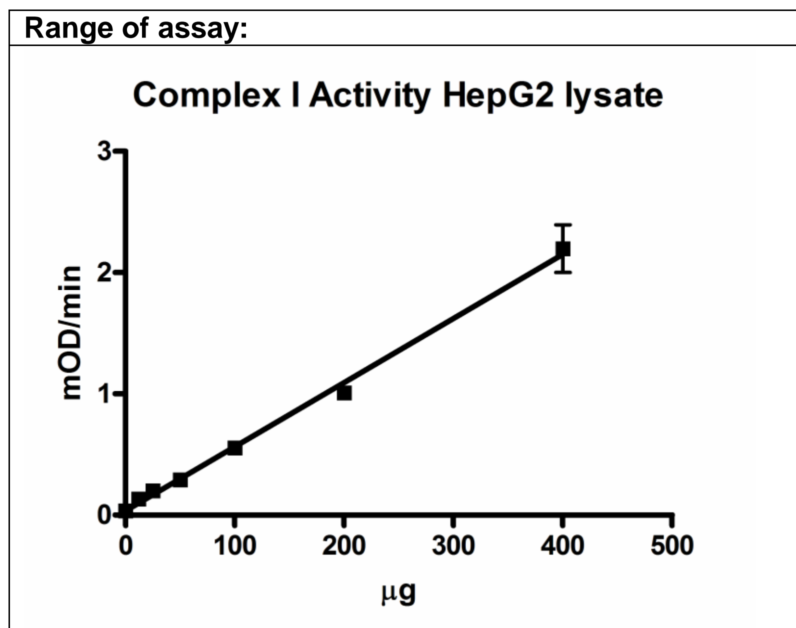
Cultured HepG2 cell lysate:

Raw data - change OD 450 nm:



This raw data can be expressed as rate (mOD/min) per µg of cell lysate added per well as shown below.

Range of assay:



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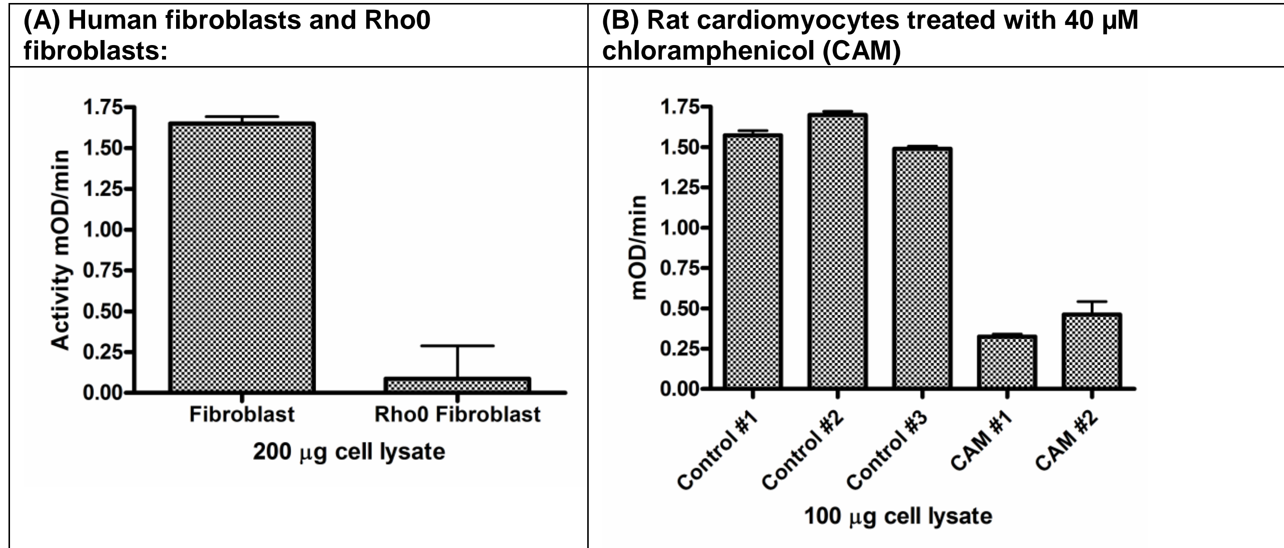
As examples: (A) the assay was used to determine the Complex I activity in normal fibroblasts and rho0 fibroblasts (cells in which the mtDNA is removed and hence essential Complex I proteins are not expressed). The rho0 cells showed no/little complex I activity. In a similar analysis (B) rat cardiomyocytes were grown for 5 days in ± 40 µM chloramphenicol (CAM) to inhibit mitochondrial protein synthesis, Complex I assembly and hence activity was greatly reduced:



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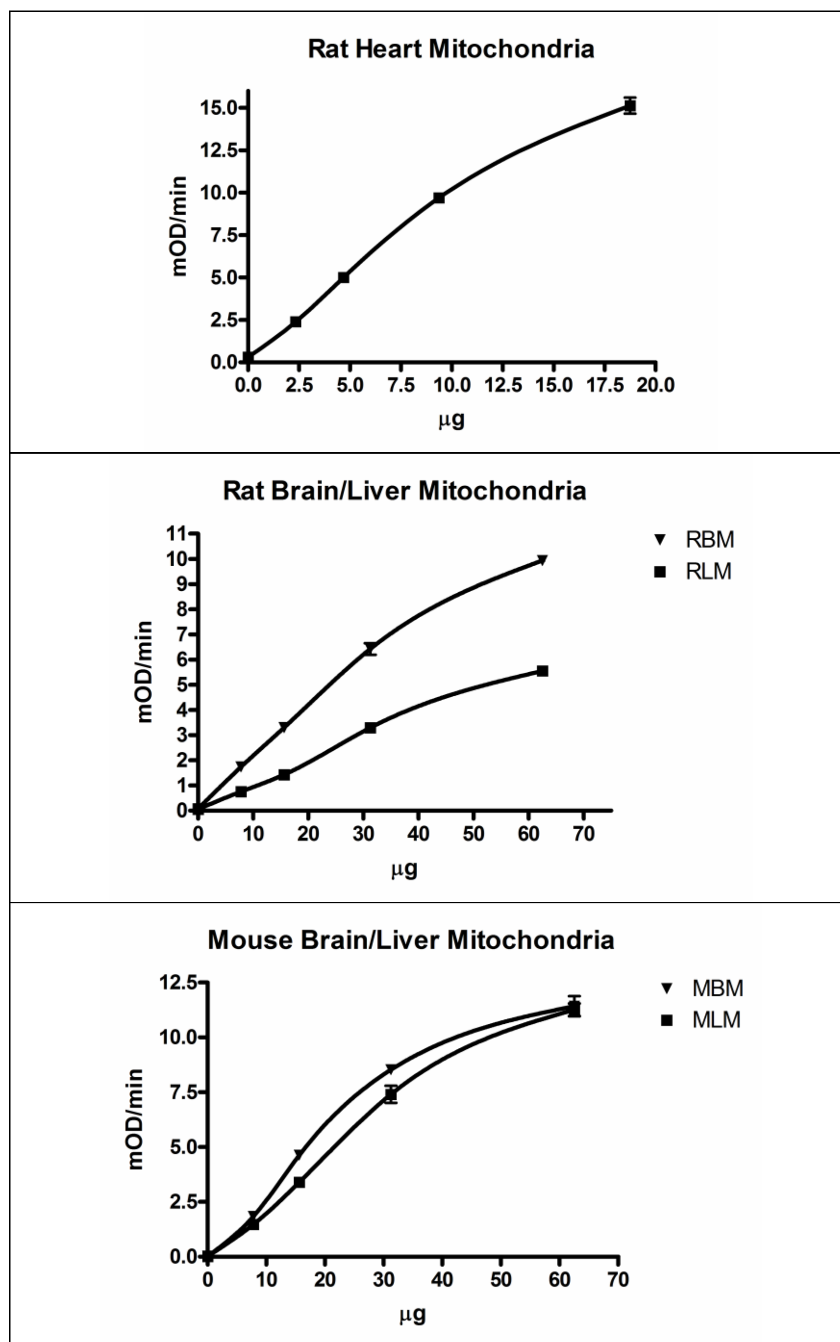
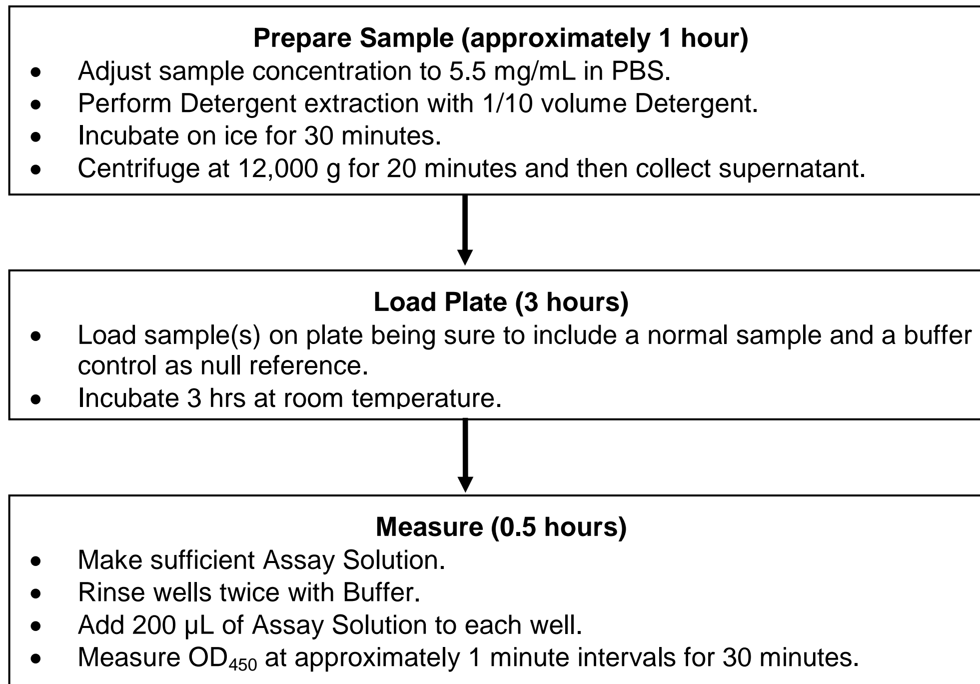
This assay measures Complex I activity in human and rat cultured cells but also in tissues/tissue mitochondria samples within the recommended ranges given on page 2. Note that these ranges depend on mitochondria preparation quality. Examples of Complex I activity measured in different rat and mouse mitochondrial samples are shown below:



FLOW CHART

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



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Frequently asked questions:

How should I store my kit?

Buffer, block, the covered microplate and the detergent should be refrigerated and are stable for at least 6 months. The 20X diluted Buffer can be stored at room temperature, however for extended periods of time (>1 month) it should be stored in the refrigerator to prevent microbiological growth.

How should I store my reagents 1 and 2?

Reagents 1 and 2 are temperature sensitive. They must be stored at -80°C and are stable for at least six months from date of receipt. It is also advisable to aliquot them before freezing to prevent repeated freeze thaw cycles.

How should I aliquot?

Divide the reagents 1 and 2 into aliquots depending on how many experiments you wish to run (consult table, step C3). The plate comes in 12 strips, therefore it is anticipated that up to 12 experiments on different days could be done. The reagent 1 is supplied as 1 mL so for 12 independent experiments you could further aliquot each tube into 12 x 83 µL aliquots and immediately freeze at -80°C for storage until use. Similarly the reagent 2 could be aliquoted into 12 separate 17 µL aliquots each.

How do I make mitochondria?

We have found that little or no optimization is necessary if crude mitochondria are made from samples. Mitochondria can be prepared by simple differential centrifugation of homogenized samples as described in our mitochondrial preparation kit handbook: http://www.mitosciences.com/isolation_kits.html

How do I grow and prepare cultured cell samples?

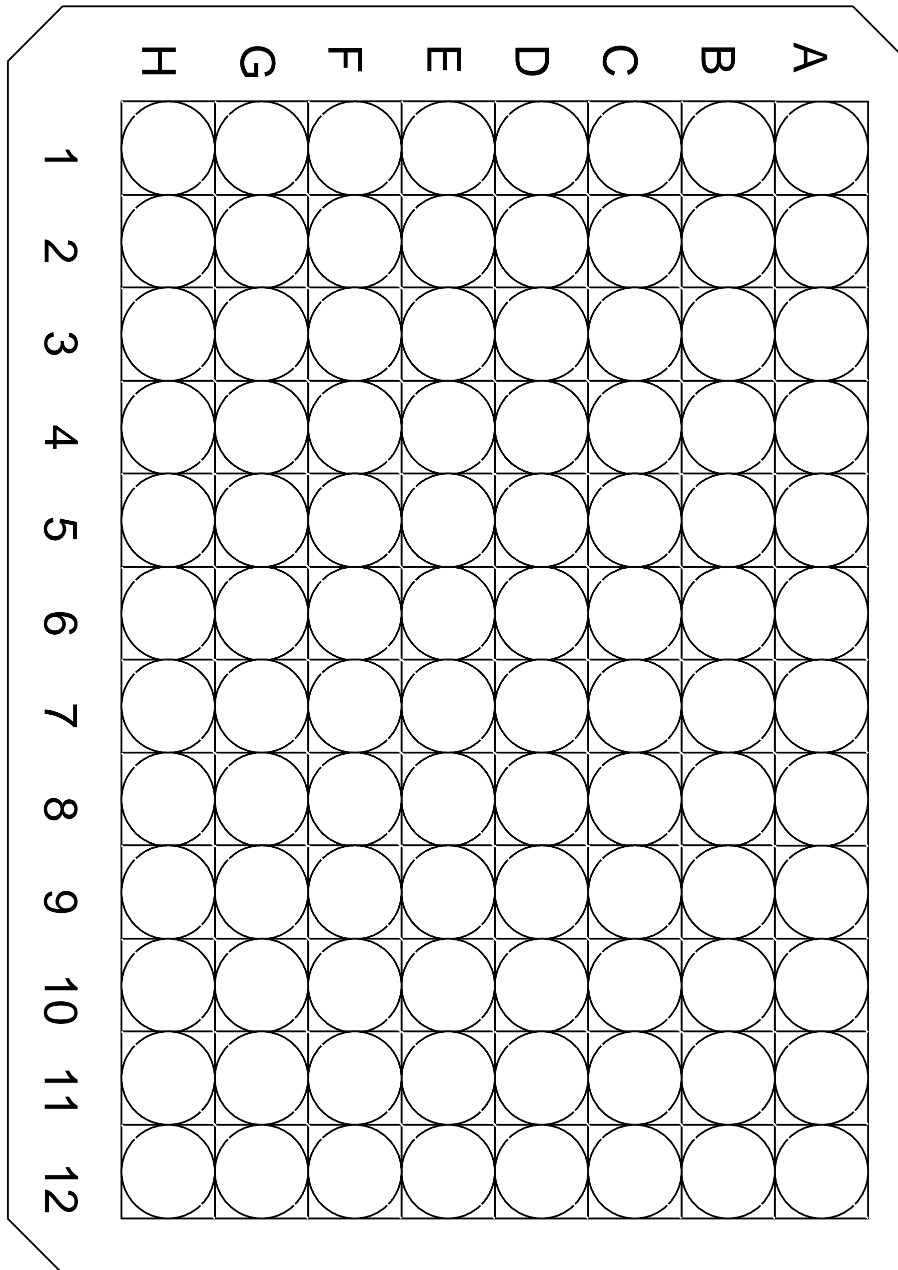
Complex I activity in cells from different origins differs greatly. Non-transformed fibroblasts have a higher activity than transformed cell lines such as HepG2 or HeLa cells. Consequently cell type and growth conditions are a large factor in Complex I activity measurement.

Approximately how much protein is yielded from my plate of cells?

We find the following typical yield of cells from a single confluent 177 cm^2 plate:

Human fibroblasts	$1x10^7$ cells	1.5mg total protein
Human HepG2	$2x10^7$ cells	3 mg total protein

It is recommended that you accurately determine from your first confluent plate the number of cells and the total protein yield.



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