Catalog #KHM2051

MitoProfile® Human Complex V Activity

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INTENDED USE

The Complex V (ATP Synthase) Activity microplate kit is used to determine the activity of this enzyme in human, rat, or bovine samples. Mouse samples are not appropriate for use. Other species have not been tested.

The activity microplate can be performed in conjunction with the Complex V Quantity kit (Invitrogen Cat. no. KHM2051). A multiplexing microplate for measuring activity and quantity is available as a separate kit (Invitrogen Cat. no. KHM2251).

INTRODUCTION

The ATP synthase complex (EC 3.6.3.14), also called Complex V or F_1F_0 ATPase, is responsible for ATP production in the oxidative phosphorylation process and can work in reverse as a proton pumping ATPase. The enzyme was thought to be localized exclusively in the mitochondria, but it has now also been identified on the plasma membrane of several cell types, including hepatocytes (where it acts as an HDL receptor), on endothelial cells (where it may act as an angiostatin receptor), and on the surface of cancer cells.

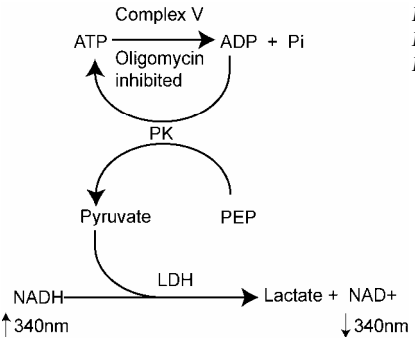
The mammalian ATP synthase is composed of 17 subunits. Five of the subunits make up the easily detached F_1 domain. The remainder subunits are part of two stalk domains and the proton pumping F_0 domain. Two of the F_0 subunits are encoded on mitochondrial (mt) DNA while all other subunits of the ATP synthase complex are nuclear encoded. Mutations in the mt-encoded subunits are implicated in OXPHOS disease.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

READ ENTIRE PROTOCOL BEFORE USE

PRINCIPLE OF THE METHOD

Complex V is immunocaptured within the wells. The enzyme functions by hydrolyzing ATP to ADP and phosphate. This production of ADP is ultimately coupled, as shown below, to the oxidation of NADH to NAD^+ which is monitored as a decrease in absorbance at 340 nm (this assay is fully described in Lotscher, DeJong, and Capaldi (1984) *Biochemistry* 23, 4134-40). The ATP hydrolysis activity and therefore the coupled reaction is inhibited by oligomycin (a specific inhibitor of Complex V). The overall reaction is as follows.



Pyruvate kinase (PK),
Lactate dehydrogenase (LDH),
Phosphoenolpyruvate (PEP)

This assay is designed for use with purified mitochondria. However, homogenized tissue and whole cells can also be used, but some sample optimization may be necessary. As described below, homogenized samples should be frozen, thawed, pelleted, and then 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 are listed below. The ranges may be extended by using a non-linear fit of the data from a normal sample.

Heart mitochondria	0.2-20 µg/well
Brain mitochondria	1-5 µg/well
Liver mitochondria	2-15 µg/well
Whole cultured cell extract	20-100 µg/well

Note: Ranges for tissue extract may vary slightly. The lowest amount indicated is the limit of detection. For sample loading use the recommended amount specified below.

REAGENTS PROVIDED

The 96-well microplate in this kit has a monoclonal antibody pre-bound to the wells. This plate can be broken into 12 separate 8-well strips; therefore the plate can be used for up to 12 separate experiments.

Item	Quantity
Buffer	10 mL
Detergent	1 mL
Lipid Mix	6 mL
Reagent Mix	20 mL
96-Well Plate (12 strips)	1

STORAGE

Store Buffer, Detergent, Lipid Mix and plate at 4°C - DO NOT FREEZE. Store Reagent Mix at -20°C or at -80°C for longer term storage.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Spectrophotometer measuring absorbance at 340 nm.
2. Method for determining protein concentration.
3. Deionized water.
4. Multichannel pipette.

PROTOCOL AND RECOMMENDED PROCEDURES

Note: This protocol contains detailed steps for measuring Complex V 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.

A. Sample Preparation

1. Prepare the buffer solution by adding *Buffer* (10 mL) to 190 mL deionized H_2O. Label this solution as Solution 1.
2. Freeze the homogenized sample (for homogenization see Notes section).
3. Once frozen, thaw the sample and pellet by centrifugation at ~16,000 rpm.
4. Resuspend the sample by adding 4 volumes of Solution 1. Determine the protein concentration by a standard method and then adjust the protein concentration to 5.5 mg/mL.

   Note: If the sample is less than 5.5 mg/mL, centrifuge to pellet again and take up in a smaller volume to concentrate the pellet and repeat protein concentration measurement. The optimal protein concentration for detergent extraction is 5.5 mg/mL.

5. 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 now 5 mg/mL.
6. Mix immediately and then incubate on ice for 30 minutes.
7. Spin in tabletop microfuge at maximum speed (~16,000 rpm) for 20 minutes. Carefully collect the supernatant and save as sample. Discard the pellet.

8. The microplate wells are designed for 50 µL sample volume, so dilute samples to the following recommended concentrations by adding Solution 1.

Sample Type	Recommended concentration
Heart mitochondria	1 µg/50 µL
Brain mitochondria	5 µg/50 µL
Liver mitochondria	10 µg/50 µL
Whole cultured cell extract	50 µg/50 µL

9. Keep diluted samples on ice until ready to proceed to Section B (Plate Loading).

B. Plate Loading

1. Add 50 µL of each diluted sample into individual wells on the plate. Include a normal sample as a positive control. Include a buffer control (50 µL Solution 1) as a null or background reference.
2. Incubate for 3 hours at room temperature.

C. Measurement

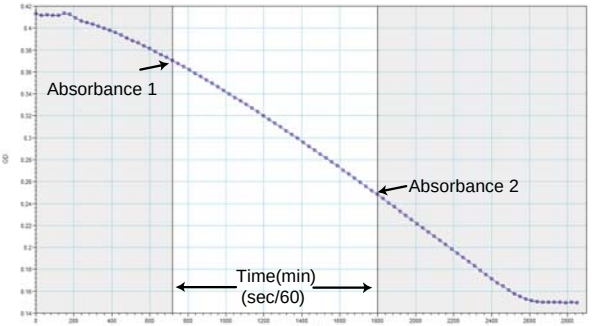
1. The bound monoclonal antibody has immobilized the enzyme in the wells. Empty the wells by turning the plate over and shaking out any remaining liquid.
2. Once emptied, add 300 µL of Solution 1 to each well used.

3. Empty the wells again and add another 300 µL of Solution 1 to each well used.
4. Empty the wells again and add 40 µL of *Lipid Mix* to all wells used.
5. Incubate the *Lipid Mix* in the wells at room temperature for 45 minutes.
6. DO NOT empty the wells. Instead add 200 µL of *Reagent Mix* into wells already containing 40 µL of *Lipid Mix*. Any bubbles in the wells should be popped with a fine needle as rapidly as possible.
7. Set the plate in the reader. Measure the absorbance of each well at 340 nm at 30°C. Using a kinetic program, take absorbance measurements for 60-120 minutes. The interval should be 1 minute between readings (though may be up to 5 minutes between readings).

SAMPLE DATA

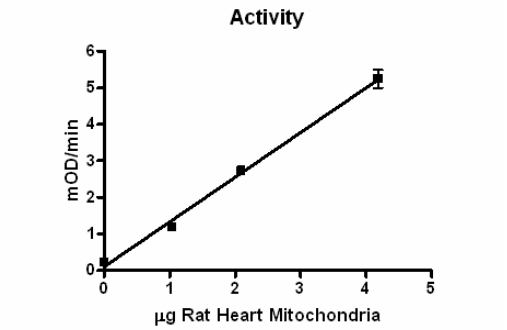
The activity of the ATP synthase enzyme is coupled to the molar conversion of NADH to NAD^+ measured as a decrease in absorbance at OD 340 nm. The activity rate is expressed as the change in absorbance at 340 nm/minute/amount of sample loaded into the well.

To do this, examine the rate of decrease in absorbance at 340 nm over time. This assay starts slowly and takes time to stabilize. The fastest, most linear rate of activity is most often seen between 12 and 30 minutes. This is shown below where the rate is calculated between these time points. Most microplate analysis software is capable of performing this function. Repeat this calculation for all samples measured.



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

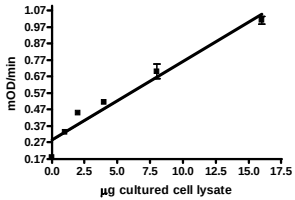
For the control or normal sample, the rate versus amount loaded can be plotted as a straight line in the linear region of the assay as shown below.

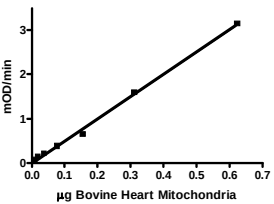


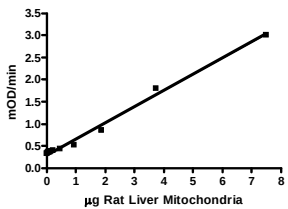
Compare the rates of the **control** (normal) sample and with the rate of the **null** (background) and with your **unknowns**, **experimental** or **treated** samples to get the relative ATP synthase activity.

Note: This assay rate should be 90% oligomycin sensitivity. If the sensitivity observed is significantly less than 90%, then sample preparation may need to be optimized to maintain the integrity of the oligomycin binding site.

Examples of activity/load relationships for other samples.







ASSAY SUMMARY

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

Prepare Sample (1-3 hours).

- Homogenize sample → Freeze → Thaw → Pellet.
- Bring up sample to 5.5 mg/mL in Solution 1.
- Perform detergent extraction with 1/10 volume Detergent followed by 16,000 rpm centrifugation for 20 minutes. Take supernatant.
- Adjust concentration to recommended dilution for plate loading.

Load Plate (3 hours).

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

Measure (2 hours).

- Rinse wells twice with Solution 1.
- Add 40 µL of Lipid Mix to wells.
- Add 200 µL Reagent Mix to the Lipid Mix in the wells.
- Measure OD_{340} at 1 minute intervals for 1-2 hours at 30°C.

A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12

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. It is recommended to use one of the Mitochondria Isolation Kits (Invitrogen Cat. no. KRM3011, KMR3021, KHM3031, KHM3041).

Sample Solubilization

Once completely homogenous, the sample must be frozen, thawed and pelleted as described above. This fractures the membranes and allows the removal of soluble non-membrane associated proteins. Once pelleted the sample should be resuspended in the supplied buffer. 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. 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 ATP synthase solubilization by the supplied detergent. The sample is incubated, centrifuged and supernatant (detergent extract) is collected.

Oligomycin sensitivity

When measuring activity and following the above steps precisely the ATP synthase F_1 and F_0 domains will be intact and coupled, maintaining the oligomycin binding site. The oligomycin sensitivity of ATP synthase bound in the wells should be approximately 90%. If significantly less oligomycin sensitivity is observed in a normal or control sample then sample solubilization and optimization must be performed: consider (i) multiple freeze-thaw cycles (ii) decreasing the amount of detergent from 1/10 volume to 1/15 or 1/20 volume.

REFERENCES

1. Lotscher, DeJong, and Capaldi (1984) Biochemistry 23: 4134-4140.

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