ab109716 –

ATP Synthase Specific Activity Microplate Assay Kit

Instructions for Use

For the quantitative measurement of ATP Synthase Specific activity in samples from Human, Rat and Cow

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

Table of Contents

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.

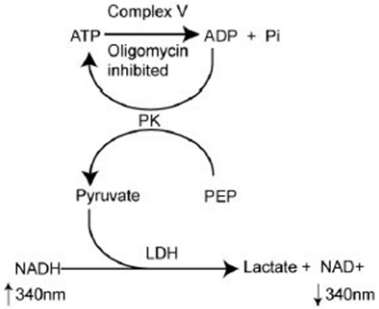
The ATP synthase complex (EC 3.6.3.14), also called Complex V or F1F0 ATPase, is responsible for ATP production in the oxidative phosphorylation process and can work in reverse as a proton pumping ATPase. The enzyme was thought to be localized exclusively in the mitochondria, but it has now also been 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.

ab109716 (MS543) measures the activity and quantity of this enzyme in a sample. First, ATP synthase is immunocaptured within the wells. The enzyme then 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. The ATP hydrolysis activity and therefore the coupled reaction is inhibited by oligomycin (a Complex V specific inhibitor).

The overall reaction is as follows:



PK- Pyruvate kinase, LDH-Lactate dehydrogenase, PEP-phosphoenolpyruvate

Subsequently, in the same well/s, the quantity of enzyme is measured by adding a Complex V specific antibody conjugated with alkaline phosphatase. This phosphatase changes the substrate from colorless to yellow (405 nm). This reaction takes place in a time dependant manner proportional to the amount of protein captured in the well.

Note: This protocol contains detailed steps for measuring ATP synthase 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. However, since the plate is modular, multiple experiments can be performed. To do this, prepare proportional amounts of buffers and solutions for the desired number of strips/wells.

Separate microplates are also available for measuring the activity of the enzyme only (ab109714/MS541). This duplexing microplate assay has been developed for use with human, rat, and bovine samples. Mouse samples are not appropriate for use. 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, 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 - 10 µg/well
Liver mitochondria	2 - 20 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.

2. Assay Summary

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.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Item	Quantity
Buffer (Tube 1)	15 ml
Detergent	1 ml
Reagent Mix	20 ml
Detection Antibody (Tube A)	1 ml
Lipid Mix	6 ml
96 – well microplate (12 strips)	1
2500X AP Label (Tube B)	0.012ml
AP Buffer (Tube 2)	20 ml
AP Development Reagent (Tube 3)	0.4 ml

4. Storage and Handling

Store Buffer, Detergent, Detector Antibody, 2500X AP Label, AP Buffer, and the microplate at 4°C. Store the Reagent Mix, AP Development Reagent and Lipid Mix at -20°C. For multiple experiments, the Reagent Mix and AP Development Reagent, and Lipid Mix may be aliquoted and stored at -80°C to minimize freeze-thaw cycles.

5. Additional Materials Required

- Spectrophotometer that measures absorbance at 340nm and 405nm.
- Method for determining protein concentration
- Deionized water
- Multichannel pipette

6. Preparation of Samples

Sample Preparation

1. Prepare the buffer solution by adding TUBE 1 (15 ml) to 285 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 5.0 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 - 5 µg/50 µl
Brain mitochondria	5 – 10 µg/50 µl
Liver mitochondria	10 -20 µg/50 µl
Whole cultured cell extract	50 – 100 µg/50 µl

9. Keep diluted samples on ice until ready to proceed.

7. Assay Method

A. Plate Loading

1. Add 50 µl of diluted sample to the appropriate wells. Include a buffer control (50 µl SOLUTION 1) as a null or background reference.

2. Incubate for 3 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 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).

The plate may be stored covered overnight at 4°C before proceeding to section C.

C. Addition of Detection Antibodies and Quantity Measurement

1. For an entire plate add 1 ml TUBE A to 5 ml of SOLUTION 1. Label this as SOLUTION A.

2. Empty the wells again by turning the plate over and shaking out the remaining liquid.

3. Add 50 µl of SOLUTION A to each well used.

4. Incubate SOLUTION A in the wells at room temperature for 1 hour.

5. Separately, for an entire plate add 2.4 µl of TUBE B (2500X AP Label) to 6 ml of SOLUTION 1. Label as SOLUTION B.

6. Empty the wells again by turning the plate over and shaking out the remaining liquid.

7. Once emptied, add 300 µl of SOLUTION 1 to each well used.

8. Empty the wells again and add another 300 µl of SOLUTION 1 to each well used.

9. Empty the wells again, then add 50 µl of SOLUTION B to each well used.

10. Incubate SOLUTION B in the wells at room temperature for 1 hour.

11. Empty the wells by turning the plate over and shaking out any remaining liquid.

12. Once emptied, add 300 µl of SOLUTION 1 to each well used.

13. Empty the wells again and add another 300 µl of SOLUTION 1 to each well used.

14. Add the contents of TUBE 3 (0.4 ml) or an aliquot of TUBE 3 (if only a part of the microplate is used) to the contents of TUBE 2 (20 ml) or proportionate lesser volume of TUBE 2 (if only part of the microplate is used). Label this as DEVELOPMENT SOLUTION.

15. Empty the wells again by turning the plate over and shaking out the remaining liquid.

16. Add 200 µl of DEVELOPMENT SOLUTION to each well used. Any bubbles in the wells should be popped with a fine needle as rapidly as possible

17. Set the plate in the reader. Measure the absorbance of each well at 405 nm at room temperature. Using a kinetic program, take absorbance measurements for 30 minutes. The interval should be 1 minutes between readings.

18. Save data and analyze as described in the "Data Analysis" section.

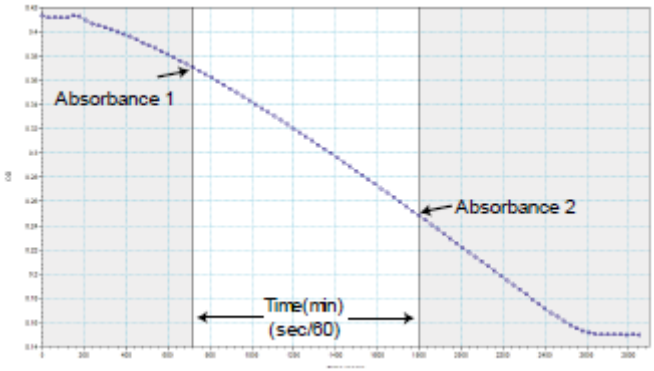
8. Data Analysis

Activity Data (from section B step 7)

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.



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

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 in Figure 1.

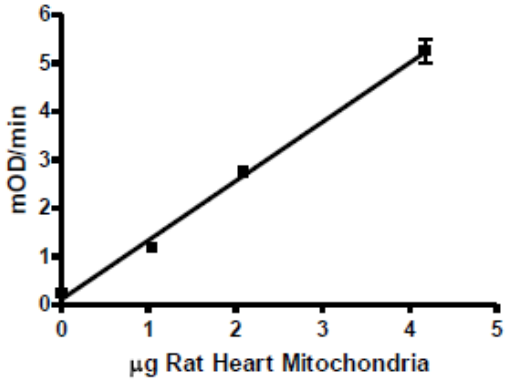


Figure 1. ATP Synthase in Rat Heart Mitochondria.

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 (see the Notes section).

Examples of activity/load relationships for other samples are shown in Figures 2, 3 and 4.

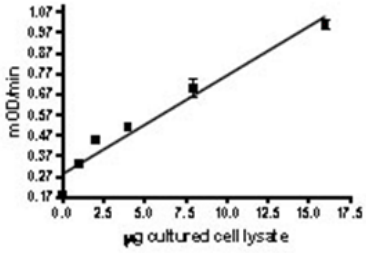


Figure 3. ATP Synthase in Culture Cell lysate

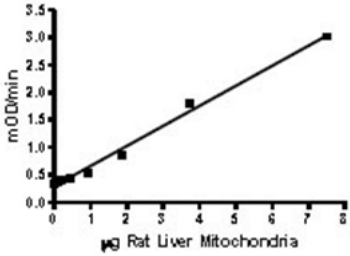


Figure 4. ATP Synthase in Rat Liver Mitochondria.

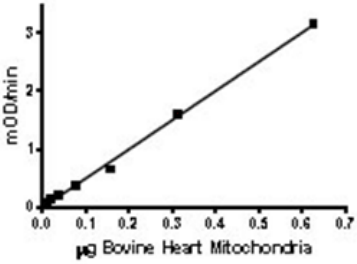
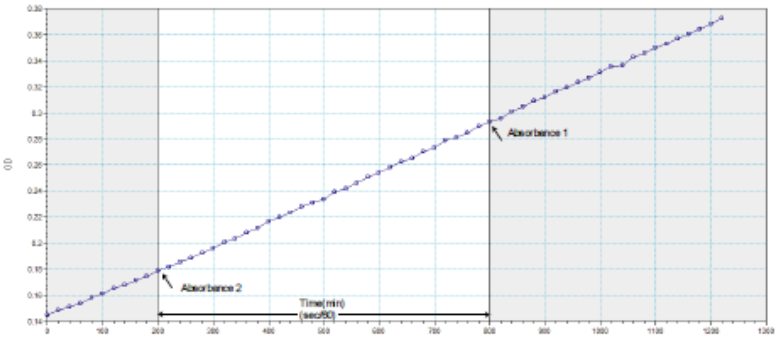


Figure 5. ATP Synthase in Bovine Heart Mitochondria.

Quantity Data (from section C step 18)

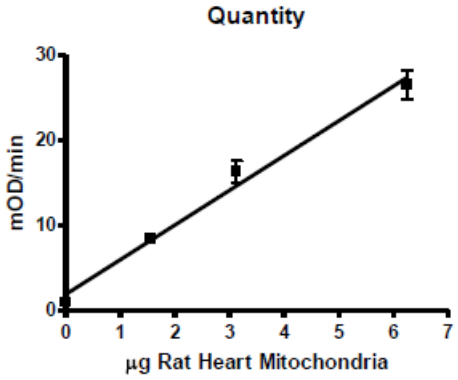
The quantity of ATP synthase captured in each well is proportional to the amount of alkaline phosphatase activity within each well. The quantity is the change in absorbance at 405 nm/minute/amount of sample loaded into the well.

Examine the linear rate of increase in absorbance at 405 nm with time. This is shown below where the rate is calculated between these time points. Most microplate software is capable of performing this function. Repeat this for all samples.



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

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.

Examples of quantity/load relationships for other samples:

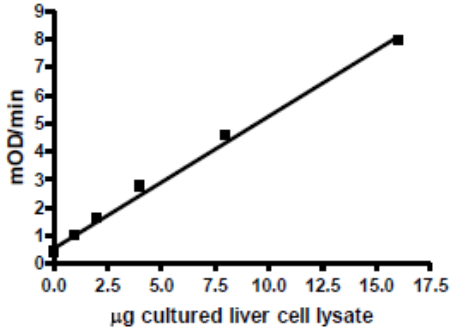


Figure 7. ATP Synthase in Liver Cell lysate

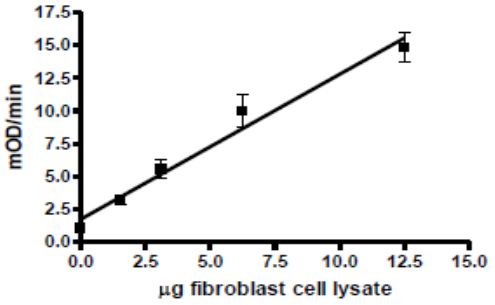


Figure 8. ATP Synthase in fibroblast cell lysate

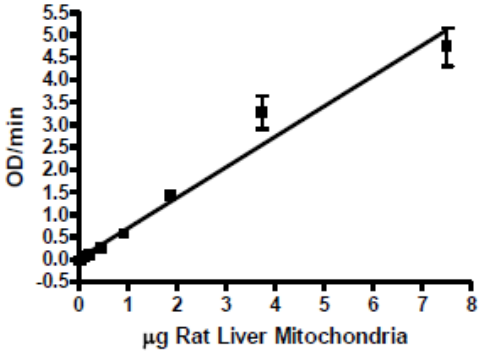


Figure 9. ATP Synthase in Rat liver mitochondria.

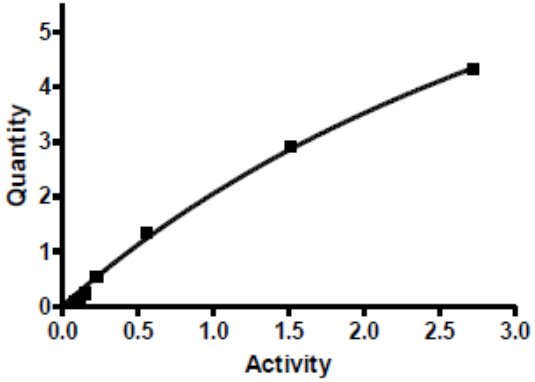
Specific Activity

By measuring both quantity and activity of ATP synthase in a sample, the ratio of the two parameters can be calculated. This is the relative specific activity and should be compared to the normal or control. The relative specific activity is an extremely useful valuable since three possibilities exist:

1) The relative activity is the same in the sample as in the normal (i.e. the amount of assembled ATP synthase has a proportional amount of activity).

2) The relative activity is lower as in the case of a catalytic point mutation, chemical inhibition or inhibitory modification.

3) The relative activity is higher which might occur if the activity if ATP synthase has been up-regulated perhaps by modification, such as phosphorylation/dephosphorylation, or as a result of uncoupling of the F1 and F0 domains.



9. Specificity

Species Reactivity: Human, rat, and bovine samples.

Mouse samples are not appropriate for use. Other species have not been tested.

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 after scraping cells from the plate in PBS. 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-ab11017171/MS850-MS853).

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. 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 F1 and F0 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 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.

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