CREATIVE BIOMART®
Assay Kit

ATP synthase Specific Activity Microplate Assay Kit

Product Information

Cat.No. Kit-0117

Product Overview

Rapid multiplexing microplate kit is used to determine both the activity and quantity of ATP synthase (Complex V) in a Human or Rat sample. The ratio of the two measurements represents the enzyme"s specific activity. The ATP synthase enzyme is immunocaptured within the wells of the microplate and the enzyme activity is measured by monitoring the decrease in absorbance at 340 nm. Specifically, the conversion of ATP to ADP by ATP synthase is coupled to the oxidative reaction of NADH to NAD+. The formation of NAD+ results in a decrease in absorbance at 340 nm. Subsequently, in these same wells, the quantity of ATP synthase is determined by adding an ATP synthase specific antibody conjugated with alkaline phosphatase. This phosphatase changes the substrate pNPP from colorless to yellow (OD 405 nm) in a time dependent manner proportional to the amount of protein captured in the wells. Included for optimal performance of the assay are buffer, detergent, antibody, substrate, lipid mix and a 96-well microplate pre-coated with monoclonal antibody.

Storage

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 freezethaw cycles.

Size

96 tests

Kit Components

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

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2500X AP Label (Tube B) 0.012ml
AP Buffer (Tube 2) 20 ml
AP Development Reagent (Tube 3) 0.4 ml

Materials Required but Not Supplied

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

Technical 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 Mitochondrial Isolation Kit.

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 aprotein 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.

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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.

Detection method Colorimetric

Compatible Sample Types

Cell culture supernatant, Cell culture extracts, Tissue, Tissue Extracts, Cell Lysate

Preparation

Sample Preparation
1. Prepare the buffer solution by adding TUBE 1 (15 ml) to 285 ml deionized H2O. 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

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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.

Assay Protocol

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.

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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.

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.

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Rate (mOD/min) = (Absorbance 1 – Absorbance 2)/ Time (min)
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).

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) = (Absorbance 1 – Absorbance 2)/ Time (min)
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.

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.