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ab109715 –

ATP Synthase Immunocapture Kit

Instructions for Use

For the isolation of ATP Synthase from small amounts of Human, Rat, Mouse and Bovine tissue

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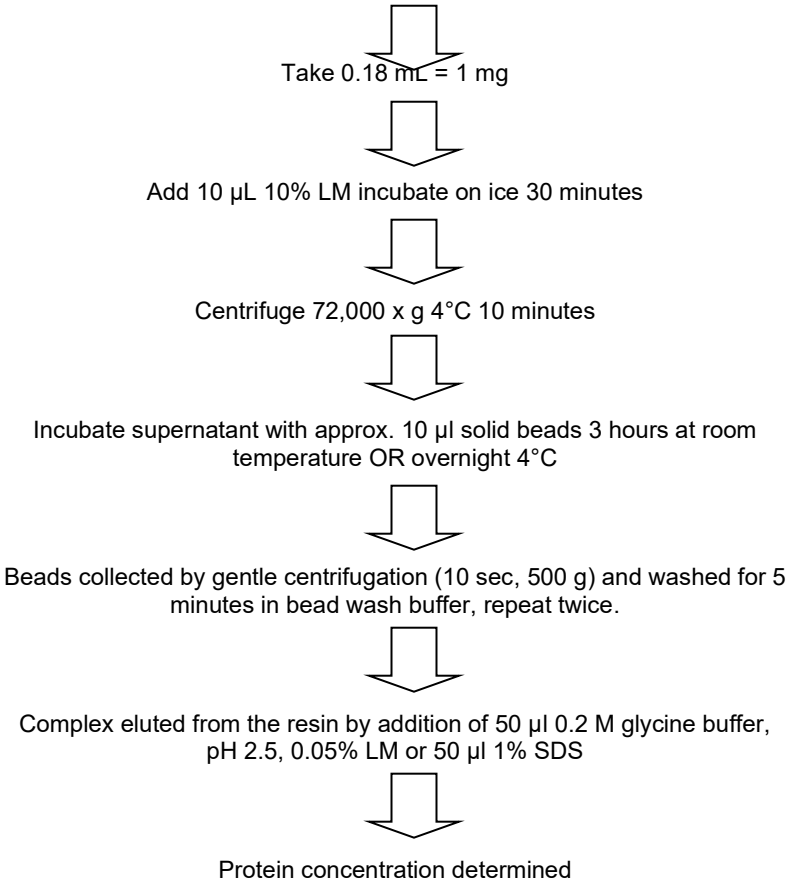
1. Introduction

ab109715 ATP Synthase Immunocapture Kit allows isolation of the ATP Synthase complex from small amounts of tissue. This facilitates subsequent analysis of assembly state and activity. Thus the enzyme retains olygomycin sensitive ATP hydrolysis activities after isolation. Finally, the extent of post translational modifications including oxidative damage can be readily analyzed by proteomic approaches or antibody detection of these modifications. Potential uses for ab109715 include but are not limited to examining alterations of ATP Synthase subunits in inherited mitochondrial diseases, Alzheimer's disease, angiogenesis, hypertension and aging.

Note: The immunocapture protocol for this kit requires Abcam detergent lauryl maltoside (ab109857/MS910).

2. Assay Summary

Resuspend mitochondria to 5.5 mg/mL in PBS, (The BHM control sample is also supplied at this concentration) Add 1X protease inhibitors



3. Kit Contents

- Immunocapture antibody coupled to agarose beads

4. Materials Required

- n-dodecyl-β-D-maltopyranoside (Lauryl maltoside, ab109857/ MS910)
- Phosphate buffered saline, PBS
- Elution buffer – Glycine, SDS or Urea elution buffer
- Protease inhibitor cocktail
- Double distilled water
- Laboratory benchtop microfuge
- Protein electrophoresis equipment
- Tube rotation equipment
- pH meter, weighing balance and other standard lab equipment

5. Storage and Handling

ab109715 may be stored at 4°C.

6. Preparation of Samples and Reagents

A. Sample Preparation:

When performing an immunocapture procedure, it is always recommended to isolate mitochondria from cells before immunoprecipitation. It is possible, however, to isolate complexes from whole tissue or cell extract, although this may

result in a weaker signal and/or additional bands resulting from non-specific cross-reactivity. Abcam immunocapture antibodies have been optimized to immunoprecipitate the OXPHOS and PDH complexes from a wide range species. However a minimal amount of starting mitochondria/cells is critical. Suggested minimum amounts of mitochondria as starting material are presented in Table 1 providing the user with the opportunity to generate the best quality product.

Sample	Minimal Starting Amount	Recommended Starting Amount
Heart mitochondria	100 µg	1-5 mg
Muscle mitochondria	200 µg	2-5 mg
Brain mitochondria	300 µg	5 mg
Cultured cell mitochondria	1 mg	5 mg

Cultured cell extract	6 mg	15 mg

Table 1. Suggested minimal starting amounts.

The total amount of OXPHOS complexes in mitochondrial samples varies greatly between species and tissue types. Therefore it is highly recommended that during the experimental planning steps an estimation is made of the total amount of the complex in the user's sample. In this way the appropriate detection strategy can then be employed. Table 2 suggests detection strategies based upon anticipated yield of immunocaptured product.

Yield of Complex	**Detection Strategy**
1 µg +	Gel staining with Coomassie
10 ng +	Gel staining with silver/sypro ruby
1 ng +	Western blotting
Any	Mass spectrometry

Table 2. Suggested detection methods by yield of product.

B. Preparation of Reagents:

Phosphate buffered saline solution (PBS)
1.4 mM KH_2PO_4
8 mM Na_2HPO_4
140 mM NaCl
2.7 mM KCl, pH 7.3

Lauryl maltoside stock
200 mM n-dodecyl-β-D-maltopyranoside (10% w/v lauryl maltoside)

1000 x Protease inhibitor stocks
1 M phenylmethanesulfonyl fluoride (PMSF) in acetone
1 mg/ml leupeptin
1 mg/ml pepstatin

Bead wash buffer
1x PBS
1 mM n-dodecyl-β-D maltopyranoside (0.05% w/v lauryl maltoside)

Glycine elution buffer
0.2 M Glycine. HCl pH 2.5
1 mM n-dodecyl-β-D-maltopyranoside (0.05% w/v lauryl maltoside)

SDS elution buffer
1% sodium dodecyl sulfate (SDS)

Urea elution buffer
4 M Urea. HCl pH 7.5

2x SDS page sample buffer
20% glycerol
4% SDS
100 mM Tris pH 6.8
0.002% Bromophenol blue
optional – 100 mM dithiothreitol

7. Assay Method

A. Sample Solubilization: The sample should be solubilized in a non-ionic detergent. It has been determined that at a protein concentration of 5 mg/ml mitochondria are completely solubilized by 20 mM n-dodecyl-β-D-maltopyranoside (1% w/v lauryl maltoside). The key to this solubilization process is that the membranes are disrupted while the previously membrane embedded multisubunit OXPHOS complexes remain intact, a step necessary for the antibody based purification procedure described below.*

1. To a mitochondrial membrane suspension at 5.5 mg/mL protein in PBS add 1/20 volume of 10% lauryl maltoside (final concentration of 0.5%).

2. Mix well and incubate on ice for 30 minutes.

3. Centrifuge at 72,000 g for 30 minutes. At a minimum, a benchtop microfuge on maximum speed, usually around 16,000 g should suffice.

4. Collect the supernatant and discard the pellet.

5. Note: Samples rich in mitochondria such as heart mitochondria, the cytochromes in Complexes II and III should give this supernatant a brown coloration.

6. Add a protease inhibitor cocktail to the mixture and keep the sample on ice until immunoprecipitation is performed.

Note: One important exception to this solubilization method is the pyruvate dehydrogenase enzyme. In order to isolate PDH at a protein concentration of 5 mg/ml mitochondria the required detergent concentration is only 10 mM (0.5%) lauryl maltoside.

The PDH enzyme should also be centrifuged at lower speed, a centrifugal force of 16,000 g is maximum for the PDH complex.

B. Immunoprecipitation: Abcam's immunocapture beads are agarose beads irreversibly cross-linked to highly specific monoclonal antibodies, which are capable of binding and retaining specific OXPHOS complexes or the PDH complex.

The loaded beads are completely saturated with antibody to ensure the maximum amount of antibody per bead volume and hence the maximum amount of immunoprecipitated product.

The smallest working amount of beads is around 5 µl of solid beads in a small microtube (e.g. a 500 µl tube). It is not practical to use a volume smaller than this for immunocapture procedure, therefore it is recommended *that when wishing to use less beads researchers dilute the concentrated Abcam's beads with unloaded agarose beads, giving a workable volume of beads. The yield from such diluted beads will of*

course be less. Plain beads for dilution are available from Abcam.

The Antibody used in this kit is available as a standalone item (ab109867).

1. Add the desired amount of antibody loaded agarose beads (i.e. at least 5 µl of Slurry) to the appropriate amount of solubilized mitochondrial supernatant.

2. Allow this mixture to mix for at least 3 hours at room temperature or overnight at 4°C. Mixing should be done on a nutator, or by turning in a tube.

C. **Elution:** After the mixing step is complete, collect the beads by centrifugation for 1 minute at a slow speed (i.e. 1,000-3,000g or the slowest setting on most benchtop microfuges).

1. Remove the supernatant from above the beads and discard. The complex of interest should now be specifically bound to the antibody coating the beads.

2. Wash the beads to remove any non-specifically bound proteins prior to elution by adding 100 volumes of wash buffer to the beads.

3. Gently mix for 5 minutes. Collect the beads by gentle centrifugation as performed in Step 1.

4. Remove the wash buffer from above the beads only and discard. Repeat bead washing process twice.

5. In the final step, all wash buffer is removed from above the beads. The complex is now ready for elution from beads collected in the bottom of the tube.

Three options are available for elution of the bound complex from the beads. The chosen elution procedure should be performed on the beads three times. This ensures complete removal of target from the beads. The three samples should be collected and analyzed, samples containing the most protein should then be pooled. Possible elution methods are:

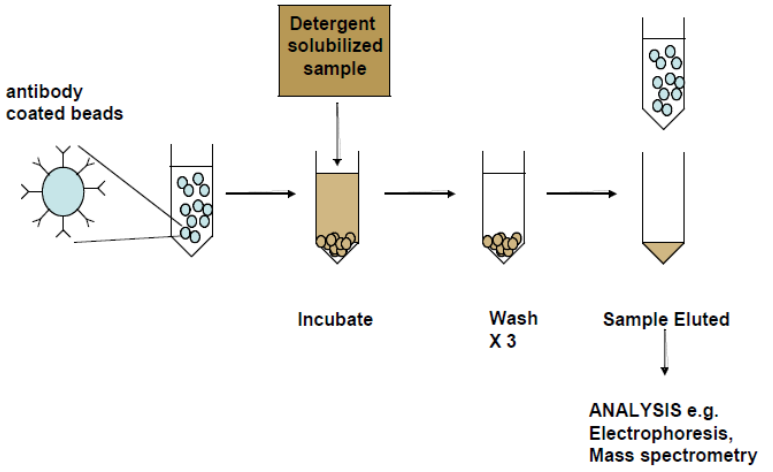
- **Glycine buffer elution** – the complex is eluted from the beads by acidification. Beads are resuspended in 2-5 volumes of glycine elution buffer (pH 2.0). This mixture is

incubated for 10 minutes with frequent agitation before gentle centrifugation. The purified complexes have now been released into the supernatant which should be collected from *above* the beads *only*. It is advised to repeat this process with fresh glycine buffer each time at least twice more to ensure that all of the captured complex has been released from the beads. This method is ADVANTAGEOUS because the beads may be reusable after removal of glycine elution buffer by washing the beads as described above. However the eluted sample has an acidic pH which may need to be neutralized before analysis.

- **SDS buffer elution** – the complex is eluted from the beads by the denaturant SDS. Beads are resuspended in 2-5 volumes of SDS elution buffer. This mixture is incubated for 10 minutes with frequent agitation before gentle centrifugation. The purified complexes have now been released into the supernatant which should be collected from *above* the beads *only*. It is advised to repeat this process at least one more time to ensure all of the captured complex has been released from the beads. This method is ADVANTAGEOUS because the extraction method is highly efficient and therefore the sample is more concentrated. However the antibody is denatured by the SDS and is no longer viable and should be discarded.

- **Urea buffer elution** - the complex is eluted from the beads by the chaotrope urea. Beads are resuspended in 2-5 volumes of urea elution buffer. This mixture is incubated for 10 minutes with frequent agitation before gentle centrifugation. The purified complexes have now been released into the supernatant which should be collected from *above* the beads *only*. It is advised to repeat this process at least twice more to ensure that all of the captured complex has been released from the beads.

  This is ADVANTAGEOUS because the sample may be digested by proteolytic enzymes for mass spectrometry. However the sample should be diluted at least four fold in order to reduce the urea concentration to levels permitting proteolysis by trypsin.



8. Sample Analysis

Samples eluted by any of the three methods can now be resolved by electrophoresis. Resolved proteins should be detected by the method chosen in Table II.

No antibody should be present in the sample once eluted from the beads; therefore prior to loading, the sample can be reduced by a reducing agent such as 50 mM DTT or 1% β-mercaptoethanol. Another optional step is heating of the sample which can be done at 95°C for 5 minutes or at 37°C for 30 minutes prior to loading onto the gel. *These steps may increase the resolution of protein bands and also reduce the complexity of the sample by breaking any disulfide bonded proteins.*

9. Specificity

Species Reactivity: Human, rat, mouse, bovine

10. Optimization Steps and General Tips

Using agarose bead slurries: Since the beads are a solid material they cannot be pipetted directly. Instead they are provided in a much larger volume of liquid (usually 40 volumes of PBS). Pipetting this solution up and down mixes the beads into a slurry allowing their transfer to the experimental tube. Since bead losses can occur on the inside of the *pipette tip* this is the only time in this protocol the beads are pipetted directly. All other steps in this protocol require sample mixing by rotation or mixing by in a large volume of buffer. Therefore the beads should not be mixed by pipetting to avoid losses. Also, avoid vigorous mixing during the elution step when the liquid volume is low since beads might become deposited around the inside tube unexposed to elution buffer leading to losses. Instead gently tap the tube to agitate the beads within the small volume of sample elution buffer.

Samples eluted by Glycine buffer: Sample eluted by glycine buffer are low in pH. In most cases it is necessary to remove this acidity with a strong buffer. For example, adding SDS-PAGE loading buffer directly to a glycine eluted sample will turn this sample from blue to a

yellow as the bromophenol blue acts as a pH indicator. Therefore the sample should be neutralized by the slow addition a 1 M Tris.HCl pH 7.5 in microliter amounts until blue color is restored.

Sample concentration: When analyzing immunocaptured products by SDS-PAGE, it is recommended to load as much sample as possible on the gel. Samples may also be supplemented with fresh reducing agent such as dithiothreitol or β-mercaptoethanol. Heating samples at 95°C for 5 minutes or at 37°C for 30 minutes is also optional.

Blot development: When analyzing immunocaptured products by Western blotting choose an appropriate method for blot development. The alkaline phosphatase (NBT/BCIP) and horseradish peroxidase (ECL) methods are recommended.

11. Troubleshooting

Problem	Solution
Antibody contamination	• When eluting pipette only the liquid sample from above the beads. This sample can be spun again and aspirated to ensure that it is free of beads • Electrophoresis - Do not include reducing agent in sample during electrophoresis, this should maintain antibody at 200 kDa during electrophoresis
Sample turns yellow with loading buffer addition	• Neutralize the acidity with Tris Base
Large CV	• Check pipettes
Weak or no signal	• Increase the bead amount • Isolate mitochondria from the sample • Increase the amount of sample • Increase sample/bead incubation time
Non-specific bands	• Isolate mitochondria to higher purity • Add a reducing agent to the eluted sample e.g. DTT • Heat the eluted sample at 95°C for 5 min before loading

Technical Support

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