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🌐 Preparation of soluble and insoluble mitochondrial protein fractions for mass spectrometry analysis

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We use this protocol and it's working

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Abstract



Preparation of soluble and insoluble mitochondrial protein fractions from HeLa cells for mass spectrometry analysis.





Day 1

25m





- 1 Thaw mitochondrial stocks on ice, and aliquot out one tube of  60 μg of mitochondria for each sample.
- 2 Centrifuge samples at  10000 rcf for  00:10:00 at  4 °C , and carefully aspirate the supernatant. 10m
- 3 Add  60 μL of ice cold 0.5% TX-100 in PBS, vortex each sample for ~5 seconds, and leave samples to incubate on ice for  00:15:00 . At the conclusion of the  00:15:00 lysis incubation, vortex each sample for ~5 sec again. 30m
- 4 Centrifuge the samples at  12000 rcf for  00:10:00 at  4 °C . 10m
- 5 Using a pipette, carefully remove  57 μL of the supernatant (representing 95% of the lysis volume) and place this sample into a clean microfuge tube on ice, being sure not to disturb any pellets that have formed on the bottom of the tube. The volume removed represents the 'soluble' protein fraction. The tube containing the pellet represents the 'insoluble' protein fraction.
- 6 Add  57 μL of 0.5% TX-100 in PBS to each insoluble protein fraction by pipetting on the side of the tube. Gently flick each tube to rinse the sides of the tube.
- 7 Centrifuge the insoluble protein fractions at  12000 rcf for  00:10:00 at  4 °C . 10m
- 8 Using a pipette, carefully remove  57 μL of the supernatant from the insoluble protein fraction, and place into a clean microfuge tube (which will function as the waste collection tube for all samples).
- 9 Repeat steps 6 – 8, which will total 2 washes
- 10 Repeat step 6. You should now have two tubes for each sample (soluble and insoluble protein fractions), and one waste tube.

11 Thaw aliquots of recombinant Ag85A on ice, and add  180 ng of Ag85A to each insoluble protein fraction, and  171 ng of Ag85A to each soluble fraction.

12 Equilibrate all samples to room temperature and then add 2x SDS solubilization buffer to a final concentration of 1x (2x: 10% w/v SDS, 200 mM HEPES pH 8.5). Vortex each samples for ~ 5 seconds to mix.

13 Sonicate all samples in a waterbath sonicator set to  21 °C for  00:10:00 .

10m


14 Add TCEP to a final concentration of  10 millimolar (mM) and chloroacetamide to a final concentration of  40 millimolar (mM) to each sample, vortex each sample for ~5 seconds to mix, and incubate samples at  37 °C for  00:45:00 (standing).

45m





Note

Make sure chloroacetamide is made up fresh from powder

15 Acidify each sample by adding phosphoric acid to a final concentration of 1.2%/sample (ensure pH <4. More phosphoric acid can be added if the pH is not low enough at 1.2%/sample)





16 Add binding buffer ( 100 millimolar (mM) triethylammonium bicarbonate, 90% v/v methanol pH 7.1 with phosphoric acid) to each sample at a ratio of 1:7, sample volume to binding buffer.

17 Vortex each sample for ~5 sec to mix




18 Load  400 µL of each sample into a S-Trap Mini column, and centrifuge at  6500 rcf at  Room temperature for  00:00:30 . Discard the flow through.

30s

19 Repeat step 18 until the full volume of each sample has been loaded on its column.












20 Wash each column by adding  400 µL of binding buffer, centrifuging at  6500 rcf at  Room temperature for  00:00:30 , and discarding the flow through.

30s

- 21 Repeat step 20 three times, for a total of 4 washes.
- 22 Move the columns to a 1.5 mL LoBind microfuge tube (Eppendorf) and add  125 μL of digestion buffer directly to the column filter (digestion buffer: [M] 50 millimolar (mM) triethylammonium bicarbonate supplemented with sequencing grade trypsin at a concentration of 1 μg trypsin to 50 μg of starting protein (which will differ between soluble and insoluble samples)).
- 23 Centrifuge samples at 1000x rcf for  00:00:30 at room temperature, and pipette the digestion buffer flow through directly back onto the column filter. Move the columns to clean 2.0 mL LoBind microfuge tubes. 30s
- 24 Seal each sample to the LoBind microfuge tube with parafilm, and incubate samples overnight for at least  16:00:00 at 37 deg C (static incubation) 16h

Day 2

3m

- 25 Remove the parafilm and add  80 μL of digestion buffer without trypsin (50 mM triethylammonium bicarbonate) to each sample. Centrifuge samples at  3200 rcf for  00:01:00 at  Room temperature. Leave the flow through in the collection tube. 1m
- 26 Add  80 μL of 0.2% v/v formic acid to each sample, and centrifuge at  3200 rcf for  00:01:00 at room temperature. Leave the flow through in the collection tube. 1m
- 27 Add  80 μL of 50% v/v acetonitrile/0.2% v/v formic acid to each sample, and centrifuge at  6500 rcf for  00:01:00. 1m
- 28 Remove and discard the S-Trap columns from each sample.
- 29 Lyophilise the total eluate from each sample, seal the samples with parafilm and store at  -80 °C until needed for downstream processing.