AUG 09, 2023

DOI:
dx.doi.org/10.17504/protocols.io.kxygx394kg8j/v1

Protocol Citation: Louise Uoselis 2023. Preparation of soluble and insoluble mitochondrial protein fractions for mass spectrometry analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx394kg8j/v1

Protocol status: Working
We use this protocol and it's working

Created: Aug 09, 2023

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

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ABSTRACT

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

Last Modified: Aug 09, 2023

PROTOCOL integer ID: 86208

Keywords: ASAPCRN

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 [M] 10 millimolar (mM) and chloroacetamide to a final concentration of [M] 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 ([M] 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 ug trypsin to 50 ug 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.