

Mitochondrial enrichment followed by Affinity Purification couple to Mass Spectrometry (AP-MS)

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Protocol description

Affinity purification mass spectrometry (AP-MS) is a high-throughput method for the isolation and identification of specific target protein interaction partners. It consists of the selective extraction of multiprotein complexes directly from cell lysates. All protein components of these complexes are then trypsin-digested and identified by nLC-MS/MS. The final output is a protein-protein interaction (PPI) data set.

Due to their dual membrane composition and high hydrophobicity, mitochondrial SLCs are not ideal candidates for the mild lysis condition we standardly used in RESOLUTE WP2 AP-MS. We, therefore, use an adapted protocol (modified from Frezza et al.) for the study and definition of protein-protein interaction network of mitochondrial SLCs. This protocol includes cell lysis and the isolation of an enrich mitochondrial fractions prior to affinity purification.

Materials

Biological materials:	<ul style="list-style-type: none"> ▪ Co-Isogenic Resolute Cell lines: Jump In™ T-REx™ HEK 293 (Invitrogen™ A15008) over-expressing the bait protein, a codon optimized SLC fused to HA-Twin-streep at C or N-terminus. ▪ SH-quant : Isotopic labeled peptide : [NH₂]AADITS(L)YK[COOH] (L): Heavy Leu (13C15N) (+15) (Wepf et al., 2009)
Reagents:	<ul style="list-style-type: none"> ▪ Dulbecco's Modified Eagle's Medium - high glucose (DMEM) Product: D5796-500mL Manufacturer: SIGMA ▪ Fetal Bovine Serum (FBS) Product: S1810-5000 Manufacturer: Biowest ▪ Penicillin-Streptomycin (10,000 U/mL) Product: 15140-122 Manufacturer: Gibco ▪ Blasticidin HCl Product: ant-bl-5b Manufacturer: InvivoGen ▪ G418 disulfate salt (Geneticin) Product: A1720-5G Manufacturer: Sigma-Aldrich ▪ Doxycycline hyclate Product: D9891-1G Manufacturer: Sigma Aldrich ▪ 1X Dulbeccos PBS without Ca & Mg 1x500mls (Sigma) Product: D8537-500ML

	<p>Manufacturer: Sigma</p> <ul style="list-style-type: none"> ▪ Sucrose Product: 1.07654.1000 Manufacturer: Merk ▪ 4-Morpholinepropanesulfonic acid (MOPs) Product: AM9570 Manufacturer: Ambion ▪ HEPES: Product: 9105.3 Manufacturer: Carl Roth ▪ NaCl: Product: s7653-5K Manufacturer: Sigma-Aldrich ▪ Ethylenediaminetetraacetic acid (EDTA) Product: E6758-500G Manufacturer: Sigma-Aldrich ▪ Digitonin: Product: D141-500MG Manufacturer: Sigma ▪ Protease inhibitor cComplete Product: 0505648900 Manufacturer: Roche ▪ PMSF Product: A0999.0005 Manufacturer: BioChemica ▪ AC-Leu-Leu-argininal x 1/2 H₂SO₄ Product: 11 034 626 001 Manufacturer: Roche ▪ Aprotinin from bovine lung Product: 10 981 532 001 Manufacturer: Roche ▪ Trypsin inhibitor from soybean Product: 10 109 886 001 Manufacturer: Roche ▪ Avidin
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	<p>Product: 2-0204-015 Manufacturer: IBA</p> <ul style="list-style-type: none"> ▪ Phosphatase inhibitor cocktail 2 Product: P5726-5ML Manufacturer: Sigma ▪ Phosphatase inhibitor cocktail 3 Product: P0044-5ML Manufacturer: Sigma ▪ Sodium lauryl sulfate solution 10% (SDS) Product: 71736-500ml Manufacturer: Merk ▪ StrepTactin Sepharose (50% suspension) Product: 2-1201-010 Manufacturer: IBA ▪ Bio-Rad Protein Assay Dye Reagent Concentrate Product: 5000006 Manufacturer: Bio-Rad ▪ Protein standard I Product: 5000005 Manufacturer: Bio-Rad ▪ DTT (DL-Dithiothreitol), for molecular biology, minimum 99% titration Product: D9779-5G Manufacturer: Sigma-Aldrich ▪ Iodacetamide (IAA), SigmaUltra Product: I1149-5G Manufacturer: Sigma-Aldrich ▪ Water, for HPLC LC-MS Grade Product: 83645.320 Manufacturer: VWR ▪ SpeedBeads™ magnetic carboxylate modified particles Product: 45152105050250 Manufacturer: GE Healthcare ▪ SpeedBeads™ magnetic carboxylate modified particles Product: 65152105050250 Manufacturer: GE Healthcare
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	<ul style="list-style-type: none"> ▪ Acetonitril for HPLC LC-MS Grade Product: 83640.32 Manufacturer: VWR ▪ Ethanol absolute Product: 1.00983.1000 Manufacturer: MERCK ▪ Trizma® base Primary Standard and Buffer, ≥99.9% (titration), crystalline Product: T1503 Manufacturer: Sigma-Aldrich ▪ Empore™ SPE Disks matrix active group C18, diam. 47 mm, pk of 20 Product: 66883-U Manufacturer: Sigma-Aldrich ▪ Trypsin/Lys-C Mix, Mass spec Grade Product: V5073 Manufacturer: Promega ▪ TFA Uvasol (trifluoroacetic acid) Product: 1.08262.0100 Manufacturer: MERCK
Equipment:	<ul style="list-style-type: none"> ▪ Mini Bio-Spin™ Chromatography Columns. Product: 732-6207 Manufacturer: Bio-Rad ▪ Magnetic rack ▪ Speed back ▪ Vials with insert ▪ Caps for vials

Reagents setup:

○ Stocks:

- **750 mM HEPES, pH8** (100 ml): Dissolve 17.87 g of HEPES in 50 mL of H₂O, adjust to pH 8 by adding 10N NaOH. Add H₂O until volume is 100 ml. Store at 4 °C.
- **5M NaCl** (250mL): Dissolve 73.05 g of NaCl in 200mL of H₂O, allow to dissolve with stirring. Then add H₂O until volume is 250 mL. Store at room temperature.
- **0.5 M EDTA, pH8** (250 mL): Add 36.53 g of EDTA to 150 mL of H₂O. Dissolve the EDTA by adjusting the pH to 8 (adding 10N NaOH under continuous stirring). Add H₂O until the total volume reaches 250 mL. Store buffer at room temperature.

- **5% w/v Digitonin in water** (10 mL). Dissolve 0.5 g of digitonin in 10 mL of H₂O at 95 – 98 °C. Mix until detergent is dissolve – solution will become transparent- under continuous stirring. Cold to RT before use it. Digitonin will be stable in H₂O at RT for at least 24 h. Precipitate digitonin can be re-dissolve by heating. Aliquots of dissolved digitonin can be stored at -20 °C.
- **100 mM PMSF**: Add 17.4 mg of PMSF per mL of Isopropanol. Aliquot in 2mL aliquot and store at -20 °C
- **Avidin 25 µg/µl** (25000x). Aliquot in 20µl aliquot and store at -20 °C.
- **Protease Inhibitor Cocktail** (1000x): 1µg/mL Leupeptin, 1µg/mL Aprotinin, 10 µg/mL Trypsin inhibitor in H₂O.
- **0.1 M Tris-MOPS, pH 7.4** (50 mL): Dissolve 0.605 g of Trizma® base in 25 ml of water, adjust pH to 7.4 by adding MOPS powder). Add H₂O until the total volume reaches 50 mL. Store at 4 °C
- **0.1M EDTA-Tris, pH 7.4** (50 mL): Dissolve 1.46 g of EDTA in 25 ml of water, adjust pH to 7.4 by adding Trizma® base powder. Add H₂O until the total volume reaches 50 mL. Store at 4 °C
- **1M Sucrose** (50 mL): Dissolve 17.11 g Sucrose in a final volumn of 50 ml of H₂O. Store at 4 °C
- **Mito-enrich-buffer:**
 - Composition: 10 mM Tris-MOPS pH 7.4, 1 mM EDTA-TRIS pH 7.4, 200 mM Sucrose. 1x Complet™ inhibitor cocktail.
 - For 50 mL: Mix 5 mL of 0.1 M Tris-MOPS pH 7.4, 0.5 mL of 0.1 M EDTA-TRIS pH 7.4, 10 ml of 1 M Sucrose, 1 tablet of Complet™ inhibitor cocktail and 34.5 mL of H₂O.
 - Prepare fresh and place on ice before starting the affinity purification experiment.
- **Mito-AP-Buffer I:**
 - Composition: 50 mM HEPES pH 8.0, 150 Mm NaCl, 5 mM EDTA, 1% Digitonin, 1 mM PMSF, 1x Proteinase inhibitor cocktail.
 - For 100 mL: mix 6.7 mL of 750 mM HEPES pH8, 3 mL of 5 M NaCl, 1 mL of 0.5 M EDTA, pH 8, 20 mL of 5% w/v digitonin, 1 mL of 100 mM PMSF, 0.1 mL of protease Inhibitors (1000x) and 83.24 mL of H₂O.
 - Prepare fresh and place on ice before starting the affinity purification experiment.
- **Mito-Lysis buffer:**
 - Composition: 50 mM HEPES pH 8.0, 150 Mm NaCl, 5 mM EDTA, 1% Digitonin, 1 mM PMSF, 1x Proteinase inhibitors, avidin (1x), Phosphatase inhibitor cocktail 2 (1x), phosphatase inhibitor cocktail 3 (1x).
 - For 50 mL: mix 49 mL of AP-Buffer I, 2 µl of avidin 25 µg/µl avidin stock, 500 µl of Phosphatase inhibitor cocktail 2 and 50 mL of phosphatase inhibitor cocktail 3.
 - Prepare fresh and place on ice before starting the affinity purification experiment.
- **AP-Buffer II:**

- Composition: 50 mM HEPES pH 8.0, 150 mM NaCl, 5 mM EDTA.
 - For 50 mL: mix 3.3 mL of 750 mM HEPES pH8, 1.5 mL of 5 M NaCl, 0.5 mL of 0.5 M EDTA, pH 8, and 44.7 mL of H₂O.
 - Prepare fresh and cool it down in ice before to start AP experiment
- **AP peptide elution buffer:**
 - Composition: 2% v/v SDS in buffer II.
 - For 2 mL: Mix 0.4 mL of 10% SDS and 1.6 mL of AP-buffer II
 - Prepare fresh before to starting Affinity purification experiment. Keep at room temperature to avoid SDS precipitation.
- **500 mM DTT** (5 mL): Dissolve 0.385 g of DTT in 5 mL of H₂O. Aliquot in 200 µL aliquot and store at -20°C
 - **1 M Iodoacetamide (IAA)** (100 µl): Dissolve 0.018 g of IAA in 100 µl of H₂O and vortex vigorously. Prepare fresh before use it and protect from the light.
 - **70% v/v EtOH in water** (100 mL): Mix 70 mL of ethanol with 30 mL of H₂O . Store at room temperature.
 - **50 mM Tris-HCl, pH8:** (200 mL): Add 1.2114 g of Trizma® base to 100 mL of H₂O. Adjust to pH 8 by adding HCl. Then add H₂O until volume is 200 mL. Store at 4 °C.
 - **30% TFA in water** (50 mL): Mix 15 mL of TFA with 35 mL of H₂O. Store at 4 °C.
 - **1% TFA in water** (50 mL): Mix 166 µl of 30% TFA solution with 49.8 mL of H₂O. Store at 4 °C.
 - **Stage tip solvent:**
 - Composition: 0.4% Formic Acid, 2% TFA.
 - For 100 mL: Mix 97.6 mL H₂O, 2 mL TFA and 400 µl FA. Store at 4 °C.
 - **Peptide elution solvent:**
 - Composition: 60% Acetonitrile 0.4% TFA
 - For 100 mL: Mix 60 mL acetonitrile, 400 µl FA and 39.6 mL H₂O. Store at 4 °C.

Procedure

A. Cell culture and induction (Day 1-9)

1. Cell line selection:
 - Thaw HEK293 Jump-in cell line in 6 well plate and incubate for 24 h in DMEM 10% FBS 1% P/S.

- Replace growing medium for selection medium (DMEM 10% FBS, 1% P/S + Blasticidin HCl-5 µg/mL and Geneticin 2mg/mL).
- Expand cells on this medium for 6 days. On the last day split cells to 8 x 15 cm dishes. Count cells and seed 15×10^6 cells / dish (40% confluent).
- After 24h of incubation at 37 °C, check cell confluency. At least 80% confluent is necessary for doxycycline induction.

2. Doxycycline induction (24 h):

- At 80% confluency replace the medium for doxycycline induction medium (DMEM 10% FBS 1% P/S + 1 µg/mL Dox) and incubate cells for 24 h at 37 °C.

B. Cell lysis and isolation of mitochondrial enrich fraction (Day 10)

1. Cell lysis and isolation of mitochondrial enrich fraction:

- Carefully remove the medium and wash the cells 2 times with cold PBS (4 °C).
- Harvest the cells in cold PBS supplemented with completTM protease inhibition cocktail by scraping. Harvest 4 x 50 mL tubes (4 dishes per tube).
- Centrifuge 600g at 4°C for 10 min.
- Discard the supernatant and resuspend cells in 6 ml of ice-cold Mito-enrich-buffer.
- Homogenize the cells using a Branson digital sonicator: 30 sec, 10% , 05 s on/0.5 s off.
- Centrifuge at 600g for 10 min at 4 °C.
- Collect supernatant, transfer it to a 15 mL polypropylene Falcon tube.
- Centrifuge at 10000g for 15 min at 4 °C.
- Discard supernatant and wash the pellet with 500 µl of Ice-cold- Mito-enrich-buffer.
- Centrifuge at 10000g for 15 min at 4°C.
- Discard supernatant and freeze the pellet at -80°C

C. Affinity Purification (Pull-down) (Day 11)

1. Mitochondrial lysis:

- Thaw mitochondrial pellet on ice for 20 min.
- Add 3 mL of ice-cold mito-Lysis buffer (1% Digitonin) per sample and resuspend pellet by pipetting up and down.
- Incubate on ice for 15 min
- Sonicate samples briefly in a Branson sonicator with microtip (10 s, 0.5 s on/0.5 s off, 10% output).
- Incubate on ice for 15 min.
- Transfer sample into 2 x 1.5 mL tubes/sample
- Centrifuge in tabletop centrifuge 14.000xg, 4°C, for 20 min
- Harvest supernatant and merge lysates (from the same sample) in a 15 mL tube
- Remove aliquot of lysate (50 µL) for later Quality Control Western Blot -> Aliquot A1
- Determine protein concentration of cell lysate by Bradford assay. Total protein concentration should be similar from sample to sample. If it is very different, measure again and normalize.

2. Selective isolation of bait protein on StrepTactin Sepharose beads (AP):

- Beads conditioning: Add 150 µL of StrepTactin Sepharose bead slurry per sample into a new 1.5 mL tube and wash the beads (2x times). Add 1 mL of ice-cold-mito-Lysis buffer

(0.5 % Digitonin), mix gentle (avoid pipetting) centrifuge @ 1000 rpm 1 min 4 °C and discard supernatant.

- Add beads to protein lysates in the 15 mL tubes and place tubes in a wheel. Let the tube roll for 2 h at 4 °C. **Note:** Use a small volume of mitochondrial lysate (i.e., 300 µl) to resuspend beads by pipetting and transfer them into the tube.

3. Beads wash:

- Discard supernatant and transfer beads to fresh 1.5 mL tubes. (Use 1 mL of Mito-Lysis buffer - 0.5 % Digitonin- to resuspend and transfer the beads)
- Centrifuge beads @ 1000 rpm 1 min 4 °C and discard supernatant.
- Wash beads 2 x 1 mL ice-cold Mito-Lysis buffer (0.5 % Digitonin) and Centrifuge @ 1000 rpm 1 min 4 °C
- Add 1 mL of ice-cold AP-buffer II to the beads and transfer to a Bio-Rad mini-columns, let buffer drop by gravity-flow from the column.
- Wash 3 x 1 mL ice-cold AP-buffer II and let buffer drop by gravity-flow from the column.

4. Protein Elution:

- Remove completely buffer II by centrifugation @ 1000 rpm 1 min 4 °C.
- Close columns on the bottom and resuspend beads in 125 µL of 2% SDS in AP-Buffer II.
- Incubate 15 min at RT in a shaker (600 rpm approx.).
- Open cap and let eluate into a 1,5 mL tube. Spin columns and tubes at 1000 rpm for 1-2 min RT.
- Save aliquot of eluate (20 µL) for later QC Western Blot. -> Aliquot A3
- Store eluates (eluted AP samples) at -20 °C until protein digestion.
- Resuspend beads in 150 µl of AP-Buffer I for later QC Western Blot. -> Aliquot A4

D. Quality control of AP by Western Blot (Day 12-13)

1. Test AP performance by immunodetection of bait SLC (HA-eppitope) in the different stages of the process (aliquots A1 to A4) by Western Blot.
2. Cross contaminated samples or not expressing bait-SLC are rejected for the rest of the procedure.

E. SP3 digestion of proteins (Day 14-15)

1. Thaw samples and let them reach Room Temperature (RT) to avoid SDS precipitation.
2. Protein Reduction:
 - Add 2.2 µL 500 mM DTT (final concentration DTT approx. 10 mM)
 - Vortex and spin each sample
 - Incubate 1h at 56 °C
 - spin samples and Cool to RT
3. Protein alkylation:
 - Prepare a fresh 1 M stock of IAA and add 6.1 µL per sample.
 - Homogenize with vortex and incubate 30 min in dark at RT. **Note:** Protect samples from light (IAA is light sensitive).
4. Preparation of magnetic beads stock
 - Prepare magnetic beads mix stock (1:1 combination) 60 µl of each Mag-beads and vortex at 300 rpm.

- Place mix on magnetic rack and incubate for 1-2 min, then remove supernatant carefully avoiding beads.
- Wash beads (x3 times) with water: Add 50 μ L water and vortex (300 rpm) place on magnetic rack (incubate 1-2 min) and remove supernatant.
- Add 105 μ L of water and store beads at 4°C until use.
Note: Final volume of beads slurry 120 μ L. This volume is enough for up to 12 samples (scale the volume depending on the number of samples).

5. Protein binding and aggregation:

- Add 8.8 μ L beads from stock solution to each sample.
- Vortex and incubate 5 min RT.
- Add 300 μ L ACN to the mix. Mix by pipetting up and down (final concentration of ACN around 70% v/v)
- Incubate on the bench for approx. 5 min. Beads should start precipitation. If not, add more ACN.

6. Wash beads:

- After precipitation immobilize beads in magnetic rack for 2 min and then remove the supernatant.
- Rinse Beads with 200 μ L EtOH 70% in water pipetting up and down: Incubate 30 sec and immobilize in magnetic rack and discard supernatant (X2 times).
- Rinse Beads with 180 μ L ACN pipetting up and down.
- Incubate 30 sec and immobilize in magnetic rack and discard all the supernatant.

7. Protein digestion

- Add 100 μ L of 50 mM Tris-HCl.
- Vortex at 300 rpm.
- Add 1 μ g Trypsin, mix and incubate at 37 °C over-night.

F. Stage tip C-18 Solid phase extraction (SPE) of digested peptides (Day 16)

1. Acidify sample:

- Vortex thoroughly and spin down digested samples by short centrifugation.
- Immobilize beads in magnetic rack and transfer the supernatant to a fresh 1.5 mL tube (avoid beads).
- Add 2 μ L 30% TFA. Mix thoroughly and check the acidified samples with pH paper strip using 0.2 μ L of sample. pH needs to be 3. Continue adding 30% TFA if necessary, until the right pH is reached.
- Spin down samples with a short centrifugation.

2. Stage tip assembly: (Original Reference: Juri Rappsilber et al, 2003)

- Place Empore disk flat on a clean hard surface and press a gauge bunt ended syringe needle into the Empore disk to core out a piece of the filter material.
- Press a second core into the syringe needle for extra loading capacity.
- Place the needle into a 200 μ L pipette tip and push the cored disk pieces into the pipette tip with PEEK or fused silica tubing. Gently pack the material into the end of the pipette tip; a gap of several millimetres should be visible between the disk and the end of the tip.
- Place Stage tips into 2 mL tubes. Use an adaptor to hold the stage tip. The end of the pipette tip should be about 1 cm from the bottom of the tube.

3. Activate and equilibrate C-18 material:
 - Activate C-18 material by adding 100 μ L of 100% acetonitrile and centrifuge at 1000-1600 rpm, RT, 1 - 2 min. Discard flow through. In case liquid don't flow through completely after centrifugation use a small syringe tool to push the liquid manually. Repeat this step 3 times.
 - Equilibrate C-18 material by adding 60 μ L of stage tip buffer and centrifuge at 1000 - 1600 rpm 1 - 2 min. Discard flow through. Discard flow through. In case liquid don't flow through completely after centrifugation use a small syringe tool to push the liquid manually. Repeat this step 2 times.
4. Load sample in stage tips:
 - Load acidified sample in stage tip, centrifuge at 1000 - 1500 rpm., RT, for 1 - 3 min. In case liquid don't flow through completely after centrifugation use a small syringe tool to push the liquid manually.
 - Collect the flow through, load again the column and repeat the previous step.
 - Transfer flow-through into empty brown glass vial. For later QC.
5. Wash:
 - Wash sample loaded onto SPE column with 100 μ L 0.1% TFA and centrifuge at 1000 - 1500 rpm, RT, for 1 - 3 min. In case liquid don't flow through completely after centrifugation use a small syringe tool to push the liquid manually.
 - Discard flow through
6. Peptide Elution:
 - Elute with 2 \times 50 μ L elution buffer in a 1.5 mL low binding tube.
 - Evaporate solvent in a vacuum centrifuge at 45 $^{\circ}$ C until 2 μ L remain. Check regularly and ensure that samples are not dried to completeness! Note: Peptides can be store dry at -20 $^{\circ}$ C until MS analysis.
7. Peptide reconstitution
 - Before MS analysis reconstitute samples in 32 μ L of 0.1% TFA 16.66 fmol/ μ L SH-quant isotopic label peptide, mix by vortex.
 - Spin down samples and sonicate for 10 min in a sonicator bath.
 - Centrifuge (14.000 rpm, 4 $^{\circ}$ C, 15 min).
 - Transfer supernatant to a glass vial with insert.
 - Samples are now ready for MS analysis. They can be directly injected in a MS device or store at -20 $^{\circ}$ C.

Additional notes

- Section: Reagent set up:
 - The volumes shown in this section are indicative. The required volume of each reagent will depend on the number of samples to be prepared.
 - Always use LC-MS grade water for the preparation of all the solvents and buffers.
 - Use only glass pipettes handling TFA and FA.
- Section: SP3 digestion of proteins. Step 4. \rightarrow Magnetic beads stock can be prepared in higher volume and store in at 4 $^{\circ}$ C until use.

Data availability

AP-MS data are accessible on our consortium website re-solute.eu under:

<https://re-solute.eu/resources/>

References

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Please write to contact@re-solute.eu in case of questions or errors.