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Sucrose Gradient_Surface Isolation

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We use this protocol and it's working

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Disclaimer

This protocol needs prior approval by the users' institutional review board (IRB) or equivalent ethics committee(s).

Abstract

Protocol for extracting the surface membrane of cell lines or PDX tumors (or patient tumors) and preparing for mass spec.

Materials

MATERIALS

- D-Sucrose **Fisher Scientific Catalog #**BP220-1
- HEPES **Millipore Sigma Catalog #**H3375
- UltraPure™ 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #**15575020
- Stericup-HV Sterile Vacuum Filtration System (0.45 um PVDF Membrane) **Millipore Sigma Catalog #**SCHVU11RE
- Sodium chloride 99 % crystalline powder PDV **VWR Scientific Catalog #**AAAA12313-0B
- Calcium chloride dihydrate **Sigma Aldrich Catalog #**C3306
- Magnesium chloride solution BioUltra (2 M in H2O) **Sigma Aldrich Catalog #**68475
- Potassium Phosphate Dibasic **Millipore Sigma Catalog #**PX1570
- Trifluoroacetic Acid Optima™ LC/MS Grade **Fisher Scientific Catalog #**A116-50
- Iodoacetamide **Sigma Aldrich Catalog #**I1149
- Sodium Deoxycholate **Sigma Aldrich Catalog #**D6750
- Dithiothreitol (DTT) 1M **Thermo Fisher Scientific Catalog #**P2325
- Ammonium hydrogen carbonate ≥99.0% **Alfa Aesar Catalog #**AA14249
- 13.2 mL Open-Top Thinwall Ultra-Clear Tube 14 x 89mm **Beckman Coulter Catalog #**344059
- Amicon Ultra-0.5 Centrifugal Filter Unit (for up to 10 KDa) **Merck Millipore Catalog #**UFC501096
- Pierce™ BCA Protein Assay Kit **Thermo Fisher Scientific Catalog #**23225
- Acetonitrile Optima™ LC/MS Grade **Fisher Scientific Catalog #**A955-500
- 18 G x 1 1/2 in (Thin wall fill) **Becton-Dickinson Catalog #**305185
- 22 G x 1 1/2 in **Becton-Dickinson Catalog #**305156
- cOmplete™ Protease Inhibitor Cocktail **Roche Catalog #**4693116001
- Sequencing Grade Modified Trypsin Frozen 5 x 20microg **Promega Catalog #**PAV5113

HOMOGENIZATION BUFFER (250 mM sucrose, 10 mM HEPES, 1 mM EDTA - for 1000 mL)

1) 85.58 g D-Sucrose
2) 2.38 g HEPES
3) 2 mL of 0.5 M EDTA
4) Bring to 900 mL with ddH_2O
5) Adjust the pH to 7.4 with either HCl or NaOH
6) Bring the final volume to 1000 mL with ddH_2O
7) Filter solution through a Durapore-Stericup 0.45 um filter

[Store solution at 4 °C]

HEPES BUFFER (115 mM NaCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.4 mM K_2HPO_4, 20 mM HEPES - for 1000 mL)

1) 6.72 g NaCl
2) 133 mg $CaCl_2$
3) 114 mg $MgCl_2$
4) 418 mg K_2HPO_4
5) 4.77 g HEPES
6) Bring to 900 mL with ddH_2O
7) Adjust the pH to 7.4 with either HCl or NaOH
8) Bring the final volume to 1000 mL with ddH_2O
9) Filter solution through a Durapore-Stericup 0.45 um filter

[Solution is light sensitive and should be wrapped in foil and stored at 4 °C]

60% D-Sucrose Solution (for 500 mL)

1) 300.00 g D-Sucrose
2) Bring to 400 mL with ddH_2O and mix on stir plate with low heat.
3) Bring the final volume to 500 mL with ddH_2O
4) Filter solution through a Durapore-Stericup 0.45 um filter

[Store solution at 4 °C]

[Make diluting sucrose concentrations with the 60% D-Sucrose solution]

Sucrose Gradient Buffers (for 50 mL)

	New % of Sucrose	60% Sucrose (mL)	ddH2O (mL)
	42.8	35.67	14.33
	42.3	35.25	14.75

	41.8	34.83	15.17
	41.0	34.17	15.83
	39.0	32.50	17.50
	37.0	30.83	19.17

Digestion Buffer (100 mM NH_4HCO_3, 10% Sodium Deoxycholate, both made with ddH_2O - for 400 uL per sample)

1) 40 uL of 10% Sodium Deoxycholate (SDC)
2) 360 uL of 100 mM NH_4HCO_3

[The 10% SDC needs to be made fresh every time you are preparing a sample]

Reduction with 5 mM Dithiothreitol (DTT) in NH_4HCO_3 (50 mM NH_4HCO_3, 1000 mM DTT - for 400 uL per sample)

1) 2 uL of 1000 mL DTT
2) 398 uL of 50 mM NH_4HCO_3

Alkylation with Iodoacetamide (IAA)

1) 92.48 g of IAA in 1000 mL makes 0.5 M IAA

[I would make a smaller 250 uL - 500 uL stock solution of 0.5 M IAA, depending on the number of samples]
[Adding 16 uL of 0.5 M IAA to one 400 uL sample makes the final concentration 20 mM]
[0.5 M IAA needs to be made fresh every time it is used]
[IAA is extremely light sensitive and needs to be stored in the dark at 4 °C]

Safety warnings

This protocol needs prior approval by the users' institutional review board (IRB) or equivalent ethics committee(s).

DAY 1

1 After all buffers are prepared, retrieve PDX/tumor samples or cells and thaw on ice. Add 1 tablet of **Roche cOmplete™ Protease Inhibitor Cocktail EASYpack** per 50 mL of homogenization buffer. Vortex to dissolve tablet.
- For cells, aim to have roughly **100 million**.
- For solid PDX tumor, aim to have **between 250 and 350 milligrams**.

2 Resuspend each sample in 10 mL of **HOMOGENIZATION BUFFER** in a 50 mL conical and use an electric TissueRuptor® II (from Qiagen) to dissociate tumor chunks into a fully fluid and homogenous mixture with no particulate or remaining solid tissue.
- **Keep samples on ice throughout!**
- Be sure to autoclave the TissueRuptor stems before use and change stems between samples.

3 Retrieve a second 50 mL conical and pass the sample through an 18-gauge needle into the second conical at least once.
- If passaging in easy, move on to the next step. If syringe becomes clogged, further electric homogenization may be necessary.

4 Pass the sample through a 22-gauge needle between the first and second 50 mL conical 3 times.
- If sample becomes clogged in the 22-gauge syringe, further electric homogenization may be necessary.

5 Transfer fully homogenized samples to 15 mL conicals and centrifuge at 1000 x g, 4°C for 00:15:00 .

6 Collect the supernatant, which is the **Post-Nuclear Cytosol (PNC)**, in a new 15 mL conical.
- **Keep samples on ice!!!**

7 Retrieve Beckman Coulter® Ultra-Clear Centrifuge Tubes, rotor (SW-41-Ti), sample holders, and necessary equipment for centrifugation. Add 2 mL of 60% sucrose as a cushion to the ultra-clear tubes slowly with a P-1000. Layer the first 2 mL of PNC supernatant from your samples on top of the 60% sucrose cushion **VERY SLOWLY** and carefully so as not to disturb the 60% gradient layer (can use a serological pipette).
- **Samples running opposite of each other on the rotor should be balanced in their respective holders to the 100th decimal with homogenization buffer!**

8 Centrifuge samples at 100000 x g, 4°C for 01:00:00 .

9 After centrifugation, use a serological pipette to carefully take out roughly 75% of the homogenization buffer supernatant making sure not to disturb the protein layer on top of the 60% cushion. Then using a P-200, harvest the **Crude Plasma Membrane (CPM)** fraction from the top of the 60% sucrose cushion and place in a new ultra-clear centrifuge tube.
- You can use a swirling technique with the P-200 to suck up the CPM fraction.
- Try to **minimalize the amount of 60% sucrose carry over** when transferring the CPM fraction to new ultra tubes.

10 Using a P-1000, set the volume to 750 uL and add 1.5 mL of each sucrose gradient on top of the sample and each other **VERY SLOWLY in a "tear drop" style fashion** starting with 42.8% sucrose, then 42.3%, then 41.8%, then 41.0%, then 39.0%, and lastly 37.0% sucrose at the top.
- You can fill the rest of the tubes (**SLOWLY**) and balance opposite facing samples to the 100^{th} decimal with 37% sucrose.
- It is **VERY IMPORTANT** to add your consecutive gradients slowly and steadily so as to have well-defined layer separation and prevent mixing of the gradients.

11 Centrifuge samples at 100000 x g, 4°C overnight for a minimum of 18:00:00.
- I would recommend setting the ultra-centrifuge to "hold" during this spin in case you are doing other experiments.

DAY 2

12 The next day recover the **Enriched Plasma Membrane (EPM)** fraction from the top of the 37% sucrose cushion using a P-200 and place in a new ultra-clear centrifuge tube.
- You can use a swirling technique with the P-200 to suck up the EPM fraction.
- Retrieve as much of the EPM fraction on top of the 37% cushion as possible, while also **limiting the amount of 37% sucrose carry-over**, and place into new ultra-clear tubes. The key is to not get greedy!

13 Fill up the ultra-tubes with **HEPES BUFFER** and balance opposing tubes to the 100^{th} decimal before spinning.

14 Centrifuge samples at 150000 x g, 4°C for a minimum of 02:30:00.
- Sometimes you will have to centrifuge your samples anywhere between 02:30:00 and 05:00:00 during this step to guarantee total pellet sedimentation of the available EPM.
- **Would recommend starting with a 3-hour spin every time.**

15 Decant the HEPES supernatant out of the ultra-clear tubes being careful not to disturb the protein pellet. **Resuspend the pellet in 400 uL of DIGESTION BUFFER** (10% SDC + 100 mM NH_4HCO_3) and vortex.

- After resuspension with digestion buffer you can store your samples in -80 °C, however, **it is ideal to complete the protocol up through the trypsin digestion**.
- It would also be helpful to run a BCA Assay or other protein quantifications to determine the protein concentration per sample and determine how much trypsin to use for each sample.
- If pellets are not solvating into the digestion buffer well, you can sonicate the samples on ice for 00:10:00.

16 Retrieve Amicon® 0.5 mL Centrifugal Filter Units (for up to 10 KDa). Apply samples to the filter held in eppendorf tubes and spin down the samples between 8000 x g and 12000 x g for 00:10:00 at 4 °C to bind protein to the filter column.

- A small portion of liquid sample might remain at the bottom of the filter unit, but this is okay.

17 Decant the flow-through waste from the collection tube and wash the filters with 400 uL of ddH_2O by spinning samples down between 8000 x g and 12000 x g for 00:10:00 at 4 °C. **Repeat this step a second time.**

- Again, a small portion of H_2O might remain at the bottom of the filter unit, but this is okay.

18 Next, you want to perform a **REDUCTION** on your protein samples using a **5 mM Dithiothreitol (DTT)** solution made in **50 mM NH_4HCO_3**. Add 400 uL of the 5 mM DTT solution to each sample's filter unit and let **incubate at room temperature for** 01:00:00 for full reduction to occur. **DO NOT SPIN DOWN AFTER**.

19 You now want to cap/block all the reduced sulfide bonds on the proteins by **ALKYLATION with IODOACETAMIDE (IAA)** in order to prevent DTT from reverse reacting and reforming disulfide bonds. You want a ratio of **4 IAA equivalents for every 1 DTT equivalent** for full deactivation. Therefore, you will need a minimum of **20 mM of IAA to combat the 5 mM of DTT per sample**. Using a **stock solution of 0.5 M IAA** (made with ddH_2O), you will need roughly **16.67 uL of IAA** per each 400 uL sample. Add directly to the filter units with a P-20 and **INCUBATE IN THE DARK FOR** 00:30:00 **AT ROOM TEMPERATURE**.

- 0.5 M of IAA should be made fresh with ddH_2O for every new experiment.

20 After the incubation, spin down the samples at 12000 x g, 4°C for 00:05:00 and decant out the waste from the collection tube.

- You may have to spin the samples for up to 00:15:00 in order for the waste to fully pass through the column into the collection tube.

21 After quantifying your total protein, make a 400 uL solution of **50 mM NH_4HCO_3** that includes the appropriate amount of **TRYPSIN** for each sample. A **ratio of 1 ug of trypsin to 20 ug of protein** is our standard ratio. Add the respective trypsin solutions to each sample and **let the reaction sit overnight at room temperature for full peptide digestion**.

- It would also be useful to test the pH of the samples after trypsin is added to make sure the solution is at a pH 8. This is ideal for optimal enzymatic activity.
- Some reagents require you to add **TEAB** to the trypsin before creating the 50 mM NH_4HCO_3 solutions for each sample. The trypsin used in the materials section does NOT require this.

DAY 3

22 Using a P-200, lightly resuspend the solution in the filter to make sure your peptides are mixed and detached from the column. Retrieve fresh centrifuge tubes and carefully tilt the sample in the filters upside-down into the new tubes. Centrifuge the upside-down filters in the new collection tubes at 12000 x g, 4°C for 00:02:00. This ensures that the total amount of peptides fall into the new collection tube.

23 In order to **promote peptide binding to the stage tips**, use **LC/MS-Grade TRIFLUOROACETIC ACID (TFA)** to acidify your samples. Start by adding 3 uL - 5 uL of TFA to the samples inside a fume hood. You want to make sure the pH of your sample is pH 2. Let the samples **incubate on the bench at room temperature** for 00:30:00.

- TFA is highly corrosive and will burn your skin. Handle with care IN A FUME HOOD.
- TFA also deactivates the trypsin by quenching the reaction as well as pulling the SDC out of the sample solution as a precipitate.
- While the samples sit for 30 minutes, you can begin **STAGE TIPPING with CARBON-18 RESIN** for your samples.

24 Spin down the samples after the incubation at **maximum speed** for 00:15:00 at 4 °C. While this occurs you can continue to **STAGE TIP with C-18 RESIN**. About 4-6 punches of the resin per sample are optimal to pack a P-200 pipette tip. Using pipette tip holders, or **ADAPTERS**, you can place the tips in fresh eppendorf tubes.

- Punches are easily made using P-1000 pipette tips that have the tip-ends snipped around half an inch.
- The C-18 resin helps to cleanse the sample of excess salt and contaminants.

25 Wash the stage tips with **100 uL of 100% Acetonitrile** and spin them down at 2000 x g for 00:02:00. Decant the waste.

26 Wash the stage tips with **100 uL of 0.1% TFA** (diluted with ddH_2O) and spin them down at 2000 x g for 00:02:00. Decant the waste.

27 Add the sample to each of their own stage tips and spin them down at 2000 x g for 00:01:00 so they bind to the resin. Decant the waste.

28 Wash the samples with **100 uL of 0.1% TFA** and spin them down at 2000 x g for 00:02:00. Decant the waste.

29 Finally, elute your samples into fresh eppendorf tubes by using **100 uL of 70% Acetonitrile** and centrifuging them at 1500 x g for 00:02:00.

- The samples will be put into the speed vacuum next, so it is okay if they start to dry out.

30 Place your samples in the speed vacuum to dry down the samples. This can take anywhere between 2-6 hours.

- Freeze back the speed vacuumed samples in -20 °C until ready to run on the mass spectrometer.