SF9 Connexons Purification by Atsu

Atsu's Membrane Lysate Prep

This protocol is used to isolate membranes from cultured SF9 cells, all steps are performed on ice. You will need to prepare the following solutions:

*Buffer*:
- 1mM NaHCO3
- 1mM PMSF

40mM NaOH Start with 4N NaOH and do a 1:100 dilution with Buffer

20mM NaOH Start with 4N NaOH and do a 1:200 dilution with Buffer

Procedure

- 1200mL of SF9 cells are used for one membrane prep, these are spun down 4000rpm, 15min, 12°C and the supernatant is removed by slowly decanting.
  - The bottles used for this centrifugation do not need to be autoclaved.
- Divide the pellets into 6 x 80mL centrifuge tubes using cold PBS.
- Add ~10mL of PBS to the 400mL tube, resuspend and divide into the 80mL tubes.
- Add about 20mL more of PBS resuspend again to get every last little bit of pellet.
- Repeat again with more PBS (total not to exceed 80mL in each 80mL tube)
- Centrifuge again, 4000rpm, 15min, 12°C.
  - The pellet is too viscous by itself so you can dilute it with “Buffer”
- Here we have 6 pellets, add 5mL buffer to bring up to 10mL.
- Combine the pellets so there are 2 pellets in 3x 80mL tubes
- Add an equal volume of 40mM NaOH to each tube.
  - Since we have combined into 3 tubes, add 10mL to each new tube.
  - This gives us a final concentration of 20mM NaOH.
  - From now on you will only use the 20mM NaOH, you will NOT need the 40mM NaOH anymore.
- Wash the empty tube that used to contain the pellet with 20mM NaOH to remove all the cells that are stuck to the walls.
- Fill each of the centrifuge tubes to the 80mL line with 20mM NaOH.
- The cells need to be broken down, sonicate each tube until mixture is homogenous (amplitude 80, for about 30 seconds).
  - If you do not sonicate enough you will have a bad recovery.
- Fast Cool the Eppendorf Centrifuge.
- Spin 12,000rpm, 7°C (if you set this particular centrifuge at 4oC while spinning at max rpm an error can happen) 25-30min.
- Decant supernatant.
- Take the “Buffer” use 12mL to suspend all 3 of the pellets together.
  - f you started with 1200mL, final will be 100mL of cells concentrated into 12x 1.5mL tubes.
- Mixture is still not homogenous so you need to sonicate it to mix.
- Sonicate at a weaker amplitude ~ 30amp for 30 seconds.
  - Aliquot 1mL in to 12 x 1.5mL tubes.

Now you can purify protein or freeze in Liquid Nitrogen and store at ~80°C, but try to use it within 3months.

Atsu’s Protein Prep from SF9 Cells

1.2L of SF9 cells were cultured and their membranes were lysed to give 12mL of sample, this is divided with 1mL each in 12x 1.5mL tubes.

3x Solution Buffer

(Final Conc.) (of stock)
DoDM 6% (12mL of 20%)
HEPES pH7.5 30mM (1.2mL of 1M)
NaCl 3M (24mL of 5M)
NaN3 (0.005%) (100uL of 2%)
H2O (2.5mL)

TOTAL (40mL)

- Add 500uL of 3x Solution Buffer to each 1mL tube
- Incubate at RT on blue rotating wheel and let mix for 10min
- Meanwhile, cool the Eppendorf 5810R Centrifuge using fast cool to 4°C
- Prepare the Nickel Resin (purchased from Qiagen = Ni-NTA Agarose)
  - Shake the bottle to make sure it is mixed up
  - We will use 100uL of nickel resin per 1mL of sample, so if we have 12mL, we will need 1.2mL
    - The nickel resin needs to be washed before use

**Binding Solution**

<table>
<thead>
<tr>
<th>(of stock)</th>
<th>Stock</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES 8.0 10mM</td>
<td>(2.5mL)</td>
<td>1M</td>
</tr>
<tr>
<td>DoDM 0.1%</td>
<td>(1mL)</td>
<td>20%</td>
</tr>
<tr>
<td>NaN3 0.005%</td>
<td>(625uL)</td>
<td>2%</td>
</tr>
<tr>
<td>NaCl 1M</td>
<td>(50mL)</td>
<td>5M</td>
</tr>
<tr>
<td>H2O (sigma)</td>
<td>(192mL)</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>~250mL</td>
<td></td>
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</tbody>
</table>

**Wash Solution + L-Histidine**

| L-Histidine 10mM | (800uL) -> stock is 5mM |
| NaOH | (70uL) -> to adjust pH |
| Wash Solution | (39mL) |
| **TOTAL** | (40mL) |

- Split the nickel resin into 2x 1.5mL tubes, with 600uL in each
- Add ~500uL Wash + L-His to Nickel Resin & Vortex
- Centrifuge at 6-7000 rpm for about 1min, pipet off supernatant
  - Repeat this 2 more times
- When the samples have finished their 10min incubation put in centrifuge eppendorf 5810R, spin at 14,000 rpm for 10min at 4°C
- While the samples are spinning place the nickel resin in Binding Solution, add ~500uL to each 600uL resin sample
- Divide the 2x 1.5 tubes into 4x 50mL tubes, ~300uL per 50mL tube
  - The resin binds to plastic so to make sure you get all of it out, at 1mL more of wash solution to ea 1.5mL tube, pipet up and down and add to 50mL tubes
  - Repeat once more
- Fill the Falcon tube to a little less than 40mL with Binding Solution
- When the samples are done spinning in the centrifuge add them to the 50mL tubes
  - We have 12x 1.5L tubes and 4x 50mL tubes, so add 3x 1.5mL tubes to each 50mL tube using a pipet
- Put tubes on the blue rotating wheel in the cold room
The metal ‘pinch’ the holds the tubes to the board is too tight so make sure you clamp it to the ‘butt’ of the tube and not near the cap or it might break

Let them mix overnight

The Next Day…

• Get the 50mL tubes from out of the cold room
• Spin down, 2500 rpm, 2min, 4°C (we used the small eppendorf in the TC room)
• Pipet off the supernatant
  • Be careful not to disturb the pellet, it is better to leave a little supernatant then to risk the pellet
• Resuspend the sample in remaining supernatant, transfer to a 1.5mL tube, put 2 pellets into each 1.5mL Tube
  • Use a very small volume on the pipet to transfer, this will help cut down the amount binding to the walls of the pipet tip
  • Save this pipet tip, we will wash it out further to get every little bit out
• Spin 7-8000 rpm for ~1min
  • Should spin at 4°C, but out tabletop centrifuge doesn’t do that, and it’s a short spin so RT is ok
• Add 500uL of Wash Solution + L-Histidine to each of the old 4x 50mL tubes, use the saved pipet tip to pipet up and down and collect every last bit of resin, add to 1.5mL tube
• Spin again 7-8000rpm ~1min (4°C or RT), remove supernatant
• Add 500uL Wash + L-His to each 50mL again, pipet up and down, add to 1.5mL tube, spin again
• Repeat this one more time

Elution Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Conc.</th>
<th>(Stock Conc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (7.4)</td>
<td>20uL</td>
<td>10mM</td>
</tr>
<tr>
<td>DoDM</td>
<td>5uL</td>
<td>0.1%</td>
</tr>
<tr>
<td>NaCl</td>
<td>200uL</td>
<td>1M</td>
</tr>
<tr>
<td>NaN3</td>
<td>2.5uL</td>
<td>0.005%</td>
</tr>
<tr>
<td>L-His</td>
<td>600uL</td>
<td>300mM</td>
</tr>
<tr>
<td>4N NaOH</td>
<td>70uL</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>110uL</td>
<td></td>
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</tbody>
</table>

TOTAL 1mL -> check that pH is between 7&8 using pH paper, vortex
(if you make 1.5mL you can use the extra 500uL as the Blank on the spectrophotometer)

• When done spinning final wash, take away as much of supernatant as possible without disturbing resin
• 1.2L culture was used so final elution will be 1.2mL
• We have 2 tubes with 500-600uL resin each, add 600uL Elution Buffer to each of these
• Vortex the samples, don’t pipet up and down since the resin will bind to the tip
• Place samples on rotating wheel in the cold room and let mix for 1hr
• Take the samples out of the cold room and spin at 7-8000rpm ~1min
• Carefully remove the supernatant from both tubes and combine into a 1.5mL tube
  • The supernatant now contains your sample
• Spin this down just in case there is still resin inside
• You can now read the supernatant on the spectrophotometer
• Store at 4°C