

First day

- Cool down rotors in Ultracentrifuge and Eppendorf centrifuge.
- Spin down the SF9 cells in the 400ml bottles no sterile:
 - 12 °C
 - 15 minutes
 - 4000 rpm
- Decant the supernatant in the sink.
- Wash the pellet with PBS + Na Azide 500µl in 500ml and put the pellet in the 80ml tubes.
- Fill up 4 tubes and centrifuge again at:
 - 12 °C
 - 15 minutes
 - 4000 rpm

Throw away the supernatant and wash the pellet with 8.7% sucrose in Tris or Hepes buffer.

- Spin down as above.
- In the while, prepare 6 ultra **clear tubes** with Sucrose gradient 27%/50%:
 - 1.5 ml of 50% sucrose
 - fill up the tube leaving 1.8 ml free for cellular pellet.
- Decant the supernatant in the sink and resuspend the pellet in 7ml of 8.7% Sucrose solution.
- Sonicate the cells at minimum amplitude, for 20 seconds.
- Put this cells lysate in the **first gradient 27%/50%**.
- Ultracentrifuge at 40 K rpm, for **1:30 h** at 4 degrees giving 3 minutes of acc. and 3 for dec.

- Recover the 27-50% interface band (aspirating away the superior bands) and put it in **polycarbonate tubes**.
- Fill up the tubes with the ***Starting Buffer*** and mix with a transfer pipette up and down.
- Ultracentrifuge at 30K for 25 minutes clear acc. and dec.
- Add 750 μ l of ***Starting Buffer*** to the pellet and 750ul of Sarcosine detergent 0.6%.

(In this way you have 0.3% of detergent final concentration)

- Let on the bench for 10 minutes after pipetted up and down to resuspend and wash the pellet.
- Ultracentrifuge at 30K for 25 minutes.
- Add 750 μ l of ***Starting Buffer*** to the pellet and 750ul of Brij detergent 0.6%.
- Wash it with 0.3% Brjie and ultracentrifuge 30k, for 25 minutes.
- Let on the bench for 10 minutes after pipetted up and down to resuspend and wash the pellet.
- Ultracentrifuge at 30K for 25 minutes.
- In the while prepare the Nickel resin (Ni-NTA agarose):
 - a) Mix the bottle, pick up 600 μ l of resin and put in one eppendorf tube, another 600 μ l of resin and put it in another eppendorf tube.
 - b) Spin down for a minute, aspirate and put 600 μ l of ***Washing buffer*** (= Starting Buffer + 0.5mM Imidazole = 0.007g in 200ml finals).
 - c) Repeat this 3 times.

- d) Put the tubes in Ice and divide the resin in 4 falcon tubes of 50ml: put 300µl of resin in each tube.
- When centrifuging is done, resuspend the pellet in 300µl of ***Starting Buffer*** and add 300µl of Saponine 0.6% for each tube.
 - Pick up the entire volume in each tube and put it in the Falcon tubes, ~900 µl for every Falcon tube.
 - Fill up the falcon tubes with ***Saponine Buffer***:
***Washing Buffer* + 0.06 g saponine.**
 - Let the membrane to attach to the Nickel resin over night at 4 °C.

The second day

Wash the resin with ***Washing Buffer ***:

(The one with 0.5mM of Imidazole, but without Saponine)

- Spin down the falcon tubes in the Eppendorf centrifuge:
4 °C
3 minutes
3000 rpm.
- Throw away the supernatant carefully to don't disturb the resin.
- Put the resin in two eppendorf tubes and centrifuge them 1 minute at 8000 rpm.
- Add ***Washing Buffer*** to the falcon tubes (600ul to each) and put them in the same eppendorf tubes.

(The scope is to recover as much resin as possible, washing it at the same time from the stuff binding it weakly)

- Repeat this step 3 or 4 times, every time spinning down the eppendorf tubes for 1 minute at 8000rpm.
- Elute the membrane from the nickel resin by adding 200mM Imidazole to the ***Starting Buffer*** (= 0.7 g Imidazole in 50 ml of Starting buffer.)
- Add starting buffer and spin down the membrane at 30K for about 25 minutes in the ultracentrifuge.