GraDeR Step-by-Step Protocol - *All procedures are carried out at 4 degree Celsius (cold room) or on ice*

1. Mix IMP solution with LMNG

Add LMNG directly to the detergent-solubilized integral membrane protein (IMP) in the buffer and detergent in which previous purification steps have been carried out. Add LMNG from a 10% stock solution to a final concentration of up to 0.05% (w/v). Detergent exchange occurs due to high IMP affinity of LMNG.

While a final concentration of 0.05 % (w/v) works fine for most complexes, the final concentration can be lower for some IMPs, allowing for loading of a higher sample volume on the subsequent gradient. Thus, an optimization of the minimum required LMNG concentration can be done optionally.

2. Incubate LMNG for detergent exchange.

Incubate LMNG for 20 minutes to exchange detergent bound to the IMPs against LMNG.

Since the off-kinetics of LMNG are several orders of magnitude slower than for other detergents, a detergent exchange will automatically occur.

With an incubation time of 20 minutes, the exchange is complete for most IMPs and detergents used in the previous purification steps. It might be, however, that certain detergents require longer incubation, or that the incubation time is longer if the final concentration of added LMNG is lower.

3. Prepare the gradient using the GradientMaster

1. Prepare light and heavy gradient solutions (light solution: buffer of choice, crowding agent of choice [e.g. 10% (w/v) glycerol] + 0.003% (w/v) LMNG; heavy solution: buffer of choice, crowding agent of choice [e.g. 30% (w/v) glycerol])

2. Prepare gradient using the appropriate GradientMaster program.

Addition of 0.003% of LMNG requires the gradient conditions to be optimized in a way that the IMP migrates lower that the lower third of the gradient. For some more stable IMPs, the addition of LMNG to the light gradient solution can be omitted.

Crowding agents which are known for their de-lipidating properties (i.e. sucrose) may harm IMPs where structural lipids are required for the integrity and functionality of the complex.

4. Loading of gradients with IMP-LMNG solution

Gently layer the IMP-LMNG solution onto the gradient.

Gradient overloading may occur if either too much IMP or too much LMNG is added: Both IMPs and stable LMNG micelles can co-migrate and hamper the separation capability of the gradient. As an effect, IMPs might be improperly separated, LMNG micelles might still be in the harvested fractions and the size of the LMNG bicelle might be enlarged.

For safely achieving an efficient separation by the density gradient centrifugation, we recommend to initially load no more than ~100 picomoles of IMP + LMNG micelles per 5 ml gradient volume. Conservatively interpolating the aggregation number of LMNG (which is not known for lacking monodispersity of LMNG micelles) from DDM, 100 µl of buffer containing 0.05% LMNG account for not more than 35 picomoles of LMNG micelles. The loading capacity of the gradient can be optimized experimentally.

5. Run gradient in swing-out rotor for 12 - 20 hours.

Gradient centrifugation speed and time should be optimized for peak fractions to arrive roughly at a position 1/3 up from the bottom of the gradient.

Software packages for the simulation of gradient centrifugation (i.e. COMPASS) can facilitate the optimization of run conditions.

6. Fractionation

Fractionate from bottom to top using a long needle and a pump. The needle is carefully immersed into the gradient tube until reaches the bottom and stands in the tube by leaning on the tube walls. The fraction size should not exceed 20 drops per fraction.

Fractioning the gradient from the bottom is imperative for a micelle-free preparation since fractionating from the top will result in a continuous carry-over of free detergent molecules and micelles from the water-to-air interface.

7. Fraction analysis

Several methods are available to evaluate concentration, intactness, monodispersity, activity etc. of the IMPs in each fraction. Generally useful methods include CN-PAGE, FSEC, dot blot and negative stain EM.

8. Remove crowding agent

Remove crowding agent from fractions by dialysis, concentrators or desalting columns. For delicate IMPs, especially mammalian multisubunit complexes, dialysis is the method of choice.

9. Concentrate

If necessary, concentrate sample to desired concentration by e.g. using centrifugal concentrators.

10. Prepare cryo grids

Cryo grids can be prepared in the same way as for cytosolic examples.

In some cases and for delicate IMPs, we have seen that blotting with nitrocellulose instead with regular cellulose results in a larger population of intact particles since the blotting process is slower, milder and better controllable.