INCORPORATION OF 3A4 into NANODISCS

1. Make a lipid stock – dissolve lipids in chloroform for storage at -20°C. Aim for 90 mM concentration.
2. Determine the concentration of the lipid in the lipid stock using Phosphorus Determination Assay.
3. Figure out how much lipid you need to dry out. This is what you need to know:
   a. You want to make nanodiscs in the excess of the 3A4. A good ratio is 15 (or 20) MSP T2 to one 3A4. In other words ~ 7 to 10 Nanodiscs to one 3A4. This insures monomeric 3A4 incorporation, and often increases the efficiency of the 3A4 Nanodisc incorporation.
   b. 3A4 is incorporated into POPC lipid (Palmitoyl oleoyl phosphatidyl choline) Nanodiscs. For POPC the correct ratio between MSP T2 and lipid is 1 MSP T2 to 65 POPC molecules. This ratio is dependant not only on the type of lipid used, but also on the type of MSP. For insertion of only 3A4 into discs the MSP used is MSP T2 without a his-tag. Since there is a variety of MSPs available make sure you have the correct one. This is important for purification of 3A4 Nanodiscs and pulling only the Nanodiscs with 3A4 in them – thus the MSP cannot have a his tag.
   The correct ratio for MSP T2 with DPPC lipids is 1:90 (85), and with DMPC 1:80.

So, decide how much 3A4 needs to be incorporated (example 25 nmol).
Then determine the amount of MSP you will need using the ratio described above (25 nmol x 15 = 375 nmol of MSP T2)
Then determine the amount of lipid you will need from the above ratio (375 nmol x 65 = 24.4 umol POPC)
Then determine how much lipid from your lipid stock this comes out to be (for a 90 mM stock of lipid this will mean you will need to dry out 271 ul – i.e 24.4 umol/90 mM).
YOU ARE READY TO BEGIN.

4. Use a Hamilton syringe to transfer the lipids from the lipid stock into a glass tube. Dry out the lipids under a stream of nitrogen (or argon). Try to form a thin film on the bottom of the tube, and make sure you dry them well without splashing onto the wall of the tube.

5. Leave the glass tube with dried lipids in vacuum overnight (no less than 4-5 hours).

6. Solubilize lipids in 100 mM cholate. The cholate can be made in a number of buffers, but 50 mM K-phosphate, and 0.3 M NaCL, pH 7.4 works. It can also be higher than this in concentration. The important thing is that the ratio of cholate to lipid is 2:1. Therefore, if you are adding 100 mM cholate, the concentration of lipids needs to be 50 mM, which means that you need to add 24.4 umol/50 mM = 488 ul of 100 mM cholate.
   a) Vortex the cholate/lipid mixture
   b) Place the mixture in very warm water, and occasionally vortex until the mixture is clear., while keeping the water warm.
   c) Sonicate the mixture once clear for ~10 min.
7. Add MSP1T2 (for a MSP1 T2 stock that is 250 uM, this would mean you need to add 375 nmol / 250 uM = 1.5 ml) for the cholate/lipid mixture after sonication.

8. Dilute 3A4 (which is stored at -80C in 20% glycerol) 3X. For example above, say that we have 1 ml of 25 uM 3A4 – then dilute to 3 ml. You can use the same buffer as above (50 mM K-phosphate, 0.3 NaCl pH 7.4), but other buffers will work as well.

The purpose of this is to dilute the glycerol concentration in the final reconstitution mixture. It interferes with the nanodisc formation, and its concentration needs to be below 5-6%. (~4)

9. Combine 3A4 and the MSP1 T2/lipid/cholate mixture.

10. Make sure the concentration of cholate in this final mixture is >= 18 mM.
In the example above the total volume is 3 ml + 0.488 ml + 1.5 ml = ~ 5 ml
18 mM x 5 ml = 90 umol
You have 488 ul x 100 mM = 48 umol
So you need another 90 - 48 = 42 umol

Use 400 mM stock of cholate and add 105 ul of it to the mixture (42 umol/400 mM = 105 ul).

10. Incubate mixture for 1 - 1½ hours at 4C.

11. Add a sample volume equivalent of biobeads to the mixture (>= 5ml in the example above), and set on a shaker at 4C to incubate for ~ 5½ hours.

12. Remove the sample from the mixture with biobeads. Add some buffer to biobeads and swirl to remove remainder of the sample. Filter through a 0.22 um filter, and store at 4C until purification.

PURIFICATION OF 3A4 NANODICS:

Your sample currently contains empty nanodiscs, 3A4 in Nanodiscs, and unless you just made the most perfect Nanodisc sample in the world ever imagined, you will have some “other junk”. For some experiments the excess of empty nanodiscs is not a problem. In this case, only use the size exclusion chromatogram to purify the Nanodisc (i.e. to obtain the ~ 10 nm fractions). Otherwise follow the below described protocol (this can be done at room temp, since 3A4 in nanodiscs is stable at room temperature):

1. Make a small (1 - 2 ml) Ni-NTA column. Ni-NTA is stored in 20% ethanol, so wash the column with water first.
We use Ni-NTA resin since MSP is prepared in a buffer containing some EDTA which strips the Ni off the Ni-NDA column, and 3A4 is stored with some DTT or BME, where DTT also interacts with the column. Ni-NTA resin handles this better than Ni-NDA.
2. Equilibrate the column with buffer (50 mM K-phosphate, 0.3 M NaCl pH 7.4)
3. Load your Nanodisc 3A4 sample.
4. Wash with 15 mM imidazole to get rid of weak and nonspecific binding (MSP can nonspecifically bind to Ni). 15 mM imidazole is in the same buffer. 2 column volumes are enough.
5. Elute the 3A4 Nanodiscs wth 0.3 M imidazole.
6. Now, inject the elution fractions onto a size exclusion column (FPLC), and collect the 10 nm fractions.
7. If all went well the $R_g$ should be close to 1 (0.8-1 is good). Otherwise is an indication of either extra empty nanodiscs present (the purification above was not done properly for whatever reason), or the incorporation of 3A4 into Nanodiscs was not optimal.
8. If there is a reason for concern, reinject the 10 nm 3A4 Nanodisc fractions on to the size exclusion column. Is the peak narrow, without any shoulders? It should be, if not, the 3A4 discs may not be stable, and something went wrong with the incorporation into Nanodiscs.
9. If the 3A4 Nanodiscs seem OK, take the fractions and look at bromocriptine binding (3A4 in Nanodiscs will bind bromocriptine and subsequently undergo a spin shift which is observed by a shift in absorbance from 417 nm to 390 nm with bromocriptine bound).