LIPID BINDING ASSAYS

1. For purification of FRB protein:

Protein Purification/Size Exclusion Buffer
40mM Tris pH 7.5
0.15M NaCl
2mM EDTA
2mM EGTA
[Note: do not use phosphate buffer, such as PBS.]

S-100 chromatography
Column from BioRad: 2.5cm x 120cm
Matrix: Sephacryl S-100 from Pharmacia
Settings:
   Flow rate = 10x, 3
   Fraction size = 8min (~1mL/min)
   Chart speed = 0.5
   Sensitivity = 1

Note: if not using S-100 chromatography as the last step (i.e., GST is only removed by glutathione column), need to change the buffer used for purification from the beginning (resuspension of bacteria) from PBS to the following:
   40 mM TrisCl, pH7.5
   0.15 M NaCl
   1 mM DTT (added within a couple of days)

2. Preparation of small unilamellar vesicles (SUVs)
(All phospholipids are obtained from Avanti Polar Lipids; need the chloroform solutions!)

- Add 25mg of POPC (phosphatidylcholine) and 25mg of the specific phospholipid (e.g., phosphastidic acid) to a glass test tube, evaporate the chloroform by blowing N2 gas into the tube while gently rotating the tube – you want the lipids to dry as a film on the wall around 1 inch from the bottom.
  [Note: POPC is used as a “filler” here. You may want to adjust the ratio to use less of the other lipid, either for economical or physiological reasons. For our FRB-PA binding assays, 10% PA (thus 90% PC) is sufficient for full binding activity.]

- Add 3mL of vesicle buffer (10mM Tris pH 8, 150mM NaCl) to the dried lipids, put the tube on ice, insert the probe from a probe-sonicator – the tip of the probe should be half way into the solution; clamp to make sure both the tube and the tip are secure, the tip should not touch the side of the tube. The total amount of lipids can be scaled down to a third (i.e., 1 mL sonication volume), but not further. For smaller volumes, see the alternative below.

- Sonicate with a microprobe on a pulsed cycle for 9min: power=4.5, duty cycle=60%, output control=4. If not enough (see below), do another 9min.
[Note: you’ll need to find out the optimal settings with each sonicator. Watch the solution, it should go from very cloudy to almost clear (but not quite), stop sonicating once the solution no longer clears up further.]
Alternatively, you can use a water-bath sonicator, and put the lipid suspension in Eppendorf tubes and float them in water to sonicate. Alternate between freezing in lipid nitrogen and sonicating for 1-2 min, until the solution no longer clears up further.

- Spin the sonicated vesicles in a small ultracentrifuge (TLA rotor) at 25°C at 60,000rpm for 2hr. Transfer the upper layer of clear solution to a new tube – this is your SUVs, and store at room temperature. Ideally you want to use the vesicles immediately or within 1-2 days, but most of them are relatively stable for 1 week.

3. Lipid binding assay

- Form the complex by incubating the mixture of protein and vesicles at room temperature for 30min: total volume: 400 uL
  - protein: 12.5uM final
  - vesicles: 200-300 uL
  - if necessary, make up the total volume using vesicle buffer.

  For rapamycin disruption of FRB binding:
  - Mix Rapamycin and FkBP12 (1:1) and incubate for 10min, then add it to FRB protein (1:1:1) and incubate for 10min. Add the complex to the lipid solution and incubate for 30min.

- Load the entire mixture (400 uL) onto the Sephacryl S-400 size exclusion column to chromatograph, collect ~1mL per fraction (see below for column settings).

- Boil samples (start before void volume fractions, can do every other or every three samples) in SDS sample buffer, and load on SDS-PAGE, followed by silver-staining.
  [Note: the bound protein will come out in void volume where the vesicles elute, and the free protein (FRB is 12 kDa) should come out later.]

Sephacryl S-400 (or S-300) chromatography:
Column from BioRad: 0.5cm x 50cm
Matrix: Sephacryl S-400 (or S-300) from Pharmacia (now GE)
Settings:
  - Flow rate = 1x, 11
  - Fraction size = 4min (~0.2 mL/min)
  - Chart speed = 0.5
  - Sensitivity = 0.5