**Purification of FRB Protein**

(1) Inoculate LB/Amp with BL21 containing pGEX-2T-FRB from single colonies or frozen glycerol stock, 10 mL for each liter of prep. Shake at 37 °C overnight.

(2) The next day, inoculate LB/Amp with the overnight culture at 1:100 dilution (i.e., 10 mL into 1 liter in 2L flasks). Typically 1-2 liter culture is sufficient to produce 10-20 mgs of purified FRB protein. Grow this culture to OD<sub>600</sub>=0.6-0.8 at 30 °C with vigorous shaking.

(3) Induce expression with 0.3 mM IPTG at room temperature (crucial!) for 6-8 hours.

(4) The cells are harvested by centrifugation using JA-10 rotor at 5000 rpm for 10 min, and resuspended in PBS containing 1mM DTT and ~10 mg lysozyme (freshly added); for each liter of culture, use 20 mL buffer. (Optional: protease inhibitors can be added at this step, but not necessary for wild-type FRB.) Transfer the cell suspension to a 50-ml Corning tube and freeze at -20 °C overnight. (Note: the cell suspension can be stored at -20 °C almost infinitely.)

(5) Thaw the frozen cell suspension at room temp. This process will take 1-2 hours. When it is completely thawed, the solution should be very viscous - an indication that the cells are well lysed. Put the solution on ice as soon as it is thawed but still cold. From here on, all steps should be taken at 4 °C or on ice unless stated otherwise.

(6) Sonicate the lysates using a microprobe at 3.5 setting, 4 x 9 seconds, or until the viscosity is eliminated – do not over-sonicate as it may damage the protein. Transfer the solution to 40 ml centrifuge tubes, and centrifuge in JA-17 rotor at 16,000 rpm for 30 min. Transfer the supernatant to a clean 50-ml Corning tube.

(7) Equilibrate a glutathione column with PBS + 1 mM DTT. Use 0.5 mL to 1 mL glutathione beads (GE Health Sciences) for each liter of culture. Load the supernatant onto the column controlled by gravity, and wash with PBS+1 mM DTT at least 10x bead volume. Alternatively (this may go faster), resuspend the matrix in the lysate in a 50-ml tube, and rock gently at 4 °C for 1-4 hours. Pack the matrix by short centrifugation (swing-bucket rotor, 2,000 rpm, 1-2 min) and wash it 3 times with 20 mL PBS+1mM DTT each time. Then transfer the matrix to a column. Wash the loaded column with additional buffer (5x volume of matrix).

(8) Elute the GST-FRB protein with elution buffer of 5-10x bead volumes. Elution buffer should be either freshly made or from frozen: 10 mM glutathione (reduced) in 50 mM Tris-HCl, pH 8.0. Take out a small aliquot of the eluate (e.g. 20 ul) for analysis by SDS-PAGE later.

(9) Add thrombin (Sigma, T4648, 1mg powder for ~10mgs protein) to the eluate together with CaCl<sub>2</sub> to 10 mM, and transfer to a dialysis bag (MW cutoff = 3 kDa), dialyze against PBS+10 mM CaCl<sub>2</sub>+1mM DTT (2 liters dialysis buffer for 10 mL eluate) overnight at 4 °C.

(10) Transfer the solution from dialysis bag to a clean tube. Check the protein by SDS-PAGE for extent of cleavage. If the protein is less than 70% cleaved, add more thrombin to the solution and continue cleaving overnight (no need for further dialysis). Save a small aliquot for SDS-PAGE later.
(11) Pass the cleaved protein through an equilibrated glutathione column and collect the flow-through. For ultra-pure protein prep (e.g., for crystallographic studies), go to Step 12. If ~90% purity is acceptable, go to Step 13.

(12) If the volume is significantly more than 12 mL, concentrate the solution in a 50-ml stir-cell ultraconcentrator (Amicon) to approximately 12 mL. Load this concentrate (12 mL or less) to an equilibrated (with PBS+1mM DTT) Sephacryl S-100 column (2.5 cm x 100 cm), fill the column with buffer and run it at a flow-rate of 70 ml/hr (P-1 pump setting at 10x 3). Start the fraction collector when starting the pump. Collect fractions at 8 min/each (~10 mL). GST peaks around #25-26, FRB peaks around #33-34. Run SDS-PAGE to determine fractions containing pure protein, pool these fractions. Determine the concentration by OD_{280}. If necessary, concentrate the solution in a 50-ml or 15-mL stir-cell to a desired concentration.

FRB: E=29220

(13) Alternatively, pass the protein solution through the glutathione column (same as the one used for purification) 1-2 more times – each time the column should be eluted with glutathione (to remove free GST) and re-equilibrated with PBS.

How to regenerate glutathione columns:
1) Elute the protein (either GST fusion or GST) with glutathione as described above;
2) Pass 2x bed volume of 0.1 M Tris-HCl, 0.5 M NaCl, pH8.5;
3) Pass 2x bed volume of 0.1 M sodium acetate, 0.5 M NaCl, pH4.5;
4) Repeat steps 2 and 3 three or four times;
5) Wash the column extensively with PBS (at least 10x bed volume).