

Extracting Recombinant CaM, AFP-CaM, and AFP-CaMBD Fusion Proteins

1. Grow overnight culture (5 to 10 ml) in LB containing 50 $\mu\text{g/ml}$ (LB-Amp₅₀ or LB-Kan₅₀) of the appropriate antibiotic and 1% glucose at 30°C. (Start *late* in the day to be certain that cultures do not over grow-- A_{600} should ideally be 1-1.5).
2. Inoculate 250 ml of fresh LB-Amp₅₀ or LB-Kan₅₀ (**pre-warmed to the growth temperature!**) with 5-ml of culture and grow at 30 to 37°C until the cells reach a density of $A_{600} = 0.6$. Grow CaM or CaM fusions at 37°C. CaMBP fusions are somewhat less soluble and usually express better at 30°C; some require growth at room temperature.
3. Add IPTG to a final concentration of 0.5 mM (30 mg/250 ml) and grow an additional 3-4 hr for cells grown at 37°C or 4 to 16 hr for cells grown at 30°C. Note that we have also used the Novagen Overnight Induction Kit with **excellent** results as an alternative to IPTG.
4. Collect cells at 5,000 *g* for 10 min.
5. Drain the pelleted bacteria well by inverting the centrifuge tube on paper toweling for a few minutes. AFP-CaM fusion protein pellets will usually be fluorescent under room light, except for BFP-CaM (visualize BFP with a long wave UV lamp).
6. Suspend the cells in 5 ml of Extraction Buffer (recipe below) by pipeting up and down repeatedly to break up clumps of bacteria. When an even suspension is formed, pipet in 5 ml of B-PERII (Pierce) with swirling.*
*If desired, the cells can be quick frozen at this stage in buffer, stored at -20°C, and thawed and processed at a later time. Note that proteins expressed in E. coli B strains (e.g., BL21(DE3)) require more extraction buffer than those expressed in K12 strains.
7. Incubate the suspension at room T with gentle shaking or rocking for 15 to 30 min.
8. Sonicate the suspension briefly to shear the bacterial DNA, if necessary; this is most commonly needed with E. coli B strains. The best way to judge the time needed is to give short bursts (10 to 15 seconds) with an equal time between each burst in an ice bath. Test after each burst whether the suspension will form drops at the end of a Pasteur pipet. When the solution viscosity has been reduced enough to allow drops to form by this test, the suspension can be cleared by centrifugation.
9. Centrifuge at 27,000*g* for 30 min at 4°C (15,000 rpm in Sorvall SS34, Beckman JA20 or equivalent).

At this point, CaM and AFP-CaM proteins are purified by Ca^{2+} -dependent hydrophobic affinity chromatography (next section), while AFP-CaMBD fusion proteins are purified by Ca^{2+} -dependent CaM-Sepharose affinity chromatography or NiNTA-agarose affinity chromatography for proteins harboring a His-tag.

Extraction Buffer

Tris base (Trizma) 6.06 50 mM
Dissolve in 800 ml H₂O and adjust pH to 7.5 with HCl.
Bring to a final volume of 1 L with H₂O.

Autoclave.

Just before using, add the following to 50 ml of Extraction Buffer:

Dithiothreitol (DTT) 15 mg 2 mM

Complete Protease Inhibitor Cocktail (Roche) without EDTA 1 tablet

Use this mixture shortly after adding the DTT and Protease Inhibitors; any unused solution can be stored at -20°C.

Purifying CaM and AFP-CaM

1. Add CaCl_2 to a final concentration of 2 mM and mix by swirling. Heat the protein extract at 65°C for 3 minutes, then immediately chill the solution by pouring the extract into a clean centrifuge tube **previously chilled in an ice- H_2O bath**.
2. Remove particulate matter (there will be a lot!) by centrifuging at $27,000g$ for 15 min at 4°C .
3. Apply the sample to a Phenyl-Sepharose (high sub) column (2.5 to 5 ml bed volume for protein extracted from 250 ml of induced cells) equilibrated with 10 bed volumes (25 to 50 ml) the following:

Equilibration/Wash Buffer I	Amount for 1 liter
50 mM Tris-HCl pH 7.5	6.06 g
200 mM NaCl	12 g
2 mM CaCl_2	20 ml of 0.1 M
0.5 mM DTT	77 mg

4. Wash the column with Equilibration/Wash Buffer I until the $A_{280} < 0.01$. Usually 5 column volumes of buffer are sufficient.
5. Wash again with Wash Buffer II until the $A_{280} \sim 0$. As in the previous step, 5 column volumes are normally sufficient.

Wash Buffer II	Amount for 500 ml
50 mM Tris-HCl pH 7.5	3.03 g
0.5 mM CaCl_2	2.5 ml of 0.1 M
0.5 mM DTT	39 mg

6. Elute calmodulin from the column with Wash Buffer III:

Wash Buffer III	Amount for 500 ml
50 mM Tris-HCl pH 7.5	3.03 g
1 mM EGTA	10 ml of 50 mM
0.5 mM DTT	39 mg

7. Pool the fractions with the highest A_{280} (or fluorescence for AFP-CaM fusion proteins) and dialyze *vs.* water (CaM only) or 10 mM HEPES/KOH pH 7. Note that the UV absorbance of CaM is **VERY LOW** because of its low content of aromatic amino acids! CaM is stable for several days to a week or 2 at 4°C . During that time, the protein can be assayed by SDS-PAGE.
8. **For CaM:** Lyophilize or store as dialyzed liquid (after quick-freezing in liquid N_2) at -80°C . If the concentrations are low, ultrafiltration in an Amicon concentrator (the stirred-cell type driven by N_2) equipped with YM10 membranes works well to concentrate the protein with minimal loss; Centricon 10 filters also work well. Lyophilized CaM is almost impossible to dissolve in distilled H_2O because it is so acidic; use either dilute buffer at neutral pH or "a few" drops of a solution of 10 mM Tris base to suspend and dissolve the dried protein.
For AFP-CaM: Store as dialyzed liquid at 4°C (one of the few proteins more stable than CaM is GFP, which is reported to be brightly fluorescent many YEARS after isolation when stored at 4°C !). Concentration using a stirred Amicon cell or a centrifugal membrane filter (Centricon 30) can be used to concentrate the protein. More recently, we've found that storage at -80°C is fine as well, particularly if the protein is snap frozen in powdered dry ice or liquid N_2 .
9. Measure protein concentration by Bradford or BCA assay. Typical yields of purified CaM are 15 to 50 mg L^{-1} of induced bacteria. AFP-CaM yields are lower, but often $>5 \text{ mg L}^{-1}$.