Antibody Production and Denaturing Purification of proteins not soluble in PBS

James Fishburn, Hahn Lab March 2006

Proteins should be submitted at 1.0 mg/ml (4 mg total) in PBS

A small quantity of purified denatured protein should be kept for testing the generated antibodies

If proteins are not soluble in PBS they can be purified under denaturing conditions, dialyzed in PBS, sonicated, and submitted as precipitates. Apparently, an emulsification process that the proteins go through before injection allows this to work.

Proteins with a 6-His N-terminal tag are expressed from pET21(a) in BL21(DE3)RIL cells

Denaturing Buffers

Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, 6 M Guanidinium-HCl, 20 mM imidazole pH 7.4

Wash buffer: 20 mM sodium phosphate, 0.5 M NaCl, 8 M urea, 20 mM imidazole pH 7.4

Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 8 M urea, 500 mM imidazole pH 7.4

Day 1

Grow 10+ ml culture overnight

Day 2

1. Inoculate 1 L media with 10 ml saturated overnight
2. Grow cells to OD600 0.6 (ca. 3hrs at 37 deg)
3. Induce cells with 1 mM IPTG
4. Continue incubation for 3 hrs at 30 deg with shaking
5. Harvest cells- 5' spin at 5000 rpm in GSA
6. Resuspend cell pellet in 30 ml Binding buffer + 10 mM beta-mercaptoethanol
7. Incubate extract at RT with gentle mixing until translucent
8. Clarify extract by centrifugation: 20' spin at 10,000 rpm in SS-34

9. Transfer supernatant to 50 ml tubes, freeze in N2, and store at -70 deg

Day 3

1. Thaw samples in RT water bath
2. Make Wash and Elution buffers during thaw
3. Prepare Ni-Sepharose: wash with 5 volumes DI water, wash with 5 volumes Binding buffer, resuspend Ni-Sepharose to 50% in Binding buffer (spins are at 500 x g for 2'-5')
4. Add 2 ml 50% Ni-Sepharose slurry to extract
5. Incubate extract with resin for 30' at RT with mixing (nutate)
6. Collect resin by centrifugation, remove supernatant and save (flow through)
7. Wash resin a total of three times using 5 volumes of Binding buffer for each wash
8. Add 2 volumes Elution buffer to resin and incubate 5' at RT with mixing (nutate)
9. Collect resin and save first elution
10. Repeat elution step 2-4 more times saving each elution
11. Analyze purification by SDS-PAGE (4-12% Bis-Tris, MES) and CBB staining- 2 ºl of each elution per lane is sufficient for gel staining along with 1 ºl each of crude lysate, clarified lysate, and the flow through
12. Combine desired elutions
13. Determine concentration of protein by Bradford assay
14. Dilute protein to 1 mg/ml in PBS + 3 M urea
15. Dialyze 5 ml of protein in 500 ml PBS + 1 mM PMSF + 1 mM DTT for 1 hr at RT
16. Repeat dialysis two more times (3 x 1hr total): protein will precipitate
17. Transfer dialyzed and precipitated protein to 12 ml Falcon tube (#352059)
18. Put protein on ice
19. Sonicate protein using small tip for 2 x 20 second pulses with 1 minute incubation on ice between pulses
20. Divide protein into 5 x 1 ml aliquots in 1.5 ml tubes
21. Freeze and store at -70 deg

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