PURIFICATION PROTOCOL FOR BARNASE xHA102

Cell Strain: M15

Plasmids: barnase (w/ or w/o cytochome b_2 targeting sequence) in pQE60 (Amp resistance)
lac repressor in pREP4 (Kan resistance)

Notes:

1. Throughout the protocol samples are taken. These should be run on gels to ensure that the protocol is proceeding as expected. Also, try to keep the number of times the samples are quick frozen to a minimum.

2. This protocol also works well to purify barstar from pQE60 in M15(pREP4) cells. However, all the buffers used for barstar should have a pH at least higher than 5. Usually, 50mM MOPS pH7.0 buffers works well.

Day 1

Streak +Amp +Kan plates with clones from -80°C freezer
-put in 37°C incubator overnight

Day 2

1. Inoculate 25 ml +Amp +Kan LB in test tubes as a pre-culture in the early in day 2 (8 am recommended).

2. Twelve hours later (8 pm) centrifuge pre-culture at 8,000 rpm, 4°C for 10 minutes.

3. Resuspend pellet in 25 mL of M9 minimal growth media. Measure OD_{600} and inoculate cultures of 1:100 and 1:200 dilutions in minimal growth media M9 + amp +kan + amino acids (minus leucine).

4. Let cultures expand in 37°C shaker overnight. Next day, determine OD_{600} at 9:00 am.

Day 3

1. First thing in morning, take the OD600 of the two cultures (1:100 or 1:200) and determine which is closest to but less than OD600 = 0.4. Continue with this culture. (in my experience it was the 1:100 dilution).

2. When this culture expands to an $O.D._{600}$ of 0.4 (Mondragon lab spec) in 37°C shaker, take a 1 mL sample, then induce with IPTG to final concentration of 200 mg/mL for 4 hrs
   -make IPTG solution directly before use
   -make 1000X concentration: 0.2 g IPTG/1 mL dH_2O

Take 1 mL sample immediately after adding IPTG. Shake at 37°C.

2. 20 minutes post induction, pulse culture with Leucine. (100X concentration of leucine is 40 mg/10mL).

3. Pellet entire culture at 5000 rpm for 30 min at 4°C in Sorvall H-4000 rotor.

4. Resuspend pellet in Breaking Buffer (See note in Buffers section), at 4 ml/wet gram cells. Add appropriate amounts of fresh PMSF and Benzamidine to breaking buffer before use.

[Can stop here if necessary. Quick freeze samples in liquid nitrogen or dry ice/EtOH. Store at -80°C]

Day 4

[Quick thaw samples in 30°C water bath, if necessary
-store on ice]

Sonicate on ice with large sonicator tip on 50% duty cycle, output 8, 8 sets of 20 pulses

Spin at 19,000 rpm for 20 min at 4°C in Sorvall SA-300 rotor

Resuspend pellet in Washing Buffer (See note in Buffers section) 4 ml/wet gram pellet

Sonicate on ice as before
-As the volume of the sonicated solution decreases, it is advisable to use the small sonicator tip. When using small tip, set output to 4, NO HIGHER THAN 5.

Spin at 19,000 rpm for 20 min at 4°C in Sorvall SA-300 rotor

Repeat [wash, sonication, and spin] once

Resuspend final pellet in fresh Extraction Buffer at 4 ml/wet gram pellet

-use hand homogenizer
-ultracentrifuge at 32,500 rpm in Beckman SW 55 Ti rotor (≈ 130,000×g) for 1 hr, 4°C

Quick freeze samples in liquid nitrogen or dry ice/EtOH. Store at -80°C]

Immerse a certain length of dialysis membrane (Spectrum® MWCO 3000) in water overnight.

Day 5

[Quick thaw samples in 30°C water bath

-store on ice]

Put the protein into the Spectrum® dialysis bag prepared as mentioned above and dialyze it against the dialysis buffer (See note in Buffers section) for three hours in the cold room.

Spin down the aggregate from dialysis at 19,000rpm with SA-300 rotor for 20 min at 4°C.

Dilute supernatant into 50 ml dilution buffer (See note in Buffers section) using pipette tips, mixing buffer vigorously, at 4°C

Spin down the aggregate from dilution at 19,000rpm with SA-300 rotor for 20 min at 4°C.

Concentrate supernatant sample with 50ml Amicon stirred-cell at 4°C. Once the volume is smaller than 10 ml, add 4-5x volume of the solution that you want to store the protein in.

- For those proteins that can form dimers through disulfide bonds, store them in the buffer containing 5mM DTT

Make a 1:10 dilution and take the $O.D._{280}$ to measure the protein concentration and flash freeze and store in the -80°C freezer.

The final protein yield is usually 8-10mg out of 1L of culture.

Buffers

Breaking Buffer
50 mM NaOAc/HOAc buffer, pH 5.0
1 mM PMSF (added freshly)
2 mM EDTA
5 mM benzamidine (added freshly)

Washing Buffer
50 mM NaOAc/HOAC buffer, pH 5.0
1% (w/v) Triton
0.2 M NaCl
1 mM PMSF (added freshly)
5 mM benzamidine (added freshly)
2 mM EDTA

Extraction Buffer (make freshly)

50 mM NaOAc/HOAc buffer, pH 5.0
6 M Guanidine Chloride
0.1% (w/v) Triton
0.5 mM PMSF
2 mM EDTA
5 mM DTT
1μg/ml Leupeptin (from 1mg/ml stock, -20°C freezer)
1μg/ml Antipain (from 1mg/ml stock, -20°C freezer)

Dialysis Buffer
50 mM NaOAc/HOAc buffer, pH 5.0
1.5 M Guanidine Chloride
0.5 mM PMSF (added freshly)
2 mM EDTA

Dilution Buffer
50 mM NaOAc/HOAc buffer, pH 5.0
2 mM EDTA
5mM DTT

ver. June 24,1999