Double Mutant (CA40,82) Barstar Purification

pMT643 plasmid in JM109 cells , tac promotor

last modified 05/20/99 by Sherry Black

You may want to try growing the plasmid in JM105 cells, to see if they grow significantly better. The barnase clones under the tac promotor grow significantly better in the JM105 cells.

Day 1

Freshly transform or streak a 2xTY + amp plate from the permanent stock (clone#745 Box 9 G8) in the late afternoon. Incubate overnight at 37C.

Day 2

Innoculate starter culture in the late afternoon with a nice large round colony from the plate and grow overnight in 2xTY + amp at 37C 200rpm. Make media for tomorrow's innoculation.

Day 3

Innoculate 1:100 with the starter culture early in the morning. Monitor the OD600 and induce when it starts to double with filter-sterilized IPTG dissolved in water to a final concentration of 1mM. With this dilution ratio the cells should start to double in a couple of hours (OD600 should be above 0.3). Take a 500uL sample of the preinduced cells. Save 50mL of your culture in a 250mL baffled flask to incubate without inducing. Monitor induction and harvest in 3-5 hours, when the uninduced cells cease to double. The induced cells should be growing significantly slower than the uninduced cells. This is a sign that the induction is working. Just before harvesting take 500uL samples from the post induction cultures. Harvest the cells by spinning at 5,000Xg, 10min, 4C. Take a 500uL sample of the supernatant and discard it. Resuspend cells in a total of 40mL/L (of original culture) of 50mM Tris buffer pH 8.0 plus 1mM PMSF. Freeze in –25 freezer overnight.

Day 4

Thaw cells and sonicate at intensity 8, 0.5 sec "on", 0.5 sec "off", 30sec total "on" for 5 or 6 times. The solution should turn darker brown and more clear and glassy. Ultracentrifuge at 22,000rpm (65,000xg) in Surespin 630 rotor for 90 minutes. Save supernantant, and also a sample of the pellet to run on a gel and make sure the barstar is in the supernatant (it should be). Precipitate barstar by adding ammonium sulfate slowly, while stirring vigourously in the cold room, to a final concentration of 80%. Set up a gel filtration column to run your supernatant sample through. Run prep samples on a polyacrilimide gel.

Day 5

Run gel filtration column with 50mM Tris/50mM NaCl pH 8.0. Syringe-filter the protein sample before applying to the column. Collect fractions and run them on a polyacrilimide gel. Combine fractions containing the barstar and prepare for an ion exchange column (Pharmacia Mono-Q)

Day 6

Run ion exchange column with Buffer A:50mM Tris pH 8.0 Buffer B: 50mM Tris/1M NaCl pH 8.0. Barstar should elute at 200-300mM NaCl.

Run column samples on a gel (5uL of each sample). Take OD260 and OD280 of barstar fraction to determine concentration. DNA contamination is minimal if OD260<OD280. Barstar ext. coeff. 280 = 22,690 M-1cm-1, A280/ext. coeff. 280 = molar concentration. Barstar M.W. = 10211.59. Should get a couple mg of barstar per FPLC run.