

PI-PLC Purification

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1. Inoculate with an overnight culture 1 liter of LB media + 1 X ampicillin. (100ug/ml)
2. Grow at 37 degrees to an OD of approx. 1.0 at 600 nm.
3. Induce by adding IPTG to 1mM.
4. Grow at **30** degrees for 16 hours.
5. Spin down cells in large bottles in GSA rotor: 4,000g x 15 minutes at 4 degrees (5,000 rpm in this rotor).
TAKE BOTTLES BEFORE USE! Do all remaining steps keeping solutions cold.
6. Dump supernatant and resuspend cells in COLD solution 0.01 M Tris pH 8.1, 0.4 M NaCl. Use 60mls/gm of cells.
7. Shake bottles on a rotator in ice bath for 45 minutes.
8. Spin cells 12,000g x 10 minutes at 4 degrees (9,000 rpm in GSA).
9. Keep supernatant and put aside in cold! Resuspend pellets in ice cold ddH₂O. Again use 60 mls/gm of cells. Shake on ice for 45 minutes.
10. Spin at 12,000g x 30 minutes at 4 degrees.
11. Add supernatant to above supernatant and discard pellet. (If you run a SDS-PAGE you can find that the water fraction has much more protein than the salt fraction, so you can just discard the salt fraction and run the water fraction through the column, in this way you need not to dialyse the solution which is very good!)
12. Concentrate the supernatant down to around 50 mls using an amicon YM10 filter. (Concentrate to ~30 mls if you don't want to go through column step.)
13. Load over a DEAE cellulose column with a flow rate of about 1 ml/ min. keep flow through. (I use DEAE sephorose fast flow column to do the purification, which is very good, and there is no need to concentrate the supernatant, you just run it through the column.)
14. Elute column with 400 ml. .02M Tris (pH 8.5) + 400 ml 0.02M Tris 0.2M NaCl gradient with same flow rate. About 100 8ml fractions will come off.
15. Read O.D. 280nm of each fraction, there should be two main peaks, run SDS-PAGE, the first peak is what we want, the MW is ~35kd.
16. Pool the fractions, concentrate down to over 1mg/ml in low salt buffer(pH8-8.5, 0.02 M Tris-Cl). Do a BCA essay to examine the exact protein concentration. Aliquot and store at -20 degree.

Alternate procedure for PI-PLC activity.

*15. Assay fractions for activity (every fifth one).

16. Pool active fractions and concentrate to around 1 ml.

Past experiences: 137 fractions-- #50-80 pooled

 III fractions-- #30-70 pooled

 100 fractions -- #15-65 pooled

17. Assay PIPLC using a 6 well dish of PI-linked cells using 1, 2.5, 5, and 10 microliters of PIPLC.

*assay: 1.To 5ul sample add 200ul substrate ----->25mM Hepes(pH 7.4)

2.5mM EDTA

0.1% DOC

0.75ul ³H PIP(inositol phosphate)

2. Incubate in 37 degree waterbath for 30 minutes. Keep 5 ul for step 4.
3. Extract the rest with 0.75 ml 1:2 chloroform:methanol
 - 0.25 ml chloroform
 - 0.25 ml 1M HCl

Vortex and centrifuge for 2 minutes

4. count in scintillation counter
 - 10ul aqueous fraction (approx: 950 ul total)
 - 10 ul organic (bottom) fraction (approx. 450 ul)
 - 5ul from step 2

The fractions with the most activity should have a higher aqueous to organic counts ratio. REMEMBER to account for different volume percentages that you are counting before you calculate ratios.