Expression of the PCR product: Thursday 3/6/03 /

(following p. 16 → on of TOPO expression kit manual) Friday, 3/7/03

- Transformation
  - The purified DNA from miniprep # 1 was transformed into One shot® BL21 (DE3) cells.
  - The entire transformation rxn was then added to 10 mL of LB media w/ carbenicillin and incubated with shaking at 37° C overnight.

- Pilot Expression
  - 10 mL of LB w/ carbenecillin were innoculated w/ 500 mL of the above overnight culture and grown for 2 hours at 37° C with shaking.
  - 1 mL was put aside for a glycerol stock
    glycerol stock: { 1.0 mL cell culture
    350 µL sterile 60% glycerol
    stored in a cryovial at -80° C
  - The culture was split into 2 4.5 mL cultures. 4.5 µmol (4.5 µL of a 1M solution) of IPTG was added to one of the cultures. Thus I have 1 induced and 1 uninduced culture.
  - Following procedure on p. 18, 5 timepoints were taken (in 500 µL aliquots) for each culture:
    t=0, t=1, t=2, t=3.5, t=5 hr

- Visualization of protein by polyacrylamide gel electrophoresis:
  - samples from above were prepared by added to the cell pellets from above 40 µL dH_2O and 40 µL 2× SDS-PAGE sample (blue loading buffer.) The pellets were resuspended by pipeting up and down and then vortexing. → then centrifuged
  - The samples were boiled for 5 minutes, about 10 seconds.
  - 10 µL were loaded into each well. Two gels were made (one for Coomasie and one for a Western blot)

- Resolving buffer + staking buffer →
  Acryl gel buffer → 1.5 M Tris HCl pH 8.8 (regular)
  → 0.5 M Tis HCl pH 6.8 (stacking)

- For about a 60 KDa protein, a 10% gel was made.

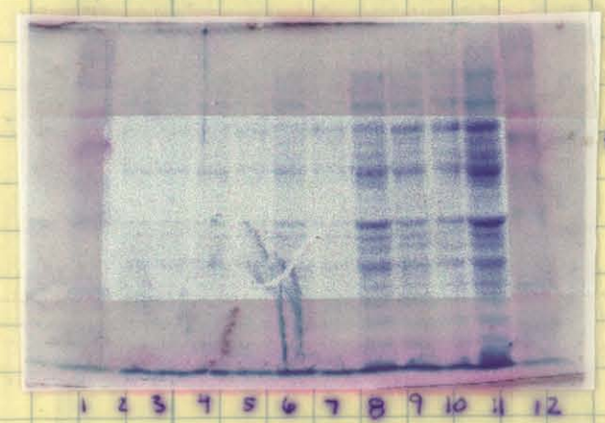
The APS should be made fresh if you are going to use a 10% solution. This is only good in solution for 1-2 weeks. I use a very small amount of the dry APS added directly to the gel solution. This works fine, but it may take a little practice. If you add too much, the acrylamide will polymerize too quickly. It does however, save you the agony of pouring a gel that will not polymerize because the APS has gone bad. Likewise, the temed can go bad, but it takes much longer.

The APS provides free radicals that drive polymerization of acrylamide. The temed acts as a catalyst for polymerization by accelerating the formation of free radicals from APS. In other words, these two should be added immediately before you are going to pour the gel. I usually add the temed first and then the APS. If you are adding the APS dry, then make sure that it all gets into solution before you pour the gel.

The following recipes are for 2 minigels. Please note that they are made with 40% acrylamide, not 30% acrylamide (the recipe given above). The 40% stock is purchased in solution from Continental Labs (#5424.500):

Percent gel:	10%	12%	15%	Stacking (4%)
40% acrylamide	3 ml	3.75 ml	4.5 ml	675 ul
Acry. gel buffer	3 ml	3 ml	3 ml	1.67 ml (stacking buff)
10% SDS	120 ul	120 ul	120 ul	67 ul
ddH_2O	5.8 ml	5.1 ml	3.74 ml	4.1 ml
10% APS	60 ul	60 ul	60 ul	33 ul
TEMED	4 ul	4 ul	4 ul	3 ul

- the polyacrilamide gel was run for 2 hours at 100 V in 1 x TANK buffer. Gel stained w/ Coomasie blue (the destained overnight)

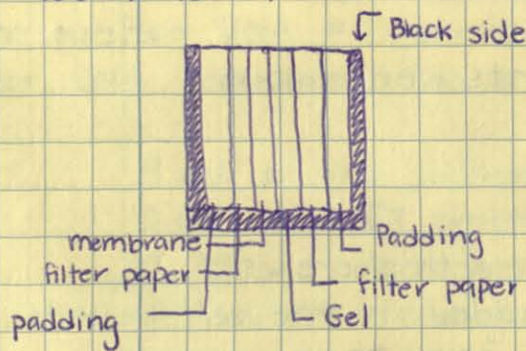


← pink = 63,000 Da

In 1	protein ladder
2	t=0 uninduced
3	t=0 induced
4	t=1 hr uninduced
5	t=1 hr induced
6	t=2 hr uninduced
7	t=2 hr induced
8	t=3.5 hr uninduced
9	t=3.5 hr induced
10	t=5 hr uninduced
11	t=5 hr induced

- The samples were stored at -20° C.

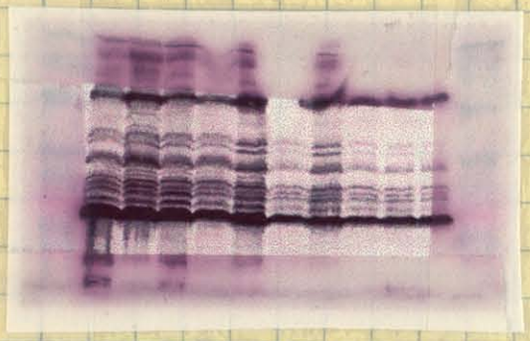
- Western Blot Analysis:
  - The gel protein was transfered to a membrane running at 100 V for 1 hour in transfer buffer



(remember to add ice pack before starting the transfer)

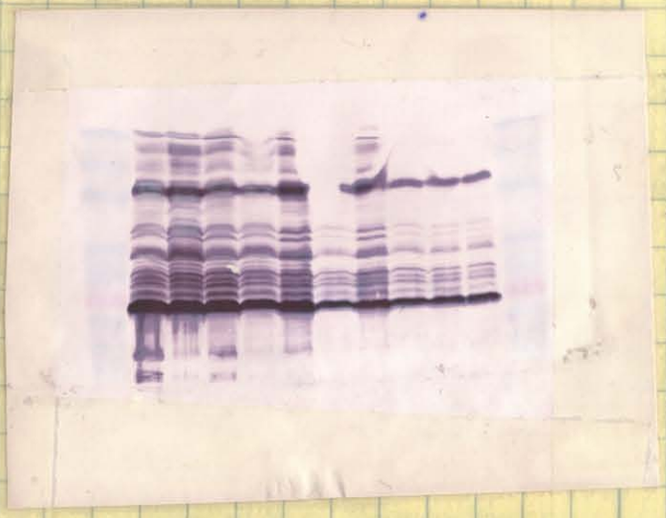
- The gel was placed in a plastic container (ie. the top to pipet container) and rocked in a concentrated solution of powdered milk in TBST buffer. The container was wrapped in saran wrap and agitated overnight.
- 1/3000 (3 uL in 9 mL) of the 1° antibody (here anti His) was added. Membrane was blocked 1 hr. [The antibody can be reused a few times if kept at 4° C. Probably shouldn't be frozen)
- washed 4 x 15 min. w/ TBST
- incubated w/ 2° antibody (goat anti mouse IGG) 1/1000 in TBST 1 hour.
- washed 4 x 15 min. w/ TBST, then 1 x 15 min w/ TBS.
- Developing agent was added.
The membrane was rinsed well w/ dH2O, then scanned.

Saturday, 3/8/03



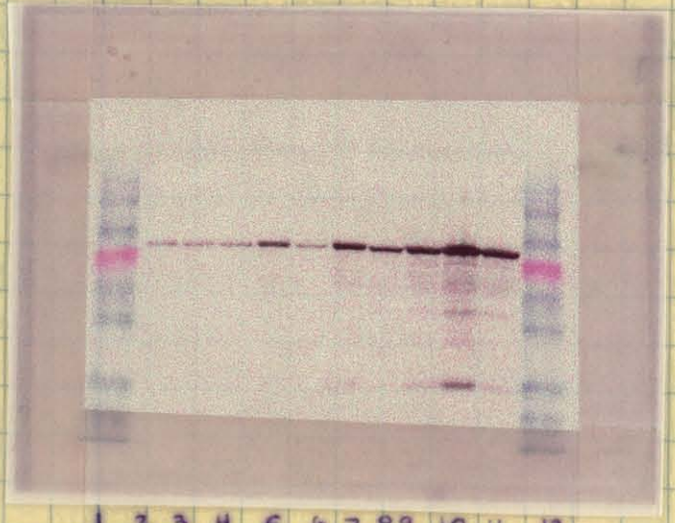
12 11 10 9 8 7 6 5 4 3 2 1

← see other pg. for lane assignments

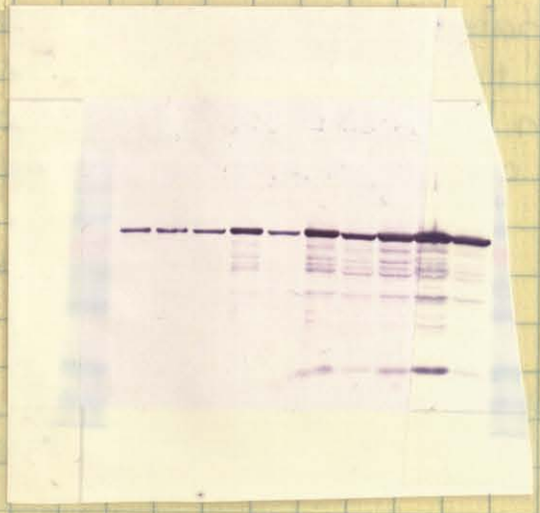


- It looks like a TON of nonspecific binding is taking place. This may in part be due to too much protein being loaded onto the gels (10 uL) and the fact that the membrane was blocked w/ a fairly diluted amount of milk in water (instead of a concentrated solution of milk in TBST.)

- New gels were made and this time loaded w/ only 5 uL of the protein / cell lysate mixture. A second Monday 3/10/03 western blot analysis was also done (this time blocking w/ alot of powdered milk in TBST.)



1 2 3 4 5 6 7 8 9 10 11 12



(4th) lysis and Purification of Pellet from p. 3-96: Lysis with 6 M Guanidine: Ni column purification using Urea at several pHs 04/09/03

Lysis: Cell pellet was removed from the -80°C freezer and thawed for several minutes at 4°C in the cold room. To the pellet in the centrifuge tube, 20 mL of the guanidine lysis solution (6 M guanadine·HCl, 300 mM NaCl in a 25 mM Tris buffer, pH 8.0) was added and stirred in the cold room ≈ 1.5 hr (until cell solution looked a bit clear.

- The lysed cells were spun down for 20 min at 15,000 rpm and then the supernatant was decanted (hopefully containing the protein) into a 50-mL tube. The solution was mixed for 15 min w/ 3 mL Ni solution (so 1.5 mL resin)

- The resin was loaded onto the column. Elution from the Ni column was achieved by lowering the pH (thus protonating the His residues such that they no longer bind the Ni). Since guanidine is not compatible with our gel procedures, this washing and elution wash done with urea. First the column was washed 15 mL 8M urea, 100 mM NaH_2PO_4, 10 mM Tris at pH 8.0. The column was then washed with 10 mL of 8 M urea, 100 mM NaH_2PO_4, pH 6.3 in 10 mM Tris.

- The protein was eluted with 10 1-mL fractions of 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris at pH 4.5

protein ladder ↑ fr1 fr2 fr3 fr4 fr5 fr6 fr7 fr8 fr9 western w/ anti-human pax

Grr! Even though the guanidine should have immediately denatured everything (including all proteases!) the gels show tons of degradation. The yield seems a bit better than before (as in something is visable on the Coomasie), but there are way too many junk bands.