ABRF 2009 Satellite Educational Workshop

(sw3) Recombinant Protein Laboratory

February 6-7, 2009

Being held at

Protein Production Facility

St Jude Children's Research Hospital

PROTOCOL HANDBOOK

Day 1.

Growth of bacteria

You will be provided with:

- an overnight culture of *E. coli* harboring the plasmid pTrcTopoHis into which the gene for yeast ADH1 has been cloned.
- A bottle of LB media
- A 2.8 L Fernbach flask
- 50 mg/ml ampicillin in 70% ethanol
- 1 M solution of IPTG

1. Place 1 L of the LB media to a 2.8 L Fernbach flask. Add ampicillin to 50 µg/ml (1 ml per L).
2. Remove 1 ml of the media and use to blank spectrophotometer with at 600 nm.
3. Add 1 ml of the starter culture to the flask and place in the 37°C shaking incubator.
4. Monitor growth of culture by ascetically removing 1 ml samples at approximately 1 h intervals and reading at 600 nm.
5. When OD reaches approximately 0.5 to 1.0:
   a. Remove a 0.5 ml sample to an eppendorf tube. Spin and save the pellet at 4 or -20°C (label "-")
   b. Add 0.5 ml of the 1 M IPTG to the flask and return it to the incubator
6. Continue growth of induced culture for approximately 3 h. There is no need to measure the OD, as induced cells will only double about once more. One group could place their cells in a 16°C incubator and continue growth overnight.
7. At the end of incubation time, remove 0.5 ml to an eppendorf, spin and save pellet (label "+"). Harvest remaining cells by centrifugation. Store pellet at 4 or -20°C until ready to use.
8. During the morning, we will also make buffers for tomorrow:

MCAC 0 Buffer

Compound	g/L	Final Conc
Trizma Base	2.42	20 mM
NaCl	29.22	0.5 M
Glycerol	100	10 % (w/v)
PMSF	1 ml	1 mM

Procedure: Weigh glycerol directly into a 1 L beaker. Add Trizma and Nacl, and enough water to make volume up to about 800 ml. Stir to get everything dissolved. Adjust pH with HCl to get 7.9. Make up volume to 1 L. Filter through a 0.22 micron bottle top filter.

MCAC-1000:

Compound	g/L	Final Conc
Trizma Base	2.42	20 mM
NaCl	29.22	0.5 M
Glycerol	100	10 % (w/v)
Imidazole	68.08	1 M
PMSF	1 ml	1 mM

Procedure: Remove 250 ml of MCAC 0 buffer. Add 17 g imidazole. Stir to dissolve and filter.

DAY 2.

Cell Lysis by microfluidizer

Place cell pellet into a small beaker.

Add 10 – 20 ml of MCAC 0 buffer per L of culture. Thoroughly suspend cells by pipetting up and down.

Microfluidizer Instructions (NOTE: psi = Gauge reading × 230)

1. Pack water bath with ice/water slurry and allow to chill before using.
2. Place beaker under drain.
3. Remove stopper and plunger.
4. Check there is isopropanol in the chamber (about half full).
5. Push plunger down halfway to liquid level. Replace stopper
6. With the valve in re-circulate, and holding gently onto the stopper, start the pump slowly (15-20 psi). Run for several cycles to remove air.
7. Stop pump, remove plunger.
8. Start pump again, observe liquid in chamber. Should rise and fall gently. If not, repeat from step 6.
9. Remove plunger. Set valve to drain. Turn on pump slowly and collect effluent. Turn pump off.
10. Fill reservoir with about 15-20 ml buffer. Turn on pump slowly and flush system. Repeat.
11. Place sample in reservoir. Adjust pressure to desired (16,000 psi = reading of 70). Mimimum volume = 14ml.
12. Turn on pump. Discard first 6 mls. Collect lysate. Stop pump.
13. Add buffer and collect one more pump of the piston (ca. 6 mls).
14. Wash entire system with water (refill reservoir several times) and then isopropanol. Leave in isopropanol. Drain water bath. Leave plunger in to prevent evaporation.

Cell lysis by detergent

1. Thaw cell pellet in beaker.
2. Add 100 ml of SoluLyse (Genlantis) per L of original culture. Mix thoroughly by pipetting but do not vortex!
3. Incubate at room temperature for about 10 min with occasional gentle agitation.

Centrifugation

1. For both methods of cell lysis, transfer to a centrifuge tube or two and balance.
2. Spin at > 27,000 × g for 15 to 30 min.
3. Remove supernatant (with soluble proteins) from pellet soon after 'fuge has stopped.
4. Filter through a 0.22 or 0.45 µm filter if the protein is going to be purified by AKTA.
5. Transfer about 100 µl of the supernatant to a clean eppendorf tube, labeled "load".

Purification by bench-top column

While cell extracts are centrifuging, start to prepare your column.

- An empty BioRad column
- A retort stand
- Beaker
- Pipettes and pipettor
- Ni-NTA resin from Qiagen or Talon resin from Clontech
- Ice bucket
- 15 ml Falcon tubes + rack
- Eppendorf tunes + rack
- Bradford Reagent

1. Attach column to retort stand and place a beaker underneath. Gently suspend the resin and pipette about 10 ml of the slurry into the bottom of the column. Do NOT run the resin down the side if possible. 10 ml of slurry will give an approximately 5 ml bed volume column.
2. Allow storage solution to drip into beaker. The columns are designed so that they will not dry out if left for short periods of time.
3. Gently layer about 25 ml of RO water onto top of column. Allow to flow through.
4. Apply about 25 ml of cold MCAC 0 to the top of the column. Allow to flow through. Cap column off until ready to load protein.
5. Apply protein carefully to top of column, being careful not to disturb the resin too much. Collect flow through in Falcon tubes.
6. Make MCAC 40 by mixing 4 mls MCAC 1000 with 96 mls MCAC 0. [MCAC 40 is 40 mM imidazole]
7. When the protein has flowed into the column, carefully layer on 20 ml of MCAC 40. Collect this wash in Falcon tubes. Check that no more protein is coming off by mixing 2 µl of Bradford reagent with 8 µl of wash. If it still turns blue, add another 10 ml of MCAC 40 and repeat.
8. Make MCAC 250 by mixing 25 mls MCAC 1000 with 75 mls MCAC 0.
9. Elute protein by carefully layering 5 ml of MCAC 250 onto column. Collect 1.5 ml fractions into numbered eppendorf tubes, or 2.5 ml fractions into numbered 15 ml Falcon tubes. Repeat with 5 ml aliquots until all protein has eluted.
10. Spot test fractions with Bradford to see where protein is.

NuPage

- 1 NuPage gel
- 20 × Running buffer
- 4 × loading buffer
- Protein Markers
- Staining solution
- Destain

1. Suspend – and + samples from cell growth in 100 and 200 µl MCAC 0, respectively.
2. Take 950 µl of the 4 × Loading buffer and add 50 µl of β-mercapto-ethanol (BME).
3. Remove 20 µl from -, +, load and each fraction that tests blue with Bradford into fresh, labeled eppendorf tubes with 5 µl of loading buffer in them.
4. Place tubes in 95°C hotblock for 5 – 10 minutes. Give touch spin to collect liquid in bottom of tube.
5. Prepare 500 ml of 1 × running buffer.
6. Assemble gel apparatus with running buffer.
7. Load 15 µl of heated sample to each well. Have at least 1 lane of marker (5 µl) per gel.
8. Run at 200 V for about 45 min.
9. Disassemble. Wash gel with water then cover with staining solution. Heat in microwave for 1 minute. Allow staining solution to sit on gel for up to 1 h. Then decant and destain gel with several changes of 30% methanol, 10% acetic acid.

Bradford Assay of Protein Concentration

- Cuvettes
- 1 mg/ml γ-globulin
- Bradford Reagent

Prepare Standard Curve.

1. Turn on the lamps. Use method file "Bradford". Use γ-globulin as the standard. Dilute to 1 mg/ml and label tube.
2. Prepare two fold series of dilutions in eppendorf tubes:
   a. Aliquot 200 µL of water into 4 tubes;
   b. Add 200 µL of 1mg/ml µ-globulin to the first tube, label as 0.5 mg/ml;
   c. Add 200 µL of 0.5 mg/ml γ-globulin to the next tube, label as 0.25 mg/ml;
   d. Add 200 µL of 0.25 mg/ml γ-globulin to the next tube, label as 0.125 mg/ml;
   e. Add 200 µL of 0.125 mg/ml γ-globulin to the next tube, label as 0.625 mg/ml;
   3. Prepare following dilutions in duplicate in glass tubes:

Final Conc. (µg/ml)	γ-globulin Dilution	µL γ-globulin	µL H_2O
0	none	0	800
1.25	0.625	20	780
2.5	0.125	20	780
5	0.25	20	780
10	0.5	20	780
20	1.0	20	780

4. Add 200 µL of BioRad dye concentrate to each standard. Vortex.
5. Read at 595 nm after 5 min, but before 1 hour.
6. Print out the standard curve and table of data.

Prepare Samples.

1. Dilute samples appropriately into 800 µL water. Final protein concentration should be between 1 and 20 µg/ml.
2. Add 200 µL of BioRad dye concentrate. Incubate between 5 and 60 min.
3. Read OD at 595 nm with method "Bradford".
4. Enter correct dilution coefficient.
5. Print out table of data.
6. Check that ODs are in the range between ~0.05 and 0.5.

Example of Dilution Factor Calculation

Given a protein of unknown concentration, set up the following tubes:

Protein + water	Use this much per assay (µL)	Dilution Factor
10 + 90	20	0.5*
	10	1
	5	2
10 + 190	20	1
	10	2
	5	4

Example: 10-fold diln x 1000 µL (vol of assay)/20 (µL/assay) = 500 to get µg/ml

Divide by 1000 = 0.5 to give mg/ml

Assay of ADH Activity

You will need:

- 100 mM Glycine, pH 8.5
- 100 mM NAD^+ in glycine buffer
- 0.5 M Ethanol
- RO Water

Procedure:

1. Equilibrate spectrophotometer at 340 nm
2. To a 1 ml cuvette add 500 µl glycine buffer; 50 µl NAD+, 200 µl 0.5 M ethanol and 200 µl water
3. Mix well and allow to equilibrate in the spectrophotometer
4. Measure a baseline absorbance for several minutes
5. Add 50 µl of purified ADH. Immediately mix well and measure absorbance.
6. Dilute enzyme and repeat step 5.
7. Convert absorbance per minute to µmoles NADH/minute using Beers law [$A=\varepsilon cl$ where A = absorbance; ε = molar extinction coefficient (6.3×10^3 $M^{-1}cm^{-1}$); c = concentration and l = length of light path (1 cm)]. Divide this by amount of enzyme added to get specific activity (mmoles NADH per minute per mg enzyme).