

**Nelson Lab Bible**  
**Revised on 9/3/2003**  
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## Common Solutions

### DULBECCO'S PBS

	g/mole	g/L	Concentration
CaCl <sub>2</sub> anhydrous	111.02	0.1	0.9mM
KCL	74.55	0.2	2.7mM
MgCl <sub>2</sub> •6H <sub>2</sub> O	203.3	0.1	0.5mM
NaCl	58.44	8	138mM
Na <sub>2</sub> HPO <sub>4</sub>	268.07	2.16	8.1mM

Dissolve all ingredients in ddH<sub>2</sub>O. Bring up to final volume of 1 liter.

### 10X DULBECCO'S PBS (8 liters)

16g KCl  
16g KH<sub>2</sub>PO<sub>4</sub>  
640g NaCl  
172.8g Na<sub>2</sub>HPO<sub>4</sub>

up to 8 liters with ddH<sub>2</sub>O

### 1M DTT (Dithiothritol) Sigma D-9779

3.85 g DTT in 25 ml ddH<sub>2</sub>O. Prepare 500µl aliquots and store at -20C.

### 5 M NaCl

146.1 grams NaCl, up to 500 mls ddH<sub>2</sub>O

### 1M KCl

37.28G KCL, up to 500ml ddH<sub>2</sub>O

### 0.5 M EDTA

Dissolve 186.1 grams EDTA·2Na or 146.1g EDTA· free acid in 750 mls ddH<sub>2</sub>O.

Add 15 g solid NaOH to solution to solubilize EDTA.

Use 5N NaOH to adjust pH to 7.5-8.0

Final volume = 1 liter.

### 1 M Tris , pH 7.5

Dissolve 60.57g Tris in ddH<sub>2</sub>O.

Adjust pH of solution to 7.5 with concentrated HCl.

Bring to final volume of 500 mls.

Cold Room Carboy:

For 4 Liters: 484.56 g Tris, pH to 7.5 using conc. HCL (approx. 277mls) and bring up to final volume of 4 liter with ddH<sub>2</sub>O.

### 1M NaN<sub>3</sub> = 0.065%

Dissolve 6.501g NaN<sub>3</sub> in ddH<sub>2</sub>O.  
Bring to final volume of 100 mls.

**0.1 M PMSF** (Phenylmethyl-sulfonyl fluoride, Sigma Cat# 7626)

Make up just before using:

0.01742g PMSF/ 1.0 ml 95% ethanol

**0.1M Pefabloc** (Roche Molecular, Cat#1429-868 100mg)

Dissolve in 4.2mls of ddH<sub>2</sub>O. Aliquot 250µl/tube and store at -20°C.

**Tris Saline (in carboy in cold room)**

4Liters:

20mM Tris, pH 7.4                      80 ml 1M Tris,pH7.4

120mM NaCl                      96 mls 5M NaCl or 28g NaCl

Up to 4 Liter with dd H<sub>2</sub>O

## Tissue Culture

### Tissue culture solutions

#### **DMEM (serum-free)**

(Gibco 31600-075, 1X5L, **low glucose**, +glutamine, with 110mg/L Na Pyruvate, w/o NaHCO<sub>3</sub>)

1 package of DMEM (Eagle's serum-free) for 5 liters of media  
5 grams sodium bicarbonate

Add 1 package of DMEM to 4500 mls. of glass ddH<sub>2</sub>O.

Stir until all material dissolves.

Add 5 grams of sodium bicarbonate.

Check pH. pH of media should be 7.0. Adjust using concentrated HCl.

Bring up to final volume of 5 liters with glass ddH<sub>2</sub>O.

Filter sterilize with a 0.22µm filter (Corning or Millipore).

Store media (500mls/bottle) in Corning 500 ml glass tissue culture bottles at 4°C.

#### **DMEM (Working media) 500 mls**

500 mls DMEM (serum-free)

50 mls FBS

5.0 mls 100X PSK

#### **LCM STOCK (serum-free, -MET or -CYST) FOR 6 LITERS**

May also be used to make phosphate-free media, by omitting the sodium phosphate.  
(MEM with EARLE'S salt conc)

KCL	2.4 g.
MgSO <sub>4</sub> .7H <sub>2</sub> O	1.1989 g.
NaCl	35.777 g.
D-Glucose(dextrose,monohy)	6.0 g.
Phenol red	0.06 g.

#### AMINO ACIDS:

L-arginine·HCL	0.756 g	
<b>**L-cystine·2HCL**</b>	<b>0.18774 g</b>	<b>Omit if preparing -cyst media</b>
L-glutamine	1.7752 g	
L- histidine HCL·2H <sub>2</sub> O	0.252 g	
L-isoleucine	0.312 g	
L-leucine	0.312 g	
L-lysine HCL	0.435 g	

L-phenylalanine	0.192 g
L-threonine	0.288 g
L-tryptophan	0.06 g
L-tyrosine	0.31188 g
L-valine	0.275 g

AMINO ACIDS NEED TO STIR AT LEAST 1 HOUR TO DISSOLVE

100X Vitamin solution      60 mls

NaHCO<sub>3</sub>      6 g

Na Hepes (10mM)    15.618 g or (14.298g HEPES acid. Acidic! pH to 7 using 1N NaOH)

100X Ca<sup>++</sup>    0.168 mls

NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O or    0.84 g

NaH<sub>2</sub>PO<sub>4</sub> (anhyd)    0.72 g

ADJUST TO pH 7.0 USING CONCENTRATED HCL

Filter sterilize. Store media (500ml/bottle) at 4°C.

**LCM (Working media) 100 mls**

88 mls LCM Stock

10 mls dFBS

1 ml 100X MET

1 ml 100X PSK (antibiotics)

**LCM-MET-CYS (100 mls) HCM-MET-CYS (100 mls)**

96.5 mls LCM Stock

2.5 mls dFBS    2.5 mls dFBS

1 ml 100X PSK

95.5 mls LCM Stock

1 ml 100X PSK

1 ml 100X Ca<sup>++</sup>

**LCM CHASE (100mls)**

85 mls LCM Stock

10 mls dFBS

2 mls 100X MET

2 mls 100X CYS

1 ml 100X PSK

**HCM CHASE (100 mls)**

85 mls LCM Stock

10 mls dFBS

2 mls 100X MET

2 mls 100X CYS

1 ml 100X PSK

1 ml 100X Ca<sup>++</sup>

**LCM + 1/10 MET+ 1/10 CYS (for overnight labeling) (100 ml)**

10 mls complete LCM (this provides 1/10 MET)  
9 mls dFBS  
1 ml 100X PSK  
80 ml of LCM Stock

**100X PSK (ANTIBIOTICS) (ICR T/C Facility)**

Dissolve in 500 mls of PBS:

Kanamycin Sulfate	6.1g	100mg/ml
Penicillin "G" Sodium	1.5g	50u/ml
(1650u/mg)		
Streptomycin Sulfate	2.5g	50mg/ml

Filter to sterilize. Dipense 50 ml per 100ml bottle. Store at -20°C.  
Freeze on a slant to prevent break on thaw.

**SHELF LIFE 6 MONTHS.**

Kanamycin Sulfate, #860-1815, Gibco, 25g  
Penicillin "G" Sodium, #860-1830, Gibco, (100 million units)  
Streptomycin Sulfate, #G-6501, Sigma, 25g

**100X CaCl<sub>2</sub>**

Dissolve 2.65g CaCl<sub>2</sub>·2H<sub>2</sub>O in 90 mls. of glass ddH<sub>2</sub>O. Bring up to final volume of 100 mls.

Filter sterilize with a 150 ml 0.22µm filter unit.

Aliquot 20 mls. each into 5 50 ml blue cap tubes.

Store at -20°C.

**100X Cystine**

310mg cystine in 100mls

pH to 8-9 using concentrated NaOH solution.

Filter sterilize using 0.22µm filter. Aliquot into sterile tubes 20ml/tube.

Store in -20 freezer.

**100X Methionine**

Dissolve 0.15g of methionine in 90 mls of glass ddH<sub>2</sub>O.  
Bring up to final volume of 100 mls.  
Filter sterilize and store at -20°C.

**HDF WASH (IRC T/C Facility)**

6 liters:           5950 mls glass ddH<sub>2</sub>O  
                      48 g NaCl  
                      2.4g KCl  
                      6g glucose (Dextrose, monohydrate)  
                      2.1g NaHCO<sub>3</sub>

Dissolve all ingredients in glass ddH<sub>2</sub>O.  
Bring to a final volume of 6 liters.

Add **1.2g EDTA**. (NOTE: EDTA takes a while to dissolve)  
Filter-sterilize using a 0.22 µm Corning filter unit or Millipore filter unit.  
Aliquot 500 mls each into 500 ml glass tissue culture bottles.  
Store at 4°C.

**TRYPsin STOCK SOLUTION**

Recipe for 20 tubes of stock:

Dissolve 6.25 g of trypsin (Difco Cat#0152-1310) in 250 mls of HDF wash.  
Let stir 20 minutes at room temperature to dissolve. (NOTE: solution will remain cloudy)  
Centrifuge for 30 minutes at 10,000 RPM 4°C in JA-20 rotor (Beckman).  
Decant supt. and save. Discard pellets.  
Filter-sterilize trypsin solution using 500 ml 0.22 µm Corning filter unit.  
Aliquot 12.5 mls each of sterile trypsin solution into 20 sterile 50 ml blue cap tubes.  
Store at -20°C.

**TRYPsin WORKING SOLUTION (0.0625%)**

Add 1 tube of sterile trypsin stock solution to 1 bottle (500 mls) of sterile HDF wash.  
Mix well.



## **DIALYZED FETAL BOVINE SERUM**

This procedure takes 5 days to complete. If it is started on Monday and if Tris-saline is changed every day, FBS will be ready by Friday.

Entire procedure is done at 4°C.

Dialyze 500 mls of FBS.

1. Prepare dialysis solution - two 4 liter batches in plastic beaker:

Tris-saline (4L):	10mM Tris-HCL pH7.5	40 mls 1M stock
	120mM NaCl	96 mls 5M

Put into 4°C cold room.

Solutions must be 4°C before you begin dialyzing.

2. Thaw 1 bottle of FBS.

3. Put FBS into 5 or 6 sections of 3/4" wide dialysis tubing which has been rinsed with ddH<sub>2</sub>O and checked for tares. Use orange dialysis clips for tubing.

4. Put dialysis tubes which contain FBS into one 4 liter beaker of Tris-saline. Add a large stir bar. STIR GENTLY.

5. Change Tris-saline solution after 24 hours. At this time make a fresh batch of Tris-saline for the next 24 hour change.

6. Change Tris-saline 2 more times.

7. Filter sterilize FBS using a 500 ml 0.22µm Corning filter unit.

8. Aliquot into 14 50 ml sterile blue cap tubes.

9. Store at -20°C.

## **DEXTRAN COATED CHARCOAL (DCC) FILTERED SERUM**

For 250 ml serum:

Prepare 500 ml of the following solution:

10mM Tris pH 8.0	5 ml
0.25% charcoal (NORIT, Sigma # C-5260)	1.25g
0.0025% Dextran (Sigma # D-4751)	0.0125g
	to 500 ml with dH <sub>2</sub> O

Mix, then spin in large centrifuge bottles:10K rpm, JA-10, 30 min, 4 °C.

Decant away supernatant.

Add 250 ml serum to pellet & resuspend charcoal.

Stir on stir plate in 45°C incubator, 45 min.

Pellet charcoal in centrifuge as above (or longer, if necessary).

Filter supernatant (serum) through 0.45 µm filter to clear charcoal, and then through 0.22 µm filter to sterilize.

Freeze in aliquots (blue caps).

(Procedure from Megan Troxell)

## **MDCK cells**

### **Thawing J-MDCK cells (Nelson lab) 1/16/97**

Remove vial from liquid nitrogen freezer and put into 37<sup>o</sup> water bath. Let thaw in water bath until only a small piece of frozen material remains. Remove from bath let thaw completely. Add contents of vial to a 175mm flask which contains 25 mls of DMEM media+10% FBS+ antibiotics. Place cells in 37<sup>o</sup>C incubator+ 5% CO<sub>2</sub>. Let cells adhere for 2-3 hours and then replace with fresh media.

### **Maintaining MDCK cells:**

Grow cells in dishes or flasks to 75% confluence in DMEM+10% FBS+ antibiotics (concentrations listed below).

Trypsinize cells for 15-25 minutes until cells round up and come off dish by pipeting up and down. We use 0.06% trypsin solution.

Place trypsinized cells into sterile tube containing small volume (6-10 mls) of DMEM+10%FBS (this neutralizes trypsin) and centrifuge low speed in clinical centrifuge to pellet cells.

Resuspend cells in desired volume of DMEM and plate as needed.

### **Media information:**

Dulbecco's Modified Eagle Medium, low glucose, with L-glutamine, with 110mg/L sodium pyruvate, without sodium bicarbonate.

**We add sodium bicarbonate-1g/L and pH media to 7.0.**

Gibco Cat# 31600-075

### **Freezing down MDCK cells**

Plate cells on 150mm dishes and grow until dish is 1/2 confluent.

Trypsinize cells, collect in tube, neutralize trypsin with media and centrifuge.

Resuspend pelleted cells in DMEM complete media (10% FBS).

Count and adjust concentration of cells to 6X10<sup>6</sup> cells / ml.

Put cells on ice.

Add sterile cell culture grade 100% DMSO (Sigma, Cat# D-2650) to cells-media to final concentration of 8 %.

Aliquot 1.0 ml of cell suspension/DMSO to labeled Nunc cryovial (threads of vial are on the inside). Let cells cool on ice for 30 minutes.

Put all vials into thick styrofoam rack or one that is packed with paper towels. The container will allow the temperature of the cells to drop gradually. Put styrofoam container into -70 freezer for 2-3 days.

After this time, transfer vials to liquid nitrogen cell freezer.

## Procedure to render MDCK cells contact naive

### **Day 1** Low density plating

Trypsinize MDCK cells and plate  $1.5 \times 10^6$  cells in 100mm cell culture dish or  $2-2.5 \times 10^6$  cells in 150mm dish in DMEM complete.

### **Day 2** Low density plating

Repeat trypsinization and low density plating cells.

### **Day 3** "Instant confluent monolayer"

Trypsinize cells and plate "instant confluent monolayer"; density= $2.7-3.0 \times 10^5$  cells/cm<sup>2</sup>. That is,  $3-3.5 \times 10^6$  cells for 35mm dish and  $2.5 \times 10^6$  cells for 24mm Costar filter. Plate cells in LCM (low Ca<sup>2+</sup> media- $5 \mu\text{M}$  Ca<sup>2+</sup>) on collagen-coated dishes, coverslips or filters. Let cells sit down for 1 hour for coverslip and dishes, and 3 hours for filters. After this time period, remove media and replace with fresh LCM media.

## **Plating densities for MDCK cells:**

//R<sup>2</sup>=surface area of circle; R=1/2 diameter

<u>dish size, mm</u>	<u>surface area, cm<sup>2</sup></u>	<u>instant confluent monolayer</u>
35	9.61	$3-3.5 \times 10^6$
30	7.07	$2.2 \times 10^6$
60	28.26	$7.0 \times 10^6$
22X22 coverslip	4.84	$1.45 \times 10^6$
100	78.5	$1.5 \times 10^6$ -single cell density
150	176.63	$2-2.5 \times 10^6$ -single cell density

## Collagen coating dishes and filters

Prepare collagen solution from rat tails as described on page 7-8 of this manual. Dilute collagen stock 1:10 with 1:1000 acetic acid solution to prepare a working solution. Put collagen on dish or filter for 2 minutes and let sit. Pour off. Put dishes or filters under UV light for 2 hours. For coverslips: put glass coverslip in dish and add collagen working solution and let sit for 2 minutes. Remove collagen and expose UV dishes for 2 hours. After UV, dishes, filters or coverslips are sterile and ready for use.

## **L-cells expressing mouse E-cadherin under Dexamethasone-inducible condition**

mouse E-cadherin cDNA was inserted into the pLK-neo vector and transfected into mouse L-M(TK-) cells ( see Gene 1992, 111(2): 199-206 for vector and JCB 1996, 134,2, 549-557 for clones).

**LP- L-cells with vector alone**

**LE- L-cells with E-cadherin**

### **Information and Procedure for thawing LE cells (E-cadherin transfected L cells)**

Angela Barth, who made this cell line and is the main stockholder in the Nelson lab, can provide detailed information about growing and manipulating the LE cells besides the general guidelines depicted here.

### **Thawing:**

Thaw quickly at 37°C. Put the vial immediately on ice and add 1-1.5ml of DMEM+5%FBS. Mix with cell solution and then transfer to a 15 ml sterile tube. Put the tube, which contains the cell solution, on ice. Wait for 1 minute or so, and then add in another 1-1.5ml of DMEM+FBS and mix. Leave the cell solution on ice for 1 minute again. Keep doing this slow addition of DMEM+FBS until there is a total of 10-12mls of media. Then put the cells in dish or flask at 37°C to grow. Do not spin cells and change medium the next day. (The rationale behind doing this procedure is to allow the DMSO to be released from the inside of the cell gradually.)

Culture in DME -medium from Gibco, Cat no. 31600-075 + 1 g/l Nabicarbonate, ph 7. For long term culture (3-4 weeks) keep cells in medium with 300 g/ml G418.

### **Induction:**

Add Dexamethasone (Sigma) to final concentration of 1 M to induce maximal expression of E-cadherin for at least 16 hours to get maximal induction. The stock for dexamethasone is prepared as 1mM in ethanol.

### **Note:**

Because of the leakiness of the promoter, and the trace amount of steroid hormone in the FBS, there will be some expression of E-cadherin even when you don't add dexamethasone in the media. One thing you need to pay attention is keeping your LE cells away from Dexamethasone until you need to induce E-cadherin expression. We thawed fresh cells regularly and cultured the cells only for 3 to 4 weeks.

### **Freezing:**

L-cells are fragile and do not like freezing and thawing. Grow them to about 70 to 80% confluency, and then harvest them by mild and short trypsination. I resuspend the trypsinized cells in medium, precool them on ice and add precooled freezing medium 1:1 stepwise (1/2 vol and after 4-5 min another 1/2 vol to resuspended cells). Freezing medium is 20% DMSO, 40% serum and 40% DMEM+10%FCS (endconc. DMSO 10%). For one p-100 plate, I usually add 2 ml of freezing solution to 2 ml of resuspended cells, and aliquoted to 4 vials. Freeze slowly at -70°C ( f.e.: wrapped in paper towels and in a styrofoam box). Transfer vials into liquid nitrogen after 2 days.

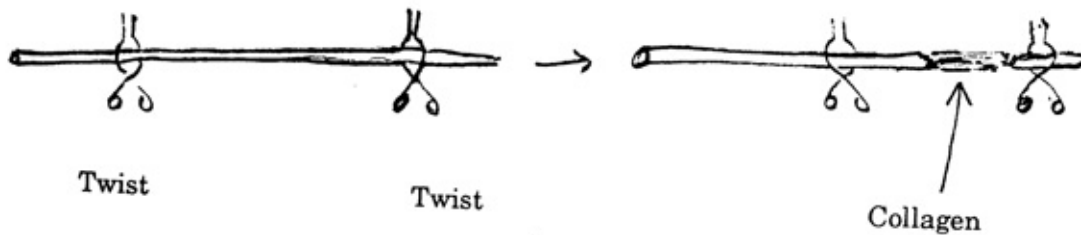
## Rat-tail collagen prep

Place 5-10 tails in 95% ethanol to thaw. Prepare a 1:1000 acetic acid solution using sterile water, a sterile beaker and a sterile stir bar. Have the dilute acetic acid solution stirring at room temperature. Keep solution covered.

When the tails have thawed, starting on the cut end of the tail, clamp 2 hemostats about 2-3 cm apart on a tail.

While holding the hemostat in your left hand, twist or rotate the right hemostat 360 degrees and then pull. Keep pulling until it breaks off.

You should have white collagen fibers at the end of the broken (2-3cm) piece of tail. Cut the white fibers off the broken piece of the tail using a sharp razor blade and place them on glass gel plate. Continue breaking and pulling 2-3 cm pieces of the tail. You get less material the closer you get to the tip of the tail. Tease the collagen fibers by holding one end of the fibers stationary with one razor blade and then use a scraping motion with a razor blade at a 45 degree angle. You want to flatten the fibers and open them up. Put the teased fibers in the stirring acetic acid solution. They should turn translucent. When finished, put the beaker of collagen at 4°C and stir overnight.



Next day:

Centrifuge 3/4 full 50ml Nalgene plastic tubes for 2 hours at 15,000RPM in SS-34 rotor. Remove supernatant and save. Discard pellets. (Portion of the pellet will be gelatinous.)

Store supernatant at 4°C and dilute 1:10 with 1:1000 acetic acid.

Put diluted collagen solution on filters, dishes or coverslips in dishes. Let sit for 2 minutes and pour off. Put dishes or filters under UV light for 2 hours.

Store in container at room temperature for 3-4 weeks.

### **Preparation of gel reconstituted rat-tail collagen**

Tissue Culture Laboratory

Division of Neuropathology

University of Pennsylvania School of Medicine

S.U. Kim, M.D.

(Ehrmann, R.L. and Gay, G. O., National Cancer Inst. J. 16:1374-1403, 1956; Bornstein, M.B., Lab. Invest. 7:134-137, 1958)

1. Freshly obtained tails from 6 month old rats are immediately stored in the deep freeze, where they may be kept until it is convenient to use them.
2. The skin is not removed. The tail is soaked in 95% alcohol for 15 minutes prior to fracturing.
3. Beginning at the tip, the tail is successively fractured into small pieces by means of two Kelly clamps. Each piece in turn is pulled free from the remainder of the tail and the long silvery tendon strands are cut free and allowed to drop into a petri dish containing distilled water.
4. With two fine forceps, the pooled tendon strands from one tail are teased apart into finer filaments and then removed en masse to a sterile 250 ml. centrifuge bottle containing 150 ml of 1:1,000 acetic acid solution. The bottle is sealed and stored for 48 hours in the refrigerator.
5. Centrifugation for 2 hours at 15,000 RPMs (Sorvall Centrifuge) separates the transparent, jelly-like solution from the remaining solid residue. About 100 ml. of viscid fluid are removed and pipetted into large test tubes.
6. Depending on the thickness of gel desired, 1-2 drops of the thickened collagen are placed on the appropriate glass or plastic coverslips and spread with a glass rod to cover the surface. The film is exposed to ammonia vapor for 3-5 minutes which gels the solution into a firm, adherent, transparent, apparently structureless coat.
7. Collagen coated coverslips were washed 10 minutes each in two changes of sterile distilled water in columbia dishes and then two changes of BBS (Hank's).
8. Equilibration against BBS (Hank's) is accomplished by placing 7 coverslips in a Columbia dish containing 4 drops serum. It may be possible to store the completely prepared coverslips for as long as 2 weeks in the refrigerator before use.

## CaPi transfection of MDCK cells

2X HBS (HEPES buffered saline):

50 mM Hepes

280 mM NaCl    adj pH to 7.10 +/- 0.05 with NaOH.

(pH needs to be re-adj before each use)

1. The day before transfection, plate  $1 \times 10^6$  cells in a 10 cm dish for each sample.

[Preparation of DNA for transfection]

2. Place 500  $\mu$ L 2xHBS in sterile tube.

Add 10  $\mu$ L 70 mM Na-PO<sub>4</sub> (pH6.8).

3. DNA sample:

20 ug non-selectable DNA +

2 ug selectable DNA (pSV2neo)

in 440  $\mu$ L 10 mM Tris pH7.5.

Add 60  $\mu$ L 2 M CaCl<sub>2</sub> to DNA mix:

4. Add DNA sample dropwise to the tube containing 2x HBS while bubbling the HBS tube using a 1 ml pipette and pipette aid. Let stand 20 min at room temperature.

5. Trypsinize cells.

Spin down.

Resuspend in 1 mL fresh DME/FCS.

Pipet cells into a new 10 cm-dish.

Add DNA ppt to cells (in suspension) dropwise.

Agitate to distribute.

Let stand for 20 min at room temperature.

6. Add 3.5 ml DME/CFS. Return to incubator for 6-9 hrs.

7. [glycerol shock]

Remove medium.

Add 15% glycerol in 1x HBS for 1 min at room temperature.

Wash twice with DME.

Add 10 ml DME/FCS.

Let cells grow for 2-3 days.

8. Split cells 1 to 4 (or higher) for selection.

9. 24 hrs (or longer), apply selection.

Selection: 500  $\mu$ g/ml G418. for G cells, 400  $\mu$ g/ml G418. for J cells

Colonies should be well formed in 10-14 days.





**Protocol for Wright's stain of cells on opaque filters**

(David Salant)

**Solutions:**

Wright stain: 0.1g Wright stain dissolved in 60 ml methanol

Buffer:	Potassium phosphate, monobasic ( $\text{KH}_2\text{PO}_4$ )	0.663g
	Sodium phosphate, dibasic ( $\text{Na}_2\text{HPO}_4$ )	0.256g
	ddH <sub>2</sub> O	100ml
	pH 6.4	

**Procedure:**

Cover cells with 12-20 drops Wright stain for 1-2 minutes.

Add equal amount of buffer for 2-4 minutes.

Flood chamber with buffer or ddH<sub>2</sub>O so that surface of fluid runs off without settling on cells. Decant buffer.

Air dry and view under 20X brightfield after cutting wet filter from holder and placing on a glass microscope slide.

## Biochemistry

### Extraction

#### **TX-100 Extraction buffer**

For 100 mls:

0.5% (v/v) Triton X-100      0.5 mls 100% Triton X-100  
10 mM Tris-HCL pH 7.5      1 ml 1M Tris-HCL pH 7.5  
120mM NaCl    2.4 mls 5M NaCl  
25mM KCL    2.5 mls 1M KCL  
2mM EDTA    0.4 mls 0.5M EDTA  
2mM EGTA    0.4 mls 0.5M EGTA

For TX-100 extraction buffer with CaCl<sub>2</sub>:

Omit EDTA and EGTA and add 1.0 ml of 180mM CaCl<sub>2</sub>/100 mls.

Combine ingredients listed above to make STOCK solution.

Store at 4°C.

Add ingredients listed below to 100 ml of stock solution. Add just before using.

0.1mM DTT    0.025 mls 0.2M DTT  
0.5mM PMSF or 0.5mM Pefabloc\*\* 0.25mls 0.1M PMSF or 0.25 ml 0.1M  
Pefabloc  
0.1mg/ml DNase  
0.1mg/ml RNase  
\*\* Pefabloc, Roche Molec., Cat # 1429 868-100mg

Extraction Procedure:

Wash cells 2 or 3 times with cold PBS or HDF buffer.

Add Triton X-100 buffer for 10 minutes on rocker at 4°C.

Extraction volumes: P-35      1.0 ml  
Filters    400µl-Apical  
800µl-Basal lateral

Scrap cells off dish or filter using rubber policeman.

Collect material and transfer into clean screw-cap tube.

Centrifuge 13K RPM in eppendorf centrifuge for 15 minutes or in Beckman JA-20 20K RPM for 10 minutes.

Transfer soluble (supt) to clean tube and freeze.

Add 100µl of SDS Immunoppt buffer, resuspend by pipeting up and down 2-3 times and then boil for 5 minutes.

Add 900µl (P-35) or 1100µl (filter) of TX-100 buffer, mix and freeze.

### **CSK Extraction buffer**

For 100 mls:

50 mM NaCl 1 ml 5M NaCl  
300 mM Sucrose 12 mls 2.5M sucrose  
10 mM Pipes, pH6.8 10 mls 0.1M Pipes, pH6.8  
3 mM MgCl<sub>2</sub> 0.3 mls 1M MgCl  
0.5% (v/v) Triton X-100 0.5 mls 100% Triton X-100  
(0.1M Pipes, pH 6.8 3.35g/100mls; MW=335.3)  
(2.5M Sucrose 85.58/100mls  
Combine ingredients listed above to make stock solution.  
Store at 4°C.

Add these just before using buffer:

1.0 mM PMSF or Pefabloc 1.0 mls 0.1M PMSF  
0.1 mg/ml DNase 1 mg DNase  
0.1 mg/ml RNase 1 mg RNase

Extract cells for 10 minutes at 4°C in 1000µl of CSK.

Scrap cells from dish using a rubber policeman. Put into a screw cap tube and centrifuge at 20K RPM in JA-20 rotor for 10 minutes.

Decant supt. and freeze using liquid nitrogen.

Add 100µl of SDS immunoprecipitation buffer. Pipet up and down 2-3 times to resuspend pellet. Boil to solubilize.

Add 900µl of CSK to solubilized pellet. Freeze.

DNase, Cat# 104159, and RNase, Cat#109126 -Roche Molec.

### **MEBC Extraction Buffer**

50mM Tris, pH 7.5 5ml of 1M  
100mM NaCl 2ml of 5 M  
0.5% NP-40 0.5ml 100%  
1mM Pefabloc 0.25mls 0.1M stock

### **Metabolic labeling procedure**

Wash cells 2 times with preincubation media (either LC-MET-CYST or HC-MET-CYST) and then incubate for 15-45 minutes at 37°C.

Label cells with 125-250μCi of <sup>35</sup>S-protein labeling mix (Amersham Cat# 2JQ0079) in LC-MET-CYS or HC-MET-CYS for 15 minutes to 4-5 hours.

If labeling overnight in either media, add 1/10 volume of LCM complete or HCM complete to add back small amount of methionine and cystine.

Labeling volumes:     24mm filter                     400μl-apical  
   900μl-basal lateral

These volumes are used if you label the cells in the 6-well dish.

                          35mm dish   500μl

Chase in either LC-Chase or HC-Chase.

### **Labelling on parafilm (Inke Nathke)**

Prepare petri dishes lined with parafilm. Either cut the parafilm round so it fits a dish or just lay a square piece of parafilm into a dish. The only important thing is that the parafilm is flat and not wrinkled.

After starving in methionine free media, wash cells on filters twice with labelling media (ie. no methionine)

Prepare labeling media with <sup>35</sup>S-methionine to 250μCi/100μl. (or use the amount of label you use in a total of 100μl). Add 100μl drop to parafilm lined petri dish and place filter right on top of the drop. I commonly use 10cm dishes and put up to three filters into each dish.

Label for desired length of time and wash filters as usual using the original tray.

### **Crosslinking with DSP**

Weigh out DSP into a separate eppendorf tube. Keep the tube in a dessicator until adding the DMSO. Right before wanting to use the DSP, prepare 20mg/ml DSP in DMSO (tissue culture grade, SIGMA in individual ampules) as stock solution. When opening the vial containing DSP make sure it is at room temperature and is always kept in the dessicator. After using the DSP, purge the vial with nitrogen (Scheller lab).

Wash cells (on filters) with PBS (with or without calcium as desired or other buffers (they should not contain amines). Dilute DSP stock solution 1:100 into PBS and add 1ml of the diluted DSP to the top and the bottom compartment of each filter. Incubate at room temperature on the belly dancer or a rocker for 20-30 minutes. (It also works at 37°C for 20 min.) Wash in PBS containing 50mM glycine once and then incubate in the same buffer for 5 minutes at room temperature (to quench excess crosslinker). Lyse and harvest cells as usual but include 10mM glycine in the lysis buffer.

To ensure complete reduction of the crosslinker, boil samples in SDS-sample buffer as usual (with DTT or beta-mercaptoethanol) and add an additional 10 $\mu$ l of freshly thawed (or prepared) 1M DTT to each well of the polyacrylamide gel.

### **[3H]-inulin Assay**

- Add 1.0  $\mu$ l of 1.0  $\mu$ Ci 3H-inulin to 1.0ml of media or HEPES-Ringers buffer.
- Add to Apical compartment and let incubate at 37°C or 4°C for 30 minutes.
- Remove 10  $\mu$ l each from the apical and basal compartments and put pipet tip into scintillation vial.
- Add 3-4 mls of scintillation cocktail and then count.

### **Evom meter instructions**

(from Tzuu-Shuh Jou email)

Regarding your question about Evom itself, I believe I left a copy of a very useful user's manual, from Millipore (They sell an almost exactly the same device as the one in our lab, and the custom service is super. I called them for one question related to the machine, and the technical support guy sent me that user manual even I am not their user!), to Kent. If Kent forgets, just ask him to check his file chest beside the sink in B107. I definitely remember I gave him one copy.

As for my personal experience, I always turn the machine on and immerse the electrodes in DMEM + 10 serum + PSK at overnight before I need to measure the resistance ( this is recommended by Megan ), and if I need to do a long time course, I would keep the meter on until I finish reading all the time course values. The other tip is I leave the cells, which are usually on a 1.1 cm diameter filter, pore size 0.4 micrometer, in the hood at room temperature before I do the measurement. This is for the equilibrium, and it is recommended by the manual. In general, polarized MDCK II strain monolayer usually has about 200-300 ohm.cm<sup>2</sup> read-out, but ask any one who had experience in using that machine. The values could be very fluctuating during a single time course study.

The cleaning of the electrodes is very important. I usually rinse the electrodes in autoclaved dd H<sub>2</sub>O several times and let the electrode sit in autoclaved dd H<sub>2</sub>O for a few hours, with the power off, before I rinse the electrode with isopropanol. Kent always said the electrodes should be kept in some kind of KCl solution, but I never tried that advice. I was also told that Millipore carries a blade type, instead of chopsticks type, electrode. Maybe this new device could be a better option.



## **Biotinylation of cell surface proteins**

### I. Steady State Biotinylation Procedure

### II. Newly Synthesized Biotinylation Procedure

#### I. Steady State

Procedure for cells grown on collagen-coated Costar 24mm, polycarbonate, 0.4µm pore filters (Cat #3412)

Entire procedure done at 4°C.

Remove media and wash cells 3 times with Ringer's buffer +/- Ca<sup>2+</sup>.

Ringer's Buffer:

<u>Final concentration</u>	<u>Stock concentration</u>	For 500mls:
10mM Hepes, pH 7.4	1M Hepes, pH 7.4	5mls
154mM NaCl	5M NaCl	15.4mls
7.2mM KCl	1M KCl	3.6mls
+/- 1.8mM CaCl <sub>2</sub>	180mM CaCl <sub>2</sub>	5.0mls

(1M Hepes, pH 7.4 26.03g Hepes /100mls)

Just before using, dissolve sulfo-NHS-biotin\*\* @ (Pierce #21217-50mg) in 100% DMSO for a 100X stock concentration of 2mg/100µl.

Dilute biotin 1:100 in Ringer's buffer to final concentration 200µg/ml and put onto cells. Biotinylation volumes for filters: 400µl-apical and 800µl-basal-lateral.

Incubate for 30 minutes at 4°C on rocker platform.

\*\*Note: The day of the experiment, preweigh biotin in small tubes and record the weight. Keep refrigerated until needed.

@Store all biotin compounds at 4°C in a dessicator. Let dessicator come to room temperature before removing bottle.

Remove biotin solution and wash 5 times with Tris-saline.

Tris-saline:	For 1liter:
10mM Tris-HCl, pH 7.4	10mls 1M Tris, pH7.5
120mM NaCl	24mls 5M NaCl

Add extraction buffer of choice (400µl-apical and 800µl basal-lateral) and incubate for 10 minutes at 4°C on rocker platform.

Scrap cell extract off filter using a modified rubber policeman (modification-cut the diagonal edge off, leaving a straight edge.) and centrifuge at 13,000 RPM in eppendorf centrifuge for 15 minutes at 4°C.

Separate the soluble (S) and insoluble (P-pellet) and freeze soluble. Resuspend insoluble by triturating in 100µl of SDS IP buffer. Boil 5 minutes.

Add 1.1ml of extraction buffer to resuspended insoluble, mix and freeze.

Process samples for immunoprecipitation, run on SDS PAG and transfer to nitrocellulose. Process nitrocellulose blots for detection of biotinylated proteins using ECL. (pages 45-46)

## II. Newly Synthesized Biotinylation

Procedure for cells grown on collagen-coated Costar filters.

Entire procedure done at 4°C.

Do not add DTT to any buffers used in biotinylation, immuno- or avidin- precipitation procedures.

Wash cells 3 times with Ringer's buffer (recipe under steady state biotinylation).

Just before using, prepare a 100X stock 3mg/100µl of NHS-S-S-biotin (Pierce #21331-100mg) in 100% DMSO.

Use this stock within 10 minutes.

Prepare working biotin solution by diluting 100X biotin 1:100 in ringer's buffer. Final biotin concentration=300µg/ml.

Add biotin solution to filters- 400µl-apical and 900µl basalateral.

Incubate for 30 minutes at 4°C on rocker.

Remove biotin and wash 5 times with Tris-saline.

Extract cells with extraction buffer for 10 minutes, scrap cells using a modified rubber policeman, and centrifuge 15 minutes at 13,000 RPM eppendorf centrifuge to separate the soluble and insoluble. Process as described in steady state biotinylation.

### **Avidin precipitation**

Precipitation of newly synthesized biotinylated (NHS-S-S-biotin) proteins.

Immunoprecipitate with antibody of choice as described on pages 19 & 20.

After immunoprecipitation washes, elute antigen-antibody complex from protein A sepharose by adding 200µl of 0.2M glycine, pH 2.6, 1% TX-100. Incubate for 25 minutes at room temperature on rocker. Centrifuge 1 minute 13K RPMs. Remove supt and transfer to clean tube. Repeat procedure again and combine supts. Total volume= 400µl.

Add 5  $\mu$ l of 1N NaOH, 25 $\mu$ l of 1M Tris, pH 7.4, 40 $\mu$ l of 10% BSA, and 60 $\mu$ l of immobilized avidin (Pierce #20219). Incubate overnight at 4°C on rocker.

The next day:

Wash with LSB. Add 60  $\mu$ l of SDS sample buffer + DTT. Boil 5 minutes and load supernatant only on gel.

## 125I-Cell surface labeling procedure

(K. Siemers)

### **SOLUTIONS**

#### Basic D-PBS + 10mM HEPES, pH 7.4 (200mls 5X)

100mls of 10XPBS

10 mls 1M HEPES, pH 7.4

#### Wash & Labeling Buffers (0.1% glucose)

with 1.8mM Ca<sup>2+</sup> 250mls:

50 mls 5X PBS/HEPES stock

2.5 mls 100X (180mM) CaCl<sub>2</sub>

0.25g glucose

up to 250 mls H<sub>2</sub>O

without Ca<sup>2+</sup> 250mls:

50 mls 5X PBS/HEPES stock

0.25g glucose

up to 250 ml H<sub>2</sub>O

#### Lactoperoxidase

12.5µg/ml lactoperoxidase

Stock= 1.0mg/800µl (100X)

#### Glucoseoxidase

1µg/ml glucoseoxidase

Stock=1.0mg/10 mls (100X)

<sup>125</sup>Iodine (Dupont/NEN, Cat #NEZ-0332)

6.5mCi/14 filters= 464 µCi/filter

#### Stop Buffers

Basic D-PBS + 10mM HEPES + 5mM KI + or - Ca<sup>2+</sup>

with 1.8mM Ca<sup>2+</sup> 500 mls:

100 mls 5X Basic PBS/HEPES stock

5 ml 500mM (100X) KI

5.0 mls 180mM (100X) CaCl<sub>2</sub>

#### LCM

#### HCM

### TX-100 Extraction Buffer + Ca<sup>2+</sup>

TX-100 extraction buffer  
0.1mg/ml DNase  
0.1mg/ml RNase  
0.5mM PMSF or Pefabloc

### **PROCEDURE**

**Entire <sup>125</sup>I surface labeling procedure MUST be done under FUME HOOD in B107. (NO <sup>125</sup>I in fume hood in B109)**

Remove media and wash cells 3 times with wash buffer +/- Ca<sup>2+</sup>.

Add 1/100 volume glucoseoxidase and 1/100 volume lactoperoxidase to labeling buffer (+/- Ca<sup>2+</sup>). Add <sup>125</sup>I-iodine to labeling buffer. Labeling volumes: 400μl=AP, 900μl=BL for each Costar filter.

Incubate for 5 minutes at room temperature on rocker.

Remove labeling buffer.

Wash 5 times with stop buffer (+/- Ca<sup>2+</sup>).

Wash 1 time with either LCM or HCM (chase media).

Add LCM or HCM to filters to be chased.

Remove chase media.

Wash 3 times with cold D-PBS.

Add TX-100 extraction buffer to filters- AP=400μl, BL=800μl for each filter.

Incubate for 10 minutes at 4°C on rocker.

Scrap cells using rubber policeman. Collect material and spin 13K RPM eppendorf centrifuge for 15 minutes at 4°C.

Transfer supt to clean tube and freeze.

Add 100μl of SDS IP buffer and resuspend. Boil for 5 minutes. Add 1100μl of TX-100 buffer, mix and freeze.

## **Immunoprecipitation**

### **IMMUNOPRECIPITATION SOLUTIONS**

#### **HIGH STRINGENCY BUFFER (HS-B)**

For 1 liter:

0.1% SDS     1 g SDS  
1% Deoxycholate     10 grams deoxycholate  
0.5% Triton X-100     5 mls 100% Triton X-100  
20 mM Tris-HCL, pH 7.5     20 mls 1M Tris-HCL, pH 7.5  
120 mM NaCl     24 mls 5M NaCl  
25 mM KCL     25 mls 1M KCL  
5 mM EDTA     10 mls 0.5M EDTA  
5 mM EGTA     10 mls 0.5M EGTA  
Add just before using:  
0.1 mM DTT     0.5 mls 0.2 M DTT

#### **HS-B + SUCROSE**

1 M Sucrose     342.3 grams sucrose  
HS-B     up to 1 liter

#### **HIGH SALT WASH BUFFER (HS-B + 1M NaCl)**

For 500 mls:

1M NaCl     29.22 grams  
HS-B     up to 500 mls.

#### **LOW SALT WASH BUFFER**

For 500 mls:

2 mM EDTA     2 mls 0.5M EDTA  
10 mM Tris-HCL, pH 7.5     5 mls 1 M Tris-HCL, pH 7.5  
Add just before using:  
0.5 mM DTT     1.25 mls 0.2M DTT

#### **SDS IMMUNOPRECIPITATION BUFFER**

For 10 mls:

1% SDS     1 ml 10% SDS  
10 mM Tris-HCL, pH 7.5     0.1 ml 1M Tris-HCL, pH 7.5

2 mM EDTA 0.04 ml 0.5 M EDTA

**PROTEIN A SEPHAROSE 4B BEADS** (Pharmacia, Cat# 17-0780-01)

Rehydrate 1.5 grams of Protein A beads in 50 mls of glass ddH<sub>2</sub>O in a 50 ml blue cap tube on rocker for 1-2 hours at room temperature or overnight at 4°C.

Wash rehydrated beads 1-2 times with HS-B.

Add HS-B to final volume of 15 mls.

Store at 4°C.

**IMMUNOPRECIPITATION PROCEDURE**

For 1.0 ml of cell lysate.

Thaw samples. Keep on ice.

Add 30µl of Pansorbin solution (Calbiochem #507858) and 5 µl of preimmune or non-immune serum to all samples. This serum should be the same species as your primary antibody.

Vortex. Leave on ice or in 4°C cold room for 30 minutes. (NOT NECESSARY TO ROCK)

Centrifuge samples 5 minutes in eppendorf centrifuge (13K RPM) at 4°C.

Transfer supts to clean screw cap tubes containing appropriate amount of immune antibody. (Volume of antibody used for IP will vary with the antibody).

**Note:** Immune antibody can be preabsorbed onto Protein A Sepharose and several of these steps can be eliminated. See page 20 for procedure.

Vortex. Let stand on ice for 30 minutes.

Add 60 µl of Protein A-Sepharose CL-4B (Pharmacia #17-0780-01) 1:1 bead solution. Recipe for Protein A on page 18. (Use large orifice pipet tips)

Incubate on rocker platform 120 minutes at 4°C. (Time may vary depending on antibody used.)

After incubation, Centrifuge 30 seconds in eppendorf centrifuge at 13K RPMs at 4°C.

Remove supt carefully using a fine tip plastic disposable pipet attached to a suction hose.

Add 750µl of HS-B buffer. Vortex. Immediately underlayer sample with 180µl of 1M Sucrose in HS-B buffer. Recipes for solutions on page 17.

**NOTE:** Underlayering may not be necessary step for all antibody immunoprecipitations. Check before omitting this step.

Centrifuge 2 minutes.

Remove supt.

Add 1000 $\mu$ l of 1 M NaCl/HS-B (recipe page 17)

Vortex and centrifuge.

Remove supt.

Add 1000 $\mu$ l of low salt buffer. (LS-B) (recipe page 17)

Centrifuge and remove supt.

Add 60 $\mu$ l of 2Xsample buffer with DTT (recipe for 4XSB, page 22) to each sample.

Vortex and then boil (100°C) for 5 minutes.

Note: Check before boiling samples. Some proteins will aggregate when boiled.

Incubating at 65°C for 5 minutes

Centrifuge 10 seconds.

Load supt and beads onto SDS polyacrylamide gel.

### **Immunoprecipitation with pre-absorbed antibody**

While samples are pre-clearing, mix protein-A sepharose (50-60 $\mu$ l/IP) with antibody (usually 5-15 $\mu$ l/IP, depending on antibody). If only preparing sample for one IP, add 200 $\mu$ l PBS (no calcium) to each tube (just to provide a little volume for mixing).

Incubate on rocker at 4°C for 1 hour and wash once with PBS. Then just add pre-cleared lysate to each prepared protein-A/antibody resin. When preparing resin for more than one IP, mix the appropriately scaled amounts of protein-A sepharose and antibody for 1 hour and wash once with PBS. Before removing the first supernatant for washing, mark volume on the tube and bring back up to that volume with PBS so that the right amounts of aliquots can be removed.

Add lysates to individual tubes containing antibody bound to resin and incubate at 4°C for 2 hours on rocker. Wash as usual (see bible).



## **Electrophoresis**

### **SDS –PAGE Solutions**

Premixed solution of 30% acrylamide/0.8% bisacrylamide (National Diagnostics, Cat #EC-890) is now being used in the lab.

**30% ACRYLAMIDE/0.8% BISACRYLAMIDE** 100mls  
(for SDS Polyacrylamide gels)

30 g Acrylamide (Bio-Rad cat #161-0103)

0.8 g Bisacrylamide (Bio-Rad cat#161-0201)

Dissolve in 40 mls of glass ddH<sub>2</sub>O.

Bring up to final volume of 100 mls.

Filter through a 0.45µm cellulose acetate filter unit.

Store in dark glass bottle or foil-covered bottle at 4°C.

**1M Tris , pH 8.7** 2 Liters

Dissolve 242.28 g of Tris base (Mallinckrodt Cat#7732) in 1.75 liters of ddH<sub>2</sub>O.

Adjust pH of solution to 8.7 by adding concentrated HCl.

Bring up to final volume of 2.0 liters.

**1M Tris , pH 6.8** 1 Liter

Dissolve 121.4 g of Tris base (Mall. # 7732) in 750 mls of ddH<sub>2</sub>O.

Adjust pH of solution to 6.8 by adding conc HCl.

Bring to final volume of 1 liter.

**10% SDS** 100mls

Dissolve 10 grams of SDS (ultra-pure grade SDS) in 90 mls of ddH<sub>2</sub>O.

Bring up to final volume of 100 mls.

**10% APS** (Ammonium Persulfate, Bio-Rad Cat#161-0880)

Dissolve 1g of APS in 10 mls ddH<sub>2</sub>O. Store at 4°C.

**100% TEMED** (Bio-Rad, Cat# 161-0700)

## SDS RUNNING BUFFER

### 18 Liters of 1X

192mM      259.65 g Glycine (BioRad, Cat#161-0718)  
25mM      54.54 g Tris (Mallinkrodt, Cat # 7732 )  
0.1%      18 g SDS (Serva, Cat# 20763)  
Up to 18 Liters with ddH<sub>2</sub>O

Dissolve glycine and Tris in 4 liters of ddH<sub>2</sub>O. (Use a 4 liter beaker)

Dissolve SDS in 200 mls of ddH<sub>2</sub>O.

Add glycine and Tris solution to carboy and bring up to 16 liters.

Add SDS solution to carboy and bring up to final volume of 18 liters.

Mix solution well.

### 4 Liters of 10X

577 g Glycine  
121.2 g Tris  
40 g SDS  
Up to 4 Liter with ddH<sub>2</sub>O

## 4X SDS SAMPLE BUFFER

20ML 40ML

SDS	1.6g	3.2g
DTT	0.62g	1.24g
1M Tris, pH 6.8	3.2 ml	6.4 ml
Glycerol (100%)	6 ml	12 ml
ddH <sub>2</sub> O to 20 mls	to 40 mls	
Bromophenol Blue	0.050 ml	0.10 ml

Aliquot and store at -20°F.

This buffer can be made without DTT. Prepare solution as listed above and store at room temperature. When you need buffer, add 1.0ml of 1M DTT to 4mls of **2XSB**. Store at -20°C.

## PROTEIN STANDARDS (Sigma, Cat# SDS-6H [29-205Kd])

Dissolve protein standards in 1.5 mls of 1XSB (SDS).

Aliquot 50µl into tubes.

Store at -20°C.

Thaw and boil 100°C for 1-2 minutes.

Load 10µl/well onto Hoefer standard (16X14 cm) gels and 5µl/well onto BioRad mini (10X10 cm) gels.

Standards:

205,000	myosin
116,000	B-galactosidase
97,400	phosphorylase B
66,000	albumin, bovine plasma
45,000	albumin, egg (ovalbumin)
29,000	carbonic anhydrase

### **COOMASSIE STAIN-STOCK 1% Stock**

Dissolve 10 g of Coomassie brilliant-blue R (Sigma Cat. # B-0630) in 950 mls of 95% ethanol.

Let stir overnight at room temperature.

Filter using Whatman #4 filter paper.

Bring to final volume of 1 liter using 95% ethanol.

### **COOMASSIE STAIN-WORKING SOLUTION 0.1%**

Final conc.

2 Liters:	200 mls Coomassie stock solution	
	800 mls 95% ethanol	38%
	800 mls ddH <sub>2</sub> O	
	200 mls glacial acetic acid	10%

### **DESTAIN SOLUTION**

Final conc.

8 Liters:	1 liter 95% ethanol	12%
	6.6 liters ddH <sub>2</sub> O	
	400 mls glacial acetic acid	5%

### **FLUOROGRAPHY** (For SDS PAG with <sup>35</sup>S labeled proteins)

Stain and destain gel.

Add enough **Amplify** (Amersham Cat# NAMP1000) to cover gel.

Incubate for 30 minutes on rocker at room temperature.

Place gel onto Whatman 3mm paper and dry on gel dryer at 80°C for 2 hours.

Expose to x-ray film.

## **Inke's Coomassie Stain and Destain**

2.2 g Coomassie  
400mls H<sub>2</sub>O  
400mls 100% methanol  
80ml glacial acetic acid

Mix and filter stain through Whatman filter.

To stain gels:

Stain gels in this solution for 30-45 minutes for thin gel (0.75mm); it might take longer for thicker gels.

Destain I (20% methanol, 7.5% acetic acid) for 30-45 minutes.

Destain II (7% acetic acid) .

## GEL RECIPES

For 1 gel:  
(1.5mm thick)

	For 2 gels					
	<u>5%</u>	<u>7.5%</u>	<u>9%</u>	<u>10%</u>	<u>12.5%</u>	<u>STACK</u>
ddH <sub>2</sub> O	13ml	10.5ml	9ml	8.25ml	5.5ml	15.25ml
1M Tris, pH 8.7	11.2ml	11.2ml	11.2ml	11.2ml	11.2ml	-----
1M Tris, pH6.8	----	----	----	----	----	2.56ml
10% SDS	0.3ml	0.3ml	0.3ml	0.3ml	0.3ml	0.2ml
Acry/Bis	5.0ml	7.5ml	9ml	10ml	12.5ml	2ml
100% Temed	15μl	15μl	15μl	15μl	15μl	20μl
10% APS	100μl	100μl	100μl	100μl	100μl	100μl

For 2 mini gels:  
3.5ml/gel  
Recipe makes 2 gels.

	<u>7.5%</u>	<u>5%</u>	<u>Stack</u>
ddH <sub>2</sub> O	2.8ml	3.47ml	3.8ml
1M Tris, pH 8.7	2.98ml	2.98ml	-----
1M Tris, pH 6.8	-----	-----	0.64ml
10% SDS	80μl	80μl	50μl
Acrylamide/Bis	2ml	1.3ml	0.5ml
100% Temed	4μl	4μl	5μl
10% APS	27μl	27μl	25μl

## Native gels

### **40% acrylamide/1.5% bisacrylamide (Bio-Rad)**

Dissolve 40g acrylamide and 1.5g bisacrylamide in glass ddH<sub>2</sub>O.

Bring up to final volume of 100 mls.

Filter with 0.45µm filter unit.

Store in dark bottle at 4°C.

### **Running buffer- 10X Stock**

800 mls 1M Tris-base (96.91g Tris/800mls)

200 mls 2M sodium acetate

80 mls 0.5M EDTA, pH 7.5

Combine above ingredients.

Adjust pH to 7.4 with glacial acetic acid.

Bring to final volume of 2 liters with ddH<sub>2</sub>O.

Store at 4°C.

### **2.5M Sucrose**

**GEL RECIPE** (For 1 gel: 2-4% Acrylamide gradient, 1.5mm thick)

2%      4%

40% Acry/Bis stock	1 ml	2 ml
10X Running Buffer	2 ml	2 ml
ddH <sub>2</sub> O	16.6 ml	13.1 ml
2.5M Sucrose	----	2.5 ml

100% TEMED	5 µl	5 µl
10% APS	281.25 µl	281.25 µl

## 2D IEF Gels

(Adapted from O'Farrell. 1975. *Journal of Biological Chemistry*. **10**: 4007-4021.)

### Setup

#### 1) Clean Tubes

Glass tubes (130 x 2.5mm inside diameter) for first dimensional (IEF) tube gels are cleaned by submerging them in concentrated acid dichromate overnight on the bench top. Make sure that no air bubbles are trapped in the tubes during this incubation. The next morning the acid dichromate is removed by flushing it out with dH<sub>2</sub>O in the sink. Care should be taken to remove all the acid. The tubes are then incubated in basic methanol (made by dumping a liberal amount of 10N NaOH into a glass casserole dish of methanol) on a rocker at room temp. for 3hr. The combination of NaOH and methanol will form bubbles during the incubation - this is normal. The basic methanol is then thoroughly flushed out of the tubes with dH<sub>2</sub>O in the sink. The tubes are then left standing on their ends to dry. It is critical for later steps that the glass tubes be absolutely clean before the IEF gel solution is allowed to polymerize within them. When the tubes are dry, seal one end with parafilm, and then securely tape them up against a shelf edge somewhere so that they are vertical.

#### 2) Make the following solutions:

2L of 0.02M NaOH and degas this extensively while stirring to remove CO<sub>2</sub>. I find that a heavy-walled brown 4L ethanol bottle works well for this. Allow the solution to degas during setup.

2.5L 0.01M H<sub>3</sub>PO<sub>4</sub> (2.9ml 85% H<sub>3</sub>PO<sub>4</sub> into 2.5L dH<sub>2</sub>O).

1ml Lysis Buffer (-DTT)

0.571g urea  
200µl 10% NP-40  
47.5µl Ampholine pH 5-7  
21.5µl Ampholine pH 4-6  
10µl Ampholine pH 3-10  
To 950µl with dH<sub>2</sub>O.

\*Add 50µl 1M DTT just prior to use.

1ml Sample Overlay solution

0.54g urea  
24µl Ampholine pH 5-7  
11µl Ampholine pH 4-6  
5µl Ampholine pH 3-10  
To 1ml with dH<sub>2</sub>O.

1ml 0.05% SDS (-DTT)

945µl dH<sub>2</sub>O  
5µl 10% SDS

\*Add 50µl 1M DTT just prior to use.

### 3) Set up tube gels.


10ml gel mixture (~0.8ml per tube gel)

5.5g urea  
1.33ml: 28% acrylamide, 1.62% bis  
2ml 10% NP-40  
1.9ml dH<sub>2</sub>O  
475µl Ampholine pH 5-7  
215µl Ampholine pH4-6  
100µl Ampholine pH 3-10  
10µl 10% APS

Degas solution for 1-2min.

Add 7µl TEMED to mix and pour tube gels to 1.5cm from top using a syringe to add the gel mix. Press the tip of the syringe against the side of the tube as you add the gel mix. Avoid introducing bubbles at all costs. Always pour extra tube gels in case you introduce bubbles into some of them. Also, always plan to have 1 extra tube gel to run as a blank so that you can check the pH gradient later. After pouring the gel mix, overlay with dH<sub>2</sub>O. Allow to completely polymerize. The water/acrylamide interface will blur at first and then become sharply defined as the acrylamide polymerizes. This gel mixture yields the following pH gradient.

pH    4.19   4.67   5.03   5.35   5.62   5.85   5.95   6.28   6.50   6.70   6.85



Gradient is determined after running first IEF dimension by removing blank tube gel from tube, cutting it into 1cm fragments, incubating each of these in 1ml of dH<sub>2</sub>O in air tight 4ml snap cap falcon tubes overnight at room temp., and then measuring the pH of the resulting solutions using the thin pH meter electrode.



#### 4) Pre-Run IEF Gels

After the tube gels have polymerized, remove the parafilm from the bottom of the tubes and set them up in the IEF apparatus. The tubes fit through the rubber gaskets of the upper reservoir insert (be careful not to snap tubes as you insert them - wetting with a little water or buffer helps in this regard). Plug unused gaskets with cone-shaped rubber plugs. Place the 2.5L of 0.01M  $\text{H}_3\text{PO}_4$  in the lower chamber. Insert the upper reservoir (with tube gels) into the lower chamber. Be careful to avoid trapping air bubbles in the bottom of the glass tubes as they contact the lower buffer (this is hard to avoid, but bubbles can be dislodged by gently squirting into the bottom of the tube gels with a pipetman or syringe filled with the  $\text{H}_3\text{PO}_4$  buffer). Layer 50 $\mu\text{l}$  of lysis buffer onto each tube gel in the upper reservoir, then fill to the top of the tubes with degassed 0.02M NaOH. Fill the upper reservoir with ~1L of the degassed 0.02M NaOH and check for leaks. Pre-run the IEF gels (constant current): 200V for 15min., 300V for 30min., 400V for 30min. Continue degassing the unused 0.02M NaOH.

#### 5) Prepare Samples

While the IEF gels are pre-running, thaw your samples (immunoprecipitated protein A beads previously prepared or otherwise). Add 50 $\mu\text{l}$  0.05% SDS + 50mM DTT to each (or if sample is in solution, add SDS and DTT to make the final solution 0.05% SDS and 50mM in DTT). Incubate samples at 40°C for 1hr. Then add 54mg of urea crystals to each to make a final solution with a final volume of ~100 $\mu\text{l}$  and a urea concentration of 9M (urea must be heated at 40°C to go into solution quickly).

#### 6) Run IEF Gels with Samples

After pre-run, empty upper reservoir and discard used 0.02M NaOH. Place upper reservoir back into lower chamber again being careful not to trap air bubbles in the bottom of the tubes (lower buffer does not need to be changed). Draw solution off the top of each tube gel using a syringe. Load samples onto tube gels. Then layer 20 $\mu\text{l}$  sample overlay solution onto each tube gel. Fill tubes to top with fresh, degassed 0.02M NaOH. Fill upper reservoir with remaining fresh, degassed 0.02M NaOH. Gels should be run for a total of 5,000 - 10,000 V·Hr with the final 1-2hr. being run at 500-800V (to focus protein spots more tightly). I generally run the IEF gels overnight at 350-400V and then turn the voltage up the next morning.

## **Next Day:**

### 7) Set up Second Dimension

Set up gel plates using 3mm spacers and pour a separating gel of the appropriate acrylamide concentration so that its top is 4.5cm from the top of the gel plates. The 1.5mm spacers are not thick enough to accommodate the 2.5mm tube gels without distorting them. After the separating gel polymerizes, pour a 2.5cm thick stacking gel. Remember to double the volume of all the solutions in comparison to the normal 1.5mm thick gels.

### 8) Prepare Tube Gels for Second Dimension

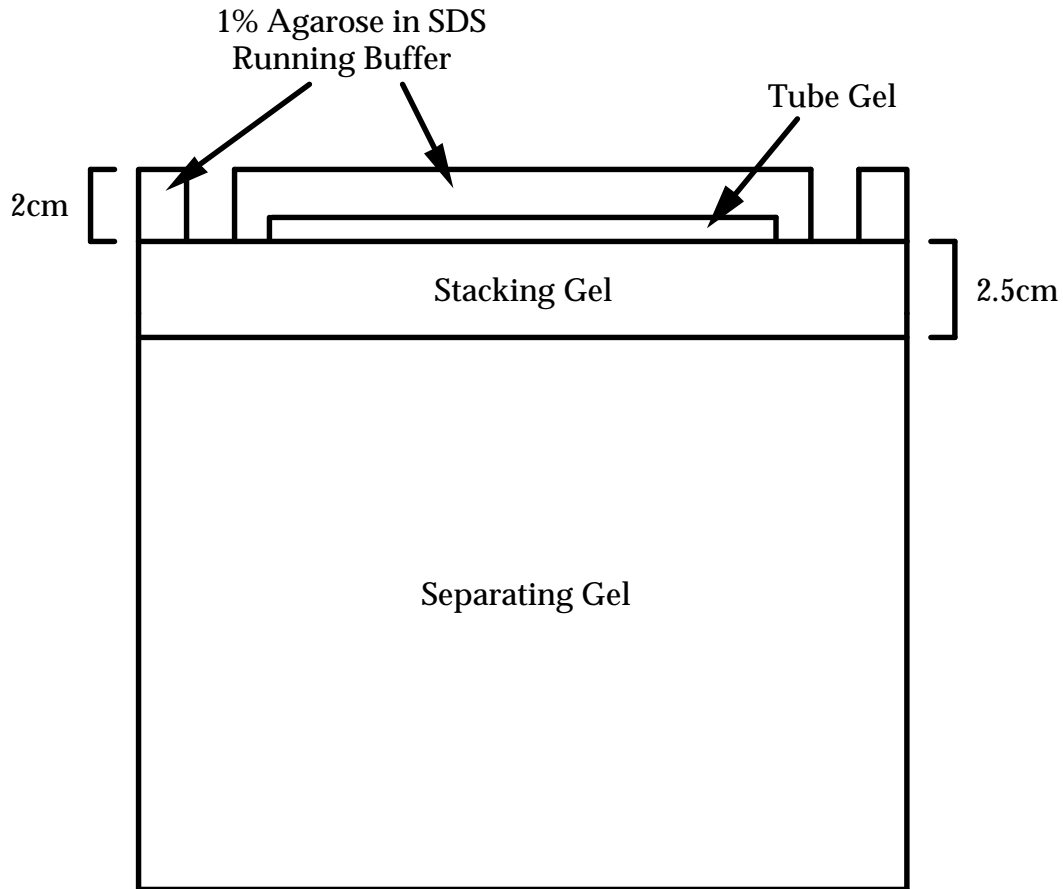
Make 10ml 1x sample buffer in a 14ml Falcon snap-cap tube for each tube gel on which samples were run.

Stop the first dimension electrophoresis. Empty the upper reservoir and remove the tube gels from the apparatus. Remove the tube gels from the glass tubes by using plastic tubing of the appropriate diameter attached to a syringe; pull back the plunger of the syringe to fill the syringe with air. Then place the end of the plastic tubing over one end of the glass tube. Slowly and carefully push in the plunger of the syringe until the tube gel starts to be forced out the other end of the tube. Then use steady pressure to continue to force the tube gel out of the glass tube onto a glass plate. Once the tube gel is free and lying on the glass plate, notch one end of it with a razor blade so that you will know which end is acidic and which basic. Pick up the tube gel with gloves or a forceps (the tube gels are sturdy enough to do this) and place it into one of the tubes of 1x sample buffer. Repeat for all tube gels on which samples were run. Place the tubes with gels and sample buffer onto a rocker at room temp. for 30min. to allow the sample buffer to completely enter the tube gels. During this time, remove the blank tube gel, cut it up into 1cm pieces and place these into 4ml airtight tubes containing 1ml dH<sub>2</sub>O. Seal and allow to equilibrate overnight on the bench top (for measuring the pH gradient - see above).

### 9) Run Second Dimension

Carefully pour off the sample buffer from each tube containing a tube gel. Pour the tube gel out onto a piece of parafilm and straighten it out. Remove any water from the top of one of the gels previously prepared. Bring the parafilm up to the edge of one of the glass plates of the gel and carefully (this part is a little tricky) allow the tube gel to slide into the slot between the plates. The tube gel will usually not slide all the way down onto the top of the stacking gel. To get it down there, use a thin spatula to push one end of the tube gel down. Try not to trap any air bubbles between the bottom of the tube gel and the top of the stacking gel. Take two 3mm thick teeth from a 15 well 3mm thick comb and place one on either side of the tube gel with enough space between them, the tube gel, and the gel spacers so that a well on either side of the tube gel can be formed. If there is not enough space, the tube gel can be carefully moved farther to one side, and only one comb tooth used. Microwave 1% agarose in SDS running buffer until it goes into solution. Allow the agarose to cool until it can be held with bare hands and then pipet enough onto each gel to fill them to the top of the gel plates. This should completely cover the tube gel and form wells around the comb teeth. See diagram below. Allow the

agarose to completely cool and polymerize. Carefully remove the comb teeth, flush out the wells, and load the wells with MW standards and/or samples you wish to run in the second dimension as a control. Assemble the gel apparatus as normal from here and run. The 3mm thick gels need twice as much current as the 1.5mm thick gels to run at the same speed. Since 35-40 mA is about the maximum at which you can run the gels without heating becoming a problem and causing 'smiling', this means the minimum run time for these gels is greater than for the 1.5mm gels. I recommend running them overnight at 15 mA per gel and then turning them up to 35 mA per gel the next morning.



#### 10) Detection of Protein Spots

Once the second dimension is complete, take the gel apparatus apart and remove the gels as one would normally do. If the proteins in the gel are to be electroblotted, the transfer should be conducted as for 1.5mm gels. If the gels are to be processed for fluorography, keep in mind that extra time will have to be allowed for each step in the process. The 3mm gels take longer to stain and destain. Stain with coomassie blue 30min. - 1hr. Destain for as long as it takes (it helps speed this process if you heat the gel + destain + kimwipes in a microwave until the whole thing is hot but not boiling; then place it on a rocker; after the kimwipes are saturated, repeat the process until the gel has cleared). For enhancing the gels, increase incubation times accordingly (this will depend upon the enhancing chemical used). When drying these gels down, make sure you have a good vacuum. These gels are especially prone to cracking and this is worse the higher the

concentration of acrylamide used. It also helps to ramp the temperature of the gel dryer up slowly. For these gels to dry completely, you may have to dry them for up to 6hr. After drying, treat as normal.

**Silver stain** (Solutions needed to stain 1 gel-1.5mm thick)

Use GELCODE SILVER STAIN KIT, Pierce Chemical Co., Cat#24597

1. Place gel into a glass dish containing 50% methanol. Incubate overnight on rocker.
2. Incubate gel in glass ddH<sub>2</sub>O for 20 minutes.
3. Prepare silver solution- Mix 9.9 mls of silver stock solution from kit with 138.6 mls glass ddH<sub>2</sub>O.
4. Discard water in glass dish and add silver solution to gel. (NOTE: orange tint will appear in solution)
5. Incubate 60 minutes at room temperature on rocker.
6. Prepare reducer base and reducer aldehyde solutions. Add 9.9 mls of reducer base to 64.35 ml glass ddH<sub>2</sub>O. In a separate container, add 9.9 mls of reducer aldehyde to 64.35 ml glass ddH<sub>2</sub>O. **Combine the two solutions just before using.**
7. Discard silver solution and wash gel for 15 seconds with glass ddH<sub>2</sub>O.
8. Mix reducer base and reducer aldehyde together. Add to gel.
9. Incubate for 9 minutes on rocker. (NOTE: solution will turn brown, and then yellow) The stain should develop at this step.
10. Prepare stabilizer solution- Mix 9.9 mls of stabilizer solution with 435.6 mls of glass ddH<sub>2</sub>O.
11. Discard reducer solution and add 1/3 of stabilizer solution (145 ml). Incubate for 1 hour on rocker. Repeat this step 2 more times with remaining stabilizer solution.

## Western blot

### TRANSFER SOLUTIONS

For large Hoefer (now called Amersham/Pharmacia) Gels

#### **TRANSFER BUFFER (Hoefer large blots)**

6 Liters:        20mM Tris-acetate, pH 8.3  
                  0.1% SDS  
                  20% Isopropanol

Dissolve 14.52g Tris in ddH<sub>2</sub>O.

Adjust pH to 8.3 using glacial acetic acid.

Add 6g SDS to Tris solution.

Combine Tris-SDS solution with 1200 mls of 100% Isopropanol for nitrocellulose membrand or 1200 mls 100% Methanol for Immobilon-P (PVDF) membrane in a 6 liter erlenmeyer. Make up to a final volume of 6 liters using ddH<sub>2</sub>O.

#### **10X TRANSFER BUFFER (without Isopropanol) 5 Liters**

121 g Tris  
50g SDS

Dissolve 121 g Tris in 4500 mls of ddH<sub>2</sub>O.

Adjust pH to 8.3 using glacial acetic acid.

Add 50 g SDS. Let dissolve.

Bring up to final volume of 5 Liters.

Put into carboy in B109.

For 1 Liter of 1X Transfer buffer:

Dilute 10X Transfer buffer using ddH<sub>2</sub>O and add 200ml of 100% isopropanol or other organic reagent.

#### **Transfer buffer for BioRad mini gels**

For 1 liter  
25mM Tris                    3.03g Tris  
192mM Glycine                14.4 g Glycine  
20% v/v Methanol, pH 8.3    200ml 100% methanol  
Do not adjust pH. pH should be approximately 8.1-8.4.

### Stains for nitrocellulose or Immobilon-P (PVDF)

#### **0.1% AMIDO BLACK ( for nitrocellulose)**

in 40% methanol

10% acetic acid

Filter.

Destain with gel destain.

**PONCEAU STAIN** (nitrocellulose or PVDF membranes)

Working solution:

Dilute Ponceau S concentrate-2% (Sigma, Cat P7767) **1:100** with ddH<sub>2</sub>O.

Destain for short time with H<sub>2</sub>O or PBS.

**0.2% COOMASSIE BLUE** ( for PVDF membranes)

**For 300 mls:**

in 50% methanol	150 mls 100% methanol
10% acetic acid	30mls glacial acetic acid
0.2% Coomassie blue	0.6g Coomassie blue R

Destain with 50% methanol, 5% acetic acid

**Incubation and Wash Buffer:**

**10X GELATIN WASH BUFFER (without gelatin)**

8 liters:

- 145.37g Tris-HCL, pH7.5
- 608g NaCl
- 16 g NaN<sub>3</sub>
- 160 mls 0.5M EDTA, pH 7.5
- 80 mls 100% Tween-20
- up to 8 liters with ddH<sub>2</sub>O

**1X GELATIN WASH BUFFER**

10 liters: 1 liter 10X GWB Stock  
9 liters ddH<sub>2</sub>O  
10g gelatin (Difco Cat#0143-01)

Dissolve 10g of gelatin in 500 mls ddH<sub>2</sub>O.

Heat on hot plate/magnetic stirrer until gelatin is in solution.

Combine gelatin and 10X GWB.

Bring up to final volume of 10 liters.

**Blocking Buffer**

3% BSA

5% Non-fat dry milk (Carnation)

2% normal serum (goat, donkey or sheep-depending on the secondary antibody that is used)

Dissolve in 1X gelatin wash buffer, if using for <sup>125</sup>I-secondary antibody. If blot will be used for ECL, prepare blocking buffer in TTBS (tween-tris buffered saline -Recipe p. 45). The sodium azide in gelatin wash buffer will interfere with ECL reaction.

## **TRANSFER PROCEDURE**

Assemble transfer cassette (one for each gel) in this order:  
(Prewet all materials with transfer buffer.)

cassette with handle  
sponge  
sheet of 3mm whatman paper  
nitrocellulose or Immobilon-P (PVDF)\*\*  
gel  
sheet of 3mm whatman paper  
sponge  
cassette

\*\*If you are using **Immobilon-P**, prewet membrane in 100% methanol for 2 minutes. Prepare transfer buffer that contains **methanol**. Incubate PVDF membrane in transfer buffer for 10 minutes prior to transfer.

Transfer gel to 0.45µm 14 X14 cm piece of nitrocellulose (S&S Cat# BA85) or Immobilon-P (Millipore) for **3 hours at 250mA** or **16 hours at 50mA**. Both conditions should be done at 4°C with constant stirring.

## **AFTER TRANSFER**

Put blot directly into stain.

### **Staining membrane after transfer:**

Stain with 0.1% india ink-1 hour or 0.1% amido black-2 minutes or 0.02% ponceau S for 1 minute for nitrocellulose membranes ( works okay with ECL) or 0.2% coomassie blue or Ponceau S for 2 minutes for PVDF membrane.

### **Destain:**

Destain amido black stained blot with gel destain.

Destain coomassie-stained PVDF membranes with 50% methanol/5% acetic acid.

Destain ponceau with PBS or water.

Rinse several times with ddH<sub>2</sub>O and 2 times with buffer before adding blocking solution.

### **Block:** (Recipe p. 41)

Add blocking buffer and incubate overnight at 4°C or for 2 hours at 37°C.



### **<sup>125</sup>I-Secondary Antibodies:**

Incubations with immune antibody and <sup>125</sup>I-secondary antibody are done in 1XGWB. Incubation time and dilution of immune antibody are different for each antibody. <sup>125</sup>I-secondary antibody is used at 1μCi/lane for both mini and large gels in 10 mls GWB for 45-60 minutes. Washes after primary antibody and <sup>125</sup>I-secondary antibody are done every 20 minutes for 1 1/2 hours with gelatin wash buffer. Dry blot, wrap in saran wrap and expose to X-ray film.

### **HRP-secondary antibody and ECL:**

Incubation times and dilution of immune antibody in blocking buffer are different for each antibody. HRP- anti-mouse IgG, anti-rabbit IgG or anti-rat IgG secondary antibodies are diluted 1:5000 in blocking buffer in TTBS for 45-60 minutes. Washes after immune and secondary antibodies are done for 1-2 hours with 4-5 changes of TTBS. Perform ECL.

### **ECL Western Blotting Detection Reagent** (Amersham, Cat #RPN2106)

Add equal volumes of ECL Reagent 1 and ECL Reagent 2 to tube and mix. Add ECL mixture to plastic tray and add blot to tray. Protein-side of blot toward the reagent. Incubate for 1 minute at room temperature with gentle agitation. Put blot into plastic bag and remove excess liquid. Expose blot to film for various times to get desired exposure.

### **DETECTION OF BIOTINYLATED PROTEINS USING ECL (AMERSHAM)**

#### **SOLUTIONS:**

Vectastain ABC Peroxidase Standard Kit,  
Vector Laboratories,  
Cat#PK-4000,  
\$105.00

Ovalbumin, chicken egg  
Sigma Cat#A-5378  
\$123.80-25 grams

ECL Western Blotting Detection System  
Amersham Cat# 323-9750  
\$195.00

## PROCEDURE

Transfer proteins from gel to nitrocellulose or Immobilon-P.

Prepare buffers (Store at 4°C. USE at room temperature.)

Buffers:

TBS (Tris buffered saline) for 500mls:	1L 10X TBS
50 ml 1M Tris-HCl, pH 7.4	500ml 1M
0.1M Tris	45g NaCl
4.5 grams NaCl	

TTBS (Tris buffered saline + Tween-20)

For 500mls:

same as TBS

0.5 ml 100% Tween-20 (conc=0.1%)

## AFTER TRANSFER:

Do not stain blot with India ink. If you need to see molecular weight standards, you can either use prestained MW standards from Gibco/BRL (Cat#CPA112), or stain the blot with ponceau, amido black or coomassie (recipe listed under transfers-page 33). The blot can be stained with india ink after the ECL procedure.

Wash blot 3 times with ddH<sub>2</sub>O.

Wash blot 2 times with TBS.

Block in 7.5% ovalbumin in TBS for 2 hours at 37°C or overnight 4°C on rocker. I usually do this part in the Kapak pouches that we use for western blot incubations or a small plastic tray. I use 8-10 mls of blocking media/blot.

The next day:

Discard blocking media.

Wash one time with TTBS.

Prepare avidin peroxidase standard:

Use 10 ml of standard/ large blot.

Vector Peroxidase std kit:

Mix 2 drops of Reagent A with 10 ml of TTBS. Mix.

Add 2 drops of Reagent B. Mix.

Let peroxidase standard incubate for 30 minutes at room temperature before incubating with blots.

Put blots in pouches or small plastic tray, and add 10 ml of standard listed in previous step. Incubate for 30 minutes at room temperature on rocker. ( I use the Belly Dancer rocker.)

After incubation, remove blot from pouch.

Wash 5 times with TTBS over a 60 minute time period.

**Prepare ECL reagent:**

Mix 4 mls of reagent 1 (white label) with 4 mls of reagent 2 (black label). This is enough to do one large blot. Do not reuse.

I usually put reagent in small plastic tray\*\*\* and then add blot-protein side down.

Incubate for 1 minute with gently mixing. I do the mixing by hand. Do one blot at a time.

Remove blot from tray and put semi-wet blot into pouch. Remove most of the moisture from the bag. Do not completely dry-Keep moist!

Expose to film. Start with 2 minute exposure and adjust as necessary. If you find that you need very short exposure times (less than 5 seconds), to get a signal that does not saturate the film, try waiting 5 minutes and then reexpose.

If you wait too long, you do lose signal. If you lose signal, just redo the ECL step and expose to film.

If the background is high, wash blot 2 hours or overnight at 4oC with TTBS and redo ECL.

\*\*\*Note: I keep one plastic tray that I use only for ECL

**ECL Plus**

Allow solutions A and B to come to room temperature.

Prepare ECL Plus reagents by mixing 2mls Rgt. A:50µl Rgt. B.

Final volume= 0.1ml/cm<sup>2</sup>.

Place protein side up on piece of saran wrap.

Pipet mixture onto membrane. Incubate 5 minutes at rt.

Expose blot to film.

**Western blot stripping solution**

100mM -Mercaptoethanol

2% SDS

62.5mM Tris-HCl, pH 6.7

Incubate @ 50°C for 30 minutes with agitation.

Wash 2 times for 10 minutes each in TTBS.

Block 5% NFDM in TTBS for 2 hours at RT or 37°C.

## **Electroelution of proteins from SDS-PAGE**

(ISCO Little Blue Tank)

### **Prepare gel sample:**

Run sample on SDS polyacrylamide prep gel-1.5mm thick & 3-well comb or 3.0mm thick & single-well comb.

After run, notch both sides of gel every 0.5 cm using a scapel or razor blade.

Remove a small section ( 1-1.5cm or 1-2 lanes) of the gel, which contains sample, and coomassie stain and destain to determine location of protein. If you use a 3mm gel, destain by heating gel in destain solution (procedure described on page 31). Wrap the unstained portion of the gel in saran wrap and store at 4°C until staining is complete. \*\*\*(Protein **must not** be fixed in gel if you plan to electroelute it.)

Once you determine the location of the protein, cut it out of the unstained gel. If you plan to electroelute at a later time, freeze the gel piece at -20°.

### **Electroelution:**

If you are using a new trap, soak in ddH<sub>2</sub>O for 30 minutes to rehydrate membrane and to remove glycerol. Soak in SDS running buffer for 15 minutes.

Test traps for leaks. Place trap in little blue tank. Be sure to keep membranes wet; do not let them dry out. All traps should be positioned in the same direction. Maximum of 4 traps in one little blue tank.

Fill little blue tank reservoirs with SDS gel running buffer to cover both membranes of the trap.

Mince gel into 0.5 cm pieces.

Place gel pieces in the large well of the trap. Do not overfill, as gel pieces will swell and may fall into concentrator well (smaller well).

Place plastic mesh over gel pieces and concentrator trap.

Carefully fill sample traps with SDS running buffer. Be careful not to get gel pieces in concentrator side of trap.

Connect the electrode/lid so that the gel piece-side of the trap is black or - electrode, and the concentrator side is red or + electrode.

Run at constant voltage 150-200volts or constant current 4-5mA/trap for 4-8 hours (from Little blue tank manual). I have used constant voltage- 100 volts for 6 hours at room

temperature. It may be necessary to precool buffer to 4°C and electroelute in 4°C cold room.

**When run is complete:**

Reverse the electrodes and run for 30 seconds.

Remove plastic mesh over concentrator trap using forceps.

Remove concentrated protein from concentrator-side of trap using a 200µl pipetman. Remove 300-400µl. (There are 2 sizes of traps: microtrap-200µl recovery and nanotrap-40µl recovery).

Sample traps can be reused. Wash traps in several changes of ddH<sub>2</sub>O and store sample traps in 0.1% NaN<sub>3</sub> at 4°C.

## **Immunofluorescence**

### **Fixation/Extraction**

#### **Extract -> Formaldehyde Fix**

(can do with any of the fixatives listed)

Wash cells 2-3X with PBS.

Add 2.0 mls of extraction buffer (CSK, 0.5% Triton X-100, 0.01% Saponin, etc.) for 35mm dish or 1.0ml-apical, 2.0ml-basalateral for filters, and incubate for 1-5 minutes at room temperature.

Remove buffer and wash cells for 10 minutes with 2 to 3 changes of PBS. If you are working with monolayer of cells that has been in HCM (DMEM) for 2 or more days, be extremely careful when extracting cells. Monolayer of cells may lift off coverslip or filter during or after extraction procedure.

Fix cells:

#### **For formaldehyde fixation:**

Add 2.0 mls of 1.75% or (1.9% formaldehyde prepared in PBS, or PLP). (Dilute 37% formaldehyde stock to 1.75% (1.9%) just before using.)

Incubate for 10-15 minutes at room temperature.

#### **For methanol fixation:**

Add 2.0 mls of -20°C 100% Methanol.

Incubate for 5 minutes in -20°F freezer.

#### **For paraformaldehyde fixation:**

Paraformaldehyde Stock (3%):

Heat 80ml PBS to 60C.

Add 3g paraformaldehyde.

Mix (at 60C) for 30 min.

Add a few drops 10M NaOH until the solution is clear.

Cool, adjust to physiological pH and make up to 100ml.

Aliquot and store at -20C.

Add 2.0ml 3% paraformaldehyde stock.

Incubate 15 min. at RT.

Remove fixative and wash 10 minutes 2-3 changes of PBS.

#### **Fix -> Extract**

Reverse order of procedure described above.

### **Blocking Cells**

Prepare block solution: PBS containing 0.2% BSA, 50mM NH<sub>4</sub>Cl<sub>2</sub> and 1% normal goat serum.

If you extracted with 0.01% Saponin, add saponin to blocking buffer as well as wash buffer.

Add 1.0 ml per coverslip. Incubate for 25 minutes at room temperature. Wash 2-3X (10 min) with PBS-BSA.

### **NOTE:**

PBS-BSA refers to PBS that contains 0.2% BSA

PBS used for blocking, primary and secondary antibody incubations and washes contains 0.2% Bovine serum albumin. (Sigma #A-7906)

## Antibodies

### **PRIMARY ANTIBODY Parafilm procedure**

Place 50-100 µl of diluted antibody solution onto parafilm in container with cover (Antibody is diluted with PBS-BSA.)

For filters, incubation is done on parafilm. 100µl-BL; 200µl-AP

Incubate for 45 minutes at room temperature or overnight at 4°C.

Remove solution and wash.

### **SECONDARY ANTIBODY**

Same procedure as described for primary antibody. Use a 1:200 dilution of secondary antibody.

Incubation time: 30 minutes at room temperature.

Secondary antibodies from Jackson ImmunoLabs:

goat anti-rabbit-IgG-FITC	Cat#111-095-144
goat anti-rabbit-IgG-RHOD	Cat#111-085-144
goat anti-mouse-IgG-RHOD	Cat#115-085-146
goat anti-mouse-IgG-FITC	Cat#115-095-146
goat anti-rat-RHOD	Cat#112-085-143
goat anti-rat-FITC	Cat#112-095-143

Remove solution and wash.

Before mounting coverslips, wash 3 times in PBS no BSA.

### **IF labeling with 2 antibodies of the same species**

Procedure from Jackson ImmunoLabs catalog

All steps performed at room temperature.

Fix, permeabilize and block as usual.

Incubate with **primary antibody**-rabbit or mouse for 45 minutes.

Wash.

Block with **GOAT anti-rabbit or anti-mouse** at 1:100 for 30 minutes.

Wash.

Incubate with **donkey anti-GOAT-FITC or RHODAMINE** at 1:100 for 30 minutes.

Wash.

Block with **GOAT anti-rabbit or anti-mouse Fab fragment** at 1:100 for 30 minutes.

Wash.

Incubate with **second antibody**-rabbit or mouse.

Wash.

Incubate with **donkey anti-RABBIT or MOUSE-Rhodamine or FITC** for 30 minutes.



Wash.  
Mount coverslips.



## **Mounting coverslips**

If you used -FITC secondary, use elvanol + 0.2% p-Phenylenediamine, pH 8.0-recipe under lab solution-page 21) or Vectashield (Vector).

If you used -RHODAMINE secondary, use elvanol:

**ELVANOL** 100 ml.

Dissolve 20 grams of Mowiol (Calbiochem, Cat# 475904) in 80 mls of PBS, pH 7.0

Stir for 16 hours at room temperature.

Add 40 mls of glycerol to 80 mls of prep.

Stir 16 hours at room temperature.

Centrifuge 12000 rpm for 15 minutes in SS-34 rotor to remove particles.

Decant material into air-tight bottle.

Store at 4°C.

**Elvanol + 0.1% Paraphenylene diamine** (anti-quench agent for FITC)

10 mls

Dissolve 0.01g Phenylene diamine in 0.5 ml of PBS pH 7.0.

Add this to 9.5 mls of Elvanol. Mix well.

Adjust pH to 8.0 by adding 5 M NaOH. Use pH paper to monitor pH.

Approximate amount of 5M NaOH needed 60µl for 20 mls elvanol/PPD.

Aliquot 0.5 ml into 1.5 ml screw cap tubes.

Store at -70°C in a light tight box.

Leave overnight to harden at 4°C or -20°C.

Mount coverslips by putting a small drop of mounting medium on labeled glass slide, and then putting coverslip (cell side of coverslip toward mounting medium) onto the drop of media. Use forceps to gently push out any air bubbles. Turn slide over onto a piece of clean bench paper, so that coverslip is facing down, and gently apply pressure to remove excess media.

For mounting filters: Cut filter from plastic insert using a sharp scapel. Place cut out filter, cell side facing up onto glass slide. Add a drop of mounting media onto filter and cover with coverslip. Apply gentle pressure to remove air bubbles and remove excess mounting media. Turn slide over onto a piece of clean bench paper, so that coverslip is facing down, and gently apply pressure to remove excess mounting media. Apply nail polish to seal.

Coverslips can be sealed by applying nail polish around the perimeter of the coverslip.

Store slides in slide box in a dark area at 4°C or -20°C until ready to view on microscope. Let slides come to room temperature before viewing.

Plan to view your slides the same day that you prepared them or the next morning. In some cases, not all, the fluorescence may decrease over time.

### **Sectioning kidneys for immunofluorescence:**

Remove tissue and cut into smaller pieces if required (tissue pieces should be no larger than 0.5 cm thick to allow adequate penetration of fixative)

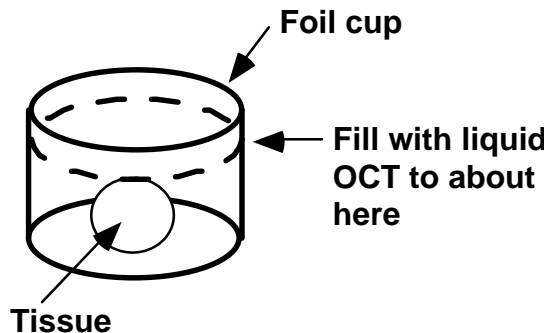
Fix tissue in PLP (refer to PLP recipe) at 4°C for 30 min. - with rocking if possible

Wash tissue 3x 10 min. with PBS at 4°C - with rocking if possible; use approximately 10 volumes of PBS for each wash (1 volume = approximate volume of tissue)

Place tissue into a container of 2.5 M sucrose in PBS at 4°C for 24 hr. - mix occasionally (by vigorously swirling or inverting); after first 24 hr., transfer to tube of fresh 2.5 M sucrose in PBS and store at 4°C [sucrose is for cryo-protection; incubation and storage allows time for sucrose to penetrate the tissue]; use approximately 20 volumes of sucrose solution for every volume of tissue; it may help to mix the tissue+sucrose a couple of times during the storage period; tissue can be stored fixed and in 2.5 M sucrose at 4°C for up to several months

Freeze tissue in OCT (Miles) cryo-embedding medium; for this, I like to make a cup-shaped container out of aluminum foil using a scintillation vial as a template; after freezing, the aluminum foil can be peeled away, leaving the frozen block of OCT with the tissue embedded in it; place tissue in bottom of foil cup, then cover with liquid OCT up to approximately 0.5 cm above top of tissue (see figure 1); grab edge of foil cup with forceps and place partially into dewar of liquid N<sub>2</sub> so that tissue and OCT freeze rapidly but not so rapidly that OCT cracks as it freezes

**Figure 1**

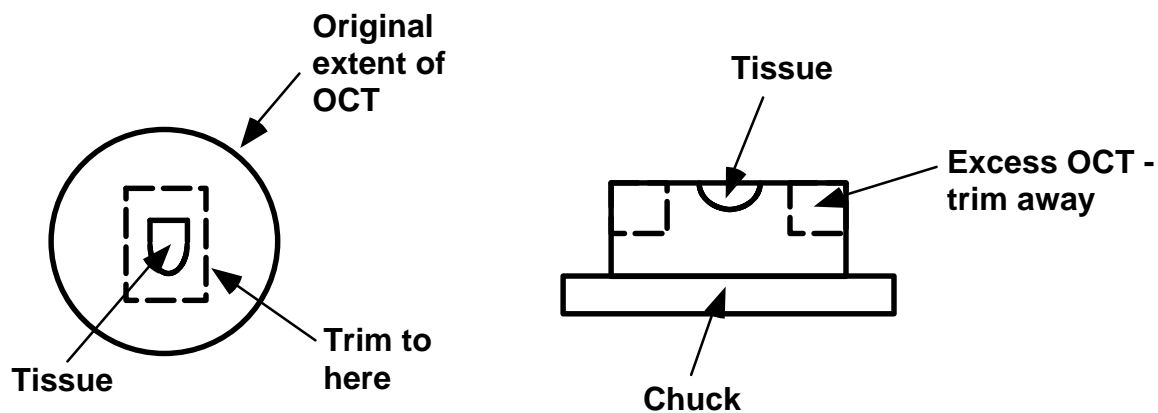


After freezing tissue in OCT, peel off aluminum foil and place on bed of dry ice; next place cryostat chuck (also called an object disk) onto bed of dry ice, face up (side with grooves); place a glob of liquid OCT onto face of chuck and before it freezes, place frozen block of OCT with tissue facing up (so you can see it) in center of liquid OCT on chuck; allow liquid OCT to freeze (this will cement tissue block to chuck); if necessary, after OCT freezes, add more liquid OCT around base of block where it meets the chuck

(tissue block should be well secured to chuck with OCT so that tissue block is not knocked off during sectioning)

Trim tissue block to get rid of excess OCT around tissue where sections will actually be cut (see figure 2 for illustration of how block should be trimmed); the smaller the cross-sectional area one sections through, the easier it is to get good sections; use a new razor blade for trimming; scrape horizontally to trim rather than trying to cut vertically; this will eliminate the possibility of shattering the block (which can be very brittle when cold enough); do not allow block to melt while trimming; if necessary, place chuck with OCT block back onto dry ice and allow to re-freeze before proceeding further

**Figure 2**

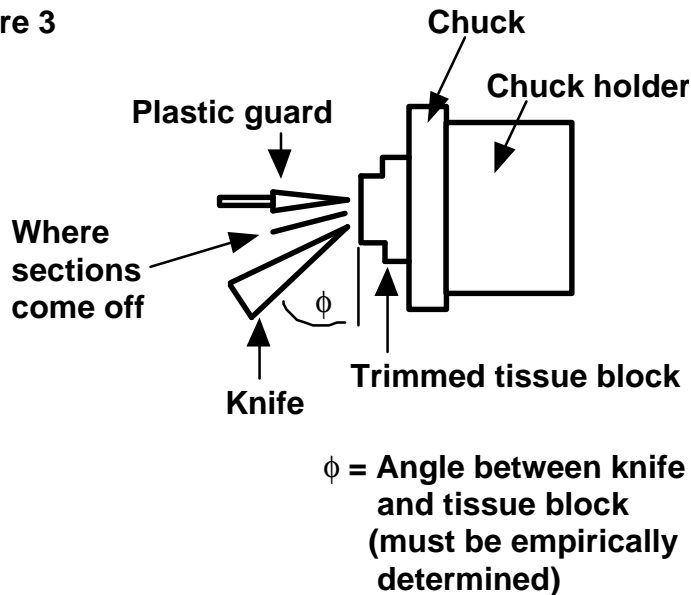


Once block is trimmed, place into cryostat which has already equilibrated to desired temperature; allow tissue block to equilibrate to desired temperature for at least 20 min. prior to sectioning; for cryo-protected tissue prepared as above, I like to section at  $-40^{\circ}\text{C}$  to  $-45^{\circ}\text{C}$ ; this is much colder than temperatures typically used for sectioning but the 2.5 M sucrose greatly decreases the freezing point of the tissue and at temperatures much higher than  $-35^{\circ}\text{C}$  the tissue melts and disintegrates as the cryostat knife passes across it (if your cryostat does not cool to around  $-40^{\circ}\text{C}$  using refrigeration alone, it may be necessary to help it by placing dry ice into the cryostat chamber and/or pouring small amounts of liquid  $\text{N}_2$  into the cryostat chamber)

Once cryostat and tissue block have equilibrated, attach chuck to chuck mount and begin sectioning; sections should be 5 - 10 mm thick (this can be set on the cryostat); it is important to set the orientation and angle of the knife blade properly - this will make a big difference in the quality of the resulting sections [in general, the thinner the sections to be cut, the larger should be the angle between the knife and the tissue block ( $\theta$ ) - see figure 3, the best angle must be empirically determined]; once the temperature and knife angle have been adjusted, the only way to get good sections is to practice (to get a feel for how fast to section through the sample); the plastic guard which rests on top of the knife blade during sectioning (see figure 3) also needs to be adjusted so that it does not

interfere with sectioning but still serves its function (which is to prevent the sections from curling up on themselves as they come off the knifer blade)

**Figure 3**



After a section has been cut, quickly transfer to a slide which has either been 'subbed' (see protocol for preparing subbed slides) or chemically treated to cause sections to adhere tightly (Fisherbrand 'superfrost plus' slides are an example) [use of 'subbed' or otherwise treated slides is necessary to keep sections from floating off the slides during the staining procedure]; transfer to slides by quickly lifting plastic guard, placing one end of a room temperature slide on the knife below the section (use thumb of one hand to hold this end of the slide on the knife blade and to apply slight pressure to it) while holding the other end of the slide up off the knife surface over the section (use thumb and forefinger of other hand for this), and then allowing the raised end of the slide to 'fall' onto the section; this immediately flattens the section out onto the slide; quickly pull the previously raised end off the knife surface (because the knife is cold and the slide is warm, the section will stick to the slide and come off with it); place the slides with sections on them onto a bed of dry ice to keep them frozen until you are ready to process them for immunofluorescence (slides with sections can also be stored at  $-80^{\circ}\text{C}$  for up to several days)

**Protocol for preparing PLP (Periodate-Lysine-Paraformaldehyde) fixative**

(From McLean and Nakane, 1974; preferred for frozen section morphology, paraffin morphology Tunel assay)

Final concentration:

- 0.075M Lysine
- 0.0375M NaPO<sub>4</sub>
- 0.1M NaIO<sub>4</sub>
- 2% Paraformaldehyde

Make two stock solutions:

**Solution A:**

Lysine solution:	50ml ddH <sub>2</sub> O	300mls:
	262 mg NaH <sub>2</sub> PO <sub>4</sub> (MW=142)	786mg
	2.17 g Na <sub>2</sub> PO <sub>4</sub> •7H <sub>2</sub> O (MW=268)	6.51g

Dissolve and bring up to 100ml with ddH<sub>2</sub>O.

Add 1.827g lysine and dissolve. Aliquot and store at -20. 5.48g lysine

**Solution B:**

8% Paraformaldehyde (PF) solution in ddH<sub>2</sub>O. (Make fresh or prepare stock at store at -20.)

In a 15 ml culture tube that can stand heat, mix 5 ml ddH<sub>2</sub>O and 1 drop (50µl) 10N NaOH, add 0.4g paraformaldehyde and vortex.

Right before use: mix 3 parts **A** with 1 part **B**. Add 21.4 mg NaIO<sub>4</sub> per 10 ml (2.14mg/ml) of final solution.

NaIO<sub>4</sub>: Sigma Cat # S-1878

This actually ends up being twice the PO<sub>4</sub> concentration specified but it works just fine and we have never bothered to change the recipe.

**Protocol for preparing 'subbed' slides**

(from Humason, Gretchen L. 1979. Animal Tissue Techniques. 661 pp.)

Dissolve 1 g of gelatin in 1 liter of hot distilled water. Cool and add 0.1 g of chromium potassium sulfate. Store in refrigerator. Dip slides several times in the solution. Drain and dry in a vertical position. Store in dustfree box.



### **Immunofluorescent staining of tissue sections:**

Thaw tissue sections on slides at room temperature until all moisture has evaporated from slides

Extract slides with CSK buffer + 1 mM Pefabloc (protease inhibitor from Bohringer Mannheim) for 15 sec. at room temperature (place slides into removable staining dish insert and extract in staining dish filled with approximately 250 ml CSK + Pefabloc)

#### CSK Buffer

50 mM NaCl

300 mM Sucrose

10 mM Pipes, pH 6.8

3 mM MgCl<sub>2</sub>

0.5% (v/v) Triton X-100

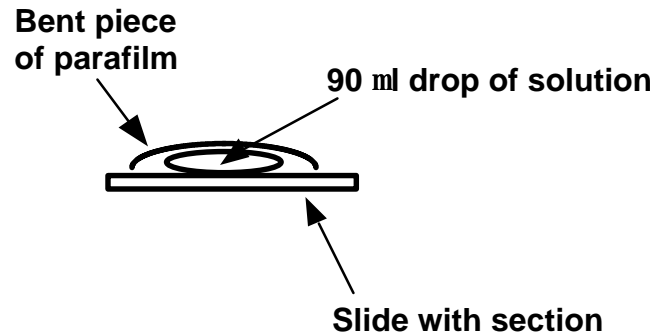
Note: Once slides have been extracted, it is extremely important not to let them dry out; so move staining dish inserts one at a time directly from one fluid-filled dish to another; in addition, when going from a wash to an incubation, remove one slide at a time from the last wash and set it up for the incubation before removing the next slide from the washing solution (it is better to leave some slides sitting in the wash for slightly longer periods of time than to risk having some of the slides dry out before you get to them)

After extraction, wash slides 2x 5 min. with PBS at room temperature in staining dishes filled with PBS (during this and all subsequent washing steps, be relatively gentle when placing inserts with slides into staining dishes or removing them so that sections do not come off slides)

Block all slides 2 hr. at room temperature with blocking solution in humidified slide incubation chamber (plastic box which can be sealed tightly with wet paper towels lining the bottom); use 90 µl / slide kept on top of section by means of a concave 1.5 cm x 1.5 cm piece of parafilm as follows (see figure 1)

- place concave piece of parafilm on bench top (make parafilm concave by slightly bending it between your fingers)
- place 90 µl drop of solution onto parafilm piece
- remove one slide at a time from the last wash and invert slide with section
- center section over drop of solution
- carefully lower inverted slide so that solution makes contact with slide surface and solution spreads out between parafilm and slide with no air bubbles trapped where section is located
- re-invert slide so that slide is right-side-up

**Figure 1**



Blocking Solution

PBS +  
50 mM NH<sub>4</sub>Cl  
25 mM L-Lysine  
25 mM Glycine  
0.2% BSA  
20% Normal Goat Serum

Note: If primary antibodies from rat or mouse are to be used to stain mouse tissue, the blocking solution should also be supplemented with a 1:10 dilution of unlabeled goat anti-rat secondary or 1:5 dilution of goat anti-mouse secondary antibody; this is required to prevent non-specific binding of these secondary antibodies to mouse tissue (in mouse kidney, this non-specific background is manifested as intense staining of all basement membranes)

Carefully remove parafilm pieces from slides with forceps; tilt slides to dump off blocking solution; wash slides 2x 5 min. with PBS + 0.2% BSA at room temperature in staining dishes

Incubate slides with primary antibodies overnight at 4°C in humidified chamber; 90 µl primary antibody solution / slide kept on top of section by means of parafilm pieces as above; primary antibodies are diluted into the following solution:

Primary Antibody Solution

PBS +  
20% Normal Goat Serum  
0.2% BSA

Next Day:

Carefully remove parafilm pieces from slides with forceps; tilt slides to dump off primary antibody solution; wash slides 2x 5 min. with PBS + 0.2% BSA at room temperature in staining dishes

Incubate slides with secondary antibodies for 2 hr. at room temperature in humidified chamber in dark (drawer works well); 90  $\mu$ l secondary antibody solution / slide kept on top of section by means of parafilm pieces as above; secondary antibodies are diluted into the following solution:

Secondary Antibody Solution

PBS +

20% Normal Goat Serum

0.2% BSA

Carefully remove parafilm pieces from slides with forceps; tilt slides to dump off secondary antibody solution; wash slides 2x 5 min. with PBS + 0.2% BSA at room temperature in staining dishes

Remove slides from last wash one at a time and mount in whatever mounting media you prefer; slides are removed one at a time to ensure that none of them dry out while sitting

## **Protein Purification**

### **GST fusion protein purification**

(Jim Marrs)

#### Media

Test induction of fusion protein  
Growth and lysis of bacteria-french press and sonication  
Prep of glutathione agarose beads  
Purification of soluble non-denatured fusion protein  
Thrombin cleavage  
Factor Xa cleavage

#### **Media:**

(from Jim Marrs)

#### **SB**

32g tryptone  
20g yeast extract  
5g NaCl  
5 ml 1N NaOH  
up to 1 liter

#### **LB**

10g tryptone  
5g yeast extract  
5g NaCl  
1 ml 1N NaOH  
up to 1 liter

#### **YT plates**

For 1 Liter:  
5 g NaCl  
16 g tryptone  
10 g yeast extract  
up to 1 Liter. Put into 2 Liter erlenmeyer and autoclave

#### **Testing induction of fusion proteins ( from Jim Marrs)**

##### **Glycerol stocks**

Pick 5 or more colonies from plate.  
Grow overnight in amp/media at 37°C with shaking.

Make glycerol stocks 50:50 bacteria: sterile glycerol. Freeze at -70.

Make a 1:100 dilution of overnight stock in 3-5 ml of amp/media. Make 2 cultures of each colony.

Grow 1 hour at 37°C with shaking.

Add IPTG to one of the two cultures. ( One is IPTG induced, the other is control)

Grow 4 hours at 37°C with shaking.

Remove 1.5ml of induced and uninduced culture. Spin 30 seconds at 13K RPMs. Remove supernatant and discard.

Vortex pellet to disrupt. Add 100µl of hot SDS-PAGE sample buffer and vortex. Heat 100°C for 5 minutes. Vortex.

Spin in microfuge 10 minutes 13K RPMs.

Collect supernatant. Run 20µl of supernatant on SDS-PAG.

### **Growth and Lysis of bacteria** (from Jim Marrs/Ken Miller 6/6/91)

Grow 100ml overnight of GST fusion protein in superbroth (SB)/amp or 2yt/amp.

Add to 1 liter (2X500ml) prewarmed media/amp in morning. Grow 1 hour.

Add 0.5ml of 100mM IPTG to each 500 ml culture (final concentration IPTG=0.1mM). Grow 5 hours.

Harvest. Spin 5K RPMs for 10 minutes in 500 ml centrifuge bottles. Discard supt.

Lyse by Sonication or French Press:

#### **Sonication**

Weigh bacterial pellet. Add (3-4X weight) volume of resuspension buffer.

	<u>Final concentration:</u>	<u>For 10 mls:</u>
Resuspension buffer: PBS	0.5% Tween 20	
	2mM EDTA	
	0.5mM Pefabloc	50µl 0.1M
	0.1 % mercaptoethanol	10µl 100%
	0.05mM Leupeptin	50µl 10mM

Add Pefabloc, -ME and leupeptin just before using.

Sonicate 3 times 30 seconds at full power with 30 second stop between each sonication.

Spin 10K RPMs for 15 minutes at 4°C to pellet. Separate supt from pellet. Save supernatant. Resuspend pellet and resonicate. Spin and separate S & P.

### **French Press**

Resuspend pellet in 10 mls of resuspension buffer ( recipe listed under sonication procedure).

Lyse by passing through French Press 2 times at 1300psi. Collect into 250 ml beaker.

Spin 10K RPMs for 10 minutes at 4°C. Save supernatant.

### **Preparation of Glutathione Agarose Beads**

(Sigma Cat#G-4510, 50ml, aliquoted into 5 ml equivalent aliquots)

Wash one 5 ml aliquot (0.37g powder) with approximately 10 ml of PBST (PBS+ 0.05% Tween 20) and wash gel onto Whatman filter in a buchner funnel to remove the maltose stabilizer. Collect gel into 15ml tube and bring volume to 10 ml with PBST. This represents "50% beads". Store at 4°C.

### **Purification of soluble, non-denatured fusion protein**

Mix 1 ml of bacterial supt with 1ml 50% Glutathione agarose beads.

Incubate rocking for 30 minutes at 4°C.

Wash beads 4 times with 10 ml of PBST in 15 ml tube.

### **Thrombin cleavage**

Wash beads one time with thrombin cleavage buffer ( 50mM Tris, pH 8.0, 150mM NaCL, 2.5mM CaCl<sub>2</sub>, 0.1% -ME).

Resuspend in 2 ml thrombin cleavage buffer. Add 6µg thrombin (Sigma T-6759, 500units, frozen in 6 µg aliquots in -70 freezer).

Incubate rocking at room temperature for 20 minutes.

Thrombin can be inactivated using PMSF.

Spin down beads and collect supt. Freeze in aliquots in -70. Quantitate on gel. Yield should be 1-3mg total in 2 mls.

**Endoproteinase Xa (Factor Xa)**  
(Procedure from USB- Cat#72250)

Factor Xa buffer:  
20mM Tris-HCL, pH 8.0  
100mM NaCl  
2mM CaCl<sub>2</sub>  
1mM NaN<sub>3</sub>

Do a test experiment with a small amount of fusion protein bound to glutathione agarose beads:

5µl fusion protein (1mg/ml)                      UNDIGESTED

20µl fusion protein (1mg/ml)                   DIGESTION REACTION  
1µl of factor Xa (200µg/ml)

Incubate tubes at room temperature.

At 2, 4, 16 and 24 hours, remove 5µl and add to tube containing 5µl 2X SDS sample buffer+DTT. Boil all samples and run on SDS PAG.

Scale up procedure based on results from small scale cleavage.



## **Serum-free hybridoma supernatant from spinner culture**

(Brigitte Angres)

Serum-free medium: HB 101, Irvine Scientific

- if cells are not yet grown in HB 101:
  - get cells gradually used to HB 101 (e.g. increase part of HB 101 in steps of 20% in the other medium with every other passage)
- grow cells in large flask in log phase
- transfer cells into sterile spinner bottle (250 ml or 500 ml bottle) and fill up half with medium
- close bottle tight and incubate cells in 37°C room on stir plate with moderate stirring speed (not too slow to avoid sedimentation of cells)
- when cells grown denser fill bottle up with medium, incubate as above
- take aliquots every day and check cells under microscope: continue culture until cells start to die (change of cell shape from round to shranked)
- pellet cells and store supernatant at 4°C (all sterile!)

## **Affinity purification of antibodies**

### **CnBr-activated Sepharose 4B Binding E-cadherin fusion protein (PAN) to Cn-Br sepharose**

1g dry=3.5ml swollen resin

Swell gel for 15 minutes in 1mM HCl.

Wash on a sintered glass filter with 1mM HCl. Use 200ml 1mM HCl/g resin in several aliquots.

Dialyze fusion protein in coupling buffer (recipe below).

Wash Cn-Br sepharose with coupling buffer (use 5ml buffer/g dry gel) and immediately transfer to ligand.

gel: buffer ratio 1:2 for coupling suspension

Concentration 5-10mg protein/ml gel

Couple at room temperature 2 hours or over night 4°C. Use end over end mixing.

Transfer gel to buffer with blocking agent- 0.2M glycine, pH 8.0-- for 16 hours at 4°C or 2 hours at room temperature.

Wash away excess absorbed protein using:

(1) coupling buffer                    0.1M NaHCO<sub>3</sub>, pH 8.3  
    0.5M NaCl

(2) 0.1M acetate buffer, pH 4  
      0.5M NaCl

(3) coupling buffer

Protein-Sepharose conjugate is now ready for use.

## **AFFINITY PURIFICATION**

**Perform all procedures at 4°C.**

### **(A) Remove GST antibodies:**

1.0ml Bacterial lysate containing GST fusion protein from Dan Stewart.

Centrifuge 13K RPMs for 20 minutes at 4°C.

Add lysate to 1 ml of glutathione agarose.

Incubate for 30 minutes at 4°C.

Spin, remove and save supt.

Wash beads 5 times with PBST. Recipe for PBST is in Bible, p.50-it is under resuspension buffer. PBST is resuspension buffer.

Incubate 2 mls of Pan-cadherin (E2) antibody serum with glutathione-GST agarose for 2 hours at 4°C.

Centrifuge at 13K RPMs and collect serum.

**(B) Affinity purify antibody:**

Run 2 mls of E2 serum (GST Abs removed) over 1 ml Cn-Br sepharose coupled to pan-cadherin fusion protein using peristaltic pump at slow flow rate or by hand at 4°C. I do it by hand. Recirculating serum over column 10 times.

Wash loaded column with 20 ml of 0.1M KPO<sub>4</sub> buffer, pH 7.4 (**I used PBS for this step**)

Elute antibody with 3-4 mls 0.1M glycine, pH 2.5. Add 1.0ml of glycine to top of the column. Wait until most of it runs into column before adding the next 1.0 ml.

For fractions: Add 70µl 0.75M Tris, pH 8.8 to 12 empty tubes. Collect 12 fractions at 1.0 ml each from column.

Dilute each fraction 1:10 and read absorbance @ 280 & 320 and/or run SDS gel to check purification.  
(OD<sub>280</sub> 1mg/ml read 1.35)

I did not dilute my fractions. I removed 5µl of sample and mixed with 5µl of 2X sds sample buffer, boiled and loaded onto a 10% acrylamide SDS mini gel.

Wash the column with another 10 mls of glycine and then run 20 mls of PBS+ sodium azide. Store column in PBS+ sodium azide. Keep at 4°C.

# Molecular Biology

## Molecular biology enzyme list

Blue Index	Box #1 Name	Company	Blue Index	Box #2 Name	Company	Blue Index	Box #3 Name	Company
1	Acc I	Promega	25	Dra I	Promega	49	Pfl MI	NEB
2	Acl I	NEB	26	Ecl I CRI	Promega	50	PshA I	NEB
3	Age I	NEB	27	Ecl136 II	Fermentas	51	<b>Pst I</b>	Promega
4	Alu I	Promega	28	<b>Eco 47 III</b>		52	Pvu I	Promega
5	AlwN I	NEB	29	Eco NI	NEB	53	Pvu II	Stratagene
6	<b>Apa I</b>	Promega	30	Eco O109	Stratagene	54	Rsa I	Promega
7	ApaL I	Stratagene	31	<b>Eco RI</b>	NEB	55	<b>Sac I</b>	Promega
8	Avr II	NEB	32	<b>Eco RV</b>	Promega	56	<b>Sac II</b>	Promega
9	<b>BamHI</b>	Promega	33	Hae II	NEB	57	<b>Sal I</b>	Promega
10	Bcl I	Promega	34	<b>Hinc II</b>	Stratagene	58	<b>Sca I</b>	Promega
11	<b>Bgl I</b>	Promega	35	<b>Hind III</b>	Promega	59	Sfi I	Promega
12	<b>Bgl II</b>	Promega	36	Hpa I	Stratagene	60	Sfu I	Roche
13	Bsa BI	NEB	37	<b>Kpn I</b>	Promega	61	<b>Sma I</b>	Promega
14	Bsm BI	NEB	38	<b>Mbo I</b>	Promega	62	Sna BI	Promega
15	Bsp 106	Stratagene	39	Mlu I	Promega	63	<b>Spe I</b>	Invitrogen
16	Bsp CI	Stratagene	40	Mwo I	NEB	64	<b>Sph I</b>	Promega
17	Bsp HI	NEB	41	Nae I	Promega	65	Ssp I	Promega
18	<b>Bst EII</b>	Promega	42	Nar I	Promega	66	<b>Stu I</b>	Promega
19	Bst XI	Promega	43	Nco I	Promega	67	Tth III	NEB
20	<b>Bsu 36I</b>	Promega	44	Nde I		68	<b>Xba I</b>	
21	Cla I	NEB	45	Nhe I	NEB	69	<b>Xho I</b>	Invitrogen
22	<b>Dpn I</b>	Promega	46	<b>Not I</b>	Promega	70	<b>Xma I</b>	NEB
23			47	Nsi I	Promega	71	Xmn I	Promega
24			48	Nsp I	NEB	72		

\* Bold enzymes: an extra tube is in Green Box #4

Green Index	Box #4: Name	Backup Enzyme Company	Yellow Index	Box #5: Modifying Enzymes Name	Company
73	Apa I	Promega	96	Alk. Phosphatase (CIP)	Promega
74	BamHI	NEB	97	Alk. Phosphatase (CIP)	Promega
75	Bgl I	Promega	98	Klenow	Takara
76	Bgl II		99	Pfu polymerase	Stratagene
77	BstEII	NEB	100	Pfu polymerase	Stratagene
78	Bsu 36I	Promega	101	Pfu polymerase	Stratagene
79	Bsu 36I	Promega	102	rRNasin (Rnase Inhibitor)	Promega
80	Dpn I	Promega	103	rRNasin (Rnase Inhibitor)	Promega
81	Eco 47III	Promega	104	RQ I Rnase-free Dnase	Promega
82	EcoRI	Promega	105	RQ I Rnase-free Dnase	Promega
83	EcoRV	NEB	106	rTdT	Gibco
84	Hinc II	Promega	107	TNT T7 Polymerase	Promega
85	Hind III	Promega	108	T4 DNA Polymerase	Promega
86	Kpn I	Promega	109	T4 Polynucl. Kinease	Promega
87	Mbo I	Promega	110	T4 Polynucl. Kinease	Takara
88	Not I	Promega	111	T4 Polynucl. Kinease	Takara

89 Pst I Promega  
90 Sac I NEB  
91 Sac II NEB  
92 Sal I Promega  
93 Sca I Promega  
94 Sma I  
95 Spe I Invitrogen  
96 Spe I Promega

112 T4 DNA Ligase HC Promega  
113 T4 DNA Ligase Buffer Invitrogen  
114 T4 DNA Ligase Invitrogen  
115 dATP 10mM  
116 dATP 100mM  
117 dCTP 100mM  
118 dGTP 100mM  
119 dTTP 100mM

**White Box:**  
Sph I Promega  
Stu I NEB  
Xba I NEB  
Xho I NEB  
Xma I NEB

**Backup  
Enzyme**

## **RNAi Protocols for MDCK cells**

### **Transfecting only siRNA to knockdown endogenous protein**

MDCK cells, split fewer than 20 times, are plated in media without antibiotics on collagen-coated coverslips in 6-well plates, so that they are 50% confluent the next day (about  $3 \times 10^5$  cells). (This may be the optimal amount, but Amy has transfected cells at a wide range of densities.) Instead of plating cells the day before transfection, transfection complexes can alternatively be added at the time cells are plated, before they have settled onto the plate; this may increase the degree of knockdown.

Next day, for each well of 6-well plate, mix:

Tube1: 15ul of 20uM siRNA (21mer duplex with dTdT 3' overhangs, in 1x annealing buffer, ordered from Dharmacon) to 250 ul optiMEM, mix by tapping with finger.

Tube2: 15ul oligofectamine(mixed) to 60ul optiMEM. Mix by tapping and wait 7 to 10min (not more than 5 minutes if using regular media without serum instead of optiMEM).

Mix Tube1 and Tube2 by *inverting* and wait 20 to 25 min. Then add dropwise to cells. Leave serum-containing media (2ml per well) on cells.

After 4-6 hours, can change or add fresh media to avoid cytotoxicity of oligofectamine (half as much oligofectamine may also be used).

Cells can be split onto new collagen-coated coverslips when confluent (OK to use PSK antibiotics here).

Fix and stain cells 2-4 days after transfection. (If knockdown is not strong enough, cells can be split and retransfected.)

### **Co-transfecting a marker plasmid and siRNA**

*(This may be a useful way to check if the siRNA is getting transfected, although transfection efficiency may be lower than with above protocol.*

*I used a GFP construct as a marker and 3 days after transfection saw GFP expression in about 10% of cells, all of which had knocked down levels of the protein targeted by the siRNA. About 35% of all cells had knockdown, that is, much more than were expressing the plasmid.)*

MDCK cells, split fewer than 20 times, are plated in media without antibiotics on collagen-coated coverslips in 6-well plates, so that they are 80-90% confluent the next day.

Next day, for each well of 6-well plate, mix:

5ug plasmid DNA (preferably Endo-free) and 15ul of 20uM siRNA to 250 ul optiMEM, mix by tapping with finger.

15ul Lipofectamine 2000 (mixed) to 250ul optimem. Mix by tapping and wait 7 to 10min (not more than 5 minutes if using regular media without serum instead of optimem).

Mix RNA dilution with Lipofectamine 2000 dilution by *inverting* and wait 20 to 25 min before adding dropwise to cells. Leave serum-containing media (2ml per well) on cells.

After 4-6 hours, can change or add fresh media to avoid cytotoxicity.

Cells can be split onto new collagen-coated coverslips when confluent.  
Fix and stain cells 2-4 days after transfection.

## **Miscellaneous protocols**

### **35mm film development**

(Tri-Pan Black & White film)

**DIAFINE A** 3 minutes, shake every 60 seconds

**DIAFINE B** 3 minutes, shake every 60 seconds

**WATER** 30 seconds, several changes

**FIXER** 15 minutes, shake every 60 seconds

**PHOTOFLO** Rinse

### **HANG NEGATIVE TO DRY**

#### **Development of Tri-X pan film (from ET Chen)**

- 1) Dilute Kodak T-max developer 1:5. Incubate film for 6 minutes at RT (21<sup>o</sup>)  
Developer is good for 2 weeks from date of dilution.
- 2) Wash for 30 seconds with water.
- 3) Incubate with Kodak fixer (179g/1liter) for 10 minutes at RT (21<sup>o</sup>). This solution is good for 2 weeks. Plan ahead- this solution requires 20-40 minutes to dissolve.
- 4) Wash film over 1-2 hour period with several changes of water.
- 5) Photoflo 1:200 dilution
- 6) Hang film to dry.



