

# **Argaves Laboratory Protocols**

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## SDS-POLYACRYLAMIDE GEL PROTOCOL

### SOLUTIONS:

Lower Gel Buffer: 0.4% SDS in 1.5M Tris-HCl pH 8.8

Upper Gel Buffer: 0.4% SDS in 0.5M Tris-HCl pH 6.8

29.2% Acrylamide, 0.8% Bis-acrylamide:

Component	Amount
acrylamide	29.2 g
bis acrylamide	0.8 g
dH <sub>2</sub> O	up to 100 ml

Note: acrylamide stock should be filtered through Whatman #1 filter paper and stored in amber bottles at 4°C.

Running Gel Buffer 5X:

Component	Amount
SDS	5.0 g
Tris base	14.5 g
Glycine	72.0 g
dH <sub>2</sub> O	up to 1000 ml

pH to 8.2-8.3 Note: Never titrate with HCl

SDS-PAGE Sample Buffer (4X, Reducing): 1 X = 50 mM Tris pH 6.8, 2 % SDS, 0.1% bromophenol blue, 10 % glycerol, 1 % β-mercaptoethanol.

Component	Amount
1 M Tris HCl pH 6.8	20 ml
glycerol	40 ml
bromophenol blue	200-400 mg
β-mercaptoethanol	4 ml
SDS	8 g
H <sub>2</sub> O	up to 100 ml

Note: Filter solution through a 0.22-0.45 μm filter before addition of SDS. Mix thoroughly, aliquot into 1.5 ml tubes and store at -20°C.

SDS-PAGE Sample Buffer (2X, Reducing):

Component	Amount
SDS	0.4 g
glycerol	2.0 ml
β-mercaptoethanol	1.0 ml
0.625M Tris-HCl pH 6.8	2.0 ml
0.2% bromophenolblue	0.3 ml

### SLAB GEL RECIPES:

Component	Separating Gel						Upper Gel
	5.0%	7.5%	10.0%	12.5%	15.0%	20.0%	
Acrylamide %	5.0%	7.5%	10.0%	12.5%	15.0%	20.0%	5.0%
Upper Gel Buffer	---	---					1.87 ml
Lower Gel Buffer	2.0 ml	2.0 ml	2.0 ml	2.0 ml	2.0 ml	2.0 ml	---
Acrylamide Stock	1.4 ml	2.0 ml	2.65 ml	3.35 ml	4.0 ml	5.35 ml	1.12 ml
Water	4.6 ml	4.0 ml	3.35 ml	2.65 ml	2.0 ml	0.65 ml	4.5 ml
10% AMPS	60 $\mu$ l	60 $\mu$ l	60 $\mu$ l	60 $\mu$ l	60 $\mu$ l	60 $\mu$ l	38 $\mu$ l
TEMED	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l	8 $\mu$ l
Total Volume	8.07 ml	8.07 ml	8.07 ml	8.07 ml	8.07 ml	8.07 ml	7.54 ml

## REDUCTION AND CARBOXYMETHYLATION OF PROTEINS FOR SEQUENCING

- Denature at least 100  $\mu\text{g}$  protein in either urea or guanidine-HCL (6-8 M final concentration) in 1 ml.
- Add 3  $\mu\text{l}$  mercaptoethanol and incubate room temperature for 2 hours.
- Add 10 mg iodoacetamide, mix and adjust pH to 8.0 with 5 M NaOH. Incubate at room temp. for 1 hour.
- Dialyze against 10 mM Tris pH 8.0 or water.
- Concentrate volume down to 50  $\mu\text{l}$  in speed vac.
- Electrophorese sample on SDS-PAGE and transfer to nitrocellulose.
- Stain with Ponceau-S and excise band for proteinase digestion (e.g., lys-C).

## SDS-PAGE FOR PROTEIN SEQUENCING

- Wear gloves, wash all components of electrophoresis apparatus with soap and rinse thoroughly.
- Pour separating gel and allow it to polymerize overnight.
- Precondition the separating gel. Place 50  $\mu$ M glutathione (dilute 200X glutathione stock (10 mM glutathione, Sigma # G-4251) into electrophoresis buffer), 1X electrophoresis buffer in upper buffer chamber and regular 1X electrophoresis buffer in the lower chamber. Run gel at half-normal voltage or current conditions for twice the normal time.
- Pour stacking gel as usual.
- Prepare samples by heating at 50-60°C for 10-15 min. See procedure for reduction and carboxymethylation of proteins for sequencing.
- Add thioglycolate running buffer (0.1 mM sodium thioglycolate, Sigma # T-0632) in standard 1X electrophoresis buffer) to upper chamber. Apply samples and run under normal voltage/current conditions at 4°C.
- Wear gloves when handling gel from this point on.
- Stain gel in 0.1% Coomassie brilliant blue R-250, 1% glacial acetic acid, 40% methanol for minimum time necessary to stain protein.
- Destain gel in 50% methanol minimal amount of time to see your polypeptide band.
- Place gel on plastic wrap and cut out band from the gel using razor blade. Carefully chop gel slice into small pieces and place pieces into a microfuge tube.
- For amino-terminal sequencing of proteins on a Hewlett Packard (model G1000S) sequencing instrument add extraction buffer (0.1% SDS, 0.1M Tris, pH 8.0) to tube using enough to just cover gel pieces (500  $\mu$ l). Incubate the tube overnight at 37°C.

### References:

- Hunkapillar et al., (1983) Meth. Enzymol. 91, 227-236.
- Spiker and Isenburg, (1983) Meth. Enzymol. 91, 214-.
- Applied Biosystems User Bulletin "Protein Sequencers", April 1990, #42.
- A Practical Guide to Protein and Peptide Purification for Microsequencing, Ed. Paul T. Matsudaira, Academic Press, (1989) ISBN 0-12-480280-X.

## ELECTROBLOT TRANSFER OF SDS-PAGE SEPARATED PROTEINS TO NITROCELLULOSE AND IMMUNOSTAINING

PROTEIN TRANSFER BUFFER: Tris (25 mM), Glycine (192 mM), methanol 20%, approximate pH = 8.3

Component	To make 1000 ml	To make 1500 ml
Tris	3.033 g	4.55 g
glycine	14.4 g	21.6 g
methanol	200 ml	300 ml
dH <sub>2</sub> O	up to 1000 ml	up to 1500 ml

Note: Mix the Tris and glycine with H<sub>2</sub>O then add methanol. Chill prior to use.

### TRANSFER PROCEDURE:

- Best results are achieved when transfer is performed immediately following SDS-PAGE, but gels can be stored in plates in cold room overnight with fair results.
- Remove one glass plate and place a dry Whatman no. 2 filter paper (the size of the Scotchbrite pads) on the gel. Rub the Whatman paper to adhere the gel to the paper, flip plate/gel/paper sandwich and slowly peel back the filter paper along with the gel.
- Place a prewet (5 min. in dH<sub>2</sub>O, 1 min. in transfer buffer) SS-BA85 nitrocellulose sheet (or PVDF membrane pre-treated according to manufacture instructions) onto the gel and with a gloved hand, rub out any air bubbles trapped between the gel and filter. This step is crucial to achieving complete transfer.
- Place a prewet (with transfer buffer) Whatman no. 2 filter paper on top of the nitrocellulose and rub out any trapped air bubbles.
- Dip the gel-nitrocellulose-Whatman filter sandwich into the electrophoresis buffer.
- Place the gel-nitrocellulose-Whatman filter sandwich into gel holder and then into the electrophoresis tank (filled with transfer buffer), taking care to orientate the nitrocellulose filter proximal to the anode (+) pole.
- Insert the cooling coil and circulate water or place transfer apparatus in ice.
- Electrophorese at 70 V, approx. 250 mA for 4-5 hours (2 hours may be sufficient for transfer of proteins ≤66 kDa).
- Peel the nitrocellulose filter away from gel using a forceps/gloved fingers.

- Stain filter with Ponceau Stain (0.1-0.2% Ponceau in 10% acetic acid) to see lanes, remove excess stain with deionized water, mark or cut filter, and destain in PBS or TBS.
- Place filter in a sealable bag.
- To block unoccupied binding sites, add 3-5% non-fat dry milk, TBS and incubate for 1 hour at room temperature.
- Dilute primary antibody in 5% non-fat dry milk, TBS, 0.05% Tween-20 (0.2 µg IgG/ml is a good starting point), add to filter in bag, seal and place on nutating platform..
- Incubate for for 1-4 hours at room temperature or 4°C overnight.
- Wash three to four times with TBS, 0.05% Tween-20 (250 ml each wash).
- Incubate with conjugated secondary antibody diluted 5% non-fat dry milk, TBS, 0.05% Tween-20.
- Wash three to four times with TBS, 0.05% Tween-20 (250 ml each wash).
- Detect bound antibodies using appropriate detection reagents (e.g., Amersham ECL kit).
- Expose to MR series of Kodak film.

## **REVERSIBLE STAINING OF FILTER BOUND PROTEIN WITH PONCEAU S**

- Following electrophoretic transfer remove filter and incubate in 0.2% Ponceau S (Sigma P-3504), 10 % acetic acid for 1-2 minutes.
- Remove excess stain by washing with deionized water. Filter can be marked or photographed at this point.
- Incubate filter in 1X PBS solution for 5 minutes to destain protein bands.

## **ALCIAN BLUE STAINING OF GLYCOPROTEINS IN SDS-PAGE GELS**

- Fix gel in 12.5% TCA for 30 minutes.
- Wash gel with water 4 times.
- Incubate gel in 1% periodate (in 3% acetic acid) for 1 hour.
- Wash gel with water 4 times. Washes should be checked by adding a few drops of a 1%  $\text{AgNO}_3$ . If a smokey color appears, continue washing.
- Incubate gel in 1% sodium metabisulfite (in water) for 30 minutes.
- Wash gel with water 4 times.
- Stain gel with 0.5% alcian blue (in 3% acetic acid) for 4 hours.
- Destain with 7% acetic acid.

## HISTOLOGICAL FIXATIVES

### 3.7% PARAFORMALDEHYDE FIXATIVE

- Heat 90 ml H<sub>2</sub>O in microwave for 1 min.
- Add 3.7 g paraformaldehyde while stirring.
- Add 1 drop of 5 M NaOH and continue stirring until paraformaldehyde dissolves completely.
- Add 10 ml 10 X PBS or Buffer F (1X= 10 mM NaPO<sub>4</sub>, 150 mM NaCl, 2 mM CaCl<sub>2</sub>, pH 7.4), and filter.
- This solution must be made fresh each day.
- 3.0% Paraformaldehyde fixative:
  - Heat 90 ml H<sub>2</sub>O in microwave for 1 min.
  - Add 3.0 g paraformaldehyde while stirring.
  - Add 1 drop of 5 M NaOH and continue stirring until paraformaldehyde dissolves completely.
  - Add 10 ml 10 X Buffer F and filter.
  - This solution must be made fresh each day.

### METHACARN FIXATIVE

60% methanol, 30 % chloroform, 10 % acetic acid.

- Reference: Warburton et al., 1982. *J. Histochem. Cytochem.* 30, 667-676.

### OMNIFIX

A non-formalin tissue fixative available from:

Zymed Laboratories Inc.  
52 South Linden Ave.  
South San Francisco, CA 94080  
(415) 871-4494  
(800) 874-4494

## SILANE COATING OF MICROSCOPE SLIDES

- Place slides in metal racks.
- Wash slides in 10% Extran 300 detergent at 50°C 2-24 hrs. (EM Science no. EX0996-1).
- Wash in running deionized water for 2 hr.
- Dry in 120-160°C oven 30 min to 1 hr.
- Dip slides for 5 seconds in 2% 3-aminopropyltriethoxysilane (Sigma A-3648) in acetone.
- Rinse 2 times in acetone.
- Rinse 2 times in diH<sub>2</sub>O.
- Dry at 42°C.

## PHOTOMICROSCOPY SIZE MEASUREMENT CALCULATIONS

Sample:

Objective = 40 X

Photoeyepiece = 3.3 X

Ext. tube = 1.25 X

- Total magnification at the plane of the 35 mm film = 165 X
- measured area of negative = 35 mm x 23.5 mm = 822 mm
- measured area of photo print = 60 mm x 40 mm = 2400 mm
- magnification from negative to print =  $2400\text{mm}/822\text{mm} = 2.92\text{X}$
- total magnification of print  $165\text{X} \times 2.92\text{X} = 481\text{X}$
- 1cm at 481 X =  $2.1 \times 10^{-3}$  cm at 1 X
- $2.1 \times 10^{-3}$  cm x  $1 \times 10^{-2}\text{m}/\text{cm} = 2.1 \times 10^{-5}$  m =  $21 \times 10^{-6}$  m = 21  $\mu\text{m}$
- A 1 cm bar on your print corresponds to 21  $\mu\text{m}$ .

## INDIRECT IMMUNOFLUORESCENT MICROSCOPY

- Coat glass Lab-Tek chamber slides (Nunc Inc., Naperville, IL) with desired protein, \_\_\_\_\_, at \_\_\_\_\_  $\mu\text{g/ml}$ , for 4 hours at room temperature, or use plastic slides with no coating.
- Seed wells with \_\_\_\_\_ cells at \_\_\_\_\_ density. (For medium/high density of gingival fibroblasts, start with one 100 mm dish of confluent cells, resuspend the cells and add half of the cells to 25 mls of media, use this to seed plates, allow cells to attach from 4-24 hours before fixing).
- Wash cells once with PBS, then fix cells for 20 minutes with 3.7% paraformaldehyde (Fluka, Buchs, Switzerland).
- Remove the fixative after 20 min and wash 3 X 5 min with dPBS while shaking.
- Incubate plates for 1 hr at room temp with 3% normal goat serum (NGS) in PBS to block.
- Dilute the primary antisera in 3% NGS/PBS and incubate 2 hr at 37°C or o/n at 4°C.
- Wash the slides gently 3 X 5 min with dPBS while shaking.
- The fluorochrome conjugated antisera (Cappel, West Chester, PA) should be centrifuged in the ultracentrifuge for 15 min at 50,000 rpm at 4°C, before being diluted 1/40 in 3% NGS/PBS, incubate with the slides for 20 min at room temp. (Be sure to keep the fluorochrome and the slides, once treated with the fluorochrome, in the dark, as the fluorochrome will quench).
- If background is a problem, the fluorochrome should be filtered, and can be kept at 4°C for short periods of time to prevent precipitation.
- Wash the slides again gently 3 X 5 min with PBS.
- Apply mounting solution (e.g. Vectasheild or 50% glycerol in dPBS) to the surface of the slides and a coverslip overlaid and fixed to the surface with clear nail polish. Reseal edges of coverslip with nail polish
- Photographs are taken with Fujichrome 1600 color reversal film.

### Preparation of 3.7% paraformaldehyde:

- heat 90 mls of di water for 1 min in microwave, add 3.7g of paraformaldehyde while mixing with magnetic stirrer.
- Add 1 drop of 4 M NaOH which will dissolve the paraformaldehyde.
- Add 10 mls of 10X dPBS and chill.
- Filter the solution and add 0.2% Triton X-100 if permeabilized cells are desired.

## IMMUNOFLUORESCENT STAINING OF LIVE CELLS

- Incubate cells (grown on coverslips) with antibody (heat inactivated and diluted in medium 50  $\mu\text{g/ml}$ ) for 1 h  $37^\circ\text{C}$ .
- Wash 3 times in buffer F (10 mM  $\text{NaPO}_4$ , 150 mM  $\text{NaCl}$ , 2 mM  $\text{CaCl}_2$ , pH 7.4).
- Fix in 3% paraformaldehyde 15 min at rt.
- Rinse with 50 mM  $\text{NH}_4\text{Cl}$ .
- Rinse 2 times with buffer F.
- Add 0.05% Triton X-100 in buffer F ( $4^\circ\text{C}$ ) and incubate on ice for 10 min.
- Add secondary antibody and incubate 30 min at rt.
- Wash 4 times (15 min each) with buffer F at rt.
- Mount coverslips using mounting medium (Sigma #1000-4) and seal with nail polish.

### Preparation of 3.0% Paraformaldehyde fixative:

- Heat 90 ml  $\text{H}_2\text{O}$  in microwave for 1 min.
- Add 3.0 g paraformaldehyde while stirring.
- Add 1 drop of 5 M  $\text{NaOH}$  and continue stirring until paraformaldehyde dissolves completely.
- Add 10 ml 10 X Buffer F (1X = 10 mM  $\text{NaPO}_4$ , 150 mM  $\text{NaCl}$ , 2 mM  $\text{CaCl}_2$ , pH 7.4), and filter.
- This solution must be made fresh each day.

## PREPARATION OF APOE-FREE HDL

- Dialyze HDL sample against imidazole buffer (15 mM NaCl, 5 mM imidazole, pH 6.5).
- Add the dialyzed HDL solution to the heparin-Sepharose column (see below) and collect 1/4-column volume fractions.
- When the sample has completely entered the resin, add additional buffer and continue collecting fractions until spectrophotometer readings at 280 nm reach zero.
- Pool peak fractions and dialyze against dPBS, 0.3 mM EDTA. Measure absorbance at 280 nm. Determine protein concentration using BCA protein assay (Pierce) or any other Lowry-based assay modified for lipoproteins.
- Filter-sterilize using a 0.22  $\mu$ m filter.
- Aliquot and store at 4°C. Capping the tubes under a stream of nitrogen will minimize oxidation.
- ApoE-containing HDL that is bound to the column can next be eluted by applying 2 column volumes of 1 M NaCl, 5 mM imidazole (pH 6.5) and collecting 1/4 column volume fractions.
- Note: Regardless of whether you want the apoE-containing HDL, you must elute the column with 1 M NaCl, 5 mM imidazole (pH 6.5) and equilibrate with 15 mM NaCl, 5 mM imidazole (pH 6.5) as part of the column regeneration procedure.

### Preparation of a Heparin-Sepharose column:

- Add imidazole buffer (15 mM NaCl, 5 mM imidazole, pH 6.5) to dry heparin-Sepharose in fritted glass funnel. Wash resin with at least 200 ml of imidazole buffer per 1 gm of heparin-Sepharose. Transfer the resin to a chromatography column.
- Use the following guideline to assist you with your chromatography procedure:

Original HDL Concentration (mg/ml)	Column bed vol d (cm) X h (cm)	Column bed volume (ml)	HDL sample Volume (ml)	Loaded protein (mg)	Fraction size (ml)
6.5 mg/ml	1.5 X 4	7	3	19.5	1.75
200 $\mu$ g/ml	0.7 X 2.5	1	1	0.2	0.25

- Note: Apply no more than 3 mg HDL per ml of resin.

## **FLOW CYTOMETRY ASSAY OF Dil-HDL UPTAKE**

### **1. BN/ F9 cell culture**

- Seed cells into 12 well dishes at  $1-1.5 \times 10^5$  cells per well and incubate at 37°C, 5% CO<sub>2</sub> in serum-containing medium (e.g., for BN cells use MEM, 10% FCS pen/strep; for Differentiated F9 cells use DMEM with 10% BCS, pen/strep.) until the cells attach (4-6 hours). Note: Differentiated F9 cells require 0.1% gelatin-coated plates.
- Remove the medium and add serum-free medium (MEM or DMEM, ITS) and grow cells overnight at 37°C, 5% CO<sub>2</sub>.

### **2. Preparation of solutions containing Dil-HDL and competitors**

- ~2.5 hours prior to the scheduled flow cytometry appointment:
- Label 1.5 ml Eppendorf tubes and place on ice.
- Add 275 µl (or more, depends on most diluted sample; normalize) of serum-free medium containing 3 µg/ml Dil-HDL (made apoE-free by absorption on heparin-Sepharose) per tube except for a control tube that gets 275 µl of serum-free medium lacking Dil-HDL.
- To each tube add the desired amounts of competitors and adjust the final volume to 500 µl with dPBS. Note: All components to be associated with the cells must be dialyzed against dPBS ahead of time. Determine the protein concentrations after dialysis using the BCA assay. Do not dilute the medium by more than 10-15% if possible. In Table I., treatments 3+4 test the system not controls for fragments but system exp 5-15.
- Incubate the solutions containing Dil-HDL for 30 min at 37°C. (Not for antibodies only, remove from chart), surface labeling, oligos

### **3. Addition of samples to cultured BN or differentiated F9 cells and flow cytometry**

- Obtain cells in 12 well dishes as describe in section 1.
- Remove medium, wash cell layer once with serum-free medium and add 500 µl of solutions prepared in step 2.
- Incubate cells at 37°C, 5% CO<sub>2</sub> for 90 minutes.
- While cells are incubating warm up trypsin/EDTA/proteinase K solution to 37°C and label two sets of tubes; one set of 1.5 ml Eppendorf tubes and another of 5 ml tubes with cell strainer caps (Falcon). Place the tubes on ice
- Carefully remove supernatants from each well (save if desired, or else discard)
- Add 400 µl of pre-warmed trypsin/EDTA/proteinase K solution to each well and incubate 1-2 mins at room temperature.
- Collect the released cells using a 1 ml pipettor.
- Wash each well with 150 µl of trypsin/EDTA/proteinase K solution and pool with previous cell suspension.

- Centrifuge at 4°C at 400 x g for 6 min (2800 rpm in Eppendorf Microfuge). Remove supernatants with a pipette.
- Resuspend the cell pellet in 350 (400-preferred)-600 µl ice cold dPBS by gently pipetting up and down based upon cell pellet size.
- Using a Pipetman, carefully filter the cell suspension through the screens of the above-labeled tubes to remove clumped cells to obtain single cell suspension
- Bring the tubes on ice to the flow cytometry laboratory. Bring the legend so that the operator can label the data files appropriately.

## **FLOW CYTOMETRY ANALYSIS of DiI-HDL UPTAKE in BN CELLS**

Designed for signaling effector experiments, 24-well plate format.

### **Day 1**

- Plate BN cells at  $0.8-1 \times 10^5/\text{cm}^2$  in complete medium (CM: MEM/10%FBS/1X non essential amino acids/1X pen-strep) in 24 well plates. Allow cells to attach for 6-12 h, then replace medium with serum free medium (SFM; MEM/ITS/ non essential amino acids / pen-strep). Grow overnight 37°C, 5% CO<sub>2</sub>.

### **Day 2**

1. Warm SFM (37°C) and assemble reagents needed for uptake assay (RAP, DiI-HDL, apoE-free HDL, DMSO, etc.) Make sure the frozen agents are thawed.
2. Aspirate medium from the cells and wash with 1 ml warmed SFM.
3. Aspirate the wash medium and add 0.3 ml SFM; incubate 1 hr, 37°C, 5% CO<sub>2</sub>
  - i. During this incubation prepare competitor and DiI-HDL dilutions in SFM. For 24 well plates, do calculations for 400 µl (total well volume) but prepare 100 ul mixtures for addition. Use DiI-HDL at 1-2 µg/ml. Warm mixtures at 37°C for 10-15 min.
  - ii. Example: 1 µg/ml DiI-HDL treatment; XS HDL, 200X = 200 µg/ml; RAP, 1µM. Prepare a dilution of DiI-HDL (0.4 µg/20 µl) sufficient for all samples.
4. Add 100 µl of each prewarmed agent or competitor to the wells. Incubate cells for 15 min, 37°C.
5. Add 20 µl diluted DiI-HDL (without aspirating agents).
6. Incubate cells for 30 min, 37°C.
7. Prepare Trypsin-EDTA/Proteinase K (50 µg/ml) and warm at 37°C.
8. Wash cells with DPBS (1 ml/well), 2 times.

9. Add 0.2 ml trypsin-EDTA/Proteinase K and incubate for 1-2 at RT. Prepare labeled eppendorf tubes with 0.5 ml cold DPBS\*\*.
10. Pipet cells to loosen from well and transfer to Eppendorf-tubes with DPBS\*\*; place on ice. From this moment, work as much as possible on ice! Keep Eppendorf-tubes cold.
11. Spin tubes: 3 min, 3000 rpm, 4°C.
12. Aspirate supernatant, add 0.5 ml DPBS and vortex gently to resuspend cell pellet.
13. Spin tubes: 3 min, 3000 rpm, 4°C.
14. Repeat DPBS wash, step 12 + 13.
15. Aspirate supernatant, and resuspend pellet in 300µl DPBS.
16. Transfer solutions into 12 x 75 mm polystyrene tubes -- if aggregates are present, strain the cells using strainer cap tubes. Store on ice. Samples are now ready for flow-cytometry.

\*\*Alternatives include using complete medium, serum-free medium, or DPBS plus trypsin inhibitor to stop trypsin digestion.

## **FLOW CYTOMETRY ASSAY OF CELL SURFACE ANTIGENS**

1. Detach cells using 2 mM EDTA, PBS or non-enzymatic cell dissociation solution.
2. Wash cells with serum-free medium.
3. Centrifuge 10 mins at 735 x g at rt.
4. Wash cells with medium containing 0.1% sodium azide, 2% BCS.
5. Aliquot  $2.5-5 \times 10^5$  cells per 1.5 ml tube. Centrifuge 8 mins at 735 x g at 4°C (Eppendorf Microfuge model 5415 3000 rpm = 735 x g).
6. Resuspend cell pellets in 250 µl medium containing 0.1% sodium azide, 2% BCS and primary antibody (10 µg/ml for purified IgG and 2 µl/ml for serum).
7. Incubate on ice for 40 mins.
8. Centrifuge 8 mins at 735 x g at 4°C.
9. Wash cells with medium containing 0.1% sodium azide, 2% BCS.
10. Centrifuge 8 mins at 735 x g at 4°C.
11. Resuspend cell pellets in 250 µl medium containing 0.1% sodium azide, 2% BCS and FITC-conjugated secondary antibody (0.1-20 µg/ml).
12. Incubate on ice for 40 mins at 4°C.
13. Centrifuge 8 mins at 735 x g at 4°C.
14. Resuspend cell pellets in 250 µl dPBS.
15. Centrifuge 8 mins at 735 x g at 4°C.
16. Resuspend cell pellets in 250 µl dPBS and pass suspension through 35 µm screen cap of Falcon 2235 tube.
17. Subject to flow cytometric analysis.

18. Controls must include cells treated with a normal IgG of the same species as the primary, cells treated with fluorochrome-conjugated secondary antibody only, and cells not being treated with a fluorochrome-conjugated secondary antibody.

## **FLOW CYTOMETRY ANALYSIS OF PERIPHERAL BLOOD LEUKOCYTE SURFACE ANTIGENS**

- 100 µl of whole blood
- add primary antibody
- incubate for 30 min on ice.
- add 2 ml of PBS
- centrifuge
- resuspend in 200 µl PBS 1-5% HSA
- add secondary fluorochrome conjugated antibody 0.1 µg/ml in 1-5% HSA or the same species that secondary is derived or both.
- centrifuge
- resuspend in 2 ml FACS lysis buffer mix end over to make even suspension
- incubate 10 min room temperature in the dark
- centrifuge
- resuspend the cell pellet in 2 ml PBS
- recentrifuge
- resuspend in 150 µl PBS and perform FACS analysis
- include control without primary
- include control without any antibody.
- include control with normal rabbit serum.

## REVERSE TRANSCRIPTION-PCR: PREPARATION OF cDNA

In a 0.5 ml microcentrifuge tube mix the following:

	Volume ( $\mu$ l)	Final concentration
dNTP (10 mM)	2.0	1 mM
random hexamer (60 ng/ $\mu$ l)	3.33	9.9 ng/ $\mu$ l
5X RT buffer	4.0	1X
RT (MMLV 200 U/ $\mu$ l)	1.0	10 U/ $\mu$ l
RNasin (30,000 U/ml)	1.0	1.5 U/ $\mu$ l
RNA* (total)	---	0.05 $\mu$ g/ $\mu$ l
H <sub>2</sub> O	up to 20 $\mu$ l	

\*Heat RNA at 65°C for 5 minutes.

- Incubate mixture at 37°C for 1 hour.
- Dilute reaction mixture 50-fold and use 10  $\mu$ l (containing ~10 ng cDNA) for subsequent PCR reaction.

## PCR AMPLIFICATION OF gt11 INSERT cDNA

In a 0.5 ml microcentrifuge tube mix the following:

Component	Volume ( $\mu$ l)
$\lambda$ gt11 phage supernatant	10 $\mu$ l
H <sub>2</sub> O	40 $\mu$ l

- Heat at 95°C for 5 min.
- Place on ice.

Add:

Component	Volume ( $\mu$ l)
dNTP (10 mM @)	2.5 $\mu$ l
lgt11 forward primer (100 ng/ $\mu$ l)	10 $\mu$ l
lgt11 reverse primer (100 ng/ $\mu$ l)	10 $\mu$ l
10X Taq buffer	10 $\mu$ l
Taq enzyme	0.5 $\mu$ l
H <sub>2</sub> O	17 $\mu$ l
Total volume	100 $\mu$ l

- Overlay with 100  $\mu$ l mineral oil.
- Run 35 cycles of: 94°C for 1 min., 55°C for 2 min., 72°C for 3 min.
- Follow this with a 10 min at 72°C extension incubation and a 4°C soak.

## COS CELL TRANSFECTION USING BIO-RAD GENE PULSER

- Place cuvette (Gene Pulser Cuvette # 165-2088 0.4 cm electrode gap) on ice before use.
- Remove COS cells from one confluent 150 mm dish of COS cells with trypsin-EDTA.
- Centrifuge and wash cells 2 times with Dulbecco's PBS.
- Resuspend cell pellet in 0.5ml of PBS plus 20-50 µg of plasmid DNA.
- Place cells in cuvette and put on ice.
- Settings for Gene Pulser: 960 mF 0.25 volts
- (Ohms resistor is bypassed when capacitance extender is used; check in the back to see that the capacitance extender is plugged into Gene Pulser. Other cells will require different settings)
- Place cuvette with cells into holder and slide to make contact with electrodes.
- Push two red buttons until beeping starts which indicates completion of discharge.
- Remove cuvette and place on ice for 10 minutes.
- Remove cells from cuvette and plate in DMEM and grow at 37°C for approximately 48 hours.

## **COTTON SWAB CLONING OF TRANSFECTED CELLS**

- When colonies are 100-500 cells in number, mark the dish under each colony (make sure that marked colonies are well isolated).
- Remove the medium from dish and wash with sterile dPBS
- Remove the wash by aspiration.
- Dip sterile cotton tip applicator sticks (Puritan, Hardwood Products Co. Guilford, Maine) in (37°C) warmed trypsin-EDTA solution (Gibco).
- Touch the tip of the swab to the colony location and gently swab the surface.
- Immediately place the swab into a well in a 24 well dish containing selection medium and twirl vigorously to dislodge cells.
- Allow the isolated cells to grow for approximately one week.

## LACTOPEROXIDASE METHOD FOR [<sup>125</sup>I]-LABELING CELLS (METHOD 1)

- Wash cells 3 times with PBS.
- Resuspend  $2 \times 10^7$  cells/ml in PBS.
- Add the following reagents:
  - 200  $\mu$ l lactoperoxidase (1 mg/ml in PBS).
  - 1.0  $\mu$ Ci sodium [<sup>125</sup>I]-iodine.
  - 20  $\mu$ l 0.12% hydrogen peroxide (20  $\mu$ l 30%/5 ml PBS).
- Incubate 5 minutes on ice with gently vortexing every minute.
- Add 20  $\mu$ l 0.12% hydrogen peroxide again.
- Incubate 5 minutes on ice with gently vortexing every minute.
- Stop reaction by adding 10 ml of 0.1 M NaI, 1 mM PMSF, PBS.
- Wash labeled cells 3 times with PBS.

## LACTOPEROXIDASE METHOD FOR [<sup>125</sup>I]-LABELING CELLS (METHOD 2)

- Wash cells 3 times with Dulbecco's PBS (dPBS).
- Remove cells using dPBS, 2 mM EDTA (15-30 min).
- Pellet cells by centrifugation.
- Resuspend cells in serum free culture medium.
- Pellet cells by centrifugation.
- Resuspend cells in dPBS.
- Pellet cells by centrifugation.
- Resuspend cells in 716  $\mu$ l dPBS, 20 mM glucose.
- Place on ice.
- Add 8  $\mu$ l of lactoperoxidase (stock 24 mg/ml in 3% non-fat milk).
- Add 266  $\mu$ l glucose oxidase (stock is 0.4 U/ml freshly made) (final conc. 0.1 U/ml).
- Add 1 mCi Na<sup>125</sup>I.
- Mix gently.
- Incubate on ice for 20 minute with gentle mixing (finger vortex) every 5 min.
- Add 2 ml dPBS, 10 mM KI.
- Centrifuge 3 min in serofuge at high speed.
- Remove supernatant. Discard radioactive supernatant appropriately.
- Resuspend pellet in dPBS and recentrifuge.
- Pelleted cells can now be extracted with appropriate detergent containing buffer.

### Reagents:

- lactoperoxidase - Boehringer Mannheim #107174
- glucose oxidase type V - Sigma cat no. G6500

## IMMUNOPRECIPITATION ANALYSIS

- From metabolically radiolabeled culture cells:
- Extract the cell layer with 1% Triton X-100, 0.5 M NaCl, 50 mM Tris-HCl pH 7.4-8.0, 1 mM PMSF using a cell scraper.
- The extract should be repeatedly drawn up and expelled through a 21-gauge needle to shear DNA.
- Clarify the extract by centrifugation at 50,000 X g in a Beckman TL-100 ultracentrifuge.
- Pre-clear the supernatant by addition of 1/50th volume of 1:1 (v/v) protein A/G-Sepharose: extraction buffer and incubate for 1 hour at 4°C.
- Pellet the protein A/G-Sepharose by centrifugation at 12,000 X g for 5 minutes at 4°C. Remove supernatant using a narrow bore pipette tip to avoid removing any of the Sepharose.
- Aliquot the extract (1-1.4 ml) into microcentrifuge tubes, add 2-4 µl antiserum and incubate for 4-18 hours at 4°C.
- To precipitate immune-complexes add 100 µl of 1:1 solution of protein A/G-Sepharose: extraction buffer and incubate 1-2 hours at 4°C.
- Centrifuge at 12,000 X g for 5 minutes at 4°C.
- Use a micropipette tip to remove supernatant (avoid the pellet) and dispose it as radioactive waste or freeze it at -20°C with radioactive tape label.
- Wash pellet 5 times 10 minutes with 1% Triton X-100, 0.5 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM PMSF.
- Wash one time with TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4) and transfer suspension to a new microfuge tube.
- Centrifuge and aspirate away supernatant.
- Add 50 µl 4 X electrophoresis sample buffer, mix and heat for 5 minutes at 95°C.
- Centrifuge and analyze 50 µl of the supernatant by SDS-PAGE.

## CELL ADHESION ASSAY

- Coat wells of microtiter dish (Falcon ProBind, not tissue culture plastic) with adhesive substrate in coating buffer either overnight at 4°C or 4 hours room temp or 2 hours at 37°C. Typically, the substrate is coated at a range of concentrations (e.g., 10 - 0.4 µg/ml, 100 µl per well).
- Block unoccupied or non-specific binding sites with 3 mg/ml BSA in PBS (150 µl per well).
- Cells were removed from culture dishes with trypsin, EDTA and washed with medium containing soybean trypsin inhibitor (0.5 mg/ml).
- Cells were resuspended in medium at 1-10 X 10<sup>5</sup> cells/ml and allowed to recover from trypsinization for 15-30 minutes.
- 100 µl aliquots of cell suspension were added to each well and incubated for 30-60 minutes at 37°C.
- After incubation add 50 µl of 8 % gluteraldehyde, PBS (final conc. 2.5%) and gently agitate the plate for 20 minutes.
- Rinse the cell layer with PBS and stain the attached cells with 0.2-0.5% crystal violet, PBS for 10 minutes at room temperature.
- Wash with dPBS. Dump each wash into sink and blot plate using paper towels. Repeat 3 times.
- Attached and spread cells can be evaluated by microscopy.

## **ASSAY TO MEASURE BINDING, INTERNALIZATION AND DEGRADATION OF RADIOLABELED LIGAND BY CULTURED CELLS**

### **BINDING**

- Seed 24 well culture dishes with  $3 \times 10^4$  to  $1 \times 10^5$  cells/well.
- Grow cells 18 hours or until nearly confluent.
- Wash cell layer gently twice with 37°C DMEM 0.5 ml.
- Add 0.5 ml DMEM containing ITS or Nutridoma serum substitute, 20 mM Hepes, pH 7.4 and 15 mg/ml BSA to each well and incubate at 37°C for 1 hour.
- Wash cell layer gently once with ice cold DMEM-HEPES.
- Add DMEM-NUT/ITS-HEPES-BSA containing radiolabeled ligand (400-500  $\mu$ l, 1 nM) and incubate at 4°C for 2-3 hours.
- Wash with 0.5-1 ml ice-cold dPBS three times (use dump method).
- Add 500  $\mu$ l 0.1 N NaOH and count bound cpm.
- Trypsinize a series of parallel wells and count cells.

### **INTERNALIZATION AND DEGRADATION**

- After step 4 add radiolabeled ligand and incubate 2-18 h at 37°C (we typically go o/n).
- Remove medium and add 50% TCA to final conc. of 10% and vortex and centrifuge 5 min in microfuge 8K X rpm.
- Count the supernatant to measure ligand degradation.
- For internalization, remove cell surface bound ligand by washing cell layer twice with PBS then treating cell layer by following the Protease Release Method below.

### **ANTIBODY INHIBITION**

- After step 4, pre-incubate cells with antibody (0.1 mg/ml DMEM, NUT/ITS, Hepes, BSA) for 1 h at 37°C.
- Spike each well with appropriate radiolabeled ligand and mix gently.
- Proceed as described above.

**DMEM-NUT/ITS-BSA:** 150 ml

- 1.5 ml ITS, from Collaborative Res. (Nutridoma can be substituted for ITS)
- 3.0 ml 1M HEPES pH 7.0
- 2.25 g BSA
- 150 ml DMEM
- mix and store some on ice and some at 37°C (see steps 4 and 6).

## **PROTEASE RELEASE OF LIGANDS FROM CELL SURFACE**

- Protease solution: 50 µg/ml each of Proteinase K (Sigma) and Trypsin (Gibco), 5 mM EDTA, in Dulbecco's PBS without calcium and magnesium, pH 7.4.
- Incubate cells with protease solution for 10-20 minutes at 4°C.
- Remove cell suspension after dispersing.
- Centrifuge 8000 X rpm in Eppendorf centrifuge.
- Remove supernatant to a new tube.
- Rinse pellet with 0.5 ml PBS, centrifuge, combine supernatants and count. **\*\*This is the cell surface-bound fraction\*\***.
- Cut the bottom of centrifuge tube containing pellet and count. **\*\*\*This is the cell-associated or internalized fraction\*\*\***.

## FLOW CYTOMETRY ANALYSIS OF DII-HDL UPTAKE IN BN CELLS

This assay is a 24-well plate format and designed for evaluating signaling effectors on HDL uptake.

### Day 1

- Plate BN cells at  $0.8-1 \times 10^5/\text{cm}^2$  in complete medium (CM: MEM/10%FBS/1X non-essential amino acids/1X pen-strep) in 24 well plates. Allow cells to attach for 6-12h, then replace medium with serum free medium (SFM; MEM/ITS/ non-essential amino acids /pen-strep). Grow overnight 37°C, 5% CO<sub>2</sub>.

### Day 2

- Warm SFM (37°C) and assemble reagents needed for uptake assay (RAP, DiI-HDL, apoE-free HDL, DMSO, etc.) Make sure the frozen agents are thawed.
- Aspirate medium from the cells and wash with 1 ml warmed SFM.
- Aspirate the wash medium and add 0.3 ml SFM; incubate 1 hr, 37°C, 5% CO<sub>2</sub>
- During this incubation prepare competitor and DiI-HDL dilutions in SFM. For 24 well plates, do calculations for 400 µl (total well volume) but prepare 100 µl mixtures for addition. Use DiI-HDL at 1-2 µg/ml. Warm mixtures at 37°C for 10-15 min.
- Example: 1 µg/ml DiI-HDL treatment; XS HDL, 200X = 200 µg/ml; RAP, 1µM. Prepare a dilution of DiI-HDL (0.4 µg/20 µl) sufficient for all samples.
- Add 100 µl of each pre-warmed agent or competitor to the wells. Incubate cells for 15 min, 37°C.
- Add 20 µl diluted DiI-HDL (without aspirating agents).
- Incubate cells for 30 min, 37°C.
- Prepare Trypsin-EDTA/Proteinase K (50 µg/ml) and warm at 37°C.
- Wash cells with DPBS (1 ml/well), 2 times.
- Add 0.2 ml trypsin-EDTA/Proteinase K and incubate for 1-2 at RT. Prepare labeled eppendorf tubes with 0.5 ml cold DPBS\*\*.
- Pipet cells to loosen from well and transfer to Eppendorf-tubes with DPBS\*\*; place on ice. From this moment, work as much as possible on ice! Keep Eppendorf-tubes cold.
- Spin tubes: 3 min, 3000 rpm, 4°C.
- Aspirate supernatant, add 0.5 ml DPBS and vortex gently to resuspend cell pellet.
- Spin tubes: 3 min, 3000 rpm, 4°C.
- Repeat DPBS wash, step 12 + 13.
- Aspirate supernatant, and resuspend pellet in 300µl DPBS.

- Transfer solutions into 12 x 75 mm polystyrene tubes -- if aggregates are present, strain the cells using strainer cap tubes. Store on ice. Tubes are now ready for flow-cytometry.

\*\*Alternatives include using complete medium, serum-free medium, or DPBS plus trypsin inhibitor to stop trypsin digestion.

## **STAINING OF CHONDROCYTE CELL CULTURES**

- Remove and discard medium from culture dishes.
- Gently add 10 % formalin and incubate 10 minutes.
- Remove and discard formalin. Wash 2 times with water.
- Add alcian green stain and incubate 10 minutes.
- Remove and discard stain and wash 2 times with water.
- Add 3 % acetic acid and incubate 3 minutes.
- Remove acetic acid and wash 2 times with water.
- Add metanil yellow and incubate 30 seconds.
- Remove and discard stain and wash 2 times with water.
- Air dry.

### **STAINS:**

**Alcian green (0.5 %):**

0.25 g/50 ml 3 % acetic acid

**Metanil yellow (0.25%)**

0.25 g/100 ml water containing 0.1 ml glacial acetic acid

## **IMMUNOLOGICAL SCREENING OF $\lambda$ gt11 cDNA LIBRARIES**

### PREPARATION OF LB AGAR PLATES

- To 500 ml of autoclaved LB, 1.5% agar molten at 56°C add;  
125  $\mu$ l tetracycline 60 mg/ml in 70% ETOH  
5 ml 1M MgSO<sub>4</sub>  
mix by gently rotating the bottle and avoid making bubbles  
then pour 150 mm plates (about 8 plates from 500 ml)
- After agar has solidified place plates in sterile hood with the lids ajar  
for 1-2 hours. This allows excess moisture to be removed from agar  
and prevents top agarose from peeling away later (plates can be stored  
at 4°C for several weeks).
- Prewarm plates at 42°C prior to use.

### GROWTH OF Y1090 CELLS

- Streak 1090 cells onto a LB, tet (15  $\mu$ g/ml) plate and grow o/n at 37°C.  
Pick a single colony and inoculate 50 ml of LB, tet (15  $\mu$ g/ml),  
maltose (0.2%) and grow o/n at 37°C.
- Centrifuge 5000 rpm for 10 min in sterile Sorval screw top cfg tubes.  
Decant supernatant, resuspend pellet in 20 ml sterile TM (10 mM  
Tris pH 7.5, 10 mM MgSO<sub>4</sub>) O.D.600 should equal 2.0.
- These cells can be stored at 4°C and used for one week.

### PLATING OF $\lambda$ gt11 PHAGE AND INDUCTION OF FUSION PROTEIN PRODUCTION

1. Dilute phage (for immunological screening use 20-50 x 10<sup>3</sup> pfu per 150 mm dish). Add  
diluted phage to 0.125 ml of Y1090 cells in Falcon 2059 tubes.
2. Allow phage to adsorb at 37°C for 20 min.
3. Add 6.5 ml 48°C top agarose (LB, tet, 0.7% agarose). Mix thoroughly by pipetting up and  
down, work rapidly to avoid agar solidifying.
4. Pour onto surface of a pre-warmed (45°C) plate and evenly distribute by gently tilting  
plate.
5. Allow top agarose to solidify approx. for 5-10 min and place plates inverted at 42°C for 3-  
5 hr.
6. When plaques appear carefully lay a dry nitrocellulose filter (previously soaked in a  
solution of 10 mM IPTG) on the surface of the agarose.
7. Incubate at 37°C for 5-12 hr.
8. Mark position of filter by making needle holes asymmetrically along the edges through  
the filter into the agar.

## IMMUNOLOGICAL DETECTION OF FUSION PROTEINS EXPRESSED BY $\lambda$ gtII CLONES

- Remove NC filters from plates and rinse away bacterial debris by washing in TBS, 0.05% Tween, then quench unoccupied protein binding sites by incubating filters in 3% non-fat dry milk, TBS for 1 hr at 37°C.
- Individually seal filters into seal-a-meal plastic bags.
- Add primary Ab diluted appropriately in 3% milk, TBS and 0.05% Tween and incubate overnight at room temperature with agitation (10 ml per 250 mm filter; 4 ml per 85 mm filter).
- Remove filters and wash 5 times for 10 mins in TBS, 0.05% Tween.
- Incubate filters in horseradish peroxidase (HRP); conjugate second antibody diluted in 3% milk, TBS, 0.05% Tween (e.g. BioRad conjugate can be diluted 1:3000) for 1-2 hr at room temperature with agitation.
- Remove filters and wash as in step 4.
- Prepare DAB\* color reagent by diluting the contents of each vial with 20 ml of 0.1 M citrate-phosphate buffer, pH 5.0, and 20 ml of 30% H<sub>2</sub>O<sub>2</sub>.
- Incubate filters in diluted color reagent solution for 1-10 min at room temperature, then rinse with deionized H<sub>2</sub>O. Positive plaques often produce donut shaped staining patterns.

\*DAB ; 3,3' diaminobenzidine tetrahydrochloride, Litton Bionetics, Kensington, MD

## ISOLATION OF $\lambda$ gt11 PHAGE DNA FROM PHAGE PLAQUES

1. Prepare LB plates with 1.5% agarose supplemented w/10 mM  $\text{MgSO}_4$  and tet. (Note: use garose not agar).
2. Plate approx.  $1 \times 10^5$  pfu per 150 mm plate.
3. Grow at 42°C until phage plaques are confluent.
4. Add 8 ml of SM to each plate and incubate at room temp. for 3 hrs. with constant shaking.
5. Remove and save SM and rinse plate surface with an additional 2 ml of SM.
6. Pool SM solutions and then centrifuge at 8 K G for 10 min. to pellet debris.
7. To supernatant, add DNase I and RNase A to 1 mg/ml and incubate for 30 min. at room temp.
8. Add chloroform to 5% of volume and vortex 30 sec.
9. Centrifuge in Sorvall at 10 K rpm for 10 min at 4°C to pellet debris, then remove supernatant and extract it with an equal volume of chloroform.
10. Centrifuge in Sorvall at 4 K rpm for 3 min.
11. To aqueous phase, add an equal volume of 20% PEG (8000), 2.0 M NaCl and incubate on ice for 1 hr.
12. Centrifuge precipitated phage in Sorvall at 10 K rpm for 10 min.
13. Resuspend phage pellet in 1 ml SM and centrifuge in microfuge 5 minutes.
14. Take supernatant phage suspension and centrifuge in TL-100 ultracentrifuge at 50 K rpm (100K X g) for 1 hr at 15°C.
15. Discard supernatant and resuspend bluish-white phage pellet in 0.5 ml SM.
16. Add 5 ml 10% SDS and 5 ml 0.5 M EDTA and incubate at 65°C for 10 min.
17. Extract with phenol/chloroform.
18. Extract aqueous phage with an equal volume chloroform.
19. To aqueous phase add 1/10 vol. of 3 M NaOAC and 2 vols. 100% EtOH and chill to -20°C for 20 min.
20. Centrifuge precipitated DNA and rinse pellet w/80% ethanol.
21. Vacuum dry pelleted DNA and resuspend in 100 ml distilled H<sub>2</sub>O for each plate used in step 2.
22. Centrifuge to remove insoluble material (e.g., agarose).

## AFFINITY SELECTION OF ANTIBODIES ON gt11 INSERT ENCODED PROTEIN IMMOBILIZED ON NITROCELLULOSE

- Prepare 85 mm LB agar (amp or tet) plates and dry in hood for 1 hour.
- Grow a 50 ml overnight culture of Y1090 cells from a single colony. The next day centrifuge cells at 5000 rpm, for 10 min in sterile screw top Sorval tube. Decant supernatant and resuspend pelleted cells in approx. 20 ml TM (10 mM Tris, pH 7.5, 10 mM MgSO<sub>4</sub>). Note: the O.D.660 should be 2.0.
- Add 1-5 X 10<sup>5</sup> pfu to 125 ul of Y1090 cells and incubate at 37°C for 20 minutes.
- Add 4.5 ml 48°C top agarose, mix and pour onto pre-heated 85 mm agar plates.
- Grow phage at 42°C. Plaque density should be quite high and uniform. It is often best to pour several plates having different pfu counts and then pick the best for subsequent IPTG induction.
- Carefully overlay selected plates with dry nitrocellulose filters (previously soaked in a solution of 10 mM IPTG in sterile water).
- Incubate at 37°C 5-12 hours.
- Remove filters and wash in TBS 30 minutes at 4°C.
- After washing, block filter in 3% BSA, TBS for 1-2 hours at room temperature.
- Incubate filter in TBS, 0.05% Tween-20 for 10 minutes.
- Seal filters in bags back to back and incubate with antiserum diluted in TBS, Tween. The antiserum dilution is empirical (I have used 1:10 and 1:50 dilutions with success). One suggestion is that you preabsorb the antiserum with some boiled Y1090 cells. In addition, parallel filters containing control phage plaques should be made.
- After the incubation, wash the filters 3 times 30 minutes in TBS, Tween.
- Stack the filters on top of one another and cut into 1 cm squares using a razor blade.
- Load the squares into a syringe and elute bound antibody by drawing into the syringe 1 ml volume of 10 mM glycine, pH 2.3, 0.5 M NaCl, 0.05% Tween-20 and vigorously shaking the syringe. Repeat one or two times.
- Rapidly neutralize the eluates using Na<sub>2</sub>PO<sub>4</sub> and then dialyze against TBS-Tween.

**Reference:** Argraves et al., 1986, JBC 261:12922-12924

## MACROPLAQUE ASSAY

- For 85mm dishes add host bacterial cells (200  $\mu$ l of OD600= 2.0) to 3.5 ml top agarose, mix and apply to surface of LB agar plate. Note: pre-dry plates as usual.
- Let top agarose solidify (plates can be stored at 4°C).
- Make dilutions of phage stocks in SM to give approximately 100 pfu/1-2 $\mu$ l (for a primary pick in 1 ml of SM make 1:250 dilution in SM and spot 1-2  $\mu$ l). Spot 1-2  $\mu$ l aliquots onto the surface of the agarose. It is best to make several dilutions and spot aliquots of each to determine the best dilution and volume. By setting the dish onto a template the spots can be placed in straight rows.
- Allow spotted aliquots to absorb into the agarose.
- Place plates in incubator and periodically examine for the appearance of plaques. Best results are obtained when individual plaques can be seen.
- When plaques appear, overlay nitrocellulose filter containing IPTG and incubate 4 hours to overnight at 37°C.
- Make asymmetrical marks on the filter by puncturing filter with a needle.
- Remove nitrocellulose filter and wash for 1 hour in TBS, 0.05% Tween.
- Block filter with 3% milk, TBS for 1 hour.
- Place filter in sealed plastic bag and add antibody diluted in 3% milk, TBS, Tween. Incubate 1 hour at room temp.
- Wash filter with TBS, Tween 3 times 5 minutes.
- Add secondary antibody conjugate diluted in TBS, Tween and incubate 1 hour.
- Wash filter with TBS Tween 3 times 5 minutes.
- Develop with chromogenic substrate.

## **FREEZE-PHENOL EXTRACTION OF DNA FROM AGAROSE**

- View ethidium stained DNA in gel with long wave UV light.
- Mark position of DNA band in gel with a razor blade.
- Excise gel containing the band and trim away excess agarose.
- Place gel slice in a 1.5 ml centrifuge tube.
- Put tube at  $-70^{\circ}\text{C}$  for 15 minutes.
- Add a volume of phenol approximately equal to the volume of the gel slice (typically 200  $\mu\text{l}$ ).
- Using a thin stainless steel spatula mince frozen gel into phenol.
- Vortex phenol gel suspension for 1 minute.
- Place tube a  $-70^{\circ}\text{C}$  for 15 minutes.
- Remove tube from freezer and centrifuge at 12 k X g at  $4^{\circ}\text{C}$  for 15 minutes.
- Transfer aqueous layer to a new tube.
- Re-extract phenol with an equal volume of TE buffer by vortexing for 1 minute.
- Centrifuge for 1 minute and combine aqueous layer with that from above.
- Extract with an equal volume of chloroform by vortexing 1 minute.
- Centrifuge and transfer aqueous layer to a new tube.
- Add 1/10 volume 3 M NaOAc and 2.2 volumes cold ethanol.
- Place tube at  $-20^{\circ}\text{C}$  of 15 minutes.
- Centrifuge for 15 minutes at 12 k X g at  $4^{\circ}\text{C}$ .
- Pour off supernatant and wash pellet by adding 1 ml of cold 80% ethanol.
- Centrifuge, pour off supernatant and dry pellet.
- Dissolve DNA with desired volume of TE or water.

## DNA FRAGMENT (>10 KB) ISOLATION FROM AGAROSE GELS

- Separate DNA electrophoretically on a normal agarose gel.
- After the fragment of interest is well resolved, use a razor blade to excise a band of agarose in front of the fragment.
- Fill the slot with melted low melt agarose (% and buffer to match the standard gel). Allow the gel to solidify (15-30 min).
- Continue electrophoresis until the fragment has entered the low melt agarose.
- Excise the low melt agarose/DNA fragment. Place in a tared container (eppie or 15 ml tube), being careful to remove any excess running buffer, and weigh the gel slice.
- Add an equal volume of TE, pH 8 (e.g., 200 mg gel = 200  $\mu$ l TE) and place the tube at 65°C until the gel is completely dissolved (5-10 min). It is suggested to get the sample into eppies for the following extractions.
- Add 1 volume of Tris-buffered phenol (RT), mix by inversion and spin 5 min 12k rpm.
- Transfer sup to new tube (inclusion of some agarose is acceptable because it will subsequently be removed), add equal volume of phenol and repeat extraction.
- Transfer sup to new tube, add 0.1 vol of 4 M LiCl, mix by inversion and place on ice for 2 min. Spin as above.
- Transfer sup to new tube being careful to avoid the agarose pellet.
- At this point it is suggested to add carrier to facilitate precipitation (e.g., 1  $\mu$ l of 20 mg/ml glycogen).
- Add 2.5 vol of ethanol, mix by inversion and incubate at -70°C for 15-30 min.
- Spin 15 min in microfuge at 12k rpm
- Decant EtOH and wash pellet in 70% EtOH.
- Dry pellet and resuspend in appropriate solvent.

This method is good for isolating large DNA fragments (>10 kb) which do not purify well (or necessarily intact) using standard frag isolation kit chromatography, but it also works for isolation of small fragments. It is derived from a couple *BioTechniques* papers. The first (Steck, *Biotech.* vol 17, no 4, 1994) describes using a standard agarose gel and running your fragments into slots of low melt agarose inserted after some initial electrophoresis. The isolation from the low melt agarose is taken the second paper (Favre, *Biotech.* vol 13, no 1, 1992). Note: inversion is recommended instead of vortexing, and some pipetting steps are eliminated in consideration for DNA fragment integrity.

## PREPARATION OF COMPETENT JM101-109 CELLS FOR TRANSFORMATION

- Using sterile technique streak out the stock of E. coli JM101 or JM109 onto a minimal agar (M9) plate. Place plate at 37°C and grow cells overnight. The colonies on this plate may be used to pick single colonies for one month if it has been stored at 4°C.
- Inoculate 2 ml of YT medium with cells from a single colony. Grow with vigorous shaking 8-12 hours at 37°C.
- Dilute this fresh culture 1:100 into YT medium and grow in shaking incubator until the culture reaches an O.D.<sub>660</sub> of 0.3-0.4 (approximately 1.5-2 hours).
- Transfer all but 0.5-1 ml of the culture to sterile Sorval screw-top centrifuge and centrifuge at 7000 X g for 5 minutes. Take the 0.5-1 ml of the culture that remains in the bottom of the culture flask and dilute it 1:100 with fresh YT medium and incubate at 37°C with shaking. During the time that it will take to complete the preparation of competent cells this culture can grow. This will provide the source of exponentially growing cells that you will need in the transformation procedure.
- Resuspend the pelleted cells in chilled 50 mM CaCl<sub>2</sub> (1/2 of the previous volume). Note: Resuspend the pelleted cells by gently pipetting the cells up and down. Incubate the cell suspension on ice for 20 minutes.
- Centrifuge the cells as above and resuspend in a volume of 50 mM CaCl<sub>2</sub> equal to 1/10 of the growth volume. Note: The cells at this point are "fragile" and should be handled gently. Avoid vortexing!
- The amount of competent cells needed depends on the number of transformations to be made. Typically, 0.3 ml of competent cells are used per transformation. Note: Competent cells are concentrated ten fold out of the growth medium.

## TRANSFECTION OF PLASMID DNA INTO COS1 CELLS

For each 100 mm dish of COS1 cell prepare the following:

X  $\mu$ l DNA (0.1-50  $\mu$ g)  
0.4 ml 1M Tris pH 7.3  
0.4 ml 10X DEAE-Dextran (DME)  
3.2 ml SF-DME

- COS1 cells should be plated the night before transfection such that the density at the time of transfection will be about 70% confluent. A good starting point would be  $5 \times 10^5$  cells/dish.
- Rinse the cells 2X (5 ml each) either SF-DME or TBS-D.
- Add 4ml per plate of the DNA-DEAE Dextran, mix and incubate at 37°C for 4-6 hr.
- Rinse the cells 1X in SF-DME (5 ml).
- Add 2 ml of 10% DMSO dropwise to each plate and let stand at rt for 1.5-2.0 min then aspirate immediately.
- Add 5 ml of DME, 10% FBS containing 100  $\mu$ M chloroquine (from 1000 X stock), incubate at 37°C for 1-2 hr.
- Rinse cells 1X with SF-DME then add 10 ml DME, 10% FBS and incubate at 37°C for 2-3 days.

### Notes:

- Cells may be split and replated 24 hr after transfection. Allow sufficient time for the cells to recover.
- Monitor the condition of the cells throughout the course of the transfection.
- The volume above may be scaled up or down proportionately for use with culture dishes of different size.

## M13 COMPLIMENTATION TEST (C-TEST)

Since the (+) strand is always the viral strand, recombinant phages with DNA originally inserted in either orientation into the RF molecule will hybridize only via the insert DNA. To test whether two recombinant phages have DNA complementary to each other:

- mix 20  $\mu$ l of supernatant from two different recombinants in a 1.5 ml tube.
- add 5  $\mu$ l of 250 mM EDTA, pH 8.0, 1% SDS, 0.1% bromophenol blue, 50% glycerol, vortex.
- heat at 65°C for 1 hour.
- centrifuge to bring down any condensation.
- electrophoresis in 0.7% agarose gel in Tris-borate buffer (include 0.5  $\mu$ g/ml EtBr) at 100 volts for 5 hours.

If the two viral DNAs hybridize via their insert they form a figure eight-like structure and migrate slower in the gel than the single viral circle. As a control run the individual viral recombinant and non-recombinant (from a blue plaque) DNA strands in separate lanes.

## DNA SEQUENCING GEL STOCK SOLUTIONS

8% Wedge Gel Stock: 600 ml

40% acrylamide stock	120 ml
urea	300 g
10X TBE	60 ml
Deionized water	180 ml
Temed	85 ul

- Combine in 1000 ml beaker immersed within a beaker containing hot water.
- Stir until the urea is completely into solution.
- Filter mixture through 10 micron filter membrane.
- Degas for 30 minutes.
- Dispense into 115 ml aliquots and store at 4°C.
- Use one 115 ml aliquot per gel.
- Each aliquot requires 1.2 ml of 10% APS to polymerize.

6% Wedge Gel Stock: 600 ml

40% acrylamide stock	90 ml
urea	300 g
10X TBE	60 ml
Deionized water	210 ml
Temed	85 ul

## PREPARATION OF mRNA - GUANIDINE HYDROCHLORIDE METHOD

### Day 1

#### Homogenization, Removal of Nucleases and Proteins

1. Prepare buffer A and buffer B
2. Weigh tissue (frozen in liquid nitrogen) or packed cell pellet.
3. Add 60 ml buffer A to 4 to 10 grams of tissue or cells.
4. Polytron 3 X 1 minute at highest speed (may need to centrifuge at 2000 rpm for 3 minutes to push down foam between grindings). Cool on ice between grindings for about 1 minute.
5. Centrifuge 15 minutes at 6500 rpm in GSA at 4°C, to remove cell debris.
6. Measure volume of supernatant by pipetting into a clean 150 ml Corex tube, add 1/2 volume of ice-cold ethanol. Mix well and place in either a -20°C bath or a -20°C freezer for 30 minutes (time and temperature are critical).
7. Centrifuge 30 minutes at 6500 rpm in GSA, at 4°C. discard supernatant.
8. Break up pellet well with a flame-sealed pasture pipette. Add 30 ml of buffer B; place on shaker to dissolve entire pellet (may take >2 hours at room temperature).
9. Centrifuge 30 minutes at 6500 rpm in GSA at 4°C to remove fine insoluble material. Transfer supernatant to clean 150 ml tube.
10. Add 0.5 volume of ice-cold absolute ethanol, mix, place in -20°C bath for 30 minutes.
11. Centrifuge 30 minutes at 6500 rpm at 4°C in GSA. Discard supernatant.
12. Break up pellet with flame sealed Pasteur pipette. Add 20 ml buffer B and place on shaker until entire pellet dissolves (may take >4 hours).
13. Add 10 ml cold absolute ethanol, mix well, and place at -20°C for 30 minutes.
14. Centrifuge for 30 minutes at 6500 rpm in GSA at 4°C. Discard supernatant.

#### Removal of Lipid Material

15. Break up pellet with flame-sealed pasture pipette. Add 10 ml 0.02 M EDTA, place on shaker until entire pellet dissolves (may take >1 hour). Removal of Lipid Material (preparation of RNA continued)
16. Transfer to a 30 ml Corex tube and centrifuge at 5000 rpm for 15 minutes at 4°C in SS-34 rotor (not longer).
17. Transfer and SAVE supernatant into a 50 ml Falcon tube, break up the pellet of fine material. Add 5 ml 0.02 M EDTA, shake until entire pellet dissolves (may take >30 minutes).
18. Centrifuge 5000 rpm for 15 minutes at 4°C in SS-34. Save supernatant, break up pellet and extract with 5 ml more 0.02 M EDTA (if no pellet, skip this extraction)
19. Centrifuge 5000 rpm for 15 minutes at 4°C. Save supernatant and discard pellet.
20. Add an equal volume of 1:4 BuOH:CHCl<sub>3</sub> to the pooled 0.02 M EDTA supernatants. Vortex 1 minute then centrifuge 1 minute at 1000 rpm (Sorval), room temperature.

### Removal of Remaining DNA

21. Remove upper (aqueous phase) layer and combine in 150 ml Corex tube. Add 3 volumes of 4 M NaOAc, pH 5.5, mix well, place at -15°C overnight.
22. Centrifuge 6500 rpm in GSA at 4°C for 1 hour. Discard supernatant.
23. Wash pellet with 20 ml cold 3 M NaOAc. Centrifuge at 6500 rpm in GSA at 4°C for 15 minutes. Discard supernatant.
24. Wash pellet in 20 ml 80 % ethanol, lyophilize about 5 minutes. Dissolve in 5 ml H<sub>2</sub>O and transfer to a 30 ml centrifuge tube.
25. Remove a 10 ul aliquot and add to 990 ul H<sub>2</sub>O. Mix and read A260, and A280.
26. Add 1 ml 3 M NaOAc and 15 ml cold ethanol. Place at -20°C for 35 minutes (sample may freeze).
27. Centrifuge at 5000 rpm in SS-34 for 15 minutes at 4°C. Discard supernatant.
28. Wash pellet gently with 10 ml cold 80% ethanol, decant ethanol and lyophilize pellet about 5 minutes.
29. Dissolve in 3 ml H<sub>2</sub>O. Re-quantify by O.D. and either store aliquots at -70°C or proceed to oligo dT affinity chromatography.

### BUFFERS FOR GUANIDINE HYDROCHLORIDE METHOD OF mRNA EXTRACTION

Stock Solutions: (enough for about 4 preparations)

1. 8M guanidine hydrochloride : Dissolve 382.12 g GuHCl in dH<sub>2</sub>O; bring up to 500 ml
2. 4M sodium acetate, pH 5.5 : Dissolve 164 g NaOAc in dH<sub>2</sub>O. Adjust pH to 5.5 with glacial acetic acid, bring up to 500 ml dH<sub>2</sub>O.
3. 0.2M EDTA, pH 7.0 : Dissolve 37.23 g Na<sub>2</sub>EDTA in dH<sub>2</sub>O, adjust pH to 7.0 with 50% NaOH; bring up to 500 ml with dH<sub>2</sub>O.
4. 1M Tris, pH 7.5 : Dissolve 60.5 g Tris base in dH<sub>2</sub>O, adjust pH to 7.5 with HCl, bring up to 500 ml with dH<sub>2</sub>O.
5. 5M NaCl : Dissolve 146.1 g NaCl in dH<sub>2</sub>O, bring up to 500 ml with dH<sub>2</sub>O.
6. 0.5M dithiothreitol : Weigh about 77 mg DTT (MW=154.25) into a sterile 1.5 ml centrifuge tube.

Calculate final volume:

$$\frac{x \text{ mg DTT}}{77.7625} = \text{ml of filtered dH}_2\text{O to be added}$$

Dissolve and store at -20°C.

## ISOLATION OF TOTAL RNA FROM CULTURED CELLS

- Two confluent 175 cm<sup>2</sup> flasks of WI-38 lung fibroblasts will yield approximately 300 mg of RNA.
- All solutions should be made from DEPC-treated water.

### PROCEDURE

1. Wash the cell monolayers 5 times with ice-cold PBS. This PBS need not be DEPC-treated. Remove as much PBS as possible.
2. Solubilize cells in situ by adding 2.5-3ml/flask of 4 M GITC solution (For preparation, see "Maniatis" p. 189).
3. Pass the cell homogenate through 22-gauge needle twice to shear high molecular weight DNA. This treatment reduces the entrapment of RNA into the band of high molecular weight DNA during ultracentrifugation.
4. Layer about 1.4 ml of the homogenate onto a 0.7 ml cushion of 5.7 M CsCl<sub>2</sub> in 0.1 M EDTA (pH 7.5) in a 2.1 ml quick-seal tube. The inner wall of the tube should be pre-washed twice with 4 GITC solution. Make 4 tubes.
5. Centrifuge at 100,000 rpm overnight at 20°C.
6. After centrifugation, cut the top of the tube with a razor blade and aspirate carefully down to just past the DNA layer.
7. Wash the wall twice with 4 M GITC solution. Carefully aspirate all of the remaining solution from the tube. RNA forms a firm pellet on the bottom.
8. Add 100-200 ml of 80% ethanol to free the RNA pellet from the tube wall. Transfer the pellets into a 1.5 ml microcentrifuge tube with a pipette tip.
9. Centrifuge and discard supernatant. Wash the pellet with 80% ethanol again. Centrifuge and remove the supernatant. Dry pellet by lyophilization.
10. Dissolve the pellet in 400 µl of: 10 mM Tris (pH 7.4), 5 mM EDTA, 1% SDS.
11. Extract twice with phenol and chloroform and with chloroform once.
12. Precipitate RNA with ethanol and NH<sub>4</sub>OAc.
13. Dissolve the pellet in 30 µl of DEPC water. Measure OD<sub>260</sub> to quantify.
14. Make aliquots of 15-50 µg of RNA and store at -80°C.

### PREHYBRIDIZATION/HYBRIDIZATION BUFFER (Buffer N)

4X SSC, 36.5% formamide, 0.1% dextran sulfate, 1X Denhardt's, 20 µg/ml salmon sperm DNA, 20 mM Tris pH 7.4. To make approximately 800 ml:

Dextran sulfate (dissolve in dH <sub>2</sub> O first)	80g
H <sub>2</sub> O	292ml
Formamide (deionized)	320ml
20 X SSC	160ml
1 M Tris pH 7.4	16ml
100 X Denhardt's solution	8ml

Salmon sperm DNA (2mg/ml) 8ml

Stir overnight and filter through Whatman No. 1  
Store at -20°C (good for months).

## 0.8% AGAROSE-FORMALDEHYDE GEL FOR NORTHERNS

### GEL PREPARATION

Add: 0.8 g Agarose  
72 ml dd H<sub>2</sub>O  
10 ml 10X MOPS (41.8 g MOPS, 3.72 g EDTA, 6.8 g NaOAc per liter H<sub>2</sub>O)

if the Na salt of MOPS is used it is necessary to pH to 6.8)  
Heat in microware until agarose goes into solution.  
Cool solution to 60°C and add 18 ml of 37% formaldehyde with continuous shaking.  
Do not add ethidium bromide.

### RNA SAMPLE PREPARATION

RNA should be in 50% formamide, 6% formaldehyde, 1 X MOPS and dye:

Component	Volume
10X MOPS	2 µl
formaldehyde (37%, 12 M)	3.5 µl
formamide (deionized)	10 µl
dye (50% glycerol, 1 mM EDTA, 0.4% bromphenol blue)	2.0 µl
RNA + H <sub>2</sub> O (0.2-0.5 µg poly A+ RNA, 10 µg total RNA)	4.5 µl
H <sub>2</sub> O	Up to 20 µl

Prior to electrophoresis, heat sample to 68°C for 5 minutes.  
Put immediately on ice to quick chill.  
Apply sample and immediately start run.

### ELECTROPHORESIS

Running buffer is 1 X MOPS (best results are achieved when formaldehyde is included at a concentration of 6%).  
Electrophorese at 15-25 V for 18 hours.

Lanes containing molecular weight standards can be cut from the gel and the markers can be stained with ethidium bromide (10 mg/ml) for 30 min and destained for several hours in H<sub>2</sub>O. Destaining can be accelerated using 0.1 M ammonium acetate and 0.01 M β-mercaptoethanol.

## BLOT TRANSFER

Remove gel and equilibrate in 10 X SSC for 45 min at r.t. (20 X SSC, 3.0 M NaCl, 0.3 M Na citrate, pH 6.8 with citric acid).

- Measure gel and cut nitrocellulose to size.
- Blot in 10 X SSC overnight.
- Mark position of wells on filter with soft pencil.
- Remove filter and bake at 80°C for 2-5 hours.
- Blot Transfer (RNA hybridization analysis cont.)
- Remove nitrocellulose filter and wash twice in 2X SSC (15 minutes each wash).
- Bake filter in vacuum oven for 2-6 hours at 80°C.
- The filter can be stored in sealed bag at -20°C.

## Filter Prehybridization

- Seal filter in plastic bag and add prehybridization buffer (Buffer N).
- To calculate the volume needed to saturate the filter: filter length (cm) X width (cm) X 25 μl (vol required to saturate 1 cm<sup>2</sup> of nitrocellulose).
- Remove filter and air dry for 1 hour.

## Probe Hybridization

- Seal filter in a plastic bag.
- Heat probe at 95°C for 5 minutes and add 0.5-1 X 10<sup>6</sup> cpm/ml hybridization buffer.
- Add solution to the side of the filter containing the RNA and incubate at 42°C for 24 hours.
- Remove filter from bag and wash in the following manner:

2X SSC at r.t. for 10 min.

2X SSC at 68°C for 10 min. (lower temperatures can be used for lower stringency)

0.2X SSC at 68°C for 10 min.

0.1X SSC at 68°C for 10 min. (this high stringency wash is optional).

- Air dry filter, then perform autoradiography.

## 20X SSC

NaCl            3.0 M

Sodium citrate      0.3 M

adjust pH to 6.8 with 0.3 M citric acid

## **STAINING RNA GELS WITH ACRIDINE ORANGE**

### **STAIN SOLUTION**

33 µg/ml acridine orange (dilute stock 6.6 mg/ml 200 fold), 10 mM Na-PO<sub>4</sub> pH 6.5-7.0

### **STAINING PROCEDURE**

- Stain agarose gel for 30 minutes at room temperature with shaking.
- Destain with 10 mM Na-PO<sub>4</sub> buffer 3 times for 20 minutes or use 0.1M ammonium acetate, 0.1 β-morcapto-ethanol
- Photograph using red filter and UV 245nm.

### **REFERENCE**

PNAS 74:4835-38, 1977.

## KARYOTYPING ES CELLS

An actively growing culture of cells is required, i.e. 2 - 3 d ES cell culture. The total number of cells needs to be between  $10^6$ - $10^7$  cells.

1. Harvest cells in the usual manner, into a conical bottom centrifuge tube.
2. Centrifuge to remove medium.
3. Resuspend cell pellet by flicking the bottom of the tube.
4. Add 8 ml of warmed KC1 solution to each tube and mix gently with a pipette.
5. Return to 37°C water bath for 10 - 14 mins, (this needs to be determined for each cell type)
6. Add 2 ml of fixative and mix by inversion.
7. Centrifuge cultures at 1500 rpm for 5 mins.
8. Aspirate most of the supernatant.
9. Using a pasteur pipette gradually resuspend the cells in ~2 mls of fixative. This needs to be done very gently. Then add fixative to 8 mls, and mix gently by inverting the tube.
10. Centrifuge cells again.
11. Repeat 8, 9, 10 3 - 4 times.
12. Gently resuspend cells at appropriate concentration.
13. Make spreads by "huffing" on slides, holding at 45°C and dropping one drop of cells onto the top of the slide. Stain slides with Leishmanns.

## STAINING

1. Stain slides with 1 ml Leishmann's stain and 5 ml pH 6.8 buffer for 7 - 9 mins (enough for 2 slides).
2. Rinse briefly in running water and air dry.
3. Clear in X2 changes of xylene.
4. Coverslip using Depex mountant.

## SOLUTIONS

### HYPOTONIC SOLUTION (0.075M POTASSIUM CHLORIDE)

Add 0.56 g KC1 to 100 ml H<sub>2</sub>O.

### LEISHMANN'S STAIN

BDH PROD 35022

0.2% w/v solution in methanol.

GURRS BUFFER TABLETS pH 6.8

BDH PROD 33193

### FIXATIVE

3 parts methanol to 1 part glacial acetic acid.

### NOTES:

- The time in KC1 is crucial. If the time is too short, the chromosomes will be too tightly packed. Too long, they will not be contained in their appropriate group.
- When staining spreads, the Leishmanns must be made up fresh for 2 - 4 slides at a time, as it precipitates out.
- Slides need to be soaked in absolute ethanol overnight and wiped with a lint-free tissue. If it is too warm to "huff" on the slides, they can be placed in the fridge/freezer.

- Fixative must be made up fresh each time. Dispose of fixative waste properly, i e., into solvent waste.
- Avoid breathing vapor.

**Reference:**

[http://grimwade.biochem.unimelb.edu.au/bowtell/cellbiol/sect57.htm#KARYOTYPING ES CELLS](http://grimwade.biochem.unimelb.edu.au/bowtell/cellbiol/sect57.htm#KARYOTYPING_ES_CELLS) by David Bowtell (bowtell@ariel.ucsf.unimelb.edu.au)

## RNA DOT BLOT PROCEDURE

- Cut nitrocellulose (SS-BA-85) to fit apparatus.
- Cut a piece of Whatman #2 filter paper the same size.
- Soak nitrocellulose in water for 10 minutes.
- Soak nitrocellulose in 10X SSC for 5 minutes.
- Soak Whatman filter in dH<sub>2</sub>O.
- Prior to assembly of apparatus make sure holes are clear.
- Place Whatman filter paper on apparatus and the nitrocellulose on top of Whatman.
- Assemble apparatus.
- Mix RNA in 100 µl of 10X SSC, 20% vol. formaldehyde (37%) and heat for 5 minutes at 68°C. Cool on ice. (apply 1-5 µg total, 0.1-1 µg poly A+)
- Apply sample using house vacuum.
- Rinse each well with 200 µl of 10X SSC, 20% vol. formaldehyde (37%).
- Remove nitrocellulose filter and air dry.
- Bake filter under vacuum at 80°C for 2-3 hours.

## PURIFICATION OF gp330/LRP-2/Megalin FROM HUMAN URINE

- Obtain morning urine from donors.
- In cold room combine urine samples into a graduated cylinder (on magnetic stirrer).
- To 1800 ml of urine add:

100 ml 1 M Tris pH 8.0  
2 ml 1 M CaCl<sub>2</sub>  
20 ml 100mM PMSF in ETOH (add very slowly while stirring) [1 mM] final  
160 µl 25 mg/ml leupeptin in H<sub>2</sub>O [2 µg/ml] final  
0.4 ml 5 mg/ml P-PACK in ETOH [1 mM] final  
33 µl 2 mg/ml pepstatin in DMSO [66 ng/ml]  
Adjust volume to 2 liters with urine or H<sub>2</sub>O.

- Stir at 4°C for 2 hours.
- Pour into 1 liter centrifuge bottles.
- Centrifuge at 3500 rpm for 30 mins at 4°C.
- Setup 2 liter side arm flask with large scinterred glass funnel containing 150 ml Sepharose CL-4B.
- Pass urine slowly through Sepharose twice.
- Pass urine over RAP-Sepharose at 4°C (can go overnight).
- Wash column with 50 column volumes of 0.5 M NaCl, 50 mM Tris pH 8.0, 1 mM CaCl<sub>2</sub>.
- Elute with 2 column volumes of 8 M urea, 50 mM Tris pH. 8.0 and collect 8 1/4 column volume fractions.

## PURIFICATION OF gp330/LRP-2/Megalin FROM RAT/PIG KIDNEYS

- Obtain 4 tubes of frozen ground pig kidney or weigh out 200 g frozen rat kidneys (purchase from Pelfreeze).
- Add 500 ml of 2 mM Tris pH 7.0, 50 mM mannitol and homogenize in blender for 2 min.
- Add 1 M  $\text{CaCl}_2$  to a final concentration of 10 mM and protease inhibitors (see below) and stir in cold room for 10 min.
- Centrifuge in GSA rotor for 20 min at 3500 rpm at 4°C (keep supernatant). Re-centrifuge supernatant again to remove any residual insoluble material (keep supernatant).
- Centrifuge supernatant at 12000 rpm in GSA for 30 min.
- Discard supernatant and bring up pellet in 100 ml of 50 mM Tris pH 8.6, 1% deoxycholate (DOC), plus protease inhibitors.
- Stir for 20 min at 4°C.
- Centrifuge at 16,000 rpm in GSA for 25 min.
- Save supernatant.
- Dilute 1:10 with 50 mM Tris pH 8.0, 0.5 M NaCl, plus protease inhibitors (see below).
- Pass over plain Sepharose and RAP-Sepharose as indicated on gp330 urine prep protocol at 4°C.
- Wash with 50 column volumes of 0.5 M NaCl, 50 mM Tris, pH 8.0, 0.1% DOC at 4°C.
- Elute at 4°C with 2 column volumes 1 M urea (deionized), 50 mM Tris pH 7.4.
- Elute at 4°C with 2 column volumes 4 M urea (deionized), 50 mM Tris pH 7.4.
- Elute at 4°C with 2 column volumes of 8 M urea (deionized), 50 mM Tris pH 7.4, 0.1% DOC and collect 8 X 1/4 column volume fractions.
- Place eluted fractions on ice and measure  $A_{280}$  nm. Take aliquots of each fraction for SDS-PAGE.
- Pool peak fractions and dialyze at 4°C against 0.1% OG, TBS.

- Measure OD<sub>280</sub> (blank against dialysis buffer). Run SDS-PAGE on sample (non-reduced).
- Add 0.5 M EDTA to final conc. of 20 mM.
- Add to heparin-Sepharose and rotate for 45-60 min. in cold room.
- Collect all flow through and add 0.5 col. vol. 0.1% OG, TBS, EDTA and collect (save).
- Repeat step 21 four more times.
- Measure OD<sub>280</sub> on each of the collected flow through fractions. Run sample from each on SDS-PAGE (non-reducing).
- Pool peak fractions and dialyze against 0.1% OG (no TBS).
- Measure OD<sub>280</sub> of dialyzed material and run on SDS-PAGE under non-reducing conditions. Make 250-500 µl aliquots and label. Store at -70°C.

**Protease inhibitors:**

per 100 ml of buffer add:

Volume	Stock	Final Concentration
84 µl	20 mg/ml PMSF in ETOH	1 mM
17 µl	25 mg/ml leupeptin in H <sub>2</sub> O	2 µg/ml
3.3 µl	2 mg/ml pepstatin in DMSO	66 ng/ml
83 µl	5 mg/ml P-PACK in ETOH	1 mM

## IMMUNOAFFINITY PURIFICATION OF FIBULIN-1 FROM PLACENTA

### Day 1: Tissue Pre-extraction

- Thaw 6x50 ml tubes of frozen ground placenta.
- Add 300 ml of TBS, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.005% digitonin, 1 mM PMSF
- Stir in beaker for 20 minutes at 4°C.
- Transfer extract to 250 ml centrifuge bottles.
- Centrifuge in GSA rotor for 30 minutes, 10,000 rpm, 5°C.
- Discard supernatant and save pellet.

### Tissue Extraction

- Add 300 ml of 4M KSCN, 10 mM EDTA, 1 mM PMSF to pellet and mix in blender for 1 minute set at blend.
- Stir in beaker at 4°C for 1.5 hours.
- Centrifuge extract for 30 minutes in GSA rotor, 10,000 rpm, 5°C.
- Transfer clarified supernatant into dialysis tubing cut to the length of a 4L beaker.
- Dialyze supernatant against 4L PBS, 10 mM EDTA pH 8.0, 1 mM PMSF, at 4°C.
- Change dialysis buffer once after 4 hours and allow dialysis overnight at 4°C.

Note: it is critical that the extracts be thoroughly dialyzed before continuing!  
Centrifuge dialyzed extract, twice, for 30 minutes in GSA rotor, 10,000 rpm 5°C.  
Extract is ready to apply to columns.

### Affinity Chromatography (Day 2)

- Equilibrate 50 ml Sepharose CL-4B with 2 col vol TBS in cold room (4°C).
- Apply extract from above to Sepharose CL-4B column and collect flowthru.
- Equilibrate IgG-Sepharose (5D12 or 3A11) column with 2 col vol TBS.
- Apply Sepharose-absorbed flowthru from above.
- Wash with 10 col vol TBS.
- Elute bound fibulin with 2 col vol 3M KSCN, 1 mM PMSF.
- Dialyze peak fractions against 1L TBS, 1 mM PMSF, changing buffer once.
- Centrifuge the dialyzed material for 15 minutes, 5000 rpm (Sorvall centrifuge).

### Day 3

- Pass dialyzed material over Gelatin-Sepharose column twice, save flowthru. Push out any remaining material by adding an additional col vol of TBS.
- Pool flowthrus and apply to Heparin-Sepharose column and collect flowthru material.

- Apply flow-through to WGA column repeatedly, 4 times. Push out any remaining material by adding an additional col vol of TBS. Pool flow through material. Wash WGA with 10 col vol TBS.
- Elute WGA with 2 col vol of 0.5M N-acetylglucosamine in TBS (1.1 g GlcNAc, 10 ml TBS). Collect 8 X 1/4 col vol fractions.
- Run an aliquot of each eluted fraction on SDS-PAGE.

## Cleaning columns

### Sepharose CL-4B:

1 col vol TBS  
 2 col vol 8M urea - resuspend by pipetting or inverting column.  
 2 col vol water - to wash out urea.  
 2 col vol TBS, 0.05% thimerosal\*

### IgG-Sepharose:

2 col vol 10% ethanol (every other use)  
 2 col vol 8M urea, 50 mM Tris-HCl pH 7.5 (every other use)  
 10 col vol TBS  
 2 col vol TBS, 0.05% thimerosal\*

### Gelatin-Sepharose:

3 col vol 8M urea, 50 mM Tris-HCl pH 7.5  
 10 col vol TBS  
 2 col vol TBS, 0.05% thimerosal\*

### Heparin-Sepharose:

3 col vol 2M NaCl  
 10 col vol TBS  
 2 col vol TBS, 0.05% thimerosal\*

### WGA-Agarose:

3 col vol 0.1M Acetic Acid  
 6 col vol 50 mM Tris pH 7.4 1M NaCl  
 2 col vol TBS, 0.05% thimerosal\*

\*Thimerosal- Ethylmercurithiosalicylic acid, sodium salt 97% Aldrich cat.#- E3,525-7. Toxic risk

## **PURIFICATION OF LRP-1 FROM PLACENTA**

### **Tissue Pre-extraction (Day 1):**

- Thaw 6x50 ml tubes of frozen ground placenta. Keep tissue as cold as possible.
- Add 300 ml of wash buffer TBS, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, protease inhibitors.
- Stir in beaker for 20 minutes at 4°C.
- Transfer extract to 250 ml centrifuge bottles.
- Centrifuge in GSA rotor for 30 minutes, 10,000 rpm, 5°C.
- Discard supernatant and save pellet.

### **Tissue Extraction:**

- Add 300 ml of 4M KSCN, 10 mM EDTA, 1 mM PMSF to pellet and mix in blender for 1 minute set at blend.
- Stir in beaker at 4°C for 1.5 hours.
- Centrifuge extract for 30 minutes in GSA rotor, 10,000 rpm, 5°C.
- Transfer clarified supernatant into dialysis tubing cut to the length of a 4L beaker.
- Dialyze supernatant against 4L PBS, 10 mM EDTA pH 8.0, 1 mM PMSF, at 4°C.
- Change dialysis buffer once after 4 hours and allow to dialyze overnight at 4°C.

## LIGAND-SEPHAROSE SELECTION OF ANTIBODY

- Dilute antiserum 1:10 to final 0.5 M NaCl, 50 mM Tris pH 7.5 (0.05% Tween optional).
- Pre-elute ligand-Sepharose column with 10 mM glycine pH 2.5 then immediately equilibrate column first with 0.5M Tris pH 7.5 then with 0.5 M NaCl, 50 mM Tris pH 7.5 (0.05% Tween optional).
- Apply diluted antiserum to column several times. Save flow through material and label.
- Wash column with 0.5M NaCl, 50 mM Tris (0.05% Tween optional) until OD<sub>280</sub> goes to zero.
- Elute with 2 column bed volumes of 10 mM (or 100 mM) glycine pH 2.5. Collect 8 1/4 column bed volume fractions into 1M Tris pH 8.0 (1/10<sup>th</sup> fraction volume). Immediately equilibrate column with TBS.
- Determine OD<sub>280</sub> of eluted fractions, pool peak fractions and dialyze against TBS.
- Re-elute the column with 8M urea and as above determine OD<sub>280</sub> of eluted material, pool and dialyze.
- Wash urea out of column with TBS buffer and store column at 4°C.
- As an alternative to the urea elution, perform elution using high pH buffer.

## **PURIFICATION OF gp330/LRP-2 ANTIBODIES BY AFFINITY SELECTION ON gp330/LRP-2/Megalin-SEPHAROSE**

- Pre-elute gp330-Sepharose column with 2 column volume 8 M urea, 50 mM Tris pH 8.0 then with 2 column volumes dH<sub>2</sub>O then with 2 column volumes 100 mM glycine pH 2.5. Equilibrate column immediately in 0.5 M Tris pH 8.0 and then with TBS pH 7.4.
- Pre-elute RAP-Sepharose with with 2 column volume 8 M urea, 50 mM Tris pH 8.0 then with 2 column volumes dH<sub>2</sub>O and then with 2 column volumes 100 mM glycine pH 2.5. Equilibrate column immediately in 0.5 M Tris pH 8.0 then with TBS pH 7.4.
- Centrifuge 2-5 ml antiserum at 10,000 X g for 15 min.
- Apply supernatant to gp330-Sepharose column. Best results are often obtained if the antiserum is incubated with the column for several hours to overnight at 4°C on rocker.
- Collect the flow-through and save. Typically there is significant amounts of residual antibody remaining that can be recovered by an additional passage over the gp330-Sepharose column.
- Wash the column with TBS until the O.D.280 goes to zero.
- Elute with 2 column bed volumes of 10 mM (or 100 mM) glycine pH 2.5. Collect 8 1/4 column bed volume fractions into 1 M Tris pH 8.0 (1/10th fraction volume). Immediately equilibrate the column with TBS.
- Determine OD280 of eluted fractions, pool peak fractions.
- Apply the pooled peak fractions to a RAP-Sepharose column and incubate for 1 hour at 4°C on rocker.
- Recover as much volume of the applied solution as possible.
- Heat inactivate by incubation at 56°C for 30 min
- Dialyze against isotonic dPBS and sterile filter.
- Determine OD280 and perform ELISA to determine titer against gp330 and RAP. Concentration may be required to elevate concentration toward 1.0 mg/ml.
- Aliquot into sterile tubes and store at -20°C (indicate selected on gp330/LRP-2-Seph. and abs on RAP-Seph on labels with date, OD, and notebook page for ref).

## STANDARD ELISA PROTOCOL

- Coat a 96 well microtiter plate with 100-200  $\mu\text{l}$ /well desired antigen at a concentration range of 1-3  $\mu\text{g}/\text{ml}$  diluted in coating buffer or TBS pH 8.0. Incubate for 4 hours at room temperature or overnight at 4°C.
- Rinse plate 3 times with PBS or TBS using either squirt bottle or pipette man (add 250  $\mu\text{l}$ /well).
- Block wells with 3 mg/ml BSA or non-fat milk in PBS or TBS, adding 250  $\mu\text{l}$ /well for 1 hour at room temperature. It is best to fill the wells with blocking solution.
- Rinse wells 3 times with PBS/TBS-Tween 20 (0.05%).
- To make a serial dilution of antibody, add 150  $\mu\text{l}$  of starting sera to first well. Add 100  $\mu\text{l}$  of PBS/TBS-Tween (0.05%) to other wells in the row. Transfer 50  $\mu\text{l}$  from first well into second well and mix 5 times. Repeat this dilution process down plate. Always perform dilution series in duplicate. Incubate for 1-4 hours at room temperature.
- Rinse plate 3 times with PBS/TBS-Tween (0.05%).
- Add 100  $\mu\text{l}$  of appropriate conjugate diluted 1:3000 in PBS/TBS-Tween. Incubate for 1 hour at room temperature.
- Rinse plate 3 times with PBS/TBS-Tween.
- Develop with PNPP, OPD or appropriate substrate.

## ELISA COATING BUFFER

500 ml:

1.38 g Sodium carbonate  
3.11 g Sodium bicarbonate  
add H<sub>2</sub>O up to 500 ml

(1X stock solution should have a pH of 9.5)

## TWO-ANTIBODY SANDWICH ELISA ASSAY

- Quantitative ELISA for determining antigen concentration in blood, urine etc.
- Coat wells with monoclonal antibody in coating buffer. Either purified IgG (1-3 µg/ml) or hybridoma culture supernatants (diluted 1:2).
- Block with 3 mg/ml BSA or milk in TBS (~200 µl per well) 1 hour.
- Titrate antigen of known concentration 1:3 in TBS-Tween (0.05%) down two rows.
- Titrate multiple urine or plasma samples 1:3 in TBS-Tween (0.05%) in separate rows.
- Incubate for 2 hours room temperature.
- Wash three times with TBS-Tween.
- Add diluted rabbit antiserum (e.g. 1:5000) in TBS-Tween (150µl per well).
- Incubate for 2 hours room temperature.
- Wash three times with TBS-Tween.
- Add goat anti-rabbit IgG HRP conjugate diluted in TBS-Tween.
- Incubate for 2 hours room temperature.
- Wash with TBS-Tween.
- Develop.

## PEROXIDASE SUBSTRATE DEVELOPMENT FOR ELISA

- Mix 25 mg ortho-o-phenyl-diamine (OPD) with 25 ml of development buffer buffer (5.0 g citric acid monohydrate, 7.0 g sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) up to 500 ml  $\text{H}_2\text{O}$  and filter, pH should be 5.0 without adjustment)
- Add 12  $\mu\text{l}$  of hydrogen peroxide, mix thoroughly
- Use 200  $\mu\text{l}$  per well.
- Measure absorbance at 450 nm.

## **TWO-ANTIBODY SANDWICH ELISA ASSAY for FIBULIN-1**

- Quantitative ELISA for determining fibulin-1 concentration in blood, lung lavage etc...
- Coat microtiter wells with 3 µg/ml polyclonal antibody 1323 IgG in coating buffer (150 mM NaCl, 50 mM Tris, pH 8.0) (TBS).
- Block unoccupied sites with 3 mg/ml ovalbumin in TBS (~250 µl per well) 1 hour.
- Titrate fibulin-1 of known concentration 1:3 in TBS-Tween-20 (0.05%) starting 500 nM in duplicate over 12 wells.
- Titrate duplicate samples with unknown levels of fibulin-1 1:3 in TBS-Tween (0.05%) in separate rows.
- Incubate for 2 hours room temperature.
- Wash three times with TBS-Tween.
- Add diluted mouse monoclonal 3A11 IgG (3 µg/ml) in TBS-Tween (150 µl per well).
- Incubate for 2 hours room temperature.
- Wash three times with TBS-Tween.
- Add goat anti-mouse IgG-HRP conjugate diluted in TBS-Tween.
- Incubate for 1 hours room temperature.
- Wash with TBS-Tween.
- Develop using TMB (Kirkegard and Perry) substrate.

## SOLID PHASE MICROTITER WELL LIGAND BINDING ASSAY

- Coat a 96 well breakaway well microtiter plate with 100-150  $\mu$ l/well desired protein at a concentration range of 1-3  $\mu$ g/ml diluted in coating buffer or TBS pH 8.0. Incubate for 4 hours at room temperature or overnight at 4°C.
- Remove coating solution by dumping or aspiration.
- Block wells with 3% BSA or 3% non-fat milk or 1% ovalbumin in TBS, adding 400  $\mu$ l/well and incubate for 1 hour at rt. It is best to completely fill the wells with blocking solution.
- Remove blocking solution by dumping or aspiration.
- Make a serial dilution of radiolabeled protein plus competitor, add 150  $\mu$ l of starting dose to first well.
- Add 100  $\mu$ l of TBS-0.05% Tween, 3% BSA, 5 mM  $\text{CaCl}_2$  radiolabeled tracer protein to other wells in the row. Typically, 1 nM tracer (200,000 cpm/well) can be used.
- Transfer 50  $\mu$ l from first well into 2nd and mix 5 times. Repeat this dilution process down plate. Always perform dilution series in duplicate. Incubate overnight at 4°C.
- Rinse plate wells 3 times with TBS-Tween.
- Break away wells and count in gamma counter.

## ISOLATION OF MOUSE PLATELETS

- Anaesthetize mouse and make cut in abdomen and move intestines to your right. You will see two large vessels that join and form a bulbous region.
- Into a 1 ml syringe with a 27-gauge needle draw up 100  $\mu$ l of 38% buffered citrate.
- Insert needle into bulbous region and draw 1 ml blood (takes about 30 seconds).
- Remove needle from syringe and squirt blood into a 1.5 ml eppendorf and mix by inversion.
- Centrifuge in clinical centrifuge 600 rpm for 25 minutes.
- Red cell layer will be at bottom and above it will be a white layer of platelets.
- Use 1 ml pipette and very slowly draw up the whitish layer leaving the red cell layer behind.
- Transfer to a new tube and centrifuge at 2500 rpm in a microfuge. Remove plasma supernatant that can be used for dilution.

## PLATELET ADHESION ASSAY

- Coat microtiter plate wells with desired adhesive proteins in TBS, pH 8.0 overnight at 4°C (10 µg protein/ml, use 100 µl/well).
- Block unoccupied binding sites with 1% BSA in 140 mM NaCl, 5.5 mM glucose, 10 mM Tris, pH 7.5 (TBS-glucose buffer, TBS-G) for 1 hr 37°C.
- Resuspend <sup>51</sup>Cr-labeled platelets at 2 x 10<sup>8</sup>/ ml in 1 %BSA, TBS-G buffer, containing 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub> in the presence or absence of FG (2-3 mg/ml).
- Add 50-100 µl aliquots of platelet suspension to each well and incubate for 60 min at room temperature.
- Remove unattached platelets by aspiration, rinse wells 3 times with BSA,TBS-G buffer or PBS.
- Extract bound platelets with 2% SDS and measure radioactivity by scintillation counting.

## [<sup>51</sup>Cr]-CHROMIUM LABELING OF PLATELETS

- Collect whole blood into anticoagulant citrate-phosphate dextrose solution (CPD-A1), (Baxter Healthcare Corporation, Deerfield, IL), use 63 ml of anticoagulant per 450 ml of whole blood.
- Centrifuge blood at 120 x g, 20 min at r.t. and collect platelet-rich plasma (PRP).
- Add prostaglandin E1 (PGE1) at a concentration of 1 µg/ml and citric acid to 4 mM and centrifuge PRP for 10 min at 120 x g at r.t. to removed residual red blood cells.
- Centrifuge PRP at 1800 x g for 15 min at r.t. to collect platelets.
- Wash platelets in 5.5 mM glucose, 120 mM NaCl, 4.26 mM NaH<sub>2</sub>PO<sub>4</sub>, 7.46 mM trisodium citrate, 2.35 mM citric acid, pH 6.5 (citrate wash buffer, CWB) 2 times.
- Resuspend platelets at 2 x 10<sup>9</sup>/ml in TBS-G buffer and add 50-100 µCi of <sup>51</sup>Cr.
- Incubate at r.t. for 45-60 min.
- Add 5 volumes of CWB and centrifuge for 5 min, 1800 x g at r.t.
- Wash platelet pellet 3 times with CWB to remove free chromium-51.
- Resuspend platelets at 2 x 10<sup>8</sup> / ml in BSA, TBS-G, and add to wells.

## FLUORESCEIN ISOTHIOCYANATE LABELING OF FIBULIN-1

- Dialyze fibulin-1C against 0.1M NaHCO<sub>3</sub> pH 9.5 o/n in cold room.
- Using 457 µg of fibulin-1C
- [Fibulin-1C]= 457 µg x 1 g/1 X 10<sup>6</sup> mg X 1 mol/78000 g = 5.8615 nmole
- Used ratio of 40:1 of FITC to protein, FITC needed = 40 X 5.8615 X 10<sup>-9</sup> mole X 389.36 g/mole = 0.000091294 g
- Weigh out 3.3 mg of FITC and dissolve in 1 ml of methanol.
- Add 27.66 ml of (3.3 mg/ml of FITC) to 457 mg of fibulin-1C in 0.1M NaHCO<sub>3</sub> pH 9.5.
- Mix and incubate in the dark at room temperature for 4.5 hr.
- Equilibrate PD10 column with TBS. Add the fluorescein-labeled fibulin-1C to the column.
- Collect 1 ml fractions and determined the O.D.280 nm. Scan the peak fractions from 550 nm to 200 nm.

### Example:

- Concentration correction of fluorescein-labeled fibulin-1C

Fr. 3 O.D.277.3 nm = 0.057  
O.D.495.5 nm = 0.133

[Protein]= [Abs280nm-(0.35 X Abs495nm)]/31200(1/M) = [0.057- (0.35 X 0.133)]/31200= 334.93 nM

FITC= 0.133/72000= 1.847 mM

Ratio = FITC/Protein= 1847 nM/334.95 nM = 5.51

Fr. 4 O.D.277.3 nm = 0.572  
O.D.495.5 nm = 1.348

[Protein] = [Abs280 nm -(0.35 X Abs495nm)]/31200(1/M)=[0.572- (0.35 X 1.348)]/31200=3.211538 mM

FITC = 1.348/72000= 18.722 mM

Ratio = FITC/Protein= 18.722 mM/3.211538 mM= 5.83

## [<sup>125</sup>I]-LABELING OF LIPOPROTEINS

(Method per Kelley Argraves)

- Perform the following steps quickly.
- Pre-equilibrate a PD-10 column with incubation medium (e.g., DMEM).
- Add 170 µl of 2 M glycine pH 10 to 300 µg lipoprotein (usually in 200-400 µl ).
- Add 2 µl 33 mM iodine monochloride (ICI) to 23 µl of 2 M NaCl.
- Add 5 µl (0.5 mCi) sodium-[<sup>125</sup>I] to the lipoprotein/glycine mixture.
- Add 5-10 µl of diluted ICI (step 3) to the lipoprotein mix, gently mix and incubate 5 min, mixing about once a minute.
- Apply iodinated mixture to the PD-10 column and allow it to enter the column, add 5 ml of incubation medium, collect fractions\*.

\*Chromatography instructions: For fractions 1 and 2 = collect 15 drops, following fractions = 10 drops. Count fractions, radiolabeled lipoprotein is usually found in fractions 4-6. Pool peak fractions, assume 50% loss of material, count 2 µl and determine specific activity based on these #'s.

## [<sup>125</sup>I]-IODINE LABELING OF PROTEINS

### IDOGEN Method

- Using IODOGEN-coated tube (see below for method of preparation).
- Use 50 µg protein in 20 µl TBS.
- Add protein solution to IODOGEN-coated tube.
- Wearing lab coat, gloves, goggles and sleeve protectors transfer the tube to fume hood with work surface lined with absorbant paper. With the exception of counting the fractions in a gamma counter the following procedures are done in the hood
- Add 0.5 mCi (5 µl) <sup>125</sup>I-Na (NEN) and mix gently.
- Incubate 5 min at room temperature.
- Apply entire volume to a Pharmacia PD-10 column pre-equilibrated with TBS or medium.
- Add 5 ml of buffer and collect 10 drop fractions (~0.5 ml). Peak fractions usually will elute around fractions 6 and 7.
- Remove a 1-2 ml aliquot from each and count using the gamma counter.
- Pool peak fractions and count an aliquot using gamma counter.
- Dispose of contaminated tubes, bench coat, etc. and survey the area for contamination.

### Preparation of Iodogen-coated Tubes:

- Prepare a 5 mg/ml solution of IODOGEN (Pierce Chemical Co.) in chloroform.
- Add 20 µl of IODOGEN solution to the bottom of glass tubes (10 x 75 mm). Many tubes can be prepared simultaneously.
- Place tubes in hood and let chloroform completely evaporate (this takes about 1 hour).
- IODOGEN-coated tubes can be stored at room temperature under vacuum.
- Equilibrate PD-10 column (Pharmacia) using ~25 ml of buffer (PBS).

- Add 50 µg protein in 200 µl to tubes coated with Iodogen. Optimal iodination can be achieved with PBS. Tris buffer interferes with the reaction, but not completely.
- Wearing protective clothing, gloves, goggles etc., take the tube to fume hood and place in the inner charcoal filtered hood.
- Add 0.5 mCi (~5 µl) mix gently and allow reaction to proceed for 2-10 minutes at room temperature.
- Apply sample to PD-10 column and collect 12 X 0.5 ml fractions.

#### **[<sup>125</sup>I]-IODINE LABELING OF PROTEINS (Continued)**

- Take 5 µl aliquots from each fraction and count in gamma counter.
- Pool the peak fractions.
- Assume that recovery is ~75%.

## APO A-1 PREPARATION

### Delipidation of HDL:

1. Dialyse HDL against 0.1% Ammonium Bicarbonate.
2. Lyophilize
3. Mix 3:1 EtOH:Et<sub>2</sub>O Mix well and put in freezer to get cold. Use absolute EtOH and Et<sub>2</sub>O which has been opened only recently.
4. Add sample (100 mg) to 50 cc glass, screw cap tube.
5. Half fill tube with EtOH:Et<sub>2</sub>O in tube.
6. Place in freezer for 2-3 hours. Mix vigorously every half-hour.
7. Fill tube with Et<sub>2</sub>O. Mix well.
8. Centrifuge at 3,000 rpm for 30 minutes at 4°C
9. Remove tubes from centrifuge one at a time and aspirate solvents, leaving 1–2 cm of solution over protein.
10. Repeat Steps 7-9 3X. Shaking vigorously with each Et<sub>2</sub>O addition.
11. Evaporate remaining solvent under N<sub>2</sub> stream while “shelling” protein residue on bottom of tube.
12. Add 10 ml AcA column buffer and stir in cold room overnight.
13. Apply to Pharmacia 26/100 AcA column

### AcA column buffer (0.2 M Tris 6M Urea pH 8.4):

600 mL 10 M Urea

24.23 g THAM (Fisher T-395-1)

QS to 1L with water

### AcA column: Pharmacia 26/100

### 10 M Urea: (Fisher T395-1)

- Make 10 M stock. (600g/L)
- Pour over Rexyn-300 (Fisher # R276-500) column(5 cm x 30cm) (BioRad 737-5031) immediately before use.
- Measure conductivity relative to distilled water.

## [<sup>35</sup>S]-SULFATE METABOLIC LABELING OF CULTURED CELLS

1. Metabolically label cells with 50-100 µCi/ml [<sup>35</sup>S]-sulfate in low sulfate Hams F12 medium (Gibco -6 µM sulfate- cat. no. 3201765, 3 µM sulfate 11765-054). Supplement medium with L-glutamine and pyruvate. Don't add Pen-Strep since it is a rich source of sulfate and use FBS or FCS that has been dialyzed against sulfate-free PBS).
2. Incubate for 16-24 h 37°C, 5% CO<sub>2</sub>.

Note: Chlorate treatment (20 mM for 18 h prior and during experiment) may only partially inhibit sulfation (~70%). see 1988 BBRC vol 150 (1) p342 uses of two classes of compounds. Other important refs: Methods of Enzymology vol 179, p428, 1989. Harper et al., 1986 JBC, 261, p3600. Hascall et al., Arch Biochem and Biophys 1986 250 202-210. *Biochem J.* 1987 241 p591-601

3. Remove conditioned culture medium and adjust to 300 mM NaCl. This will prevent loading of the DEAE with non specific molecules, essentially only PGs will bind.
4. Wash cell layer with dPBS (wash can be combined with above conditioned culture medium).
5. Extract cell layer (e.g. 75 cm<sup>2</sup> flask) of cells of 1-2 ml detergent buffer (included proteinase inhibitors). Scrape and collect extract into 2 ml microfuge tubes. Correct ionic strength to 300 mM NaCl. Centrifuge to remove insoluble material.
6. 50-100 µl packed vol of DEAE Fast Flow Sepharose. Add 0.3-0.5 volume of extract to each microfuge. Incubate at room temperature on Nutator for 30 mins.
7. Centrifuge 30 seconds in microcentrifuge at speed 3 to pellet beads. Remove the supernatant. Wash beads 4-5 times with buffer containing 300 mM NaCl. Discard washes in radioactive waste container.
8. Add 1-2 beads volumes of 1-1.5 M NaCl, vortex, centrifuge, and remove supernate and save. Repeat the elution twice and combine the supernatants. Count an aliquot of pooled supernatant.

For gel analysis run as many counts as possible per gel lane.

## DETERGENT EXTRACTION OF CULTURED CELLS

### Extraction buffer:

1% Triton X-100, 0.5% Tween-20, 0.5 M NaCl, 50 mM Hepes, pH 7.5, 1 mM PMSF (add PMSF just prior to performing extraction).

### Procedure:

- Wash the cell layer three times with PBS or TBS. Carefully remove wash solution by aspiration. Do not allow the cells to dry.
- Place the cell culture plate on ice.
- Add 0.5 ml (per 150 mm plate) of ice-cold extraction buffer in the center of the plate.
- Scrape cells with a scraper from the periphery towards the center.
- Tilt the plate and scrape the cells with the extraction buffer. You may add another 0.25-0.5 ml of extraction buffer to wash all the cells down.
- Pass the cell suspension through a 21-gauge needle 15-20 times.
- Centrifuge the cell extract at 100,000 x g for 1 hour at 4°C.
- Remove and save supernatant.

## **ASSAY TO MEASURE UROKINASE (uPA) ACTIVITY IN CONDITIONED MEDIUM**

- Add duplicate aliquots of conditioned culture medium to 1.5 ml eppendorf tubes.
- Add plasminogen to final concentration of 0.3  $\mu$ M (0.625 U/ml).
- Incubate for 10-20 min at 37°C.
- Add plasmin substrate S-2251 (DiaPharm Group Inc., Franklin, OH, Tel.:800-526-5224) to a final concentration of 0.5 mM.
- A standard curve can be generated using known amounts of human urokinase (Calbiochem, La Jolla, CA).
- Transfer to wells of a microtiter plate measure the O.D. at 405 nm..

## ASSAY TO MEASURE RAP EFFECTS ON CELLULAR UROKINASE (uPA) ACTIVITY

- Grow cells to 70-80% confluence in 12 well plates.
- Wash cell layer with DMEM and add serum free medium containing 1% Nutridoma. Incubate 30 min at 37°C, 5% CO<sub>2</sub>.
- Remove medium and add DMEM, 1% Nutridoma containing either 1 µM RAP or ovalbumin. Incubate at 37°C, 5% CO<sub>2</sub>.
- At various time intervals (e.g. zero time and then every thirty minutes up to 8 hours) remove and save medium from designated well(s). Wash the cell layer twice with dPBS. Centrifuge the conditioned culture medium at 14,000 X g for 30 minutes in microcentrifuge (at 4°C).
- Add 0.25 ml Triton X-100 extraction buffer (e.g. 0.1% Triton X-100, 50 mM Tris-HCl pH 7.4) and scrape cell layer using a cell scraper. Remove and save the extract and add 0.25 ml extraction buffer and repeat process. Combine both extracts.
- Pass combined extracts 10 times through a 18 gauge needle to completely disrupt cells and shear DNA.
- Centrifuge extract at 14,000 X g for 30 minutes in microcentrifuge (at 4°C).
- Add duplicate aliquots of the extract to wells of a microtiter plate. To separate wells add aliquots of the conditioned culture medium.
- Add plasminogen (EACA- and lysine-free, human plasma. Cal-Biochem #528178) to final concentration of 0.3 µM (0.625 U/ml).
- Incubate for 10-20 min at 37°C.
- Add the plasmin substrate S-2251 (DiaPharm Group Inc., Franklin, OH, Tel.:800-526-5224, make stock 1-2 mg/ml) to a final concentration of 0.5 mM and measure the O.D. at 405 nm. Development may take up to 6 hours or longer!
- Optional: A standard curve can be generated using known amounts of human urokinase (Calbiochem, La Jolla, CA).

## DEIONIZATION OF UREA AND FORMAMIDE

- Urea and formamide are inherently unstable and will spontaneously decompose to form free ions during storage:

Formamide: HCOO<sup>-</sup> and NH<sub>4</sub><sup>+</sup>

Urea: CN<sup>-</sup> and NH<sub>4</sub><sup>+</sup>

To minimize the free ion content the following procedure is suggested:

- Mix 50 ml formamide or 150 g of urea (in 1-8M solution) with one gram of the mixed-bed, ion exchange resin Dowex X68 or BioRad AG501-X8 (20-50 mesh) and stir for thirty minutes at room temperature.
- Filter twice through standard filter paper, aliquot and store at -20°C.
- Upon thawing, keep preparations at 4°C and discard after one week.

Note: Tris buffer should be added to urea solutions after deionization.

## **THAWING CELLS FROZEN IN LIQUID NITROGEN**

- Wear protective eyeglasses since the vials may explode during thawing procedure.
- Remove vial from liquid nitrogen and place in a floating rack in 37°C water bath.

Thaw for 2-3 minutes.

- Sterily remove cell suspension and layer onto the surface of 9 ml of complete medium in a 15 ml centrifuge tube.
- Centrifuge and resuspend the cell pellet in 10 ml complete medium and transfer to 100 mm dish or into two T-25 flasks.

## HAPTOTACTIC TRANSWELL FILTER ASSAY

1. Add 100  $\mu$ l of fibronectin (FN) (100 mg/ml in TBS, pH 7.4) to the plastic wells that the filter inserts come packaged; place into a humidified plastic container and incubate overnight at 37°C.
2. Rinse filters by submersion into sterile dPBS, and aspirate away residual dPBS.
3. Remove FN solution from the wells of the plastic packaging and wash by adding 2 ml of sterile dPBS. Aspirate to complete dryness and then add 100  $\mu$ l BSA (1 mg/ml). Place filters into BSA solution and incubate for 1 h at rt.
4. Rinse the filters by submersion into sterile dPBS, and aspirate away residual dPBS.
5. Remove BSA solution from the wells of the plastic packaging and wash by adding 2 ml of sterile dPBS. Aspirate to complete dryness and then add 100  $\mu$ l of either fibulin-1 or BSA (100  $\mu$ g/ml in dPBS, 1 mM CaCl<sub>2</sub>). Place filter inserts into either solution and incubate for 4 h at 37°C.
6. Rinse filters by submersion into sterile dPBS, and aspirate away residual dPBS.
7. Place inserts into wells of 24 well plates containing 0.4 ml of serum-free culture medium, ITS (0.5  $\mu$ l/ml) and either fibulin-1 or BSA (50  $\mu$ g/ml).
8. Detach cells using trypsin-EDTA, centrifuge and resuspend pellet in 10% serum-containing medium to neutralize trypsin. Repeat. Finally, resuspend the cell pellet in serum-free medium containing ITS.
9. Count cell number and dilute to  $4.0 \times 10^5$  cells/ml.
10. Add 250  $\mu$ l of cell suspension to the upper chamber of each filter insert (mix cell suspension between applications) and incubate 22-24 h at 37°C.
11. Remove filter inserts and scrape away cells from upper surface using a cotton swab. Do not apply too much pressure to the filter with the swab; it will break. Repeat swabbing.
12. Fix the cells attached to undersurface with 10% formalin for 10 min and stain with Wright-Giemsa stain for 10 min and then rinsed with dH<sub>2</sub>O.
13. To assure that all cells have been removed from the upper surface, repeat the swabbing.
14. Dry membranes and count cells in five different 400 x optical fields.

Note that triplicate or quadruplicate inserts are required for each experimental treatment.

## USE OF BOEHRINGER MANNHEIM SPIN COLUMNS

- Invert column several times to resuspend gel.
- Remove top then bottom cap and drain column.
- Prespin column at 1100 X g for 2 min.
- Empty the collection tube and repeat centrifugation.
- Add sample 25-50  $\mu$ l
- Spin for 10 min at 1100 X g.
- Save sample in collection tube.
- Discard column in radioactive waste.
- Remove 1  $\mu$ l aliquot from collection tube and determine cpm by scintillation counting.

Compute cpm incorporated per  $\mu$ g by the following equation:

$$\text{cpm}/\mu\text{l} \times \text{total volume}/\text{DNA ng} \times 1000 = \text{cpm}/\mu\text{g} \text{ (should be } 1\text{-}5 \times 10^8 \text{ cpm}/\mu\text{g)}$$

**EXTINCTION COEFFICIENTS** - E 280 nm, 0.1% solution = 1 mg/ ml

To calculate an extinction coefficient for a protein:

$$\text{Extinction Coefficient (280 0.1\%)} = 5690 * (\text{no. of "W"}) + 1280 * (\text{no. of Y}) + 60 * (\text{no. of C})$$

Phenylalanine doesn't absorb much at 280, so it is not in the calculation

<b>Protein</b>	<b><math>E_{280\text{nm}}</math>, 0.1% solution</b>
bovine serum albumin	0.66
fibronectin	1.28
fibulin-1C	0.46
IgG	1.4
plasminogen	1.8
RAP	0.926
urokinase (uPA)	
uPA kringle	1.81
$\alpha_2\text{M}$	0.89
LRP-1/ $\alpha_2$ macroglobulin receptor	1.35
ovalbumin	0.71
fibrinogen	1.51
GST (MW 23.87)	0.59

## FORMULAS

### VOLUME OF A CYLINDER

$$\pi r^2(h)$$

### RECTANGULAR HYPERBOLA

$$Y = A \cdot X / (B + X)$$

This equation is also known as a saturation binding curve or a binding isotherm. Y is zero initially and increases to a plateau value of A (B<sub>max</sub> or V<sub>max</sub>). B is the dissociation constant (K<sub>d</sub> or K<sub>m</sub>). When X=B, Y=0.5\*A. A is expressed in the units of the Y axis; B is in the units of the X axis (usually concentration).

## PROTEINASES

<b>Protease</b>	<b>Type</b>
Chymotrypsin	Serine
Thermolysin	Metallo
Trypsin	Serine
Pronase	Mixture
Papain	Cysteine

## **COMMONLY USED SOLUTIONS**

### **TRIS BUFFERED SALINE (TBS) - 1X**

50 mM Tris pH 7.4, 150 mM NaCl

500 ml:

25 ml 1M Tris pH 7.4

15 ml 5M NaCl

- add deionized H<sub>2</sub>O up to 500 ml

### **TRIS BUFFERED SALINE - 10X STOCK**

0.5 M Tris pH 7.5, 1.5 M NaCl

1000 ml:

60.57 g Tris

87.66 g NaCl

- add H<sub>2</sub>O up to 950 ml and stir into solution.
- add 30-35 ml HCl (conc.) to adjust to pH 7.4
- add H<sub>2</sub>O up to 1 liter.

### **ELISA COATING BUFFER**

500 ml:

1.38 g Sodium carbonate

3.11 g Sodium bicarbonate

- add H<sub>2</sub>O up to 500 ml

(1X stock solution should have a pH of 9.5)

### **PHOSPHATE BUFFERED SALINE (PBS) - 10X STOCK**

1000 ml:

2.0 g Potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>)

2.0 g Potassium chloride (KCl)

80.0 g Sodium chloride (NaCl)

11.44 g Sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>)

- add H<sub>2</sub>O up to 1 liter (1X solution should have a pH of 7.4 without any adjustment).

### OCTYLGLUCOSIDE EXTRACTION BUFFER

Buffer composition: 50 mM octylglucoside, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM PMSF, 150 mM NaCl, 50 mM Tris pH 7.4.

To prepare 100 ml:

- 5 ml 1 M Tris pH 7.4
- 3 ml 5 M NaCl
- 0.1 ml 1 M CaCl<sub>2</sub>
- 0.1 ml 1 M MgCl<sub>2</sub>
- 2 ml 100 mM PMSF (titrate in slowly)
- 1.47 g octylglucoside (Calbiochem F.W. 294.4)
  - Add water up to 100 ml.
  - Store at 4°C.

### SDS-PAGE SAMPLE BUFFER (4X, REDUCING)

Buffer composition: 1 X = 50 mM Tris pH 6.8, 2 % SDS, 0.1% bromophenol blue, 10 % glycerol, 1 % β-mercaptoethanol.

To make 100 ml of 4 X:

- 20 ml 1 M Tris HCl pH 6.8
- 40 ml glycerol
- 200-400 mg bromophenol blue
- 4 ml β-mercaptoethanol

Filter solution through 0.22-0.45 μm filter

- Add 8 g SDS
- Add H<sub>2</sub>O up to 100 ml
- Mix thoroughly, aliquot into 1.5 ml tubes and store at -20°C.

### SDS-PAGE COOMASSIE STAIN

To prepare 1000 ml:

- 0.3 g Coomassie blue R250
- 450 ml methanol

- Mix for 1 hour then add the following:

- 450 ml H<sub>2</sub>O
- 100 ml glacial acetic acid

- Mix thoroughly and filter through Whatman 3M filter paper.

## INHIBITORS OF MICROBIAL GROWTH

Sodium Azide ( $\text{NaN}_3$ ) 0.02%, is widely used to prevent microbial growth.

Thimerisal (ethylmercuricthiosalicylate e.g., Merthiolate) 0.005%, is a very effective anti-microbial agent. It binds to and is inactivated by substances containing thiol groups.

## DAB or OPD DEVELOPMENT BUFFER

500 ml:

5.0 g citric acid monohydrate

7.0 g sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ )

add  $\text{H}_2\text{O}$  up to 500 ml

(solution should have a pH of 5.0 without adjustment)

## DAB WORKING SOLUTION

DAB; 3,3'diaminobenzidine tetrahydrochloride, Litton Bionetics, Kensington, MD

- Resuspend the contents of 1 vial in 20 ml citrate-phosphate buffer.
- Add 20  $\mu\text{l}$  of  $\text{H}_2\text{O}_2$  and mix thoroughly.
- After use discard DAB as hazardous waste.

## **BACTERIAL MEDIA**

### SOB MEDIUM STOCK

tryptone	40g
yeast extract	10g
5M NaCl	4.0ml
1M KCl	5.0ml

- Add dH<sub>2</sub>O up to 2000ml and thoroughly mix.

Distribute into either:        2 x 4000ml erlenmeyers  
   4 x 2000ml erlenmeyers  
   4 x 50ml screw top bottles

- Autoclave in liquid cycle (121°C for 30 min.)

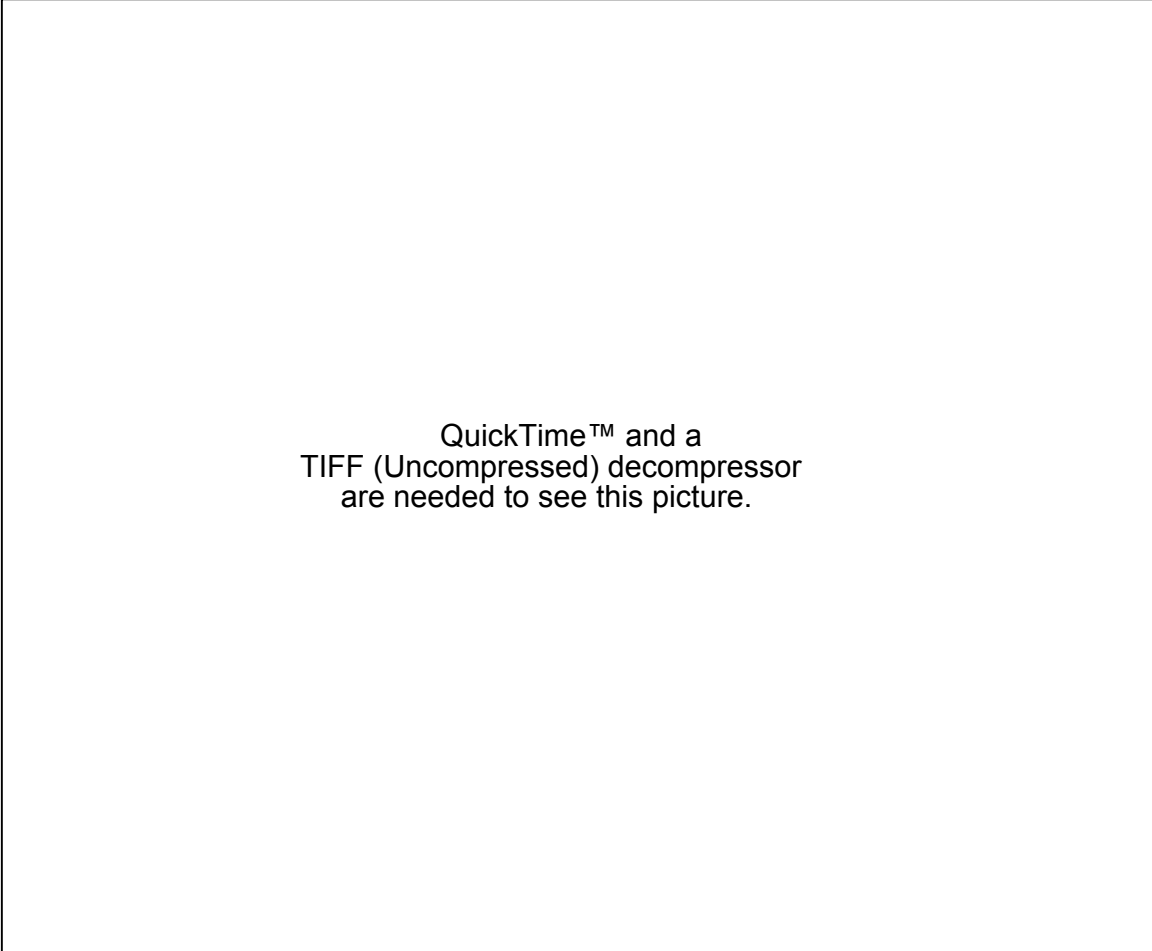
### SOB COMPLETE

- add 1/100th volume of 2M Mg (20mM final conc.)
- filter if necessary

### SOG COMPLETE

- add 1/100th volume of 2M Mg
- add 1/100th volume of 2M glucose - then sterile filter.

Shown below are excitation and emission spectra for 8 different dyes used in FACS immunofluorescence experiments. Spectra are uncorrected for detector sensitivity and are scaled for presentation purposes.



QuickTime™ and a  
TIFF (Uncompressed) decompressor  
are needed to see this picture.

## Table of Excitation and Emission Wavelengths

Fluorochrome	Excitation	Emission
3-Hydroxypyrene 5,8,10-Tri Sulfonic acid	403	513
5-Hydroxy Tryptamine	380-415	520-530
5-Hydroxy Tryptamine (5-HT)	400	530
Acid Fuchsin	540	630
Acridine Orange (bound to DNA)	502	526
Acridine Red	455-600	560-680
Acridine Yellow	470	550
Acriflavin	436	520
AFA (Acriflavin Feulgen SITSA)	355-425	460
Alizarin Complexon	530-560	580
Alizarin Red	530-560	580
Allophycocyanin	650	661
ACMA	430	474
Aminoactinomycin D	555	655
Aminocoumarin	350	445
Anthroyl Stearate	361-381	446
Astrazon Brilliant Red 4G	500	585
Astrazon Orange R	470	540
Astrazon Red 6B	520	595
Astrazon Yellow 7 GLL	450	480
Atabrine	436	490
Auramine	460	550
Aurophosphine	450-490	515
Aurophosphine G	450	580
BAO 9 (Bisaminophenyloxadiazole)	365	395
BCECF	505	530
Berberine Sulphate	430	550
Bisbenzamide	360	600-610
BOBO 1	462	481
Blancophor FFG Solution	390	470
Blancophor SV	370	435
Bodipy FI	503	512
BOPRO 1	462	481
Brilliant Sulphoflavin FF	430	520
Calcien Blue	370	435
Calcium Green	505	532
Calcofluor RW Solution	370	440
Calcofluor White	440	500-520
Calcophor White ABT Solution	380	475
Calcophor White Standard Solution	365	435
Cascade Blue	400	425

Catecholamine	410	470
Chinacrine	450-490	515
Coriphosphine O	460	575
Coumarin-Phalloidin	387	470
CY3.1 8	554	568
CY5.1 8	649	666
CY7	710	805
Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid)	340	525
Dansa (Diamino Naphtyl Sulphonic Acid)	340-380	430
Dansyl NH-CH3 in water	340	578
DAPI	350	470
Diamino Phenyl Oxydiazole (DAO)	280	460
Dimethylamino-5-Sulphonic acid	310-370	520
Diphenyl Brilliant Flavine 7GFF	430	520
Dopamine	340	490-520
Eosin	525	545
Erythrosin ITC	530	558
Ethidium Bromide	510	595
Euchrysin	430	540
FIF (Formaldehyde Induced Fluorescence)	405	435
Flazo Orange	375-530	612
Fluorescein Isothiocyanate (FITC)	490	525
Fluo 3	485	503
Fura-2	340-380	512
Genacryl Brilliant Red B	520	590
Genacryl Brilliant Yellow 10GF	430	485
Genacryl Pink 3G	470	583
Genacryl Yellow 5GF	430	475
Gloxalic Acid	405	460
Granular Blue	355	425
Haematoporphyrin	530-560	580
Hoechst 33258 (bound to DNA)	346	460
Indo-1	350	405-482
Intrawhite Cf Liquid	360	430
Leucophor PAF	370	430
Leucophor SF	380	465
Leucophor WS	395	465
Lissamine Rhodamine B200 (RD200)	575	595
Lucifer Yellow CH	425	528
Lucifer Yellow VS	430	535
Magdala Red	524	600
Maxilon Brilliant Flavin 10 GFF	450	495
Maxilon Brilliant Flavin 8 GFF	460	495
MPS (Methyl Green Pyronine Stilbene)	364	395

Mithramycin	450	570
NBD Amine	450	530
Nile Red	515-530	525-605
Nitrobenzoxadidole	460-470	510-650
Noradrenaline	340	490-520
Nuclear Fast Red	289-530	580
Nuclear Yellow	365	495
Nylosan Brilliant Flavin E8G	460	510
Pararosaniline (Feulgen)	570	625
Phorwite AR Solution	360	430
Phorwite BKL	370	430
Phorwite Rev	380	430
Phorwite RPA	375	430
Phosphine 3R	465	565
Phycoerythrin R	480-565	578
Pontochrome Blue Black	535-553	605
Primuline	410	550
Procion Yellow	470	600
Propidium Iodide	536	617
Pyronine	410	540
Pyronine B	540-590	560-650
Pyrozal Brilliant Flavin 7GF	365	495
Quinacrine Mustard	423	503
Rhodamine 123	511	534
Rhodamine 5 GLD	470	565
Rhodamine 6G	526	555
Rhodamine B	540	625
Rhodamine B 200	523-557	595
Rhodamine B Extra	550	605
Rhodamine BB	540	580
Rhodamine BG	540	572
Rhodamine WT	530	555
Rose Bengal	540	550-600
Serotonin	365	520-540
Sevron Brilliant Red 2B	520	595
Sevron Brilliant Red 4G	500	583
Sevron Brilliant Red B	530	590
Sevron Orange	440	530
Sevron Yellow L	430	490
SITS (Primuline)	395-425	450
SITS (Stilbene Isothiosulphonic acid)	365	460
Stilbene	335	440
Snarf 1	563	639
sulphO Rhodamine B Can C	520	595

Sulpho Rhodamine G Extra	470	570
Tetracycline	390	560
TRITC (Tetramethyl Rhodamine Isothiocyanate)	557	576
Texas Red	596	615
Thiazine Red R	510	580
Thioflavin S	430	550
Thioflavin TCN	350	460
Thioflavin 5	430	550
Thiolyte	370-385	477-484
Thiozol Orange	453	480
Tinopol CBS	390	4.3
TOTO 1	514	533
TOTO 3	642	661
True Blue	365	420-430
Ultralite	656	678
Uranine B	420	520
Uvitex SFC	365	435
Xylene Orange	546	580
XRITC	582	601
YO PRO 1	491	509

## AMINO ACID ABBREVIATIONS AND CODONS

3 Letter Code	1 Letter Code	Full name	mRNA nucleotide triplets (codons)
Ala	A	Alanine	GCA, GCC, GCG, GCU
Arg	R	Arginine	AGA, AGG, CGA, CGC, CGG, CGU
Asn	N	Asparagine	AAC, AAU
Asp	D	Aspartic acid	GAC, GAU
Cys	C	Cysteine	UGC, UGU
Glu	E	Glutamic acid	GAA, GAG
Gln	Q	Glutamine	CAA, CAG
Gly	G	Glycine	GGA, GGC, GGG, GGU
His	H	Histidine	CAC, CAU
Ile	I	Isoleucine	AUA, AUC, AUU
Leu	L	Leucine	UUA, UUG, CUA, CUC, CUG, CUU
Lys	K	Lysine	AAA, AAG
Met	M	Methionine	AUG

Phe	F	Phenylalanine	UUC, UUU
Pro	P	Proline	CCA, CCC, CCG, CCU
Ser	S	Serine	AGC, AGU, UCA, UCC, UCG, UCU
Thr	T	Threonine	ACA, ACC, ACG, ACU
Trp	W	Tryptophan	UGG
Tyr	Y	Tyrosine	UAC, UAU
Val	V	Valine	GUA, GUC, GUG, GUU
STOP			UAA, UAG, UGA

**CODON TABLE II:**

Codon	3 Letter Code	1 Letter Code
AAA	LYS	K
AAC	ASN	N
AAG	LYS	K
AAT	ASN	N
CAA	GLN	Q
CAC	HIS	H
CAG	GLN	Q
CAT	HIS	H
GAA	GLU	E
GAC	ASP	D
GAG	GLU	E
GAT	ASP	D
TAA	STOP	STOP
TAC	TYR	Y
TAG	STOP	STOP
TAT	TYR	Y
ACA	THR	T
ACA	THR	T
ACC	THR	T
ACG	THR	T
ACT	THR	T
CCA	PRO	P
CCC	PRO	P
CCG	PRO	P
CCT	PRO	P
GCA	ALA	A
GCC	ALA	A
GCG	ALA	A
GCT	ALA	A

TCA	SER	S
TCC	SER	S
TCG	SER	S
TCT	SER	S
AGA	ARG	R
AGC	SER	S
AGG	ARG	R
AGT	SER	S
CGA	ARG	R
CGC	ARG	R
CGG	ARG	R
CGT	ARG	R
GGA	GLY	G
GGC	GLY	G
GGG	GLY	G
GGT	GLY	G
TGA	STOP	
TGC	CYS	C
TGG	TRP	W
TGT	CYS	C
ATA	ILE	I
ATC	ILE	L
ATG	MET	M
ATT	ILE	L
CTA	LEU	L
CTC	LEU	L
CTG	LEU	L
CTT	LEU	L
GTA	VAL	V
GTC	VAL	V
GTG	VAL	V
GTT	VAL	V
TTA	LEU	L
TTC	PHE	F
TTG	LEU	L
TTY	PHE	F

## PROCEDURE FOR QUALITY CONTROL OF RECEPTOR PREPARATIONS

For each receptor preparation that you prepare:

1. Label receptor preparations with the following information:
  - a. complete legible description (e.g. human, gp330 or human  $\alpha_2$ MR).
  - b. date of final purification step.
  - c. OD<sub>280</sub>.
  - d. your initials along with page number of gel.
  - e. buffer composition.
2. Run an aliquot of pooled peak fractions on gel and put into notebook on a separate page with date and O.D. information and the total yield per preparation.
3. Perform an ELISA RAP binding assay titrating RAP starting from 150 nM and diluting three fold down the plate.
4. Store preparations in individual box for each receptor at -20°C.
5. Label affinity columns with date of coupling.

### Procedure for Quality Control of Affinity Selected Antibodies:

After affinity selection pool peak fractions and dialyze against TBS, pH 8.0

1. Measure OD<sub>280</sub> and aliquot into 1.5 ml tubes.
2. Label each tube with the following information:
  - a. complete description ( $\alpha_2$ MR selected, rb 777)
  - b. date.
  - c. notebook page number.
  - d. OD<sub>280</sub> value.
  - e. buffer composition (eg. TBS)
3. Perform an ELISA on each preparation coating plates with ligand and BSA as neg. control.
  - a. determine titer.
4. Store aliquots in an individual box at -20°C.
  - Label ligand-Sepharose columns with date of coupling.

**SDS PAGE GEL SAMPLE LOADING FORMS**

Date \_\_\_\_\_  
Lane No.      Sample Description  
1. \_\_\_\_\_  
2. \_\_\_\_\_  
3. \_\_\_\_\_  
4. \_\_\_\_\_  
5. \_\_\_\_\_  
6. \_\_\_\_\_  
7. \_\_\_\_\_  
8. \_\_\_\_\_  
9. \_\_\_\_\_  
10. \_\_\_\_\_  
11. \_\_\_\_\_  
12. \_\_\_\_\_  
13. \_\_\_\_\_  
14. \_\_\_\_\_  
15. \_\_\_\_\_  
Gel Type \_\_\_\_\_  
Reducing \_\_\_\_\_ Non-Reducing \_\_\_\_\_

Date \_\_\_\_\_  
Lane No.      Sample Description  
1. \_\_\_\_\_  
2. \_\_\_\_\_  
3. \_\_\_\_\_  
4. \_\_\_\_\_  
5. \_\_\_\_\_  
6. \_\_\_\_\_  
7. \_\_\_\_\_  
8. \_\_\_\_\_  
9. \_\_\_\_\_  
10. \_\_\_\_\_  
11. \_\_\_\_\_  
12. \_\_\_\_\_  
13. \_\_\_\_\_  
14. \_\_\_\_\_  
15. \_\_\_\_\_  
Gel Type \_\_\_\_\_  
Reducing \_\_\_\_\_ Non-Reducing \_\_\_\_\_

Date \_\_\_\_\_  
Lane No.      Sample Description  
1. \_\_\_\_\_  
2. \_\_\_\_\_  
3. \_\_\_\_\_  
4. \_\_\_\_\_  
5. \_\_\_\_\_  
6. \_\_\_\_\_  
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