PREFACE

This book contains a compendium of protocols currently in use in our laboratory, and is an update of our previous methods book. Many of these methods did not originate in this laboratory, but were obtained from other groups and are presented with little or no modification. We hope that by collecting them in one volume, we will be providing a useful service to other Drosophila groups. We would be grateful for any corrections or suggested modifications to the protocols. Please feel free to copy and distribute this manual as you wish.

The Rubin Lab
November 1990
Berkeley, California

This book was edited by Lynn Rubin, and was composed on a MacII running Microsoft Word 4, and an Apple Laserwriter. The typeface is Palatino. The hot fudge sundaes were delicious.

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Todd Laverty

QUARANTINE STOCKS

The following is a general outline of steps which should be followed when new stocks are brought into the lab. It is important that stocks go through the entire quarantine procedure to prevent a mite infestation. To be safe, one should order stocks from Bloomington or Bowling Green Stock Centers whenever possible. Stocks should never be ordered from a lab known to have mites. If you don't know if a particular lab has mites, ask someone before you order it.

When handling quarantine stocks, it is a good idea to treat them as if there were mites present. Therefore, all stocks should be kept in vials with tight cotton plugs, not bottles. New vials should be set up shortly after the adults eclose, and most importantly, quarantine stocks must be kept isolated from all other stocks.

1) New stocks must be brought directly to an isolated spot. Do not bring them near any lab stock.

2) At a quarantine microscope, transfer the adults to a fresh vial (P_0) and keep the original vial for a few days.

3) When it is obvious that the P_0 vial is going fine, discard the adults and the original vial.

4) Shortly after the adults eclose from the P_0 vial, transfer them to a fresh vial (F_1).

5) Keep the stock isolated for two more generations (F_1 and F_2).

6) After you have set up the F_2 vial, check the P_0 vial for mites.

7) When the adults eclose from the F_2 vial, put them in a fresh vial and check the P_0 and F_1 vial for mites.

8) If no mites are found, the stock may be taken out of quarantine.

9) If mites are found at any time or suspected of being in a stock, transfer the adults from the suspect vial into a vial containing a Tedion strip (strips are made by soaking filter paper in a 0.5% (w / v) solution of Tedion in acetone and then allowed to air dry).

10) Autoclave the old vial.

11) Every two days, transfer the adults to a new vial containing Tedion and autoclave the old vial. Continue this for one week.

12) Place adults in a new vial containing Tedion and follow steps 3-7 as outlined above. The only exception is that you allow two additional generations (F_3 and F_4) in isolation.

The Curator of Drosophila Stocks
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Jordan Hall A503
Indiana University
Bloomington, IN 47405
(812) 855-5783
(812) 855-2577 (FAX)
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Mid-America Drosophila Stock Center
Dept. of Biological Sciences
Bowling Green State University
Bowling Green, OH 43403
(419) 372-2631

Todd Laverty

FLY FOOD RECIPE

1) Prepare the following mixture:

water	14,000 ml
molasses, unsulfured	1,000 ml
agar	148 g
cornmeal	1,000 ml
yeast, baker's	412 g

Note: If the cornmeal and yeast are mixed together dry, you will have fewer problems dissolving the yeast in the water.

2) Boil for 10 minutes.

3) Turn off the heat and slowly add:

tegosept (10% (w / v) methyl p-hydrobenzoate in 95% ethanol)	225 ml
propionic acid	80 ml

4) Mix well and begin pumping.

5) This recipe yields approximately 10,000 ml of food, enough for 1,000 vials or 200 bottles.

References: Wirtz, R. A. and H. G. Semey, DIS 58: 176-180.

Todd Laverty

POPULATION CAGES

Starting a population cage:

A new cage should be started from 80 synchronous bottles. In a few days the flies should become acclimated to the cage and start laying a substantial number of eggs.

Every day the flies in the cage should be fed two to four collecting plates covered with a thin layer of yeast paste. An empty weigh boat with a couple of moist paper towels in it will help keep the cage humid, and should also be changed daily.

Setting up fly boxes:

Fly boxes are made from plastic food storage boxes (9" x 6") with a nitex covered opening (4" x 2") cut in the top. A monolayer of absorbent cotton is placed on the bottom and the following solution is poured evenly over it:

	1 box
water	200 ml
propionic acid	0.13 ml
phosphoric acid	0.75 ml
live yeast	54g
sucrose	27g

Eggs for the fly box are collected in the following manner:

1) Collect eggs on freshly yeasted plates overnight.
2) Under gently running water brush the eggs off the plate into a nitex sieve.
3) Wash the eggs thoroughly with water to remove any excess yeast.
4) Fold a filter paper circle (9 cm) into a cone and place it in a funnel. Using 70% ethanol, rinse the eggs off the nitex into the filter paper cone.
5) Place a filter paper circle (9 cm) on some paper towels. Transfer some eggs onto the circle. Then using a small brush and 70% ethanol spread the eggs uniformly over the circle in a monolayer.
6) Repeat step 5 until all the eggs collected are on the filter paper circles.

7) Allow the filter paper circles to briefly air dry then place on the moist cotton, two circles per box. Cover the box securely.
8) Incubate the boxes at 25°C. Flies should eclose in about 10 days.

Once the flies eclose they will only live for a day or two in the fly box. The box can be easily emptied in the following manner:

1) Hold the box upside down and place the CO_2 outlet next to, but not through, the nitex until all the flies stop moving.
2) With the box still upside down, remove the cover and dump the flies onto an open manila folder.
3) Quickly dump the flies off the folder into an empty fly bottle sitting on ice.

Maintaining the population cage:

To maintain the population cage, three fly boxes are set up to start a new cage. If possible the boxes should be set up from one overnight collection. If this is not possible, some effort should be made to synchronize all three boxes so that they all eclose within 24 hours of each other.

The following is a typical schedule for a fly cage:

1) On Monday start the cage with fresh flies. Between 50-100 ml of flies is desirable for normal uses.
2) On Friday set up three fly boxes.
3) On the following Friday the cage is cleaned of old flies, washed and dried.
4) On Monday add the new flies from the fly boxes to the cleaned cage and begin the cycle again.

This method is almost impervious to mite problems since fresh flies are used every two weeks. It is important to thoroughly clean the cage and fly boxes after each use with hot soapy water to prevent any contaminates from being passed on.

Collecting eggs for DNA or RNA extracts:

1) Eggs are collected and washed into a nitex sieve as described above.
2) Place the sieve on a flat surface and fill with 50% bleach. Let stand for 2 minutes to dechorionate the eggs.
3) Rinse the eggs with 2 liters of 0.7% (w / v) NaCl in water.
4) Place the eggs in an eppendorf tube and freeze in liquid nitrogen.
5) Store at -80°C.

Egg collecting plates:

1) Prepare the following mixture:

water	2500 ml
molasses	360 ml
agar	88g

2) Autoclave for one hour.
3) Slowly add:

tegosept (10% (w / v) methyl p-hydrobenzoate in 95% ethanol)	0.5 ml
ethyl acetate	20 ml

4) Mix well and pour into plastic weigh boats (5" x 5").
5) Let cool and store at 4°C.
6) This recipe yields approximately 18 plates.

SOME MODIFIED DROSOPHILA TRANSFORMATION VECTORS

Modified Carnegie Vectors	D. Mismer
Modified Neomycin Selection Vector	D. Mismer
Vectors Permitting Promotion Fusions with Chloramphenicol Acetyltransferase Transcriptional Module	D. Mismer
sevenless Expression Casettes	D. Bowtell
pCAWC Vector	D. Bowtell
HSS Derivatives	Kevin Jones

Kevin Moses

G_{418} SELECTION

G_{418} selection

I have found that a concentration of 1 mg / ml G_{418} cleanly selects between one dose of an insertion of pHSNEO and wild-type. However there is some batch variation in the effective percentage of the drug. Therefore I recommend a pilot run with a known transformed stock and wild-type, at perhaps 0, 0.5, 1.0, 1.5 and 2.0 mg / ml G_{418}. I also recommend running such controls in each experimental run.

1) G_{418} is made up as 100 mg / ml stock solution and stored at -20°C in 5 ml aliquots.

2) Yeast-glucose Drosophila medium is made by boiling the following for 5 minutes:

10% weight / volume dry yeast
10% weight / volume glucose (sucrose works also)
1.6% weight / volume agar
3 % volume / volume nipagen (≈tegosept, 10% w / v in ethanol)

3) Bring the food to ~ 50°C in a water bath.

4) Add the G_{418} with a dash of bromophenol blue (in water) and mix with a flea (the blue dye both demonstrates that the mixing is complete and marks the food as it will be poisonous to most flies).

5) Pour tubes or bottles in the normal way and store the food at 4°C.

6) Before use bring to room temperature and add a minimum quantity of wet yeast (so that the adults will completely consume it in two days.

7) Allow the adults two days on the food and then sub on.

8) If using heat-shock inducible vectors, raise the cultures once to 37°C for 30 minutes on the next day. This should suffice.

Don Rio

DROSOPHILA TISSUE CULTURE

Maintenance

We mainly use the Schneider line 2 cell line and the Kc cell line. Both are diploid and originally derived from embryos. Schneider L-2 grow in M3 medium (see recipe) supplemented with 10% heat inactivated (56° C for 30 min) fetal calf serum and antibiotics (penicillin and streptomycin). Kc cells grow in D-22 medium with antibiotics (penicillin and streptomycin) but don't require serum. Both lines grow at 25°C without CO_2 and are maintained in 25 cm^2 T-flasks (Corning). Routinely cells are grown at 1-5x10^6 cells / ml and are split into fresh medium at a 1 to 4 or 5 dilution every 3 days. The cells don't attach well to a solid substrate and so are resuspended by pipeting for subculturing. No trypsin is required.

Freezing and thawing

1) Grow cells to 3-5x10^6 cells / ml (log phase) in a 30-50 ml of medium in a 150 cm^2 T-flask.
2) Resuspend cells by pipeting (sterile) and transfer to 50 ml sterile polypropylene tube. Spin in IEC table top centrifuge (at setting 5) for 1-2 min.
3) Remove medium by aspiration and resuspend in 1.5 ml freezing medium. Use either M3 (for L-2) or D-22 (for Kc) and 10% DMSO (Schwartz-Mann, spectropure) and 20% H.I. FCS.
4) Aliquot 0.5 ml cells into 2 ml nunc vials or provials. Label and transfer to freezer box with foam inside (to allow slow cooling).
5) Transfer to -70°C freezer overnight (or longer).
6) Transfer to liquid nitrogen tank for permanent storage.

To thaw:

1) Remove vial from liquid nitrogen and warm in water bath at 25°C (or room temperature).
2) As soon as the contents are thawed, transfer to 25 cm^2 T-flask with 5-10 ml medium.
3) Allow cells to attach (3 or more hrs) and replace medium with a fresh aliquot.
4) Incubate at 25°C for 3-5 days.
5) The cells may take a while to fully recover.

DROSOPHILA TISSUE CULTURE CELL TRANSFECTION

A. Solutions

1. 0.25 M CaCl2 : **for 100 ml:**
   3.68g $CaCl_2$ 2 H_2O
   ddH_2O to 100 ml

   Sterile filter, aliquot into 15 ml polypropylene tubes. Store at -20°C.

2. 2XHEBS

	for 1 L:
NaCl	16g
KCl	0.7g
Na_2HPO_4	0.4g
dextrose	2g
Hepes (free acid)	10g

   pH to 7.1 with NaOH
   ddH_2O to 1 L
   Sterile filter, aliquot in 50 ml tubes
   Store at -20°C.

   NOTE: When thawing for a transfection, re-pH to 7.1 and re-sterile filter before use.

B. Procedure

1) Seed 5 ml medium in 60 mm dish (Falcon) with 0.2 - 0.3 ml of cell culture (5-8x10^6 cells / ml; e.g. 2-3 day old culture).
2) Incubate at 25°C at least 6 h or overnight before transfection.
3) Mix 10 µg plasmid DNA with 0.4 ml 0.25 M $CaCl_2$ and add to 0.4 ml 2X HEBES pH 7.1 dropwise with swirling in 17X100 mm polycarbonate tubes (Falcon).
4) Incubate at room temperature for 20-30', the solution should become lightly cloudy.
5) Add 0.8 ml ppt / 60 mm dish, swirl and incubate at 25°C.
6) For selection: (e.g. for transformed cell lines) after 24 h: split cells 1 Æ 4 into new medium. After another 24 h add selective drug. G-418: (for pcopneo and derivatives) at 1 mg / ml; Hygromycin (pcophyg and derivatives) at 200 µg / ml.
7) Split cells every 7-10 days into selective medium.
8) Grow cell lines as mixed cultures in selective medium.

ECHALIER'S D-22 MEDIUM FOR Kc CELLS (HIGH PO_4) - 10L - 2X.D22

1) Dissolve in less than 500 ml

L-glutamic acid	79.4g
glycine	40.4g

Adjust to pH 7.0 with KOH pellets (about 37.5 gm; not much more, if any!)

2) Dissolve in less than 500 ml

L-glutamic acid	138.18g
glycine	70.32g

Adjust to pH 7.0 with NaOH pellets.

3) Dissolve in 250 ml or less

$MgCl_2$ 6 H_2O	20g
$MgSO_4$ (anhydrous)	36g

4) Dissolve in ~150 ml

Na H_2 PO_4 H_2O	8.32g

5) Dissolve in less than 500 ml

L-malic acid	13.4g
succinic acid	1.2g
sodium acetate 3 H_2O	0.5g
glucose	40g
phenol red (sodium salt)	0.2g

6) Dissolve in 1 L

$CaCl_2$	17.8g

7) Vitamin de Grace - 500X 40 ml

8) Dissolve in 1 L

yeast extract	30g

9) Dissolve in 4 L
Heat to dissolve. Cool and add slowly to media.

Lactalbumin hydrolysate	300g

Mix in this order:

1) Add $CaCl_2$ (6) to $MgCl_2$ and $MgSO_4$ (3) slowly with stirring on stirrer.

2) Add K-glutamic / glycine (1)
Na-glutamic / glycine (2) and Na H_2 PO_4 H_2O (4)
malic acid / succinic acid / glucose / NaOAC (5)
yeast extract (8) and lactalbumin hydrolysate (9)
500X graces vitamins (7) then add PEN / STREP

for 10 L 2 X - add 1.0g streptomycin sulfate
0.63 g penicillin G 1585u / mg

3) Adjust to pH 6.7 with KOH
4) Adjust volume to 10 L
5) Filter sterilize and dispense into sterile 1L bottles.
6) Store at 4°C.

500X GRACE'S VITAMINS FOR ECHALIER'S D22 MEDIUM

Vitamin	mg/L
thiamine hydrochloride riboflavin	10
calcium pantothenate	10
pyridoxine hydrochloride	10
p-aminobenzoic acid	10
folic acid	10
niacin	10
isoinositol (myo-inositol)	10
biotin	5
cholin chloride	100

M3 MEDIUM - FOR SCHNEIDER LINE-2 CELLS: 10L (1X)

	g		*g*
a-alanine	15	oxaloacetic acid	2.5
b-alanine	2.5	phenylalanine	2.5
arginine-HCl	6..04	proline	4
asparagine	3	serine	3.5
aspartic acid	3	threonine	5
cysteine-HCl	2	tyrosine	2.5
glutamine	6	tryptophan	1
K glutamate	71.5	valine	4
Na glutamate	65.3	glucose	100
glycine	5	bactopeptone	25
histidine	5.5	yeastolate	20
isoleucine	2.5	$CaCl_2$	7.6
leucine	4	$MgSO_4$	21.5
lysine-HCl	8.5	Na H_2PO_4 H_2O	7.8
methionine	2.5	BIS-Tris	10.5

Dissolve in 9 L dd H_2O

Add 1 mg choline and 10 mg $KHCO_3$

Adjust pH to 6.8 with NaOH, adjust volume to 10 L

Filter and store at 4°C

For complete medium add heat inactivated fetal calf serum and pen/strep.

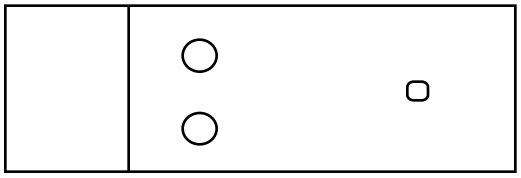
To make radiolabeling medium (for ^{35}S-met): omit methionine, yeastolate and bactopeptone.

Todd Laverty

CYTOLOGICAL EXAMINATION OF POLYTENE CHROMOSOMES

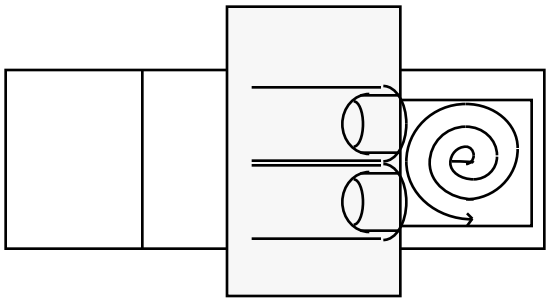
This method of preparing polytene chromosomes is slightly different from traditional methods. In the past chromosomes were spread by applying pressure to the slide with one's thumb or with a vise. Described below is a modified technique of Johng K. Lim's of the University of Wisconsin-Eau Claire, in which the chromosomes are gently spread by a wave of stain flowing under the coverslip.

1) For the best results larvae should be grown in well yeasted media at 18°C and care should be taken not to overcrowd the bottles.
2) On a clean siliconized slide place two drops of 45% acetic acid and a small drop (2-3 mm) of lacto-aceto orcein stain.

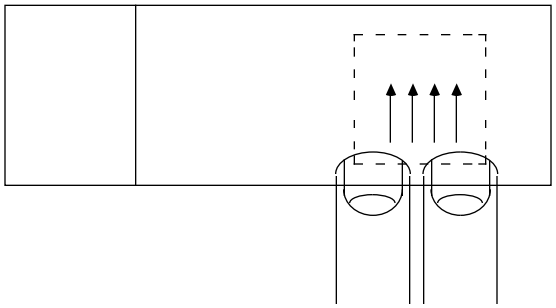


3) In the first drop of 45% acetic acid dissect out the salivary glands from a third instar larva.
4) Transfer the glands to the second drop of 45% acetic acid and cut off the anterior ends of the glands. Quite often this will allow the glands to separate from the membrane and the attached fat body. If this does not occur, carefully dissect away the fat body.
5) Transfer the "fat free" glands into the staining solution and incubate for 2-3 minutes.
6) Place a clean siliconized coverslip (18 mm square) over the glands in the stain.
7) Place the slide on several sheets of paper towel. With another sheet of paper towel hold the edge of the coverslip to prevent it from moving.

8) Using a stiff, pointed probe, gently press once over the glands. Then gently tap the coverslip, starting over the glands, working your way toward the edge in a spiral pattern.

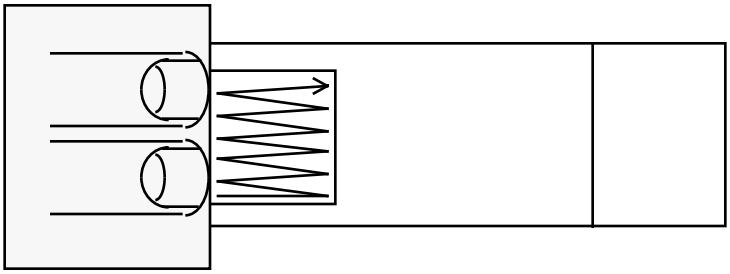


9) Turn the slide over so that the coverslip is between the paper towel and the slide. Press gently on one edge of the slide so that the stain flows to the opposite edge.



10) Turn the slide back over and again hold the edge of the coverslip with a paper towel.

11) Using the probe, gently streak the coverslip with a rapid back and forth motion at one edge and then gradually move to the opposite edge.



12) Blot off excess stain and examine under the microscope using phase contrast. If spreading is not sufficient, gently restreak the coverslip.

13) To make the slide permanent, keep the slide overnight at room temperature.
14) Freeze the slide in an ethanol-dry ice bath for 60 minutes)
15) Pop off the coverslip and immediately place the slide in 95% ethanol (room temperature) for 2-3 minutes.
16) Place the slide in 100% ethanol (room temperature) for 10 minutes.
17) Place a drop of mounting media over the spread and cover with a 22 mm square coverslip.

Lacto-aceto orcein stain:

Incubate a 2% (w / v) solution of "Gurr's orcein natural" in concentrated lactic acid at room temperature for several hours, then filter. Heat, but do not boil, a 2% (w / v) solution of "Gurr's orcein natural" in concentrated glacial acetic acid for several hours, then filter. Combine the filtered solutions with water in a 1:1:1 ratio.

Gurr's Orcein Natural
Cat. no. 22100
Bio / Medical Specialties
Box 1687
Santa Monica, CA 90406
(213) 829-2373

Todd Laverty

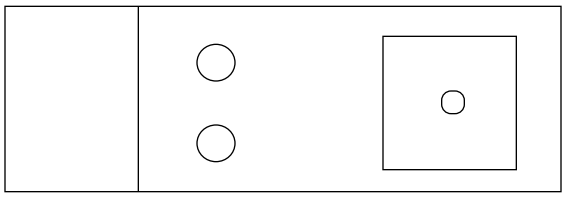
CHROMOSOME *IN SITU* HYBRIDIZATION USING BIOTIN LABELED PROBES

Preparation of Chromosome Squashes:

This method of preparing polytene chromosomes for use with *in situ* hybridization is very similar to the protocol described in the previous chapter. It is an easily learned technique and with a little practice will yield consistent results.

1) For the best results larvae should be grown in well yeasted media at 18°C and care should be taken not to overcrowd the bottles.

2) On a clean siliconized slide place two drops of 45% acetic acid and a clean siliconized 18 mm square coverslip. On the coverslip place a small drop (2-3 mm) of fixative(1/2/3 mixture of lactic acid / water / glacial acetic acid).



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3) In the first drop of 45% acetic acid dissect out the salivary glands from a third instar larva.

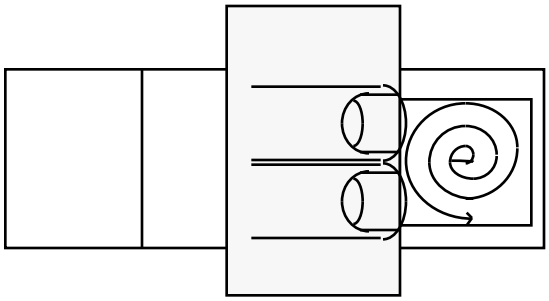
4) Transfer the glands to the second drop of 45% acetic acid and cut off the anterior ends of the glands. Quite often this will allow the glands to separate from the membrane and the attached fat body. If this does not occur, carefully dissect away the fat body.

5) Transfer the "fat free" glands into the fixative and incubate for 4-5 minutes.

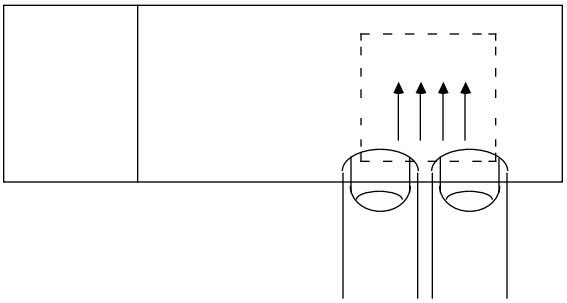
6) Pick up the coverslip and glands with a clean slide by carefully touching the slide to the drop of fixative.

7) Place the slide, coverslip up, on several sheets of paper towel. With another sheet of paper towel hold the edge of the coverslip to prevent it from moving.

8) Using a stiff, pointed probe, gently press once over the glands. Then gently tap the coverslip, starting over the glands, working your way toward the edge in a spiral pattern.

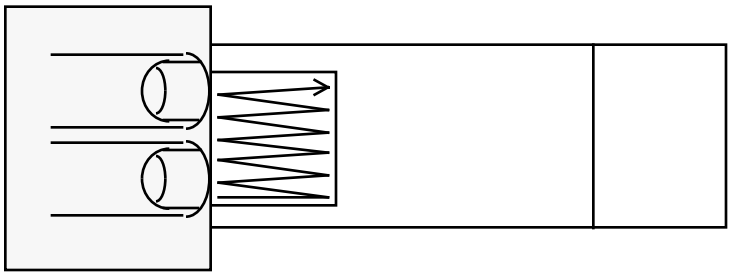


9) Turn the slide over so that the coverslip is between the paper towel and the slide. Press gently on one edge of the slide so that the fixative flows to the opposite edge.



10) Turn the slide back over and again hold the edge of the coverslip with a paper towel.

11) Using the probe, gently streak the coverslip with a rapid back and forth motion at one edge and then gradually move to the opposite edge.



12) Blot off excess fixative and examine under the microscope using phase contrast. If spreading is not sufficient, gently restreak the coverslip. On a good slide the chromosomes should appear low contrast, cytoplasmic debris should be vague, and no fixative should be flowing between the slide and the coverslip.

13) Incubate the slide over night at a temperature between 4° and 18°C. Leaving the slides at room temperature for a couple of hours before incubating them will help when incubating at or near 4°C.

14) Place the slides in a dry ice / ethanol bath for 60 minutes. During or before this time cool down some 100% ethanol in a staining dish to -60 to -80°C.

15) Remove the slides individually from the dry ice / ethanol bath and quickly flip off the coverslip with a razor blade and place the slide immediately into the chilled ethanol.

16) Allow the ethanol to warm up to room temperature, then air dry the slides. Chromosome squashes can be stored at this stage for several months before hybridization.

Pretreatment of Chromosomes for Hybridization:

The chromosomes used for hybridization must be non-refractile after ethanol dehydration (A [16]). The chromosomes on the slide should look the same at this point as they did when the slide was made or it is not worth using. In general one slide is enough for a single probe if the chromosomes have good morphology. After the pretreatment the slides should be hybridized within 24 hours.

1) Place the slides in 2X SSC at 65°C for 30 minutes.

2) Wash for 2 minutes in 2X SSC at room temperature.

3) Acetylate the slides as follows: Prepare 500 ml of 0.1 M triethanolamine-HCl (1M stock pH 8.0) in a staining dish containing a magnetic stirring bar. Rapidly agitate the solution and add 0.625 ml of acetic anhydride. Turn the stirrer off and immediately place the slides into the solution. Incubate for 10 minutes. (Some people claim this step is unnecessary, but it has not been tested in this lab.)

4) Wash the slides in 2X SSC two times for 5 minutes each.

5) Ethanol dehydrate two times 5 minutes in 70% ethanol and 5 minutes in 95% ethanol. Air dry.

6) Denature the chromosomes by incubation in freshly prepared 0.07 N NaOH for 3 minutes.

7) Repeat steps 4 and 5.

Biotinylation of DNA:

The following protocol is for 0.5 µg of DNA or less. The quality of the DNA is not important. Quick prep DNA and DNA fragments isolated by gel purification work equally well as CsCl purified DNA. Probes can be stored at -20°C for several months.

1) Prepare a 1:400 dilution of a 1 mg/ml stock of DNAse I (Worthington, DPFF grade) in 10 mM Tris, pH 7.5/10 mM $MgCl_2$ (TM10). The DNAse I stock should be prepared in TM10 and should be stored in 20µl aliquots at -20°C.

2) Mix:

10X nick-translation buffer	2.5 µl
dATP, dGTP, dCTP mix (0.3 mM each)	2.5 µl
Bio-16-dUTP	2.0 µl
^{32}P-dATP (1mCi/ml)	1.0 µl
DNA	~ 0.5 µg
DNAse I (1:400)	1.5 µl
distilled water to	25 µl

add 10 U of DNA polymerase I and incubate at room temperature for 60 minutes.

3) Stop the reaction by adding 25 µl of 50 mM EDTA, pH 8.0.

4) Remove unincorporated nucleotides by separation over a P 10 spin column.

5) Check the percentage of incorporation by comparing the signals of the column and the flow through on a Geiger counter. 5 - 10% incorporation is sufficient. Probes that incorporated more than 40 - 50% might not do so well in the hybridization, since oversubstitution of the Bio-16-dUTP renders the forming hybrids less stable.

6) Add 20 µg of carrier DNA (Herring Sperm or equivalent) and 5 µl of 3 M sodium acetate. Precipitate the DNA by adding 2.5 volumes of ethanol and incubate in dry ice/ethanol for 20 minutes or at -20°C overnight.

7) Recover the DNA by centrifugation and dry under vacuum for 2 minutes.

8) Resuspend the pellet in 75 µl of hybridization buffer.

Hybridization and Washes:

1) Denature the hybridization probe by boiling for 3 minutes and chill on ice.

2) Apply 5 µl of hybridization solution on the slides and distribute the solution over the chromosomes with a 18 x 18 mm coverslip. Try to avoid trapping air bubbles.

3) To prevent evaporation of the probe seal the edges of the coverslip with rubber cement.

4) Incubate in a moist chamber at 58°C for 12-18 hours.

5) Place the slide in a beaker containing 2X SSC and pry off the coverslip and rubber cement with a scalpel blade.

6) Wash the slides 3 times 15 minutes in 2X SSC at 53°C. Proceed directly with the signal detection. It is important not to let the slide dry out during the signal detection procedure.

Signal Detection:

The age or storage conditions of the Detek I-hrp kit can have a dramatic effect on the kit's proficiency to detect signals. Kits that are over six months old or that have not been kept cold (2-8°C) can give weak or inconsistent signals. On the other hand, under ideal conditions probes can be diluted 10-fold and still yield strong signals.

1) Wash the slides in 1X PBS 2 times for 5 minutes each at room temperature.

2) Wash for 2 minutes in 1X PBS / 0.1 % Triton-X-100.

3) Rinse in 1X PBS.

4) Place the slides horizontally on two 5ml pipettes in a tray lined with moist paper towels. Apply 100µl of a 1:250 dilution of the Streptavidin-biotinylated peroxidase complex in 1X dilution buffer (all the components are supplied in the Detek I-hrp kit by ENZO Diagnostics, Inc.) Distribute the solution by placing a 22 x 40 mm coverslip on the slide.

5) Cover the tray with plastic wrap and incubate at 37°C for 30 minutes.

6) Float the coverslip off in a beaker of 1X PBS and repeat steps 1 through 3.

7) Prepare a solution containing 0.5 mg / ml of diaminobenzidine in 1X PBS. Add 1 / 100 volume of 1% hydrogen peroxide (prepared from a 30 % stock in distilled water). The staining solution should always be prepared fresh. **Diaminobenzidine is a carcinogen. Wear gloves when handling the solution.** Diaminobenzidine can be inactivated by bleach which should be used liberally when disposing of unused diaminobenzidine and contaminated materials.

8) Place the slides horizontally in a tray and add 50 µl of staining solution onto each slide, cover with a 22 x 40 mm coverslip and incubate at room temperature for 1 - 10 minutes. The staining reaction can be observed by blotting off excess solution and examining the slide under the microscope using a low magnification phase contrast lens (e.g. 16x).

9) Stop the reaction by dipping the slides in distilled water three times and then place them in distilled water until staining.

10) Prepare a 4% dilution of Giemsa stain in 10 mM sodium phosphate buffer, pH 6.8. Stain the slides for 30 seconds, rinse in water and allow the slides to air dry. It is important not to overstain the chromosomes for this may obscure the diaminobenzidine precipitate.

11) Place a drop of mounting media over the chromosomes and cover with a 22 mm square coverslip.

12) Examine the chromosomes using phase-contrast optics. Hybridization signals appear as black bands. Weaker signals are brown.

Solutions:

1) 10X PBS
   1.3 M NaCl
   0.07 M Na_2HPO_4
   0.03 M NaH_2PO_4

2) 10X Nick-Translation Buffer
   0.5 M Tris-HCl, pH 7.5
   0.1 M $MgSO_4$
   1 mM dithiothreitol (DTT)
   500 µg / ml bovine serum albumin (BSA Pentax Fraction V)

3) Hybridization Buffer
   0.6 M NaCl
   50 mM Sodium phosphate buffer, pH 6.8
   1X Denhardt's (0.02% BSA / 0.02% Ficoll / 0.02% polyvinylpyrrolidone)
   5 mM $MgCl_2$

Reagents and Suppliers:

1) Bio-16-dUTP
   Cat. No. 42811
   ENZO Diagnostics, Inc.
   325 Hudson St.
   New York, NY 10013
   1-800-221-7705

2) Detection Kit
   Detek I-hrp
   Cat. No. 43820
   contains: Streptavidin-biotinylated peroxidase complex / 10X dilution buffer
   ENZO Diagnostics, Inc.
   325 Hudson St.
   New York, NY 10013
   1-800-221-7705

3) Diaminobenzidine
   3',3' diaminobenzidine tetrahydrochloride
   Cat. No. D 9015
   Sigma Chemical Company
   P.O. Box 14805
   St. Louis, MO 63187
   1-800-352-3010

4) DNAse I (Grade DPFF)
   Cat. No. LS 6330
   Worthington Biochemicals
   Halls Mill Road
   Freehold, NJ 07728
   1-800-445-9603

Matthew Freeman

IN SITU HYBRIDIZATION TO EMBRYO SECTIONS USING ^{35}S RIBOPROBES

Introduction

The determination of the tissue distribution of a transcript of interest has proved to be of great use. Originally the probes were made with tritium. Although this gave very good localisation, the exposure times were rather long (a month was not uncommon). Several protocols were then published using ^{35}S as the labelling isotope, and the exposure times necessary have been cut 5 or 10-fold.

Recently, it has become possible to do non-radioactive *in situs* (described elsewhere in this manual). These have several major advantages, the main one being the ability to localise the expression with single-cell resolution. However, there are cases where radioactive *in situs* might still be the method of choice: they may be a bit more sensitive, and tissues deep within the embryo seem a bit less accessible to digoxygenin-labelled probes than to ^{35}S labelled probes. I would try a non-radioactive whole-mount in situ first, and if that was unsuccessful, resort to the old-fashioned way. In time, as the non-radioactive protocol is refined, this one may become obsolescent.

This protocol is very largely that of Phil Ingham.

Fixing the embryos:

1) Use as many embryos as it is practical to collect. Less than about 500-1000 is probably much harder to work with than a gram or two.

2) Wash embryos from collection plate into a sieve with a fine enough mesh to retain the embryos. Rinse them with water to remove excess yeast.

3) Dechorionate in 50% household bleach for about 2 minutes.

4) Rinse the dechorionated embryos thoroughly with water containing 0.1% Triton X-100.

5) Transfer the embryos to a tube with a tightly fitting lid that contains a 1:1 mixture of 4% paraformaldehyde in PBS[1], and heptane. For about a gram of embryos I usually use a 50ml tube, with 20ml each of formaldehyde and heptane. For fewer embryos, scale down the sizes appropriately.

6) Shake the tube vigorously for 15 minutes. Then remove as much as possible of the aqueous (bottom) layer with a pipette, being careful not to remove the bulk of the embryos that will be at the interface.

7) Add a volume of methanol equal to the heptane layer. Shake the tube very hard for about 30 seconds, and then allow the two phases to separate. The devitellinised embryos will sink to the bottom of the tube, and the rest will remain at the interface

[1]This must be made fresh. Heat it until near boiling, and then add a drop or two of 1M NaOH. It should go into solution within a few minutes.

with the empty vitelline membranes. The yield at this stage is variable - anything over 50-60% is fine; if there are fewer than that, try shaking harder and longer.

8) Remove as much liquid as possible and all the interface, leaving the embryos at the bottom of the tube.

9) The rest of the treatments are carried out in the same tube as the embryos are now in; each liquid is added to the tube, the embryos allowed to settle, and the liquid removed with an aspirator.

10) Rinse the embryos in methanol for 2 minutes.

11) Rehydrate them as follows:

2 minutes in 9:1 methanol:4%paraformaldehyde
2 minutes in 7:3 " "
2 minutes in 5:5 " "
2 minutes in 3:7 " "

12) Post-fix the embryos in 4% paraformaldehyde for 20 minutes.

13) Wash once in PBS.

14) Dehydrate in an ethanol series for 5 minutes each: 30%, 50%, 70%.

15) The embryos can be stored in 70% ethanol at -20° for several weeks.

Embedding the embryos:

1) Fully dehydrate the embryos as follows:

90% ethanol, 5 minutes, twice
95% ethanol, 5 minutes, twice
100% ethanol, 3 minutes, four times

2) Transfer the embryos to a glass test tube, and incubate them in xylenes, twice for ten minutes each.

3) Incubate in a 1:1 mixture of Paraplast Plus (Polysciences):Xylenes[2] at 61° for ten minutes, twice. Keep the tubes in a hot block for the rest of the procedure.

4) Incubate in Paraplast Plus at 61° for ten minutes, twice.

5) Set up the molds[3] on a hot plate set just hot enough to ensure that the wax does not set when a little is put in the bottom of the mold.

6) Quickly dispense some of the embryos in wax into the bottom of the mold. Use a pasteur pipette with the end broken off for this operation. I warm it briefly in a

[2]Paraplast Plus melts at 56°, and must not get hotter than 62°. Melt some in a conical flask in a water bath, and add an equal volume of pre-warmed xylenes. The solid wax takes quite a long time to melt, and should be set up at least an hour in advance. Also set up the water bath and the hot-block for the test tubes of embryos far enough in advance that the temperature has completely stabilised at 60-61°.

[3]The molds I use are from Polysciences, and are called 'Peel-A-Way' (Cat. no. 18986).

bunsen flame, bring it to the temperature of the wax sitting in the water bath, and then pipette the embryos. You want to put in enough wax/embryos to cover the base of the mold in a thin layer. The number of embryos should be enough to form a monolayer in the mold: fewer will mean that there are fewer embryos in each section; more will stop the embryos from settling horizontally in the wax.

7) Allow the embryos to settle for about one minute, and then try to make them move into the centre of the mold. I do this by blowing the surface of the wax with a pasteur pipette, and thus pushing the embryos away from the sides. This takes a little time to perfect - it does not really matter if the embryos are spread around the whole base.

8) Fill the mold with wax by slowly and gently, running more wax down the side; try not to disturb the embryos. The wax should fill the mold to about two thirds of its volume. Don't let the wax solidify before adding more, as this will form a weak region, and the block may break.

9) Carefully remove the mold from the hotplate and allow it to set undisturbed for about 15 minutes.

10) The blocks can now be stored at 4° for several months before cutting sections.

Subbing slides:

1) The microscope slides are subbed in poly-lysine to make the the sections stick to them well. The slides can be subbed in large batches, and stored in a dust-free environment.

2) Wash the slides by soaking them in a large container of hot water with some detergent. Gently agitate them. Rinse thoroughly (e.g. running water for 30 minutes), and finally rinse in distilled water.

3) Load the slides into racks, and dry in an oven.

4) Soak each rack of slides in a solution of 50µg/ml poly-D-Lysine[4] in 10mM Tris pH8 for about 10 minutes.

5) Air dry in a dust-free place; this can take 24 hours.

6) Store the slides in boxes at room temperature.

Cutting sections:

1) The exact procedure used will depend on the microtome that you use. The following notes should provide a guideline.

2) Push the wax block out of the mold, and mount it onto a wooden microtome block, using molten Paraplast Plus to stick it.

3) Trim the block with a razor blade so that the face to be cut is a trapezoid shape.

4) Mount the block onto the microtome, and cut 6 micron sections. The number that you can fit onto each slide depends on the size of the sections and the size of the coverslip that you intend to use for the hybridisations. I use 22mm^2 coverslips, and use about

[4]I use Sigma #P-0899, which has a molecular weight range of 70-150,000.

four sections per slide. I usually cut individual sections rather than ribbons, but both are OK.

5) Place a subbed slide onto a hot-plate set at 45°. Put a drop of clean water onto the slide, and carefully place the section onto the water drop[5]. It is important to put the newly cut (shiny) side of the section downwards. The section will spread flat over the water, and will be lowered slowly on to the slide as the water evaporates. After you have put enough sections onto a given slide, leave it on the hot-plate, undisturbed.

6) Leave the slides on the hot-plate overnight (or in a 45° oven).

7) The sections can be stored at room temperature in a dry box for several weeks prior to hybridisation.

Making the ^{35}S riboprobe:

1) The template DNA should be linearised downstream of the insert. In order to remove any contaminating RNAses, treat the digest with 200µg/ml proteinase K for 30 minutes at 37°, phenol extract once, and chloroform extract. Ethanol precipitate and resuspend at 1µg/µl.

2) I use the Stratagene transcription kit, and follow their protocol:

1µg DNA
1µl 10mM rATP
1µl 10mM rCTP
1µl 10mM rGTP
1µl RNAse Block[6]
1µl 0.75M DTT
4µl 5X transcription buffer (Stratagene)
5µl ^{35}S UTP 40µCi/µl, 800Ci/mmole (SP6/T7 grade)
1µl of a 1:5 dilution of T3 or T7 polymerase in 1X transcription buffer
Final volume = 20µl

3) Incubate at 30° for 1 hour.

4) Add 10X MS to 1X, and 1µl of DNAse (use RNAse-free DNAse). Incubate at 37° for 30 minutes.

5) TCA precipitate a tiny aliquot, to discover the percentage incorporation of label, which allows to you calculate the amount of probe transcribed.

6) Add tRNA (phenol extracted, ethanol precipitated, and resuspended in DEPC treated water). Since you are transcribing new RNA, the total amount made depends on the amount of the hot (limiting) nucleotide in the reaction, and the incorporation. To get the right probe and tRNA concentration for the hybridisation, use the following example as a guide. For 75% incorporation, using 200µCi of UTP in the reaction, add 500µg tRNA. For different amounts of transcripts, add an amount that will give the

[5]A small paintbrush is an easy way to handle the sections.

[6]Stratagene's RNAsin

same relative amount (i.e. for 50% incorporation of the same amount of label, add 333µg).

7) Phenol extract once; back extract with an equal volume of 10mM DTT; chloroform extract once; add an equal volume of 4M ammonium acetate (DEPC treated), and 2.5 volumes of ethanol.

8) Chill, spin, rinse pellet, and resuspend in 50 µl of 10mM DTT.

9) Add 50µl 2X carbonate buffer, and incubate at 60° for 140 minutes[7].

10) Add equal volume of 0.2M sodium acetate, 1% acetic acid; add 2.5 volumes of ethanol, chill, spin, rinse pellet, and resuspend in 50% formamide[8]. Aim for 5 x 10^5 cpm / µl in the formamide. (For the example above - 75% incorporation of 200µCi - use 96µl of formamide).

-The probe is now 5X concentrated. Store it at -20° till use.

Prehybridisation Treatment of the Slides:

1) Load slides into a rack.

2) The following treatments should be carried out in glass dishes: the dishes I use hold 400ml.

3) Dewax in xylenes, twice, for 10 minutes each.

4) Rehydrate in ethanol series: 100%, 95%, 80%, 60%, 30%, 2 minutes each.

5) Incubate in 0.2M HCl for 20 minutes at room temperature.

6) Rinse in H_20, 5 minutes.

7) Incubate in 2X SSC, 30 minutes, 70°.

8) Rinse in H_2O, 5 minutes.

9) Digest with 0.125mg / ml protease in P buffer at room temperature for 10 minutes[9].

10) Incubate in 0.2% glycine in PBS for 1 minute.

11) Rinse in PBS, twice, 1 minute each.

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[7]This is what I've found best to give transcripts with a mass average of around 100 bases. It is wise to run a small aliquot on a sequencing gel to check the size. Aim for no smaller than 50 bases, and no larger than 200 bases (average size).

[8]The formamide used for resuspending the probe, and that used in the hybridisation buffer, should probably be of the best quality available. It should be deionised, and perhaps even recrystallised. The formamide used in the wash buffer can come straight out of the bottle.

[9] I use Sigma protease (#P-5147). It should be made up at 40mg / ml in water, and predigested at 37° for 4 hours. It is then stored in 1ml aliquots. It may need to be titrated: I use the above conditions, but if these seem incorrect, use half the amount that completely removes the sections from the slides.

12) Post-fix in 4% paraformaldehyde in PBS[10], 20 minutes, room temperature.

13) Rinse once in PBS, 1 minute.

14) Acetylate the sections in 0.5% acetic anhydride in 0.1M triethanolamine, pH8.0 (I use 3ml in 600ml), 10 minutes, room temperature, in the hood, with vigorous stirring. Acetic anhydride is very unstable in water - add it to the triethanolamine at the same time as the slides.

15) Rinse in PBS, 2 minutes.

16) Dehydrate in ethanol series: 30%, 60%, 80%, 95%, 100%, two minutes each.

17) Air dry.

-The hybridisations should be set up soon after this pretreatment - the sections are probably not very stable at this stage.

Hybridisation:

1) The probe concentration during the hybridisation should be around 100,000cpm / µl.

2) Boil the probe (which is in 50% formamide, 5mg / ml tRNA) for 2 minutes (3µl for each slide to be hybridised).

3) Chill on ice and add 4 volumes of hybridisation buffer. Mix well, and spin for 30 seconds.

4) Place 15µl on to each slide, near the edge of the tissue sections.

5) Carefully lower a $22mm^2$ clean (but not necessarily siliconised) coverslip over the liquid in such a way as to make the probe cover the sections. Try not to allow too many bubbles.

6) Seal the edge of the coverslip with rubber cement; use plenty. I find it easiest to pour some into a 5ml syringe and use it from the syringe.

7) Hybridise overnight in a humid chamber at 50° (not neccessary, but not a bad idea).

Washing:

1) Carefully peel off the rubber cement, using a sharp pair of forceps; try not to dislodge the coverslip while doing this.

2) Place the slide in a rack in a tank of wash buffer at 50°. Do this, and all subsequent washes in the hood - the wash buffer is very smelly. Suspend the rack so that there is

[10]This must be made fresh. Heat it until near boiling, and then add a drop or two of 1M NaOH. It should go into solution within a few minutes.

space for the coverslips to slide off, which should happen within a few minutes. If needed, stir the wash buffer gently[11].

3) When the coverslips are off, move the rack into a fresh tank of wash buffer. Wash for about 4 hours at 50°, with hourly changes of buffer.

4) Wash the slides in 1X NTE at 37°, for 5 minutes.

5) Incubate in 20µg / ml RNAse A (the stock solution is 10mg / ml, boiled for 5 minutes, allowed to cool slowly, and stored at -20°) in NTE at 37° for 30 minutes. This step removes single stranded RNA, and reduces background.

6) Wash in NTE at 37° for one hour with 4 changes.

7) Dehydrate through ethanol series: 30%, 60%, 80%, 95%, 100%. Dilute the ethanol with 0.3M ammonium acetate instead of water.

8) Air dry.

Autoradiography:

1) I use Kodak NTB2 emulsion, diluted 1:1 with water[12], and aliquotted into 5ml scintillation vials, which are stored in absolutely light tight conditions at 4°[13]. This is the right quantity to fill the dipping chamber that I use, which is made of perspex and is just larger than a slide: any vessel that is deep enough to allow the slide to be dipped so that the emulsion easily covers the sections is suitable, but adjust the aliquot size accordingly.

2) Set up a water bath at 45° in the dark room and melt an aliquot of emulsion. I put the vial inside a film developing tank which has some water in it, and put the whole thing in the water bath. This is then light tight, and you can come and go safely. Alternatively a dark room with a properly sealed rotating door can be used. The emulsion takes about 15 minutes to melt.

3) While dipping the slides and drying them, they must be kept out of all light other than weak safelight of the appropriate kind for the emulsion used (Kodak no. 2 for NTB2). The way that you organise the dark room depends on your set up, just make sure that it is really light-tight.

4) Pour the melted emulsion into the dipping chamber, which should be sitting in the water bath, to keep it warm, and thus to stop the emulsion from solidifying. Dip each

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[11]The wash buffer is 50% formamide, and therefore should not be put down the sink. Furthermore, the first couple of washes contain significant amounts of radioactivity, and should be treated with appropriate care. I bottle everything and leave it to EH&S.

[12]The new batch of emulsion is melted in the dark at 45° (which takes about 45 minutes) and mixed in a conical flask with an equal volume of distilled water, also prewarmed to 45°. The mixing must be done gently, and bubbles should not be allowed to form: vigorous stirring increases the background. It is worth checking the background of a new batch, by dipping a clean slide and developing it. Very few silver grains should be visible. Also this same control can be done with an experiment, which helps to trace the source of any high background.

[13]Also make sure that there is no radioactivity stored in the same fridge, as the emulsion is very sensitive, and will develop a high background.

slide briefly into the dipping chamber and remove it without rubbing off the layer of liquid emulsion. Place the slide into a rack so that it stands vertically with the end that you held while dipping, downwards. This allows excess emulsion to drain away from the sections, and causes there to be a thin, uniform layer over them.

5) Allow the slides to dry for at least two hours before putting them in light tight boxes, which should have some drying gel (e.g.. Drierite) in them. Tape the boxes shut, and wrap them extensively in aluminium foil. Expose at 4°, away from any radioactivity.

6) It is convenient to have three or four slides for each probe used, and to put one of each type into each box. This way when you want to develop them, you need only open one box, and you need not re-wrap it.

7) Try developing the first set of slides after about 4 or 5 days - moderate to strong signals should be exposed enough by then. Judge how much longer to leave the other slides from the amount of signal seen at this time.

Developing the slides:

1) It is crucial that all the solutions used in the developing and staining of the slides are at the same temperature, which should be below 20°. If they are not, the emulsion, which is very delicate when wet, may come off the slides during their treatment, thereby ruining the whole experiment. To avoid this I make up the solutions the day before using them, and let them all equilibrate in an 18° room. When they are to be used they can then be taken into the dark room, and it is OK if they all warm up slowly together. The solutions are:

Kodak D-19 developer
2% acetic acid
1:3 dilution of Kodafix fixer[14]
3 litres distilled water
10mM sodium phosphate buffer, pH6.8

2) Allow the box of slides to come to room temperature while still sealed (about 1 hour).

3) In a dark room with appropriate safelight, load the slides into a rack.

4) Immerse them in developer for 2 minutes.

5) Transfer them to 2% acetic acid for 30 seconds (this acts to stop the developer, and is probably not crucial).

6) Incubate the slides in fix for 5 minutes.

7) Rinse them in distilled water for 15 minutes with two changes. They are safe in the light from this point onwards.

8) The final process is to stain the slides with Giemsa. The degree of staining wanted varies with the intensity of the signal: if the signal is strong, stain for about 15 or 20 minutes, but if it is weak, stain for only 30 seconds to 1 minute. I usually stain the first batch to be developed weakly, and then decide how to treat the next batch.

[14]The type of fixer is not crucial, but is should have a hardener in it. Use the dilution suggested for films rather than paper.

9) Make up a 5% Giemsa solution in 10mM $NaPO_4$ immediately before staining the slides.

10) Incubate the slides in the Giemsa solution for the desired amount of time.

11) To remove the stain, gently pour water into the container until the film that forms at the surface of the Giemsa solution is poured away. This is important so that the film does not coat your slides.

12) Rinse the slides two or three times in water for about 30 seconds. While wet, they can be viewed at low power, and if they appear overstained, they can be rinsed more extensively: normally this is unnecessary.

13) Air dry the slides, and then mount them in DPX mounting medium under a coverslip.

Solutions:

P buffer:

50mM Tris 7.5, 5mM EDTA.

5X Transcription Buffer:

200mM TrisHCl, pH8, 40mM $MgCl_2$, 10mM spermidine, 250mM NaCl.

10X MS:

100mM Tris pH7.5, 100mM $MgCl_2$, 500mM NaCl.

2X Carbonate Buffer:

80mM $NaHCO_3$, 120mM Na_2CO_3, which should be pH10.2.

Hybridisation Buffer:

The hybridisation mix can be made in a large volume, and stored at -20°[15]. The recipe for 1.25X buffer is:

50% formamide
12.5% dextran sulphate
0.375M NaCl
12.5mM Tris pH7.5
12.5mM sodium phosphate buffer pH6.8
6.25mM EDTA
1.25X Denhardt's
12.5mM DTT

Wash buffer:

1X salts, 50% formamide, 14mM mercaptoethanol.

10X salts:

3M NaCl, 0.1M Tris, 0.1M $NaPO_4$, 50mM EDTA, pH6.8.

Quantities for 1 litre:
175.3g NaCl
14.04g Tris HCl
1.34g Tris base
6.78g $NaH_2PO_4.H_2O$
7.1g Na_2HPO_4
100ml 0.5M EDTA

10X NTE:

5M NaCl, 100mM Tris pH7.5, 10mM EDTA.

References

-Cox et al., Dev Biol, **101**, 485 - 502 (1984)
-Ingham et al., Nature 318, 439 - 445 (1985)

[15]The solutions used to make this buffer should probably be DEPC treated, and the whole thing should be filtered through a millipore filter (which will be slow, because of the dextran sulphate).

Marek Mlodzik & Nipam Patel

WHOLE MOUNT *IN SITU* HYBRIDIZATION WITH A NONRADIOACTIVE PROBE

This protocol describes a procedure for the localization of RNA transcripts within whole *Drosophila* embryos using a non-radioactive detection method. The embryo hybridization procedure is essentially as described by Tautz and Pfeifle. Most of the modifications are in the procedure for the production of the probe. We have employed two techniques: a modified version of the standard oligo priming procedure and a protocol using PCR that generates single-stranded DNA probes.

Recently, Boehringer Mannheim released digoxigenin-UTP for the production of RNA probes. This could increase the sensitivity of the detection and replace the production of DNA probes. However, since this method has not been used yet, it is not included in this protocol. The pretreatment and detection as described here are the same for DNA or RNA probes.

Preparation of Whole Mount Embryos:

This protocol is a slightly modified version of the protocol of Tautz and Pfeifle which is based on the fixation protocols of Ken Howard, Phil Ingham and Alfonso Martinez-Arias and the whole mount hybridization protocol of Paul Mahoney and the Boehringer Genius Kit.

1) Collect the embryos (anywhere between 0-15 hrs) and rinse thoroughly with water.

2) Dechorionate in 50% "Clorox" (commercial bleach) for 2-5 min.

3) Wash with water.

4) Transfer the embryos (up to 3ml volume of embryos) into a polypropylene tube containing:

16 ml	0.1 M Hepes, pH 6.9 2 mM Mg-sulfate 1 mM EGTA
4 ml	20% paraformaldehyde (dissolve paraformaldehyde in boiling water. You may have to add NaOH to get it into solution)
20 ml	heptane

5) Place on a rotator for 15 to 20 min.

6) Remove the lower phase (fixative) and add 15 ml methanol and shake the tube vigorously for 10-30 sec. The devitellinized embryos will sink to the bottom (heptane will be on top and the vitelline membranes will stay at the interface).

7) Transfer embryos together with some methanol into a new tube. Wash with MeOH:EGTA (90% methanol;10% 0.5 M EGTA, pH 8.0).

8) Prepare 4% paraformaldehyde in PBS (PP; made by diluting 20% paraformaldehyde solution with 1X PBS) and MeOH:EGTA (ME) solution (see above). Rehydrate the embryos as follows:

5 min	in 7 parts ME / 3 parts PP

5 min in 5 parts ME / 5 parts PP
5 min in 3 parts ME / 7 parts PP
20 min in PP

9) If you want to store the embryos at this point, wash them in PBS and dehydrate them through 30%, 50% and 70% ethanol and store at -20°C; rehydrate before pretreatment.

II. Pretreatment:

All the following incubation steps are performed at room temperature. Avoid potential RNAase contaminations. The PBS can be treated with DEPC and autoclaved and then supplemented with 0.1% Tween 20 (=PTw). We have found that the DEPC treatment is not absolutely necessary.

1) Wash the embryos 3 times for 5 min each in PTw

2) Incubate the embryos for 7-8 min in PTw plus 50 µg / ml non-predigested Proteinase K (Boehringer Cat# 745 723; you may have to determine the optimal extent of treatment for your batch of proteinase K; the optimal time seems to be 2-3 min before the embryos begin to show any noticable damage).

3) Stop Proteinase K digestion by incubating for 2 min in 2 mg / ml glycine in PTw.

According to Tautz andf Pfeifle, the Proteinase K digestion is a critical step. If it is too short, you lose sensitivity and increase the background. If it is too long, the embryos tend to disintegrate during hybridization.

4) Wash 2 times for 5 min each with PTw.

5) Refix for 20 min with PP.

6) Wash 5 times for 5 min each in PTw.

Preparation of DNA Probe

I. PCR-labeled Single-stranded Probe from Cloned DNA:

Using this PCR technique, single stranded DNA probes containing digoxigenin can be made for in situ hybridization. The biggest advantage of this technique over the random oligo priming method is that a much larger quantity of probe can be made with the same amount of starting nucleotide. In addition, the ratio of labelled DNA to unlabelled starting material (which will compete for hybridization) is much higher. In situations where transcripts are not very abundant, these single-stranded probes seem to work better than the oligo primed probes. The major disadvantage is that it is difficult to control the probe size. The PCR reaction will generate full length strands for inserts up to 2-3 kb. We simply boil the DNA to reduce its size, but have not made any estimates of how effective this really is nor have we investigated other alternatives to this problem. Empirically, however, this labelling technique works extremely well for the in situs. In addition, we have found that biotin-16-dUTP can also be incorporated this way and used for in situs using streptavidin-alkaline phosphatase for the detection step. This method works okay, but is clearly 2 to 3- fold less sensitive than the digoxigenin. Also, PCR can be used to generate very large amounts of biotin-labelled double-stranded DNA by the use of two primers as long as the distance between primers is less than 2-3 kb. Finally, we have observed that biotin-labelled DNA runs

normally in agarose gels, but digoxigenin-labelled DNA will run as though it is larger than it actually is.

1) Prepare the following stock solutions:

10 x React (comes in PCR kit)	0.5 M KCl 0.1 M Tris-HCl, pH 8.3 15 mM $MgCl_2$ 0.01% (w / v) gelatin	
5 x dNTP Mix (equivalent to 1 mM dCTP tube 6 of Genius kit)	1 mM dATP	7.1 µl of 10mM
	1 mM dCTP	7.1 µl of 10mM
	1 mM dGTP	7.1 µl of 10mM
	0.65 mM dTTP	4.6 µl of 10mM
	0.35 mM Digoxigenin-11-dUTP (Dig-dUTP can be purchased as a 1mM soln from Boerhinger)	25.0 µl of 1mM
		20.5 µl of H_2O
		71.4 µl total vol.
SK primer	30 ng / µl (approx 5.3 µM)	
KS primer	30 ng / µl (approx 5.3 µM)	

Linearized DNA containing insert. Linearize Bluescript DNA as you would to make an RNA runoff probe. For example, you might have a 2.0 kb EcoR1 fragment cloned into the R1 site of Bluescript. You might linearize by cutting one aliquot with Kpn and another with SacII (assuming there are no Kpn or SacII sites in the insert). The Kpn cut DNA can be used with the SK primer to create one strand which might be the anti-sense strand. The SacII cut DNA can be used with the KS primer to create the sense strand which can be used as a control in the hybridization. T3, T7, M13, or internal primers can be used in place of the KS / SK primers. We have on occasion cut DNA and then not bothered to phenol extract and precipitate afterwards, but have simply heat inactivated the restriction enzyme and then successfully used it for the PCR reaction. You will want to dilute your DNA with water to a final concentration of about 100-200 ng / µl. Keep in mind that if your insert is much over 3 kb that the probe produced will probably not be fully representative of the entire insert .

2) Set up the following reaction (25 µl volume):

water		8.5 µl
10 x React		2.5 µl
5 x dNTP mix	5.0 µl	
KS (or SK) primer		5.0 µl
DNA (100-200 ng / µl)	2.0 µl	

Add 40 µl mineral oil and centrifuge. Boil 5 min then add Taq:

Taq I DNA Polymerase 2.0 µl (1.25 units total; a 1:8 dilution in 25.0 µl water of 5U / µl Taq stock)

3) Mix the contents and then centrifuge for 2 min. Incubate for 30 cycles in the PCR thermal cycler under the following conditions:
   95°C for 45 seconds
   55°C for 30 seconds
   72°C for 1 minute

4) After the PCR run, add 75 µl dH_20, then centrifuge.

5) Remove 90 - 95 µl of the reaction from beneath the oil.

6) Do two ethanol precipitations as follows. Add NaCl to 0.1 M and 3 vols of 100% EtOH. You can use 10 µg of glycogen or tRNA as a carrier (0.5 µl of a 20 mg / ml stock). Mix well, -70^{O}C for 30 min, and centrifuge. Wash with 70% ethanol. Speedvac dry. 2nd precipitation is optional.

7) Resuspend pellet in 300µl of hyb buffer (hyb. buffer of Tautz and Pfeifle protocol below). For efficient penetration and hybridization to the embryos, the average probe length should be about 50-200 bp. To reduce the size of the single-stranded DNA, boil the probe for 40-60 min. The probe can be diluted as much as ten-fold before use, but this varies quite a bit depending on the abundance of your transcript and how much of a problem you have with background staining. I recommend that you use the probe anywhere from undiluted (original 300µl) to three-fold diluted for your first attempt.

8) Check of probe (solutions are same as those described in the embryo protocol below):
   1. Remove 1 µl of probe. Add 5 µl of 5X SSC. Boil 5 min. Quick cool on ice.Centrifuge.

   2. Spot 1-2µl onto a small nitrocellulose strip that will fit into an eppendorf tube or 5 ml snap cap tube.

   3. Bake between two sheets of Whatman paper in an 80°C vacuum oven for 30 min. The residual formamide may cause the nitrocellulose to warp. If this is a problem, reduce the time in the baking oven or do this spot test before the second precipitation. Note: unincorporated nucleotide binds only slightly to the nitrocellulose.

   4. Wet the filter with 2X SSC, wash 2X 5 min in PBT. Place strip into eppendorf tube or 5 ml snap cap tube.

   5. Block 30 min in PBT. Incubate in PBT + antibody (1:2000) for 30-60 min.

6. Wash 4 x 15 min in PBT.

7. Wash 2 x 5 min in NaCl/$MgCl_2$/Tris/Tween solution (Levamisole is not needed).

8. Develop with NBT and X-phosphate as described for embryos. Spots should be visible within a few minutes and dark by 10-15 min.

II. <u>Preparation of probe by oligolabelling</u>

An alternative to PCR-generated probes is to create probes by the random oligo-priming technique. This is the modification by Charlie Oh (Kornberg lab) of the protocol that is described in the Genius Kit. By increasing the random primer concentration and lowering the temperature, smaller probe fragments are created resulting in a higher signal and less background.

10X LB475 µl 1M Pipes pH 6.6
25 µl 1M $MgCl_2$
3.3 µl ßME

Labelling reaction:

1) Prepare 200ng of purified insert DNA in 9 µl of water.

2) Boil 10 min.

3) Quick freeze in dry ice/ethanol or liquid nitrogen.
4) Add following components on ice.

10X LB	2.0 µl
hexamer	6.0 µl of a 10 mg/ml soln. (Random hexamers; Pharmacia # 27-2166-01)
dNTP	2.0 µl (tube 6 of Genius Kit)
Klenow	1.0 µl (5 Units)

5) Incubate at 15°C for 1 hr.

6) Transfer to room temp for 3 hrs.

7) Precipitate probe and resuspend in 200µl of hyb buffer.

Hybridization and detection:

1) Hyb solution:
   50% formamide
   5x SSC
   100 µg / ml sonicated salmon sperm DNA
   50 µg / ml heparin
   0.1% Tween 20

2) Wash embryos 10 min in a 1:1 mix of hyb soln. and PTw.

3) Wash embryos 10 min in hyb soln.

4) Prehybridize in heat denatured hyb soln for about 1 h at 45°C. We have found that embryos can be stored in the hyb soln at -20°C for 1-2 weeks.

5) Boil the probe for 10 min and then cool on ice. Remove most of the prehyb soln from the embryos and add 100-200µl of probe. About a 30µl volume of embryos in the 100-200 µl of probe works well.

6) Mix and hybridize overnight in a 45°C waterbath. Round bottom Nunc tubes work well for this. After the hybridization, the probe can be recovered and used repeatedly. We have been able to use the same bit of probe up to six times. The repeated boiling may improve the signal by further reducing the size of the probe.

7) Wash embryos 20 min in Hyb soln. All washes in steps 7 and 8 should be done with agitation at 45°C. You will want to pre-mix and pre-warm all the solutions.

8) Wash embryos 20 min each in:
   4 parts Hyb. soln. / 1 part PTw
   3 parts Hyb. soln. / 2 parts PTw
   2 parts Hyb. soln. / 3 parts PTw
   1 part Hyb. soln. / 4 parts PTw
   2 times 20 min in PTw

9) Wash embryos 2 times 20 min each with PBT. This and all subsequent steps are done at room temp.
   PBT = 1X PBS, 0.1% BSA, 0.2% Triton X-100.

10) Dilute the alkaline phosphatase conjugated goat anti-digoxigenin antibody (vial 8 of the Genius Kit) to 1:2000 - 1:2500 with PBT. Incubate the embryos for 1 hour with 200-400µl of the dilute antibody soln.

11) Wash 4 times 20 min each in PBT.

12) Wash 3 times 5 min each in 100 mM NaCl
50 mM $MgCl_2$
100 mM Tris, pH 9.5
0.1% Tween 20
1 mM Levamisol (Sigma #L-9756; add fresh just before use.

Levamisole is an inhibitor of potential endogenous phosphatase. Nipam has done experiments where it has been left out and there does not seem to be any background problem.)

Prepare a solution of NaCl / $MgCl_2$ / Tris / Tween / Levamisole containing 4.5 µl of NBT (vial 9 of Genius kit) and 3.5 µl of X-phosphate (vial 10) per ml of solution. Add 0.5-1.0 ml to the embryos after the last wash. You will see the signal appear anywhere between 10-60 min. Stop by washing several times in PBS. We have tried alternative substrates. Vector Kit II substrate produces a brown / black product that is insoluble in xylene and can therefore be mounted in permount. This substrate diffuses less and allows for the resolution of sub-cellular localization, but it is 2-3 fold less sensitive than the standard method. We have not had any luck using an HRP enzyme detection system.

13) The embryos reacted with NBT / X-phosphate should be transfered to 50% glycerol in PBS for 1-2 hrs and then transfered to 70% glycerol in PBS. They can be stored at 4°C for several weeks.

Some Final Notes:

If the embryos are prepared properly, the morphology can be excellent. The embryos can be dissected in the 70% glycerol soln if needed. One additional possibility is to reduce the level of background by digesting with a single-stranded nuclease after the hybridization with a single-stranded probe. This would be equivalent to the RNAse A digestion of ^{35}S RNA in situs. This should destroy the unbound probe and leave the RNA / DNA hybrids intact. It may be tricky as the single-stranded nucleases can be difficult to control. The protocols described above are based on experiments performed by me, Nipam Patel, Yash Hiromi and Elinor Fanning using a variety of different gene probes.

REAGENTS:

Genius Kit. Boehringer Cat# 1093 657. Alternatively, the components can be purchased individually. Several are listed below.

Digoxigenin-11-dUTP Boehringer Cat# 1093-088, 25 nmol / 25 µl (1mM)

Anti-Digoxigenin-AP Conjugate Boehringer Cat# 1093-274

NBT is 4-Nitro blue tetrazolium chloride, Boehringer, Cat# 1087-479

X-phosphate is 5-Bromo-4-chloro-3-indolyl-phosphate, Boehringer, Cat# 760-994

Marek Mlodzik

FIXATION AND PRETREATMENT FOR IMAGINAL DISC WHOLE MOUNT NONRADIOACTIVE *IN SITU* HYBRIDIZATION

The following protocol is based on the method described for whole mount embryo hybridization (see above).

1) Dissect discs from late 3rd instar larvae in PBS.
2) Fix dissected material for 15'-20' in 4% paraformaldehyde, PBS, 0,5% Triton X-100 on ice in Eppendorf-tube, while you continue to dissect more discs.
3) Fix for additioinal 20' at RT (same fixative as above) after you have finished dissecting.
4) Following fixation, wash the discs 3 times for 5' in PTw at RT.
5) ProteinaseK digestion:

   12.5 µg / ml enzyme in PTw (1 / 4 of the concentration that is used for embryos)
   usually between 4' and 10' at RT is fine, depends on batch of proteinaseK
   when new batch: titrate time !

   The longest digestion that still retains reasonable morphology gives the best results.
6) Wash digested discs in PTw, 2mg / ml glycine: 2 times 5' at RT, followed by two 5' washes in PTw.
7) Postfixation: 20' at RT in 4% paraformaldehyde
   0.2% glutaraldehyde
   PTw
8) Wash fixed discs in PTw 4 times 5' at RT.

Prehybridization:

1) PTw : hybridization buffer (1:1) for 10' at RT
2) Hybridization buffer, 10' at RT
3) Hybridization buffer, 1 hour at 45 C
4) Hybridize o / n at 45 C, in Eppendorf tube without shaking

Buffers:

PBS (10X stock)	76.1 g NaCl
	18.8 g $Na_2HPO_4 \cdot 7H_2O$ (9.9 g if anhydrous)
	4.1 g $NaH_2PO_4 \cdot H_2O$
	H_2O to 1 litre
PTw	PBS containing 0.1% Tween

Hybridization Buffer same as for embryos (see above)

Probe and Detection:

Probe can be made either by random-oligo priming or alternatively by PCR generating single stranded labelled DNA (see above).
More recently, a digoxigenin-labelling kit for RNA probes is also available.

Detection is done as described for embryos (see above), all steps in Eppendorf tubes.

Comments:

The success of the method depends largely on the abundance of the respective transcripts. In our experience, detection of transcripts that are found at 1:50,000 (or more abundant) in the eye disc cDNA library usually work fine, e.g. *glass* (1:5000) or *scabrous* (1:20,000). For transcripts with lower abundance, e.g. *seven-up* (1:100,000) or *sevenless* (1:100,000), this method works in about 1 out of 2 experiments.

GOOD LUCK !

Matthew Freeman

IMMUNOSTAINING DROSOPHILA EMBRYOS

Fixation and permeabilisation.

1) Collect embryos on grape juice agar collection plates, and rinse them onto a Nitex mesh sieve, rinsing them well with water containing 0.05% Triton-X-100 (optional) to remove excess yeast.

2) Dechorionate in 50% household bleach (2.5% sodium hypochlorite final conc.), by dipping the whole Nitex sieve into a beaker of bleach for 2-4 minutes and swirling the embryos once or twice. Rinse well in PBS / 0.05% Triton-X-100 to remove the bleach. Do not let the embryos dry out at this stage.

3) Transfer the embryos with a soft paintbrush to a vessel with a tight-fitting lid containing: 50% n-Heptane
50% PEM-formaldehyde

PEM-formaldehyde is:
0.1M PIPES
2mM EGTA
1mM $MgSO_4$
pH to 6.95 with HCl
mix 9:1 with stock solution of 37% formaldehyde

The size of the vessel, and the quantity of fixative should be adjusted according to the number of embryos used. For about 50-500 emryos I use a 5ml glass vial with a screw-cap; for 0.5-1g of embryos I use a 50ml polypropylene tube with a screw cap. It is a good idea to use enough fix / heptane to fill the container to at least 70-80%, as this stops too many embryos being lost by sticking to the sides.

This fix is a good all-purpose one, but there may be some antigens which are destroyed by it. The most likely source of problems is the formaldehyde solution, which contains up to 10% methanol as a stabiliser. Some antigens (for example 24B10 antigen) are methanol sensitive, in which case, either buy expensive and unstable formaldehyde without methanol, or make it by dissolving paraformaldehyde in water. To do this heat the water to near boiling and add a drop or two of 1M NaOH. You can substitute PBS or Balanced Salt Solution (for recipe see Wilcox, in Roberts: Drosophila, A Practical Approach. IRL Press. 1986) for the PEM, but I like PEM the best for most cases. Other fixes are possible: anything you know preserves your antigen should work, though there might be penetration problems if you use too high a glutaraldehyde concentration.

4) Vigorously shake the embryo / heptane / fix for 15-30 minutes at room temperature.

5) With a pipette, remove as much of the aqueous (bottom) layer as possible, without taking the embryos at the interface.

6) Add 1 volume of 100% methanol to the embryos / heptane, and shake very hard for 30 seconds-1 minute. Allow the two phases to separate. The devitellinised embryos will sink to the bottom, and the embryos with their vitelline membranes still attached and the empty vitellines will remain at the interface. This step is not always very efficient: anything over 50% sinking is fine, and on a good day it should be 80 or 90%.

7) Remove the heptane (top layer), all the interface, and the methanol above the embryos with an aspirator.

8) All the subsequent steps are carried out in the same tube, which can be filled with a solution, the embryos allowed to settle, and the solution removed.

9) Treat the embryos with:

100% methanol	10 mins (at this stage the embryos can be stored at -20°C for months)
PBS / 0.2% saponin	10 mins
" "	10 mins

The embryos can be stored at 4° like this for a week or two.
Before staining:

PBS / 0.2% saponin / 5% serum (normal goat or foetal calf) 10 mins

As discussed above, some antigens are methanol sensitive, and in this case the vitelline membranes have to be removed by hand. In this case, substitute the following for 6-9 above:

6a) Remove the aqueous (lower) phase. Take some embryos in heptane with a pipette, and spread them dropwise onto a microscope slide covered with double sided sticky tape. Let the heptane evaporate, and <u>just as it does</u>, immerse the slide in PBS / 0.2% saponin in a petri dish; do not let the embryos dry out on the slide. Using a fine tungsten needle, gently tease the embryos out of their (already cracked) vitelline membranes; this step is made much easier if the embryos are initially fixed for longer than normal (about 45 minutes). Transfer the embryos to an Eppendorf tube of PBS / 0.2% saponin / 5% serum, using a Gilson pipette with a yellow tip cut off to make a wide enough opening.

Antibody Staining:
I stain the embryos in Eppendorf tubes or, for large numbers of embryos, 12x75mm polypropylene tubes (Falcon 2063).

1) Incubate the fixed embryos in primary antibody diluted in PBS / 0.2% saponin / 5% serum. Typically monoclonal supernatant is diluted about 1:5, polyclonal serum about 1:500, and ascites even more, but you should determine the best for your antibody. Incubate for 2 hours at room temperature, or overnight at 4° (which is probably a little more sensitive).

2) Rinse in 3 changes of PBS / 0.2% saponin for 10 minutes each (longer washing may reduce background).

3) Incubate in secondary antibody diluted in PBS / 0.2% saponin / 5% serum for 2 hours to overnight. In most cases, I use peroxidase conjugated 2nd antibody, and do a DAB reaction; this is the most sensitive detection system available, and can be made even more so with silver-gold intensification. However, a fluorescent conjugated 2nd antibody may be used, in which case, I prefer rhodamine or Texas red to fluoroscein, since they are less prone to quenching under epifluorescent illumination.

4) Rinse in 3 changes of PBS / 0.2% saponin for 10 minutes each, and one rinse of PBS. For fluorescent labelled embyos mount in 85% glycerol containing 2.5% isopropyl gallate, and view with epifluorescent illumination. An optional Hoechst 33258 stain can be added to the penultimate rinse (1µg / ml; **caution - mutagen**); this will strongly label

all nuclei, and is viewed with ultraviolet epifluorescence. Slides mounted in glycerol dry out after a few days unless the edge of the coverslip is sealed with nail varnish.

5) For the DAB reaction, when using peroxidase-conjugated 2nd antibody, incubate the embryos in 400µl of 0.5mg / ml diaminobenzidine in PBS. After 10 minutes, add 2µl of 3% hydrogen peroxide and watch the development of the brown reaction product under a stereomicroscope. When the reaction is sufficient (usually 5-15 minutes), stop it with 2 or 3 rinses in PBS. (DAB can be made up in 10X aliquots (5mg / ml in water) and stored at -70°. **USE GREAT CARE WHEN WORKING WITH DAB. IT MAY BE A VERY POTENT CARCINOGEN.** Use copious amounts of bleach to inactivate it (leave overnight), and then rinse it away with large amounts of water. Treat gloves, tips, and tubes in the same way.)

6) Either take the embryos through silver-gold intensification (see Liposits et al. Neuroscience **13**, 513-525. 1984 and the previous edition of the Rubin Lab Manual) or mount them in 85% glycerol, or dehydrate them through an ethanol series, clear them in methyl salicylate until they sink, and then mount them in the methyl salicylate (this gives the best results, but is less straightforward than aqueous glycerol).

Note:

In this protocol, I have suggested using 0.2% saponin throughout the staining. This is a good, very mild detergent which should not extract many antigens. However, if it does not work, you could try using no detergent (which makes the embryos sticky, and reduces the ease of antibody penetration), or substituting another detergent. If your antigen is nuclear, 0.1% Triton-X-100 might be better.

Reference:

The best overall discusssion of these, and many other, techniques is:

Immunocytochemistry: Theory and Practice. Lars-Inge Larsson. CRC Press (1988)

N.E. Baker

STAINING EYE IMAGINAL DISCS

A. Labelling with antibodies:

Labelling eye discs with antibodies does not differ in principle from labelling any other tissue. However, there are several manipulations that aid treatment of this tissue. The following protocol is from Tomlinson and Ready, Devl. Biol. **120**, 366-376 (1987). A second section gives detailed comments and tips on some of the steps, with the aim that the protocol could be followed by an inexperienced worker without further instruction.

1) Dissect eye imaginal discs from larvae in 0.1M $NaPO_4$ (pH 7.2). It is convenient for subsequent handling to leave the discs attached to the mouthparts. (See detailed instructions.)

2) Fix the discs. Usually we use PLP for 45 min on ice. PLP gives fixation results comparable to glutaraldehyde but preserves antigenicity. Of course the interaction of each antibody with its antigen is unique and may require individual treatment. Less commonly we use 4% paraformaldehyde in PEM for 30 min at room temperature, particularily with nuclear antigens (PLP is a better membrane fix and we guess may reduce accessibility of the nucleus to antibodies). (See detailed instructions.)

3) Wash discs in 0.1M $NaPO_4$ (pH7.2), 0.1% saponin for 15 min on ice.

4) Dissect away the peripodial membrane. Removing the peripodial membrane increases accessibility of the epithelium to antibody and visibility of the stained tissue under the microscope. For some antibodies the improvement is neglegible (eg: Mab BP104). For others this step is essential (eg: Mab22C10). (See detailed instructions.)

5) Incubate in primary antibody, 1-2 hr on ice. Make up antibody in 0.1M $NaPO_4$ (pH7.2), 5% normal goat serum, 0.1% saponin (store frozen or filter sterilized at 4°C). Use monoclonal supernatants at 1:1 dilution (but Mab22C10 at 90%), ascites or sera at 1/250 - 1/5000 as appropriate. Final saponin concentrations of 0.01% - 0.1% are satisfactory for light microscopy. (See detailed instructions.)

6) Wash 3X in $NaPO_4$ + serum + saponin.

7) Incubate in secondary antibody, 1-2 hr on ice. Generally we use HRP-coupled goat anti mouse/rabbit from BioRad at 1/200 - 1/500.

8) Wash 3X in $NaPO_4$ + serum + saponin.

9) Incubate in diaminobenzidine (for HRP coupled secondary). The reaction is generally complete after 15-30 min, and for many antibodies staining can be much briefer. Staining solution is 0.5 mg ml^{-1} DAB, 0.1% saponin, 0.1 M $NaPO_4$ pH7.2, 0.003% H_2O_2. (See detailed instructions.)

10) Wash in 0.1M $NaPO_4$.

11) Transfer to a screw-cap vial in 0.1M $NaPO_4$, such as the 1 dram/3.7 ml bottles from Fisher.

12) Postfix and intensify in 2% OsO_4 in 0.1M $NaPO_4$, from 2min on ice to 10 min at room temperature, as desired. (See detailed instructions.)

13) Wash in several changes of 0.1M $NaPO_4$. Discs can be stored at 4°C at this stage.

14) Using a pasteur pipet, transfer to 50% ethanol in a petri-dish (60x15mm). After 5 min transfer to 100% ethanol, and then again to fresh 100% ethanol.

15) Mount in DPX. (See detailed instructions.)

Detailed Instructions for steps numbered:

1) To remove eye-antennal discs, hold the larva firmly about halfway down the body, using forceps. With a second pair of forceps, grasp the mouthparts and pull them out of the head. Usually the eye-antennal discs come out with the mouthparts. Other tissues may also remain attached; still holding on to the mouthparts, remove the brain and salivary glands, if present. Then grab the *internal* part of the mouthparts (forceps B in Fig. 1) and remove this and the eye-antennal discs from the *external* part of the mouthparts (still held by forceps A in Fig. 1). All other tissues should get left behind at this step.

   To make 1 liter 0.1 M $NaPO_4$ pH7.2 use:
   10.22 g $Na_2HPO_4.H_2O$ **OR**
   12.82 g $Na_2HPO_4.2H_2O$ **OR**
   19.30 g $Na_2HPO_4.7H_2O$ **OR**
   25.79 g $Na_2HPO_4.10H_2O$ plus 3.86 g $NaH_2PO_4.H_2O$ **OR**
   4.37 g $NaH_2PO_4.2H_2O$

FIGURE 1

2) The manner in which the discs are transferred to fixative is important. Flatter preparations, better for both analysis and photography, will be obtained if discs are spread on the surface meniscus, apical surface downwards. Use a wire hook (0.51 mm dia. tungsten wire from Ted Pella) to pick up the tissue where the antennal discs are attached to the mouthparts, and lower it into the fix.

- PLP is 2% paraformaldehyde, 0.01 M $NaIO_4$, 0.075 M lysine, 0.037 M $NaPO_4$, pH7.2. For light microscopic work, we do not pH the final solution, but instead make up PLP as follows. Make an 8% formaldehyde solution fresh from solid without boiling (a trace of NaOH will put the formaldehyde into solution with only moderate heating). While this is cooling, dissolve 0.36 g lysine in 10 ml H_2O, 7.5 ml 0.1 M $NaPO_4$ pH 7.2, 2.5 ml 0.1 M Na_2HPO_4 on ice. Immediatley before use, mix 15 ml buffered lysine solution, 5 ml formaldehyde solution, 50 mg $NaIO_4$ to give PLP.
- PEM is 0.1 M PIPES pH7.0, 2 mM $MgSO_4$, 1 mM EGTA, and is conveniently made up as a 2X stock to be diluted with 8% formaldehyde.

4) We dissect the peripodial membranes on a transparent dissecting dish. One can be made by setting a thick layer of "Sylgard 184" silicone elastomer (Dow Corning) in a 150x15mm glass petri dish. Transfer a pair of discs to a large drop of wash solution on the dissecting dish. Arrange fibre-optic illumination against a black background so that the peripodial membranes are visible (low-angle illumination from the side opposite you is best).

Good quality forceps and scalpel will help the dissection itself. Use Dumont #5 Biologie grade forceps (expensive, but can be resharpened on a stone when necessary), and a #11 scalpel blade with a good point (not all brands are the same, e.g. only about 1 in 5 Feather blades is as sharp as the typical Bard-Parker blade).

To remove the peripodial membrane, grip the antennal disc to position the eye disc at the correct angle. Once the antennal disc has been flattened it will not be easy to reposition the eye disc, so first remove other tissue (eg pharynx) that may interfere, and run the forcep tips between the two eye-antennal discs to ensure they can be moved separately. Rotate the eye-antennal disc so the antennal disc is towards you, the eye disc is on edge, and the peripodial membrane faces right(for a right hander). Hold the antennal disc firmly to maintain this orientation (see Fig. 2). Use the scalpel blade to pin the peripodial membrane to the dissecting dish between the eye and antennal discs, where it is furthest separated from the epithelium (see Fig. 2). Using your grip on the antennal disc, pull the eye disc away from the blade (pull down and away, about 7 o'clock in Fig. 2). Ideally, the torn peripodial membrane will spring back from the eye disc and curl up near the optic stalk. At other times all or part of the membrane remains closely apposed to the epithelium, and it is necessary to peel it off, using forceps to grasp the cut edge.

FIGURE 2

The apical surface of the epithelium that is now exposed will often adhere to wire transfer hook, so henceforth always pick up the discs from the basal side.

At first most people find this dissection difficult. General hints that might apply to any dissection include: 1) Avoid disturbance, stress or stimulants; 2) Brace every available digit for stability; 3) Put down dissecting implements that are not in use, lest they dissect something unexpectedly; 4) Know your limits and don't destroy a good dissection trying to make it perfect. In case a target is useful, with practice the membrane can be dissected and a pair of discs transferred to the next solution in well under a minute, on average.

5) Antibody incubations, washes, and DAB reactions are carried out in the 60-well (conical bottomed) microtest plates manufactured by Nunc. Use a wire hook to transfer the discs from one solution to the next. Each well takes 13 µL. Incubations can be left overnight at 4°C if the lids are sealed with parafilm to reduce evaporation.

9) **Unsubstituted benzidene is one of the most potent chemical carcinogens known.** *Diaminobenzidene is believed to be safer but should be handled carefully*. Sigma sells DAB in sealed "isopacks" which are convenient for making stock solution. Aliquots should be stored at -70°C. *All materials that contact DAB should be soaked with bleach prior to disposal* (I like to handle DAB inside a heatseal bag, which the waste never leaves).

12) Osmium intensification produces a background staining of the tissue that we find useful as a counterstain. Cleaner intensification can be obtained by adding Co and Ni to the DAB reaction (add 30 µl 50 mM $CoCl_2$ 50 mM $NiCl_2$ to 1 ml of buffer immediatly before the DAB. Ignore the clouding due to precipitation of the phosphates)(Adams, J.C., *J. Histochem. Cytochem.* **29**, 775, 1981). In this case a different postfixation will be required before mounting. 2% glutaraldehyde can be used (and results in slight destaining). Alternatively a graded series of ethanols is possible (30%, 50%, 70%, 90%, 100%), but this will not preserve delicate structures such as the apical photoreceptor-cell membranes. Of course EM-intensification protocols can also be used, such as the silver-gold method (Liposits et al., *Neuroscience* , **13**, 663, 1984).
SAFETY. **Both osmium tetroxide and glutaraldehyde are volatile neurotoxins with cumulative effects, and should be handled in a fume-hood.** *Osmium tetroxide fixes the brain via the nasal mucosa, repeated glutaraldehyde exposure can cause blindness.* Osmium tetroxide has a pungent odour, somewhat like garlic. Glutaraldehyde has only a mild smell. Waste solutions should be inactivated by pouring onto excess "protein" (eg glycine or powdered milk). OsO_4 is sufficiently volatile that solid waste, eg gloves, is clean if left overnight.

15. Mount as follows: Take up discs in 100% ethanol using pasteur and drop onto a clean microscope slide. Wipe away excess ethanol with a tissue. Add one drop of DPX mountant (FLUKA) before the discs dry, but when little ethanol remains. Remove the mouthparts and orient the discs apical side upwards using fine forceps. If desired, remaning peripodial membrane or other debris can be knocked off at this point. Carefully lower a 22x40 mm coverslip down from one side, watching all the while to see that the discs do not turn over. To some extent disc rotation can be controlled by moving the coverslip. If this is unsuccessful, remove the coverslip and reorient the discs. Another drop of DPX will be necessary before replacing the coverslip. As soon as you are satisfied, flatten the

discs with smooth firm pressure on the coverslip (eg: with the butt end of a pair of forceps). Releasen the pressure equally smoothly. Do not attempt to remove the coverslip once the discs are flattened.

B. Cobalt Sulphide staining:

CoS staining offers a rapid method for visualizing the apical borders of disc cells. The protocol given here is that of Tomlinson & Ready (Dev. Biol., **120**, 366, 1987).

1) Dissect discs in 0.1 M $NaPO_4$ pH7.2.

2) Fix in 2% glutaraldehyde in 0.1 M $NaPO_4$ pH7.2 (handle in the fume hood), 30 min, room temperature.

3) Wash in 0.1 M $NaPO_4$ pH7.2

4) Wash in H_2O.

5) Dissect away the peripodial membrane as for antibody staining, except that the discs are still in H_2O. Also it is necessary to *remove* the membrane, not just peel it back, or else it will snap back over the disc in subsequent steps.

6) Incubate in 2% $CoNO_3$ in H_2O. 96-well microtest plates are useful here too.

7) Transfer to 1% $(NH_4)_2S$ in H_2O, until the disc is coal-black (typically about 30s)[1].

8) Transfer to H_2O. Watch the disc destain. It is about done as the black becomes dark grey and some details can be seen (typically about 30 s). Slightly *before* this point, transfer to a drop of glycerol on a microscope slide and mount under a coverslip. Do not squash the disc, allow it to be gently flattened by the cover-slip. The staining is not stable and data should be recorded as soon as possible. If the coverslip is sealed with nail polish and the slide stored at 4°C the staining seems to be OK for a few weeks, otherwise 24 hr is about the safe limit.

The stain is essentially a precipitate coating the apical disc surface and is very delicate. It is easily destroyed if the discs are handled roughly. Once the peripodial membrane has been removed it is important not to handle the apical surface during transfers. Make sure to get all the glycerol off the wire hook before starting each new prep.

C. Basic Fuchsin staining

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[1]Make ammonium sulphide solution fresh from a 20% stock (Fisher Scientific). Keep the ammonium sulphide well away from the cobalt nitrate and use a fresh well as soon as any precipitate forms on the surface of the solutions. Ammonium sulphide gives off H_2S gas, which is more toxic than hydrogen cyanide, but the smell becomes intolerable way below dangerous levels. Nevertheless, handle the 20% stock in a hood and get worried if you can no longer smell the stuff.

Basic fuchsin is a nuclear stain. Mitotic chromosomes and, at other stages of the cell cycle, spindle pole body associated material are especially well stained. Basic fuchsin is useful for following cell division and cell death (in dying cells the whole cell is labelled). The version used here was adapted from Weischaus and Nüsslein-Volhard, p 223 in *Drosophila, a Practical Approach* , (ed D.B. Roberts), IRL, Oxford / Washington, 1986. Stainless steel mesh baskets, of the kind used for embryonic cuticle preps. are useful for handling the discs.

1) Dissect discs in 0.1 M $NaPO_4$ pH7.2.

2) Transfer to Fix A (4 ml 95% EtOH, 0.5 ml CH_3COOH, 1.6 ml 20% paraformaldehyde) at room temperature for 10 min.

3) Transfer to Fix B (4 ml 95% EtOH, 0.5 ml CH_3COOH, 0.4 ml 20% paraformaldehyde) at room temperature for 1 h.

4) Wash thoroughly in 70% ethanol.

5) Incubate in 2 M HCl for 10 min at 60 °C.

6) Wash with H_2O.

7) Wash with 5% acetic acid.

8) Stain in 1% basic fuchsin in 2.5% acetic acid for 20 min at room temperature. The fuchsin solution should be made up fresh every few weeks.

9) Destain in 5% acetic acid. Do not overdo this step - usually a few minutes is enough. A few salivary glands can be included as a control, as their nuclear staining is easily visible with the dissecting microscope.

10) Dehydrate in 70 %, 95% and 100% EtOH, and mount in DPX.

Bruce Kimmel

ANTIBODY AND COBALT STAINING OF PUPAL EYE DISCS

Dissection of pupae greater than 40 hrs. old:

1) Obtain a bottle or vial of flies which has wandering third instar larvae on the sides of the vessel. Identify all of the white prepupae in the vessel and circle them with a noneraseable pen noting the time at which each individual was in the prepupal stage. Incubate the prepupae at 20°C

2) Wait the appropriate amount of time before dissecting the pupae. Pupae that are less than 40 hrs. old are more mushy and therefore more difficult, but not impossible, to dissect than those that are older. There is an excellent discussion of the various stages of pupal retina development described in Cagan and Ready (Dev. Bio. 136, pgs. 346-362).

3) Remove the hard exterior cuticle by cutting the posterior 1/6-1/8th of cuticle off. This can be done with a surgical blade by carefully cutting through only the hard exterior cuticle all the way around the pupae somewhat like using a can opener.

4) Inside the hard exterior is a varying degree of mush encased in a soft transparent cuticle layer. After step 3 the posterior end of the pupae will be visible through the hole made in the exterior cuticle. Grab both the posterior end of the pupae and the anterior end of the hard external cuticle with #5 forceps gently pulling the pupae from inside the hard external cuticle. Some mush will probably leak out from the posterior of the pupae during this step but the anterior end will remain intact.

5) Place the liberated pupae immediately into PBS or 0.1 M phosphate pH 7.2 for further dissection. Using a surgical blade cut a hole, from dorsal to ventral, in the anterior edge of only the transparent soft cuticle. Be careful not to smash the anterior end too much. Once a small hole is created in the cuticle it can be enlarged by inserting the tip of the surgical blade in the hole and cutting in the dorsal and ventral directions.

6) With #5 forceps, give one or more forceful squeezes to the pupae starting near the middle and working toward the anterior. After the first couple of squeezes, the brain with attached retina and alot of mush should pop out the hole created in the anterior of the pupal cuticle. Wash the mush away with a yellow pipet tip attached to a P20. The brain and retina will not be obvious until the mush has been washed away. At 40 hrs. of pupal development the brain and retina look sort of like two mushroom tops attached by their stalks.

7) In order to transfer the brain retina complex from one vessel to another, spear the complex in the middle of the brain with a very fine tungsten needle.

Antibody Staining of Pupal Retina/Brain Complexes:

1) The pupal retina / brain complex is dissected as above in 0.1 M PO_4 or PBS and then transfered into an eppendorf tube containing 700 µl of fixation solution. Transfer the complex by spearing the middle of the brain with a fine tungsten needle. Some people prefer to transfer the complex by pipetting it into a P20 tip. Two fixation solutions which work well on fly retinas are (a) PLP (see eye disc staining protocol) and (b) PEMP (0.1 M Pipes pH 7.0, 2.0 mM EGTA, 1.0 mM $MgSO_4$, 4% paraformaldehyde). Fix the retina / brain complex for 30-40 min. on ice.

2) Remove the fixation solution by aspiration and add 1 ml of ice cold PBT (1 x PBS, 0.2% BSA, and 0.1% Triton) or PSB (0.1 M PO_4, 0.1% saponin, 0.2% BSA). The PBT buffer will provide better penetration of antibodies as triton is a stronger detergent than saponin. However, some membrane antigens are soluablized by triton so be careful in your choice of wash solution.

3) Wash the retina / brain complexes at least 30-40 min. with a minimum of 5 more 1 ml changes of wash solution (PBT).

4) Remove the last wash and replace it with 100-200µl of your primary antibody dilution in PBT + 5-10% serum. The appropriate primary antibody dilution required for your antibody must be determined empirically. Incubate retina / brain complexes in primary antibody on ice in the eppendorf tube for at least 2 hrs.

5) Remove the primary antibody and perform 3 x 10 min. washes with 1 ml of ice cold PBT solution.

6) Replace the last wash with 100-200 µl of diluted secondary antibody in PBT + 5-10% serum. Our lab uses a Goat anti-mouse HRP conjugated second antibody obtained from New England Bio Labs. We use this antibody diluted 1 / 250-1 / 500. Jackson Immuno Research sells comparable reagents conjugated with biotin, HRP, Alkaline Phospatase, and fluorescent chromophores. The choice of secondary antibodies should be based on the sensitivity required and the application. Incubate complexes in secondary antibody for 1-2 hrs.

7) Wash the complexes 3 x 10 min. with 1 ml of ice cold PBT per wash.

8)[1] During the last wash prepare the HRP staining solution which should be 1 x PBS containing 0.5mg / ml dimethylaminoazobenzene (DAB), 0.02% $CoCl_2 \cdot 6H_2O$, and 0.003% H_2O_2. The $CoCl_2$ and H_2O_2 should be added just before use of the staining solution. Remove the final PBT wash and add the staining solution to the retina / brain complexes. Follow the staining reaction under a microscope and stop it by adding two 1 ml washes of cold PBS. Unused DAB solution should be disposed of in bleach.

9) Dehydrate the stained complexes by passing them through an ethanol series (30%, 50%, 70%, 90%, 2 x 100%) and then mount the retinas in DPX (Fluka Chemicals).

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[1] This protocol describes a staining procedure which uses cobalt intensification of the DAB precipitate. Other intensification methods are also possible and are described in the eye disc staining protocol.

Once placed in DPX the retinas can be dissected from the brain hemispheres and mounted flat.

Cobalt Sulfide Staining of Pupal Retinas:

1) Dissect the pupal retinas as described above in 0.1 M PO_4. The dissection and all the other fixing and staining steps are carried out in puddles on a Sylgard dissecting dish. Transfer the retina / brain complexes from one puddle to the next by spearing the brain with a tungsten needle.

2) Fix the retina / brain complexes for 5-15 min. at r.t. in 2% gluteraldehyde, 0.1 M PO_4 (pH 7.2).

3) Dip the speared retina in a puddle of 0.1 M PO_4 briefly and then soak the retinas in 2-4% $Co(NO_3)_2.6H_2O$ for 5 min. at r.t.

4) Dip the speared retina / brain complex into water for a brief wash.

5) Transfer the retinas into 1-2% $(NH_4)_2S$ and incubate at r.t. until the retinas turn black. If the retinas do not turn black use the higher concentrations of the cobalt and $(NH_4)_2S$ reagents.

6) Wash the retinas briefly by dipping the speared complexes into water.

7) Mount the retinas in 80% glycerol or Aqua-Poly / Mount (Polysciences). Dissect the retina from the brain and mount it flat.

8) See Cagan & Ready (Dev. Bio. 136, pgs. 346-362) for an excellent description of pupal retina development.

Sylgard 184 Silicone Elastomer Kit (Dow Corning) is available from
K.R. Anderson,
2800 Bowers Ave.
Santa Clara, CA 95051
408-727-2800.
You can add India ink for a dark work surface.

revised & edited by Marek Mlodzik

ß-GALACTOSIDASE DETECTION IN EMBRYOS

The following collection contains several protocols provided by Yash Hiromi, Christian Klämbt and others.

ß-Galactosidase Activity Staining using X-gal

The protocol from Y. Hiromi contains several staining solutions that allow adjustment to the staining intensity in particular genotypes. The use of cacodylate as buffer in the fixative and DMF as solvent for X-gal can be omitted and replaced with less toxic solutions; PBS as buffer during fixation and DMSO as solvent for X-gal.
However, the more recent protocol by Ch. Klämbt appears much easier to use (no / or chemical devitellinization of embryos) and is certainly the one of choice when screening large numbers of different genotypes. It also allows counter staining with antibodies following the ß-gal activity reaction.

Protocol from Yash Hiromi

1) Dechorionate embryos with 50% bleach. Wash well with water and collect on a Nitex mesh. Blot mesh on tissue paper to remove excess water.

2) In a deep depression slide, fix embryos in 0.5 ml of heptane which is saturated with the fixative for 15 minutes at room temperature. Cover the wells with a glass slide to prevent heptane from evaporating. Embryos should turn light yellow. Whitish embryos are not well fixed and will not stain well.

3) Take embryos with a Pasteur pipette and transfer onto a slide glass. Use a piece of filter paper to remove excess heptane. After all the heptane has evaporated, take embryos by gently touching with a double stick tape and stick the tape on a slide glass, embryo side up (Siliconized slides are easier to work with.) Cover embryos with a drop of PBS.

4) Devitellinize using a dissection needle (a tungsten needle is not necessary). You can use a sewing needle whose tip is sharpened with a sand paper. Well fixed embryos should be stiff and should come out from the membrane by gently scratching the vitelline membrane. Transfer the embryos into an Eppendorf tube.

5) Remove the PBS and add ~300 ml of staining solution without X-gal (i.e. Fe / NaP or Fe / CP). Leave at room temperature for ca. 5 minutes.

6) Remove liquid and replace with staining solution. Incubate from 2 hours to overnight at room temperature or 37° C.

7) Remove the staining solution. Rinse embryos by vortexing once in 70% ethanol and once in 100% ethanol.

8) Store the embryos in 90% glycerol / PBS at 4° C. They can be kept for months at this stage. Mount using two #1 coverslips as spacer.

Comments:

a) Staining with Fe / NaP pH 7.2 and higher temperature (37° C) results in stronger staining, presumably because the conditions are closer to the optimum for the enzymatic activity. For ftz / lacZ fusion gene transformants, staining using Fe / CP overnight at room temperature is sufficient for visualizing stripes in the extended germband stage embryos and for CNS staining. To stain stripes at the blastoderm stage, use Fe / NaP pH 7.2 and stain overnight at 37° C. In this case, do not include older embryos in the same tube, because they will stain too strongly and may transfer the blue reaction product to the other embryos in contact. For best resolution, try to use conditions that allows you to stain overnight, rather than stopping the reaction after a short incubation period. Staining at 37° C will increase the size of reaction product crystals and will make identification of stained cells more difficult.

b) White precipitate (X-gal crystals ?) will form in the staining solution during incubation. This does not affect staining reaction, but will stick to the embryos. Vortexing in ethanol helps to remove it from the embryos.

c) Embryos can also be dehydrated and mounted in Epon for permanent preparations. GMM can not be used because blue dye appears to dissolve (although slowly) in this medium.

Solutions:

Fixative (prepare fresh solution every day):

0.1 M Na cacodylate buffer pH 7.3	1 ml	
50% glutaraldehyde (EM grade Fluca #49631)		1 ml
heptane		2 ml

Shake well and let the phases separate. Use the heptane (upper) phase for fixation. Fixative (lower) phase can be reused by adding heptane to 2 ml.

Fe / NaP-solution (pH 7.2):

0.2 M Na_2HPO_4	1.8 ml
0.2 M NaH_2PO_4	0.7 ml
5 M NaCl	1.5 ml
1 M $MgCl_2$	50 ml
50 mM $K_3(Fe(CN)_6)$	3.05 ml
50 mM $K_4(Fe(CN)_6)$	3.05 ml
H_2O	to 50 ml

Store in dark at room temperature.

Fe / CP-solution (pH 8):

50 mM $K_3(Fe(CN)_6)$	5 ml
50 mM $K_4(Fe(CN)_6)$	5 ml
CP pH 8	40 ml

Store in dark at room temperature.

CP-solution (pH 8):

0.1 M citric acid	27.5 ml
Na_2HPO_4	27.61 g
H_2O	to 1 l

Staining solution:

Warm up Fe / NaP or Fe / CP to 37° C. Add 1 / 30 volume of X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) solution (8% in DMSO, store at -20° C) . Use 200 to 300 ml per tube of embryos.

Modification for weak ß-Galactosidase Expression

With weak ß-galactosidase expression, long incubation of glutaraldehyde fixed embryos in staining solution results in an undesirably yellow embryo. It is possible to avoid this with the following modification

1) Fix dechorionated embryos for 20 minutes in a monolayer in heptane previously equilibrated with 18% formaldehyde in 0.1 M cacodylate buffer, pH 7.4.

2) As in other X-gal protocols, dry embryos and mount on double sided tape, inside a silicone sealant well on a slide.

3) Wash with staining buffer, then incubate for up to 3 days at 30° C in staining solution with Xgal. Refresh solutions half way through.

4) Wash out buffer and replace with fixative (for example 4% formaldehyde in PBS). Poke a hole in the vitelline, let fixative penetrate for at least 10 minutes, and then devitellinate embryos. Store in 80% glycerol / 20% PBS until photographed.

Protocol from Christian Klämbt.

All steps are performed in custom made multi well plates unless otherwise noted.

I. Preparation of embryos still in their vitelline membrane:

1) Collect embryos and wash with PBT.

2) Dechorionate in 50% bleach for 4 minutes and wash for 2 to 5 minutes with PBT.

3) Blot the embryos dry and place them in n-heptane saturated with 2.5% glutaraldehyde / PBS. The PBS / glutaraldehyde solution can be kept and used for the saturation of fresh n-heptane for a long time (up to 8 months at 4° C).

4) Fix for 7 minutes while gentle agitating at RT.

5) Blot away excess heptane and wash embryos with PBT until they sink to the bottom and do not stick to each other any more.

6) Wash and permeabilize for another 2 hours with several changes of PBT (Even slight contamination with heptane on the vitelline membrane will cause diffuse blue background staining.)

II. Staining:

1) Heat the staining solution to 65° C, or over a flame until the solution gets cloudy (This will greatly reduce the formation of crystals during the staining process). Add 1 / 50 volume of 10% X-gal solution in DMSO to the warm staining solution.

2) Blot away excess PBT and place embryos in the staining solution.

3) Stain for 2 to 4 hours at 37° C in a humid chamber and stop the reaction by washing with PBT.

III. Preparation of devitellinized embryos for X-gal staining:
Methanol and also ethanol will destroy lac-Z activity. However, if the devitellinization step is done fast, the methanol as well as the ethanol do not significantly destroy the enzyme's activity.

1) Wash, dechorionate and fix the embryos as in **(I.)**.

2) Blot away excess heptane fix and wash the embroys in n-heptane.

3) Transfer the embryos in 1 to 2 ml heptane into a 5 ml glass tube, add 1 to 2 ml methanol.and shake vigorously.

4.)After 10 to 20 seconds the interphase becomes visible. Aspirate the heptane phase and half of the methanol phase off (at this point not all the embryos will have made it to the bottom of the glass tube. You will lose 30 to 50% of the embryos).

5) Add 2 ml methanol for a wash. Aspirate all methanol and wash twice with 80% ethanol. Do not let the embryos sit in methanol or ethanol for longer times than neccessary.

6) Wash 3 times for 5 to 10 minutes with 4 ml PBT on a rocking platform.and wash for an additional 2 hours in PBT.

7) Transfer the embryos back into a multi well plate and proceed with the staining as in **(II.).** *Do not let the embryos dry at this point.* If you want to look at the X-gal stained embryos, wash briefly in PBT and mount them in PBS/50% glycerol.

If counter staining of the embryos with specific antibodies is desired, wash away staining solution with several changes of PBT for 2 hours. Subsequently wash embryos with PBT containing BSA and 5-10% goatserum for 1 h and proceed with the usual staining protocol for antibodies.

Solutions:

Staining solution (200 ml):

10 mM PO_4-buffer pH 7.2	2.0 ml 1M
150 mM NaCl	6.0 ml 5M
1 mM $MgCl_2$	0.2 ml 1M
3 mM $K_4[FeII(CN)_6]$	0.3 g
3 mM $K_3[FeIII(CN)_6]$	0.2 g
0.3 % Triton X-100	0.6 ml

PBT: PBS with 0.5 % Triton X-100 (but without BSA)

Visualization of ß-Galactosidase for EM Histology (Protocol from Roger Jacobs)

1) Process for X-gal staining as described above for light microscopy. After staining, transfer to 0.1 M cacodylate buffer, pH 7.4, and select the embryos you wish to embed.

2) Fix in primary EM fix (2% glutaraldehyde, 2% paraformaladehyde in 0.1 M cacodylate buffer, pH 7.4) for 30 minutes.

5) Wash in cacodylate buffer with at least 3 changes for 5 minutes.

6) Fix in 1% osmium tetroxide in cacodylate buffer for 30 minutes

7) Wash in cacodylate buffer, followed by distilled water.

8) Stain in 5 % uranyl acetate for 30 minutes.

9) Dehydrate and infiltrate as in standard EM protocol. Older whole-mount embryos need longer infiltration times. Infiltrate in pure plastic for 24 to 48 hours (Note dissections infiltrate through a graded ethanol-plastic series!). Use for example Epon-Araldite plastic. You may also use methacrylate based plastics, but this will make semi-thin sectioning (0.5 µm) more difficult. It is much easier to embed whole-mount embryos in the middle rather than the bottom of the block. Cook plastic in half filled molds for about 4 hours, then add embryos in new plastic to the top half of the mold. You can orient the embryos in the mold with a fine probe.

Sectioning and Staining:

Trim and orient your tissue by conventional technique. Sections of 1.0 µm or thicker can be picked up individually off a dry glass knife and placed on a small drop of water on a subbed (1% gelatin and 0.1% chromium potassium sulphate x 12 H_2O) slide. For serial sectioning, use a glass knife with a small water boat behind it [made with mylar tape, or LKB's prefab plastic boats (#2208-100)]. 0.5µm is the easiest to handle. Ribbons of sections can be spread with a chloroform wick, and transferred to a dry subbed slide using the wire loop technique. Merely touch the loop containing the ribbon and water to the slide, and remove the water by touching a sliver of filter paper to the edge of the loop.

Best tissue contrast is obtained with 0.5% Toluidine Blue O in 1% Borax buffer. This stain is preferred when you are trimming and orienting, but will not allow you to visualize X-gal product.

To preserve contrast with X-gal, counterstain with saturated aqueous Basic Fuchsin. This stain penetrates epon slowly, but there are two tricks which will increase permeability. First, you might soak your unstained slide in xylene for 20 to 30 minutes, wash in acetone, and dry before staining. Secondly, add a drop or more of acetone containing 1% Basic Fuchsin to the slide when staining with saturated aqueous Basic Fuchsin on a hotplate (65° C).

Slides should be permanently mounted with permount and coverslip, an viewed under oil immersion optics. You can now enhance X-gal staining contrast by using appropriate filters (for example a yellow filter or in extreme cases, a red filter will darken the image of the crystals relative to the rest of the tissue).

In EM, X-gal crystals make large ugly needles that actually penetrate cell walls, and fill vacuoles inside cells. It is therefore useful mostly in low magnification work.

Immunohistochemical Detection of ß-Galactosidase

The detection of ß-galactosidase with an antibody and immunohistochemical staining gives the best possible single cell resolution. However, it is more laborious than activity staining.

I usually follow the standard protocol for immunohistochemistry in embryos as described in the relevant section of this methods book. The following reagents and dilutions are normally employed:

primary antibody:	anti-ß-gal monoclonal from Promega use at 1/200 to 1/500 dilution in PBT(PBS, BSA, Triton; for recipe see immunohistochemistry protocol) cont. 5% goat serum (The quality of the anti-ß-gal monoclonal is obviously important. However, we have sometimes had problems obtaining consistent results due to lower quality of the primary antibody. Nevertheless, Promega still provides the best monoclonal commercially available.)
secondary antibody	HRP-conjugated goat-anti-mouse polyclonal from Biorad use at 1/200 dilution in PBT/goat serum (see above)
HRP reaction	0.5 mg/ml DAB in PBT add 1µl of 3% Hydrogenperoxide and 8µl 8% NiCl(2) 7H(2)O for each ml of DAB solution

Embryo pretreatment, incubations, washes, staining reaction and mounting of the embryos is performed as described by the standard immunohistochemistry protocol.

Marek Mlodzik

ß-GALACTOSIDASE DETECTION IN IMAGINAL DISCS

The following protocols for ß-Galactosidase activity staining with X-gal and immunohistochemical staining in 3rd instar larval imaginal discs are based on the protocols for detection in embryos.

Activity staining using X-gal

1) Wash 3rd instar larvae (wandering stage) in PBS for a few minutes. Dissect in PBS.

2) Dissect anterior mouth complex by grabbing larvae at each end (Use Dumont #5 forceps). Hold larva at the posterior end and pull at the anterior end holding the mouth parts with the forceps. Usually, tissue still attached to the mouth hooks contains (most) imginal discs (the wing discs get sometimes lost), the brain / CNS complex, the salivary glands, some fatbody and anterior parts of the gut. Dissect about 5 to 6 larvae for each genotype.

3) Leave all this tissue with the mouth hooks (it can be dissected at the end of the staining procedure) and fix tissue in 1% glutaraldehyde in PBS for 15 min at RT.

4) Remove fix and wash tissue with PBS for 10 min, 2 changes of buffer.

5) Prewarm staining solution without X-gal for 10 min at 37 C.

6) Add X-gal (1 / 30 of a 8% stock in DMSO) to staining solution and incubate tissue in this solution for several hours to overnight, use 100-200µl per reaction (genotype).

7) After staining is completed remove staining solution and rinse discs once with PBS (optional) and resuspend them in 80% glycerol / PBS. Leave tissue for a few hours in glycerol solution before mounting.

8) Transfer tissue in a drop of glycerol (with cut off yellow tip) onto slide. Dissect away unwanted tissue (e.g. mouth hooks) before mounting under a cover slip. For permanent storage seal cover slip with nail polish.

Comments:
Some people prefer NaPO(4)-Buffer (pH 7.4) to PBS in dissecting and wash steps.
The staining solutions are the same as for the embryos (see embryo ß-gal protocol).

Due to the small overall size of the eye disc and the small cell size in the disc, diffusion of the ß-gal staining is more a problem in discs than in embryos.
In lacZ-lines with strong expression the following alternative staining solution can be used; it reduces diffusion but also reduces the sensitivity of the staining.

Alternative staining solution (based on A. Fire's protocol, moidified by U. Gaul):

1 ml of solution contains:

695 µl	H(2)O
1 µl	MgCl(2), 1M

200 µl	NaPi (0.5m, pH 7.2)
100 µl	Redox. Buffer 100mM K(3)Fe[CN]6; K(4)Fe[CN]6
4 µl	1% SDS (made fresh from 10% stock)

immediately before staining add 1/30 vol of 8% X-gal

Immunohistochemical Detection

Eye imaginal disc:

Dissections are performed as described in eye disc antibody staining protocol.
The antibody incubations and staining are also virtually identical to the protocol for eye disc antibody stainings (see specific section).

The primary antibody, anti-ß-gal monoclonal (from Promega), is used at 1/200 to 1/500 dilutions; the secondary, HRP conjugated goat-anti-mouse (from BioRad) is used at the same dilutions. For the incubations and washes, I usually use PBT (PBS, BSA, Triton X-100; see embryo protocols) instead of 0.1m NaPO(4), 0.1 % saponin. However, there is no significant difference in staining intensity or background between those two alternatives.

There are several options for intensification of the signal during and after the peroxidase/DAB staining reaction. The eye disc antibody staining protocol describes the osmium tetroxide postfixation and the use of Co and Ni in the DAB reaction (for details see eye disc staining protocol). I prefer to add just NiCl(2) to the DAB reaction (no Cobalt) as described in the ß-gal detection protocol for embryos. Since this enhancement method does not postfix the tissue, an ethanol dehydration serie, 30%, 50%, 70%, 90% and 100% EtOH, each for 10 min, is necessary to avoid shrinking of the tissue (this is quite important because the disc is already small enough!). A postfixation in glutaraldehyde as an alternative is only suitable for strongly stained discs, because part of the signal fades out during this process.

The dehydrated tissue can be stored in 100% ethanol for several days. Mounting of the discs in DPX mounting medium is as described in the general eye disc staining protocol.

M. Fortini

ADULT HEAD FROZEN TISSUE SECTIONS

ß-GALACTOSIDASE ACTIVITY STAINING

1) Embed up to 12 fly heads in O.C.T. Tissue Tek compound (Miles Scientific) under a dissecting microscope. For horizontal sections, which are generally the best for examining compound eye and optic lobe structures, the heads should be oriented upright in the block, so that the ocelli are pointing towards the microscope objectives. Slow freezing by periodic immersion of the block support in a dry ice/ethanol bath allows several heads to be precisely positioned in a single block with fine forceps.

2) Pretrim the block and place it in the cryostat for 20-30 minutes prior to cutting to equilibrate the block to the cryostat temperature. Cut 10-14 µm sections on the cryostat and transfer section ribbons onto slides (subbed slides not necessary). All the sections from one block may usually be fit on a single slide.

3) Warm the slides at 50-60°C for 2-3 minutes and then allow them to air dry for about 1 hour. In my experience, slides may be kept for several days sitting on a benchtop at this stage with no apparent loss of enzyme activity.

4) Fix the tissue sections in 2.0 % glutaraldehyde in PBS for 15-20 minutes at room temperature in a coplin jar.

5) Wash the slides two or three times in PBS for 5 minutes/wash.

6) After the last wash, remove excess PBS with a tissue and place the slides on a level surface in a moist airtight chamber. A Tupperware box with water-soaked blotting paper works well for this purpose. To keep the slides off the paper, they should be placed on two lengths of plastic 5 ml or 10 ml pipettes which rest on top of the paper block.

7) Allow the remaining PBS on the slides to dry (5-10 min at room temp). The slides may be left to dry for a few hours at this point with no apparent loss of enzyme activity.

8) Apply 50 µl of a prewarmed staining solution + X-gal to each slide and cover with a cover slip (50 µl suffices for a 22 x 40 mm cover slip - adjust accordingly for different sizes).

Staining solution: 50 ml total (store at room temperature in a foil-wrapped 50 ml conical tube in the dark - stable for months).

1.8 ml of 0.2 M Na_2HPO_4
0.7 ml of 0.2 M NaH_2PO_4
1.5 ml of 5 M NaCl
50 µl of 1 M $MgCl_2$
3 ml of 50 mM $K_3(Fe(CN)_6)$
3 ml of 50 mM $K_4(Fe(CN)_6)$
bring to 50 ml with ddH_2O

X-gal 30X stock solution: store at -20°C
10% X-gal in N,N-dimethylformamide

IMPORTANT: To avoid instantaneous precipitation of X-gal crystals when applying solution to slides, it is necessary to prewarm these solutions. Generally, while the slides are in the fixative or washes, I aliquot out the required amount of staining solution into an eppendorf tube and incubate in a 42°C temp block for about 5 minutes. I then add 1/30 volume of the X-gal stock solution to this tube, quickly vortex or shake, and place it back in the 42°C temp block. I apply the staining solution + X-gal onto the dried slides as quickly as possible, put on all the cover slips, and immediately put the sealed chamber in a 37°C incubator.

9) Allow the staining reaction proceed for as long as necessary (from a few minutes to overnight).

10) Examine the slides periodically under a dissecting microscope. When the staining appears to be complete, float the cover slips off in PBS and wash the slides 2 x 5 minutes in PBS.

11) At this point, the slides may be dehydrated through a graded ethanol series and mounted in DPX (Fluka) or other suitable mountant. The ethanol series also helps remove X-gal crystals deposited on the tissue sections during the staining reaction. Alternatively, the slides may be simply mounted in 70% glycerol in PBS after the final PBS wash (in my experience, the glycerol mounting gives better morphology for photomicroscopy but is less suitable for long-term storage of slides).

12) For Hoechst labeling of nuclei, do not dehydrate the sections. Instead, pipette 0.5-1.0 ml of a 1 µg/ml solution of Hoechst 33258 in PBS directly onto each slide. After 1-2 minutes, drain the dye solution off, rewash the slides in PBS a few times (5 minutes each) and mount in 70% glycerol/PBS.

ANTIBODY STAINING

The protocol provided below has been found to work well for antibody stainings of frozen sections of *Drosophila* head tissues, and it should also be suitable for staining of other tissues. The sucrose infiltration step (2) provides improved cryoprotection of the tissue sections but may be omitted if good morphology of the tissue is not required.

1) Fix the tissue of interest in 2.0% formaldehyde in PBS at 4°C. For *Drosophila* heads, first remove the proboscis and fix the head capsule for 60-90 minutes. For other types of tissues, adjust the fixation time according to their relative size and estimated efficiency of infiltration.

2) Wash the tissue in PBS and transfer to 12% sucrose in PBS at 4°C. Allow the sucrose solution to infiltrate the tissue for 16 hrs.

3) Remove the tissue from the sucrose solution and submerge it in a drop of O.C.T. Tissue Tek Compound (Miles Scientific). Allow the tissue to be permeated by the O.C.T. compound for 10-30 minutes at room temperature and then embed the tissue in frozen O.C.T. compound using an ethanol-dry ice bath (see preceding protocol.)

4) Place the frozen block containing the tissue to be sectioned in the cryostat chamber for at least 20 minutes to equilibrate it to the cryostat temperature (-14°C to -18°C).

5) Cut 10-14 µm sections on the cryostat and load onto freshly gelatinized slides.

6) Heat slides briefly at ~40°C on a drying plate for no more than one minute to dry the sections onto the slides. Overheating may adversely affect the antigenicity of the protein of interest and the morphology of the tissue.

7) Fix the sections immediately in 0.5% formaldehyde in PBS for 20-60 minutes at room temperature. Slides may be stored in this fixative at 4°C for a few days if necessary.

8) Wash the slides two or three times for 2-3 minutes / wash in PBS.

9) Block the slides for 30 minutes in PBSG (0.2% BSA, 1% goat serum, 0.01% saponin in PBS).

10) Wash the slides through several changes of PBS / 0.01% saponin.

11) Apply 75-150 µl of the primary antibody at the appropriate dilution in PBSG to each slide. Unless an excess of antibody solution is used, gently place cover slips on the slides to evenly spread the antibody solution over the tissue sections (100 µl of solution suffices for a 22 x 40 mm cover slip). Incubate the slides in the primary antibody for 30-60 min at room temperature or overnight at 4°C in a moist, airtight chamber (ie. a Tupperware box containing wet blotting paper).

12) Wash the slides through several changes of PBS / 0.01% saponin (float off the cover slips in the first wash).

13) Incubate the sections with the secondary antibody at the appropriate dilution in PBSG exactly as described in step 11 for the primary antibody. For Bio-Rad Laboratories HRP-conjugated goat-anti-mouse IgG, a 1:200 dilution for 30-60 minutes at room temperature works well.

14) For an HRP-conjugated secondary antibody, the slides are again washed through several changes of PBS / 0.01% saponin and then incubated in 0.5-1.0 ml of staining solution per slide without cover slips or 100-150 µl per slide with cover slips. The staining solution consists of: 0.5 mg / ml diaminobenzidine (DAB), 0.003% H_2O_2, 1.5 mM $CoCl_2$, and 1.5 mM $NiCl_2$. The $CoCl_2$ and $NiCl_2$ are optional components; in their absence, a brown product forms, whereas in their presence, a more intense blue-black product is obtained. Since the extent and localization of background staining can vary considerably between intensified and non-intensified preparations, it is often worth trying both methods in parallel.

NOTE: *DAB is thought to be a potent carcinogen and is to be handled with care. All DAB-containing tubes, pipette tips, used DAB solutions and similar materials should be immersed overnight in 100% bleach and disposed of according to the proper regulations.*

15) Monitor the staining reaction under a dissecting microscope. When the reaction has proceeded to completion (usually 5-30 minutes), it is stopped by washing the slides through a few changes of PBS.

16) For compound microscope viewing and photomicroscopy, the sections may either be dehydrated through a graded ethanol series and mounted under cover slips in a stable mountant such as DPX (Fluka Biochemie), or they may be mounted under cover slips in 70% glycerol / PBS after air-drying without dehydration. In general, the glycerol mounting is superior for photomicroscopy but is not suitable for stable, long-term storage of samples.

17) For Hoechst flourescent counterstaining of nuclei, do not dehydrate the sections after step 15. Instead, pipette 0.5-1.0 ml of a 1.0 µg / ml solution of Hoechst 33258 in PBS directly onto each slide, allow to sit for 1-2 minutes, then rinse off through a few changes of PBS and mount in 70% glycerol / PBS as described above.

Yash Hiromi

WHOLE MOUNT STAINING OF DROSOPHILA RETINA FOR β-GALACTOSIDASE ACTIVITY

1) Prepare postfix solution in wells of a microtiter plate, and fixative-saturated heptane in a well of a **deep** depression slide.

2) Fix whole adults in 0.5ml of fixative-saturated heptane for 20 minutes at room temperature. Cover the wells with a sideglass to prevent heptane from evaporating.

3) Remove adults from heptane and place on a piece of blotting paper for a few seconds to evaporate heptane. Place in a drop of postfix solution in a shallow depression slide. Remove body from the head and discard.

4) Using two tungsten needles, dissect retina from head as follows. First, cut along the edge of the lens and remove retina + cornea from the rest of the head. In most cases a thin pigmented layer of cells located between the retina and the lamina will come off the retina. Since in many lacZ lines this layer stains heavily and masks the expression in the retina, it needs to be removed by scratching with a fine needle. Then, remove the lens from the retina by inserting the needle between the retina and the cornea. Removal of the cornea is not absolutely necessary for staining, but it accelerates bleaching of the pigments and will also make the preparation hydrophilic. Transfer the dissected retina to postfix solution in a microtiter plate and fix for an additional 10 minutes.

5) Remove the postfix solution using a drawn out Pasteur pipet and rinse once with PBS, and then replace the PBS with staining solution without X-gal (i.e., Fe / NaP or Fe / CP). Leave at room temperature about 5 minutes.

6) Remove liquid and replace with staining solution. Incubate 2 hours to overnight at room temperature or 37°C.

7) Remove stianing solution. Rinse retina once in 70% ethanol and once in 100% ethanol. This step helps to remove X-gal crystals that are adhering to the sample.

8) Remove ethanol and add 50% glycerol / PBS. Take retina out into a drop of 50% glycerol in a depression slide and remove X-gal crystals with a fine needle. Mount in 50% glycerol using one #1 coverslip on one slide.

Staining with Fe / NaP pH7.2 and higher temperature (37°C) results in stronger staining, presumably because the conditions are closer to the optimum for the enzymatic activity. For *Rh4/lacZ* fusion gene transformants, using Fe / NaP overnight at room temperature allows you to stain overnight, rather than stopping the reaction after a short incubation period. Staining at 37°C will increase the size of reaction product crystals and will make identification of stained cells more difficult.

Fixative-saturated Heptane (Prepare fresh solution every day)

0.1M Na cacodylate buffer pH7.3	1ml
50% gluteraldehyde (EM grade, Fluka #49631)	1ml
heptane	2ml

Shake well and let the phases separate. Use the heptane (upper) phase for fixation. Fixative (lower) phase can be reused by adding heptane to 2ml.

Postfix Solution

0.1M cacodylate buffer pH7.3	2ml
water	80µl (1% final concentration)
50% gluteraldehyde (EM grade, Fluka #49631)	20µl (0.05% final concentration)
10% Triton X-100	

Fe/NaP (pH7.2)

0.2M Na_2HPO_4	1.8ml
0.2M NaH_2PO_4	0.7ml
5M NaCl	1.5ml
1M $MgCl_2$	50 µl
50mM $K_3(Fe(CN)_6)$	3.05ml
50mM $K_4(Fe(CN)_6)$	3.05ml
H_2O	to 50ml

Store in the dark at room temperature.

Fe/CP (pH8)

50mM $K_3(Fe(CN)_6)$	5ml
50mM $K_4(Fe(CN)_6)$	5ml
CP pH8	40ml

Store in dark at room temperature.

CP (pH8)

0.1M citric acid	27.5ml
Na_2HPO_4	27.61g
H_2O	to 1 liter

Staining Solution

Warm up Fe/NaP or Fe/CP to 37°C. Add 1/30 volume of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (8% in DMF (*N*,*N*-dimethylformamide), store at -20°C).

Kevin Moses

EMS MUTAGENESIS

EMS

(*ethane methyl sulfonate* or *methanesulfonic acid ethyl ester* as *Sigma* calls it) A chemical mutagen that most commonly causes transitions by methylation of G residues but can yield a spectrum of mutations such as deletions etc. Mr = 124.2g ρ = 1.17 g/ml. EMS can be ordered from *Sigma* (catalogue No. M-0880).

Safety

EMS is a mutagen and a carcinogen. It should therefore be handled in a hood with gloves. EMS solutions can be deactivated in a solution of 4g NaOH and 0.5 ml thioglycolic acid in 100 ml. We store contaminated dry waste in sealed plastic bags under a hood, which are periodically removed by University safety officers.

Screen design

Normally one treats males and then crosses them to untreated female virgins. In a simple screen these females will be marked with the mutation of interest and the F1 progeny can be screened for new alleles. There are many more sophisticated types of screen. In order to distinguish the chromosome bearing the new allele from the maternal chromosome one of these may be marked with another (at best closely linked) mutation, ideally a recessive also present on the appropriate balancer. It may be desirable to screen only the F1 sons in order to avoid recombination between the marker and new allele. It is best to screen for some other RK1 marker while screening for the gene of interest, (probably the best are eye colour mutations such as *white, cinnabar* or *scarlet*) to give you something to find and as a running positive control for the effectiveness of the treatment.

Males

It is best to use more males than you would normally as their virility can be affected by the treatment. Avoid the marker *yellow*, which reduces virility and increases mortality during treatment. Ideally treat 3 day old males. Those that are too young will not yet be fertile, those that are too old may suffer from Portnoy's complaint.

Dosage

Dosage is controlled by EMS concentration and can be monitored by scoring for X-linked lethals. This is best done by crossing some of the treated males *en masse* to virgins bearing an X-chromosome balancer (such as FM6). The resulting F1 daughters are allowed to mate freely to their brothers, and after several days are placed in tubes individually and allowed to lay for a few days, and are then discarded. The F2 sons must inherit the X-chromosome from their F1 mothers which are now a single isolates (called *) from the original mutagenised grand-fathers. The F2 males can thus be either FM6/Y or */Y. In some lines * can carry a newly induced lethal mutation and thus only FM6/Y sons will appear. Thus the % of lines with no Bar^+ F2 sons is the % newly induced X-linked lethals. Typically 25 mM EMS will yield about 30% X-linked lethals, which equates to roughly one hit per autosome on average, and 1 hit in 2000 to 5000 for most loci. The effective dosage of EMS may vary with different lab conditions or stocks, so a pilot run to test several EMS concentrations may be worthwhile.

Mosaicism

Orthodoxy holds that EMS mutated post-meiotic sperm undergo DNA repair in the embryo. This may well occur after the first cleavage division so it is quite common

for the F1 progeny to be somatic and sometimes germ-line mosaics for a new allele. Thus often apparently mutant F1 individuals can yield a mixture of F2 progeny, or no F2 individuals bearing the new allele. This is tiresome but unavoidable, and the rate of such "false positives" will depend in part on the distance on the zygotic fate map between the organ which is the focus of your mutation (e.g. the eye for *white* mutations) and the germ line. This rate of "false positives" can easily be 50%.

Clusters

If progeny are recovered from cells that were pre-meiotic during EMS treatment, several identically mutant sperm could result from their subsequent mitotic division. Thus the same allele could be recovered multiply. As spermatogenesis requires 5 days at 25°C, so long as the parents are discarded on the fourth day after treatment this should not occur. It may be that the mutagenesis rate is not constant in these different daily cohorts of sperm.

References

These topics are well discussed in Tom Grigliatti's chapter 2 *Mutagenesis* in David Roberts' book *Drosophila a practical approach* (IRL press, Oxford, Washington DC **1986**), and in Michael Ashburner's *Drosophila, A Laboratory Handbook* (Cold Spring Harbor Press, Cold Spring Harbor New York, 1989).

Treatment :

1) Make solution I (100mM Tris Ph7.5 and 10% sucrose). Autoclave and store at room temperature.

2) On the day of use make deactivating solution (4g NaOH + 0.5 ml thioglycolic acid in 100 ml). Put this in the hood along with plenty of paper towels etc. and a plastic bag for dry waste such as gloves and yellow tips.

3) **In the hood, on a diaper,** dilute solution I 1+9 and add EMS. 26.5 µl EMS in 10 ml yields a 25mM EMS solution.

4) Make up glass tubes with wadded kimwipes or filter papers in the bottom, and cotton bungs on top. Make two such for every 100 males to be treated.

5) Transfer the males to empty bunged vials and allow to dehydrate for 30 minutes.

6) **In the hood, on a diaper**, pour the EMS solution from tube to tube so as to thoroughly soak the paper in half of your prepared tubes and dispose of the residue by dilution in deactivating solution. Do not allow the cotton bung to become wet (this will prevent the tube from ventilating when the males are in it and they will die).

7) **In the hood, on a diaper,** transfer the dehydrated males to the EMS tubes, and leave them there over night. Leave a sign up on the hood to notify others of the presence of EMS.

8) In the morning transfer the males on to the dry tubes to allow them to wipe their feet and hopefully shit-out most of the EMS.

9) After 30 minutes transfer the males to freshly yeasted ordinary fly food tubes and properly dispose of all of the dry waste. The males may now be removed from the hood. Allow them a couple of hours to recover, then cross them.

10) Sub on the crosses every day for four days and discard the adults on the fifth.

Marek Mlodzik

LOCAL MUTAGENESIS BY IMPRECISE P-ELEMENT EXCISION

This method is based on the fact that in most cases P-elements excise imprecisely when transposing via the P transposase. Most of these imprecise excision events leave sequences from the P elements behind. However, in a reasonable proportion of excisions (~10%) flanking genomic DNA is removed with the P elements, creating small deletions around the original P element insertion point.

The use of this approach allows to create local mutations in genes neighboring the original P insertion, e.g. in lines of the P[lacZ] insertions that do not have a phenotype by itself.

The following crossing schemes are using the Δ2-3 element at 99B as a source of the transposase. The given schemes are designed for P-elements with *rosy* as selectable marker. Nevertheless, very similar schemes can be used for other common markers such as the *white* gene.

II. Chromosome P Insertions
1st generation:

MM		mm
P[ry$^+$] / P[ry$^+$] ; ry / ry	x	CyO / Sp ; Sb, Δ2-3 / TM6, Ubx

2nd generation (here occurs the excision):

M (single males)		mm
P[ry$^+$] / CyO ; Sb, Δ2-3 / ry	x	CyO / Sco ; ry / ry

3rd generation:

M (single males, *ry* eye color)		mm
E{P} / CyO ; ry / ry	x	CyO / Sco ; ry /ry

4th generation (brother-sister cross):

MM		mm
E{P} / CyO ; ry / ry	x	E{P} / CyO ; ry / ry

established stock, if excision is lethal; check homozygous flies for visible phenotypes.

III. Chromosome P Insertions

1st generation:

MM		mm
P[ry^{+}], ry / P[ry^{+}], ry	x	Ki, p^{p}, Δ2-3 / Ki, p^{p}, Δ2-3

2nd generation (here occurs the excision):

M (single males)		mm
P[ry^{+}], ry / Ki, p^{p}, Δ2-3	x	TM3, ry / rf10 (ftz, e, ry)

3rd generation:

M (single males, *ry* eye color)		mm
E{P}, ry / TM3, ry	x	TM3, ry / rf10

4th generation (brother-sister cross):

MM		mm
E{P}, ry / TM3, ry	x	E{P}, ry / TM3, ry

established stock, if lethal; check for visible mutations in homozygous flies

X Chromosome P Insertions:

1st generation:

mm		MM
P[ry^{+}] / P[ry^{+}] ;; ry / ry	x	+ / -- ;; Ki, p^{p}, Δ2-3/ Ki, pp, Δ2-3

2nd generation (here occurs the excision):

M (single males)		mm
P[ry^{+}] / -- ;; Ki, p^{p}, Δ2-3 / ry	x	FM6 / dnd ;; ry / ry

3rd generation:

m (single virgins, *ry* eye color)		MM
E{P} / FM6 ;; ry / ry	x	FM6 / -- ;; ry / ry

established balanced stock, if excision is lethal; check for visible phenotypes in homozygous flies.

Richard Carthew

X RAY MUTAGENESIS

Collect 0 to 2 day old adult males and let them age a further 2 days at 25 C. Aliquot the flies into groups of 50 each. Place each group of 50 into a gelatin capsule (Lilly, No.000) which has been perforated with a 23 gauge needle. Put the capsules in a petri dish on ice. Make sure the flies get cool. Take care that the flies and capsule do not get wet. Place the dish in the X ray machine and give the flies a 4000 rad dose (115 kV; 5 mA). Alternatively, place males in empty vials and lay vials on their sides in x-ray machine before giving them a 4000 rad dose. Immediately transfer the flies to a vial with plenty of yeast and allow them to recover for a few hours. Let the males mate with an equal number of 3 day old virgin females. I like to put 30 of each sex in a bottle. Knock the flies into fresh bottles on successive days and on the fourth day discard the flies. Starting with 400 males, one can easily get ten to twenty thousand F1 flies to screen.

Richard Carthew

MICROSCOPIC ANALYSIS OF THE ADULT RETINA

Fixation and embedding for microscopy:

Dissect the head away from the body. With a number 11 scalpel blade, gently cut away one of the eyes to expose the inside of the head. Avoid putting pressure on the head when cutting (making the other eye 'bulge'). Place the dissected head into 2% glutaraldehyde in phosphate buffer. This solution should be on ice. Often it is necessary to tap the head down into the solution as it has a tendency to float. Alternatively, you can give them a 5 second spin in a microfuge to submerge it.

Add an equal volume of 2% OsO_4 in phosphate buffer. Only handle OsO_4 in the hood and wear gloves; it is volatile and extremely toxic. Incubate the mixture for 30 min on ice. Remove the glutaraldehyde / OsO_4 mixture and wash the tissue with cold phosphate buffer by filling the tube completely. Dispose of waste glutaraldehyde and OsO_4 by adding an excess of dry milk powder to the waste solutions in a bottle. The deactivated solution can then be safely removed. Add fresh OsO_4 to the tissue and incubate on ice for 1-2 hours. Discard the OsO_4 and dehydrate the tissue by successive 10 min incubations on ice in the following ethanol solutions: 30%, 50%, 70%, 90%, 100%. For preparing tissue for electron microscopic analysis, also include an 80% EtOH wash. Repeat the final 100% EtOH incubation.

Replace the final alcohol treatment with propylene oxide and incubate for 10 min at room temperature. Handle propylene oxide with gloves and only use it in the hood. Repeat the propylene oxide wash twice. Add an equal volume of durcapan resin to the last propylene oxide wash and thoroughly mix. Handle resin with non-latex gloves as it is carcinogenic when unpolymerized. Incubate overnight at room temperature. Use soft resin for semi-thin and thick sectioning and use hard resin for thin sectioning.

Replace the resin / propylene oxide mixture with pure resin and incubate at least 4 hours. Place tissue in a drop of resin on a slide. Examine the head under a dissecting microscope and discard any poorly fixed heads. Dissect away unwanted tissue from the head. Using a needle, transfer a fixed head to a mold which has been filled with resin. Useful molds are BEEM or silicone rubber flat embedding molds (Ted Pella Co.). Place the head very close to the edge of the mold (Figure 1). Orient the head so that the head is anterior up and the eye to be sectioned is closest to the edge. Make sure that the head is flat and resting on the bottom of the mold. Bake the resin at 70°C overnight. Bake any waste resin before discarding.

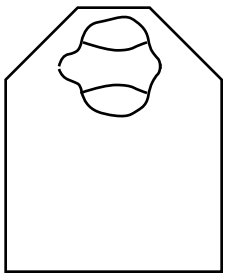

Figure 1

Sectioning and Staining Specimens.

Examine the embedded heads to ensure that they did not shift position while the resin hardened. Only section those which maintained their correct orientation. Trim the resin block with a razor blade, preferably the kind which are Teflon coated. Trim away excess resin which covers the eye. This is diagrammed in Figure 2. Start by trimming resin to form the face of the block which will be cut. Then cut away resin to form a trapezoid shape, so that the final form resembles a pyramid with its top lopped off.

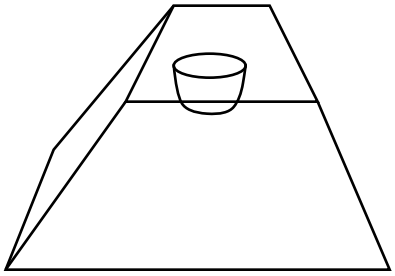

Figure 2

Cut sections with your favorite microtome. Section thickness can range from 0.5 to 5 microns for tissue embedded in soft resin, and from 50 to 300 nm for tissue embedded in hard resin. Sections can be collected either with a wire loop or a shaved wooden stick. The stick is best for collecting serial sections when section order must be preserved.

Transfer the sections to a puddle of water on a subbed slide. Place the slide on a heating block (about 80°C) and allow the water to evaporate. If you wish to examine unstained sections, add a drop of DPX (Fluka) and a cover slip. If you want to stain the eye sections, use toluidine blue. Place slides with sections on them onto a heating block which is at a temperature of 70-80°C. Allow the slides to warm up. Add several drops of toluidine blue solution (enough to only cover the sections) to the slide, and incubate. The incubation time is variable: it depends on the temperature of the block, how much toluidine solution is added, etc. Therefore, make an empirical test by staining for several different amounts of time. Around 30 seconds is usually optimal. After incubation, remove the slide and rinse thoroughly with water Examine under the microscope for how well the staining worked. If necessary, you can repeat the procedure until staining is optimal. Take care; it is possible to overstain tissue with this procedure. Allow the slides to completely dry before mounting in DPX.

Solutions:

1) Phosphate buffer. This is 0.1 M Sodium Phosphate pH 7.2. It can be made by mixing 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 in a 72:28 ratio, respectively, and then adding an equal volume of water.

2) Durcapan Resin. This can be ordered as Durcapan ACM from Fluka. It is based on the formula for Araldite resin, and is stored for long periods of time at room temperature as four separate components: epoxy resin (A), hardener (B), accelerator (C), and plasticiser (D). These components can be mixed together and either used immediately or stored in aliquots at -20°C. If stored at -20°C, you can freeze-thaw an aliquot for up to three times without affecting the resin. The resin is quite toxic, so bake any waste at 70°C overnight before discarding. The recipes for soft and hard resin are:

	SOFT (grams)	HARD (grams)
Resin A	54	50
Hardener B	44.5	50
Accelerator C	2.5	1.75
Plasticiser D	10	0.75

3) Glutaraldehyde and Osmium Tetroxide can be purchased as concentrated solutions from Ted Pella Co. and diluted into phosphate buffer. Store both solutions at 4°C in a sealed container. 2% glutaraldehyde solutions are good for one month, and 2% OsO_4 solutions are good for several months. See page 73 for instructions on disposal of waste glutaraldehyde and OsO_4.

4) Toluidine Blue Solution. Dissolve toluidine blue and borax to 1% final concentration for each in water. Store it in a wide barrel syringe with a filter attached so that the solution will be filtered immediately before use.

5) Preparing Subbed Slides. Heat 500 ml water to 80°C. Add 5g gelatin (Sigma) and 0.5g chromium potassium sulfate. Stir on a hot plate until dissolved (about two hours). Place new slides in racks, wash briefly in a warm hemosol solution, rinse with water. Dip slides in gelatin solution, and dry covered overnight.

Richard Carthew

SOMATIC MOSAIC ANALYSIS IN THE EYE

Somatic clones in the eye can be generated by X ray-induced mitotic recombination and can be genetically marked by the cell-autonomous *white* gene. For any gene you want to analyze by generating clones, you want to use the marker *white* gene at a cytological map position which is close to the gene of interest. This is conveniently done by choosing an appropriate P-element transformant stock in which the P vector contains the *white* gene and has integated in the genome cytologically close to the gene of interest, which will be called gene X. Three criteria should be considered before choosing the transformant stock. One, the *white* gene should be located cyologically close to gene X. Two, it is preferable if the *white* gene is more proximal to the centromere than gene X. Third, one copy of the *white* gene in this transformant stock should produce enough pigment in the photoreceptor cells that it can be easily seen in semi-thin or thick sections. This last consideration can be rapidly surmised by looking at flies hemizygous for the *white* gene under a dissecting microscope. Their eye color should be indistinguishable from wild type. Alternatively, you could fix and section eyes from hemizygous flies and score pigment in photoreceptor cells. However this last criterion is most important when analyzing mosaic ommatidia and is not important if you just want to look at the phenotype of the entire clone.

Make a fly stock which is homozygous for a mutant allele of gene X and which is homozygous *white* minus. We use the null allele of *white*, w-1118. Obviously if the mutant allele of gene X is lethal, it has to be balanced over a chromosome with a dominant marker.

The strategy is to put the chromosome with mutant gene X in trans to the chromosome containing the *white* gene. Recombination events between sister chromosomes during mitosis are induced by X irradiation. If recombination occurs between the centromere and the *white* gene it will produce a daughter cell that is homozygous mutant for *white* and gene X. Replication will expand cell numbers to the point that in the eye a white patch is seen in a red eye.

Make a mass fly cross between w^-; X^- and w^-; P[w^+]. I prefer to mate 400 of each genotype putting 100 pairs in one bottle. Incubate 24 hours at 25°C. Transfer flies to fresh bottles. These can either be egg laying bottles or regular bottles. I prefer egg laying bottles because the parameters for irradiation are more consistent. If you use egg laying bottles, use plates containing yeast-glucose medium (see end of section). Collect embryos for 12 hours at 25°C. Repeat collection. Incubate plates or bottles for a further 42 hours at 25°C. The flies will now be 48 $^+$/-6 hours old and will be late first instar larvae. Inducing recombination at this time gives a balance between reasonable clone size and frequency of clones (about 1 in 20 flies). If you want to generate larger clones, irradiate at an earlier time, but be prepared to generate clones at a lower frequency.

Irradiate tlarvae in plates or unstoppered bottles with 1000 rads (115 kV, 5 mA). If using egg-laying plates, cut the plates in halves or quarters and place each piece in a bottle. When the flies have eclosed, they can be screened for clones. Most white clones appear black under a dissecting microscope. Fix and section the eyes as described in Section 19. However, before the flies are sacrificed, shine a bright light on them for a few minutes. This causes the pigment granules in the photoreceptor cells to migrate apically and makes it easier to score for *white* pigment in cells R7 and R8. Cut 1 to 4

micron thick sections and collect all of the sections serially. This is important as pigment may only be visible in R7 and R8 cells for one or a few sections. Do not stain the eye sections but mount in DPX and view under phase contrast. Pigment granules appear as black specks adjecant to each cell's rhabdomere.

Recipe for Yeast-Glucose Plates:

10% dry yeast
10% glucose
1.6% agar
3/100 (v/v) 10% tergocept

Boil water and add solids. Boil for 5 minutes while stirring. Pour into 15x60 mm plates.

Bruce Kimmel

PREPARATION OF ADULT DROSOPHILA FOR SCANNING ELECTRON MICROSCOPY

This protocol describes two different methods for dehydrating adult Drosophila in preparation for S.E.M. For S.E.M. the water in a specimen must be removed without collapsing it by the surface tension forces which occur as the liquid evaporates. Air drying is unsatisfactory for adult Drosophila. In addition to providing better preservation, the two methods described below have the added advantage of rinsing the flies with an organic solvent and thus removing exterior dirt and grime. The first method requires the use of a critical point drying apparatus which most E.M. laboratories will have. The second protocol employs the use of a low surface tension solvent such as Freon 113.

1) Anesthetize the adult flies with CO_2 or your favorite anesthetic unless of course you are a cruel and sadistic person.

2) Place ten flies into 5 mls of 25% ethanol and incubate for 12-24 hrs at r.t.

3) Further dehydrate the flies through 50%, 75% and 2 x 100% ethanol as in step 2 above. Once in 100% ethanol the exterior morphology of the fly eye is stable for at least one month.

FURTHER STEPS FOR CRICTICAL POINT DRYING

4) Crictical point dry the samples. (Critical point drying is a process of exchanging the ethanol for liquid CO_2 under high pressure. Once the ethanol has been replaced with liquid CO_2, the temperature of the critical point drier can be raised such that the CO_2 becomes gaseous. The transition from liquid to gas at temperature X and pressure Y is the critical point. The CO_2 gas can then be vented to the atmosphere thus escaping from the sample with very low surface tension and leaving the sample dry. Check with your local E.M. person for details on how to **safely** use a critical point drier. There is a good reason for calling these things bombs.)

FURTHER STEPS FOR LOW SURFACE TENSION SOLVENTS

4) Pass the samples through a graded series of Freon 113 in ethanol (25%, 50%, 75%, 2 x 100%) with 12-24 hr incubations in each step of the series at r.t. After the last 100% step, remove the Freon 113 and vacuum dry the samples. (Because of the effects which fluorcarbons like Freon 113 have on the ozone layer you may want to use an alternative low surface tension solvent. The solvent hexamethyldisilazane is reported to be such an alternative solvent. The following reference claims that dehydration through this solvent can give better results than critical point drying (J. Microscopy **121:** 185-189).

COMMON STEPS

5) The dried samples can be stored indefinitely under vacuum with dessicant.

6) The samples are then mounted onto S.E.M. stubs using T.V. tube coat (Ted Pella Inc.). This adhesive is nice because it provides a good black background for photography. Care should be taken to mount the samples with the appropriate orientation so that the external structures of interest can be observed on the scope.

7) Sputter coat the samples with a 25-nm-thick coat of platinum.

8) The samples are now ready for viewing and should be stored over dessicant.

Nick Baker

PHOTOMICROGRAPHY

The purpose of this section is to collect together information about the types of film used in the Rubin lab., with brief comments on their processing and uses. Information on the use of compound microscopes in general, for photography or otherwise, is not included and can be found in publications such as the handbooks published by the Royal Microscopical Society. Useful titles include:

Handbook #1 "An introduction to the optical microscope", by S. Bradbury, Oxford University Press, 1989 ISBN 0-19-856419-8.

Handbook #13 "An introduction to photomicrography", by D. J. Thomson and S. Bradbury, Oxford University Press, 1987 ISBN 0-19-856414-7.

Black-and-white negative film

Kodak Technical Pan is the film of choice for most purposes. Technical Pan can be processed to give a wide range of contrasts according to need. When used at lower contrasts a large range of tones are retained in the image; at higher contrasts small differences are accentuated that pictorial films have more difficulty recording. Table 1 contains a set of developing conditions for Technical Pan at different contrast levels, and typical examples of their uses. When in doubt, lower contrast will preserve more information and can be followed by printing on high contrast paper.

TABLE 1

Contrast Level	Speed rating (ASA)	Developer	Typical Use
A Low(=pictorial)	25	Technidol LC[1] 15 mins (20°C) 11 mins (25°C) 8 mins (30°C)	pictorial applications, e.g., copying figures, photo-graphing autoradiographs. Also good for eye imaginal discs after DAB staining
B medium	50	HC110, dil. F[2] 8 mins (20°C)	most other uses, e.g. sectioned material, *in situs* or embryonic cuticle preps in dark field
C high	100	HC110, dil. D[2] 6 mins. (20°C)	Polytene chromosomes[3]
D very high	150	D19, undiluted 4mins (4°C)	embryonic cuticle preps. in phase contrast

A cautionary note: processing schedule 4. results in negatives of extremely high contrast. Small gradations in lighting not visible to the eye will be recorded. Typical problems that can arise include diffraction patterns caused by dirt outside the plan of

[1] Technidol LC must be made from powder and used within 24 hours.

[2] HC110 is stored as a working stock solution. Dilution D= 1 part working stock + 9 parts H_20. Dilution F = 1 part working stock + 19 parts H_20.

[3] See use of monochromatic light below.

focus (eg: on the top of the coverslip or undersurface of the slide), by air-bubbles in immersion oil, or imperfect centering of the microscope light source. With short exposures the time taken for the shutter to traverse the field may be enough to produce an exposure gradient across the negative. It will be appreciated that only an exceptionally well-maintained microscope can be used for such work.

Technical Pan is less useful when very fast exposure ratings are required, eg for photographing fluorescence images. Instead use Kodak Tmax P3200. Processing conditions for this film with HC110 are given in Table 2.

TABLE 2
HC110 dilution B

	Speed Rating (ASA)	21°C	24°C	27°C	29°C
A	1600	7.5	6	5	4.5
B	3200	10	7.5	7	5.75
C	6400	12	9.5	8	6.75

Use of monochromatic illumination with B&W film

The signal-to-noise ratio of black-and-white films can sometimes be increased by using a monochromatic light source. This is because parts of the spectrum not absorbed by the specimen (and which therefore contribute only "noise") are not used. Also a narrow bandwidth should increase lens resolution. Examples are the use of a green bandpass filter (550-600nm) to photograph orcein-stained chromosome preparations, or a blue bandpass filter (450-500nm) to highlight pigment granules in *Drosophila* eye sections.

Colour negative film

Colour negative films have some advantages over black-and -white. They contain more information, and are developed and printed commercially both rapidly and with little effort to yourself. We have preferred Fujicolour 100, but are trying the new high-definition Kodak Ektar films. The main disadvantage is loss of control over the printing process. Only the standard (low) contrast level is available, cropping and enlargement are expensive, and variation in colour balance and exposure are hard to avoid. These last two points result from the strong red colour of the negatives, which must be filtered out by the printer. Inexpensive commercial labs invariably expose and balance prints automatically with respect to some standard. Consequently there is little point bracketing the exposure of your negatives or using colour filtered illumination, since the printer will likely correct all of this. Printing your own requires equipment and skill.

Colour slide film

Colour reversal (slide) films do not suffer these disadvantages, since what you get back is the film you originally exposed, and the processing is invariant. Accurate prints can readily be made from slides if required. The most used films are Kodachrome and Kodak Ektachrome. These two have a different construction that affects their properties. Kodachrome comprises three separate light-sensitive layers, each of which is processed separately to generate one of the primary colours. Also a non-reflective backing reduces halation. The resolution and colour-rendition of Kodachrome are unparalleled. Also the thorough nature of the processing effectively removes uncoupled dyes, increasing the stability of the image. In Ektachrome films all the colour reactions occur within the same layer, limiting the accuracy of the colour reproduction, but making processing easier. Ektachrome films are available in a variety of speeds. Also, Ektachrome slides are supposedly more stable in *intense* illumination, such as found in a slide-projector. We tend to use Ektchrome 160ASA routinely, because of its speed and ease of processing, Ektachrome P800/1600 for fluorescent images, and Kodachrome only when the very highest standards are required.

Daylight/Tungsten films

Ektachrome is availible balanced for daylight or tungsten lamp (3200K) illumination. The colour balance of a lamp changes with its output. With an Axiophot it is therefore best to use the tungsten light source always, and use the neutral-density filters to adjust the light level. An 87A filter (which approximately corresponds to the blue microscope filter) converts tungsten illumination for use with daylight film.

"**Professional**" **films**

Both Kodachrome and Ektachrome are availible in a "Professional" grade that is slightly more expensive. These offer better quality control, more natural colour in the case of Ektachrome, and more accurate speed rating. These films are sold and stored refrigerated, and there is little point paying the extra unless you intend to do likewise.

Reciprocity

At low light levels the response of photographic films (like X-ray films) ceases to be linear. The exposure time necessary increases out of proportion to the decrease in illumination, an effect called "reciprocity failure". Typically this becomes significant for exposures >1sec. The axiophot control panel will correct for this effect if a reciprocity factor, specific for each film, is entered. Some reciprocity factors recommended by Zeiss are:

KODAK Technical Pan	3
EKTACHROME 160	5
EKTACHROME 400 (=P800/1600)	6

For colour films, however, reciprocity failure will be variable with different wavelengths, a problem not completely overcome by these exposure compensations.

Kevin Moses

GENOMIC DNA PREPS

Large Scale:

1) Freeze flies in liquid Nitrogen, in disposable polypropylene tubes, store at -70°C.

2) Set a porcelain mortar in dry ice, pour in liquid Nitrogen, and immerse the pestle until cold.

3) Add frozen flies, grind as a slurry in liquid Nitrogen, then allow the Nitrogen to boil off.

4) Chill a 50 ml polypropylene Oak Ridge tube and a spatula in liquid Nitrogen, then add up to 5 ml of the frozen fly powder to the cold tube on dry ice.

5) Remove the tube from the dry ice and immediately add 15 ml Homogenization Buffer (HB) and 15 ml 1:1 Phenol/Chloroform.

6) Cap the tube and mix by gentle inversion (to minimize DNA shearing) until well mixed and then for 30 minutes on a lab-quake or similar.

WARNING - EXPLOSION HAZARD

AS THE FROZEN POWDER DISPERSES IT WILL RELEASE NITROGEN GAS. YOU MUST PERIODICALLY CRACK THE CAP TO RELEASE THE PRESSURE AS THE POWDER IS DISPERSED, UNTIL IT IS ALL DISSOLVED. OTHERWISE THE TUBE MAY EXPLODE AND SPRAY NOXIOUS CHEMICALS ON YOU.

7) Spin at 18,000 rpm at **20°C** for 10 minutes (if at 4°C the Urea and SDS in the HB will precipitate).

8) Take the aqueous (top) phase, avoiding the interface, to a new tube and repeat the extraction twice more.

9) Take the aqueous (top) phase, avoiding the interface, to a new tube and add two volumes of ethanol (don't add salt, it's already in the HB), mix and spin at 18,000 rpm at **20°C** for 10 minutes (if at 4°C the Urea and SDS in the HB will precipitate).

10) Resuspend the pellet in 3 ml TE by gentle inversion on a lab-quake or similar (this will take some time).

11) Add 3g CsCl and 0.3 ml 10mg/ml ethidium bromide, fill a 5ml ultracentrifuge tube (top up with the same solution less the DNA pellet), check the density ($\rho = 1.56$) and spin at 45,000 rpm, 15°C for 16 hours.

12) Remove the DNA band by side-puncture (avoiding the pelleted RNA) and remove the ethidium by repeated extraction with CsCl saturated butan-2-ol.

13) Dilute 3 fold with TE, add 1/10 volume 5M NaCl and precipitate with 2 volumes of ethanol.

14) Wash the pellet in 70% ethanol, and resuspend in TE by gentle inversion.

15) Quantify by UV absorbance.

HB Buffer:

7 M	Urea
2 %	SDS
50 mM	Tris pH 7.5
10 mM	EDTA
0.35 M	NaCl

Notes-

This protocol works by keeping the material frozen until it hits the combined HB and phenol/chloroform. Thus the cells "wake-up" into a chemical environment that immediately denatures all membranes and proteins. In other words, the nucleases never get a chance. This method works well as an RNA prep also, just vortex at each mixing step, extract more exhaustively (until the interface is totally clean) and don't Caesium band. The resulting total nucleic acids prep can be used to make polyA+ RNA on an oligodT column, for Northerns, but if it is important to remove the DNA completeley (ie for cDNA cloning) then some measure must be taken to achieve this. This prep can be scaled down to 20 flies or fewer in a microfuge tube. For the mini-prep don't freeze, just homogenize in 0.5 ml HB and then extract, twice with phenol/chloroform, ethanol precipitate twice, wash in 70% ethanol and resuspend in TE. When you digest the mini-prep DNA with restriction enzymes add boiled RNAse to 0.1 mg/ml.

David Bowtell

RNA ISOLATION USING GUANIDINE HYDROCHLORIDE

1) For adult flies that are frozen, first grind to a fine powder in a mortar and pestle under liquid nitrogen. Use a large pestle to prevent "blasting" the powder out when the nitrogen is added. Some of us have found that the addition of liquid nitrogen is unnecessary.

2) Add the powder to room temp 6M GHCl, 0.1M NaOAc or 6M guanidine isothiocyanate, 0.1M NaOAc. I generally use 10-20ml per scintillation vial of flies. It is better to add too much than too little liquid but first consider the number of samples you are spinning in step 4. (A SW41 can take about 42ml of GHCl lysate per run). **NOTE.** If you add the powder to a 50ml tube be careful shaking it up once the lid is screwed on. Release of the dissolved nitrogen can blow the lid off. A beaker with the guanidine stirring in it is probably a safer alternative.

3) Shear the lysate through an 18ga. needle and then preclear twice at 10-15K in the SS34. It is important to get rid of all the particulate material. The first preclear has a lot of cuticle floating on the top.

4) Pretreat polyallomer tubes with 0.1M NaOH and rinse with DEPC DDW. Add 5.0ml of 5.2M CsCl, 10mM EDTA and then layer the supernatant over the top. Spin (SW41, SW40) 30K 16-18hr at 20° C.

5) Remove supernatant with a vacuum aspirator down to the last centimeter of liquid. Often a prominant band is visible ~0.5cm above the pellet. This is (?) tRNA. The pellet is usually not obvious until all the liquid is removed. Cut the tube off with a scalpel blade above the liquid and invert the tube. The pellet should be clear and gelatinous. Resuspend in ~200µl 10mM EDTA by shunting up and down with a p200. It will take several minutes. Be careful not to let the suspension touch the end of the pipette. Remove 5-10µl into 400µl of 10mM EDTA for OD. Add the remainder to ethanol / NaCl. It is best to store the RNA as a fine precipitate in ethanol at -70° C from which aliquots can be removed.

6) To oligo-dT select, spin down RNA (very briefly if you have a lot otherwise the pellet can be hard to resuspend). Resuspend in 10mM Tris, 1mM EDTA, 0.5M NaCl, 200µg / ml proteinase K and 0.1% SDS (it is not necessary to phenol extract) and either oligo-dT select batchwise or over a column.

7) This works fine for adult flies, embryos, discs and probably all other stages.

Marek Mlodzik

PLASMID RESCUE FROM P[lacZ] LINES

All of the commonly used P[lacZ] vectors contain sequences that allow plasmid recovery (rescue) including genomic sequences from the transformed flies. Sequences required for plamid rescue are a bacterial origin of replication and an antibiotic resistance gene (e.g. Ampicillin or Kanamycin resistance). In addition, unique restriction sites are required that cut in the transformation vector only on one side of the ori / resistance sequences; the second site is provided by flanking genomic DNA, so that the rescued plasmid contains DNA originating from both the transformation vector and the fly's genome.

Depending on the vector, you can rescue genomic sequences upstream and / or downstream of the P[lacZ] insert.

1) Extract DNA from 40 to 50 flies with your favourite procedure. An easy method is described in the protocol for "Rapid small scale isolation of Drosophila DNA and RNA", p. 183 in this volume.

2) Check quality of DNA with desired restriction enzymes and Southern blotting. Usually 2 to 4 fly equivalents are enough to see a "good smear" on agarose gel. This step is optional, but K. (Mosey) Moses would not ommit it.

3) Digest 2 to 10 fly equivalents of DNA with an enzyme that fullfills desired criteria for rescue (If several different sites are suitable, try with more than one.) If possible use an enzyme that can be heat inactivated, otherwise include phenol / chloroform extraction before precipitation of DNA after digest.

4) Resuspend digested DNA in 20 to 50 µl TE. Use half of the DNA for the ligation. To avoid intermolecular ligation events, perform ligation reaction in a larger volume; 200 µl are usually fine. It is not necessary to add more than 2µl (e.g. 2 units of N.E. Biolabs enzyme) T4 DNA ligase. Incubate reaction mix for 4 hrs to overnight.

5) To 200 µl ligation mix add 20 µl of 3 M sodium acetate and precipitate with two volumes of ethanol. Spin, wash, dry and resuspend pellet in 10 to 20 µl TE. The DNA is now ready for transformation.

6) For bacterial transformation, I have used XL1-blue cells (Stratagene) and a high efficiency transformation protocol as described by Hanahan (see below for details). However, since more recently the electroporation procedure has been commonly used and gives even higher transformation efficiency, this might be the method of choice for plasmid rescue bacterial tranformation. When using electroporation, the cuvettes should be sterile to avoid "surprises". Plate cells on Ampicillin or Kanamycin plates depending on transformation vector used.

Comments:

The number of colonies per fly equivalent of genomic DNA varies greatly, ranging from 0 to 30. The use of XL1-blue cells (10 to 100 times greater transformation efficiency than other commonly used bacterial strains) and 25 C / Hanahan transformation protocol (see below) allows usually plasmid rescue from any transformant line. Difficulties in obtaining clones from particular lines seems most likely due to an unusual distribution of enzyme sites near the insert (try with different enzymes if possible).

When rescue of genomic upstream sequences is employed, the rescued plasmid still contains the lacZ gene. For such plasmids, it is worthwhile to plate on plates containg X-gal. Usually all XL1-blue colonies containing such plasmids will turn blue. This might be usefull to destinguish the desired plasmids from potential contaminants.

High efficiency (Hanahan) competent cells

1) Pick a single XL1-blue colony to 5 ml LB, grow to saturation at 37°C.
2) Inoculate 50 ml 2xYT + 10mM $MgCl_2$ and 0.1 % glucose with 100ml of the seed culture in a 250 ml flask and grow at 25°C to A_{600} = 0.3 (about 12 hr).
3) Spin down cells in a 50 ml orange cap tube, resuspend cells in 20 ml ice cold TFB and incubate on ice for 15 min.
4) Spin down cells and resuspend in 4 ml TFB.
5) Add 140 ml DnD and incubate on ice for 10 min.
6) Add 140 ml DnD, aliquot 200 ml per transformation and incubate on ice for 20 min.
7) Add up to 20 ml DNA and incubate on ice for 20 to 40 min.
8) Heat shock at 42°C for 90 seconds then return to ice for two min.
9) Add 800 ml 2xYT (+ 10mM $MgCl_2$ and 0.1 % glucose), incubate at 37°C for one hour.
10) Plate, (if M13 phage wash the cells with two changes of medium first)

Solutions for Hanahan Cells:

		FINAL
TFB (for 500 ml)	make 100 ml 0.5 M MES pH 6.3 (9.76 g MES, pH with KOH, filter sterilise)	
	3.70 g Ultrapure KCl	100 mM
	4.45 g $MnCl.4H_2O$	45 mM
	0.75 g $CaCl_2.2H_2O$	10 mM
	0.40 g Hexamine $CoCl_3$	3 mM
	10 ml 0.5 M K-MES pH 6.3	10 mM
	Distilled Water to 500 ml, filter sterilise, store at 4°C.	
DnD	1.53 g DTT	1 M
	9 ml DMSO	90 %(v/v)
	100 ml 1 M Potassium Acetate (pH 7.5)	10 mM
	aliquot and store at -70°C.	

Reference: Hanahan, D., *Techniques for transformation of E. coli* Ch 6 in *DNA cloning, Vol I, A practical approach* Ed Glover, D.M., Oxford, IRL Press

Ulrike Heberlein

PREPARATION OF PROBES FOR FOOTPRINTING EXPERIMENTS

Probes are essentially labeled as for Maxam and Gilbert sequencing; one end of the DNA fragment is end-labeled with ^{32}P-γATP. The general strategy is to cut the plasmid with a restriction enzyme at the site to be labeled. The 5'-phosphate is removed with alkaline phosphatase and replaced with ^{32}P. The labeled DNA is then cut with a second restriction enzyme and the fragment of interest is isolated. It is important to make sure that a combination of digests will produce fragments that can be separated from each other by gel electrophoresis. Restriciton sites that result in a 5' overhang should be used; however, blunt ends can also be labeled with lower efficiency.

1) **First digest:** Digest ~5-10µg of CsCl-purified DNA (any RNA contamination will greatly reduce the efficiency of the labelling!!) with the restriction enzyme of choice in a 50µl volume. Check the digest on a gel to make sure it is complete (partial digests can cause nightmares).

2) **Removal of 5' phosphate:** To the above digest add: 45µl of H_2O + 2.5µl of 2M Tris/HCl pH9.5 + 2.5U of calf intestine alkaline phosphatase (from Boehringer Mannheim, 1U/µl, cat. #713023). Incubate at 37°C for 30-45 min. Extract with 1 volume of 1:1 phenol/chloroform twice (this is important to get rid of all the phosphatase before labelling). Extract with 1 volume of chloroform and ethanol precipitate with 2.5 volumes of ethanol in the presence of 0.3M NaOAc. Resuspend the washed and dried pellet in 16µl H_2O (this volume is based on the volumes and concentrations of reagents used in the kinasing reaction described below; adjust it if you need to).

3) **Addition of ^{32}phosphate:** To the phosphatased DNA in a 16µl volume add: 2µl 10X kinase buffer (0.5M Tris pH 8, 0.1M $MgCl_2$, 50mM DTT, 1mM EDTA pH 8, 10mM spermidine; store at -20°C) + ~1µl ^{32}P-γATP (~150µCi, ICN crude, 7000Ci/mmole) + 1µl polynucleotide kinase (10U/µl). Incubate at 37°C for 30-45 min. followed by 15 min. at 65°C to kill the enzyme.

4) **Second digest:** To the labeled DNA add: 5µl of 10X restriction enzyme buffer, 10-20U of the appropriate enzyme, and H_2O to a final volume of 50µl and incubate at 37°C for 1-2 hrs. If you want to reduce the volume of radioactive liquid waste, you can precipitate the digest at this point to eliminate the non-incorporated ^{32}P-γATP.

5) **Isolation of labeled fragments:** Separate the labeled fragments by gel electrophoresis. If you need to resolve small fragments use a 5% or 7% native acrylamide gel. Otherwise use a regular agarose gel (TAE buffer). **Remember that these gels a screaming hot!!**

A) Acrylamide gels: ~20cm long gels, 1.5mm thick are adequate. Add sucrose dye to the DNA, heat at 65°C for 5 min., load onto the gel and run for 2-3 hrs at ~200V. When finished, take off the top plate and cover the gel together with the bottom plate with Saran Wrap. Expose to film for 1-2 min. The wells are usually visible on the autradiogram, allowing easy alignment of film and gel; however you may want to use radioactive markers. Cut out the band of interest and electroelute in 0.5X TBE for 30-60 min. Precipitate the eluate with 1 volume of isopropanol at -20°C for 1-2 hrs. Spin for 15 min in a microfuge at ~15,000RPM. Resuspend the DNA in 50-100µl of TE and store at -20°C. About 0.1-0.5µl of this DNA should be enough for one footprinting reaction. If the concentration of DNA-binding factor to be assayed is low (as in most cell or tissue extracts) the minimum amount of probe, hopefully of high specific activity, necessary to be detected in an overnight exposure should be used. 1µl of labeled probe should give 50-200 cps on a minimonitor.

B) Agarose gels: Run a 0.8-1.2% TAE/agarose gel, stain with ethidium bromide, cut out desired band and isolate the DNA using Gene-Clean. (I presume that low melt agarose would also work, but I have never tried it.) Resuspend and store as above.

Purine cleavage sequence markers
(for footprinting experiments):

- Take 8µl of footprinting probe (~80,000 cpm Cerenkov) labelled as described.
- Add 5µl of 1mg/ml sonicated calf thymus DNA and dry in the speed-vac.
- Add 6µl of 2% formic acid (diluted fresh from 88% Malinckrodt stock stored at 4°C)
- Incubate at 37°C for 12 minutes and put on ice immediatly. This time is optimal to obtain an evenly cleaved ladder (~1 hit per molecule) of a 300bp long probe.
- Speed-vac for 2 hrs.
- Add 100µl of a 1:10 dilution of piperidine (Sigma, prepared fresh in the hood!!).
- Incubate at 92°C for 20 minutes. Put a weight on top of the tube(s) or they will pop open and release very toxic piperidine.
- Speed-vac overnight.
- Add 50µl ddH_2O, dissolve and speed-vac until dry.
- Resuspend in 20µl of 0.1N NaOH + 40µl formamide dye.
- Use 1-5µl for each lane.

Ulrike Heberlein

DNAaseI FOOTPRINTING

This technique exploits the fact that a protein bound to a specific DNA sequence will interfere with the digestion of that region by the endonuclease DNAaseI. In summary, an end-labelled DNA probe is incubated with a protein extract or a purified DNA-binding factor. The unprotected DNA is then partially digested with DNAaseI such that on average every DNA molecule is cut once. Digestion products are then resolved by electrophoresis. Comparison of the DNAaseI digestion pattern in the presence and absence of protein will allow the identification of a footprint (protected region) or hypersensitive sites (sites at which digestion is enhanced due to protein binding).

Binding reaction:
Mix in a 50µl final volume:

0-25µl	protein extract in HEMG (see below)
10µl	10% polyvinyl alcohol (store 4°C)
1µl	1M Hepes pH 7.6 (pHed with KOH)
~2fmoles	of end-labelled DNA probe (~20cps on minimonitor)
	competitor DNA (see below)
	ddH2O to a final volume of 50µl

If the amount of protein used is less than 25µl, add 0.1M HEMG to make up the difference. Mix components gently and incubate ~10 minutes. Incubation on ice is usually required when dealing with relatively crude protein extracts which contain endogenous nucleases and/or phosphatases. With purified proteins the incubation temperature can be increased.

It is advisable to add non-specific competitor DNA, such as sonicated calf thymus DNA, synthetic poly d(I-C) or poly d(A-T) (Pharmacia) to the binding reaction. The optimal competitor concentration needs to be determined empirically, however 0.1-0.5µg of sonicated calf thymus DNA or 0.01 OD units of synthetic DNA are usually adequate.

DNAaseI digestion:

To the binding reaction add 50µl Ca / Mg solution (10mM $MgCl_2$, 5mM $CaCl_2$) at room temperature and 1-10µl of DNAaseI solution (see below). Mix quickly and incubate at room temperature for 1 minute. Stop the reaction by adding 100µl of stop solution (0.2M NaCl, 20mM EDTA pH 8, 1% SDS, 0.25 mg / ml carrier RNA; store at room temperature) and vortexing immediately. Digestion of three samples can be carried out simultaneously with a bit of practice.

Samples are then extracted with 200µl of 1:1 phenol:chlorophorm and precipitated with 1ml of 100% ethanol. Pellet is washed with 70% ethanol, dried and resuspended in 6µl of formamide dye. Run on 6-8% sequencing gel.

HEMG:
0.1M KCl
25mM Hepes pH 7.6 (pHed with KOH)
0.1 mM EDTA pH 8
12.5mM MgCl2
10% glycerol
1mM DTT (fresh)

DNAaseI: Make a 2.5mg / ml DNAaseI (Worthington or Cooper Biomedical, bovine pancreas, DPFF, 2000U / mg) solution in ddH2O and freeze 2-5µl aliquots; keep at -70°C. Make a fresh dilution of this stock solution in ice cold ddH2O and keep on ice. For the no-protein control use ~1µl of a 1 / 1000 dilution. For crude protein extracts the amount and dilution of DNAaseI needs to be determined; 5µl of a 1 / 100 dilution are usually adequate.

Ulrike Heberlein

PRIMER LABELLING FOR PRIMER EXTENSION ASSAY

Mix in a 20µl final volume:

2µl 10x kinase buffer
1µl oligonucleotide primer (5pmoles/µl)
200µCi 32P-γATP (crude, ICN, 7000Ci/mmole)
10 U polynucleotide kinase
ddH_2O to 20µl

Incubate at 37°C for 30-45 minutes. Heat at 65°C for 15 minutes.

To remove unincorporated label precipitate as follows:

Add 60µl TE + 320µl 2.5M NH4OAc + 20µg RNA carrier + 650µl 100% ethanol (follow this order of addition; don't add the carrier RNA to the reaction first or some RNA will get labelled). Chill on dry ice for 5-10 minutes. Spin in the cold for 15 minutes. Discard supernatant (**very hot!!)**. Resuspend pellet in 180µl TE and add 20µl 3M NaOAc + 500µl 100% ethanol. Chill on dry ice for 15 minutes, spin in the cold for 10 minutes, wash with 70% ethanol, dry and resuspend in 100µl TE (should be ~200cps/µl on minimonitor).

<u>10x kinase buffer</u>:

500mM Tris pH 8
100 mM $MgCl_2$
10mM spermidine
1 mM EDTA
50mM DTT (add fresh before using)

Ulrike Heberlein

PRIMER EXTENSION

All solutions, eppendorfs and pipet tips need to be RNAase-free and sterile. Use gloves and work carefully (don't sneeze in your tubes).

1) Precipitate the RNA with 5' end-labelled primer in 0.3M NaOAc with 2.5 volumes of ethanol. If working with low amounts of RNA add some yeast RNA carrier (approx. 10µg). Wash pellet with 70% ethanol and dry.
2) Resuspend RNA in 8 µl ddH_2O and add 2 µl of 5X annealing buffer (1.25M KCl, 10mM Tris-HCl pH 7.9, 1mM EDTA). Double this volume if more than 20 µg of RNA are used, i.e., use 16 µl ddH_2O and 4 µl of 5X buffer.
3) Incubate at 60° for 90 minutes. Spin briefly every 30 minutes to avoid drying of the RNA. The annealing temperature depends on the length and GC content of the primer. The above is O.K. for 20-30 base primers with 50-70% GC content.
4) Add 23 µl of PE mix (10mM $MgCl_2$, 5mM DTT, 100 µg / ml actinomycin D, 0.33mM dNTP's, 20mM Tris pH 8.7 at room temp., pH 8.3 at 37°C, store at -20°C in the dark) and 10 U of reverse transcriptase (Life Sciences). Mix carefuly, spin briefly, mix again. (Use twice these quantities if annealing was done in 20 µl.)
5) Incubate at 37°C for 1 hour.
6) Add 0.3 ml chilled ethanol. Put in dry ice bath for 15 min. Spin at 4°C for 10 min. Wash in 70% ethanol. Dry.
7) Dissolve in 2 µl of 0.1N NaOH, then add 4 µl of formamide dyes. Boil for 2 min., chill on ice and load on sequencing gel.

Matthew Freeman

OLIGO-LABELLED DNA PROBES

This technique is an alternative to nick translation, and is generally superior. I find it less fussy, and it routinely gives considerably hotter probes. It also has the advantage that even quite dirty DNA preps work fine, and it is possible to label fragments in LGT agarose with no purification.
The 5X oligo-labelling buffer (OLB) can be made in large batches and stored at -20° for at least a year. If you do this, keep it in aliquots of a size such that you thaw and refreeze a particular tube for not more than a month or two - after this it may become less effective.

For all normal uses it is only necessary to use one hot nucleotide (I use ^{32}P-dATP). The OLB has the necessary cold nucleotides, so you need to make another one if you want to use two hot ones.

The OLB is made from several component solutions.

Solution O:	1.25M Tris HCl, pH8.0 1.25M $MgCl_2$
Solution A:	2mls Solution O 36µl ß-mercaptoethanol 10µl 100mM dATP 10µl 100mM dGTP 10µl 100mM dTTP
Solution B:	Random hexamers (Pharmacia), 90 OD Units / ml in TE
10mls 5X OLB:	2mls Solution A 5mls 2M HEPES, pH6.6 3mls Solution B.

DNA labelling:

1) Boil DNA in 32µl (made up with water) for 5-10 minutes, then incubate the DNA at 37° for 5 minutes (this is clearly black magic, and may not be necessary).

2) Set up the reaction as follows:

DNA	32µl
5X OLB	10µl
10mg/ml BSA	2µl
^{32}P-dATP (3000 Ci/mmol)	5µl
Klenow	1µl (2-5U)

The reaction is done at room temperature; most incorporation has ocurred by one hour, but it continues to increase till about 3-4 hours. Unlike nick translation, it does not then diminish, and if it's convenient, it's fine to leave it overnight.

3) Separate unincorporated nucleotides by your favourite method: mine is small spin columns in 0.5ml Eppendorf tubes.

4) You will find routine incorporation of between 50% and 80%, unless you used less than 20-30ng of DNA. The specific activity can reach about $3x10^9$ dpm/µg.

To label a DNA fragment in LGT agarose substitute the following for step 1 above.

1a.)Cut the band from an LGT gel and add 3mls of water for every gram of agarose (Eppendorf tubes weigh 1g). Boil for 10-15 minutes, then place at 37° for 10 minutes. Use 32µl of this mix in the normal reaction. The remaining DNA/gel mix can be frozen and reused many times (I boil it each time, but am not sure that it's necessary).

Notes:

a) The quantity of DNA: anything over about 5ng works; theoretically 20-40ng gives the hottest probe in the above reaction; over 100ng will reduce the final specific activity.

b) I have found one or two cases (out of many) where a supercoiled plasmid doesn't label well. This can be helped by a longer boiling step or, in desperation, linearising the plasmid before labelling.

Refs:

A.P. Feinberg and B. Vogelstein. Anal. Biochem. **132**, 6-13 (1983)
Anal. Biochem. **137**, 266-277 (1984)

Matthew Freeman

NORTHERN BLOTS USING RIBOPROBES

The advantage of using riboprobes for northerns is that you can make very hot probes, and the extra stability of RNA:RNA hybrids may increase the sensitivity. They are therefore useful if you are looking for similar, but not identical, transcripts, or if your transcript's abundance is very low. There are significant disadvantages also. In order to make the hottest possible probe, a relatively large amount of radioactivity is used, and also there can be a problem with spurious bands (for example I often see the same band when I have used Bluescript vectors as the probe, presumably because there is a degree of similarity between some vector sequences and some Drosophila transcript). The latter problem is usually solved by increasing the stringency of the final washes: the different signals melt off very efficiently at different temperatures, and you could normally expect that the most stable hybrid represents the 'real' transcript. Nevertheless, this could clearly cause problems in some situations, as well as actually being an advantage in others (such as deciding which of several transcripts was most similar to a given probe). It should be noted that these probes are RNA, and are therefore sensitive to RNAses, which contaminate most glassware, and all hands. Use normal precautions for working with RNA and all will be well. The protocol below takes a very cautious approach; in practice you can usually be more sloppy.

Labelling the Probe:

The following recipe gives the hottest probe that can be made from available nucleotides. It uses 750µCi to make a single probe, and therefore should only be used in extreme situations. You can reduce the amount used by using ribonucleotides of lower specific activity and/or reducing the reaction volume; the critical factor is that the concentration of the hot nucleotide is at least 8µM. The whole procedure is based on the one in the instructions that come with the Stratagene Transcription Kit; there is a discussion of all the stages in that booklet.

1) Dry down 750µl of 3000Ci / mmol, 1mCi / ml rATP in the Speedvac (I split it into two tubes, thereby halving the time taken).

2) Resuspend the hot nucleotides in the following reaction:

5µl 5X Transcription Buffer* (Stratagene)
1µl 10mM rCTP
1µl 10mM rGTP
1µl 10mM rUTP
1µl 0.75M DTT
1µl RNAse Block (25U, Stratagene; RNAsin is OK too)
1µg DNA†
10U T3 or T7 Polymerase
Water to 20µl

*5X - 200mM TrisHCl, pH8, 40mM $MgCl_2$, 10mM spermidine, 250mM NaCl.

† The DNA is Bluescript cut down stream of the insert, treated with 200µg / ml proteinase K, 15 minutes, 37°, phenol extracted, ethanol precipitated, and resuspended in DEPC-treated water.

3) Incubate at 37° for 30 minutes.

4) Add 100µg RNAse-free tRNA, an equal volume of 4M ammonium acetate, and 2.5 volumes ot ethanol. Precipitate. By measuring the radioactivity in the pellet and the supernatant, it is possible to get a very approximate idea of the incorporation; it should be greater than 50%. Resuspend in boiling salmon sperm DNA just before setting up the hybridisation.

Northern Blot

The northern is run under standard conditions.

Hybridisation

1.Prehybridise for at least 4 hours at 65° in:

50% formamide
5X SSC
1X PE*
150µg / ml salmon sperm DNA (phenol extracted)

*5X PE is:
250mM Tris-HCl, pH7.5
0.5M sodium pyrophosphate
5% SDS
1% polyvinyl pyrolidine
1% ficoll
25mM EDTA

Make in DEPC-treated water, heat to 65° to dissolve. Cool to about 37°, and add 5% BSA (fraction 5) to 1% final. Heat again to 65° for 15 minutes. Store indefinitely at room temperature.

2) Hybridise in the same mix with the probe (I resuspend the probe in a little just-boiled salmon sperm DNA). Hybridise at 65°, overnight or longer.

3) Wash in 0.1X SSC, 0.1% SDS at 65°. This is rather low stringency (see above), and any signal you see should be checked by rewashing the filter (which should not be

allowed to dry completely) at increasing temperatures. For example I have found three bands on a northern that only gives one with DNA probes. One of the spurious bands comes off between 65° and 70°, and the other by 75°. In this case the real band still hybridises up to 85°.

Using this protocol, a transcript that is normally visible in about 12 hours (using oligo-labelled probes), is visible in 1 - 2 minutes, suggesting that these really are very sensitive northerns.

Smadar Admon

POLYCLONAL AND MONOCLONAL ANTIBODIES

POLYCLONAL ANTIBODY

Immunization of Mice:

Injections should be made at intervals of at least two weeks.. We have been using either of the two following adjuvants with success: Freund adjuvant and MPL+TDM adjuvant (RIBI ImmunoChem Research, Inc.), the latter being less hazardous than the first.

1) 1st injection: 15 µg Antigen in 200 µl adjuvant, injected subcutaneously.

2) 2nd injection: 2 weeks later; boost with 15 µg of Antigen in adjuvant. (If time permits, boost again a month later).

3) 7 - 10 days later bleed the mouse and test serum using the assay which will be used for screening. If titre is not high enough, boost again two weeks after previous boost.

Serum Preparation:

1) After collecting, blood should be allowed to clot for 60 min . at 37°C or O.N at 4°C.

2) Separate the clot from the sides of the tube (ringing) using a pasteur pipette. Place clot at 4°C O.N.

3) Spin at 10000 x g for 10 min. at 4°C to separate the serum.

4) Serum can be stored at -20°C after adding Glycerol to 50%.

If monoclonal antibodies are desired, once you have a "good" polyclonal and a reliable screening method proceed to the next step.

MONOCLONAL ANTIBODY

If titre is positive do one of the following 2 weeks later:

I- Boost with 50 µg - 100 µg of Antigen in PBS and proceed with fusion 4 days later.

OR

II- Boost 3 days in a row with 15 µg Ag in PBS and proceed with fusion on 4th day.

Cell Lines:

1) Myeloma P3X63-Ag8.653
Origin: BALB/c, non secreting, 8-azaguanine resistant, HPRT $^-$.

2) Myeloma fox-NY
Origin: Robertsonian, 8-azaguanine resistant, HPRT $^-$, APRT $^-$.
(mice have resistance to drug and expression of heavy chain on the same chromosome).

3) Macrophage-derived J774A.1

Maintenance of the cells:

Stock solutions:

- IMDM: JRH Scientific 51-47178.

- FCS: Fetal Bovine Serum- defined, Hyclone A-1111-L (no heat-inactivation needed) or any other brand that has been sampled and checked in advance.

- Insulin: 100X stock= 20 I.U./ml
  Dissolve 80 mg of Insulin (25 I.U./mg) in 90 ml of ddH_2O by adjusting to pH 3.0 with 1-2 drops of 6N HCl. After Insulin has dissolved readjust pH to 7.2 with 0.5 M Na_2HPO_4, bring volume to 100 ml and filter sterilize with 0.2 µ membrane, keep frozen.

- Oxaloacetic Acid: 100X stock = 0.1 M
  Filter sterilize, keep frozen.

- D(+) Glucose: 100X stock = 45%
  Filter sterilize, keep frozen.

- Transferrin: 1000X stock = 1 mg/ml
  Dissolve 1 mg human transferrin for each ml of IMDM. Make a 2 mg/ml $FeCl_3.6H_2O$ or 1.2 mg/ml $FeCl_3$ in 0.001 M HCl. Add 1µl Fe solution to each ml of transferrin solution. Mix, let stand on ice for 30 min. Filter sterilize, store at 4°C.

- HT supps: 100X stock = 1.36 mg/ml (H) , 0.73 mg/ml (T).
  Weight out 68 mg hypoxanthine (Sigma H-9377) and 36 mg of thymidine (Sigma T-9250), combine. Add 1N NaOH (a few drops to 1 ml) and stir until the

powder is in solution. Bring to a final volume of 50 ml with ddH_2O. Filter sterilize, store at -20°C.

- Supplements: 25X stock (store at -20°C).
  Mix equal volumes of the following:
  L- Glutamine (200mM = 100X) , Gibco 320-5030.
  Penn-Strep (100X), Gibco 600-5070PG.
  Non-essential amino acids (10 mM = 100X), GIBCO 320-11404G.
  Sodium pyruvate (100 mM = 100X), Sigma S 8636.

- 2-Mercaptoethanol: 1000X stock (5×10^{-2}). (Store at 4°C.)

- AT supplement : 50X stock , Sigma A-7422. (Store at -20°C.)

- PCM: Parents Conditioned Medium.
  Medium is collected from the the apropriate parent myeloma after the cells have reached high density, then spun and filtered. Aliquot and keep at -20°C.

- MCM: Macrophage Conditioned Medium. (used instead of feeder cells)
  Seed macrophages at a density of 1.5×10^5 cells / ml in the medium described on next page. Add 2.5 µg / ml LPS which induces differentiation.
  Collect sup after 2-3 days, or when medium is getting too yellow. Induce 2 more times , each time with 1 µg / ml LPS and collect sup after 2 days each.
  Pool the sups, filter and use as recomended. (Could be aliquoted and stored at -20C.)

Preparation of Media:

for P3X63-Ag8.653: **IMDM complete**
to 400 ml of IMDM add:
5 ml 100X Insulin
5 ml 100X Oxaloacetic acid
5 ml 100X D(+) glucose
0.5 ml 1000X transferrin
0.5 ml 1000X 2- Mercaptoethanol
5 ml 100X HT
20 ml 25X supplements
75 ml FCS (final 15%)
100 ml PCM (Parent conditioned medium).

for fox-NY: **IMDM** or **RPMI** (JRH Scientific, 51-50178).
10 % FCS
1X AT supplement
transferrin
supplements
PCM, when available from this cell line .

for J774A.1: out of convenience we have been using the same medium as the one for the P3X63-Ag8.653 (= Ag8), without the PCM.

Growth conditions:

All cell lines mentioned above grow at 37°C, 7% CO_2 .

The Myelomas optimal density is $3.5x10^5$ / ml.

The Macrophages optimal density is $1.5x10^5$ / ml. When expanding them , use a "policeman" to scrape them; this is easier to do if they are growing in petri dishes at this stage.

Freezing Hybridoma / Myeloma / macrophages.

Freezing solution: 90% FCS + 10% DMSO, ice cold.

1) Spin down 10^7 cells (10^6 minimum) at 1200 rpm for 5 min.
2) Aspirate medium.
3) Resuspend in 1 ml of ice cold freezing solution.
4) Transfer vial to an insulated freezing box and place at -70°C for at least 1 hr. (could be for a couple of days).
5) Transfer the vial from the -70°C to the liquid nitrogen tank and log the entry in the freezer log.

Thawing cells:

1) Take vial out of liquid nitrogen tank and thaw it immediately in a 37°C bath (about 1 min).
2) When there is still a small piece of ice left, dilute the cells by transfering them into a conical tube containing 10 ml of the growth medium at 37°C.
3) Spin at 1200 rpm for 5 min.
4) Aspirate medium and resuspend cells in 5 ml of medium, in a $25cm^2$ flask.

Cell Fusion and Selection

Solutions:

- IMDM
- IMDM complete with MCM instead of PCM.
- PBS
- PEG 50% (w / v): (PEG 4000 "gas chromatography grade" Art.9727 - Merck). Sold by Bryant Laboratories, (415)526-3141, or MC / B Manufacturing Chemists, Inc.
- Aminopterin: Sigma, A 5159 (50X), use it as 100X. (final = $2x10^{-7}$ M).

1) Prepare a T-150 flask with 170ml of IMDM complete with MCM instead of PCM (= IMDM - m), and keep it in the incubator for fused cells.

2) Isolation of spleen cells :

a) Sacrifice mouse by cervical dislocation. Immerse mouse in 70% Ethanol.
b) Remove spleen (on the left side) and transfer into a small petri dish which contains IMDM at room temp. Clean fat from the spleen and transfer the spleen into an empty dish.
c) Using sharp tipped forceps, one end is punctured. A curved forceps is used to hold down the intact end, and the spleen is gently rubbed towards the opened end with another set of forceps. The cells from inside the spleen will ooze out with very little damage. Stop the process when you are left with a nearly empty, transparent skin. Collect the cells by rinsing with IMDM.
d) Transfer cell suspension to a 15ml conical tube and let the cell debris settle out (approx. 5 min.).
e) Remove the cell suspension (without disturbing the settled cell debris) and transfer to a 50ml conical tube. Add an additional 30ml of IMDM and pellet the cells at 1200 rpm for 5 - 10 min.
f) Aspirate the medium, resuspend pellet and wash again with 30ml of IMDM. One immunized spleen has aprox. 10^8 cells. After this wash the cells are ready for the fusion.

3) Myeloma cell preparation:

It is essential that the myelomas be free of debris, rounded and refractive under phase contrast, and that they are harvested in log or late log phase growth (between 3.5 and 9 $x10^5$ cells / ml).

a) Thaw cells 7 days before scheduled fusion. Myeloma do not grow well after being in culture for more than a few weeks.
b) Make sure you refreeze cells for future use.
c) We have been using a ratio of 2 spleen cells : 1 myeloma cell. However, workers have been using a ratio from 1 to 10 spleen cells per myeloma successfuly. For one spleen we harvest $5x10^7$ cells. It is advisable to do this spin at the same time that the second wash of the spleen cells is done.

4) The fusion:

a) The washed myeloma and spleen cells are pooled in 30ml of PBS (room temp.) and spun gently at 1000 rpm for 10 min.
b) Aspirate the PBS and resuspend pellet gently by tapping the tube. Volume should be approx. 0.8 ml.
c) Set a timer.
d) Add an equal volume of PEG solution, slowly, dropwise, with gentle tapping, over 1.5 min. at room temp. Then gently wiggle the tube for 1.5 min.at 37°C. Some cell clumping will be evident.
e) The suspension is spun at 1000 rpm for 3-4 min. (at this point you should see the different layers of cells with PEG on top).
f) **Slowly** add 37°C IMDM to 10 ml, without disturbing pellet. After adding , swirl the tube gently to mix and dilute the PEG. Do not disturb the cells.
g) Spin at 1000 rpm for 5 min.
h) Aspirate medium, resuspend cells by tapping. Slowly add 5 ml of 37°C IMDM-m.
i) Bring to 20 ml and add to the flask in the incubator. (If using feeder cells, add them at this point; 10^6 cells / ml).
j) Add Aminopterin (2ml to 200 ml of medium). (Some workers will leave the cells at this point for 24 hours before adding Aminopterin. We add the drug immediately.)
k) Seed the cells in 96 well microtiter dishes, 250 µl per well, 8 plates per fusion.

First clones may be seen in 7-10 days. First screen will usually start after 2 weeks, with a second and third, if necessary, a few days later.

Screening

ELISA

Materials:

- Plates - 96 well Dynatech Immulon, type 2. (Fisher 17-0221-199).
- Adsorbtion buffer 10X = 1M $NaHCO_3$ pH 9.6 (80ml 2M Na_2CO_3 + 170ml 2M $NaHCO_3$ + 250ml ddH_2O) or PBS.
- PBS, TBS
- PBS + 0.1% Tween 20 or TBS + 0.1% Tween 20.
- Blocking solution = 2% BSA (type V) in PBS. (Add 0.02% azide for longer storage.)
- Elisa buffer = 2% BSA + 0.1% Tween 20 in PBS (azide optional).
- Diethanolamine Ph 9.8 (48.5 ml diethanolamine + 0.1 gm Na Azide + 0.05 gm $MgCl_2.6H_2O$ in 400 ml ddH_2O. Adjust pH to 9..8 with concentrated HCl. Bring to 500 ml., store at 4°C in dark bottle).
- Enzyme linked antibody = Alkaline phosphatase Goat anti mouse. (Zymed 62-6422). [1]
- Substrate = Sigma 104 phosphotase substrate tablets (Sigma 104 -105).[1]

Protocol:

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[1] This is the system we have been using mostly, but there are other linked antibodies available which can be used with the same procedure.

1) ADSORBTION OF ANTIGEN
   a) Dilute Ag to 10 µg / ml in 1X adsorbtion buffer.
   b) Add 100 µl of Ag solution to each well. (A good control would be to leave an empty well with no Ag for every well that contains it.)
   c) Leave O.N covered with saran wrap , at 4°C.

2) BLOCKING
   a) Wash unbound Ag by inverting the plates and flicking the wells dry.
   b) Rinse by adding PBS to each well and inverting it again (use squirt bottle).
   c) Repeat the rinse twice.
   d) Add 100 µl of blocking solution to every well, leave 1 hr at room Temp or O.N at 4°C.

3) PRIMARY ANTIBODY
   a) Add the antibody to be tested :
      Sup of cells = 25 µl , mix well by pipeting up and down (10 times).
      serum, ascites = 1:500 to 1:1000 dilution. Do dilutions in blocking solution.
   b) Leave 1 hr at room temp or O.N at 4°C.

4) SECONDARY ANTIBODY
   a) Wash unbound antibody 4 times with PBS + 0.1% Tween 20.
   b) Add 100 µl of enzyme linked antibody to all wells. Do the appropriate dilutions in the Elisa buffer. (ex: AP is 1000X).
   c) Leave 1 hr at room temp or O.N at 4°C.

5) SUBSTRATE
   a) Dissolve substrate in Diethanolamine (for AP substrate, warm 37°C, 5 min.) to concentration of 1 mg / ml.
   b) Wash plate 4 times with PBS + 0.1% Tween 20 (use TBS instead of PBS for AP).
   c) Add 100 µl of substrate to every well.
   d) Watch color development. This could take from a few seconds to 20 min.
   e) If needed, stop the reaction by adding 50 µl of 4M NaOH.
   f) Read absorption in Elisa reader at correct wavelength (for AP system 405 nm).

Expanding Hybridoma Clones

1) Transfer the srongest positive clones to a 24 well plate by scraping the adhering cells with a truncated yellow tip.
2) Bring the volume to 1 ml using the IMDM-m medium.
3) When cells reach confluency, freeze them (about 10^6 cells per full well). Scrape them using a blue tip.
4) Add 1 ml of IMDM-m to cells left in the well, let them grow and repeat the freezing.
5) While the cells are growing, save their sup at each freezing. Use the sup to do the other tests required such as western, immunofluorescence and others. (If sup is not used under sterile conditions, add 0.02% azide and keep at 4°C for months.)
6) Transfer the cells to a 25 cm^2 flask in 4 ml of IMDM-m and continue to expand as desired. The cells at this point are "addicted" to the macrophage supplement. It is possible to wean them by gradually reducing the amount of MCM. We do it only if very large volumes are needed.
7) After deciding which one are the best clones, it is advisable to reclone them to ascertain their homogeneity (next chapter).
8) The subclones should be checked by all the assays which were used to test the originals . The subclones should be frozen at least twice.
9) If convinced that the subclone is monoclonal, checking the isotype is possible . (We have been using a kit from Zymed - MONOAB-ID).
10) If not convinced that the hybrydoma is monoclonal, reclone a second time.

Cloning of Hybridoma

1) Make an accurate cell count of cells in log phase (3×10^5 - 1×10^6 cells / ml).
2) Determine the volume needed which will give you 100 cells.
3) Add the 100 cells to 25 ml of IMDM-m and plate 250 ml of the suspension in each well. Therefore, each well should get 1 cell delivered into it.
4) Clones should be visible and ready to screen in 10 to 14 days.

Ascites Production

1) 7 days before injection of cells, inject 0.5ml of Pristane (Tetramethyl - pentadecane, Sigma T-7640), intraperitoneally.
2) Prepare cells in log phase.
3) On day of injection, spin 1-2 $x10^6$ cells per mouse.
4) Resuspend in IMDM or PBS at 1-2 $x10^6$ cells / 0.5ml (do not leave the cells without serum for more than 1 - 2hr). Transfer cells to syringe using a 16G needle.
5) Inject 0.5ml per mouse, using a 20G needle, intraperitonealy.
6) Collect ascites when animals are ready (swollen abdomen), 10 days to 15 days after injection.
7) Spin fluid at 3000g for 10 min. to remove the cells. If there is an oil layer, remove it and discard. Carefully remove the supernatant from the cells.
8) For storage, add sodium azide to 0.02%. Store at -20°C.

Literature

1. Antibodies - A Laboratory Manual; D. Lane, Ed Harlow.
2. Monoclonal Antibodies: Principles and Practice; James W. Goding.
3. Monoclonal antibody technology; Ailsa M. Campbell.

David Bowtell

ALKALI PLASMID PREPS

Large scale prep:

1) Spin down cells and resuspend in **5ml** (for a 50-200ml culture) of 25mM Tris, 10mM EDTA pH 8.0.

2) Add **10ml** of freshly prepared 0.2M NaOH, 1% SDS and mix thoroughly. Leave for 2-3min. MAXIMUM.

3) Add **7.5ml** KoAc solution. Vortex.

4) Spin 10 min. at 7K in Sorval.

5) Pour supernatant through a piece of cheese cloth and add **11ml isopropanol**. Spin 10 min. 7K in Sorval (the large amount of RNA acts as a carrier).

6) Resuspend pellet in 3ml of TE. Add to tube and make up to 4.3ml with TE. Add 100µl of 10mg / ml ethidium bromide and 4.4g of CsCl.

7) Band for 6hr (minimum) to o / n in VTi65 at 50-55K 20°C.

8) + / - reband by pulling band and adding to fresh tube and topping up with CsCl density 1.6 (is 1g CsCl per ml TE, no extra E'Br required). Spin as above.

9) Remove ethidium bromide by extracting with saturated butanol 3-4 times. Isolate plasmid DNA by adding an equal volume of TE and 5 volumes of room temperature ethanol. Spin down plasmid immediately and resuspend in 400µl TE. Reprecipitate in an ependorf tube by adding 1ml ethanol and 35µl 5M NaCl.

Mini preps ("1 tube method")

1) Grow overnight of **1.5**ml.

2) Pour into eppendorf tube and spin down cells at 7-8K for 2min (low speed makes them easier to resuspend).

3)Aspirate supernatant and resuspend cells in **50µl** 25mM Tris pH 8.0, 10mM EDTA. You can leave the lids off for all the following steps till you add the phenol.

4) Add **100µl** of (freshly prepared) 1% SDS, 0.2M NaOH. Add it forcefully and you don't need to vortex. It is not necessary to pause between steps 4, 5, 6.

5) Add **75µl** KOAc solution and vortex.

6) Add **100µl** of phenol/CHCl3. Close lids and vortex.

7) Spin in ependorf 13K for 2min.

8) Add supernatant to **500µl** ethanol and spin 13K for 5min.

9) Aspirate supernatant, removing all the ethanol. Resuspend in **50µl** TE (you don't need a 70% rinse unless you are going to cut with a very salt sensitive enzyme).

10) Dispose of phenol into appropriate waste before discarding tubes.

I normally digest 2-5µl of this prep for an analytical gel. This DNA contains very little chromosomal and is fine for cloning.

KOAc solution is:
60ml 5M potassium acetate
11.5ml glacial acetic acid
28.5ml of DDW.
Not necessary to pH it (it should be around pH 6).

<u>No phenol method</u> according to Gilles Morelle in Focus (11.1). At step 5 of mini-prep. Add 75µl 7.5M ammonium acetate instead of KOAc (pH 7.8 without adjustment) and then leave the tube on ice for 10 min.. The RNA and chromosomal will precipitate. Spin 3min 10K and add 0.6vol. (100-125µl) isopropanol, leave on ice 10 min. and spin 15K 10 min., 70% rinse and resuspend pellet in 100µl TE.

Dan Kalderon

SMALL-SCALE PLASMID DNA PREPARATION

The fastest method is the "boiling method" but alkaline lysis (see large-scale preparation of plasmid DNA) using 100µl sol'n I, 200µl sol'n II and 150µl sol'n III also works well.

Boiling Method
Works for plasmid and M13 RF DNA

1. Grow saturated culture of plasmid-containing bacteria in 1.5ml L-broth or 2XYT containing appropriate antibiotic with good aeration at 37°C or inoculate a 1:20-1:50 dilution of TG1(or DG98) O/N with a tooth-picked M13 plaque and grow for 5 hr.

2. Transfer most of the bacterial culture to a microfuge tube and pellet bacteria(30s). Aspirate supernatant (store supernatant as a stock for M13 phage).

3. Resuspend bacteria in 100µl STET by pipette or vortex
   STET is: 8% sucrose
   0.5% Triton-X100
   50mM EDTA pH 8.0
   10mM Tris-Cl pH 8.0

4. Add 100µl lysozyme (2mg/ml) in STET to each tube, mix and leave at room temp. for 1-5 min.

5. Place tubes in boiling water bath (switch off heat to prevent tops popping) for 1 min.

6. Spin 10 min at room temp. in microfuge.

7. Remove pellet with a flat toothpick.If the pellet occupies a large proportion of the volume of the solution continue but add STET to 200µl and, next time, use more lysozyme; if the pellet is very small and brittle, use less lysozyme.

8. Add 30µl 4M ammonium acetate, 250µl isopropanol, mix and spin 10 min at room temp in microfuge. Wash pellet with 70% ethanol, dry and dissolve in 20-50µl TE. Enough DNA is recovered for at least 5 digests but the bulk of nucleic acid is RNA which should be removed with DNase free RNase (made by boiling RNAase A at 2mg/ml for 30 mins. and cooling slowly) to see small DNA fragments. M13 R.F. preparations usually contain a number of bands of lower molecular weight than expected that presumably represent deletion products.

9. Plasmid DNA prepared in this way can be used for

   (i) digestion with most enzymes
   (ii) filling-in sticky ends with Klenow
   (iii) purification of DNA fragments for ligation or for use as probes
   (iv) nick-translation (with ^{32}P or biotin)
   (v) double-stranded sequencing
   (vi) template for riboprobe synthesis

   Plasmid DNA prepared as described cannot easily be succesfully used for

   (i) digestion with several restriction enzymes, although digestion is normally fine if the DNA is phenol($CHCl_3$/IAA) extracted and ethanol precipitated (although some members of the lab find this to be untrue).
   (ii) Bal 31 digestion (except with good RNase treatment as RNA inhibits Bal 31 activity on DNA)
   (iii) Drosophila embryo injection
   (iv) *in vitro* transcription

Alternative methods from step 3:

A) Add 350µl STET containing 1mg/ml lysozyme, resuspend thoroughly (1 tube at a time), boil 2 min, spin, pick out snot(sic), add amm. acetate (this is optional) and 350µl isopropanol, spin, wash in 70& ETOH and use 1/20 in a digest.

B) As in A, but use 400µl isopropanol, skip the 70% ETOH wash, and use 1/50 per digest.

Ulrike Heberlein

MAXI PLASMID PREPS

For 500 ml cultures:

1) Grow 500ml cells at 37°C until $OD_{600\ rm}$ = 0.6. Add 1.65ml of 50mg / ml chloramphenicol in 100% ethanol to give a final concentration of 167µg / ml. Incubate overnight (12-15 hours) at 37°C.

2) Harvest the cells at 5K for 10 min. Drain well.

3) Suspend the pellets in 18ml of Solution I. Add 2 ml of Solution I containing 20mg / ml lysozyme (fresh). Incubate at room temperature for 10 min. (Note: if the cell culture was not amplified with chloramphenicol, use 40mg / ml lysozyme.)

4) Add 40ml of Solution II. Mix gently by swirling. Place in an ice water bath for 5 min.

5) Add 20ml of pre-chilled Solution III. Mix the slurry by swirling. Incubate on ice for 15-20 min.

6) Centrifuge at 10K in a GSA rotor for 10 min. Carefully decant the supernatant from the pelleted cell debris. Filter through a Kimwipe in a funnel to remove any debris.

7) Add 0.6 volume of isopropanol (43.5ml). Mix by swirling. Let stand on ice for 20 min.

8) Centrifuge for 15 min. at 10K in a GSA. Discard supernatant. Let the pellets dry briefly (until the isopropanol has evaporated).

9) Dissolve the pellet in 9.3ml TE. Add 10g CsCl and 0.7ml EtBr (10mg / ml).

10) Centrifuge at 38-40K, 20°C for 48 hr in 70.1Ti rotor.

11) Remove the bands (visualized by either long UV light or visible light) containing plasmid DNA. Extract repeatedly (minimum 6X) with isopropanol (saturated with CsCl and H_2O) until the orange-red color of ethidium bromide is no longer visible.

12) Remove the CsCl by exhaustive dialysis overnight against TE at 4°C.

13) Add 1 / 10 volume of 3M NaOAc and 3 volumes of 100% ethanol. Chill at -70°C for 10 min and centrifuge at 17,000 x g for 15 min. Decant supernatant. Drain well.

Solution I (50mM glucose, 25mM Tris pH8 10mM EDTA pH8)
9.01g Glucose
25ml 1M Tris pH8.0
40ml 250mMEDTA pH8.0
DD H_2O to 1 liter
Autoclave for 20 min.

Solution II
0.2M NaOH
1% SDS
Make fresh.

Solution III
600ml 5M Potassium Acetate (pH4.8) (= 300g / 600ml)
115ml Glacial Acetic Acid
285ml DD H_2O
Autoclave for 20 min.

Steve Mount

GROWTH OF M13 PHAGE

Growth and plaque-purification of M13 phage is generally performed as described in the MRC protocols (Bankier and Barrell-1983-"Shotgun DNA Sequencing" in Techniques in the Life Sciences, B5; *Nucleic Acid Biochemistry*, B508, 1-34; Elsevier Scientific Publishers Ireland Ltd.). The host strain is JM101 or TG1, which is isogenic with JM101 except that hsdΔ5 has been crossed onto it, rendering it Eco K r- m-.

Selection for retention of the F factor by growth on minimal media is essential for JM101 and TG1. One should always grow fresh overnights in 2xYT from a colony on a minimal plate.

For preparation of RF, I quote: "When growing a bulk culture of M13 infected TG1, 10 ml. of a 1/100 dilution of an overnight culture is grown with shaking at 37° for 4 h. [toothpick a plaque into 1.5 ml. of a 100-fold dilution of an O/N culture of TG1 in 2xYT broth; shake standing vertically in a rack at greater than 300 rpm until it is visibly turbid (about 2 - 3 hours). Pour this infected culture into a 50 ml. tube holding 10 ml. of a growing culture of TG1, set up in parallel with the infected culture. Shake for a further 4-5 hours.] After centrifuging for 10 min. at 2000 rev./min, the supernatant containing the phage particles can be stored at 4° overnight [We have found that this sort of culture supernatant can be stored frozen at -20° following growth in 2xYT for at least two years without significant loss of plaque-forming-units] and used to infect a fresh culture of cells at A_{660} of ~ 0.2. This is grown with shaking at 37° to A_{660} ~ 0.6 before proceeding with the chosen DNA purification technique." We've had good results allowing two 500ml. cultures to grow to A_{600} ~ 0.5 before inoculating at 5 ml. per flask, allowing growth with vigorous shaking for another 4 hours, then adding chloramphenicol at 25 µg/ml for the final 30 min. of growth.

Plaques are generally obtained by transfection with phage DNA. Dilute an overnight 1/100 into 2xYT and grow for 1.5-2 h with shaking at 37° to A_{660} ~0.3. Pellet cells at 4° and resuspend in one-half volume cold 50 mM $CaCl_2$. Keep on ice 15-30'. Spin again and resuspend in one-tenth of the original volume cold 50 mM $CaCl_2$. These competent cells do not freeze well, give the best results between 1 and 12 hours after being made, and are not useful after 48 h. To transfect 200 µl of these cells are aliquoted out into 13x100mm glass test tubes on ice, and ligation reactions or DNA (1 µl of a 1/100 dilution of a standard single-stranded M13 miniprep works well) is added directly, folowed by a 25-30 min. incubation. During this time top agar (0.7% in 1xYT) should be melted (at least 3 ml/plate). After the incubation the cells are heat-shocked by placing the tubes in a 42° temp-block for 5 minutes. During the heat-shock 3 ml. top agar (50° C), 25 µl of the TG1 O/N, 25 µl BCIG ("X-Gal", 25 mg/ml in dimethylformamide) and 25 µl IPTG (25 mg/ml filter-sterilized in water) per plate are combined at 50° [of course, the indicator and inducer are only needed when screening for inserts]; 3 ml. of this mixture is added to each tube, and the tubes are quickly mixed and poured. Generally we use standard lambda plates, but plates of 1.2% agar in 1xYT may give slightly better growth.

Single-stranded template DNA is prepared from plaques toothpicked into 2 ml. aliquots of a 1/100 dilution of a fresh overnight placed in 13 x100 mm glass tubes. These (multiples of 24 are most convenient) are grown standing in a vertical rack,

shaking at greater than 300 rpm, for 4.5-6 h. at 37°. Transfer 1.5 ml. into a microfuge tube and spin 5'. Carefully pour or pipet the supernatant into a fresh microfuge tube containing 200 µl of 20% PEG, 2.0 M NaCl (polyethylene glycol 8000 from Sigma; this solution should be filtered), being very careful not to carry over any of the pellet. Mix, and allow this to stand for at least 10 minutes. Spin 10 minutes in a microfuge. Aspirate the supernatant, spin again for ~ 1 min, aspirate again. Resuspend in 100 µl TE (be sure the pellet is suspended before adding phenol). The most rigorous extraction procedure is to vortex well, spin for 5 min, and transfer the supernatant, using 1) 50 µl chloroform 2) 50 µl buffer-saturated phenol 3) 50µl of a 1:1 chloroform:phenol mixture. The simplest procedure is to use only phenol, and many choose to do only the first two extractions. Following extraction, the aqueous phase is brought to 150 mM NaOAc and precipitated with 250 µl of ethanol (usually overnight at -20°). The pellet is washed once with 95% ethanol and resuspended in 30-40 µl of TE. This volume can be adjusted somewhat in response to the size of the PEG pellets.

Mark Fortini

GEL PURIFICATION OF OLIGONUCLEOTIDES

I generally purify 25-mer and larger oligonucleotides using the following protocol. For smaller oligonucleotides, I use the TLC purification protocol (see follwoing protocol). For many purposes, such as making primers for DNA sequencing, oligonucleotides may be purified sufficiently by passage through a G-10 spin column after lyophilization.

1) For gel purification, first dry down unpurified oligonucleotide solution in a SpeedVac or lyophilizer.

2) Redissolve the pellet in 50-100 µl 0.1 N NaOH. Add 1/3 volume of loading buffer (formamide/bromophenol blue dye).

3) Prepare a 12% polyacrylamide gel (20:1 acrylamide:bis) as follows:
-for 250 ml of gel solution (suitable for a 30 cm x 40 cm x 0.75 mm gel)
115 g urea
12.5 ml 20X TBE
63 ml ddH_2O
75 ml 40% acrylamide solution (20 acryl:1 bis)
750 µl 25% ammonium persulfate
150 µl TEMED (add just prior to pouring gel)

4) Cast gel using spacers of 0.75 mm thickness and wide-tooth combs (wells should be 2-4 cm wide).

5) Load all or part of each oligonucleotide sample (from 2 above) onto the gel. Due to diffusion of the sample during electrophoresis, it is advisable to leave an empty lane between each sample. Run gel at 30-50 watts until the bromophenol blue dye front is ~75% down the gel.

6) Separate the gel plates and transfer the gel onto Saran wrap. The oligonucleotide samples are visualized by short-wave UV shadowing onto a TLC plate placed under the Saran wrap and gel. The area of each gel lane containing the oligonucleotide appears as a large dark spot against the green illuminated background of the TLC plate.

7) Cut out the region of the gel containing the oligonucleotide with a razor blade.

8) To elute the oligonucleotide out of the gel slices, cut each slice into ~1 cm^2 pieces and place all the pieces from each slice (usually 4-6) into 5 ml of elution buffer:
Elution buffer:
500 mM NH_4OAc
1 mM EDTA
10 mM MgOAc
0.1% SDS

9) Elute oligonucleotide out of gel for 4-6 hrs at room temp (gentle agitation is optional). To check that elution is complete, examine gel pieces by short-wave UV shadowing as described above.

10) Extract the oligonucleotide eluate with phenol and ethanol precipitate. Wash the pellet 2X with 70% ethanol, dry and redissolve in a small volume (eg. 100-200 µl) of TE, ddH_2O or other solution.

11) Measure the concentration of the purified oligonucleotide solution using a spectrophotometer. For oligonucleotide solutions, I assume that an OD_{260} of 1.0 is equivalent to 40 µg/ml.

Charles Zuker

PURIFICATION OF OLIGONUCLEOTIDES BY THIN LAYER CHROMATOGRAPHY (TLC)

For practical purposes TLC-purified oligonucleotides are as good as gel-of-HPLC-purified oligos. TLC, however, is much cheaper.

1) Lyophilize oligonucleotide (100-300µg); resuspend in 100-300µl of distilled water.

2) Load oligo on a Silica Gel TLC plate (Silica Gel 60 F-254, EM Reagents, Cat. 5765-7, 20x20 cm) as a thin line across the plate approximately 2 cm from the bottom.

3) Insert plate on a chromatography tank (30x30 cm) containing 100 ml running buffer (55ml n-propanol, 35ml NH_4OH, 110ml H_2O). Run until front is at or near the top.

4) Remove plate and let dry inside a hood for a few hours (or until completely dry). Visualize oligos using short-wave UV lamp and use a razor blade to scrape off your oligonucleotide (usually the lowest and most intense band of the ladder of oligos).

5) Add silica powder to eppendorf tube containing 300-600µl of water, vortex for a few min and spin down for 10 min. Re-extract the silica with 1/3 volume, spin, mix supenatants and respin for 5 min.

6) Read O.D. of your purified oligo (260 nm).

(D. Coit, P. Barr, P. Valenzuela)F. Laski

PROCEDURE FOR SITE-DIRECTED MUTAGENESIS

The following procedure is a modification of the primer-directed mutagenesis technique developed in the Smith, Itakura and RajBhandary labs. It has been found very useful and is repeated here intact. Following the procedure are comments and modifications which may be useful.

1) Kinase the synthetic primer as follows: Mix 25 µl of primer in H_2O (0.1 $O.D._{260}$ units, ~700 picomoles), 3 µl of 10X kinase buffer (500 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 100 mM DTT), 1 µl of 100 mM ATP and 1 µl of T_4 polynucleotide kinase. Incubate at 37° for 30 min. Stop reaction by heating for 10 min. at 65°C.

2) For annealing, mix 3 µl (2-3 µg, ~1 picomole) of M13 template DNA (or Bluescript ssDNA - see comments below) containing the sequences to be mutated, 3 µl of kinased primers (~70 picomoles) (directly from the above solution), 1 µl of a solution containing 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl and 10 mM DTT and 3 µl of H_2O to complete 10 µl final volume. Heat at 55° for 5 min, then at 23° for 5 min.

3) For extension and ligation, mix: 10 µl of the above annealing reaction, 10 µl of a solution containing 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each deoxynucleotides and 0.1 mM ATP, 1 µl of a 1:2 dilution of BRL large fragment E. coli DNA polymerase (10 units/µl) and 1 µl of Biolab's T_4 DNA ligase (400 cohesive end ligation units/µl), in a total volume of 22 µl. Incubate at 16° for 18-24 hrs.

4) OPTIONAL: For treatment with nuclease S_1 mix: 2 µl of the solution after extension and ligation, 1 µl of 10X S_1 buffer, 1 µl of a 1:100 dilution of BRL S_1 nuclease diluted in 1X S_1 buffer, and 6 µl of H_2O to complete 10 µl final volume. Incubate at 37° for 15 minutes.

5) For transformation, mix: 10 µl of the S_1 reaction mix and 100 µl of JM-101 competent cells. Let sit on ice for 60 min. For plating, add 200 µl of log phase JM101 in YT medium and 3 ml of 0.8% agarose in YT, preheated to 45°. Mix and plate over preheated (37°C) agar plates. Incubate at 37° overnight.

6) For screening by plaque hybridization: Cool plates in the cold for 60-90 min. Overlay a nitrocellulose filter over the plaques, press slightly and leave for 10 min. Carefully lift filters away from plate and leave them drying at room temperature for 60 min.

7) For lysis and fixing place filters over 3 M filter paper moisted with the following solutions: 5 min over 0.5 N NaOH-1.5 M NaCl, 5 min over 3 M NaCl-1M Tris-HCl (pH 7.5), 5 min again over 3 M NaCl-1 M Tris-HCl (pH 7.5) and 5 min over 2X SSC. Bake filters for 2 hrs at 80° under vacuum.

8) For pre-hybridization, incubate each filter in 5 ml of 0.2% SDS, 10% Denhardt's solution, 6X SSC at room temperature for 60 min.

9) For hybridization wash filters in 6X SSC for 5 min and incubate each filter in 5 ml of 10% Denhardt's, 6X SSC and $2x10^5$ cpm of probe fragment (probe DNA fragment is kinased with γ-ATP to specific activity of $1\text{-}4x10^7$ cpm / µg). Incubate at room temperature for 2 hrs.

10) Wash filters in 6X SSC at room temperature for 5 min. Expose to X-ray film for 2 hr with screen. To differentiate specific from non-specific hybridization wash again at higher temperatures, i.e. 40°, 55° etc. (depending on the length and composition of the probe), and expose to X-ray film.

11) For plaque purification, take the agar region containing the positive plaque and suspend in 5 ml of YT. This should contain a total of 10^8 to 10^9 pfu. Dilute this solution 200 times in YT to make a stock of approximately 10^6 pfu / ml. Dilute this again 100 times in YT to approximately 10^5 pfu / ml. Plate 10, 50 and 100 µl as described in step 5.

12) Repeat steps 6 to 10 as necessary.

13) Take isolated plaque and suspend in 1.5 ml of a 1:40 dilution of log phase JM101 in YT. Grow for 5 hrs at 37°. Centrifuge in 1.5 ml Eppendorf tubes. Save supernatant for DNA sequencing and pellet for alkaline-SDS miniscreens.

Comments and Changes on Procedure

Purification of M13 and Bluescript Single Stranded DNA

This is my procedure for preparing M13 single strand DNA, but feel free to use your normal procedure. One ml of a TG1 overnight is added to 300 ml of 2xYT. Thirty minutes later add 20 ul of your M13 recombinant phage. Shake for ~ 7 hours, then spin down bacteria for 10' at 6,000 RPM. Add to supernatent (300 ml) 20 ml of 5 M NaCl and 10g PEG. After PEG goes into solution let it sit for 10 min. on ice. Spin 10 min. at 6,000 RPM, pour off supernatent and let the pellet air dry. Resuspend the pellet in ~5 ml of TE, phenol 2 times, chloroform 1 time and ethanol precipitate. Resuspend the pellet in TE (as small a volume as easily possible, usually between 400 and 800 µl).

Many people use this M13-recombinant single strand DNA directly for mutagenesisis. I however, first clean it up over an alkaline sucrose gradient. The above single stranded DNA is contaminated with short fragments of DNA and / or RNA that can prime the single stranded DNA in the Klenow reaction, resulting in increased background and possibly double mutations. These short fragments are removed on the alkaline sucrose gradient, which results in DNA that is primed only if an exogenous oligonucleotide is added.

The alkaline sucrose gradient procedure is as follows: one half ml of a solution that is 50% sucrose, 1 M NaCl, 0.2 N NaOH 1mM EDTA is added to a 1 / 2 inch diameter x 2 inch polyallomer tube. A 5% to 20% gradient is then poured ontop of the 50% sucrose solution. This is done by putting 2.3 ml of a 20% sucrose solution (that is 1M NaCl, 0.2 N NaOH, 1 mM EDTA) into one slot of a gradient former (the slot closest to the spout) and 2.3 ml of a 5% sucrose solution (1 M NaCl, 0.2 N NaOH, 1 mM EDTA) into the other. The gradient is then poured, don't worry if it isn't perfect. 50 to 200 µl of the single stranded DNA solution (100 to 200 µg) in TE is pipetted onto the top of the gradient. The tubes are then spun for 5 hours at 45,000 RPM at 16° C in a pre-cooled SW 50.1. The gradient is then dripped from the bottom in 200 to 250 µl

fractions and the fractions assayed by O.D. The peak 2 to 4 fractions are usually at fraction 9 or 10. The purified DNA is neutralized by adding 1/10 volume of 1M tris pH 8.0 and 1/10 volume of 2 M HCl, and thenn 2.5 volumes of ethanol added and the DNA precipitated. The DNA is resuspended in TE and brought to a concentration of 1 mg/ml. Final recovery is usually between 30-50% of input DNA.

This mutagenesis protocol also works well for single-stranded templates in the Bluescript vector system (Stratagene, Inc.). Single-stranded Bluescript DNA is prepared as for DNA sequencing according to the Bluescript protocol booklet. The yield from a small-scale ssDNA prep (1.5 ml culture) suffices for 2-3 mutagenesis reactions.

Comment on Kinasing Oligonucleotide

My procedure is as follows: The oligonucleotide is diluted into the 30 µl reaction so that the O.D. 260 of the oligonucleotide in the kinase reaction is 4.0. As an example, if the O.D. 260 of a 1/100 dilution of the oligonucleotide is 0.295, the the O.D. 260 of the oligonuleotide is 29.5 and I want to dilute it by a factor of 0.135 (4.0/29.5) and therefore will add 4.05 µl (0.135 x 30µl) to the 30 µl reaction. I will of course round the 4.05 µl to 4 µl. Everything is then done as above except that I only do one half hour reaction and I don't bother to kill the enzyme at 65°C.

Comment on Annealing

I do exactly as they suggest except that I heat the sample at 65° instead of 55° (I don't have a 55° water bath) and I allow hybridization at room temperature instead of 23° (I don't have a 23° water bath).

Comment on Extension and Ligation

I add 1/2 µl of both Klenow and ligase without diluting it.

Comment on S_1 Treatment

Instead of just treating all the DNA with S_1 nuclease for 15 min., I always perform a titration, removing 2.5 µl aliquots of the reaction at 0, 1, 5 and 15 min. I find that for some reactions, a 15' incubation is too long and you get no plaques. The titration allows you to knock down the background but still retain enough plaques to work with. I should also add that you can skip the S_1 treatment altogether, although this will usually decrease the number percentage of phage carrying the mutation. However, when using an oligonucleotide that will create an insertion or deletion I sometimes find that S_1 treatment will decrease the percentage of plaques that contain the desired mutation, presumably because the S_1 directly digests the extended and ligated DNA at the loopout caused by the oligonucleotide.

Comment on Transformation and Plating

I follow my normal transformation procedure: mix 2.5 µl of the S_1 reaction mix and 100 µl of TG1 competent cells. Let sit in ice for 10 min. Heat at 37° for 5 minutes. Add 1 ml of 2xYT at room temp. For plating, mix 200 µl of the M13-transformed TG1 cells with 100 µl of TG1 overnight and 3 ml of 0.8% agarose in YT, preheated to 45°. Mix and plate over agar plates. Incubate at 37° overnight. Once the plaques are visible, plate out all or some of the remaining M13-transformed TG1 cells (which have been stored at 4°C) so that the plaques will be well separated. I find that between 40 to ~150 plaques per plate are ideal.

For Bluescript templates, you should do a standard bacterial transformation of the S_1 reaction mix and plate out on Amp plates to obtain colonies rather than plaques.

Properly mutagenized templates are then detected by colony hybridizations rather than plaque hybridizations, using the same conditions as described in steps 6-10 above.

Comment on Making Probe

I mix 1 µl of oligonucleotide (diluted to O.D. 260 = 1) with 1 µl of 10x kinase, 2 µl of ^{32}P-ATP (10mCi in ~50µl, carrier free from ICN), 6µl of water and 1/2 µl of kinase. Incubate for 30 min. at 37°C. I use 1/2 of this mixture directly for screening, you don't have to put it over a column.

Comment on Plaque Purification

Even if it is obvious which plaque contains the transformant I always plaque purify it because some of the plaques contain a mixture of mutated and wild type genomes. Instead of diluting to plaque purify I streak out the plaque in the same manner you would a bacterial colony and then very gently add 3.5 ml of top agar containing 100 µl of a TG1 overnight.

Comment on Repeating

This is usually not needed.

Comment on Sequencing

I usually prep 4 plaques for sequencing.

from Grace Gill via Betsy O'Neill

SITE-DIRECTED MUTAGENESIS

Use of the strain CJ236 (*ung- dut-*)to make the single stranded template DNA site-directed mutagenesis causes dUTP to be incorporated in place of dTTP. Later in the procedure after the mutation containing primer is annealed, the second strand is synthesized *in vitro* and the plasmid is transformed into TG-2 cells, the repair mechanisms in the cell tend to repair the uracil-containing parental strand, thus increasing the frequency of appearance of the desired mutant. Reports indicate an increase from about 5% to about 70% (Biotechniques vol. 5, pp786-791, 1987).

1) Purify oligo by TLC or electrophoresis.
2) Kinase 50 pmole of oligo in a 25µl reaction.
3) Set up 2 annealing reactions, one with primer and one without primer.

   ssDNA 1µl (see other protocol)
   primer 1 or 0 µl (2pm kinased oligo)
   10x anneal 1 µl
   ddH_2O to 10 µl

4) Heat to 70°C / 10 minutes. Cool slowly to 30°C, then place on ice.
5) For extension reaction, add to primer annealed template:

   10x extens 2µl
   dNTPs 4µl (2.5mM each)
   ligase 1µl (dilute concentrated NEB stuff 4x)
   T4 Pol 1µl
   ddH_2O 2µl

6) Leave on ice 5 minutes. Transfer to RT 5 minutes. Incubate at 30°C / 90 minutes. Lower temperatures may be necessary for primers with a lot of mismatches.
7) Bring volume to 100ul with TE. Phenol / cholroform extract, ethanol precipitate.
8) Transform 1 / 10th of reaction into TG2.

<u>10x Annealing Buffer</u>

200 mM Tris, pH 7.5
20 mM $MgCl_2$
500 mM NaCl

<u>10x Extension Buffer</u>

10mM ATP
100 mM Tris, pH7.5
50 mM $MgCl_2$
20 mM DTT

Ulrike Heberlein

DOUBLE STRANDED SEQUENCING

Minilysate DNA:

1) Prepare DNA using either alkaline lysis or STET / boiling protocols from 1.5 ml of saturated bacterial culture. (I have found that DNA isolated from strains HB101 or TG-2 sequences well, whereas DNA isolated from TG-1 doesn't work as well). Never freeze the bacterial pellet before isolating the DNA!!! Before denaturing, clean the DNA by consecutive phenol:chloroform and chloroform extractions. Precipitate with 2.5 volumes of ethanol in the presence of 0.3M NaOAc and wash DNA pellet with 70% ethanol. Resuspend in 25µl of TE.

2) To 5µl of the cleaned DNA add 0.5µl of 0.5mg / ml RNAse A and incubate at room temperature for 2-5 minutes.

3) Add 5µl of 1M NaOH (make up from 10M stock about once a month) and incubate at room temperature for 5 minutes.

4) Neutralize by adding 3µl of 3M NaOAc pH 5.0.

5) Add 7µl of a ~1pmole / µl solution of primer (MW is~330g / mole / base; absorbance of a 1mg / ml single stranded DNA solution is ~25).

6) Precipitate with 75µl of 100% ethanol at -70°C for 15-30minutes (or in dry ice for 5-10 minutes). Spin in a cold microfuge for 20 minutes. Wash the pellet with 70% ethanol and dry (the pellet should be visible).

7) Resuspend thoroughly in 8µl of water, add 2µl of Sequenase buffer and anneal as usual. Follow Sequenase instructions.

CsCl purified DNA:

- Use 2-3µg of DNA in a 5µl volume. Denature with 5µl of 1M NaOH for 5 minutes at room temperature and follow protocol described for minilysate DNA.

Don Rio

PREPARATION AND PURIFICATION OF E. COLI β-GALACTOSIDASE FUSION PROTEINS AND THEIR USE AS IMMUNOGENS

Introduction

We use the pUR series of plasmid vectors (Ruther and Müller-Hill, EMBO J. (1983)). These plasmids carry polylinkers in the C-terminal portion of the β-galactosidase gene beyond the Eco RI site used for insertion in lgt11. Therefore, the fusion proteins will generally retain β-galactosidase enzymatic activity. This could be useful for following purification or for affinity chromatography. In any case, select the appropriate vector and clone your open reading frame segment (generally 200-500 bp) into the polylinker using E. coli strain 71-18. Analyze the clone by restriction mapping and/or DNA sequencing to confirm insert orientation and maintenance of the open reading frame.

Analytical analysis of fusion proteins:

The idea here is to examine the size of your fusion protein and to determine its abundance and stability.

1) Grow 1 ml overnight culture in L-broth with 100 µg/ml ampicillin (no glucose).

2) Inoculate 2 fresh 1 ml cultures with 50 µl of o/n. Grow until A_{590} ~0.2-0.4 at 37°C (~2 h).

3) To one culture add IPTG to 1 mM (1:100 of 25 mg/ml). Incubate at 37° for 2 h.

4) Spin down cultures in 1.5 ml Eppendorf tube and drain liquid.

5) Resuspend pellet in 100 µl 2X SDS gel sample buffer (use Eppendorf mixer and resuspend completely: 20-30 minutes).

6) Boil 2-4 min in boiling H_2O broth.

7) Spin 5 min Epp centrifuge.

8) Load 10 µl on 7.5% SDS polyacrylamide gel.

9) Stain gel with Coomassie blue.

10) Destain gel and visualize bands.

Preparative fusion protein purification

Plan A: Preparative SDS-gel electrophoresis (this is suitable for unstable fusion proteins).

1) Grow 100 ml IPTG-induced culture, harvest by centrifugation and resuspend in 3-5 ml 2X SDS-gel sample buffer.

2) Boil 5 min and spin at 12,000 x g for 10 min.

3) Remove supernatant and load ~1 ml onto 1.5 mm thick 7.5% preparative SDS gel.

4 Stain gel with 0.25 M cold KCl. The fusion protein band appears as a white band where K^+-SDS complexes precipitate. Alternatively, the gel can be stained with Coomassie blue without acetic acid and then detained in H_2O.

5) Excise band and either pulverize it for direct injection or electroelute the protein and concentrate it by acetone precipitation.

Plan B: Insoluble aggregate purification. (This is suitable for abundant fusion proteins and when you want a lot of fusion protein, e.g. for coupling to CNBr-Sepharose for affinity purification).

1) Grow 1 L IPTG-induced culture; harvest by centrifugation and freeze at -70°C.

2) Thaw cell pellet on ice in 50 ml Buffer A (50 mM Tris-HCl pH 7.9, 200 mM NaCl, 2 mM EDTA, 2 mM b-mercaptoethanol, 100 µg / ml PMSF, 100 µg / ml PMSF, 1 µM pepstatin and 1 µM leupeptin).

3) Add solid lysozyme to 0.2 mg / ml - incubate on ice for 20 min.

4) Add triton X-100 to 1% - incubate on ice for 10 min.

5) Add Zwittergent 3-14 (CalBiochem) to 0.5% . Incubate on ice for 10 min.

6) Sonicate 3 x 15 seconds on ice using a Branson sonicator with a microtip.

7) Layer the lysate onto 10 ml of 40% sucrose in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 M NaCl.

8) Centrifuge 30 min at 13K rpm in a Sorvall HB-4 swinging bucket rotor.

9) Resuspend aggregate pellet in 2 ml PBS.

10) Add 20 ml urea extraction buffer (8M urea, 0.5 M NaCl, 0.5 M Tris-HCl, pH 7.9, 1 mM EDTA, 30 mM b-mercaptoethanol, 1 µM pepstatin and 1 µM leupeptin).

11) Vortex vigorously until pellet is dissolved.

12) Dialyze at room temperature for 2 h against 2 L of 50 mM Tris-HCl pH 7.9, 0.5 M NaCl, 10% glycerol, 200 µg / ml PMSF with 1 change.

13) Remove insoluble material by centrifugation at 10,000 x g for 10 min.

14) The fusion protein is present in the supernatant at ~1-4 mg / ml and is ~80% pure. It can be used directly for immunization and should be stored at -20°C.

Immunization:

1) We use 3-5 lb. female New Zealand white rabbits.

2) Inject 50-200 µg fusion protein in 0.5 ml PBS + 0.5 ml complete Freund's adjuvant (Gibco) at multiple intradermal sites or subcutaneously.

3) At 3-week intervals - a boost of the same amount of antigen with incomplete Freund's adjuvant. After 2 boosts, the serum is usually high titer. The rabbits are bled and serum prepared.

4) I have kept boosting for 3-4 months and can still get usable (high titer) serum.

Preparation of Serum from Blood:

1) Bleed rabbit from central ear artery into glass Corex tube. Allow blood to clot at room temp for 1-2 h.

2) Use pipette to dissociate clot from sides of glass tube and leave o / n at 4°C for clot to contract.

3) Use pipette to remove serum (should be approx. 1 / 3 of the total blood volume). Spin at 1000 rpm in benchtop clinical centrifuge or 5K in Sorvall SS34 for 5 min to pellet RBC. Store at -20°C in conveniently sized aliquots.

Kevin Jones

FUSION PROTEINS FOR ANTIBODY PRODUCTION

This method for the production of specific antisera includes purification of a fusion protein produced in bacteria by isolation of an insoluble protein fraction (fusion proteins expressed at high levels frequently form inclusion bodies which are insoluble), further purification by preparative gel electrophoresis (SDS-Laemmli method), electroelution of the protein, and injection into mice (or other appropriate animal — the general experience of members of the Rubin lab has been that mice often produce quality sera having low background). Mice are also a good choice if one might be interested in generating a monoclonal.

With respect to the fusion protein vector selection, there are many possible choices and the ideal choice may be quite dependent on your protein. The pATH fusion vectors (trpE fusion) have worked quite well for me, I have obtained a mg of pure protein from 50ml of culture with several different constructs using these vectors and the method described below. Other reports of similar yields with trpE fusions are present in the literature. The pUR vector series (lacZ fusion) has been successfully used by many people in the Rubin lab, the primary advantage of this and other lacZ fusion vectors is that they lead to a very large fusion protein (116kd + what you add) which is substantially larger than most of the other proteins which are present in a bacterial extract, facilitating purification by gel electrophoresis. lacZ fusion proteins are also often insoluble or if soluble can be purified with a substrate analog affinity resin. Yields per ml of culture will generally be substantially lower than those obtainable with trpE fusions. Several lacZ fusion vector series other than the pUR series are availible at this writing, and they may provide superior yields, but I have not had any experience with these. The T7 vector system (pET vectors) deserves a mention — this vector system is useful for producing protein initiated at the bona-fide initiation codon, or for making fusion proteins (to a portion of a T7 coat protein). Levels of protein production comparable to that obtained with trpE fusions can be achieved. In summary, with regard to selecting vectors my suggestion is that you choose two different vectors which have convenient restriction sites and which produce protein having the desired characteristics (i.e. intact or fusion, methods of purification). It is useful to have fusions to different proteins (e.g. lacZ and trpE) so that a sera raised against one fusion can be tested with the other, or one fusion protein can be used for affinity purification of sera raised against another.

The choice of regions in your protein to use as antigen is somewhat arbitrary but there are several points worth mentioning. First, hydrophilic regions are often more strongly antigenic than are hydrophobic regions. Second, it is wise to try several different (preferably non-overlapping) regions if possible. Yields of fusion can be quite variable with different constructs, and one may also be a lot more antigenic than others. Also, if you have several sera directed against different portions of the protein which give identical results in histochemical, western experiments, etc. it is likely your sera are recognizing the protein you hope they are (in the absence of better controls).

The protocol below is based upon methods described for purifying insoluble trpE and lacZ fusions. Therefore, while the conditions for the growth of cells and induction are described for trpE fusions, it should in principle be applicable to purification of any insoluble protein fusion. For general information about production and use of antibodies, the laboratory manual by Harlow and Lane is highly recommended.

FUSION PROTEIN PURIFICATION-trpE fusions

1) Pick colony, grow overnight in L-broth with 100mg / ml AMP.

2) Inoculate M9-Casamino Acids media (100mg / ml AMP) 1:100; grow to $O.D._{600}$ of approximately 0.2 and add a 1:1000 dilution of 10mg / ml β-indoleacrylic acid (Sigma). Let grow overnight (8-16hr).

3) Chill cells on ice, harvest 4krpm, 10'.

4) Store cell pellet at -80^{o}.

(Volumes given in parentheses after instruction are for 25ml, 50ml, and 500ml cultures, respectively.)

5) Thaw cells on ice in 1 / 10 vol TEN (culture vol=1 vol), resuspend by vortexing. (2.5ml, 5.0ml, 50ml)

6) Add lysozyme to 0.2mg / ml. (50µl, 100µl, 1ml)

7) Let sit on ice 20'.

8) Bring to 0.2% NP-40,10' on ice. (add 100µl, 200µl, 2ml)

9) Add .15 x culture vol 1.5M NaCl, 12mM $MgCl_2$. (3.75ml, 7.5ml, 75ml)

10) Add DNase I to 4 µg / ml, leave on ice 1hr, stir regularly.

11) Layer onto sucrose cushion (at least 1 / 100 culture vol). Spin 30' 13krpm in Sorvall HB-4 (swinging bucket) or equivalent.

12) Resuspend aggregate pellet in TEN and re-spin (to wash). This wash can be repeated several times if desired.

13) The protein can be run over a prep gel; at the end of step 12 it should be at least 50% pure.

14) An extra step number, for luck.

Solutions:
TEN: 0.2 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA
sucrose cushion: 40% sucrose in TEN (filter sterilize and store at $4^{o}C$)

Assorted Notes:

1) Sonication can be substituted for DNase treatment (steps 9 and 10), both appeared to work equally well in my hands. I found DNase treatment to be easier.

2) Protein can be electroeluted from the gel slice in protein gel running buffer and then dialyzed against 1xPBS. After quantitation, this material can be used for

immunization. Generally, about 20mg / mouse is considered plenty for each immunization.

3) I found the use of protease inhibitors to be unnecessary. You may need them for your fusion, but since they are often nasty or expensive, I'd omit them if possible. It would seem likely that protein in inclusion bodies might be somewhat resistant to proteolysis, so any degradation products you see (you probably will see some) may be a result of degradation of protein during the period the cells are actively synthesizing it, and not during your purification.

REFERENCES

Science 214:1125 (1981)
J. Virol. 49:132(1984)
Cell 44:21 (1986)
Antibodies-A Laboratory Manual; Harlow, E., Lane, D. (1988) Cold Spring Harbor Laboratory Press

Kevin Moses

MAKING Trp E FUSION PROTEINS FOR ANTIBODY PRODUCTION

This procedure aims to make a novel protein with the N-terminal 37 kD of the *E. coli Trp E* gene product and any other protein at the C-terminus in order to inocculate animals for the production of anti-sera. In outline you must insert DNA bearing your open reading frame into the polylinker of one of the pATH vectors such that your protein will be appended to the first 37 kD of the bacterial *trpE* gene product. You will make a **translational** fusion which must therefore be in frame. There are several pATH plasmids with various polylinkers (see map/sequences below) and at this time all are unpublished. Details may be obtained from one of their creators, Alex Tzagolof (Columbia (212) 854-2920). I have used the trio pATH 1, 10 and 11 which supply EcoR1 sites in each of the three frames:

pATH 11:	GAA TTC CAG
pATH 1:	CGA ATT CGA
pATH 10:	CCG AAT TCG

Anecdotally pATH11 gives the highest protein yields. You can choose appropriate cDNAs that end at a 5' synthetic Eco R1 site, or engineer one in by site directed mutagenesis, or use a naturally occurring site. Otherwise there are many other restriction sites in the polylinkers to choose from. Don't worry about the C-terminus, as all three frames stop soon after the polylinker. All of these vector DNAs grow very poorly, so be prepared to put all of your miniprep plasmid DNA in one track to see it on a gel. Including tryptophan in the growth medium may help avoid selecting non-expressing mutants. Once you have your subclone in *E. coli HB101* do the following:

1) Grow a 5ml seed culture in LB + 50 µg/ml ampicillin shaken at 37°C to saturation (overnight).

2) Inoculate 250 ml of M9CA + 50 µg/ml ampicillin in a 2 liter flask with 2.5 ml of the seed culture and grow shaken at 37°C to A_{600} = 0.2 (about two hours).

3) Add 250 µl of 10 mg/ml 3β-indole-acrylic acid (*Sigma* No.I-1625) in propan-1-ol and continue to incubate shaken at 37°C for at least four hours (I usually go overnight).

4) Make up 50 ml of TEN+ and store on ice.

5) Spin down the cells and resuspend in 25 ml TEN+ and transfer to a 50 ml disposable polypropylene tube, add a dash of lysozyme (*Sigma* No. L-6876) and incubate on ice for 25 minutes.

6) Add 2.5 ml of a 10% solution of triton X-100 (*Sigma* No. T-6878) in water, vortex and incubate on ice for 10 minutes.

7) Add 1.25 ml of a 10% solution of Zwitergent (*Calbiochem* No. 693017) in water, vortex and incubate on ice for 10 minutes.

8) Sonicate at maximum microtip energy in repeated 10 second bursts with cooling on ice for a total of 60 seconds, or until the viscosity drops significantly. This step breaks

E.coli DNA, which otherwise reduces the effects of the subsequent wash steps and can interfere with the running of preparative gels.

9) Keep 50 µl of the total extract and load the rest carefully (so as to avoid mixing) onto a 10 ml sucrose cushion (40% sucrose in TEN) in a 50 ml polycarbonate tube. Mark the position of the interface.

10) Spin for 30 minutes at 4°C at 12,000 rpm in an HB-4 rotor (or similar).

11) Take off the supernatant above the interface, keep a 50 µl aliquot handy and store the rest frozen at -20°C. Then remove and discard the sucrose.

12) Resuspend the pellet in 5 ml TEN+ and transfer to a 15 ml corex tube on ice. To break up the pellet you will need to thoroughly beat up on it, vortex it, draw it through a pipette tip or use any other violent means.

13) Spin at 9,000 rpm at 4°C for 5 minutes, and remove and discard the supernatant.

14) Repeat steps 12 and 13 until the supernatant becomes clear or you think you will run out of TEN+, or you get bored. I usually do three such washes.

15) Resuspend the pellet in 2 ml TEN+, keep a 50 µl aliquot handy and store the rest at -20°C.

16) Pour a 9% acrylamide SDS gel at pH 8.8 with a pH 6.8 stacker. For my size apparatus these are as below, but can be scaled to fit:

9.6 ml 30:0.8 acrylamide:bis	1.66 ml 30:0.8 acrylamide:bis
12.0 ml 1 M Tris pH 8.8 (from base)	1.25 ml 1 M Tris pH 6.8 (from base)
10.0 ml distilled water	6.92 ml distilled water
320 µl 10% SDS 100 µl 10% SDS	
160 µl fresh 10% Ammonium persulfate	50 µl fresh 10% Ammonium persulfate
30 µl TEMED	12 µl TEMED

17) Load 15 µl each of the total and supernatant and 5 µl of the pellet in equal volumes of 2X Lämmli buffer. Also run appropriate size standards (such as *Sigma* No. SDS-6H). It is a good idea to do a parallel prep on induced vector alone so that you can see the 37 kD *TrpE* protein.

18) Run the gel as fast as you can while keeping it cool in 1X PGB and then stain for one hour in coomassie stain.

19) Destain repeatedly until satisfied with the result (overnight works well), and dry the gel down onto 3MM paper.

20) Comparison between the vector and insert preps should immediately reveal your fusion protein when the gel is stained, hopefully at the correct molecular weight. If you can't see anything quit now and get a steady job. If you have what you want, scale up the gel and run the pellet out on a prep gel.

21) Stain the prep gel for only two minutes in aqueous coomassie, and destain extensively in water.

22) Put the gel in a pyrex dish and view it on a white light box. Cut out the band and electroelute in your favorite apparatus in 1XPGB in a cold room. You can follow the passage of the coomassie blue.

23) Dialyse extensively (at least four changes over 24 hours) against 1X PBS + 2mM β−mercapto ethanol (140 µl per 1 liter) + 1.74 µg / ml PMSF.

24) Run an aliquot adjacent to a dilution series of a known protein (eg BSA) and assess its concentration.

25) Store in aliquots at -20°C. This is your antigen.

Buffers (in order of use)

LB - see buffers / media section

M9CA - see buffers / media section

TEN+ (50 ml)
0.5 ml 1 M Tris pH 8.0
0.2 ml 250 mM EDTA
2.0 ml 5 M NaCl
7 µl β-mercapto ethanol
50 µl 1.74 mg / ml Phenylmethane-sulfonyl, in propanol (Boehringer Manheim)
50 µl 1 M Benzamidine in water (Sigma B-6506 stored at -20°C in 1 ml aliquots)
50 µl 2 mg / ml aprotinin in water (Boehringer Manheim stored at -20°C in 1 ml aliquots)
50 µl 1 mg / ml leupeptin in water (Boehringer Manheim stored at -20°C in 1 ml aliquots)
50 µl 1 mg / ml pepstatin in methanol (Boehringer Manheim stored at -20°C in 1 ml aliquots)
distilled water to 50 ml

10% triton X-100 (*Sigma* No. T-6878) in water

10% Zwitergent (*Calbiochem* No. 693017) in water

40% sucrose (in TEN)

1.0 ml 1 M Tris pH 8.0
0.4 ml 250 mM EDTA
4.0 ml 5 M NaCl
40 g sucrose
distilled water to 100 ml, filter sterilise and store at 4°C.

2X Lämmli buffer

20 % glycerol
4 % SDS
250 mM Tris pH 6.8
10 % β-mercapto ethanol
0.05% bromophenol blue

5X PGB (Protein Gel Buffer)

30.27 g Tris base
144.1 g glysine
10 g SDS
water to 2 liters, store at room temperature

stain - 50 ml glacial acetic acid (to 10%)
125 ml Ethanol (to 25%)
0.25 g coomassie brilliant blue (*Sigma* No. B-1131)
distilled water to 500 ml, store at room temperature (reusable)

destain - Same as stain less the coomassie

aqueous coomassie

0.25 g coomassie brilliant blue (*Sigma* No. B-1131)
distilled water to 500 ml, store at room temperature (reusable)

1X PBS

0.1 M sodium phosphate buffer pH 7.2
0.7 % NaCl

Zhichun Lai

GST FUSIONS FOR ANTIBODY PRODUCTION

pGEX is a protein expression vector for use in *E.coli* that carries the GST (glutathione S-transferase, 26 Kd) gene from the parasitic helminth *Schistosoma japonicum*. A gene encoding a foreign protein is fused at the terminus of the GST gene at a polylinker (BamHI, SmaI, EcoRI). Each of the three vectors, pGEX-1, pGEX-2X and pGEX-3X, generates fusions in a different ORF. The GST gene is under control of the *tac* promoter and is induced by IPTG. Plasmid derived lac^q product is sufficent to inhibt the GST gene expression in the absence of inducers.

Most GST fusion proteins are soluble, and one may purify these GST fusion proteins by affinity to glutathione, the substrate of the enzyme, linked to agarose beads. Supernatant containing GST or GST fusion protein is mixed with the beads. After extensive washing, the adsorbed protein is released by adding free glutathione as competitor. This one step purification gives pretty pure material suitable for injection into an animal, or for biochemical analysis. GST fusion proteins are assumed to keep their native comformation, since no denaturation steps are involved.
The yield is about 1.6-15 mg/liter culture.

Mini Prep:

1) Screen TG2 transformant.

2) Take 100 µl overnight culture into 900 µl 2xYT with 100 µg/ml ampicillin.

3) Grow at 37°C for 1.5 hours; add IPTG to final concentration of 1.0 mM; grow 1.5 hours at 37°C. Spin down cells, resuspend in 300 µl PBT buffer (PBS + 1% Triton X-100) with 0.2 mg/ml lysozyme; sonicated about 5" (not more than 10"); spin down debris; mix supernatant with 50 µl 50% glutathione-agarose beads; leave at room temperature for a few minutes; wash 3x 1ml PBT; add 30 µl 3x SDS sample buffer and boil for 5 min. before loading to SDS protein gel.

Comments: If you don't see any fusion protein with expected size, you may run total protein on the gel. Use the one without IPTG as a control. It is possible that fusion protein is insoluble. If you still don't see anything, just simply give up, try other constructs, or other expression systems.

Maxi-Prep:

1) Dilute overnight culture 1:10 into LB or 2xYT broth with 100 µg/ml ampicillin. Grow at 37°C until OD600 reaches 0.6-1.0. It takes about 1-3 hours.

2) Add IPTG to final 0.5 mM, continue growth for 2-3 hours.

3) Spin down cells; freeze at -70°C for 5 min.; thaw in 1:100 to 1:50 culture volume of PBS containing 1 mM PMSF, 10 mM β-mercaptoethanol, 0.2 mg/ml lysozyme. Leave at 4°C for 20 min..

4) Add Triton X-100 to 1%; sonicate cells for 30-60" (do not over-sonicate; it may cause denaturation. Multiple bands will be seen on the SDS gel because of the association of denatured GST fusion protein with other proteins.)

5) Spin 10,000 x g for 5 min. at 4°C.

6) Mix supernatant with 1 ml 50% glutathione-agarose beads (For 500 ml culture) ; leave at room temperature for 10 min., while slowly shaking; collect beads at 500 x g; wash 3x 50 ml PBS with 1% Triton X-100; elute fusion protein 2x 2 min. with 1 bead volume with freshly made 5 mM reduced glutathione in 50 mM Tris-Cl pH 8.0 buffer.

- 50% Glutathione-agarose beads (Sulphur linkage, Sigma G-4510) is preswollen in PBS + 1% Triton X-100, washed twice and kept at 4°C.

- Beads can be reused after washing in 3 M NaCl.

- Smaller fusion proteins (50kD or less) tend to give better yields; it is also best to avoid regions of th gene that encode hydrophobic peptides.

Reference:

Smith, D. B. and Johnson, K. S. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67:31-40, 1988.

SOLID PHASE RADIOIMMUNE ASSAY FOR IgG

1) Apply antigen (usually 0.1-1 mg) into polyvinyl chloride well of microtiter plate (96-well) in 50 ml final volume in PBS, pH 7.4 (Costar vinyl assay plates, cat. no. 2596).
2) Allow solution to evaporate (RT overnight, or 37°C incubator for 1-2 h).
3) Apply 150 ml 5% BSA, PBS, pH 7.4. (PBS = 0.01M $NaPO_4$ pH 7.4, 0.1 M NaCl).
4) Replace tray lid; place at 37°C for two hours.
5) Remove BSA-PBS blocking solution.
6) Apply antibody in 50 µl final volume, diluting in PBS, pH 7.4 if necessary. Unconcentrated hybridoma supernatants are used without dilution.
7) Replace tray lid; place at 37 °C for one hour.
8) Remove antibody solution.
9) Wash three times with 1% BSA, PBS, pH 7.4; use about 100-150 µl per wash and discard wash solutions between applications.
10) Apply 10^5 cpm [^{125}I] - protein A or appropriate [^{125}I] labeled second antibody in 50 µl final volume, using PBS, pH 7.4 as diluent.
11) Replace tray lid; place at 37 °C for one hour.
12) Remove [^{125}I] solution.
13) Wash three times with 100-150 µl PBS, pH 7.4.
14) Aspirate any residual liquid in microtiter wells and wipe the top of the plate dry.
15) Place plate without lid between two glass plates with X-ray film and screens in place and use binder clips to clamp.
16) Autoradiograph with XAR-5 or equivalent film being certain the well bottom is against the film; use one intensifying screen; place at -70 °C for 16 hrs or overnight.

PBS: 10 mM $NaPO_4$, pH 7.4
0.1 M NaCl

The pH is very important as the reactions are not efficient below pH 7.0 and are less efficient at pH 7.0. Therefore, adjust the pH of the PBS and the PBS containing BSA. Store the BSA solutions at -20 °C.

NOTE: It is necessary to adjust pH of BSA / PBS solutions as pH drops when BSA is dissolved in PBS.

Don Rio
Revised by Smadar Admon

PROTEIN GELS - SDS POLYACRYLAMIDE

Stock Solutions: (1) 30% Acrylamide / 0.8% Bis
(2) 1M Tris, pH 6.8
(3) 1M Tris, pH8.8
(4) 10% SDS
(5) 10% APS
(6) Laemmli buffer (sample buffer) (prepare fresh):
For 1 ml: 100 µl glycerol
200µl 10% SDS
125µl 1M Tris, pH 6.8
50µl BME
0.05% BPB
525µl ddH_20
(7) Running buffer (for 4 liters): 57.6gr Glycine
12.1gr Tris base
4.0gr SDS

Recipes for gels:

% running gel	**5%**	**7%**	**9%**	**10%**	**12%**	**14%**	**16%**
30%/0.8% AA	2.66ml	3.7ml	4.8ml	5.33ml	6.4ml	7.46ml	8.53ml
ddH_20	7.1ml	6.0ml	5.0ml	4.43ml	3.36ml	2.3ml	1.23ml

Add: 6ml 1M Tris, pH 8.8
160µl 10% SDS
80µl 10% APS
8-15µl Temed

Stacking gel (5%, 5ml): 0.625ml 1M Tris, pH 6.8
0.83ml 30% / 0.8% AA
3.46ml ddH_2O
50µl 10% SDS
25µl 10% APS
6µl Temed

Staining Gels:

Staining Solution: 10% acetic acid
50% methanol
0.25% Coomasie brilliant blue R-250

Destaining Solution: 10% acetic acid
20% methanol

Smadar Admon

WESTERN BLOTS

Stock solutions:

(1) Transfer solution (4 liters):
57.6gr glycine
9.7gr Tris base
800ml methanol

(2) TBS: 25mM Tris-HCl
150mM NaCl
pH 7.4

(3) TTBS : TBS + 0.5% Tween 20

(4) Blocking solution: 2% Carnation non-fat dry milk in TBS, pH 7.4.

(5) Ponceau's S stain: 0.5gr Ponceau S + 1ml acetic acid + H_2O to 100ml.

Transfer of proteins to nitrocellulose:

After running the acrylamide gel, transfer it to nitrocellulose by electrophoresis. The gel is put in the transfer apparatus with the nitrocellulose on top of it, facing the positive electrode. The transfer should be done in the transfer solution, at 4°C, 20 volts, overnight, or at 50 volts for 4-5 hours.

After the transfer, folow the procedure below:

1) If you want to check the transfer you could stain the filter with Ponceau S stain (reversible):

   (a) Stain for 5 min.

   (b) Destain in water for 2 min.

   (c) Wash off the stain in PBS for 10-30 min.

2) Place nitrocellulose in a Seal-a-Meal bag with blocking solution. Incubate and shake for at least one hour at room temperature (can be left overnight).

3) Rinse briefly in TTBS.

4) Incubate with primary antibody diluted in blocking solution for 1-2 hours at room temperature (shaking). Proper amount of antibody is determined empirically. Supernatants from cells, no dilution - 1:5; Ascites or serum - 1:100 - 1:500.

5) Wash twice for 10 minutes in TTBS.

6) Incubate with secondary antibody diluted in blocking solution at room temperature for 1 hr. For AP or HRP conjugates, dilute 1:500 - 1:1000. For ^{125}I-labelled protein A or anti-IgG use 2 x 10^5cpm / ml.

7) Wash as in step 5.

8) (a) If using ^{125}I, expose nitrocellulose using Kodak XAR5 film and intensifying screen overnight at -70°C.

(b) If using AP conjugate, rinse once more for 5 minutes with TBS (no Tween). Develop blot using the BCIP + NBT substrate system (Bio-Rad kit).

Joe Heilig

ELECTROELUTION OF PROTEINS USING ISCO APPARATUS

1) Run preparative Laemmli SDS/PA gel as desired.

2) Remove gel from plates, do not fix, and stain for approximately 10 min.

3) Destain gel.

4) Rehydrate gel in water for 5-10 minutes.

5) Cut out region of gel containing band of interest and mince gel slice into ≈1mm cubes. This can be done conveniently in a weigh boat.

6) Prepare elution cup by securing a single layer of dialysis membrane beneath each of the wells with the retaining rings. Place a plastic mesh screen over the collection (smaller) well.

7) Prepare buffer chamber by placing a piece of Whatman 3mm paper in the divider slots of each electrode chamber and fill all chambers with 1x Laemmli gel running buffer, containing SDS, to approximately 1cm below the top of the central divider/cup holder. Buffer must not rise over this divider when the elution cups are placed on it. The paper dividers prevent bubbles generated at electrodes during elution from collecting under the wells and preventing current flow.
If a very small amount of protein is being eluted (in the pg range) such as might be recovered from a biologically significant source, not a fusion protein, it may be useful to add protease inhibitors to the elution buffer. For example, add PMSF to 1mM and aprotinin to 1/1000.

8) Pile minced gel pieces into sample (larger) well. Be careful not to trap air in the bottom of the wells or create air pockets in the mass of minced gel.

9) Place elution cups in the chamber straddling the center divider. Be certain not to trap air bubbles underneath the wells.

10) Elute in cold-room at 150 V, constant V, for ≈12 hrs. Be certain that there is current flowing through the chamber. The Coomassie Blue dye will collect in the collection chamber and provide an indicator of completion of elution.
If no current flow is detected the most likely cause is a bubble either in or under one of the wells. It can sometimes be difficult to see these bubbles so inspect closely, removing the gel slices if necessary.

11) When elution is complete, remove buffer from elution cup with pasteur pipette, remove gel slices from collection well side of cup and remove remaining buffer down to rim of the collection well.

12) Remove screen and recover eluted protein from collection well with Pipetman or equivalent. The volume of the collection well is 200µl. The collection well can be washed by pipetting solution up and down, gently so as to prevent foaming, or with fresh buffer.

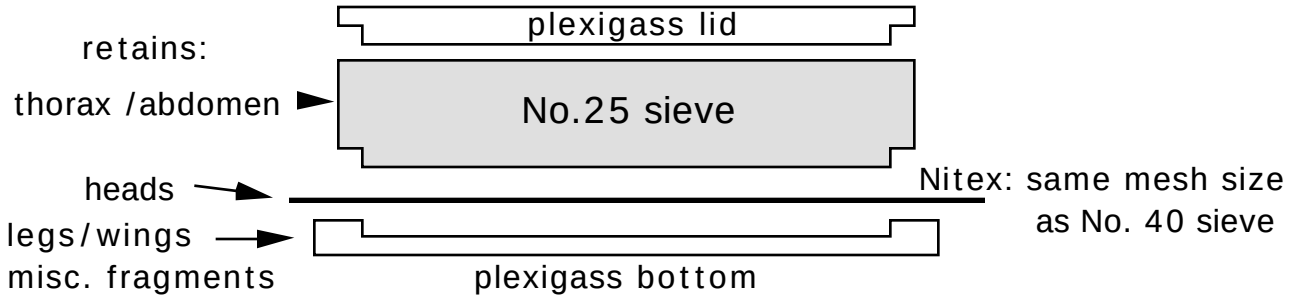
13) Dialyse the sample as necessary.

14) Clean the apparatus thoroughly.

Betsy O'Neill

MAKING FOOTPRINTING EXTRACTS FROM DROSOPHILA HEADS

This is a protocol which I adapted from the standard Tjian lab embryo nuclear prep. I generally make extracts from 200-500g of adult flies at a time. I harvest them into disposable 250 ml Corning centrifuge tubes, and store them at -70 until I'm ready to use them (up to a few weeks at least). I separate the heads by placing the tubes into liquid nitrogen for a few minutes and then shaking the tubes vigorously. This causes the heads, wings and legs to fall off. I then put this material through two sieves-a number 25, which retains the abdomen and thorax, and then a number 40 which retains the heads, but allows the legs and wing fragments to go through. This also requires vigorous shaking to get good separation. I had an apparatus constructed as diagrammed below which I place into a multivortexer to acheive separation more easily (I had to do this because my arms got tired before I had acheived good separation).



I then place the still frozen heads into about 1ml of high sucrose homogenizing buffer per 3g of adult flies. I put this mixture through a Yamato continuous flow homogenizer set at 700 rpm. I collect the flow through and pass it through the homogenizer another 4-5 times. I periodically examine samples of the flow through under a dissecting scope to monitor the efficency of homogenization. This makes it easy to determine when all of the heads have been broken up. Next, I pass the homogenate through prewetted (ddH_20) miracloth (Calbiochem #475855) placed in a large funnel, which removes large particulate matter, but allows nuclei to pass through. Nuclei are then isolated by spinning at 24K for 45 minutes at 4 C in an SW28 rotor. Since the SW28 tubes must be filled completely for the spin, I usually bring the volume of the material that comes through the miracloth up to something divisible by 40ml, since the tubes hold about 38ml. I experimented initially with spinning through a sucrose cushion on this step (hence the SW28 rotor etc.), but had difficulty isolating the nuclei--they seemed to be at the interface rather than in the pellet as described in the protocol I was trying to adapt, so I eventually settled on the 24K spin. There is generally a large layer of lipid at the top of the tube after this spin, which I usually scoop off before attempting to pour off the cytoplasmic fraction.

The pellet (nuclei) from this spin is resuspended in 0.1ml of Buffer A+B per gram of flies. The pellet itself is usually composed of a softer upper layer, the nucei, and a hard, dark lower layer. I usually resuspend only the top layer and discard the lower layer. Transfer this material into Ti60 tubes. The nuclei are lysed (in the Ti60 tubes, so that there is no need to transfer the viscous solution containing lysed nuclei) by adding 0.1 volume of 4M ammonium sulfate pH8.0. I usually add this a little at a time, and mix well, but gently, in order to get even lysis. I then place the tubes on a rotating wheel at 4 C for 20 minutes. Pellet the chromatin by spinning at 35K for 30 minutes in a 60Ti rotor at 4 C. The supernatant from this spin is a >10% AS cut. The crude extract that I

generally work with is a 10-60% AS fraction which I prepare by adding 0.3g of finely ground ammonium sulfate per ml of extract. We have a coffee bean grinder that I use to grind the AS, but I'm not sure that's necessary. I place the extract in a small beaker in an ice bath on a stir plate in the cold room, add the AS gradually, and stir for 15 minutes. I then transfer this material to a Sorval tube, and spin at 15K for 30 minutes. I resuspend the pellet in 2ml of 0.1M HEMG per 100g flies, and dialyze against 0.1M HEMG until the conductivity is equal to that of 0.1M HEMG (takes 4-6 hours). This is my final extract which I aliqout and freeze in liquid nitrogen, and then store at -70 C. The protein concentration of the extract is usually around 15 mg/ml, using BSA as a standard, or about 30mg protein per 100g flies. I have experienced quite a bit of variability in yield, which I think is due mainly to variation in the efficiency of the early steps (i.e., harvesting heads). For example, if you don't shake the frozen flies long enough to get all the heads off, the yield will be lower.

I see good footprints on the rhodopsin promoters using in the range of 10-100 ug extract in a standard footprinting reaction.

Protocol Summary:

1) Harvest heads from adult flies by using appropriate sieves.

2) Place heads into a beaker containing 1 ml homogenizing buffer per 3g adult flies.

3) Homogenize in continuous flow homogenizer at 700 rpm. Collect flow through and rehomogenize until all heads are broken open.

4) Pass homogenate through prewetted miracloth to remove particulate matter.

5) Bring the total volume of this filtrate up to an amount divisible by 40 with homogenizing buffer, mix well, and transfer to Ultra Clear SW28 tubes (about 38 ml per tube). Spin at 24K/4 C/45 minutes.

6) Remove upper layer of lipid and pour off supernatant (cyto). Resuspend pellet (nuclei) in 0.1ml Buffer A+B per gram of adult flies.

7) Transfer this material (noting volume) to 60Ti tubes. Lyse nuclei by adding 1/10 volume 4 M ammonium sulfate pH 8.0. Add the AS a little at a time, and mix gently by capping the tube and inverting it several times. This should cause the mixture to become quite viscous.

8) Place the tubes on a rotating wheel at 4 C for 20 minutes.

9) Pellet the chromatin by spinning at 35K/4 C/30 minutes.

10) Place the supernatant from this spin (note the volume) into a small beaker containing a stir bar, and place the beaker in an ice bath on a stir plate in the cold room. Gradually add 0.3g finely ground ammonium sulfate per ml of supernatant to the stirring extract. Stir for an additional 15 minutes.

11) Transfer this material to a Sorval tube and spin 15K/4 C/30 minutes.

12) Resuspend the pellet in 2ml 0.1M HEMG per 100g adult flies and dialyze against 0.1M HEMG until the conductivity of the extract equals that of 0.1M HEMG.

13) Aliquot a portion of the extract into small aliquots, and then freeze in liquid nitrogen.

SOLUTIONS

		additions per 1 liter
Buffer A+B	15mM HEPES pH 7.6	15 ml of 1M (pH w / KOH)
	100mM KCl	7.46g
	5mM $MgCl_2$	5ml of 1M
just before use add:		
	1mM DTT	1:1000 dilution of 1M
	0.5mM sodium metabisulfite	1:1000 dilution of 95mg / ml
	0.1mM PMSF	1:200 dilution of 0.2M (in EtOH store at 4 C)

		additions per 1 liter
Homogenizing Buffer	10mM HEPES pH 7.6	10 ml of 1M
	25mM KCl	1.87g
	0.15mM spermidine	174 mg
	0.5mM spermine	38 mg
	1mM EDTA pH 7.9	2 ml of 0.5M
	2M Sucrose	685g
	10% glycerol	100ml
just before use add:		
	1mM DTT	1:1000 dilution of 1M
	0.5mM sodium metabisulfite	1:1000 dilution of 95mg / ml
	0.1mM PMSF	1:200 dilution of 0.2M

		additions per 2 liters
0.1M HEMG	25mM HEPES pH7.6	50 ml of 1M
	100mM KCl	14.93g
	12.5mM $MgCl_2$	25 ml of 1M
	0.1mM EDTA pH 7.9	0.4 ml of 0.5M
	10% glycerol	200 ml
just before use add: (to 2 liters)		
	1mM DTT	2 ml of 1M
	0.5mM sodium metabisulfite	190 mg
	0.1mM PMSF	1 ml of 0.2M

4M ammonium sulfate

Add 52.8g ammonium sulfate per 100 ml. Adjust the pH with NaOH. This solution is almost saturated at room temperature. Filter any undissolved crystals and store at room temperature.

Zhichun Lai

HIGH EFFICIENCY ELECTRO-TRANSFORMATION OF *E.COLI*

Preparation of Cells:

1) Inoculate 1 liter of LB broth with 1/100 volume of a fresh overnight culture.

2) Grow cells at 37°C with vigrous shaking to an OD_{600} of 0.5 to 1.0 (the best results are obtained with cells that grow rapidly; the appropriate cell density, therefore, depends on the strain and growth condition).

3) To harvest, chill the flask on ice for 15 to 30 minutes, and spin in a cold rotor at 4000x g max for 5 minutes.

4) Remove as much of the supernatant as possible. Resuspend pellets in a total of 1 liter of cold water. Spin as in step 3.

5) Resuspend in 0.5 liter of cold water. Spin as in step 3.

6) Resuspend in about 20 ml cold 10% glycerol. Spin as in step 3.

7) Resuspend in a final volume of 2 to 3 ml in cold 10% glycerol. The cell concentration should be $1\text{-}3x10^{10}$ cells/ml.

8) The suspension could be frozened in aliquots on dry ice, and stored at -70°C. The cells are good for at least 6 months under these conditions.

Electro-transformation

1) Gently thaw the cells at room temperature and place them on ice.

2) In a cold, 1.5 ml polypropylene tube, mix 40 µl of the cell suspension with 1-2 µl of DNA (in either TE or water). Mix well and let sit on ice for about 1 minute.

3) Set the Gene Pulser apparatus at 25 uF and 2.5 kV. Set the Pulse Controller to 200 om.

4) Transfer the mixture of cells and DNA to a cold, 0.2 cm electroporation cuvette, and shake the suspension to the bottom of the cuvette. Place the cuvette in a chilled safety chamber slide. Push the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber.

5) Pulse once at the above settings. This should produce a pulse with a time constant of 4-5 msec. (The field strength will be 12.5 kV/cm.)

6) Remove the cuvette from the chamber and immediately add 1 ml of SOC medium to the cuvette and quickly resuspend the cells with a pasteur pipette.

7) Transfer the cell suspension to a glass culture tube and incubate at 37°C for 1 hour with shaking.

8) Plate on selective medium.

For 100 ml SOC medium:
Take 94 ml LB medium which contains:
2% Bacto tryptone
0.5% Bacto yeast extract
10 mM NaCl

Add:

2.5 mM KCl	1 M	0.25 ml
10 mM $MgCl_2$	1 M	1 ml
10 mM $MgSO_4$	1 M	1 ml
20 mM glucose	10 %	3.57 ml

Ref: The Bio-rad Instruction Manaual.

Ulrike Heberlein

BROMODEOXYURIDINE (BrdU or BUdR) INCORPORATION AND VISUALIZATION

BUdR incorporation is a convenient technique for labelling cells undergoing DNA synthesis. At low concentrations it will not block cell division and will be incorporated into replicating DNA with little apparent damage.

Preparation of BUdR food and feeding:

- Make a BUdR stock solution by dissolving 10 mg of BUdR in 350µl of 50% ethanol (28.6 mg / ml) and store at -20°C (it forms a suspension; be sure to vortex before using an aliquot). BUdR is quite toxic; handle and dispose of responsibly.
- For *in vivo* labelling weigh and melt some fly food (regular corn meal / agar food) in the microwave until just liquified. After letting the food cool a bit add the BUdR suspension in ethanol to a final concentration of 0.5 mg / ml. For short pulses (2-8 hrs) the concentration can be increased to 1.0 mg / ml. Return the BUdR-food into the vial and let solidify. In addition (optional) make up some yeast paste and add BUdR to 0.5 mg / ml final concentration. Put this paste on top of the corn meal / agar food.
- Put larvae of the desired age onto this food. To look at eye discs I found that it is necessary to pull third instar larvae out of the food in their original vials or bottles. If larvae are already crawling up the walls they don't feed anymore, and obviously won't incorporate any BUdR.

BUdR visualization:

1) Dissect cephalic complexes in PBS and fix for 30 minutes at room temperature in Carnoy's fix (60 ml ethanol + 10 ml glacial acetic acid + 30 ml chloroform).

2) Rehydrate by incubating for 3 minutes each in 70%, 50%, 30% ethanol in PBS-TX (1xPBS, 0.3% Triton X-100). Finish by incubation in PBS-TX for 5 minutes on ice.

3) Denature the DNA by incubating for 1 hr in 2N HCl / PBS-TX (1:1 mix of 4N HCl and PBS-TX). Wash twice, 5 minutes each, in PBS-TX on ice.

4) Incubate for 30 minutes in 10% goat serum in PBS-TX.

5) Incubate overnight at 4°C (I imagine that for eye-discs, in contrast to whole embryos or larval CNSs, a few hours is sufficient) in the primary antibody (1:200 dilution of monoclonal anti-BUdR from Becton-Dickinson).

6) Wash in PBS-TX on ice six times for 10 minutes each (again, this might be an exageration when staining eye discs).

7) Incubate at 4°C for several hours to overnight in a 1:200 dilution of biotinylated goat anti-mouse IgG (Vectastain) in PBS-TX.

8) Wash in PBS-TX on ice six times for 10 minutes each.

9) Incubate for 30 minutes in Vectastain ABC reagent (10µl of reagent A + 10µl of reagent B + 980µl of PBS).

10) Wash in PBS on ice two times for 10 minutes each,

11) React with DAB / H_2O_2 / $CoCl_2$ (100µl 5mg / ml DAB + 900µl PBS + 1µl 3% H_2O_2 + 20µl 1% $CoCl_2$) until stain becomes evident, usually a few minutes.

12) Dehydrate discs by a graded series of 30, 50, 70, 90, 100% ethanol.

13) Mount in DPX.

Melissa Cobb

LAMBDASORB LIQUID PHAGE PREP

1) Inoculate 200µl cells in a 50 ml sterile tube with either 2 plaques or 2 million pfus. Incubate for 20 minutes at room temp to allow adsorption. Add 15 mls of L-Broth containing 10mM $MgSO_4$ and shake at 37°C until lysis (5-7 hrs). Add 150µl chloroform and shake an additional 15 min to lyse the remaining cells.

2) Spin at 3,000 for 5 minutes to pellet the cell debris and transfer the supernatant into a clean tube. Set aside 100µl as high titer stock. Add 100µl of LambdaSorb place tube on a rotator for 30 minutes at room temp or leave at 4°C overnight and rotate briefly in the morning.

3) Spin at 3,000 for 5 minutes. Discard supernatant and drain the tubes well.

4) Resuspend pellet in 1ml TM10 and transfer to eppendorf tube.

5) Spin very briefly (30 sec), discard supernatant and resuspend pellet in 500µl TE.

6) Add 5µl 10%SDS, 10µl 250mM EDTA and incubate at 65-70°C for 10 minutes.

7) Spin 1 minute to pellet protein debris and transfer the supernatant to a new tube. Add 50µg / ml of Proteinase K and incubate for 30 minutes at 37°C.

8) Phenol-Chloroform extract twice. Add 50µl 3M NaOAc and 500µl isopropanol and mix well. A DNA precipitate should form readily but, if not leave at -70°C for 15 min, thaw and centrifuge. If the precipitate is readily visible, centrifuge for only a minute so it won't be so hard to resuspend, otherwise spin for 10 minutes.

9) Resuspend DNA pellet in ~50µl TE.

NOTE: Some members of the lab find steps 7 and 8 to be unnecessary.

Dan Kalderon

SMALL-SCALE PREPARATION OF BACTERIOPHAGE LAMBDA DNA

Growing Up Phage

Plate lysate

Make lambda agarose plates (preferably in NZCYM, although LB+ 10mM $MgSO_4$ is OK; 1.5% agarose).Add 20µl (gt10) or 200 µl (Charon 4) of single plaque eluate (from 2ml in TM10+chloroform) to 100µl of indicator bacteria in TM10, mix, incubate 20' at 37°C and spread on a dry 9cm agarose plate in soft agarose (0.7% 48°C).Incubate for about 12 hr. Optimally the plaques should just touch each other after about 8 hr but the yield of phage is reduced less by adding too much than too little phage. As lysis is normally complete it is best also to have one plate with no added phage to be sure that the plates could support a good lawn of bacteria. Add 5 ml of TM10 to each plate and elute at room temperature for at least 2 hr (or O/N at 4°C) with gentle agitation. Collect eluate, adding a further 2 ml TM10 to maximise yield. At this point a significant proportion of phage still remains on the plate and can be eluted with a further, say, 5ml of TM10+gelatin to give high titre phage stocks(stored with a little,0.2%,chloroform at 4°C)

Liquid culture

Plate lysates are preferred to liquid culture because fairly reproducible yields of phage are usually obtained without accurate determination of phage titre. Nevertheless , some people find it convenient to grow 50-100ml liquid cultures by innoculating with 1ml of a C600 O/N per 100ml NZCYM and infecting with 0.5-2 X 10^7 phage (more with Charon 4 than gt10). Grow for 4-7 hr or until lysis occurs.

Preparation of DNA

1) Spin down bacterial (and other) debris from plate lysates or liquid culture (5000 rpm 5' Sorvall, 10 ml polypropylene tubes).

2) Add RNase A & DNase I each to 1µg/ml to supernatant and incubate at 37°C for 30'.

3) Add an equal volume of 20% PEG (polyethylene glycol),2M NaCl in TM10, mix and stand on ice for at least 1-2 hr.

4) Spin down phage (7000 rpm 20' Sorvall SS34), pour off liquid, spin again for 30 secs and aspirate fluid.

5) Resuspend pellet thoroughly in 0.5ml TM10 and transfer to a microfuge tube.

6) Extract with 0.5ml chloroform

7) Add 5µl 10% SDS, 10µl 250mM EDTA pH 8 and incubate at 65-70°C for 10mins.

8) Add 0.5ml phenol (equilibrated with TE) and shake to mix (but do not vortex henceforth); centrifuge for 2 min and remove aqueous phase which will probably drag some interphase along due to high viscosity.

9) Extract with phenol / chloroform / iso-amyl alcohol (25:25:1).

10) Add 0.5ml isopropanol and mix. A DNA precipitate should form but if not, leave at -70°C for >15 min, thaw and centrifuge. If DNA precipitate is readily visible spin for only a minute (to help subsequent dissolution), otherwise 10 min. Wash pellet in 70% ethanol, dry and dissolve in 50-200µl TE by gentle shaking or with the aid of a Gilson. The yield of gt10 recombinants is usually greater than for Charon 4 but the latter normally gives enough DNA for at least 5 restriction enzyme digests. As a large amount of RNA is normally present in the DNA preparation it is worth treating with boiled RNase A (at ~100µg / ml) during or prior to digestion, so that (i) small DNA fragments are not obscured and (ii) the size of fragments can be more accurately estimated from their electrophoretic mobilities. (In the presence of excess RNA, all fragments will migrate faster than they should).

from Grace Gill via Betsy O'Neill

PREPARATION OF SINGLE STRANDED DNA (for *in vitro* mutagenesis)

Day 1

1) Innoculate an ON into LB amp of desired plasmid contained in bacterial strain CJ236, inserts in LB amp.

Day 2

1) The next day, transfer 50µl of the overnight into 2 ml of LB amp. Shake at 37°C for about 3 hours, until OD_{600} is about 0.7.
2) Add 25µl of helper phage (10^9 phage for an moi of about 10). Shake at 37°C for 1 hour. (The helper phage we use is VCS-M13, available from Stratagene.)
3) Dilute 400µl into 10 ml of LB amp kan, and shake at 37°C overnight. (Phage is kan^R)

Day 3

1) Pellet cells in eppendorfs (1.5ml per tube) / 10 min.
2) Carefully remove only 1 ml of the supernatant to a new set of tubes.
3) Add 400µl 20% PEG 8000, 2.5M NaCl. Vortex and leave at RT for 15 minutes.
4) Pellet phage / 10 minutes in microfuge. A loose pellet should be visible.
5) Resuspend each pellet in 100µl TE, and combine into two tubes. Phenol / chloroform extract and ethanol precipitate ss DNA.
6) Resuspend each pellet in 100µl TE and run 3µl on a gel.

Dan Kalderon

BACTERIOPHAGE LAMBDA LIBRARY SCREENING: SUMMARY

1) INDICATOR BACTERIA: Grow O/N of C600 or Q358, spin down and resuspend in an equal volume of TM10+gelatin (can be stored for a week at 4°C).

2) TITRE: Make dilutions of phage library in TM10+ gelatin such that 10-100 plaques will form on at least one plate. Mix 100µl of indicator bacteria with an appropriate volume (10-300µl) of phage dilution, incubate for 20 min at 37°C, add 3ml top agar at 48°C, mix by gentle vortexing or inversion and pour evenly on a small (9cm) lambda plate that has been dried well (4-12 hr at 37°C, 2-4 hr at 42°C if fresh). Incubate at 37°C for >8hr and count plaques.

3) PLATING LIBRARY: To 0.3 ml indicator bacteria, add appropriate amount of phage library stock or dilution (~5-10,000 per plate for genomic, 20-50,000 for cDNA), mix, incubate 20 min at 37°C, add 8ml top (0.7%) agarose at 48°C, mix and plate on well dried 15cm plates. Incubate at 37°C for 8-16 hr (cDNAs in lambda gt10 grow much faster than genomic DNAs in Charon 4).

4) LIFTS: Cool plates (>1 hr at 4°C), label nitrocellulose circles with plate names, layer smoothly on agarose surface, stab holes through filter and agar with syringe needle (narrow guage for accuracy) dipped in water-insoluble ink and peel filter off after 30s (first lift), 60s (second lift), 3 min (third lift), 5min (fourth lift), any time (last lift). Immerse successively in denaturing solution (5-10 min), neutralising solution (10 min) and 2XSSC (to rinse) with occasional agitation. Store plates in sealed packages at 4°C. Lay filters on 3mm paper to air dry for at least 30 min before baking 2 hr at 80°C. Remove moisture from vacuum oven as required.

5) HYBRIDISATION: Pre-hybridise for 2-24 hr in aqueous buffer (5 or 2XSSCP, 10X Denhardt's, 250µg/ml salmon sperm DNA, (0.1-0.5% SDS)) at 65°C or in same buffer with 15-50% de-ionised formamide at 42°C (no SDS when using formamide) in bags with ~6 filters each. Add denatured probe (nick-translated, single-stranded, riboprobe, oligonucleotide) to fresh buffer ($<10^6$cpm/ml) and replace pre-hybridisation fluid with ~25ml of this per bag. Incubate at 42°C or 65°C (with shaking if possible) for 8 (nick-translated probe) to 36 hr (single-stranded probes). Save probe (store at -20°C) for re-screen. Wash filters in 0.1-0.5XSSC, 0.1% SDS at 65°C (high stringency) or 0.5-6XSSC, 0.1% SDS, at 42°C (low stringency) for a total of 3-4 hr with 3-4 changes. Wrap filters in Saran wrap (facing a uniform direction for convenience), mark phosphorescent spots and expose for between 2 and 24 hr.

6) PLAQUE PURIFICATION Align filters with film and mark syringe needle positions. Align film with plates and take a small plug (diameter ~2-3mm) of agarose+agar from around each positive and elute phage in 2ml TM10 (+/-gelatin)+2 drops chloroform for >2hr at room temp with shaking. Dilute 1µl into 2ml TM10 and plate 2-10µl (gt10 libraries) or 20-100µl (Charon 4 libraries) as before in top agarose on dry small or large plates to give 100-1000 plaques. Make lifts and repeat screening procedure using same probe (boiled for 5-10 min before use) and pick isolated positives, or purify a further round if necessary.

7) RE-USE OF FILTERS: If filters are kept in Saran wrap at 4°C they may be re-probed after pre-hybridising, probably any time within several weeks (but check the status of the plates). As the position of positives is already known it is not really necessary to boil the first probe off.

SCREENING BACTERIOPHAGE LAMBDA LIBRARIES

(A) LIBRARIES

There exist a number of bacteriophage lambda libraries carrying genomic DNA sequences or cDNA copies of poly A^+ RNA isolated from various stages of development or different body parts of D. melanogaster.

Considerations in Choice of Library:

(1) REPRESENTATION

The Maniatis (Maniatis et al., 1978) and EMBL (for vector see Frischauf et al., 1983; Oregon R library, Pirrotta et al., 1983; Canlon S library, V. Pirrotta, unpublished) libraries were made by sonication and partial Sau3A digestion, respectively, of high molecular weight DNA. The DNA content of each library ($6x10^5$ x16 kb ; Maniatis) far exceeds ($1.65x10^5$ kb) that of the Drosophila genome. Theoretically, therefore, the genome should be fully represented by each of these libraries, with the possible exception of DNA that is not readily extracted, DNA that has an unusual structure (e.g. heterochromatic) and DNA that contains sequences which cannot be propagated in rec A^+ hosts due to recombination or "poisoning". Practical experience has confirmed these expectations. It has been possible not only to clone a large number of specific genes but also to establish an extensive series of overlapping phage in chromosomal "walks" in either of these two phage libraries. Difficulties in cloning have been encountered for sequences that include a long inverted repeat (head to head defective P elements at the sn locus). Less severe differences in the ability of recombinant phages to grow have been inferred from under- and overrepresentation of particular characterised phage. This effect is rarely important but may be minimised by the use of unamplified libraries.

The lambda EMBL vectors tolerate a large insert (23 kb) and recombinants apparently grow slightly more vigorously than Charon 4 recombinants. These benefits are to be weighed against the greater body of gene cloning history with the Maniatis library and the more random nature of the latter library (from sheared DNA as opposed to partial Mbo I digestion).

The representation of particular sequences in cDNA libraries is subject to many more variables than pertain to genomic libraries. Amongst these are the number of phage with inserts of reasonable size, the abundance of the specific RNA in the source material for the library, the possible absence of a polyA tract, the possible presence of strong stop sequences for reverse transcriptase and the under-representation of sequences derived from the 5' end of the RNA. It is therefore strongly recommended that the initial isolation of any particular gene should be attempted using genomic rather than cDNA libraries even if the proportional representation of the sequence is expected to be higher in the latter library. (Of course

this does not apply when the use of specific cDNA libraries is a crucial step in the protocol used to identify a particular gene).

The expected frequency of occurrence of a specific cDNA in a library can be roughly predicted from the abundance of the corresponding RNA in the source for the library. As most cDNA libraries include at least 10^5-10^6 and up to $5x10^7$ independent recombinants it should be possible to isolate cDNAs for RNAs of abundance down to about 0.0001% (assuming that the majority of packaged phage include *bona fide* inserts of reasonable length).

(2) LENGTH OF INSERTS

For genomic DNA libraries the constraints of packaging limit the size of inserts to between 15 and 23 kb.

For cDNA libraries the size of inserts depends upon a large number of experimental variables and is thus best judged *a posteriori*. However, it should be noted that the method of construction can influence the quality of inserts in a predictable fashion. (i) The use of hairpin formation to prime the second cDNA strand rather than G-C tailing makes the presence of full-length or nearly intact 5' ends very unlikely (G-C tailing is now generally used). (ii) Most cDNA libraries include EcoRI linkers at each end. Thus, methylation of internal EcoRI sites is required before addition of linkers to prevent internal cleavage. Although treated with EcoRI methylase during construction, most libraries nevertheless contain some (~20%) recombinants that have been cleaved at internal EcoRI sites. A large proportion of cDNAs that have been examined have additional or 3' derived sequences of 20-30 bp at their 5' ends. It is not clear if this is a general problem or whether it is specific to those libraries that have been examined.

PLATING OUT BACTERIOPHAGE LAMBDA LIBRARIES

1. Number of plaques to screen

a. Genomic library

The number of recombinants of average insert length 16.5 kb that must be screened to detect a particular sequence in a Drosophila genomic library is given in the following Table:

Number of recombinants screened	**Probability of finding at least one phaage**	**Probable number of phage containing target sequence**
5,000	39%	0.5
10,000	63%	1
20,000	86%	2
40,000	98%	4
80,000	99%	8
120,000	100 - 6 x 10^{-4}%	12

Although the main priority is to isolate at least one recombinant containing the "required sequence" the isolation of several in the first screen is helpful in that (i) it aids restriction mapping of the region (ii) it will often delimit the location of the

desired sequence as a relatively small region of overlap common to the different lambda phage and (iii) sufficient DNA may be cloned immediately to include all relevant sequences both 5' and 3' of the gene.

Thus, a genomic library is generally screened by plating out 6-12 15 cm plates, each with 5-10,000 phage.

b. cDNA library

As the number of cDNAs in a library for a given mRNA species cannot be accurately predicted, it is best to screen as large a number of recombinants as reasonable. The maximum number of phage that can be plated on one 15 cm plate and still give a reasonable hybridisation signal is in the order of 20-50,000. Theoretically, any number of plates can be screened simultaneously. However, for an amplified library, the number of new (as opposed to clonally related) recombinants being screened is greatly diminished as the number of plaques being screened approaches the complexity of the library. Hence it is usually better to screen several libraries up to a fraction (1/3 or 1/2) of their complexity than to screen the equivalent number of plaques from just one library.

1) CELLS : Make a saturated culture of the appropriate bacterial strain (C600 or Q358) in O/N broth + 0.2% maltose. The culture may be grown from single colonies but can conveniently be grown in only a few hours from (0.1-1 ml) aliquots of frozen cells. The saturated culture can be stored at 4°C for at least a week but is best stored after pelleting the cells and resuspending in an approximately equal volume of TM 10 + gelatin. This avoids any potential problem of phage destabilisation during absorption due to absence of Mg^{2+} (O/N broth contains no Mg^{2+})

2) PHAGE : Dilute the bacteriophage lambda stock in TM10 (+/- gelatin) so that the required number of phage per plate (5-50,000) can be measured accurately (10 µl- 0.5 ml). The titre of the phage should first be accurately measured by making dilutions in TM10+gelatin and plating as below. Titres are generally stable for phage stored at 4°C inTM10 and chloroform.

3) Mix 2-500 µl of the indicator bacteria in TM10 (~4-20x10^8 cells; 1 OD_{600} = 8 X 10^8 cells/ml) with the appropriate volume of phage dilution. When plating phage at very high density it is best to use slightly more cells (500 µl of 3x concentrated cells) so that small plaques form, allowing a more even representation of phage.

4) Incubate for 15-20 min at 37°C

5) Add 8 ml top agarose at 48°C to the mixture of cells and phage. Mix by inversion or gentle vortexing and pour evenly on a 15 cm lambda agarose plate. Even spreading of top agarose is facilitated if plates are warm (37°C, 42°C). Allow ~10 mins. to set.

6) Incubate inverted at 37°C for 8-16 hrs.

IMPORTANT POINTS

(i) Check for contamination of solutions or bacteria with phage by plating one plate as for the others but with no added phage.

(ii) All solutions and media in which phage are stored, mixed or incubated must contain Mg^{2+} (~10 mM) otherwise the bacteriophage particles fall apart.This may be seen as a reduction in titre (possibly down to zero) and can give rise to plaques of variable size.

(iii) Top agarose (at the right concentration) must be used for plating (as opposed to agar) to minimise the chance of peeling this layer off when taking lifts.

(iv) Top agarose should not be too hot (>50°C) otherwise small plaques in reduced titre result.

(v) Plates must be very dry (no gloss and preferably with ridges) to prevent problems when taking lifts.

MAKING LIFTS FROM PLATED-OUT LIBRARIES

Multiple lifts from one plate :

By leaving the nitrocellulose filters on the plate for successively increasing periods of time it is possible to make several lifts from one plate that contain reasonably similar amounts of phage. Duplicates are to be recommended whenever the number of filters to be screened is not already excessive and particularly in screens at low stringency where spurious signals (not due to DNA-DNA hybridisation) may appear. For the times listed below for making four lifts, the first two lifts usually remove considerably more phage than the third and fourth. It is not possible to reduce the contact time for the first lift if the plates were originally very dry as the filters require some time to become evenly wetted.

1) Cool the plates down to 4°C (>1 hr, exposed in the cold room) so that top agarose surface is as hard as possible.

2) Label nitrocellulose circles according to plate designations in non water-soluble ink (most ball-points are OK).

3) Layer individual filters smoothly on the surface of the plates making contact from the centre outwards. If the plates were very dry, a gloved hand will be required to ensure that the entire filter wets in a reasonable time.

4) With a syringe needle dipped in water-insoluble ink mark at least three (five is better) asymmmetrically disposed spots on the filter by stabbing vertically through to the agar.The spots should be easily visible but fine enough to allow accurate alignment of filter and plate (25G5/8 needle is good). Peel the filter off from one edge and immerse, plaque side up in denaturing solution. When making multiple lifts peel the filter off after fixed periods of time (after making alignment spots):

Lift 1 - 30 sec
Lift 2 - 1 min
Lift 3 - 3 min
Lift 4 - 5 min

To maximise signal strength, leave the filter on the top agarose for as long as possible for the last lift (some phage will always remain).

5) Agitate denaturing solution occasionally to ensure filters are being wetted.

6) Transfer filters in same orientation after 5-10 mins. (excess is better than too little time) to neutralising solution. Leave for 10 mins. with some agitation to allow mixing of solutions at the surface of the filters.

7) Transfer to 2xSSC to rinse off high salt solutions (2-3 min with agitation or longer if convenient).

8) Lay filters, DNA side up on 3 mm paper to air dry for at least 30 min.

9) Sandwich the filters between 3mm or other (Blot block) paper and bake in the vacuum oven for 2 hr at 80°C. No matter how long the filters were air dried they will retain a considerable amount of moisture. For the sake of the vacuum pump, seal off the evacuated chamber and wipe off condensation on the door intermittently.

10) The filters can now be stored for several days or weeks or used immediately. The plates from which the lifts were taken should be stored at 4°C, sealed in a plastic bag and labelled. When using nitrocellulose filters straight from the pack (not sterile) it is common to see some contaminant bacteria on the plates after a few days. However, such contaminants are generally lost rather than amplified on subsequent re-screening and amplification of phage.

IMPORTANT POINTS

1) Filters should peel off easily from the top agarose without removing any agarose. If some very small pieces of agarose stick they should be removed while in the denaturing solution. If a lot of the top agarose peels off, stop. Even after extensive cleaning it is likely that there will be a high hybridisation background and no guarantee of an even signal. Repeat plating out, checking that plates are very dry, top agarOSE is of correct composition (should set at ~45°C), plates are chilled before lifts are made (and that nitrocellulose wets easily).

2) If filters are not neutralised for long enough they will turn yellow and become brittle upon baking. Whether this affects anything other than the ease of handling the filters is debatable.

HYBRIDISATION:

PROBES

1. Nick-translated or random primed plasmid DNA or fragments
2. Single-stranded probes from ss phage or cDNAs
3. RNA probes
4. Oligonucleotide probes

The choice of probe is sometimes dictated by the nature of the experiment, e.g. screening for genes that are differentially expressed (cDNA) or finding genes from amino acid sequences (oligonucleotides). However, when there is a choice the following advantages and disadvantages of each type of probe should be considered.

1a) Nick-translated or random primed plasmid DNA: Easiest probe to make reproducibly sufficiently hot for library screening. Do not use if libraries are contaminated with plasmid sequences.

1b) Nick-translated or random primed fragments: Although the Drosophila genomic libraries do not contain sequences highly related to commonly used plasmids there is some benefit to using fragments as opposed to plasmids to screen at low stringency. Background hybridisation is generally lower (perhaps due to impurities in plasmid DNA preps.) and the possibility that a strong signal is due to plasmid cross-homology is immediately eliminated. Perhaps surprisingly, the use of fragments purified only once (twice is OK) on an agarose gel does not eliminate strong hybridisation to plasmid-containing phage in contaminated libraries.

2) Single-stranded probes: Presumably because all radioactive material added to the hybridisation is as a defined DNA molecule, the use of M13 derived single-stranded probes gives the cleanest signals. Such probes can also be made of higher specific activity than nick-translated probes. However, it is necessary to start off with a lot of radioactive dNTPs to incorporate the same amount of radioactivity into single-stranded as nick-translated probes (because in the nucleotide excess that is used to generate probes of maximal specific activity, a lot of hot dNTPs are incorporated into vector rather than probe DNA). Hence, starting with the same amount of radioactive dNTPs the final signal achieved (under equivalent conditions) is generally lower (up to 5-fold) using single-stranded probes but much cleaner. The use of single-stranded probes is therefore highly recommended for low stringency hybridisations (sensitivity can be increased by scaling up from the standard protocol and by hybridising for longer than for nick-translated probes).

M13-derived "double-stranded" probes can be made as for single-stranded probes but omitting the fragment purification step and can be used as a substitute for nick-translated probes. However, (i) if anything, the cold strand will be in excess over the hot strand; (ii) the probe will be long (which in some people's view is not desirable); and (iii) the probe cannot be used at low stringency (certainly for the Maniatis library) as ~100 signals per 5000 plaques are seen, presumably as a result of sequences cross-homologous to M13.

3) RNA probes: RNA probes have not been widely used for screening libraries. It is straightforward to make very hot probes and backgrounds appear to be lower than for nick-translated probes. However, the increased stability of RNA/RNA and RNA/DNA hybrids relative to DNA/DNA hybrids can lead to two problems. First, some regions (or all) of the probe may be unavailable for hybridisation, particularly at low stringency, and second, capricious cross-hybridisation may (and has) been seen, requiring hybridisation at several stringencies to assess the significance of such signals.

4) The highest theoretical specific activity of a terminally labelled oligonucleotide (~10^4 Ci/mmol) is only about 3-fold lower than for an average nick-translated

probe of average length 400 bp (assuming 50% of probe is newly synthesised from a mixture with 2 hot nucleotides at 300 Ci/mmol).

Thus oligonucleotide hybridisation is potentially of similar sensitivity to standard screens with nick-translated probes. This should allow for the use of degenerate oligonucleotides.

The appropriate conditions to use for the hybridisation of oligonucleotides can be worked out from the following formula (quoted in Meinkoth & Wahl 1984)

$$T_d = 4(G+C) + 2(A+T)$$

where T_d is the temperature at which 50% of duplexes dissociate and A,G,C,T are the numbers of the corresponding bases in the oligonucleotide. Any mis-match leads to a reduction of about 5°C in T_d. Hybridisation is normally conducted at 5°C below T_d.

In practice oligonucleotides of 30 bases with 2-4 mis-matches have successfully been used by hybridising in 7XSSCP at 37°C and washing in 7XSSCP at 42°C.

PRE-HYBRIDISATION

Filters should be pre-hybridised for as long as possible but 2-4 hrs. is usually sufficient. As the conditions for low stringency hybridisation allow more non-specific attachment of probe to the filters, 12-24 hr pre-hybridisation is suggested in this instance. It is possible to pre-hybridise in one solution and hybridise in another but logic would suggest that it is best to use the same composition of solution in the two cases. Thus, filters should be pre-hybridised in aqueous solution (see below) at 65°C or, for low stringency in formamide solutions at 42°C in the same bags to be used for hybridisation. Six filters per bag is certainly not excessive. Large volumes (~50 ml per bag with 6 filters) should be used for pre-hybridisation.

HYBRIDISATION

a. High stringency

Hybridisation will only take place with perfect or near perfect homologues at 65°C in the following solution

2X or 5X SSCP
10X Denhardt's
250 µg/ml herring/salmon sperm DNA (should be boiled immediately prior to use to denature)
(0.1-0.5% SDS) - optional

Using 250 ng nick-translated probe (~10-25 X 10^6 cpm) in 25 ml in each bag with 6 filters, 8hr is probably sufficient (12-16 hr is generally used) for hybridisation to approach completion. (Theoretically, a probe of 2 kb should be 50% renatured by 8 hr). Under these conditions a signal should be observable in 4-12 hr (at room temp.- Kodak XAR-5). When using single-stranded probes (generallly at lower concentration

than nick-translated probes) longer hybridisation times (24-36 hr) should improve the signal but are not necessary.

b. Low stringency

Although it is possible to screen libraries simultaneously at several stringencies (but take care that all lifts have roughly equal amounts of DNA), it is best either to ascertain the optimal stringency for screening by performing genomic Southerns at a variety of stringencies or to use the lowest possible stringency under which signals stand out relative to background. Another strategy, that of hybridising at very low stringency and washing at successively increasing stringencies is not recommended as (i) it takes a long time and uses a lot of film; (ii) sometimes probe becomes irreversibly stuck to filters even though they are being kept ostensibly wet throughout; and (iii) this procedure does not allow for optimal conditions of hybridisation.

In 5 X SSCP
10 X Denhardt's
250 µg / ml salmon sperm DNA
25% formamide at 42°C, background can be consistently as low as for high stringency hybridisation; 20% formamide is generally also clean but in 15% formamide at 42°C (and appropriate washing conditions) it is very hard to get clean filters. Under the latter conditions, probes that recognise essentially unique sequences in the genome will illuminate a smear of DNA on a genomic Southern that in sum exceeds specific hybridisation.

Each 1% formamide lowers the T_m of a hetero- (or homo-) duplex by between 0.6 and 0.7°C (McConaughy et al., 1969; Meinboth and Wahl, 1983). Thus a range of 15-50% formamide at 42°C covers a T_m range of at least 21°C, the upper limit representing standard conditions for high stringency hybridisation. The T_m of imperfectly matched duplexes is apparently decreased by about 1°C per 1% in the number of mis-matched base pairs (Bonner et al., 1973).

The plaques containing DNA with the best homology to the probe will always give the strongest signals. However, this signal will decline (and background will increase) if the stringency becomes too low. Thus, it is best to screen at the highest stringency at which a signal can be clearly seen on a genomic Southern. The best general conditions for low stringency hybridisation are to use a single-stranded probe and to hybridise for 20-36 hr. Although on theoretical grounds it may seem best to substitute salmon sperm DNA with e.g. E.coli DNA when using probes derived from (for example) a mammalian source at low stringency, this has not been necessary in practice.

WASHING

After hybridisation, cut a corner off the bag, pour off the fluid into a 15 / 50 ml plastic tube (to be used for the re-screen) and immediately cut the bag open and immerse filters in wash solution.The conditions of washing have to be adjusted to the hybridisation conditions, taking into account that hybridisation is optimal at 25°C below T_m whereas hybrids, once formed, are stable under conditions much closer to the T_m. Thus the following set of conditions may be used :

Hybridization					**Washing**				
% FORM	XXSCP	T_h /°C	T_m /°C	T_m-T_h -25/°C	∂T_m= %mis	XSSC	T_w /°C	T_m /°C	T_m-T_w -10°C
0	2	65	91.3	1.3	2.6	0.2	65	72.8	-2.2
0	2	65	91.3	1.3	2.6	0.5	65	80.1	5.1
0	5	65	98.6	8.6	17.2	2	65	91.3	16.3
40	5	42	69.8	2.8	5.6	1	65	85.7	10.7
30	5	42	77.0	10.0	20.0	0.2	42	72.8	20.8
25	5	42	80.6	13.6	27.2	0.5	42	80.1	28.1
20	5	42	84.2	17.2	34.4	2	42	91.3	39.3
15	5	42	87.8	20.8	41.6	5	42	98.6	46.6
0	5	42	98.6	31.6	63.2	5	20	98.6	68.6

NOTE

The above table is based on the following equations and assumptions.

(i) The T_m of a perfect homoduplex in 1XSSC is given by

$$T_m = 69.3 + 0.41(\%G+C)$$ Marmur & Doty (1962)

For most vertebrates and Drosophila the G-C content of the genome is about 40%, so that the average T_m at 1XSSC is 85.7°C.

(ii) The dependence of T_m on ionic strength, μ is given by

$$(T_m)_{\mu 2} - (T_m)_{\mu 1} = 18.5 \log_{10} (\mu_2 / \mu_1)$$

(iii) The T_m of duplexes is lowered by 0.72°C for every 1% of formamide in solution (Bonner et al. 1973).

(iv) The optimal temperature for hybridisation is 25°C below T_m. Hence the fifth column should be close to zero for perfect duplexes. It has been found (Bonner et al. 1973) that optimal rates of reassociation for two imperfectly matched strands occurs at a temperature that is lower than for a perfect duplex by approximately half the difference between the two T_ms. Thus conditions that give a (T_m-T_h-25)=n are ideal for a heteroduplex in which the T_m is 2n lower than for a homoduplex (∂T_m= 2 X (T_m-T_h-25)).

(v) The T_m of a heteroduplex that contains n% mis-matches is lowered roughly n°C relative to a homoduplex (Bonner et al. 1973).

(vi) The washing conditions given are such that the T_m of a perfect duplex is roughly 10°C higher than the washing temperature (T_w) and correspondingly higher for imperfectly matched heteroduplexes (columns 6 and 10 should be roughly the same)

(vii) All figures given are derived from solution hybridisation data. These conditions have been succesfully used in practice (actually, with wash conditions shifted up by one position on the table).

All washing solutions normally include 0.1-0.5% SDS

After the last wash, replace with a little fresh washing solution; take filters from this clean solution and wrap between sheets of Saran wrap while still moist. This allows subsequent washing at higher stringency or removal of probe prior to re-use of filters. Put phosphorescent markers on Saran wrap for alignment and expose.

Important Points:

1) Formamide used in hybridisations should be of good quality: freshly de-ionised or stored frozen.

2) As it can be a relatively long time before the reason for a hybridisation signal is elucidated, it is worthwhile clarifying some of the possibilities in the initial screen by

(a) using a probe that does not include any vector sequence.

(b) including a genomic Southern in the hybridisation (particularly at low stringency, so that the number of different DNAs being illuminated can be judged).

(c) using two or more different probes either to circumvent possible vector contamination or to define more precisely the nature of the hybridising DNA.

3) Background

The inclusion of a genomic Southern should help to distinguish the source of the problem.

(a) Filters: Any agarose sticking to the filters while making lifts will give rise to background. Batches of filters can vary but this has not often proved to be a problem. It is also not clear that yellow filters (due to incomplete neutralisation) lead to high background; there is a consensus that rinsing the filters before baking is important.

(b) Probe: Nick-translated probes used at high radioactive concentration (10^6cpm / ml-do not exceed this) will always give some (uniform) background, but signals are also strong and very easily visible. The parameter most strongly favoured for adjustment to optimise signal / noise is the DNase I concentration used in nick-translation. Single-stranded probes in general do not give any significant background (when made under standard

conditions); rather the only problem here is the strength of the signal. If the signal is very low (or zero) make sure the probe really was single-stranded.

(c) Hybridisation conditions: As the kinetics of the hybridisation are generally not a problem it is best to use large hybridisation volumes to allow easy flow over the (multiple) filters and to keep the concentration of counts below 10^6/ml. Also, use plenty of pre-hybridisation fluid as there is a large surface area to coat and pre-hybridise for as long as possible, particularly at low stringency.

PICKING POSITIVES & PLAQUE PURIFYING

1) Align autoradiographs with filters and mark the position of the syringe needle holes on the film.

2) Mark positives with unique labels, so that after plaque purification it is possible to return to any plaques that did not check out as expected. To distinguish positives from background due to cassette contamination, cosmic rays and bubbles on the original plate the following criteria can be used :

(i) Shape: the source of radioactivity should be diffuse (as opposed to a point source) and should be roughly circular. Sometimes plaques will streak when lifts are taken giving a comet (a circular spot and a tail, not necessarily connected); this is a clear indication of a real positive. Duplicates help to identify the original position of the plaque but, irrespective of relative intensity, the circular region of a comet signal is the place to pick.

(ii) Intensity: Partial overlap between probe and recombinant phage DNA as well as variable plaque size means that wide variation in signal intensity is possible. Nonetheless, intensity is the best available guide to distinguish positives from background and also for low stringency hybridisation to distinguish between the quality of homology between the probe and various phage.

(iii) Clustering of positives is a clear indication of artifactual signals.

(iv) Number: Expected numbers of positives can be worked out for genomic libraries and, less reliably, for cDNA libraries. If there are an excessive number of positives, consider the possibilities that (i) the probe contains some repetitive sequences or (ii) the probe includes some "vector sequences" that are also present in the library (pUC, pBR, polylinker, ß-galactosidase sequences).

3) Align syringe needle marks on plates with their positions on the films and take a plug of agarose (down to the bottom of the plate) that overlies each positive. Because of the inaccuracy in lining up the orientation marks and because the filters generally contract a little during hybridisation and washing (if not pre-autoclaved) it is best to pick a small area around the positive such that you are confident that the positive must have been picked even given a maximal error in alignment. This can be done by using the wider end of a Pasteur pipette but is better done with multiple stabs of the other end to pick a (roughly) circular area of about 1/3-1/4 the size of the large end.

4) Elute the phage from the agar/agarose plug in 2ml of TM10 (+/-gelatin) with 1-2 drops of chloroform for at least 2 hr. Elution for less than 2 hr will lead to uneven titres. The titre of phage in the eluate is somewhat variable, depending on plating conditions, the number of lifts taken and (reproducibly) the type of bacteriophage lambda.

For the Maniatis library each plaque should yield a titre of 10^6-10^7 pfu. For cDNA libraries in gt10 the titre is 5-10 fold higher. The method of plaque picking described above removes 10-20 plaques from plates with 10-20,000 plaques. Thus, the expected number of infectious phage in the eluate will be in the range $1X10^7$-$1X10^8$ (Maniatis) and $1X10^8$-$1X10^9$(most cDNA libraries). It should only be necessary to plate out about 100 phage to find several positives; however, because of variability in the growth rate of phage and the possibility that the required plaque was initially comparitively small on a crowded plate it is often worth plating out a large number (500-1000) to be certain of not losing a positive. In the latter case a large plate must be used. Thus, the following dilutions are recommended for plaque purification with an error margin that may lead to overcrowding rather than a loss of a positive signal. Dilutions are made in TM10.

Maniatis:
[Plug in 2ml] 1µl→[2ml] Plate 50-100µl → ~500 plaques

gt10 libraries:
[Plug in 2ml] 1µl→ [2ml] Plate 5-10µl → ~500 plaques

Phage are plated out as for the initial plating but using (1/3) fewer bacteria and 3 ml top agarose if using small rather than large plates. Should the number of plaques be too great or too small return either to the original eluate or dilution, both of which should be of stable titre if kept at 4°C (or room temp. for a day). However, if the titre was initially lower than expected due to incomplete elution, it will now be higher than "expected" as elution will now be complete.

5) Make lifts, pre-hybridise, hybridise and wash as for the initial screen. The same probe can be used for initial and second screen but as most will have renatured during the first hybridisation it should be boiled for 5-10 min immediately prior to second use. (Precipitation of, presumably, BSA occurs if boiling is for too long but this does not affect the results whether additional hybridisation solution is added or not).

6) Signals from positives should be more numerous and stronger than in the initial screen – it is therefore not worthwhile making duplicates for a re-screen if only one probe is being used. Positive plaques to be amplified should be well isolated in order to avoid contamination by neighbouring, diffusing phage. The potential problem of contamination is less serious for cDNAs where, in general, a single EcoRI insert will immediately be sub-cloned than for genomic lambda DNAs where mapping or use as an *in situ* hybridisation probe may be complicated by small amounts of contaminating phage.

7) Stocks of phage can be kept in TM10+gelatin+0.2% chloroform at 4°C or in 7% DMSO, TM10+gelatin at -70°C after snap-freezing. Such stocks can be made from single plaque eluates (10^6-10^7pfu/ml) or, more conveniently if they are to

be used as a source for large scale growth of phage, as high titre eluates of plate lysates (10^{10}pfu / ml).

ARRAYS

It is sometimes desirable to investigate the nature of a large number of positively-hybridising phage by further hybridisation before preparing DNA. For this purpose it is convenient to generate multiple copies of a set of positives. Put 100µl aliquots (in duplicate if space permits) of each single plaque eluate in a well of a micro-titre dish. Pour large plates with indicator bacteria (but no phage) in top agarose. Transfer the phage array to such plates (after the top agarose has set) by dipping the "clonemaster" (an array of metal prongs) in the micro-titre dish and then resting it on the surface of the plate. Allow liquid to be absorbed and incubate at 37°C for >8 hr. Make lifts and screen as described above but expect much stronger signals (and sometimes rings rather than spots; rings are usually observed using phage in gt10 and can potentially be eliminated by dilution of the phage).

RE-USE OF FILTERS

If moist filters (stored between sheets of Saran wrap) and plates are stored at 4°C, the filters can be re-used several times for reasons of economy and in some cases, such as chromosome walking, as part of the experimental design.

STRAINS AND MEDIA

C600	(F^-, thi-1, htr-1, LeuB6, lacY1, tonA21, supE44, λ^-)
Q358	($hsdR^-_R$, $hsdM^+_R$, supF, $\phi80^r$)
Lambda plates	NZCYM or LBM+ 1.5% agar
Top agarose	NZCYM or LBM+ 0.7% agarose

NZCYM per litre	10 g	NZamine
	5 g	NaCl
	5 g	yeast extract
	1 g	casamino acids
	1.2 g	$MgSO_4$

LBM per litre 10 g	Bacto-tryptone	
	5 g	Bacto yeast extract
	10 g	NaCl
	1.2 g	$MgSO_4$

REFERENCES

Bonner, T.I., Brenner, D.J., Neufeld, B.R. and Britten, R.J. (1973) Reduction in the rate of DNA reassociation by sequence divergence

Frischauf, A.M., Lehrach, H., Poustka, A. and Murray, N. (1983) Lambda replacement vectors carrying polylinker sequences. **J. Mol. Biol. 170** 827-842

Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connell, C., Quon, D.,Sim, G.K. and Efstradiadis, A. (1978) The isolation of structural genes from libraries of eukaryotic DNA. **Cell 15** 687-701

Marmur, J. and Doty, P. (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. **J.Mol.Biol. 5** 109-118

Marmur, J. (1961) J.Mol.Biol. 3 208

McConaughy, R.L., Laird, C.D. and McCarthy, B.J.(1969) Nucleic acid reassociation in formamide. **Biochem. 8** 3289-3295

Meinkoth, J. and Wahl, G. (1984) Hybridization of nucleic acids immobilized on solid supports. **Analyt.Bioch. 138** 267-284

Pirrotta, V., Hadfield, C. and Pretorius, G.H.J. (1983) Microdissection and cloning of the *white* locus and 3B1-3C2 region of the *Drosophila* X chromosome. **EMBO J. 2** 927-934.

Hermann Steller

RAPID SMALL SCALE ISOLATION OF *DROSOPHILA* DNA AND RNA

DNA:

The following protocol describes the isolation of DNA from adult flies but can be used equally well to extract DNA from other developmental stages. DNA prepared according to that method is readily digested by restriction enzymes and has an average size of 40-60 kb. In order to remove RNA contaminating the DNA preparations DNase free RNase should be included when digesting with restriction enzymes.

1) Anesthesize flies with CO_2 or ether and put 1-20 flies in an eppendorf tube. Keep on ice until next step.

2) Add solution A containing 0.1 M Tris-HCl, pH 9.0; 0.1 M EDTA; 1% SDS and 0.5-1% DEPC (added directly before use) and homogenize gently with a 3 mm diameter glass or metal rod . Use 100 µl of solution A for extracting DNA from 1-5 flies, 200 µl for 6-10 flies and 500 µl for up to 50 flies. Incubate for 20-30 minutes at 70°C.

3) Add 14 µl of 8 M potassium acetate for each 100 µl homogenate and leave on ice for 30 minutes.

4) Spin in the eppendorf centrifuge at 4°C for 15 minutes. Transfer the supernatant into a fresh eppendorf tube being careful not to disturb the pellet. If you get flakes in the supernatant respin.

5) Precipitate DNA by adding 0.5 volumes of isopropanol at **room temperature** and spin for 5 minutes at RT. Wash the pellet carefully with 70% EtOH, respin, dry and redissolve in 10 (1 fly) to 100 (50 flies) µl TE.

RNA:

This is a modification of the method of Chirgwin et al. (Biochemistry **18**, 5294-5299, 1979) which can be used for the rapid extraction of total RNA form all developmental stages of Drosophila.

1) Homogenize tissue (e.g. 1 fly or 50-100 embryos in 50 µl and up to 200 flies in 1 ml) GHCl buffer (7.5 M guanidium hydrochloride; 0.025 M NaOAc, pH 7.0; 5 mM DTT) + 0.5% N-laurylsarcosinate + 0.5% DEPC.

2) Extract once with an equal volume of phenol-chlorororm and separate phases by centrifugation.

3) Transfer the aqueous (upper) phase into a new tube and precipitate the RNA by adding 1 µl 1 M acetic acid and 25 µl ethanol for each 50 µl of GHCl-solution. Leave at -20°C for 3-24 hours. Pellet the RNA by centrifugation for 5 min., 8-12K.

4) Remove supernatant as complete as possible and redissolve the pellet in half of the original volume (but minimally 50 µl) GHCl buffer.

5) Reprecipitate the RNA as in step 3. (The reprecipitation serves to remove DNA which will not precipitate under these conditions).

5a) **optional:** reprecipitate the RNA for 1-2 more times by repeating steps no. 3 and 4. This is only necessary if large amounts of DNA have to be removed, e.g. when analysing the transient expression of DNA injected into embryos.

6) Wash (and store) the RNA pellet in 100% ethanol, room temperature. For Northern analysis the RNA is best dissolved directly in Northern sample buffer (e.g. 50% formamide, 2.2 M formaldehyde, 1x MOPS).

Mary Mullins

CAT ASSAY: DROSOPHILA TRANSFORMANTS

1. Freeze flies to be assayed in an Eppendorf tube (1.5 ml) at -80°C for 15 min.
2. Add l00 µl of 0.25M Tris (pH 7.8) and homogenize.
3. Freeze the homogenate, thaw, and sonicate (optional) for 1 minute.
4. Heat at 65°C for 5 minutes. This precipitates most other proteins including some inhibitor(s) of CAT activity.
5. Spin in cold (rm. temp O.K.) microfuge for 10 minutes.
6. Transfer supernatant to new tube. For quantitative assays, do Bradford assays on all extracts so equal amounts of protein can be used in each assay.
7. Set up reactions:
   Extract: 1-90 µl
   0.25M Tris-7.8 + ^{14}C-chloramphenicol(CAM): 30 µl=29 µl Tris + 1 µl CAM (0.2µCi)
   4mM Acetyl CoA (Lithium) : 20µl
   0.25M Tris: adjust final volume to 140µl
8. Incubate at 37°C from l5 minutes to several hours. For good quantitation, a time course can be done to determine the linear range of the assay.
9. Stop the reaction and extract the chloramphenicol by adding 0.5ml ofethyl acetate. Vortex well and spin 1 minute in microfuge. Remove the top organic phase and place in new tube.
10. Dry in Speedvac (about 15 minutes) or overnight in the hood.
11. Resuspend in about l5 µl ethyl acetate.
12. Spot onto TLC plates (American Scientific Products, Baker-Flex TLC Silica Gel 1B Plates, 20x20cm, Cat. # 4462).
13. Run in 95:5 chloroform:methanol for l.5-2.5 hours. in a **tightly** sealed chromatography tank.
14. Dry plate and expose at room temp.
15. Four spots may be seen. The lowest is the non-acetylated CAM, the next two spots are two different monoacetylated CAM forms, the top spot (which may not be seen depending on the duration of the reaction) is diacetylated CAM. For quantitative assays, the spots can be cut out and counted in a scintillation counter.

Materials:

- ^{14}C-Chloramphenicol(CAM): Amersham, CFA515, 54mCi / mmole

Kevin Jones

CHROMOSOME WALKING USING COSMIDS

I will divide this protocol into two sections. The first section will describe the screening of the cosmid library and isolation of positive colonies. The second section will describe the endcloning method used to isolate end fragments of the cosmids for chromosome walking.

A. Screening of Cosmid Libraries

1. Preparation of Nitrocellulose Filters

i) Wash the desired number of nitrocellulose filters (we use 82 mm diameter Schleicher and Schuell) in two changes of distilled water. This removes detergent and other impurities which can retard colony growth and/or reduce the titer.

ii) Stack the filters, separating them with sheets of Whatman 3MM paper. Put two sheets of blotter paper on each end of the stack and wet the blotter sheets with distilled water. Package the resulting stack in aluminum foil and autoclave 20 minutes. This helps prevent shrinkage of the filters from occurring during the hybridization and washing process, which can make aligning signals with colonies on the master plates difficult.

iii) Number the filters and place face down on 30µg/ml ampicillin plates. This equilibrates the filters with the media. After a few minutes, flip the filters.

iv) Plate the titered library at a density of 2-4000 colonies per plate. At 37°C it typically takes about 10 hr for the colonies to reach the proper size for replica plating.

2. Replica Plating

i) Allow colonies to grow to about 0.5 mm in diameter. Larger colonies are more likely to smear, smaller ones don't always transfer evenly.

ii) Pre-equilibrate filters to be used as replicas as in step 3 above. Up to two replicas can be reliably made with one master filter. Additional replicas can be made from a master after the colonies are re-grown.

iii) Replica plate by setting the pre-equilibrated filters onto a couple sheets of the sterile Whatman 3MM paper (generated in step 2 under "Preparation of Nitrocellulose Filters"), laying the master filter on top of the replica, and putting your weight on the filters using a replica plating tool. Use a 25 ga needle to put holes through the filters while they are stuck together. This will allow alignment of signals with colonies later. It is a good idea to press on the filters with a pair of forceps near where the holes are being made with the needle to prevent the filters from slipping against each other.

iv) Grow the colonies back up. The masters usually take 3-5 hr at 37°C, the replicas anywhere from 4-8 hr. The colonies can then be lysed directly or the cosmid DNA amplified by placing the filters on 100µg/ml chloramphenicol plates for 16 hr. Chloramphenicol amplification generally improves signal to noise.

3. Lysis of Colonies, Hybridization and Washing

i) Lyse the colonies by laying the filters on blotter saturated with 10% SDS 3 minutes, then 0.5M NaOH / 1.5M NaCl 5-10 minutes, followed by 1M Tris / 3M NaCl, pH 7.4 for 5-10 minutes. The SDS step is reputed to sharpen signals.

ii) Lay the filters on top of, and then submerge them in 2xSSCP / 0.1% SDS. Wipe the colony debris off the submerged filter gently with a folded Kimwipe. Excessive "polishing" will remove the cosmid DNA, the right amount will reduce background. Just wipe hard enough to remove the debris.

iii) Bake the filters, separated by sheets of Whatman 3MM, under vacuum at 80 degrees Celsius for 2-4hr. Longer baking times help reduce DNA loss from filters that are to be repeatedly hybridized, washed, and boiled.

iv) Prehybridize the filters in 3xSSCP / 10xDenhardts / 250µg / ml carrier DNA / 0.1% SDS. A 1hr prehybridization is usually sufficient, unless an unusually large amount of probe is to be added or the hybridization is to last unusually long.

v) Hybridize the probe to the filters in the same type of buffer as used for the prehybridization. We usually hybridize nick translated ^{32}P probes at a concentration of about $2x10^5$ cpm Cherenkov per ml for 12-16 hr.

vi) Wash the filters 5 minutes in 2xSSCP / 0.1% SDS at room temperature, then 15 minutes in 2xSSCP / 0.1% SDS at 65^{o}C, then in 2xSSCP / 0.1% SDS until background cannot be heard on the filters with a monitor. We usually let this last wash go about 2hr. Sandwich the filters between two pieces of Saran wrap and apply to X-ray film.

4. Isolation of positive colonies

i) Align the autoradiogram with the filters and mark the positions of the holes.

ii) Ethanol wipe a piece of cellophane (or something similar). Spot about 0.2 ml of L-broth onto this in a number of droplets. Lay the master filter (with colonies) onto the L-broth, set the cellophane on the autoradiogram. Align the holes, sometimes background on the autoradiogram helps in the alignment. You never thought background could be useful, right? Get the alignment as precise as possible.

iii) If you're lucky you'll have one good candidate for the signal. If not, pick each colony individually with a sterile toothpick and transfer to a plate in a grid pattern. If there are double colonies, etc. pick these and streak them to get single colonies which can be put in a grid. Grow up the grid, lift the colonies from the plate using a nitrocellulose filter (make holes in the filter with an india ink filled hypodermic needle before removing the filter to help align it with the plate later) and treat the filter as above.

iv) Cosmid DNA from the resulting positive colonies can be isolated using a number of methods. The most rapid I've used is to make a boiling miniprep (see Quigley and Holmes, Analyt. Biochem. 114, 193 (1981)) of 1.5ml of a saturated culture (I use L-broth with 100µg / ml ampicillin). This generally yields roughly 5-10 µg of DNA. I've heard that alkaline lysis miniprep methods can give better yields but after determining that the cosmid is what I want using this miniprep DNA I just go ahead

and grow a 500ml or 1 liter culture and purify the cosmid by alkaline lysis followed by a CsCl gradient.

B. Isolation of cosmid end fragments by subcloning

The idea here is to isolate cosmid end fragments as rapidly as possible when chromosome walking. Many cosmid libraries are constructed using vectors where end fragments from either end of the cosmid can be easily subcloned. For example, the cosPneo vector has a polylinker containing a BamH1 site flanked on one side by an Eco R1 site and on the other side by an Xba 1 site. If the cosmid library in this example is made by inserting genomic DNA into the Bam H1 site, the two ends of the cosmid can be isolated by intramolecular ligation of complete Xba 1 and Eco R1 digests.

Miniprep DNA is sufficient for this procedure as long as you can digest it to completion. Digest 0.5-1.0 µg of cosmid DNA in a 50 µl reaction. Check half of the digest on a gel to be sure it is complete. Heat kill the enzyme if possible. Otherwise, bring the reaction to 50 µl and add diethylpyrocarbonate (0.5 µl of 10% DEP in EtOH), heat to 37° for 20 min. Add 2.5 µl 1M Tris (pH 8.0) and heat to 65°C about 10 min. to ensure that the DEP is inactivated. The digest can be run over a P10 spin column at this point to ensure high cloning efficiency, but this is usually unnecessary. In any case, bring the reaction to 100 µl and add ligase buffer and 100 units T4 DNA ligase. Transform competent cells as usual. The resulting endclones can be hybridized to cosmids from the previous step in the walk to determine which one extends the walk. They can also be used for in situ hybridization to polytene chromosomes to get an accurate picture of the progress of the walk.

Tyler Cutforth

DEEP PSEUDOPUPIL IN WHITE- AND RED-EYED FLIES

The deep pseudopupil is an optical phenomenon arising from the regular arrangement of ommatidia in the compound eye and the nature of the rhabdomeres in Diptera, which are open and can transmit light (N. Franceschini in *Information processing in the visual systems of arthropods* [ed R. Wehner], pp. 75-82, 1972). By focussing in a plane below the outer surface of the eye, virtual images of the rhabdomere patterns from several ommatidia are superimposed and the stereotypic trapezoid arrangement of photoreceptor cells R1-R7 is apparent. We use the deep pseudopupil to score living flies for defects in photoreceptor arrangement which may be masked by a normal external morphology.

Observation of the deep pseudopupil of red-eyed flies requires illumination from below (antidromic), using a narrow, bright light source (such as the Magnalite T-150) with a dissecting microscope and a transparent, elevated stage for the flies. Positioning the light source is a matter of trial and error, however a general guideline is to place the tips of the light pipes just inside the field of view under low power and angled upwards to illuminate the centre of the field. The pseudopupil itself is best viewed under high power (5X), with the anesthetized flies lying on their side, backs toward the observer. This position permits scoring of the posterodorsal quadrant of the eye, and the light pipes may need adjustment at this point to maximize the contrast between the pseudopupil and the surrounding eye surface. Other regions of the eye may be scored by tilting the head, but this is more problematic since slight motions of the hand blur the image. The deep pseudopupil of white-eyed flies is viewed with illumination from above (orthodromic), but in all other respects is similar to that of red-eyed flies.

Gerry Rubin

MAKING SEMINAR SLIDES

1. Color Slides

These are quick to make and give accurate color reproduction; good for half-tones such as autoradiograms. Black and white diagrams have a tendency to come out dark gray on pale yellow, but are certainly usable.

Film: Kodak Ektrachrome 50(Tungsten)

Shoot: Using light meter: no filter on camera. Exposure time is accurate for half tones. For originals having a high percentage of white area, the light meter will not give the correct exposure and you must correct by using a longer exposure, for example, two stops longer for a typewritten table (e.g. 1/15 sec. vs. 1/60 sec.). Always shoot a range - one stop on each side of what you judge to be the correct exposure.

Development: E6 procedure. Ziba does two runs a day, Monday – Friday: 9:30 - 1 pm and 12 noon - 4 pm.
Presto Prints (on Telegraph) does processing with a two-hour turnaround and is open on weekends. Latest time for having film developed that day is 3:30p.m.

2. White on blue slides

Good for black and white diagrams. Can also get some additional colors:

Original	Slide
black	white
white	dark blue
dark red	light blue
dark green	pink
light blue	light purple

Film: Kodak SO-279 veracolor slide film.

Shoot: A range from f8 - f11 with a 1 sec exposure with our camera setup. Use HOYA 85B and Nikon Y52 filters.

Development: C-41 processing to negatives takes about 1 hour at Ag Photo (Shattuck Center across from Andronico's) ~$3/36 exp roll.

3. Computer slides on slide maker

Using a computer program that alows you to save in PICT format (Dreams, Canvas, MacDraw, but **not** Word), make your diagram and save a copy as a PICT file. Follow instructions in Imager manual to print slides on Slidemaker.

APPENDIX I: REAGENTS

Acrylamide stock (38%/2%) (Hazardous to your health. Wear gloves. Work in hood.)

76 g acrylamide
4 g methylene bisacrylamide

Make up to 200 ml with DDH_2O. Store in dark at 4°. (Wrap bottle with tin foil.)

Amp Plates

5 g NaCl
10 g Bactotryptone
5 g yeast extract
15 g agar
DDH_2O to 1 liter
Autoclave
Add 0.3 ml amp stock just before pouring.

Ampicillin

Stock = 250 mg / ml
1 g ampicillin sodium (pharmaceutical grade) in 4 ml sterile DDH_2O
Store in aliquots at -20°

10X BU Buffer

70 g Na_2HPO_4 or 132 g $Na_2HPO_4 \cdot 7\ H_2O$
30 g KH_2PO_4
DDH_2O to 1 liter
Autoclave

Benton and Davis Solutions

Denat.	0.1 N NaOH	4 g / l or 10 ml of 10N
	1.5 M NaCl	87.5 g / l
Neut.	0.2 M Tris pH 7.5	24.2 g / l
	pH to 7.5 with HCl	= ~25 ml conc HCl per 2 l

Optical Grade CsCl Step Grade Sol'n

	CsCl	TM10
1.3 g / ml	3.1 g	6.9 ml
1.4 g / ml	3.9 g	6.1 ml
1.5 g / ml	4.5g	5.5 ml
1.6 g / ml	5.1 g	4.9 ml

Vortex. Should go into sol'n easily.

50 mM $CaCl_2$

3.7 g $CaCl_2 \cdot 2 H_2O$
Dissolve in 500 ml DD H_2O.
Autoclave.

Casamino Acids for Sinsheimer's

9.9 g casamino acids in 837 ml DDH_2O
Autoclave.
(Use 1 liter Bellco bottles.)

Chloramphenicol Stock - 45 mg/ml

9 g chloramphenicol
200 ml EtOH
Store at -20°C. Use 3.5 ml/liter.

20 mM DTT

40 µl 1 M DTT stock sol'n
1960 µl chilled sterile water
Aliquots of 100 µl/Eppendorf tube. Freeze.

Dialysis Bags

sodium carbonate 400g (10%)
EDTA 74.4 g (50 mM)
H_2O to 4 liters
boil 4X

250 mM EDTA pH 8.0

186.1 g Na_2 EDTA $\cdot 2 H_2O$
Dissolve in ~1700 ml DD H_2O. Adjust pH with 50% NaOH to pH 8.0 (~28 ml 50% NaOH).
EDTA won't dissolve until the pH is increased. Make up to 2 liters with DD H_2O.
Autoclave.

Ficoll/Bromphenol Blue Loading Buffer

6 g Ficoll
43 g H_2O
Heat with stirring.
Add pinch of Bromphenol blue
5 ml 250 mM EDTA

Fly Egg Laying Medium (for 18 trays 5" x 5")

2225 ml H_2O
88 g agar
360 ml molasses

Autoclave 30 min.
Add 0.5 ml conc. Tegosept (16 g / 150 ml 95% EtOH)
20 ml of ethylacetate, after autoclaving.

Freezing Medium (2X)

3.0 g NaCl
12.6 g K_2HPO_4 (anhydrous)
0.9 g NaCitrate • 2 H_2O
0.18 g $MgSO_4$ •7 H_2O or 0.09 g $MgSO_4$
1.8 g $(NH_4)_2SO_4$
3.6 g KH_2PO_4 (anhydrous)
88 g (70 ml) glycerol (glycerin)
Dissolve and adjust volume to 1 liter. Autoclave.

Gelatin - 10 mg/ml or 1%

2 g gelatin
200 ml DD H_2O
Heat and stir to dissolve
Autoclave.

10% Glucose

200 g glucose
Dissolve in 2 liters DD H_2O
Autoclave.

Hot Fudge Sauce

1/2 cup butter
3 squares baking chocolate
1-1/2 cups sugar
1/2 cup cocoa
dash of salt
1 cup light cream or evaporated milk
2 teaspoons vanilla

Melt butter and chocolate over low heat. Mix in sugar, cocoa and salt until smooth. Add cream and bring to a boil, stirring constantly. Remove from heat and stir in vanilla. Store at 4°C. Heat before using in microwave or by placing container in a pan of hot water.

Kan Plates

5 g NaCl
10 g Bactotryptone
5 g yeast extract
15 g agar
DD H_2O to 1 liter
Add 1 ml Kanamycin stock before pouring.

Kan Amp Plates

As for Kan plates, but add 0.4 ml amp stock.

Kamamycin Stock

Stock: 5 g Kanamycin (Sigma) in 100 ml H_2O
Filter sterilize
add 1 ml / liter plates

1 M KCl

37.28 g KCl
Dissolve in 500 ml DDH_2O
Autoclave.

5 M KOAc

49 g / 100ml H_2O

L Broth

5 g NaCl	10	20
10 g bactotryptone	20	40
5 g yeast extract	10	20
1 liter H_2O	2 liter	4 liter

Autoclave.

Lambda Plates

Make up in 4 liter flasks. Autoclave 75 minutes with stir bar. Stir while cooling.

	1 l	2.5 l
NaCl	5 g	12.5 g
Bacto-Tryptone	10 g	25 g
Yeast extract	5 g	12.5 g
Agar	11 g	28 g
MgSO4	1.2 g	3 g
water to:	1 liter	2.5 liter

Lysis Mixture

500 ml 250 mM EDTA, pH 8
100 ml 1 M Tris, pH 8
2 ml Triton X-100

Dilute to 2 liters with DD H_2O.

N.B. Adjust the volume before mixing, due to sudsing.
Autoclave. This will be cloudy after autoclaving but will clear up as it cools.

20% maltose

400 g maltose
Dissolve in 2 liters DDH_2O.
Autoclave.

Marker Stock

4 µg l Hind III
5 µg FX Hae III
TE to 400 µl
200 µl Ficoll-BPB with 25 mM EDTA

1 M $MgCl_2$

101.6 g $MgCl_2$ • 6 H_2O
Dissolve in 500 ml DDH_2O.
Autoclave.

0.1 M $MgSO_4$

12g $MgSO_4$
DDH_2O to 1 liter
aliquots of 100 ml/bottle
Autoclave

Minimal Plates

for 500 ml: Autoclave 7.5g agar in 419 ml DDH_2O with stir bar.
Add following sterile solutions:

50 ml 10xM9 phosphate
5 ml 10% NH_4Cl
5.5 µl 0.1 M $MgSO_4$
20 ml 10% glucose
1 ml 10 mg/ml thiamine HCl (add when ready to pour)

10X M9 Phosphate

30 g KH_2PO_4

113g $Na_2HPO_4 \cdot 7\ H_2O$ / to 1 liter H_2O
Autoclave

M9 Medium

per liter:
100 ml 10X phosphate
10 ml 10% NH_4Cl
11 ml 0.1 M $MgSO_4$
40 ml 10% glucose
2 ml 10 mg/ml thiamine HCl
837 ml M9 CAA

5 M NaCl

146 g NaCl
to 500 ml with DD H_2O
Autoclave.
(Note: heat to dissolve salt as concentration is near saturation.)

10% NH_4Cl

200 grams NH_4Cl
DD H_2O to 2 liters
Autoclave.

4X Nick Translation Buffer

84 µl 1 M Tris pH 8
336 µl 1 M Tris pH 7.5
42 µl 1 M $MgCl_2$
40 µg Pentex BSA
1498 µl H_2O.

O/N Broth

20g Tryptone
10g NaCl
Yeast extract 10g
DD H_2O to 1 liter
Autoclave.
add maltose to 0.1% before use if desired (0.5 ml 20% maltose/100 ml O/N).

10% SDS

100 g SDS (Biorad)
DD H_2O to 1 liter
DO NOT AUTOCLAVE

20X SSC

350.6 g NaCl
176.5 g NaCitrate
Dissolve in 2 liters DD H_2O.
Autoclave.

20X SSCP

800 ml 1M sodium phosphate pH 6.8
176.4g NaCitrate
280.5g NaCl
to 2 liters with DD H_2O.
Autoclave.

Sinsheimer's Medium

1.5 ml 5 M NaCl
9.0 ml 10% NH_4Cl
36 ml 10% glucose
20 ml 20% maltose
5 ml 1 M $MgCl_2$
2 ml 50 mM $CaCl_2$
840 ml Sinsheimer CAA

3 M sodium acetate

246.1 g sodium acetate (anhydrous) / 1 liter H_2O.
Autoclave

1 M sodium phosphate pH 6.8

138g $NaH_2PO_4 \cdot H_2O$
268g $Na_2HPO_4 \cdot 7\ H_2O$

Adjust to 2 liters with DD H_2O.
Autoclave.

Southern Blot Sol'ns

		4 liters
Denat.	0.5 M NaOH	200 ml 10N NaOH
	1.5 M NaCl	350.6 g NaCl
Neut.	1 M Tris pH 7.4	484.4 g Tris pH 7.4
	3M NaCl	701.3 g NaCl
		pH with >250 ml conc HCl

Stabs

Bactotryptone 8g
NaCl 5g
Difco agar
6g
Yeast extract 1g
DDH_2O to 1 liter

Adjust pH to 7.2-7.4 with about 4 drops of 6 M NaOH per liter. Autoclave 5 min to dissolve agar. Fill vials with 3 ml each. Autoclave. Leave upright.

25% Sucrose (w/v) in <u>50 mM</u> Tris HCl, pH 7.5

500g sucrose
100 ml 1 M Tris HCl, pH 7.5
DD H_2O to 2 liters
Autoclave.

20X TBE (Tris-borate, electrophoresis buffer)

432 g Tris base
220 g Boric acid (crystal dissolves easier than powder)
40.8 g Na_2 EDTA • 2 H_2O

Dissolve in 2 liters DD H_2O
Check: pH = 8.35 for a 1:20 dilution
Autoclave. (Use Bellco 1 liter bottles)

TCM

5 ml 1 M Tris HCl, pH 7.5 (10 mM Tris HCl, pH 7.5)
100 ml 50 mM $CaCl_2$ (10 mM $CaCl_2$)
5.0 ml 1 M $MgCl_2$ (10 mM $MgCl_2$)

Dilute to 500 ml with DD H_2O.
Autoclave.

TE pH 8.0 (10 mM Tris; 1 mM EDTA)

10 ml 1 M Tris pH 8.0
4 ml 250 mM EDTA, pH 8.0

Adjust volume to 1 liter with DD H_2O.
Autoclave.

TE/0.2 M NaCl

11.6 g NaCl
Dissolve in 1 liter TE.
Autoclave.

Tetracycline

5 g tetracycline (tetracycline HCl, pharmaceutical grade)
100 ml 50% Ethanol / 50% sterile DDH_2O
Want 25 µg/ml

Add 0.25 ml/500 ml L-broth
Let agar stir a while after adding antibiotic.

TM10 with Gelatin

2.41 g $MgSO_4$
20 ml 1 M Tris pH 7.5
20 ml 1% gelatin (or 0.2g dry gelatin. Heat to dissolve.)

DD H_2O to 2 liters.
Autoclave.

TM10 - 10 mM $MgSO_4$ and 10 mM Tris pH 7.5

2.41 g $MgSO_4$
20 ml 1 M Tris pH 7.5

Make up to 2 liters with DD H_2O. Autoclave.

Top Agar

5 g NaCl
10 g bactotryptone
5 g yeast extract
1.2 g $MgSO_4$
7 g Agar DD H_2O to 1 liter.

This must be heated to almost boiling. Aliquot 50 ml per milk dilution bottle. Autoclave.

Top Agarose

5 g NaCl
10 g bactotryptone
5 g yeast extract
1.2 g $MgSO_4$
1 liter DD H_2O
7 g Agarose (low EEO)

This must be heated to almost boiling. Aliquot 50 ml per milk dilution bottle. Autoclave.

300 mM Tris Base

36.34 g Tris base
to1 liter with DD H_2O
Autoclave.

1 M Tris pH 8

177.6 g Trizma HCl
106 g Trizma base

Dissolve in 2 liters DD H_2O.
Autoclave.

1 M Tris pH 7.5

254 g Trizma HCl
47.20 g Trizma base

Dissolve in 2 liters DD H_2O.
Autoclave.

50 mM Tris pH 8
50 mM EDTA pH 8
15% sucrose (w/v)

100 ml of 1 M Tris pH 8
400 ml of 250 mM EDTA
300 g sucrose

DD H_2O to 2 liters.
Autoclave.

2X YT Broth

16g bactotryptone
10g yeast extract
10g NaCl
H_2O to 1 liter
Autoclave.

1X YT Agar

Top	Bottom	
8g	8g	Bactrotryptone
5	5	yeast extract
5	5	NaCl
7	15	Agar
1 liter	1 liter	H_2O

Autoclave.

Carrier DNA

10 g herring testis DNA (Sigma Type XIV)
DDH_2O to 2 liters

Stir until all DNA is no longer opaque. Autoclave. Freeze until use.

100X Denhardt's Solution

20g polyvinylpyrolidone
20g bovine serum albumin (Sigma Type V)
20g ficoll
10ml 0.25M EDTA

H_2O to 1 liter

DNase for Nick-Translation

10^{-4} mg / ml Worthington DNase (DPFF) in 50% glycerol, 1X NT buffer.

Ethidium bromide

500 mg ethidium bromide (Sigma) / 100 ml DDH_2O

Loening Buffer

4.36 g Tris base (36 mM)
4.14 g $NaH_2PO_4 \cdot H_2O$ (30 mM)
0.336 g Na_2EDTA (1 mM)
DDH_2O to 1 liter

Nucleotide triphosphates

To 25 mg nucleotide triphosphate add 350µl sterile water. Bring to pH 7 by adding 1N NaOH (20-50 µl), using pH paper to test after each addition. Make a 1 / 2000 dilution into a buffer of the appropriate pH and measure the optical density at the appropriate wavelength. The final concentration should be about 100mM.

Nucleotide	pH of dilution buffer	Wavelength	Extinction coefficient
dATP	7	259 nm	15.2×10^3
ATP	7	259	15.4×10^3
dCTP	2 (0.01 M HCl)	280	13.1×10^3
CTP	2	280	12.8×10^3
dGTP	7	253	13.7×10^3
GTP	7	253	13.7×10^3
dTTP	7	267	9.6×10^3
UTP	7	262	10.0×10^3

NOTE: conc. = optical density / extinction coeff. x dilution factor. So for a 100 mM solution of UTP, diluted 1 / 2000 in pH 7 buffer, the optical density at 262 nm will be 0.5: 10^{-1} M = (0.5 O.D. units / 10×10^3 M / O.D.) x (2×10^3).

Restriction Enzyme Buffers

See Maniatis

10X Reverse Transcription Buffer

for 5 ml:

2.5 ml 1M Tris HCl, pH8.3 (500mM)
0.6 ml 5 M NaCl (600mM)
0.3 ml 1M $Mgcl_2$
12.5 ml 2 M KCl

Make 100µl aliquots. Add 1µl 1M DTT / 50µl reaction.

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