# KLYMKOWSKY LAB MANUAL

**Labnote books:** Scientists keep a lab notebook to document their work and to remind them of the details involved, so that it can be repeated by themselves and others. In the notebook record the design of each experiment, how you deviated from that design and the resulting data. The better you are at keeping you notebook up to date, the easier your life will be. Without a physical record of your experiment, it is as if you never did the experiment.

**Your lab note book is the property of the lab You can copy it when you leave, but it must be left with me.**

**Dealing with Mike:** If I have learned one lesson over the years, if you are not prepared to work for the furtherance of your own career, nothing I can do or say will make any difference. Since I like having a small lab, I expect people who come to work with me to have a love of science. If I find that you do not have it, I will ask you to leave -- basically for your own good. Without it, you cannot succeed and you are wasting your time. This does not mean that you are a failure, you may well find that you will move to another lab and be very successful, or you may leave academic science and become wealthy and wise, but I have no other choice, since letting you stay will only sour my appreciation of your good traits.

As a way of self-testing your interest in science (or rather your project) consider that the more you work on a problem, the more immersed you become in its intellectual background and technical realization, the more experiments you should be thinking up, the more work there is to do, the less time you have to complete everything that you would like to do. If you find yourself with nothing to do, you are probably not really engaged in your work.

Finally, it is worth remembering that I may be called on to write letters of recommendation for you. In general, my letters are quite frank. Try to realize what you are doing and the impression you are creating in me, and you will not be surprised by the result.

| **Recipe for LB media:** | **Recipe for LB media:** |
|---|---|
| 5g NaCl | add 15g agar (sigma A7002) and autoclave |
| 10g casamino acids (or tryptone) | |
| 5g yeast extract | add 1ml 100mg/ml ampicillin (AMP) per litre |
| 1ml 10M NaOH | after cooling to ~60°C |
| water to 1 litre - use distilled water | pour plates (0.5cm thick) |

**Preparation of competent cells - standard transformation**

(from Ausubel et al 1985).

**Notes:** typically we use DH5α cells since they are tolerant to the source of the DNA (B or K strain). The same protocol will work with BL21 cells (for protein synthesis) and other bacterial strains.

0. streak out the cells from a –80°C stock on an LB plate
   (do not use an LB-AMP plate; since the bacteria do not have plasmids in them they will not grow in the presence of AMP).
1. Inoculate a single colony of *E. coli* cells into 50ml LB media
   grow overnight at 37°C with moderate shaking (250rpm)
2. Inoculate 4ml of the culture into 400ml LB media in a 2L sterile flask
   grow at 37°C with shaking until $OD_{590}$ is ~0.375
3. Aliquot into ten 40ml sterile polypropylene tubes and leave on ice for 10 min.
4. Centrifuge cells for 7min at 3000rpm, 4°C, in Sorvall
5. Discard the supernatant; resuspend each pellet in 8ml ice-cold calcium chloride solution.
   8 cells are resuspended by gentle swirling! 8

**Calcium chloride solution**: 60mM $CaCl_2$
15% glycerol
10mM PIPES, pH 7.0
(filter-sterilized).

6. Centrifuge cells for 5 minutes at 2000 rpm at 4°C -- Discard supernatant
   Resuspend cells as before with calcium chloride solution (8ml per tube).
7. Centrifuge cells for 5 minutes at 2000 rpm at 4°C -- Discard supernatant
   Resuspend by swirling gently in 1.8ml of ice cold calcium chloride solution

*Pellets must be resuspended completely.*

8. Dispense 0.25mL cells into prechilled, sterile 1.5ml microfuge tubes and freeze at -70°C.

**Preparation of competent cells - electroporation**

**Notes:** Use this protocol for tricky ligations (blunt ends, etc).
1. Grow a colony of cells to 0.5-1.0 OD in LB broth
2. Chill on ice
3. Pellet 1L culture at 3000rpm at 4C for 15 minutes in Sorvall RC5B
4. Resuspend in 1L of sterile, cold water / spin down
5. Resuspend in 0.5L of sterile, cold water / spin down
6. Resuspend in 0.5L of sterile, cold water / spin down
7. Resuspend in 20mL of sterile cold water / spin down
8. Resuspend in 3mL sterile cold 10% glycerol
   -- make 40mL aliquots and freeze (-70C)(mark "EP").

**Standard transformation:**

1. add 1mL of miniprep DNA or 15mL of a ligation reaction to a vial of competant cells
2. let sit on ice for 30-60 minutes.
3. transfer to a plastic-capped tube / heat shock at 42°C for 1 min
4. add 0.8 ml LB media - let grow for more than 20 minutues at 37°C
5. plate out onto LB + AMP plates

typically for a simple transformation of cells with intact plasmid, I would make two plates, using 50uL and the other with 200uL of cells.

for a ligation reaction, I would plate the entire transformation onto two plates.

Always flame the glass spreader and allow it to cool before using it to spread cells out on a plate. If it is too hot, it will kill the cells.
If you do not flame it, you will cross contaminate cultures (a bad thing to do!).

**Electroporation transformation:**

Dilute miniprep or ligation 1:5 in distilled water
add 1mL of DNA to a vial of competent cells
add to electroporation cuvette / let sit on ice for 10 min
Zap cuvette (used apparatus in the Boswell lab) with 1500V
IMMEDIATELY ADD 1ml LB MEDIA
let grow for 1h at 37°C
plate out onto LB + AMP plates; incubate at 37°C

Because of the cost of the electroporation cuvettes, we only use this method for tricky applications, such as blunt end ligations or the preparation of libraries.

Colonies should appear within 12 to 14 hours at 37°C. They should appears as discrete; if they are surrounded by "satellites" then it is likely that the ampicillin in the plate is lossing its effectiveness.

**Growing up colonies:**

Use a yellow pipette tip and spear a colony.

Only take colonies that are well separated from others on the plate.

Eject the tip into a sterile / capped glass tube

Add 5ml LB broth and 100$\mu$L of ampicillin* (100mg/ml stock)

Incubate in tube roller (third floor warm room) overnight.

* Note: if you yield of plasmid DNA is poor, the most likely problem is that your ampicillin has "gone off". Make new ampicillin stock and stay aware of this possibility.

**AMPICILLIN** STOCK: 100mg/ml in distilled water

**Wizard (Promega) minipreps:**

1. Pellet 1.5ml of bacteria from an overnighter (3 min. spin in minifuge).
2. Discard supernatants / resuspend in 200mL resuspension buffer.
3. add 200mL cell lysis buffer - invert until clear.
4. add 200mL neutralization buffer - invert and then spin in microfuge for 5 minutes.
5. take supernatant - transfer to a new tube then add 0.8ml of resin
   - invert and let sit for 1 min.
6. attach mini-column to 3ml syringe / or manifold (syringes can be reused!)
   and load with resin/cell supernatant.
7. push material through the mini-column
8. remove syringe, pull out plunger, replace syringe. add 2ml wash solution -
   push through the mini-column.
10. take mini-column and place in microfuge tube - centrifuge for 20 sec.
11. take mini-column and place on fresh tube, add 50mL 65°C water, centrifuge again for 20 sec. -- your DNA is in the microfuge tube!

**Resuspension buffer:** 50mM Tris pH 7.5
10mM EDTA
100µg/ml RNAase A

**Lysis buffer:** 0.2M NaOH
1% SDS

**Neutralization buffer:** 1.32 M Kacetate pH 4.8

**Ethanol wash buffer:** 0.2M NaCl
20mM Tris pH 7.5
5mM EDTA ADD 70ml 100% ethanol / 50ml wash buffer

**Restriction analysis:**

typically, you will set up a set up reaction in 20mL (total volume) reaction
you should generally run uncut plasmid, as it provides an important point of comparison.

Set up the following type of chart:

| Sample | DNA | Buffer | Water | Zym | Buffer |
|---|---|---|---|---|---|
| 1. plasmid 1 | 2 | 2 | 16 | | - |
| 2. " | 5 | 2 | 11 | | enzyme |

etc.

For simple diagnostic digests (to determine if you are dealing with the correct plasmid) you will normally use 2mL of uncut DNA and 5mL for the digest

1mL of enzyme should be much more than enough to digest plasmid (1 h @ 37C).
NEVER ADD MORE THAN 2mL of enzyme to a 20mL reaction
the glycerol will inhibit the reaction!
Also check temperature of the digest, some enzymes work at temperatures other than 37C!)
Each enzyme has its own specific buffer optima -- check chart in Promega or NEB catalogues to determine which is best for your reaction.

**PLEASE** be careful to take only the amount of enzyme require -- they are expensive.
**ALWAYS KEEP ENZYMES IN THE BIG PIG**
warming them leads to a loss of activity and wasted time and energy!

**ALWAYS CHANGE PIPETTE TIPS TO AVOID CROSS-CONTAMINATING ENZYMES**

**Partial digests:**
In some cases you may need to do a partial digest. This can be tricky.
Always carry out a complete digest to determine the final pattern.
A good starting point is to use the enzyme (1mL) diluted 1:10 in its appropriate buffer + acetylated BSA) and reduce the time and temperature of the incubation.
I suggest 5-10 min. at r.t.

**DNA gel analysis:**

For a large gel: use 0.75g agarose melted into 100ml TAE buffer
add 50mL 1mg/ml Ethidium bromide solution
For a small gel use 0.375g agarose melted into 50mL TAE buffer
add 25mL ethidium bromide

***** COOL agarose to below 60C before pouring gel *****
otherwise you will warp the gel tray and they cost ~$60 each.

Run gel at betwee 70-100V (running slower will give you better bands.

NOTE: Gels can be stored in the cold room for a day or two, so if you pour a large gel, it can be used to analyze a number of samples.

**Quigen purification of gel bands.**

**Modifying pieces of DNA:**
**Klenow reaction:** used to fill in the overhang left by enzymes that leave a 5' overhang.
this leads to the destructive of the enzyme site.

Following restriction reaction add 5uL of 2.5 mM dNTPs & 5uL of Klenow enzyme.
Incubate at room temp for 20 minutes and then for 5 minutes at 37°C.
ligate and trans whatever.

**Ligation reaction:** take DNA (plasmid + insert or whatever) isolated from gel bands or miniprep resuspend in water (~34uL) add 4uL T4 ligase buffer and 2mL T4 DNA ligase -- incubate > 2h at 16°C and then transform into competant bacteria.

**Polymerase chain reaction:**

This is my standard set up for simple amplification of a DNA region

2µL of plasmid (miniprep) DNA
10µL of each of the two oligonucleotides*
10µL polymerase buffer
4µL $MgSO_4$ stock (100mM)
1µL Vent or 2mL Taq polymerase
15µL nucleotides
50µL water (nuclease-free)

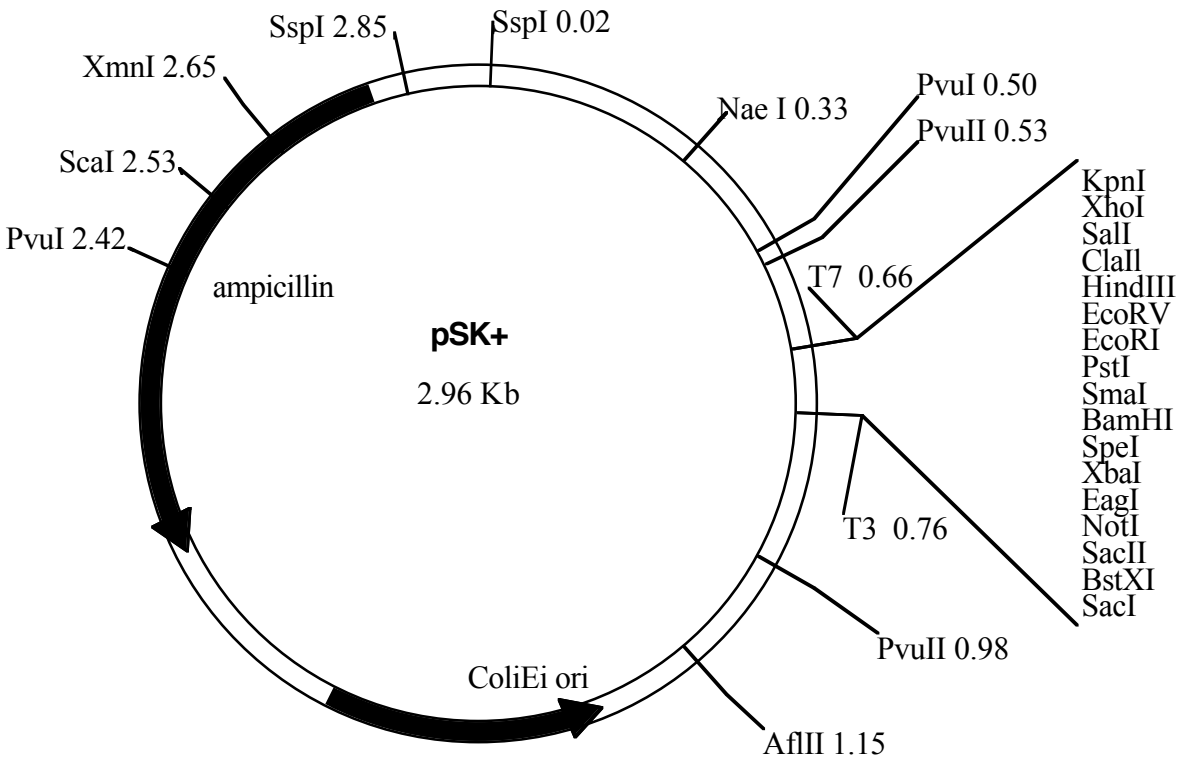
**RT PCR:**

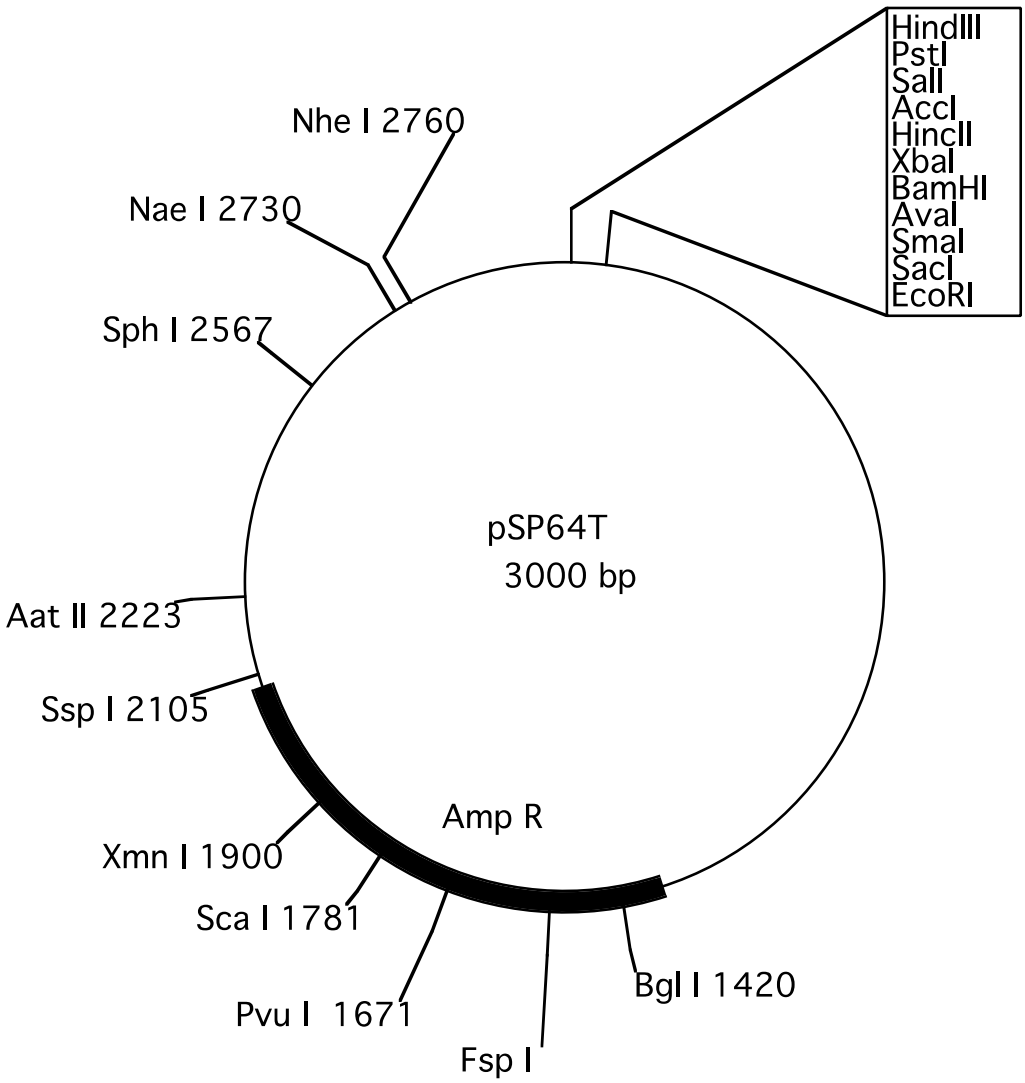
HERE -- Replace !

**Plasmids maps**

for more information, see sequence folder on the people's computer

maps are constructed using the McPlasmap program / Sequences are analyzed using DNA Strider.






**Preparation of Genomic DNA form Xenopus liver**

- Drop freshly excised tissue into liquid nitrogen into a waring blender.
  grind at top speed until tissue is a powder
- Allow liquid nitrogen to evaporate and add powdered tissue to 10 volumes of extraction buffer

**Extraction buffer**: 10mM Tris-Cl (pH8.0)
0.1M EDTA
20mg/ml pancreatic RNAase
0.5% SDS

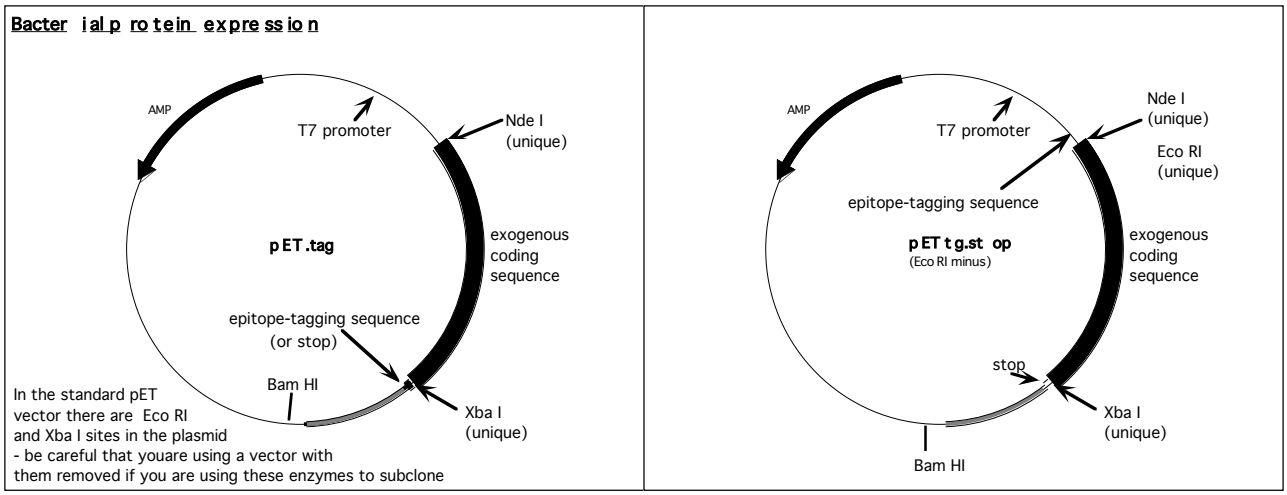
- Let digest for 1h at 37°C
- add proteinase K to a final concentration of 100mg/ml - mix using a glass rod.
  incubate at 50°C for 3h
- extract with equilibrated phenol - do not vortex, invert tube gently.
  separate phases by centrifugation at 5000g for 15 minutes.

- transfer aqueous (viscous) phase to a fresh tube with a large bore pipette and repeat the phenol extraction (2X).
- to isolate DNA in the 100 to 150kB size range:
  take aqueous phases and add 0.2V 10M ammonium acetate
  add 2V ethanol and mix the solution
- remove precipitated DNA with a sealed, U-shaped pasteur pipette.
- wash pellet 2X with 70% ethanol / allow ethanol to evaporate
- redissolve in TE pH 8.0 (1ml per 5 million cells) and allow to rock gently until DNA has completely dissolved. (12 to 24 hours)
- measure OD260 and OD2809 - ratio should be greater than 1.75
  1OD 260 unit is approximately 0.05mg DNA/ml - store at 4°C

## PURIFICATION OF PROTEINS EXPRESSED IN BACTERIA

### Growing & inducing plasmid expression:

Our bacterial expression system is based on the pET plasmid from Studier's lab. This plasmid contaion a T7 promoter. To make protein, the plasmid is transformed into a cell line (BL21DE3) in which the the gene encoding the T7 RNA polymerase has been inserted into the cellular genome. The T7pol gene is under LacZ control -- addition of IPTG induces T7pol synthesis and therefore synthesis of the exogenous protein. It should be noted, however, that a number of the plasmid we have made seem to express the exogenous protein consitutatively -- we are not sure why.




To grow up a large scale prep. for the purification of exogenous protein, begin with bacterial colonies -- start up 5ml LB/amp cultures (37°C with aeration). It can often be a good idea, before embarking on an extended purification, to test i) whether the protein is inducible and whether it is begin made. Do this by spinning down ~1ml of bacterial culture.

Remove the supernatant and resuspend the bacteria in 2% SDS - heat to 90°C and then dot 2mL onto nitrocellulose - dry and block -- incubate with antibody against exogenous protein -- 30 minutes is sufficient, wash and incubate with secondary antibody, usually anti-MIgPO (1:1000) for 30 minutes and then react with DAB (see Western blot protocols for more details).

**For large scale culture**, inoculate 0.1-1L LB media (+200mg/ml AMP) with 10ml of overnight culture -- grow to $OD_{550}$ of 0.6

**Induction of exogenous protein synthesis**

Make culture 0.4 mM IPTG - incubate at 37°C for 2h.

**IPTG:** 100 mM in $H_2O$ (sterilization not necessary)

**tagged IF tail purification**

Take cells containing either pET3tg grow up 1L LB + 100µg/ml Amp o/n 37°C
(these appear to be only modestly inducible)

Centrifuge 7000 rpm x 15min GSA

Resuspend pellet in 250 ml of 2 mM EDTA, 100 mM Tris-HCl, pH 8.0 (1/10 purification buffer A)
Centrifuge 7000 rpm x 15min GSA Freeze pellet -70°C

Thaw and then resuspend pellet in 20 ml of 7M Guanidine-HCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0 Freeze at -70˚C

Thaw and then add 80ml 50mM Tris HCl, 1 mM EDTA pH 8 (1/20 purification buffer A)
Centrifuge 7000 rpm x 30min GSA

Discard pellet Take supernatant and precipitate with 200ml of 95-100%EtOH
- Time of ppt at -70˚C depends on the volume -- large scale approximately 2h =
Thaw and centrifuge 7000 rpm x 30min GSA

Discard supernatant and resuspend pellet in 25ml of 0.2 mM EDTA, 10 mM Tris pH 8.0
(1/100 purification buffer A) Centrifuge 15,000 rpm x 30min SS34
The pellet still contains approx. 50% of the peptide but we normally discard it.

Take supernatant and run on monoQ column.

**MonoQ conditions:** Buffer A: 15mM Tris HCl, pH 8.8

run 0-15% buffer B gradient. count 45drops/fraction
tgVT elutes around fraction 10 tgDesT elutes around fraction 13.

(insert here specification of monoQ program).

**Assaying for tg tail polypeptides:** These small polypeptides do not bind well to nitrocellulose. The simple method to assay them is to dot them onto prewashed and dried nitrocellulose. Dry them under a heat lamp and fix for 10 minutes in 0.5% glutaraldehyde in PBS -- then block with low fat dried milk in TBS (10 minutes) -- incubate in 9E10 antibody (10mg/ml) for 1 hour, wash as for western blot, incubate in anti-MIgPO secondary antibody for 1 hour, wash and react with DAB.

**Concentration and rechromatography of tg tail fractions:** The peptide is too small for dialysis. To change buffers or concentrate samples use ethanol precipitation. If you are have a large volume, it is convenient to reduce the volume by n-butanol extraction. This is fast and and also removes other chemicals, eg. urea or guanidine. Any concentrated sample of the tail peptides can be precipitated with 2 or 3 volumes of ethanol, 30 min at -70°, and microfuged 10 min.

**Vimentin purification** (see Domingo et al 1992)

spin down bacteria - 7,000 rpm x 20 min and discard supernatant

resuspend pellet in 1/10 purification buffer A (100mM Tris-HCl, 2mM EDTA, pH 8.0)
(any volume that fits in a single centrifuge tube/bottle)

**Purification Buffer A**: 1 M Tris-HCl, 20 mM EDTA, pH 8.0

spin down cells - 7,000 rpm x 20 min -- discard supernatant
disperse pellet by hand shaking the tube/bottle
freeze at -70°C (this is a good point to store/accumulate material for later purification)

thaw - resuspend pellet in buffer G -- used 50ml/liter initial culture volume

**Purification Buffer G:**
50 mM Tris-HCl, 1mM EDTA, pH 8.0 (1/20 purification buffer A)
7M guanidine-HCl, 2mM DTT

- **do not** try to break cell walls in any way
- freeze at -70°C (another place where you can stop if you need to).

Thaw and place into SS-34 tubes
spin down - 15,000 rpm x 20 min -- take supernatant, discard pellet

the following is valid for vimentin -- other proteins may behave differently
a quick pilot experiment wiht 100µL of the supernatant and dot blot may be a good idea

add two volumes of ethanol and mix well -- freeze at -70°C or dry ice until viscous
spin down - 5,000 rpm x 20 min GSA - or 15,000 rpm x 20 min SS-34
take supernatant, discard pellet (mostly DNA)

add four more (original) volumes of ethanol -- freeze at -70°C or dry ice until viscous
spin down - 7,000 rpm x 20 min GSA - or 18,000 rpm x 20 min SS-34 discard supernatant

resuspend pellet in ethanol:water 6:1 (in order to wash out the rest of the guanidine)
spin down - 7,000 rpm x 20 min GSA - or 18,000 rpm x 20 min SS-34 - discard supernatant

resuspend pellet in the adequate buffer for the next purification step of your protein
usually a urea containing buffer

**Keratin purification**

Follow the general protocol for IPTG induction and isolation of inclusion bodies.

**@ All Buffers Must Be Filtered Through 0.2mm filters (solvent resistant)**

**Purification Buffer U:**
8M urea
50 mM Tris-HCl, 1mM EDTA, pH 8.0 (1/20 purification buffer A)
2mM DTT

**Buffer UG:**
8M urea, 1M guanidine-HCl
50 mM Tris-HCl, 1mM EDTA, pH 8.0 (1/20 purification buffer A)
2mM DTT

**20% Ethanol in MilliQ water**
**MilliQ water**

**Isolation of inclusion bodies:** see Domingo et al 1992.

solubilize crude protein from inclusion bodies in buffer U use 10ml
repeated freeze/thawing helps in the solubilization

spin down - depending on your preferences
eppendorf x 15 min
15,000 rpm x 20 min SS-34
35,000 rpm x 30 min 60Ti

take supernatant carefully, discard pellet

prepare FPLC system (see below)
wash pumps with your MilliQ (who knows what is in there before)
wash Mono-Q column (5ml) with 5ml at 0.1ml/min
wash pumps: A with buffer U, B with buffer UG
wash column with 5ml of 0% B at 0.1 ml/min

program the elution
sample volume + 5 ml, 0% B, 0.1 ml/min, 1 cm/ml recorder, 1 ml/fraction collector
main gradient 25 ml, 0% B to 30% B, 0.1 ml/min
safety-column maintenance gradient:
(3ml, 30% B to 100% B) + (1ml, 100% B) + (3ml, 100% B to 0% B)
inject sample loop, take the money and run

check all the fractions by dot blot

if greater purity is required, run the pooled fractions through MonoQ in the same conditions. In order to concentrate the sample and as an extra purification step, fractions containing type I and type II CKs can be mixed and polymerized by dialysis. Tetrameres can be purified by MonoQ in 4 to 6 M urea, keeping the rest of the buffer components and running conditions unaltered. The advantage is that the final fraction contains a 1:1 ratio of the two keratins and the ellution is different from both keratins, so the contaminants are efficiently removed by this second run. Exact procedures for this protocol were taken from Coulombe and Fuchs

The purification of CKs is usually not problematic because the induction in pET is very efficient, and the correct protein represents the majority of the Inclusion bodies fraction. Minor degradation (?) bands are however difficult to eliminate. The Guanidine extraction protocol was never tryed for CKs but it may help reducing degradation.

notes from the experience:
In some cases the flow rate may be raised to 0.5 ml/min, but produces over pressure easily. Flow rates as low as 0.01 ml/min some times also produce over pressure. All of this seems to be related to DNA in the sample and the fact that the column has been used for very concentrated-dirty samples and viscous buffers.

In case of serious problems with the flow, invert the column and wash it or try to continue with the run if the sample is already inside.

I also do an extra wash with 2 or 5 ml of buffer G (7M guanidine) injected using the sample loop.

**FPLC - Size exclusion chromatography**

**Washing superose :**

1. remove bottom piece and empty column by pumping through the top with MilliQ water.
2. wash the gel on a glass filter with 0.2M NaOH, then water and then 20% ethanol.
3. resuspend gel in MilliQ water (5 times gel volumn)
4. let it sediment and pour off supernatant
5. repeat once or twice more.

**Repacking a Superose column**

1. check that filters are undamaged and all parts are clean
2. assemble the column with a wetted bottom filter
3. drop the plunger into the column with the large diameter end first
4. shake the column up and domwn a few times so that the pl;unger pushes the bottom filter into place.
5. remove the plunger
6. attach the packing reservoir and vertically mount the column on a stand
7. close the outlet tubing
8. wet the tube with eluent and pour the gel suspension down the inside wall of the column -- pour all of the gel in one operation.
9. fill the reservoir to the top with eluent
10. assemble and fasten the top of the packing reservior
11. connect it to the valve pump
12. open the outlet
13. pack the column in two steps

    step 1 - 2ml/min  step 2 - 3ml/min

    allow gel to settle during step one and then pack for 1h at step 2 setting.
14. stop the pump and disconnect packing reservior
15. adjust the bed height
16. place a filter on the top of the gel bed
17. insert and adjust adaptor
18. pack for 5 minutes with a flow rate giving the following back pressure

**$^{35}$S-Met labeling of bacterial proteins**

Grow bacteria in LB-Amp ON -- Inoculate 1 to 200 ml of M9 minimum media (with glycerol) + 100µg/ml Amp with an small volume of the LB culture (5-50µl)

Add $^{35}$S-Met to the media (different amounts were used in each experiment) and grow until the density of cells is acceptable for purification. Then follow an scaled down protocol for purification as above, using eppendorf tubes if possible.

**RNA in vitro transcription** according to Kreig & Melton, 1987.

reagents: 10X transcription buffer:
400mM Tris-HCl pH 7.5; 60mM $MgCl_2$ and 20mM spermidine
100mM dithiotheritol
2mg/ml bovine serum albumin
ribonucleoside triphosphates (rATP, rCTP, rGTP and rUTP) each at 20mM human placental ribonuclease inhibitor (20U/mL)
SP6 RNA polymerase > 5U/mL
linear template DNA (0.5-1.0 mg/ml)
DEPC-treated water
RNAase-free DNAase I (1mg/ml)

**Protocol:**

1. take 5mL of linearized DNA solution (2.5 to 5mgs) -- add to tube
add 18.5mL DEPC water
5mL DTT
2.5 mL BSA
2.5mL each of 10mM rATP, rCTP and rUTP at 10mM
0.5mL 10mM rGTP
2.5mL cap
5mL transcription buffer
1.25mL RNA inhibitor
2mL of SP6 polymerase (15-25 units)

mix gently and incubate at 40°C for 1 hour. (to increase yield you make add second aliquot of SP6 polymerase at 40°C for 1 hour.

2. increase volume to 95mL with water
digest DNA by adding 5mL DNAase and incubate at 37°C for 10 minutes.
3. extract with an equal volume of phenol/chloroform/isoamyl alcohol
repeat with chloroform/isoamyl alcohol only
4. precipitate RNA with ammonium acetate (final concentration 0.7M) and 2.5V ethanol.
5. resuspend pellet in 0.7M ammonium acetate, reprecipitate with ethanol

expected yield - 8-10 mol RNA per mol DNA.

**RNA ISOLATION: OOCYTE RESIDUES**

Place 20 to 40 oocytes into a 2ml microfuge tube & remove excess liquid
can be stored frozen in lq. nitrogen until use.

Homogenize in 2ml SOL buffer + VRC (10mM).

SOL buffer 140mM KCl
2mM $MgCl_2$
5mM EGTA
10mM $NaPO_4$
5mM NaF
0.5% Triton pH7.0

Make up SOL + VRC (10mM) first and then add to oocytes.

Centrifuge for 15 minutes at 4\(deC. Remove supernatant & yolk/ Resuspend pellet in XEX + VRC using a pasteur pipette (baked).

XEX buffer: 1.5M KCl
0.3M sucrose
50mM NaF
10mM EGTA
10mM Tris pH 7.4 millipore and then add 0.5% NP40.

add 10mM VRC to XEX buffer prior to use.
be careful not to splash stuff out of the microfuge tube - it is easy to do!

centrifuge in microfuge for 15 minutes (in cold room) remove supernatant -- jump to @

**RNA FROM WHOLE OOCYTES OR EMBRYOS -- old method**

All solutions made from DEPC-treated water / RNAase-free reagents

1. Dejelly eggs and embryos

2. add 15l of proteinase K stock (final concentration 150mg/ml) and then 985ml PK buffer

homogenize with a pasteur pipete  Digest for 1h at 55°C

**for whole oocyte, 20 oocytes or embryos per ml max!**

3. extract 2X with phenol:chloroform:isoamyl alcohol (pH 6.5/7.5)

4. precipitate RNA by adding equal volume of 8M LiCl (on ice for 1 h)

5. recover by centrifugation at 10 minutes in microfuge

6. wash RNA pellet once with 100% ethanol (carefully - pellet is fluffy!) and then with 70% ethanol

7. speed-vac pellet for 20 minutes and dissolve in water (40 to 50l).

**Alternate large (or small) scale RNA prep** Hazel Sive

1. De-jelly embryos.
2. For ~4 mls settled embyros add: 16 mls proteinase K buffer
   1 ml 10 mg/ml proteinase K(final = 0.5 mg/ml)
   Incubate 37°C for 90 min.

3. Phenol/Chloroform extractions: -Divide to two 50 ml corning plastic tubes
   -Add 10 mls phenol, 10 mls chloroform & vortex
   - Spin 3K 5 mins
   - Remove aqueous layer to clean tube
   - re-extract w/ 5 mls TE & combine aqueous layers
   - Repeat extraction 2X (without back extractions) - should be no interface between layers
   - Extract with equal volume chloroform (15 ml/tube)
   - Add 35 ml ETOH.  Keep at -20°C overnight.

4. Spin 4K, 20 mins.  Resuspend pellet in 360 ul TE + 40 ul 10X NEB4 buffer
   -Add 1 ul DNAase I (Ambion, 2 u/ml)  Incubate 20 min. RT
   - Phenol extract, chloroform extract

5. Add 135l 10M LiCl (autoclaved)  --  Keep at 4°C several hours or O/N

6. Spin 4K 20 mins and Resuspend in 200 - 500l dH2O

7. ETOH ppt. with 1/10 volume 3M Na acetate/2.5 volumes ETOH.
   Resuspend in 500l dH2O.  Read $A_{260/280}$.

**Proteinase K Buffer**: 20 mM Tris pH 7.6
100 mM NaCl
30 mM EDTA
1% SDS

**Measuring RNA concentration**

spectrophotometer for 320nm to 220nm scan.
dilute RNA 1:100 - 1 OD 260nm = 37mg/ml

**Formaldehyde/agarose gel for RNA**

for minigel:

0.83g agarose
61ml DEPC water / 60°C.
add 7ml 10X MOPS running buffer
add 2.1ml formaldehyde (pH > 4) pour into gel mold / allow 1 hour to harden.

**premix**: 50mL 10X MOPS running buffer
87.5 mL 37% formaldehyde
250 mL formamide
20 mL dye mix

make 250 1X MOPS (running buffer)

sample - equal volumes sample and premix / denature at 60°C for 10 minutes.
load and run at 65V (nucleic acid migrates toward "+" end)! until rapidly migrating dye is half way across gel

**Staining:** wash gel three times (5min each) in water
stain with 5mg/ml ethidium bromide in 1X MOPS for 5 minutes
destain in water for 15 min to 2 h.
photograph on short wave uv box f4.5 1/2 sec with red filter.

**RNA SOLUTIONS***(*

**BSA:** We usually use commercially available acetylated BSA e.g. from *Promega Biotech* or *NEB*. If it is too concentrated, just make a 1mg/ml stock of your own in DEPC-treated water.

**Cap Analog:** 7m(5')Gppp(5')G from NEB. Resuspend according to directions on package. A 10mM solution is convenient.

**$CHCl_3$:isoamyl alcohol (24:1)** Use the pure stuff right out of the bottle - don't inhale. Chloroform is RNAse-free.

**DEPC-treated $H_2O$:** Use distilled water or milleQ water. Add 0.2ml DEPC for every 100ml of water or other aqueous solution, stir overnight and autoclave. *Note:* Solutions containing Tris CANNOT be treated with DEPC. It is useful to store DEPC-treated solutions or water in aliquots (e.g. 1ml) to reduce the risk of contaminating a large volume.

**DNAse I:** from Ambion, typically 2units/ml and stored in the "Other Enzymes" box in freezer 4.

**DTT:** Dithiothreitol is essential for RNAse Inhibitor function and polymerase function as well. Make a 1M stock in DEPC-treated water; aliquot and store frozen (-20℃).

**EtOH:** Use absolute ethanol at -20℃ that you treat as RNAse-free only (i.e. only put RNAse-free tips into the bottle). 70% EtOH is made by mixing absolute ethanol and DEPC-treated water in an RNAse-free container; store at -20℃.

**Phenol:** Should be equilibrated with Tris-HCl at pH ~7.5-8.0 (see separate protocol, e.g. in the Red Book) and stored at 4℃. Add 8-hydroxyquinoline.

**Plasmid DNA:** Can be stored at -20℃ in an aqueous solution such as TE, or in RNAse-free water.

**5X rNTPs:** or ribonucleoside triphosphate, 5X concentrated. We buy individual rNTP solutions from Pharmacia (pH 7.5). A 5X stock solution consists of all four nucleotides in DEPC-treated water: the concentrations of ATP, UTP, CTP and GTP in the stock solution are 2.5mM

**RNAse Inhibitor:** from Ambion, typically 40units/ml, and stored in the "Other Enzymes" box in freezer 4.

**3M Sodium Acetate:** 3M NaOAc. Prepare by dissolving enough sodium acetate crystals in distilled water to make a 3M solution, and pH with glacial acetic acid until the pH is 5.2. Treat with DEPC and store in aliquots (at room temp. is fine).

**2.5M Ammonium Acetate:** 2.5M $NH_4OAc$. Prepare by dissolving enough RNAase-free ammonium acetate in DEPC-treated water to make a 2.5M solution; pH with glacial acetic acid to 5.2.

**SP6 polymerase:** from Ambion. Typically 20 units/ml and stored in the "Other Enzymes" box in freezer 4.

**Sephadex G-50:** Swell Sephadex in RNAse-free buffer in an RNAse-free bottle overnight at room temperature or at 65℃ for a few hours. Recommended buffers are TE (10mM tris, lmM EDTA) or STE (100mM NaCl, 10mM tris, 1mM EDTA).

**5X Transcription Buffer:** commercially available stuff or can make it yourself with RNAse free stock solutions and RNAse-free water:

*A note about RNAse-free water:* If RNA is destined for injection into oocytes, it can be dissolved in DEPC-treated water. If RNA is going to be injected into oocytes, it should be dissolve in water which is taken straight from the milleQ filter and put into an RNAse-free bottle and autoclaved.

*Making RNAse-free solutions:* when weighing powders for solutions, wear gloves and shake out of container or use a baked spatula. Use RNAse-free pipette tips for mixing solutions from concentrated stock solutions.

*RNAse-free containers, etc.:* Bottles with caps can be baked at 110℃ overnight. Eppies right out of the box are RNAse-free if you keep gloves on when you take them out. I autoclave them in baked (at high temp.) beakers. Spatulas and stir bars can be baked at high temp. RNAse-free pipette tips are ordered pre-packaged and autoclaved 20 min.

## Screening a lZAP phage library
## Titering phage stocks

make a serial dilution series in SM buffer

**SM buffer**: 5.8g NaCl
2g $MgSO_4$
50ml M Tris HCl pH 7.5
5ml 2% gelatin per litre -- autoclave.

add 200mL of an$OD_{600}$ = 0.5ml host cell (XL-1 blue) culture per tube. inoculate with diluted phage.
add 3ml 48°C top agar and plate on NZY plates -- incubate overnight at 37°C.

**NZY broth:** 5g NaCl
2g $MgSO_4$ . $7H_20$
5g yeast extract
10g NZ amine (casein hydrolysate) per litre

to make plates add 15g agar / litre of broth
to make **top agar** add 0.7% agarose - both are autoclaved.

count the number of plaques and determine the pfu/ml concentration.

### Screeing the library for protein-reactions (antibodies or peptides).

plate on 150mm NZY plates (two day old plates) to 50,000 pfu/plate
use 600mL of $OD_{600}$ host cells per plate and 6.5ml top agar/plate
(typical screening - one million plaques).

incubate overnight and then refrigerate the plates for 2 hours at 4°C.
transfer to nitrocellulose sheets.

### Plaque screening

1. Pour NYZ agar plates and let sit > 24 h prior to use.
2. melt top agar and place in 50°C water bath
3. combine bacteria with phage -- for a 150mm plate take 5 x $10^4$ pfu to 600 l E.coli (XL1 blue -- grown to OD600 = 0.5) plus 7.5ml top agar
4. Add top agar to prewarmed plates -- distribute mixture evenly across the plate surface and allow to solidify at room temperature
5. incubate at 42°C until small plaques just become visible (approximately 3.5h)

6. treat nitrocellulose filtures with IPTG

make sterile 10mM IPTG solution and wet the nitrocellulose filters in that solution. After they are **completely** wet, allow them to dry and then number the filters with pencil.

7. number the plague plates and apply the IPTG-treated nitrocellulose filters.
8. allow the nitrocellulose filter to wet and then incubate at 37°C for 3.5h
9. using a needle dipped in waterproof ink, mark the orientation of the nitrocellulose filter in relation to the plate by several marks.
10. **carefully** remove the filter with forcepts and remove any adhereing top agar using a gloved hand or a smooth glass rod. Block filter with 2% lowfat dried milk in TBS + 0.02% azide for at least 1h prior to protein or antibody-binding. Filters can be stored in blocking solution at 4°C in a sealed plastic bag..

p it is generally advisable to make a duplicate transfer by applying a second IPTG-impregnated nitrocellulose filter and allowing the plate to go overnight at 37°C.

**Rescreening "positive plaques"**

Align filter and remove positive plaque using a sterile pasteur pipette. Place into 1ml SM buffer.

A typical plaque should have something like $10^6$ phage in it. From the SM solution, dilute 1:100 and 1:10,000. Take 100mL of diluted phage and combine with 200mL of XL1 blue cells (grown to OD600 = 0.5) plus 3ml top agar and plate as before -- let agar set at r.t. and then incubate at 42°C for 3.5h (small plaques should be visible). Then overlay with IPTG-soaked nitrocellulose filter and incubate for 3.5h at 37°C.

Block filters and rescreen.

**Binding reactions:**

antibodies

wash filters and react with primary antibody for 2 h at r.t.
wash filters 3 times in TBS
incubate filters in secondary antibody for 2h at r.t.
wash and react as in western blot protocol.

proteins

wash filters and incubate with purified bacterially synthesized protein you will need to determine the exact binding conditions. begin with

low physiological buffer (LPB)

50mM KCl, 2mM $MgCl_2$, 1mM DTT, 2mM EDTA, 10mM Tris-HCl (pH to 7.4 with NaOH). you may also want to try a more physiological buffer by raising KCl to 150mM

wash filter 3 times (quickly) in binding buffer -- at this point you may or may not want to gently fix the filters with 0.2% glutaraldehyde -- if you do fix, be sure to reblock the filter.

incubate in 9E10 and antiMIgAP antibodies.

HFGHH**OOCYTES**HGHHJ

## I. Oocyte Isolation

i. Anethesize female using benzocaine
- put ~5ml 6% benzocaine (stock solution in 100% ethanol) into 1L water.
- leave animal until it is limp and completely unresponsive.
- place animal onto paper towel

If you are going to keep the female alive, make small incision on flank
- cut first through skin / next through muscle layers
- ovary should be near the surface and can be teased out with a wooden stick.
- suture incisions in muscle wall first and then skin. Make sure that you **do not sew the skin to the muscle**, as this will tear during normal frog life. Depending upon the size of the initial incision, use between 2 to 3 sutures

place animal in dry container and allow it to stick to bottom
place container at an angle and carefully add 100mM NaCl solution so that animal is partially covered, but does not come free of bottom - this ensures that the animal does not dehydrate and at the same time does not drown.

There is a 4M NaCl stock solution near the frog recovery area - use 12.5ml per 500ml water to make recovery solution.

## II. Long term culture of oocytes

i. wash ovarian tissue with $Ca^{2+}$, $Mg^{2+}$ free Ringers

ii. dissociate in 0.1% collagenase in 5mg/ml ovalbumin in Ca/Mg-free Ringers dissociate overnight at 16°C. Use rocker. Typically for a dissection, I would use 5ml of enzyme solution. To dissociate a complete ovary, use 20ml.

iii. recover oocytes and wash twice in oocyte media:

either L15 based: L15 50% mammalian strength
16.7 mM HEPES, pH 7.9

MRS or OR2 (each can be supplemented with 5% dialyzed calf serum for long t e r m culture) Each media should supplemented with antibiotics - either gentamycin (50mg/ml) or penicillin/streptomycin (from 100X stock).

**Modified Ringers Solution (MRS)**

110mM NaCl
2mM KCl
1mM $MgCl_2$
2mM $CaCl_2$
2mM $NaHCO_3$
5mM HEPES pH 7.8

**OR2 media:** 82.5mM NaCl
2.5mM KCl
1mM CaCl2
1mM MgCl2
1mM $Na_2HPO_4$
5mM HEPES pH 7.8

## III. Oocyte maturation:

Make oocyte media 5mg/ml progesterone (progesterone made as 5mg/ml stock in 100% ethanol). Animal pole pigment clearing should be apparent by 3 to 4 hours, but may take as long as 6 to 8 hours to become evidence. Even in cases where animal pole spot does not appear, the oocytes can have entered M-phase, as judged by disappearance of nucleus, breakdown of keratin filaments.

## IV. Oocyte enucleation

i. transfer oocytes into MRS
ii. use a pair of blunt forceps, gently hold oocyte and puncture with a unblunted 26/27 gauge needle.
iii. gently squeeze oocyte until germinal vesicle appears. you can use the needle t o tease GV out. use only enucleated egg in which GV is removed untorn.

**note:** usually the GV remains fairly round throughout the enucleation process, but sometimes it is almost stringy. I would not use such oocytes.

iv. place enucleated oocytes into MRS - allow to heal for 30 to 60 minutes. even some very ugly looking oocytes will heal completely under these conditions!

## VI Oocyte injection (see below - Embryo section)

## VI. Preparation of insoluble and soluble fractions from oocytes & embryos

Eggs and embryos must be dejellied with 2% cysteine pH 8.0 / wash 3x with Ringers.

Place oocytes, eggs or embryos into microfuge tube & remove excess liquid. typical experiment uses 5 to 20 oocyte/embryos per gel lane.

wash once in MSB buffer

**Medium salt extraction buffer**

150mM NaCl
50mM NaF
10mM EDTA
10mM Tris-base pH 7.4

**XEX buffer:**

1.5M KCl
0.3M sucrose
50mM NaF
10mM EDTA.
10mM Tris pH 7.4
0.5% NP40

homogenize first is MSB using 1.5ml in a 1.7ml microfuge tube.

carefully pipette up and down with pasteur pipette.
be careful not to splash stuff out of the microfuge tube-it is easy to do!. -

centrifuge in microfuge for 15 minutes (at 4°C).

aspirate supernatant from the top of the tube

take care to remove yolk from the top of the "pellet" microfuge tube

re suspend pellet in 1.5ml XEX

centrifuge resuspended pellet for 15 minutes at 4°C.

solubilize recovered pellet in either SDS-page or IEF sample buffer.

**Other related topics - immunoprecipitation / western blotting/RNA isolation**

## I. Preparation for in vitro fertilization:

**Hormone injection schedule** -- adapted from the Gerhart lab;
updated May 1992 by L. Maynell.

You will need:-gonadotropin from pregnant mare serum (PMS) (Calbiochem)
-human chorionic gonadotropin (HCG) (Sigma).
-26 1/2 gauge needles and 1cc disposable syringes
- 2 to 3 adult female Xenopus.

Females are injected one day prior to fertilization with 50 I.U. PMS in the morning, and with 250 - 500 I.U. HCG[8] ~ 8 - 12 hours later.

After the second injection, females should be left in the 16°C incubator overnight. They will start dropping eggs sometime during the night.

For oocytes (difficult to harvest in winter), PMS can be injected into female 8-24 hours prior to HCG injection.

For recalcitrant females, give PMS injections ten days, then again two days prior to the HCG injection.

8 **The amount of hormone varies with the season.** 8
**In the summer try 200-250 I.U.**
**and in the winter ~400-500 I.U.**

**Males:** Males are injected two days prior to use with 50 I.U. PMS.

Dissect testes and hold at 4°C in a small sterile petri dish in sterile testis buffer (10% fetal calf serum in 1X Ringer's + 50mg/ml gentamycin).

Sperm should stay alive 10-14 days.

PMS and HCG are dissolved in sterile milliQ $H_2O$.

The resulting concentration in the vials should be PMS: 2500 I.U. per ml,
HCG: 1000 I.U. per ml.

PMS can be further diluted one part in ten with $Ca^{2+}$, $Mg^{2+}$ free Ringers, resulting in a concentration of 250 I.U. per ml, or 50 I.U. per 0.2 ml. Make 1ml aliquots in sterile eppendorf tubes and store frozen

HCG is ready to use out of the vial; 500 I.U. = 0.5 ml.

**II. In vitro fertilization** adapted from the J.C. Gerhart laboratory

**Dissection of Testes**

Anesthetize male (while in a small volume of water) with ~ 3 mls of 6% benzocaine in absolute EtOH. Add more if needed.

After approx. 10 minutes the legs will relax. Make sure the frog is completely sedated (i.e. dead).

Dissect both testes from the ventral side of the frog and place into a sterile petri dish in sterile Testis Buffer (see above). Testis can be stored in buffer at 4°C for up to fourteen days.

**Eggs**

Strip eggs from the female into a clean petri dish.

Fertilize with a small piece of testis tissue by shredding the tissue with straight forceps and rubbing it over the eggs, or add 33% Ringers to the shredded tissue and wash the eggs in the resulting solution.

Fertilization should be apparent after a few minutes as eggs rotate animal pole up.

At 5-7 minutes after fertilization, dejelly eggs in two changes of 2% cysteine pH 8.0.

It is very important to watch the eggs closely during removal of the jelly coat, as leaving them in cysteine too long increases chances of them deteriorating. Leave in cysteine no more than five minutes (you can change the cysteine solution once or twice during this time).

Rinse three times in 33% Ringers and then allow them to develop in 20% Ringers containing gentamycin or penicillin/streptomycin.

## III. Egg Activation and Inversion Experiments

For prick activation experiments, dejellied eggs were positioned in small wells made in agarose plates (2% type V high gelling temp., in 1x Ringers). They were pricked quickly using a micromanipulator with a clean glass needle while held in a solution of 5% Ficoll in 20% Ringers.

Inversion experiments were done in the same Ficoll solution. Dejellied fertilized eggs were inverted gently with forceps 180° and held in wells on agarose plates. Inversions were complete by 30' post-fertilization, and embryos were left to develop upside down throughout the experiment.

The calcium ionophore A23187 was used for ionophore activation. Dejellied eggs were placed in a solution of 10mg/ml A23187 supplemented with fetal calf serum. Activation was confirmed by observing the slow contraction of the animal pigment cap at approx. 10'-15' post-fertilization and the upward rotation of the animal hemisphere. Eggs were transferred to 20% Ringers to develop for the remainder of the experiment.

## IV. Injection of Oocytes and Embryos

**Needles:** Needles are pulled from 1.2mm internal diameter, internal capillary glass tubes (WPI). You will need to cut the 6in glass tubes into two 3in halves. Each 3 in tube will give you 2 needles. The setting on the Narashige needle puller will change depending upon who smashed the electrode last, etc. You will need to be shown the appropriate of shape of an injection needle. injection needles are different cytoplasmic transplantation needles.

Needles are pulled immediately prior to use, they are not to be stored. Needles are loaded from the back end using a 10mL Hamilton syringe. The internal thread will pull the liquid into the tip by capillary action. For protein (antibodies etc.) the syringe should be washed with distilled water between samples. For RNA, I typically wash the syringe first with DEPC-water, then with acetone, then with chloroform, then with acetone, and finally with DEPC-water again. Between RNA samples, I wash the syringe with DEPC-water only. To avoid cross-contamination, set the syringe at 1l and then pull up your sample. Load a needle with 1.0-2.0 l of the solution to be injected. If you empty the needle, it can be reloaded with more of the same stuff.

Place the needle into the injection system. You will need to break the needle with a pair of sharp forceps, do this under 12X or 25X mag. Test whether this has opened the tip by applying a 0.1sec air pulse. You can then determine the injection volume by injecting into paraffin oil.

Use the ocular lens scale to measure the volume of the liquid sphere. An oocyte can accommodate up to 40-50nl of injected stuff. Fertilized eggs can handle up to 30 nl, blastomeres can handle less.

To adjust injection volume you can:

- the needle opening
- the timer setting (times from 0.1 to 1.0 sec work well)
- the bleed valve underneath the table - this determines how much air escapes out into the room. Closing it will increase airflow to the needle
- the pressure regulator (normally set at 20psi, you can decrease the pressure if necessary).

**Timing for fertilized egg injection:**

The endoplasmic rotation begins at approximately 0.4 of the cell cycle and is over a 0.7 of the cell cycle. -- normally all injections should be complete before the beginning of the rotation.

At 20°C the first cell cycle is 90 min. long. 0.4 is 36 min. after fertilization.

At 24°C the first cell cycle is 75 min. long. 0.4 is 30 min.

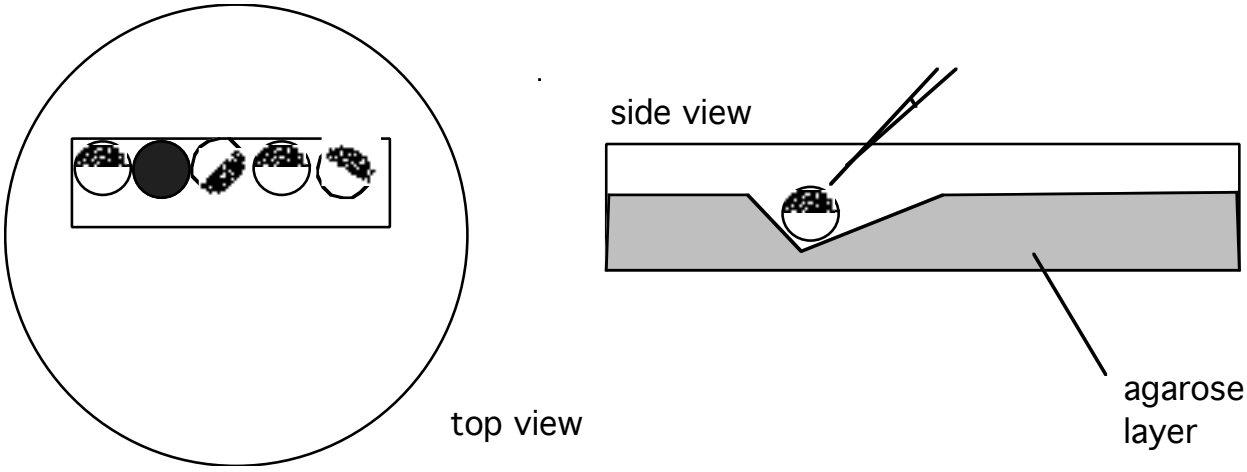
Normally, fertilized eggs are maintained in the injection room with the air conditioner on - this lowers the temperature of the room to 18-20°C.

It is important to check often to make sure the injection needle is still flowing. This can be seen by the flow into the oocyte/embryo. If you are unsure, the needle can be moved out of the liquid and looking for the flow of liquid out of the needle tip.

**Injection Plates:** Both oocytes and fertilized eggs are injected in "wedge plates" made from 2% agarose (Sigma, Type V). Injection plates can be washed after use and reused many times. With the "spear chucker" system (designed by Alberto Domingo), it is easy to inject many oocytes/eggs.

Oocytes and eggs are injected in 5% ficoll in 100% Ringers. After injection, oocytes are transferred to normal, ficoll-less media. Embryos are transferred to 5% ficoll in 20% Ringers. Embryos should be transferred to 20% Ringers between stages 5-6.

Ficoll (400,000 molecular weight, dialyzed and lyophilized - from Sigma) is relatively expensive. For injection you will need no more than 5ml. Do not make more than you need, please.




**INJECTION PLATE SCHEMATIC**

**Miscellaneous:** Remember to check the tubing pieces inside the manipulator and replace them if they become clogged with glass chips. Avoid scraping the inside of the glass needle with the Hamilton syringe when loading it. Tiny pieces of glass could clog the needle.

**Embryonic development:** A complete description of Xenopus development is found in Neiuwkoop and Faber. A copy of this book can be found on the lab reference shelf. If you

need to copy a specific section, do it -- but do not copy the entire book, it is expensive. The lab copy is not to be removed from the lab.

**I. Isoelectric gels (First dimension of a 2D gel).**

These can be run as slabs for simple isoelectric point separations if desired.

**Pouring IEF gels:**

1. Mark clean gel tubes to indicate desired height of the gel.
   Gel tubes are cleaned in chromerge, washed with tap water, distilled water and then bakes overnight.
2. Place a piece of parafin folded over twice on the bottom of the gel tube pouring apparatus.
3. prepare the gel solution

   4.12 g urea (ultrapure)
   3ml water
   1ml acrylamide stock (30% acrylamide :0.8% bis-acrylamide)
   200l 3-10 ampholines
   200l 4.5-6 ampholines
   dissolve urea and then degas under a vacuum for 2 to 3 minutes.

4. add 150l NP-40 (pipeting both ampholines and NP40 is easier if you cut off the pipet tip with a pair of scissors.
5. Filter solution through a syringe (0.22m) filter. *rinse the filter with water, it can reused.*
6. add 25l 10% ammonium persulfate and the 2.5l TEMED.
   (This starts polymerization, so once added you have to keep moving).

7. Pull up solution using a syringe fitted with thin tubing. Put the tubing into the gel tube and expel solution up the mark you may in step 1.
   **Rinse syringe with water when you are done.**

8. Leave gels to polymerize for 2 hours - check for leaks at the bottom. these can often be fixed by pressing down on the gel tubes.

**Sample preparation:**

IEF samples are dissolved in urea/NP40 buffer
This buffer is made up and stored at -20°C as a common stock. Take an aliquot and use it, discard what you do not use. DO NOT REFREEZE.

**IEF sample buffer:**

27g urea
2ml NP40
5ml of stock 9-11 ampholine

1ml b-mercaptoethanol
water to 50ml - aliquot and store at -20°C

Typically, I solubilize 1 oocytes (stage VI) or embryos in 10 l of IEF sample buffer - if the IEF sample buffer is warm (60°C) the sample will dissolve more readily. After vortexing, the sample can be incubated at 60°C for 5 minutes. **Samples must be microfuged for 15 min at room temperature prior to loading**. When you begin this centrifugation, you should also start degassing the running buffers. Take 2L of distilled water and degas it.

Place gel tubes in the gel tube holder.

Place 1.5L of water in bottom tank, add 1.5ml phosphoric acid (from stock).
Place 0.5L of water in top tank

Load gel tubes with 50 l of the sample; use Hamilton syringe and be sure to knock the air bubbles out as you load the samples. You can use the top tank solution to wash out the syringe between samples.

Add 1ml 10M NaOH to the top tank. Add 1ml of neutral red solution to top tank.

Connect to power supply, red to red and black to black. Run at 600V constant voltage. Current should begin to drop within 5 to 10 minutes of running (if not sooner). Run gel overnight.

In the morning, increase voltage to 800V for 1 hour.

You can either run the second dimensions immediately or freeze them at -70°C for future analysis. If you want to run the second dimensions immediately, start to pour the second dimension SDS-PAGE gels right after you increase the voltage on your gels from 600 to 800V. (see below of SDS-PAGE gel methods). For second dimension analysis, you will pour wide, thick gels. If you know where the protein you are looking for migrates, you can run up to four IEF gels on a single second dimension gel.

IEF gels are removed from the gel tubes by heating the gel tubes under running hot water for 30 second and then applying air pressure to the basic (upper) end. Generally the gel will start moving out. It is easiest to collect the gel in a scintillation vial. The same scintillation vial can be used for gels loaded with the same sample (I typically run two gels of each sample as insurance against accident).

If the gel does not come out easily, you may want to use a Hamilton syringe to loosen it. Take a 100l syringe and fill it with water. place the tip in the gel tube and gently run it around the periphery of the gel, expelling water as you go. Do the same thing to each end of the gel. This usually does the trick.

Once the gel is expelled, freeze it (whether or not you are planning to run the second dimension immediately). Complete the extrusion of all gels.

**Running the second dimensions:**

Recover your tube gels from the freezer and add 5ml of gel equilibration buffer and 0.2ml b-mercaptoethanol to each vial.

**equilibration buffer:** 200ml water
7.5g Tris-base
50ml glycerol
10.5g SDS

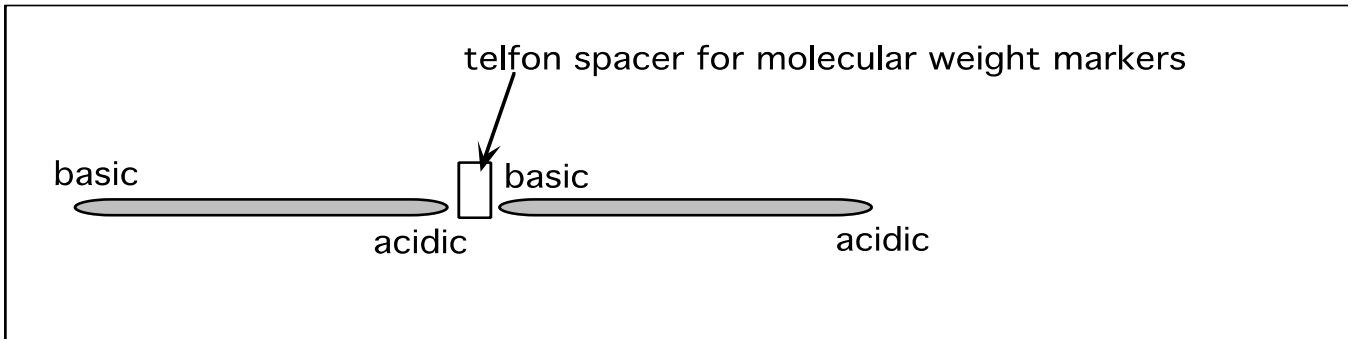
By this time you have already poured the SDS-gels, both the separating and stacking gel layers. Overlay the stacking gel layer with a enough agarose solution to make it smooth.

**agarose solution:** 0.25g agarose (Sigma type V)
50ml SDS-gel running buffer (with SDS)

agarose solution is melted by placing it in a beaker with water and microwaving it for 2 to 3 minutes.

Place telfon spacers for molecule weight markers. Cut tube gels such that unnecessary regions are removed. For wild type keratins, you can remove 2cm from each end of the gel.

Position gels on top of slap gel, cover with agarose. Typically, I orient gels in a uniform manner.




Run gel until bromphenol blue of standard is within a cm of the bottom. Depending upon the size of the protein you are looking for you cut away low or high molecular weight regions of the gel. This can be used to conserve nitro-cellulose and to gel four gels on a single blot.

## II. SDS-polyacrylamide gel electrophoresis (2nd dimension of a 2D gel)

**Stock solutions:**

1.5M Tris pH8.8
0.5M Tris pH6.8
20% SDS
30% acrylamide:0.8% bisacrylamide--Use milliQ water & filter (0.22mm)
water-saturated butanol
10% ammonium peroxysulphate (APS) / store at r.t. in capped tube

**10X Running buffer:** 1.92M glycine / 0.25M Tris-base

**Sample buffer:** 125mM Tris (pH6.8)
5% SDS
10% glycerol
5% bME
10mM EGTA
0.01% bromphenol blue / 0.01% phenol red

**Separating gel** (50ml is sufficient for a small gel or a large gel using thin spacers)
13.5ml for a 8% gel
**15ml acrylamide for 9% gel** (standard)
16.5ml for 10%
27ml for a 16% gel (used for analysis of tail polypeptides)
12.5 ml 1.5M Tris (pH8.8)
1ml 20% SDS
0.5ml APS water to 50ml.

Take 10ml of solution - add 10mL TEMED and use to seal sides and bottom of gel.
allow 5 minutes to polymerize.
add 40mL TEMED to remaining solution - pour gel to desired height.
overlay with water-saturated butanol - allow 5 minutes to polymerize.

**Stacking gel** (10ml -- use 20ml for large gel):
1.5ml acrylamide
2.5ml 0.5M Tris (pH6.8)
100mL APS
200mL 20% SDS water to 10ml

Remove butanol from top of gel / add 10mL TEMED to stacking gel solution.
Pour and allow polymerization to continue for at least 20 minutes.

**Gel running:** make running buffer 0.2% SDS

run standard gel with 0.75mm spacers at 40-50mAmps constant current with cooling fan. run double sized (or thick single sized) gel at 80 to 100 mAmps constant current

running time approximately 3-4 hrs.

**Molecular Weight Markers:** Typically we use SIGMA SDS-7B prestained standards.

211 Kda - myosin 64.4kDa- fumarase
119 kDa - b-galactosidase 44.6kDa - lactic dehydrogenase
98 kDa - fructose-6-phosphate 38.9kDa - triosephosphate isomerase
80.6kDa - pyruvate kinase

also used in Tris-tricine gels: glucagon - 3kDa and lyzozyme - 14kDa.

**Coomassie gel staining** (overnight with rocking)(you can speed it up by microwaving the gel/stain 3 to 5 minutes).

stain: 1.25g coomassie brilliant blue
225ml 100% methanol
50ml glacial acetic acid water to 500ml / filter through whatman #1 prior to use!

**Destain** in 5% acetic acid : 10% 1-butanol / **cover with plastic wrap**.

**Silver staining** (from Harlow and Lane) *take care - handle gel with gloves and touch it as little as possible. -- be sure that the tray you use is clean -- acid wash if necessary*

1. fix 3h or overnight in 250ml 30% ethanol, 10% acetic acid on the shaker

2. wash in twice in 250ml 30% ethanol for 30 minutes each time.
wash three times in 250ml of distilled water (10 minutes each)

3. add 200ml 0.1% $AgNO_3$ and incubate for 30 minutes

4. wash for 20 second in distilled water

5. add 250ml 2.5% sodium carbonate 0.02% formaldehyde (filtered first).

6. stop development with 1% acetic acid and then rinse gel in distilled water and dry.
reaction can be intensified by repeating steps 3-7
surface film can be removed using a photographic reducer.

**Silver staining II -** (Lee's German Friends)(may be better for tgTail polypeptides)

1. fix overnight in 50% methanol:12%TCA:2% $CuCl_2$:1%$ZnCl_2$ 0.1% formaldehyde
2. wash 15 minutes in 10% ethanol:5% acetic acid
3. incubate 10 minutes in 0.01% potassium permangante: 0.01% KOH
4. wash 10 minutes in 10% ethanol:5% acetic acid.
5. wash 2 X in distilled water

6. incubate 10 minutes in 0.1% silver nitrate
7. wash for 20 seconds in distilled water and then 1 minute in 10% K carbonate
8. develope in 2% K carbonate with 0.01% formaldehyde
9. stop with 10% ethanol:5% acetic acid

**Tricine-SDS-PAGE (for low Mw peptides).** Anal. Biochem. 166:368-379

Stock solutions

| Buffer | Tris (M) | Tricine (M) | pH | SDS (%) |
|---|---|---|---|---|
| Anode buffer | 0.2 | — | 8.9 (HCl) | — |
| Cathode buffer | 0.1 | 0.1 | 8.25 | 0.1 |
| Gel buffer | 3.0 | — | 8.45 (HCl) | 0.3 |

| | Acrylamide | Bisacrylamide | T = (A+B)g in 100ml sol<br>C = Bg in 100g (A+B) |
|---|---|---|---|
| Acrylamide / Bis | 30% | 0.93%<br>add 0.13g Bis to 100 ml of 30:0.8 | 31% T<br>3% C |

Separating spacer and stacking gels

| | Separating<br>16.5% T | Spacer<br>10% T | Stacking<br>4% T |
|---|---|---|---|
| Acryl/Bis 31% T, 3% C | 8ml | 4.8ml | 1.3ml |
| Gel buffer | 5ml | 5ml | 2.5ml |
| Glycerol | to 15ml (2g) | — | — |
| $H_2O$ | — | to 15ml | to 10ml |
| APS 10% | 75µl | 75µl | 100µl |
| TEMED | 7.5µl | 7.5µl | 10µl |

Gel is run overnight at 20mA constant current.

Fix gel for 30 minutes in 50% methanol and 10% acetic acid -- stain gel in 0.025% Coomassie R250 in 10% acetic acid.

**Western blot protocol: Peroxidase / Alk. Phosphotase / ECL**

After electrophoresis, cut away teeth of stacking gel and cut gel for staining and blotting.

Place gel in 20% methanol and wet blotting paper and nitrocellulose paper.

*if you are using immobilin P -- you have to wet it with 100% methanol - be carefulmethanol will melt nitrocellulose).*

Set up blot cassette - blot at full power for 2h with cooler on!

**block blot**: 2% low fat dried milk in TBS for 15 minutes.

**TBS:** 200mM NaCl / 50mM Tris-HCl pH7.4

incubate with first antibody, o/n at r.t. in 20% calf serum

wash 1X in TBS, 1X in high salt TBS, 2X with TBS + 0.5% NP40, 1X with TBS (5 min. each)

incubate 2 to 4h in 1:1000 to 1:2000 in secondary antibody diluted in serum

wash as above

**For HRP conjugates:**

React with 0.5mg/ml DAB in TBS 0.02% $H_2O_2$

react 5 to 10 minutes / wash with distilled water - dry in the dark drying causes some fading

CAUTION- DAB is carcinogenic -- it can be destroyed with bleach!

**For Alkaline phosphotase conjugates**

final wash - 50mM glycine pH9.6 / 4mM $MgCl_2$

reaction buffer includes

100mg/ml p-nitroblue tetrazolium chloride (NBT)
50mg/ml 5-bromo-4-chloro-3-indoylphosphate, p-toluidine salt (BCIP)

reaction can go overnight (in the dark) for extra sensitivity, usually complete in 10 to 20 minutes.

**For ECL visualization:**

Take care to titre antibodies: antibodies and conjugates that work fine for other methods can generate very high background with ECL.
standard dilutions: secondary HRP conjugates (1:10,000)

After incubation with antibodies: incubate blot in reaction buffer in a standard bag.
(made by mixing reagents 1 and 2) if takes ~3ml to cover a minigel blot.

incubate for 1 min. -- pour off the reagent -- seal the bag
expose (protein side up) to X-ray film (XAR5 is fine)
(this should be done within 5 - 10 min. MAX)
initial test 15 sec and 1 min. -- develop film

**stripping blots:** wash blot with 100mM b-mercaptoethanol
2% SDS
62.5 mM Tris-HCl pH 6.7

for 30 min. @ 65C (with occasional aggitation

wash membrane 2X 10 min. with TBS-tween
reblock the membrane with LFDM in TBS-tween

**Immunoprecipitation --** Protocol for extractable IF proteins in oocytes:

Homogenize 20 oocytes in 1ml of SOL Buffer pH8.0/PMSF

**SOL buffer** 300mM sucrose
140mM KCl
50mM NaF
10mM EDTA
2mM $MgCl_2$
0.5% Triton X-100
10mM Tris-base pH 8.2
10mM sodium phosphate

Spin 15 min at 4° and extract supernatant with 0.5ml of freon. Remove aqueous phase, avoiding yolky material at interphase.

Add purified antibody to appropriate concentration; for the mouse monoclonals I have used, a final concentration of 5 mg /ml purified Ab seems to work well.

Preblocking with SAC will, of course, remove quite a bit of background, although the extensive washes described below seem to yield samples that are about as clean with or

without preblocking. SAC preblocking will, in my hands, pellet some of the extractable IF and thus reduce yield if quantitation is important. Blocking with 10mg/ml cold BSA has not seemed to reduce background at all. The IF component that SAC pulls down is washed away during the washes described below. This means that an SAC pellet by itself will contain some extractable IF (and **alot** of other radiolabelled oocyte proteins as well), but a washed SAC pellet contains little, if any.

Incubate 1hr at 4° with rocking

If desired, add 20 ml of 1:40 dilution of RbŸMouse Ab. This will improve recovery of mouse $IgG_{1,}$ but the rabbit IgGs I've used also seem to contain cross reacting antiIF antibodies that can potentially confuse interpretation.

Add 50 ml of 10% SAC washed twice in incubation buffer -- Incubate 30 min at 4°C with rocking

Spin down and wash 5X in:

| | |
|---|---|
| SOL Buffer supplemented with | 0.5M NaCl |
| | 0.1% SDS |
| | 0.5% deoxycholate |

**It is important to resuspend the pellet during each wash completely by vortexing.**

My experience using this methodology is that about 70% (or more) of extractable IF is removed from the lysate, as judged by reprecipitation.

**resuspend final pellet in SDS-sample buffer.**

**Gel/blot photography**

low contrast: Ektapan-yellow filter f22 1/4s/ develop in D19-7min. m
high contrast: Ektapan-yellow filter f22 1s / develop DK50 - 5 minutes

## Purification of proteins using immunomatrix

### Preparation of antibody protein A Matrix

1. Mix protein A acrylic beads with antibody in 3M NaCl in 50mM sodium borate buffer, pH 8.9, for 30 min. at 37°C and then overnight at 4°C (beads must swell).

2. Wash beads with 0.1M Naborate buffer pH 8.9

3. Wash sepharose with 0.2M triethanolamine, pH 8.2 8Collect unbound antibody from initial binding, borate and triethanolamine washes -- to calculate binding efficiency use Coomassie binding assay, not $OD_{278}$.

4. Resuspend beads in 20 volumes of 50mM dimethyl pimelimidate dihydrochloride freshly made up in 0.2M triethanolamine, readjust pH to 8.2, agitate gently at room temp. for 45 min.

5. Stop reaction by centifugation of the beads (500 x g for 1 min.) and resuspend in an equal volume of 50mM ethanolamine (pH 8.2).

6. After 5 min., wash cross-linked beads 3 times in 0.1M borate buffer, pH 8.2. Store column in 0.1M sodium borate 0.05% sodium azide

7. Add lysate to column and rock at 4˚ for 2 hours

### Elution of Antigens bound to matrix:

1. Remove the beads by gentle centrifugation and wash with the following buffers in sequence:

i. 0.5M NaCl, 0.05M Tris-HCl, pH 8.2, 1mM EDTA, 0.5% Nonidet-P40; ii. 0.15M NaCl, 0.05M Tris-HCl, pH8.2, 0.5% Nonidet P-40, 0.1% SDS; iii. 0.15M NaCl, 0.5% sodium deoxycholate (two times)

2. Wash column with an equal volume of .05M diethylamine, pH 11.5, containing .5% sodium deoxycholate for 2 min. Collect supernatant and immediately bring to near neutrality (≈pH 7.4) with the addition of 1/10 $NaH_2PO_4$.

2. Repeat step 2 and pool supernatants, freeze at -20°C

3. Wash beads several time in borate buffer and store in borate buffer containing .02% sodium azide

## Methodology for cultured cells (growing on glass cover-slips)

The simplest way to prepare sterile glass coverslips is to cut up 18mm square coverslips into fours. Place these into a 10cm glass petri dish and bake in oven overnight. Tissue culture cells can be passaged directly into the petri plate.

1. Wash cover slip briefly in PBS or TBS.
2. Fix -- this can be done using either cold or warm methanol, acid alcohol or an aldehyde-based fixative (see below for fixative recipes).
   For alcohol-fixed cells, after 10 minutes you wash the coverslip with TBS
   In acid alcohol-fixed cells, be careful to wash thoroughly.
   For aldehyde-fixed cells, you may want to extract the cells with methanol and then wash with TBS.
3. Incubate in primary antibody for 20 to 30 minutes - typically we use hybridoma tissue culture supernatants undiluted. If your background is too high, try diluting the primary antibody 1:5 to 1:25 in TBS + 0.05% triton X-100.
4. Wash the coverslip by immerson into TBS, do this 3 to 5 times, wicking off the excess liquid onto a paper towel between immersions -- also be sure to release the liquid held in the forceps by opening the forceps.
5. Incubate in secondary antibody, again for 20 to 30 minutes. Typically, we use fluorescein- and rhodamine-conjugated secondary antibodies. These generally work well at a 1:100 dilution. Again, the dilution is into TBS + triton.
6. Wash as above and place coverslip on a drop of lab mounting media.

**lab mounting media**

10g airvol 205 dissolved into 40ml 50mM Tris pH.8.0
after this is dissolved, add 20ml glycerol and 1.2g n-propyl gallate.
aliquot (10ml each) and store at 4°C

7. Gently squeeze coverslip with a paper towel -- allow slide to dry.
8. Look!

## Methodology for looking at the surface of Xenopus oocytes/embryos

1. Fix oocytes/embryos in Dent's fix (20% DMSO:80% methanol). Overnight or longer.
2. Remove Dent's and wash briefly in TBS
3. Incubate overnight in primary antibody (primary antibody incubation can be as short as 2 hours).
4. Wash 5 times (1 minute each time) in TBS
5. Incubate overnight in secondary antibody (again, can be as short as 2 hours).
6. Wash and mount in mounting media.

7. When dry, look!

## Methodology for whole-mount immunocytochemistry of Xenopus

1. Fix specimens overnight in Dent's fix.
2. Bleach specimens for 2 to 5 days in Dent's Bleach (1/3 30% hydrogen peroxide solution: 2/3 Dent's fix). Be patient and wait to the specimen is fairly white.
3. Incubate overnight in primary antibody diluted into calf serum and 10% DMSO.
4. wash 5 times over 1 hour each in TBS
5. Incubate overnight in secondary antibody (diluted into calf serum and 10% DMSO). typically we use anti-MIg-peroxidase conjugate diluted 1:200 to 1:500 -- some batches of conjugate need to be used 1:1000 to 1:2000 to avoid high backgrounds).
6. wash as in step 4.
7. dehydrate 2 x in 100% methanol for 15 minutes each.
8. clear in BABB: 1 part benzyl alcohol and 2 parts benzyl benzoate
9. mount in either a deep depression glass slide or a brass slide -- the brass slides have coverslips attached by melted wax. Do not put BABB solutions into tissue culture plastic, since it will dissolve it.

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## Preparation of *Xenopus* embryos for section based immunocytochemistry

The following protocol has been used successfully for immuno-labeling of proteins carrying the *c-myc* epitope tag (using the 9E10 monoclonal antibody) at the EM level (gold conjugated secondary Ab) as well as the light level (FITC conjugated secondary Ab).

**Required materials:**

- L. R. White (London Resin Co. Ltd.)
- 16% paraformaldehyde (Electron Microscopy Sciences)
- 25% Glutaraldehyde (Electron Microscopy Sciences)
- 2M CaCl2
- 2M MgCl2
- 0.5M Sodium Cacodylate, pH7.4
- 150mM Sodium Cacodylate
- 150mM glycine in 150mM Sodium Cacodylate, pH 7.4

---

Prepare fix from commercially prepared stocks of paraformaldehyde and glutaraldehyde (see above).

We have been using EMS products with good luck. These products are shipped in 5ml glass ampoules that can not be resealed. I transfer unused portions to glass scintillation vials and store at 4°C for no more than 2 weeks. The fix described below gives good ultrastructure when employed at 4°C over night.

Unfortunately, this extended incubation results in a somewhat reduced signal so if expression levels are low this may be over kill. 2-4 hours of fixation results in markedly better labeling but ultrastructural preservation is considerably less impressive.

**Fix:** 4% paraformaldehyde
0.1% glutaraldehyde
10mM MgCl2
10mM CaCl2
150mM Sodium Cacodylate, pH 7.4

This fix is for tissues that will be immuno-labeled. If you want the best ultra structure chemical fixation can provide and are not concerned about retaining antigenicity increase the glutaraldehyde concentration to 2.5%. Rinse in buffer and post-fix in 2% osmium tetroxide for 2 hours. Rinse twice in water (10min/ wash), dehydrate in an acetone gradient and embed in epon/araldite.

Fix in Eppendorf tube at 4°C for 2-4 hours (or over-night, ie 8-12 hrs). I always put the tubes on a rocker.

Rinse briefly 3x in 150 mM Sodium Cacodylate at room temperature

Rinse 2x in 150mM glycine in 150mM sodium cacodylate, 15 minutes, room temperature.

For LR White embedding dehydrate in ethanol gradient (20%, 40%, 70%, 90%, 100%, 100%). 15-30 minutes each, room temperature.

Alternatively, the 70% EtOH step can be performed overnight at 4°C. With tissue that has been fixed O/N I have not noticed any appreciable increase in lipid extraction, etc. when an O/N 70% step is performed, in fact a more thorough dehydration from 70% EtOH forward appears to offer some reduction in shrinkage. For example overnight in 70% EtOH @ 4°C followed by 1 hour incubations in 90% and 2x 100% EtOH at room temperature.

**Embedding fixed & dehydrated embryos in LR White**

Infiltration of the rather large Xenopus embryo is easily accomplished with LR White due to the low viscosity of the resin at room temperature. Even so, care must be taken to ensure complete penetration of the tissue. I have, therefore, devised an infiltration schedule that attempts to strike a balance between good infiltration and ultrastructural damage from excessive extraction (a serious concern with LR White).

***N.B.*** *L. R. White will not polymerize in the presence of oxygen. Therefore some special measures must be taken: 1) Do not introduce oxygen to the resin by shaking, bubbling air from a pipette etc. 2) Use only oxygen impermeable polymerization vesicles, eg gelatin capsules NOT "BEEM" capsules. 3) Polymerize in a vacuum oven.*

Replace 100% EtOH with a 2:1 mixture of 100% EtOH : 100% LR White. Incubate at room temperature for 1 hour.

Rock embryos in 1:1 mixture of 100% EtOH with 100% LR White for 2 hours at room temperature.

Rock embryos in a 1:2 mixture of 100% EtOH:100% LR White. Room temperature for 2 hours.

Rock embryos in 100% LR White at room temperature for 2 hours.

Rock embryos in fresh 100% LR White overnight at 4°C.

Rock embryos in fresh 100% LR White at room temperature for 1hr.

Transfer individual embryos to gelatin capsules with a minimum amount of old LR White. Fill capsules with fresh LR White, cap and place in eppendorf rack. After all embryos are in capsules place eppie rack in vacuum oven with another (empty) eppie rack on top to prevent capsule caps from popping off. Pull a vacuum with the vacuum pump (not the house vacuum-- its way too weak to be of use). Cook for 24 hours. The temperature range for polymerization is 50-55°C.

I have found 24 hours at 50° gives a block a little on the soft side (desirable for thick sections), while 24 hours at 55° gives a somewhat more brittle block with more desirable thin sectioning properties Overly brittle blocks can possibly be rescued for thick sectioning by soaking in a beaker of water for a short period of time (on the order of an hour or less) but this technique is still under investigation.

**Thick Sectioning L. R. White and Fluorescence based immunochemistry**

**Required Materials:**

- Glass knives
- Polylysine or gelatin coated microscope slides or coverslips
- Fish Block (0.8% BSA, 0.1% fish gelatin, 0.02% Tween-80 in PBS, pH7.4)
- Concentrated primary antibody

•FITC conjugated secondary antibody
•PBS (phosphate buffered saline;)
•Gelvatol (see Klymkowsky and Hanken (1991) Meth. Cell Biol. 36:419-41)

Thick sections greater than 3mm can be cut on a dry glass knife and transferred with forceps to a drop of water on a glass slide or coverslip. A slide warmer set to 50° not only speeds the drying process but also helps flatten the section. Be sure the slide or coverslip has been coated with either a gelatin based media or poly-lysine (to prevent section removal during staining).

Staining:

Block sections by incubating in Fish Block (see above and Reagent Prep Sheet) 15 minutes in a humid chamber (room temperature). Most easily accomplished by pipetting a small amount of Fish Block directly on to sections.

Replace Fish Block with primary antibody diluted in Fish Block. Incubate at room temperature in humid chamber for 1 hour. For 9E10, an antibody I typically use for whole mount at 2mg/ml, I use a 20mg/ml-- that is to say for staining sections a higher antibody concentration is in order

Rinse sections by repeated (5x) immersion in a beaker of PBS.

Incubate sections in secondary antibody for 1 hour. I use Sigma's FITC conjugated anti-mouse diluted 1:100 in Fish Block.

Rinse in PBS and mount in Gelvatol.

**Remounting Thick LR White Sections for Subsequent Thin Sectioning**

**Required Materials:**

•SuperGlue® (cyanoacrylate)
•Epon blanks
•Coverslips

Before thick sections can be remounted blank blocks must be "faced off" with a diamond knife. The perfectly flat surface left by the diamond allows the thick section to lie flat enough to be thin sectioned.

If more than one thick section on a coverslip is to be remounted it is best to move each thick section to an individual coverslip. This allows each section to be glued on to a blank without concern for damaging other important sections. Moving thick sections is easily accomplished by submerging the coverslip in water in a Petri dish. Using a syringe needle or dissecting needle the thick sections are carefully prodded off and transferred to a drop of water on another coverslip. The sections are picked up by simply lifting the needle up from beneath a floating section-- as the needle comes out of the water the section folds over the needle. As the needle drops down on to the surface of the water on the recipient coverslip the section unfolds onto the bead of water.

Once dry, thick sections can be mounted onto faced off blanks.

Place a small drop of SuperGlue® or similar cyanoacrylate based glue on the face of the blank. I use a wooden applicator stick broken so as to form a wedge shaped end.

Quickly, and without hesitation, invert the coverslip onto the drop of glue. Press lightly with forceps for a few seconds.

After all desired coverslips/thick sections are glued place block/coverslip assembly in a 50° oven for 30min.

Place warm block/coverslip assembly face down in ice. Wait about 5min. Coverslip should snap off easily leaving a perfectly flat thick section on the block.

**Immuno-Gold Labeling of Thin Sections**

**Required Materials:**

•Nickel Grids carrying thin sections
•Multiple grid staining chamber (Polysciences, Inc.)
•Primary antibody
•Gold conjugated secondary antibody (Amersham, Inc.)
•1% glutaraldehyde in PBS
•Fish Block (0.8% BSA, 0.1% fish gelatin, 0.02% Tween-80 in PBS, pH7.4)

Place nickel grids carrying thin sections in drops of Fish Block on Parafilm®. This blocks non-specific binding of antibody-- the length of the incubation appears to be non-critical. I usually keep the grids in block while I prepare the antibody solutions and grid box.

Place a piece of "time tape" (common colored lab tape) over the back of the Polysciences multiple grid staining chamber. 2.5cm tape is just wide enough to cover all four holes in a

row. This should be done carefully since the tape will be responsible for sealing the grid wells.

Introduce primary antibody solution to the tape sealed wells. The volume will be between 20 and 30ml/well. The concentration of the antibody for best signal to noise must be determined empirically but I have found 9E10 works best at 2mg/ml. Be sure to dilute antibody in Fish Block.

Move grids from Fish Block drops to antibody filled wells quickly to avoid any drying.

Place grid staining chamber in a humid chamber to prevent drying. Allow staining to proceed for 1 hour at room temperature. I have noticed marked increase in background if grids are left in antibody for too long.

Replace the staining chamber sliding cover before attempting to remove tape. Remove tape and submerge staining chamber in 100ml beaker of Fish Block. Swirl GENTLY. Blot bottom on paper towel and repeat rinse in 100ml beaker of PBS. Rinse in a total of 3 beakers of PBS.

Remove grids and place in drops of Fish Block.

Rinse and dry grid box avoiding dust etc. Tape back of box with fresh tape and fill wells with secondary antibody. I have been using Amersham's goat anti-mouse IgG-10nm gold at 1:10 in Fish Block but again empirical determination of antibody concentration is required.

Place grids in secondary antibody for 1 hour at room temperature.

Rinse in Fish Block followed by PBS, DO NOT REMOVE GRIDS.

Submerge freshly rinsed (**PBS rinsed**) grid box in 10ml of 1% glutaraldehyde in PBS for 10min. Rinse copiously with water.

Heavy metal stain grids and view.

**Heavy Metal Staining of Grids**

**Required Materials:**

- 2% Uranyl acetate (in $H_20$, store in dark)
- Reynold's lead citrate

Submerge grids (in Polysciences multiple grid staining chamber) in 10ml of 2% uranyl acetate for 8min.

Rinse 4x in $H_20$ in 100ml beakers by gentle swirling.

Submerge in Reynold's lead citrate for 3min.

Rinse as before.

Dry and view.

**Preparing Formvar Coated Grids**

**Required Materials:**

•0.5% or 0.7% formvar in ethylene dichloride (Fullam)
•Grids (Pellco)
•Microscope slides
•50ml conical tube (polypropylene, stockroom variety)
•Razor blade (degreased by wiping with 100% ethanol)
•Good forceps (non-magnetic if using nickel grids)
•10cm petri plates
•9cm Whatman filter paper
•Lens paper

For use in microscopes of 100kV or less use 0.5% formvar in ethylene dichloride (Fullam sells this pre-mixed-- its clearly the choice of champions). For use in the high voltage scope use 0.7%.

It is pointless to write this protocol down accept as a materials reference-- get someone to show you how it is done (Remember: "To lose one's mind. It is a terrible thing to lose one's mind. Isn't that so.")

**Reynold's Lead Citrate**

**Required Materials:**

•Lead nitrate $(PbNO_3)_2$
•Sodium citrate $(Na_3(C_6H_5O_7)•2H_2O$

•Boiled $H_2O$
•1M NaOH (made fresh with boiled but relatively cool $H_2O$)

Boil water for 20 minutes. Use this boiled water for all subsequent steps.

For **50ml** of Reynold's citrate:

1.33g Lead Nitrate
1.76g Sodium citrate
30ml $H_2O$

Mix with magnetic stir bar for 20min. A white slurry will form.

Add 8.0ml 1M NaOH. Solution should clear (if it does not add more NaOH and worry)

Bring volume to 50ml.

**Poly-L-lysine Coating Coverslips**

**Required Materials:**

•Poly-L-lysine solution (Sigma)
•Coverslips
•1% acetic acid in methanol

Wash coverslips to be coated in acid alcohol and allow to air dry.

Dilute poly-L-lysine solution 1:10 in $dH_2O$. This working solution can be re-used.

Place dry washed coverslips in diluted poly-L-lysine for 5min. Drain coverslips and allow to dry in a 60° oven for 1 hr or O/N at room temperature.

**Gelatin Coating coverslips**

**Required Materials:**

•Gelatin

•Chromium potassium sulfate
•1M NaOH

Prepare gelatin solution:

1g gelatin
0.1g chromium potassium sulfate

Dissolve gelatin in 1L $dH_2O$ with gentle heating. Add chromium potassium sulfate. Allow solution to cool to room temperature before using.

Soak coverslips in 1M NaOH for 15min, rinse with $dH_2O$ and blot. Dip coverslip into gelatin solution (2x) and allow to air dry.

**Epon Embedding *Xenopus* for EM Ultra Structural Analysis (NOT for immuno-labeling)**

**Required Materials:**

•Araldite embedding kit (Polysciences, Inc. Mallenhauer):
    Araldite resin
    Dodecenylsuccenic anhydride
    Poly/bed 812 embedding media
    DMP-30
•Acetone dehydrated and fixed Xenopus embryos/oocytes

Use only acetone dehydrated tissue.

Weigh and mix embedding kit components in fume hood:

11g Araldite resin
36g Dodecenylsuccenic anhydride
20g Poly/bed 812 embedding media
1g DMP-30

The size of the batch of resin may, of course, be doubled or halved depending on your needs. The volume of resin prepared using the above formulation is rather large but after the various

dilutions in acetone for infiltration and so forth are finished you will have used a considerable amount of resin.

Replace 100% acetone with 1:4 mixture of resin:100% acetone. Rock at room temperature for 1 hour.

Change resin to a 1:1 mix of resin to 100% acetone. Rock for 3 hours.

Change to 75% resin in acetone. Rock for 12 hours.

Change to 100% resin. Rock for 24 hours.

Change to fresh 100% resin. Rock for 5 hours.

Embed embryos in molds and put in 60° oven for 48 hours.

**Growing hybridomas**

1. Thaw hybridoma cells quickly in 37°C water bath
2. dilute in 1ml serum and spin down cells in IEC centrifuge 3 min at setting 3.
3. discard supernatant and resuspend in 2ml of hybridoma media with 20% FCS.

Hybridoma media: RPMI 1640 base media (with bicarbonate
10 % (or 20%) heat-inactivated fetal calf serum
non-essential MEM amino acids
50mM b-mercaptoethanol
antibiotics (gentimycin or pen/strep).

4. place in a small flask and set on side, with a tilt to keep cells in bottom corner -- place in tissue culture incubator.
5. watch each day for dividing, bright cells - this is the best sign that the culture is growing.
6. As cells begin to grow they will turn the media yellow, once this begins to happen, begin feeding the cultures. The best rule is to increase add one volume of media per day until you reach the max. volume.

Typically, I would add media until a flask is at 10ml -- the next day, I would add another 10 ml and divide the culture into another flask. When the culture is ready to be fed again, I would place both cultures into a 1L spinner and add 20ml fresh media. Refeed each flask with 10ml -- don't worry, there are enough cells left in the flasks to begin growing well.

As the flasks begin to grow again, their cells can be added to the spinner or you can use these flasks to freeze down cells.

7. Our spinners can accomodate a max. volume of 700ml each. Typically, I use w spinners to make enough antibody for injection, etc. Harvest spinner when no living cells are visible in a small aliquot.

**Freezing down hybridomas**

1. Take a exopential culture of hybridomas, spin down cells in IEC
2. discard supernatant and resuspend cells in 8% DMSO/92% FCS (DMSO from the bottle is sterile).
3. Aliquot cells into sterile, screw top 2ml vials -- place in foam block and surround by foam, secure with rubber bands -- place in the -70°C freezer overnight.
4. next day, transfer to liquid nitrogen tank.

**Purify and concentrating antibodies**

1. Measure volume of spent tissue culture supernatant. Add solid NaCl to a final concentration of 3.3M, then add boric acid to a final concentration of 100mM. Adjust the pH to 8.9 and add 0.05% sodium azide.
2. After all this is dissolved, filter the solution through two layers of Whatman paper.
3. Split solution into 500ml aliquots to run over protein A column.
4. **Prior to use, clean the column by running over it**
   25mL 2M urea
   25mL 1M LiCl
   25ml 100mM glycine (pH 2.5) (column is run at room temperature).
5. requilibrate column with 3M NaCl, 50mM borate, pH 8.9 and begin to run supernatant.
6. wash column with 25 to 50mL 3M NaCl, 50mM borate buffer
   followed by 25 to 50mL 3M NaCl, 10mM borate buffer
7. elute column with 100mM glycine pH. 3.0 -- collect fractions in tubes -- typically I collect 80drops per fraction. Each tube has 1ml of 1M Tris pH. 8.0 to neutralize glycine.
8. pool fractions containing high concentration of antibody, repeat using other aliquots of tissue culture supernatant. After all have been run through once, you should re-run the supernatants again to recover remaining antibody.
9. Wash column with PBS with azide to store.

**Concentrating antibodies:** we use centricon 100 concentrator cells. Be careful to monitor antibody concentration, if you go to far concentrated antibody will precipitate out. - once antibody is near the desired final concentration, wash the antibody 3 to 5 times in injection buffer.

**list of available cells & culture conditions**

| Species | cell name | culture conditions |
|---|---|---|
| **Xenopus:** | A6 (kidney epithelia) | |
| **Mammalian:** | BHK (baby hamster kidney - muscle-like) | |
| | 3T3 (mouse fibroblast) | |
| | C2 (mouse myogenic) | |
| | HeLa (human epithelial) | |
| | SW13.clone 1 (human adenocarcinoma - vimentin +) | |
| | SW13.clone 2 ( " " vimentin -) | |
| | SW13.T7K (derived from clone 2 - express keratin) | |
| | PtK (rat kangaroo epithelial) | |

**Preparation of whole cell and cell residues** (modified from Klymkowsky et al 1987).

**Whole Cell:**

wash tissue culture flask 3X with modified Ringers supplemented with 5mM EDTA (REd buffer)

scrape cells off in REd buffer

spin cells down in micofuge and resuspend pellet in SDS or IEF sample buffer

The amount of sample buffer depends on cell number -- samples must be sheared to break up the DNA -- otherwise it is no fun trying to load them onto the cell.

**Cell Residue:**

Wash tissue culture flasks 3X with modified REd buffer

extract cells with REd buffer supplemented with 0.5% NP40 -- cells should release from the substratum in this buffer -- collect cells, rinse flask and spin down in clinical centrifuge.

Rinse pellet again in REd+NP40 buffer

solubilize pellet in gel sample buffer.

**intracellular and intranuclear injection**

**time-lapse methods**

**Using the microscopes**

**Using the video camera**

**Interacting with the computer**

**Using the confocal microscope & taking pictures**

**Taking pictures on a regular microscope**

**Developing film and printing pictures**

**Yeast D-broth** (per liter)

| | |
|---|---|
| 10g | Bacto-yeast extract |
| 20g | Bacto-peptone |
| 20g | Dextrose |

for plates: add 20g Agar.

**Minimal "drop-out" media (1L)**: drop out trp for bait plasmids / leu for prey

| | |
|---|---|
| Milli Q water | 930nL |
| 50X Drop-out stock | 20ml |
| 25X Tyr stock | 40mL |
| Bacto-yeast Nitrogen base (w/o amino acids) (w NH4SO4) | 6.7g |
| Dextrose | 20g |

Autoclave and cool to 55C before adding trp or leu.

**50X Drop-out stock**: (trp-, leu-) for 500mL

| | |
|---|---|
| adenine | 150mg |
| uracil | 100mg |
| arginine | 150mg |
| aspartic acid | 500mg |
| histidine | 100mg |
| isoleucine | 100mg |
| lysine | 200mg |
| methionine | 100mg |
| phenoalanine | 250mg |
| threonine | 750mg |
| valine | 325mg |

25X Tyr stock 50mg / 100ml (can't be autoclaved)

100X Trp 300mg / 100mL (can't be autoclaved)

100X Leu 400mg / 100ml

**Transforming miniprep DNA into yeast:**

**Making yeast stocks:**

**SDS-PAGE of yeast (whole cell) (method #1)**

1. spin down yeast cells / rinse once in sorbital media.

2. resuspend in 8M urea , 1M guanidine HCl, 50mM Tris (pH 7.6), 10mM EGTA, 5% bME.
   add scoop of glass beads
   vortex for 2 to 4 minutes

3. add 1 volume SDS Sample buffer and heat at 90C for 5 minuts

4. centrifuge for 10 minutes at room temperature

5. run on gel

**Plasmid rescue (into coli) from yeast**
**(Gene 57:267).**

1. Grow ~2ml culture overnight in selective media

2. Fill 1.5ml microfuge tube - collect by 5 sec. centrifugation

3. Remove supernatant and vortex cell pellet

4. Add 0.2ml lysis buffer

**lysis buffer**: 2% Triton X-100
1% SDS
1mM NaCl
10mM Tris pH 8.0
1mM EDTA

5. add 0.2ml phenol-chloroform and fill tube to 100uL mark with acid-washed 0.45um glass beads. vortex for two minutes.

6. spin 5 min. in microfuge. Remove the top half of the aqueous phase into a clean tube. use 1 to 5uL for electroporation.

**Yeast transformation: high efficiency method.**

1. grow up colony of yeast cells in YPD media (5ml culture at 30C with aeration).

2. next day -- use this culture to innoculate a a 300ml culture of YPD media supplemented with 30mg/L adenine hemisulfate (made 200X stock of adenine). OD600 ~ 0.3-0.5

3. harvest cells by centrifugation 5 minutes at 5000rpm
resuspend in 10ml sterile milliQ water

4. pellet cells for 5 minutes at 7000rpm (small sorvall rotor).

5. resuspend in 1.5ml buffered lithium sulfate (prepare fresh).
1 vol. 10X TE buffer
1 vol. 10X Lithium acetate stock
8 vol. sterile water -- these cells can be stored for ~ 2 weeks on ice in the cold room. -- be sure to date the tube. Note: transformation efficiency falls off with time.

***Transformation***

6. add 200mg carrier DNA with less than 5mg transforming DNA -- keep volume below 20mL.

7. add 200mL yeast suspension

8. added 1.2mL PEG solution (prepared freshly)
8 vol. 50% PEG
1 vol. 10X TE
1 vol. 10X lithium acetate stock

shake 30' at 30C.

9. heat shock exactly 15 minutes at 42C. Microcentrifuge 5 sec. at room temperature.

10. resuspend yeast in 1ml of 1X TE buffer
spread upto 200mL onto an appropriate plate.
-- make plates using DIFCO AGAR ONLY.

11. 30C until transformants appear (typically 2 to 5 days).

**10X TE buffer**: 100mM TrisHCl
10mM EDTA

**10X Lithium acetate stock**: 1M lithium acetate
pH to 7.5 with acetic acid

**Protocol for testing yeast mating types:**

1. take a glump of yeast, dilute into 1ml YPD media

2. plate out 200mL of the yeast onto a dry minimal plate
   (minimal plates are leu, trp and his minus)
3. replica plate strains onto YPD and then onto minimal plates
   (careful to use a new velvet for each of the tester plates)
   (avoid placing two much yeast on the tester plate.)
4. incubate at 30C overnight!

notes: tester strain should complement all mutations in testees;
testees will complement the his 4 mutation of the tester.
only mated (diploid) cells should grow on minimal plates.