Embryonic Stem Cell Culture Techniques

Pierce Lab
F.M. Kirby Center for Molecular Ophthalmology
University of Pennsylvania School of Medicine

Eric Pierce, M.D., Ph.D.
F.M. Kirby Center for Molecular Ophthalmology
305 Stellar Chance Labs
422 Curie Boulevard
Philadelphia, PA 19104

215-573-3919
epierce@mail.med.upenn.edu

Culture Media /Components and Supplementation 3
FEEDER/STO MEDIUM (DMEM 7+PS) ... 3
EMBROYNIC STEM CELL MEDIUM ... 3
2X FREEZING MEDIUM ... 4
MITOMYCIN C (SIGMA) 2MG/VIAL ... 4
10 X PBS (WITHOUT CA AND MG) ... 4
SEEDING DENSITIES ... 5
FOR TRANSFECTIONS OR CONVERSIONS : ... 5
FOR PASSAGE : ... 5
FOR PASSING INACTIVATED FEEDERS : ... 5
TRANSFECTION REAGENTS ... 6
OLIGOFECTAMINE- INVITROGEN/LIFE TECHNOLOGIES # 12252-011(1 ML) ... 6
GENE JUICE- NOVAGEN # 70967-3 (1 ML) ... 6
LIPOFECTAMINE- GIBCO #18324-012 (1ML) ... 6
LIPOFECTAMINE 2000 – GIBCO # 11668-027 ... 7
X-GAL STAINING ... 8
COMPONENTS ... 8
X-GAL STAINING SOLUTION ... 8
FIXATIVES ... 8
USE OF THE HEMACYTOMETER FOR THE DETERMINATION OF CELL NUMBERS ... 9
PREPARATION OF SNLP FEEDERS. ... 10
A. GELATINIZING PLATES : ... 10
B. FREEZING/THAWING SNLP FEEDERS ... 11
C. EXPANSION OF THE GROWTH PLATE OF SNLP FEEDERS ... 12
D. PREPARING SNLP STOCKS (FOR INACTIVATION ~3DAYS LATER) ... 13
E. TO **INACTIVATE** STOCKS (15 CM DISHES) OF FEEDERS (SNLP). ... 14
EMBRYONIC STEM CELLS ... 15
A. THAWING OF ES CELLS (QUICK THAW) ... 15
B. PASSAGE OF ES CELLS ... 16
FREEZING ES CELLS (SLOW FREEZING) ... 18
ES CELLS ELECTROPORATION ... 19
PICKING EMBRYONIC STEM CELL CLONES. ... 20
SPLITTING ES CELL CLONES FOR GROWTH (FREEZE) PLATES AND DNA PLATE. ... 21
FREEZING EMBRYONIC STEM CELL CLONES (IN 24 WELL PLATES) ... 22
DNA PREPARATION ... 23
A. FREEZE ... 23
B. MAKE DNA ... 23

DIGESTING DNA FOR SOUTHERN ANALYSIS **24**

SOUTHERN BLOTTING **25**

A. GEL ELECTROPHORESIS : (SEPARATES MOLECULES ON THE BASIS OF THEIR SIZE). 25
B. GEL PREPARATION : DNA IN THE GEL MUST BE TRANSFERRED TO A SOLID SUPPORT BEFORE HYBRIDIZATION. BEFORE THE GEL IS TRANSFERED IT MUST BE PREPARED WHICH INVOLVES 3 STEPS. 25
C. TRANSFER TO SOLID SUPPORT : 25
D. PREPARING THE PROBE : 26
E. PROBING BLOTS : 27
F. HYBRIDIZATION. 27
G. WASHING: 27
H. DETECTING : 28

THAWING EMBRYONIC STEM CELL CLONES (IN 24 WELL PLATES) **29**

PREPARATION OF ES CELLS FOR BLASTOCST INJECTION. **30**

CULTURE MEDIA /COMPONENTS AND SUPPLEMENTATION

Abbreviations:

DMEM = Dulbecco's Modified Eagle's Medium-Gibco # 11965-084
purchased with L-glutamine and glucose (4.5g/L already added), no pyruvate
FBS = Fetal Bovine Serum (Use serum certified for ES cells)
PS = Penicillin-Streptomycin Gibco # 15140-122
BME = b-Mercaptoethanol
DMSO = Dimethylsulfoxide-Sigma (hybri-max)# D 2650
NEAA = Non-essential Amino Acids Gibco # 1114-050[i]
Trypan Blue solution (0.4%) Sigma # T 8154
Trypsin (0.25%) Gibco # 25200-056
Mitomycin C (2mg) Sigma # M 0503
Geneticin (1g) Gibco # 11811-023

Feeder/STO medium (DMEM 7+PS)

35 mls FBS (7% FBS)
5 mls PS (1X PS
QS to 500mls with DMEM

Standard Embroynic stem cell medium

75 mls FBS(15% FBS)
5 mls of 100x NEAA
3.4 µl of 14.3 M BME (1X BME)
QS to 500 mls with high glucose DMEM.
Sterile Filter
Then add 50 µl of 10 7/ml ESGRO if needed
Culture ES media O/N prior to use

CMT Feeder medium

50 mls ES screened FBS (10% FBS)
5 mls of 100x NEAA
QS to 500mls with DMEM

CMT ES Cell Medium

75 mls ES screened FBS(15% FBS)
5 mls of 100x NEAA
5 ml 100X Nucleosides (from Specialty Media)
0.5 ml of 1000X BME*
QS to 500 mls with high glucose DMEM.

Sterile Filter
Then add 50 µl of 10 7/ml ESGRO
Culture ES media O/N prior to use

1000X B-ME solution

"Neat" B-ME is 14.3M
Final concentration in ES cell media is 0.1mM.
1000X stock = 0.1M or 100mM
To make 0.1M B-ME from 14.3M, dilute 143X
eg. 0.1 ml B-ME to 14.3 ml with dH_20
    sterile filter
    aliquot to 0.5 or 1ml, store at -20 ºC

2X Freezing medium

This should be freshly prepared for each use. Wear gloves when working with DMSO. Filter before use.

2.1 ml (60% DMEM)
0.7 ml(20% FBS)
0.7 ml(20% DMSO)

Mitomycin C (Sigma) 2mg/vial

Constitute with 2ml of 1 x PBS. Sterile filter . Store aliquots at 4 °C.
Use 100 µl /10 ml of media.(Dilute 100 x in media to use)Final concentration= 10 µg/ml

Geneticin (G 418)

It comes as a crystalline powder.The amount of active ingredient per total weight is printed on the bottle label. Make a stock solution of 100mg/ml of the active ingredient in 0.1 M Hepes.eg active ingredient is 719 µg /mg. Dilute in 7.2ml of Hepes. After mixing filter sterile. Make aliquots and sore at 4 C or at –20 C.

For 275 µg/ml conc.use 1.4ml of stock sol./500ml of media
For 400 µg/ml conc.use 2.0 ml of stock sol./500ml of media
For 600 µg/ml conc.use 3.0 ml of stock sol./500ml of media.

10 x PBS (without Ca and Mg)

137 Mm 80g NaCl
7.7 Mm 2g KCL
10 Mm 26.8g $Na_2HPO_4.7H_2O$
1.4 Mm 2g KH_2PO_4

PH to 7.4 or PH to 8.0 if using for X-gal staining.

SEEDING DENSITIES

For transfections or conversions :

CHO cells : 5 x10^{4} / well of a 6 well plate.
ES cells : 2 x 10^{5} / well of a 6 well plate for same day transfection.
: 1x 10^{5} / well for next day transfection.

For passage :

CHO cells : 1 x 10^{5} / T75 flask.
ES cells : 1 x 10^{6} / 10-cm dish for weekend.
: 2 x 10^{6} / 10-cm dish for weekday.

For passing inactivated feeders :

SNLP : 3 x 10^{6} /10-cm dish.
: 4.0 x 10^{6} /6 well plate.
: 4.2 x 10^{6}/ 24 well plate.

TRANSFECTION REAGENTS

Total transfection volume for each well of a 6 well plate is 1 + ml. For a 10 cm dish it is 4-5 ml. Refer to seeding densities for cell type.

Oligofectamine- Invitrogen/Life technologies # 12252-011(1 ml)

Used for transfection of oligonucleotides into cells. Quantities are for each well of a 6 well plate; multiply as needed.

Tube 1- Diluted oligo :185 µl of optimem + **5 µg of oligo**
Tube 2- Diluted oilgofectamine: **5 µl of oligofectamine** + 10 µl of optimem. (Total volume should be 15 µl).

PS. The amount of oligofectamine (µl) should be the same as the amount (µg) of oligo.

Mix tube 1 & 2 contents. Incubate RT for 20- 25 minutes.

Add to cells with 1 ml of optimem. Transfect 6hrs-overnight. Change media am.

Gene Juice- Novagen # 70967-3 (1 ml)

For transient and stable transfections. Has unique composition of non toxic cellular protein and polyamine.

Optimal ratio of gene juice to DNA is **3 µl gene juice to 1 µg DNA** but can be varied from 2 µl-12 µl/ µg of DNA.e.g.

Tube 1: Add 200 µl of optimem. Add 6 µl of gene juice. Mix, incubate 5 minutes.
Add 2 µg of DNA.

Incubate 15-20 minutes at RT. Add to cells with 1 ml of optimem.Transfect 6hrs-overnight. Change media am.

Lipofectamine- Gibco #18324-012 (1ml)

For transient and stable transfections. Liposome formulation of a polycationic lipid.
Optimal ratio : Use 1 µg of Lipo for 1µg of DNA.
Tube 1 : Add 100 µl of optimem + 2 µg DNA .
(if using CM9 peptide add 50 µgof peptide/µg of DNA and incubate 10-15 minutes)

Tube 2 : Add 100 µl of optimem + 1 µl of lipofectamine.

Mix tube 1 & 2. Incubate for 45 minutes.

Add to cells with 1 ml of optimem. Transfect 6hrs-overnight. Change media am.

Lipofectamine 2000 – Gibco # 11668-027

Suitable for stable transfections. Can transfect in presence of serum.e.g.

Tube 1 : Add 100 µl of optimem + 1-2 µl of LF2000.
Tube 2 : Add 100 µl of optimem + 2 µg of DNA.

Mix, incubate 20 minutes. Add to cells with 1 ml of optimem. Transfect 6hrs-overnight. Change media am.

X-GAL STAINING

Components

1- X-Gal	25 mg/ml			
2- K Ferrocyanide (K_4FeCN_6)	100Mm	in 0.1 M Na-P	pH 8*	(2.1 g/50 ml)
3- K Ferricyanide (K_3FeCN_6)	100Mm	in 0.1 M Na-P	pH 8*	(1.65 g/50 ml)
4- $MgCl_2$	1M stock			

* To prepare 100 ml of Na-P solution pH 8, mix 5.3 ml of solution NaH_2PO_4 0.2 M (27.6 g/l) and 94.7 ml of solution Na_2HPO_4 0.2 M (53.65 g/l) .

X-Gal Staining Solution

5Mm K Ferro	(20X dilution)	1.25 ml	0.625 ml	
5Mm K Ferri	(20X dilution)	1.25 ml	0.625 ml	
2Mm MgCl2	(500X dilution)	50 µl	25 µl	
1mg/ml Xgal	(25X dilution)	1 ml	0.5 ml	
		---------	-----------	
		25 ml	12.5 ml	in PBS PH-8

Fixatives

Fixed with 2% formaldehyde 0.2% glutaraldehyde in PBS PH 8.

Formaldehyde 37%
Glutaraldehyde 50%

2% formaldehyde	(18.5X dilution)	1.35 ml	0.67 ml	
0.2% glut.	(250X dilution)	0.1 ml	50 µl	
		---------	--------	
		25 ml	12.5 ml	in PBS PH 8

For 6 well plates :

1-Wash plates with PBS. Add fresh PBS PH 8 IX.
2-Cool on ice.
3-Remove PBS fix 5 minutes on ice.
4-Wash 3X with PBS.
5-Add 2 ml of the stain per well.
6-Seal plate.
7-Stain ON @ 37 °C in bacterial incubator.
8-Next day,remove stain,wash with PBS PH 8 ,view.
9-Post fix if desired with 3.7% formaldehyde for 5 minutes RT. Wash twice with PBS. Store at 4°C in PBS.

USE OF THE HEMACYTOMETER FOR THE DETERMINATION OF CELL NUMBERS

The hemacytometer consists of two chambers, each of which is divided into nine 1.0 mm squares. A cover glass is supported 0.1 mm over these squares so that the total volume over each square is 1.0 mm x 0.1 mm or 0.1 mm^3, or 10-4 cm^3. Since 1 cm^3 is approximately equivalent to 1 ml, the cell concentration per ml will be the average count per square x 10^4.

Cell distribution in the hemacytometer chamber depends on the particle number so always mix the cell suspension thoroughly before sampling.

Hemacytometer counts do not distinguish between living and dead cells. A number of stains are useful to make this distinction. Trypan blue among others (Erythrosin B, Nigrosin) can be used: the nuclei of damaged or dead cells take up the stain. If more than 20% of the nuclei are stained, the result is probably significant.

1. Dilute 300 µl of Trypan blue with 600 µl of media. Add 100 µl of cell suspension to make 1 ml (or 10X dilution of cells).

2. Place cover glass over hemacytometer chamber.

3. Transfer agitated cell suspension with a Pasteur or transfer pipet to fill both chambers of the hemacytometer (without overflow) by capillary action.

4. Using the microscope with a 10X ocular (and a 10X objective), count the cells in **both** sets of 9 squares . If over 10% of the cells represent clumps, repeat entire sequence.

5. Calculate the **number of cells per ml** in the original culture as follows : (Cells/ml = average count per square x 10^4)

- Divide **total** number of cells by the number of counted squares
- Multiply by 10^4
- Multiply by 10 (dilution factor)

6. **Total number of cells** = cells per ml x total volume of cell preparation from which the sample was taken.

7. Repeat count to check reproducibility (+/- 15%).

PREPARATION OF SNLP FEEDERS.

A. Gelatinizing Plates :

Prepare plates by covering surface with 0.1% Gelatin solution*. (1g/1L)

Plate Size (cm)	Amount of Gelatin (ml)
3	1
6	3
10	5
15	10

* To prepare gelatin solution, dissolve 1 g of gelatin in 1 liter of water and sterilize it by autoclave.

Swirl the plates to cover the entire surface. Allow plates to gel @ room temp. for at least 2 hours. Aspirate off all of the gelatin. Then the plates can be immediately used or stored at 4 °C for up to a week.

B. Freezing/Thawing SNLP feeders

1. Thaw 1 vial of SNLP feeders onto 1, 10cm dish. Change media next morning and expand as needed.

2. Freeze 1, 10cm growth plate as one vial. Label with passage number and date.

C. Expansion of the growth plate of SNLP feeders

1. Aspirate off media.
2. Add 2ml of trypsin. Incubate @ 37 °C ~ 2 minutes.
3. Stop trypsin reaction with 2.5 mls of complete media. Resuspend cells.
4. Bring the volume upto 30 ml with media in 50 ml conical tube.
5. Add 10mls of suspension to each 10-cm **gel coated** dishes.

A typical schedule can look like this:

Confluent 10 cm growth plate ←
↙↓↘
expand to 3 -10 cm dishes,

↓ ± 2 days after

3 dishes are split into 6 stock plates (15 cm)and 1 growth plate (10 cm)

± 3 days after ↙ ↘ ± 2 days after

Inactivation of the 6 stock plates growth plate is used in a new cycle

Friday : **Inactivate** 15 cm plates of SNLP those plated on Wednesday. Later Friday afternoon pass 3, 10 cm dishes of SNLP into 6 stock plates (15 cm) for inactivation on Monday and 1 growth plate (10 cm).

Monday : **Inactivate** 15 cm plates of SNLP those plated on Friday and expand 1 growth plate to 3 ,10 cm dishes.

Wednesday : Pass 3, 10 cm dishes of SNLP into 6 stock plates (15 cm) for inactivation on Friday and 1 growth plate (10 cm).

Thursday : Expand 1 growth plate to 3,10 cm dishes.

Cells are ready to pass when they are 90% confluent. There should be no spaces between the cells. For inactivation however cells should not be overgrown.

D. **Preparing** SNLP Stocks (for inactivation ~3days later)

Use a confluent growth (10 cm) plate to prepare stock (15 cm) plates for inactivation . Gel 15 cm plates for the stocks and one 10 cm plate for the passage plate.

1. Aspirate off all of the media from the 10 cm plate.

2. Add 1.5 ml Trypsin to plate; swirl to cover the entire surface. Incubate @ 37˚C for 2 minutes.

3. Add 2 ml of media to the plate to inactivate the trypsin. Vigorously pipette up-and-down to suspend the cells.

4. Determine the total volume of media that will be needed for the growth / stock plates based on the following:

Size of Plate (cm)	Amount of Media to Aliquot (ml)
15	15
10	10

5. Add 0.5 ml of the cell suspension to 10 cm dish for growth . Pass the rest into 2, 15 cm dishes for inactivation (1.5 ml/dish).

6. Place the stock plates in the 37 °C incubator, labeling the tray with the clone number (e.g., SNL), the passage number, and the date.

NOTE: The 15 cm plates (stock plate) will be used upon confluence to prepare the inactivated feeder plates. The one 10 cm plate (growth plate) will be used to maintain the cell line through a number of passages

E. To **inactivate** stocks (15 cm dishes) of feeders (SNLP).

1. Remove 15 mls of the media from each 15 cm plate.

2. Add 150 µl Mitomycin C stock solution (1mg/ml) to 15 ml of media .Add to plate and swirl to cover surface.

3. Incubate @ 37 °C for 2 hours.

4. After incubation, aspirate off all of the media and wash the cells twice with PBS, 10 ml per wash (enough to cover the surface). Aspirate off all of the PBS.

5. Add 2 ml of trypsin to each 15 cm plate. Swirl to cover the entire surface. Incubate @ 37 °C for 2 minutes.

6. Add 3 ml of media to each plate to inactivate the trypsin. Suspend the cells by pipetting up-and-down and transfer the suspension to sterile 50 ml centrifuge tube. Repeat the suspension process, pooling all of the suspensions into the tube, until all of the cells have been harvested.

7. Count the cells. Plate the inactivated cells accordingly as required (remembering to use plates that have been pretreated with the gelatin). Swirl the plates to uniformly distribute the cells.

   Size of plate **plating density** (amount of media to aliquot)

   100 mm dish 3 x 10^{6} cells (10 mls/plate of media)
   6 well plate 4 x 10^{6} cells (12 mls/dish of media)
   24 well plate 4.2 x 10^{6} cells (24 mls/dish of media)

8. Freeze rest of the inactivated cells @ 5 x10 6 per vial.(At this freezing density one vial of inactivated cells can be thawed into 10 cm dish, 6well plate or 24 well plate). Freeze cells as per protocol.

9. Place the feeders in the 37 °C incubator, labeling the plates with "inactivated SNLP" and the date.

10. Check appearance with the microscope.

11. Change media every   and before adding ES cells.

NOTE ; These feeder plates can be used up to one to two weeks depending on how they look like (the fresher the better).

EMBRYONIC STEM CELLS

If you are embarking in growing ES cells, be prepared to refeed them DAILY. All procedures should be carried out using sterile techniques. The growth and maintenance media for ES cells is DMEM (no pyruvate, high glucose) 15% FBS (ES cell media). Handling ES cells growth, maintenance, passing, freezing and thawing is conducted in a manner to protect and maintain the quality of the cells and keep them in a pluripotent state. Serum quality is critical for successful growth of ES cells and especially true for blastocysts. The quality of the feeders is very instrumental. Remember also that in passing, freezing, and electroporating ES cells; it is best that the cells are still at exponential growth (80% confluence) for optimal results.

A. Thawing of ES cells (quick thaw)

1. Remove cells from the freezer and quickly thaw in a 37 °C waterbath.

2. Transfer the cell suspension to a sterile 15 ml tube. Add 10 - 12 mls of ES media to 1 ml of cell suspension.

3. Gently mix and pellet the cells by centrifuging @ 1100 rpm for 7 minutes.

4. Aspirate off supernatant and resuspend cells into 10 mls of ES media, and plate out cells in a 10-cm feeder plate.

5. Refeed cells daily with fresh media. Upon 80-85% confluence, cells need to be passed or frozen.

B. Passage of ES cells

ES cells typically should be passaged every 2-4 days (apart from colonies under selection).If passaging is neglected the cells will differentiate and you will select for variants that might have lost totipotency. Cells must be fed when media begins to turn orange. Yellow media (acid pH) is very bad for ES cells and should be avoided at all costs. If you are planning to passage and believe that the cells might turn yellow overnight feed last thing in the evening and again the next morning before passaging. DO NOT PASSAGE CELLS WHEN MEDIA IS YELLOW.

1. Check cells under the microscope for 80-85% confluence.

2. Refeed cells 3 - 4 hours before passing them. (VERY IMPORTANT)

3. Aspirate media off. Add 2.5 ml of trypsin to a 10-cm plate.

4. Incubate @ 37 °C for 10 minutes.

5. Add 7.5 ml of ES media to inactivate the trypsin.

6. Pipet up and down several times to separate the cells and break any colonies. Collect the cells and "pan" them i.e pass the cell suspension onto 2 gel coated 10 cm dishes. Icubate for 30-45 min. Swirl gently the dishe and collect non adherent cells. Count cells. Pass : For a 10 cm dish : 2×10^{6} cells on Monday and Wednesday 1×10^{6} cells on Friday. For a 6 well plate: 1×10^{5} cells/well for next day transfection 2×10^{5} cells/well for same day transfection

7. Determine the number of feeder plates you need, depending upon the passage you are doing. Add fresh ES media to the feeder plates (to 1 x 6 cm feeder dish: 6 ml of media; 1 x 10-cm feeder: 10 ml of media). Split ratios for ES cells can vary from 1:1 to 1:10. Do not exceed 1:10. pass 2×10^{6} cells on Monday and Wednesday and 1×10^{6} cells on Friday.

The area relationships for the various dishes are as follows:

Dish	Media	Trypsin	Area (cm2)	Diameter (actual)	
96 well	200 µl/well	30-50 µl	0.32	0.6 cm	(6 mm)
24 well	1.0 ml	200 µl	2.0	1.5 cm	(10 mm)
6-well plate	2 mls	400 µl	9.6	3.5 cm	(15 mm)
60 mm dish	6 mls	0.6 ml	21.2	5.2 cm	(6-cm)
100 mm dish	12 mls	1.5 ml	55.0	8.7 cm	(10-cm)
150 mm dish	30 mls	2.5 ml	148.0	14 cm	(15-cm)

Some typical passaging ratios:

1:6 = 1 x 60 mm to 2 x 90 mm
1:6 = 1 x 30 mm to 1 x 90 mm
1:4 = 1 x 30 mm to 2 x 60 mm
1:5 = 1 x 24 well to 1 x 30 mm (6-well plate)
1:6 = 1 x 96 well to 1 x 24 well

8. Aliquot the cell suspension into plates in the volume specified for each plate. Remember to use Feeder plates. Always check the feeders before using them. They should be confluent, no gaps, not contaminated and not dividing. Use feeders that are older, (1-2 weeks old), the advantages are many: any contamination is assessed, also any dividing run-away cells can be detected, and the passage will be earlier. Also, older feeders have settled nicely and flattened.

9. Mix to have a uniform cell distribution. Return plates to the TC 37 °C incubator.

Freezing ES cells (slow freezing)

1. Check cells under the microscope for 80-85% confluence.

2. Refeed cells 3 - 4 hours before passing them.

3. Aspirate media off. Add 250 µl of trypsin to a well of a 6-well plate, or 2.5 ml of trypsin to one 10-cm plate.

4. Incubate @ 37 °C for 10 minutes.

5. Add media, ES media to inactivate the trypsin. About 750 µl to a well of a 6-well plate, or 7.5 mls to a 10-cm dish.

6. Pipet up and down several times to separate the cells and break any colonies. Collect cell suspension in a centrifuge tube for counting.

7. To count take a 100 µl aliquot of cells in 600 µl of media and 300 µl of trypan blue to calculate the cell number/ml. Total number of cells = number of cells /ml x total volume.

8. Pellet cells by centrifuging @ 1100 rpm for 7 minutes.

9. Aspirate off supernatant and resuspend the pellet in media with 1/2 the volume calculated. A typical freeze aliquot would have 1 ml of ES cells 3×10^{6} cells - 4×10^{6} cells total/vial.

10. Add 1/2 the volume with 2X Freezing Media (60% DMEM, 20% FBS, 20% DMSO, freshly prepared); the cell suspension is diluted as a result: 10% DMSO is the final conc. Add the freezing media dropwise, mixing well after each addition. e.g You have 1×10^{6}/ml in a total volume of 8 ml. So the total number of cells is 8×10^{6}. Resuspend pelleted cells in 1 ml of media (that is half the final freezing volume). Add 1 ml of freeze media (which is half the volume). Freeze 1 ml/vial.

11. Aliquot the suspension into sterile NUNC freezing vials, pre-labeled with the cell type (AB2.2, AB1, etc.), clone number, passage number, freezing density and the date.

12. Place vials into a freezing container. It is critical that the freezing rate is not faster than 1 °C/minute. Do not use any untested STYROFOAM container since freezing rates vary greatly and this will most likely result in death of most of your cells. Freeze cells overnight at -70 °C, (24 hours).

13. Next day, transfer cells to the Liquid Nitrogen freezer (or -135 °C freezer). Freezing and thawing is counted as one passage.

ES CELLS ELECTROPORATION

1. On the second day after passaging recently thawed or split ES cells in 100 mm dish, harvest the cells by adding 2 ml of trypsin/EDTA and incubating them @ 37 °C for 15 minutes (it is a little longer than usual to obtain single cells).

2. Stop the digestion by adding 4 ml of complete ES media and gently pipette up and down the cells to break any clumps.

3. Pellet the cell by 5 minutes of centrifugation at 1100 rpm (± 270 x g).

4. While the cells are spinning, turn on the electroporator and place two 4 mm cuvettes on ice.

5. Remove the media and resuspend the cells in a minimal volume of **DMEM** (± 1 ml)**.**

6. Count the cells using the hemacytometer and adjust the concentration to ± 1 x 10^7 cells/ml with **DMEM.**

7. Mix 0.8 ml of the cell suspension with 25 to 40 µg of linearise vector in one of the 4 mm cuvettes. Feel the other cuvette with 0.8 ml of DMEM. Replace them on ice.

8. Set up the electroporation conditions as follow:
   - 0.250 kV
   - 500 µF

9. Try first with the cuvette without cells. Somme littles bubles should appear after the electrical pulse and no electrical spark or sound must be heard. Then deliver the electrical pulse to the cells after gently tipping the cuvette.

10. resuspend the ES cells and place them on **3** 100 mm dishes with feeders in **non-selective** ES media. Change the media 12 hours after for a **non-selective** ES media.

11. After 24 to 36 hours, change the media for a ES media with G418 250 to 300 µg/ml (neomycin).

12. Change the media with G418 every 24 hours.

PICKING EMBRYONIC STEM CELL CLONES.

Once resistant colonies are visible in plates of tranfected ES cells they need to be isolated for growth and analysis for targeted homologous recombination events. Ideally colonies should be fairly circular and well defined with indistinguishable individual cells. Flat , large colonies with jagged perimeters are most likely undergoing differentiation and should preferably not be picked.

1. Colonies are picked into **round** bottom 96 well plates, trypsinized and passed into 24 well plate with feeders for growth.

2. Fill a sterile disposable reagent reservoir with ES media. With a multichannel add 800 µl of media to each well of a 24 well plate. Return plates to 37 °C until ready to use.

3. Fill another reservoir with trypsin.With a multichannel add 25 µl of trypsin into each well of the 96 well plate. For each 24 well plate you will need only 3 rows (24 wells) of the 96 well plate.

4. Aspirate off media from the 10 cm dish of tranfected/electroporated ES cells. Rinse the plate once with 5 mls of PBS. Then add 5 mls of PBS to the plate.

5. Pick colonies using a P-20 set at ~15 µl. To pick gently detach the colony by gently going around it and pull it into the tip with as little volume as possible and place into 96 well plate, one colony per well. Pick 24 colonies at a time. Icubate @ 37 °C for 10 minutes.

6. Add 75 µl of media/well to stop the trypsin reaction. Using the multichannel pipette to mix cells(~20 times). Transfer cells from each well into the 24 well plate. Mark each plate as A,B ,C etc. Mark the clones 6-12-18 and 24 just to keep track of the orientation of the plate.

7. Feed cells daily, until ready to passage for freeze and DNA.

SPLITTING ES CELL CLONES FOR GROWTH (FREEZE) PLATES AND DNA PLATE.

1. ES cell clones in 24 well plates need to be split when they are 60-70 % confluent (takes 2-3 days after picking) into 2 , 24 well plates. Mark 1 plate as **growth plate** which will eventually be for freeze(will need 24 well plate with feeders). Mark the other plate as the DNA **plate** (will need 24 well gel coated plate) which is used for southern analysis later.

2. Feed 2-3 hrs before passing them.

3. Aspirate off media . Add 250 µl of trypsin /well. Icubate 10 minutes at 37 °C.

4. Add 750 µl of media to stop the trypsin reaction. With a multichannel pipette up and down to create a single cell suspension.

5. Take 0.5 ml/well of cell suspension and add to growth plate and 0.5 ml/well to the DNA plate each with 0.5 ml of ES selection media already added to them. When adding cells to the feeder plate it is not necessary to mix the contents of the well by pipetting, which could detach the feeder monolayer from the bottom of the dish.

6. Once all the colonies have been split return the plates to the incubator. Change media in AM, feed until ready to freeze or make DNA.

FREEZING EMBRYONIC STEM CELL CLONES (IN 24 WELL PLATES)

1. Cells should be in log phase (~ 70% confluent) to ensure good viablity on thawing.

2. Feed plates 2-3 hrs before freezing.

3. Aspirate off the media. Add 250 µl of trypsin per well. Incubate 10 minutes @ 37 °C.

4. Add 250 µl of ES media to stop the trypsin reaction.

5. Pipet up-and down to get a cell suspension.

6. Gently add 500 µl of the cool 2x freeze media. Mix.

7. Use sterile (rainin) plate covers to seal the plate and transfer immediately to styrofoam container and -80 °C.

DNA PREPARATION

When the 24 well DNA plates are confluent they can either be frozen or used to make DNA.

A. Freeze

Wash the cells 2X with PBS. Remove residual PBS. Cover plate with tape pads. Store at – 20 °C until ready to make DNA.

B. Make DNA

1. Wash plate 2X with PBS.

2. Add 0.5ml of the lysis buffer/well.

   15 ml of lysis buffer :

   150 µl 1M Tris pH 7.5
   300 µl 0.5 M EDTA
   600 µl 5M NaCl
   188 µl 20mg/ml Prot.K(do not freeze thaw)
   0.075 g sarosyl(N-Lauroylsarcosine)
   13.762 ml H_2O

3. Seal plate with QIAGEN tape pads. Put lid on. Place plate in ZIPLOC bag with a moist paper towel. Place in 55 °C oven overnight.

4. Next day remove the digested material (new pipette tip for each sample) and place into a labeled 1,5 ml microtube. Add 0.5 ml of isopropanol. Mix by inverting the tube several times till you see fluffy DNA.

5. Spin 14K for 10 minutes at RT. Remove the supernatant. Wash pellet with cold 70% ethanol. Remove ethanol . Air dry pellet.

6. Add 50 µl of TE (10Mm Tris-Cl+ 1Mm EDTA) . If you have less DNA add less volume of TE.

7. Put at 55 °C overnight.

8. Next day digest 10 µl for Southern.

DIGESTING DNA FOR SOUTHERN ANALYSIS

After the DNA has been resuspending overnight, mix and set up a 50 µl reaction.

e.g EcoR1 digest (37 °C overnight)

2.5 µl of 10 X EcoRI buffer
1 µl *Eco*R1 enzyme
10 µl DNA
0.75 µl spermidine(3 Mm final conc)
10.75 µl dH_2O

e.g Bam H1 digest (37 °C overnight)

2.5 µl 10X Bam HI buffer
1 µl *Bam*H1 enzyme
10 µl DNA
0.75 µl spermidine
0.25 µl BSA
10.50 µl dH_2O

SOUTHERN BLOTTING

A. Gel electrophoresis : (separates molecules on the basis of their size).

Run the digested samples in a 0.8% agarose gel (7.5 µl of ethidium bromide (10 mg/ml) in 150 ml gel) with appropriate DNA markers . Gel picture should be taken with a ruler laid alongside the gel so that band positions can later be calculated.

B. Gel preparation : DNA in the gel must be transferred to a solid support before hybridization. Before the gel is transfered it must be prepared which involves 3 steps.

a- Depurination:(this results in partial depurination of the DNA fragments, which in turns leads to strand cleavage).

Rinse gel in milliQ H2O . Shake gel slowly in 0.25 M HCl* (21ml of HCl 12 N /L) for 30 minutes. Check the xylene cyanol and bromophenol blue dyes. These should change to green and blue respectively.

b- Denaturation : (the DNA molecules in the gel are double-stranded, so they must be made single stranded in order for the probe to hybridize to them. To do this, the DNA is transferred using a strongly alkaline buffer, which causes the DNA strands to separate and bind to the filter as single-stranded molecules).

Pour off HCl. Rinse with milliQ H2O. Add denaturation solution* and shake gel for 20 minutes. Replace with fresh denaturation solution and shake for another 20 minutes.

c- Neutralization : (to bring the gel pH down to <9.0.so that the transfered DNA will bind to the nitrocellulose membrane).

Take off denaturation solution . Rinse gel in milliQ H_2O. Add neutralization *solution. Shake 20 minutes, replace with fresh solution and carry on another 20 minutes.(The neutralization step can be prolonged several hours).

C. Transfer to Solid Support :

Transfer is done overnight using the Turbo blotter rapid downward transfer systems*. To transfer, soak the membrane sequentially in milliQ H2O ~5 minutes and then in 10X SSC ~2-3 minutes. Follow instructions for setting up the transfer.

Fill the transfer tray with 10X SSC:
150 ml for 11x14 cm transfers.
250 ml for 20x25 cm transfers.

Cover transfer with saran wrap.

When transfer is complete, take it apart carefully. Remove the top layers of the paper. Before removing the gel, mark the wells on the membrane with a pencil to keep track of lanes. Remove gel. Cut one corner of the membrane. Write the date and the blot number on that corner. Place the blot in 2X SSC for 2-3 minutes.

Place the wet membrane on a clean Whatmann paper and UV crosslink DNA to membrane using Stratalinker 1800. Use 1200 ujoules, autocrosslinking. Do not allow the blot to dry.
At this point the blot can either be probed or stored at 4 °C sealed in a bag.

D. Preparing the probe :

The objective is to create a radioactive copy of a double-stranded DNA fragment.

The DNA fragment (template) is labeled by Random Labeling :

- The template DNA is denatured.

- A mixture of DNA nonamers (9 nucleotides of ss DNA) of random sequences is added to the denatured template and allowed to anneal.

- DNA polymerase is added along with dATP, dGTP, dTTP, and radioactive α[^{32}P]dCTP. Usually, the phosphate bonded to the sugar (the α-phosphate, the one that is incorporated into the DNA strand) is synthesized from [^{32}P], which is radioactive.

- The mixture is boiled to separate the strands and is ready for hybridization.

For each sample:

Take ~ 25 ng of DNA fragment
Bring total volume up to 24 µl with dH_2O
Add 10 µl random primers from Prime it – kit*
Total volume should be 34 µl.

Heat all samples in boiling water for 5 minutes
Cool at room temp. Spin a few seconds .

To each sample add :
10 µl of 5X dCTP
5 µl labeled [^{32}P]
Mix well by pipetting up and down.

To start reaction add :
1 µl of Exo(-) Klenow to each sample.
Incubate samples at room temperature for one hour, then
Incubate samples at 37 °C for 2 minutes.

Proceed to purification by NuncTrap column*.

The samples prepared as above have a volume of 52 µl.
Bring each sample to 70 µl total volume with 18 µl of STE.
Pre – wet Nunc Trap column with 70 µl of STE.
Pass 70 µl sample through column using shielded support apparatus to collect flow through.
Count each sample in duplicate using 1 µl of sample in 5 ml of scintillation fluid. The normal activity should be in a range of 300 000 to 1 000 000 cpm/ µl.

E. Probing blots :

Pre-warm hybridization oven to 65 °C.

Prepare prehyb. solution and pre-warm to 65 °C.

	20 ml	10 ml	final conc.
1M Pipes, pH 6.5*	1 ml	0.5 ml	50 mM
dH_2O	11.4 ml	5.7 ml	
5M NaCl	0.4 ml	0.2 ml	100 mM
0.5M Na phosphate, pH 7*	2 ml	1 ml	50 mM
0.5M EDTA, pH 8	40 µl	20 µl	1 mM
20 % SDS	5 ml	2.5 ml	5%
10 mg/ml ss DNA (boiled)*	120 µl	60 µl	60 µg/ml

Put the blot in 2X SSC, roll the blots so that the open edge faces you, with the cap on the left.

Add prehyb. solution 2.5 ml (small bottle), 4.5 ml (big bottle) and prehyb for at least one hour at 65 °C in oven.(can prehyb. for several hours)

F. Hybridization.

The labeled probe is added to the membrane and incubated for several hours to allow the probe molecules to find their targets.

Calculate the amount of probe needed. 1x 10 6 counts/ml. Add that amount to 0.5 ml of the prehyb solution., and boil for 5 minutes.

Add probe to the bottom of the bottle carefully and mix gently. Hybridize ~ 16 hrs at 65 °C.

G. Washing:

To remove any probe on the membrane that is not stuck to the target molecules.

Wash with 5% SDS, 0.5X SSC at 65 °C :
1 X 10 minutes
3 X 20 minutes.

Rinse the washed blots in 2X SSC, remove excess liquid and wrap in saran avoiding air bubbles. Can check with Geiger counter here if desired. If want to decrease stringency, increase concentration of SSC.

H. Detecting :

Expose the membrane to the phosphor image screen for 1-2 days.
To strip membrane for reuse: boil gently for 2 minutes in 0.1X SSC, 5% SDS. Can use a prehyb bottle.
Store blots in 2X SSC in sealed bags.

Materials :

0.25 M HCl = 21 ml of 12 N HCl to 1L

Denaturation solution : 1.5M NaCl/0.5M NaOH = 87.66g of NaCl + 20 g of NaOH to 1L

Neutralization solution : 1.5M NaCl/0.5M Tris-Cl pH 7 = 87.66 g of NaCl + 60.5g of Tris-HCl to 1L. Adjust pH to 7 with 12 N HCl.

1M Pipes : 30.2 g/100 ml. Adjust pH to get into solution.

Na Phosphate buffer, pH 7 : For 100 ml – NaH_2PO_4-H_2O 3.45 g
- Na_2HPO_4 3.55 g, adjust pH , autoclave

10 mg/ml salmon sperm DNA: boil frozen stock as needed x 5 minutes, add to prehyb.

Turbo blotter from Schleicher & Schuell

Systems and refills : 11 x 14 cm 10416304 refills only : 10416306
Systems and refills : 20 x 25 cm 10416324 refills only : 10416326

20X SSC : Gibco # 15557-036

Prime it II Random priming– kit : Stratagene # 300385 (30 rnx)

NuncTrap column purification : Stratagene # 400702

THAWING EMBRYONIC STEM CELL CLONES (IN 24 WELL PLATES)

After screening for homologous recombination, the chosen clone has to be thawed.

1- Pre-warm ES + G418 media to 37 °C.

2- Thaw whole 24 well plate@ 37 °C for ~5 minutes.

3- Add 1 ml of pre-warmed media to each well.

4- Mix to resuspend cells (may need additional thawing)

5- Transfer cells from each well to a 15 ml tube with 2 ml of fresh media.

6- Spin 5 minutes @1100 rpm.

7- Remove the supernatant.

8- Plate on 24 well plate with feeders with 0.5 ml of ES + G418 media.

PREPARATION OF ES CELLS FOR BLASTOCST INJECTION.

1. Feed cells at 8.00 am.

2. Trypsinize at 10.30 am as follows :

3. For clones in 6-well plate trypsinize with 0.5 ml for 5-10 minutes.

4. Add 1.5 ml of ES media, mix well.

5. Break up clumps into a single cell suspension with a 200 µl tip of a 5 ml pipette (200 µl tip is attached to the 5ml pipette). It is important to generate a single cell suspension for blastocyst injections. Avoid foaming of the medium.

6. Check on the microscope to make sure you have a single cell suspension.

7. Pan each well in 2 wells of gelatin coated 6well plate . Add 1ml of cell suspension to well with 1 ml of media.

8. Pan 45-60 minutes.

9. Call Jean Richa to verify time of delivery(the harvested cells need to be ready for injection by Friday noon).

   Lab number : 215-573-3023.
   Office number : 215-898-6064.

10. Collect the supernatant, pool, spin 5 minutes, remove supernatant, resuspend in 1 ml of ES media.

11. Remove 0.5 ml for freeze (optional step).

12. Give Jean Richa 0.5 ml of cells in 15 ml tube .Also provide 5 mls of ES cell media in a 15 ml conical.