Growing R1 cells and their derivatives (ala the Conlon lab)

The main reference for growing these cells is chapter 2 by Wurst and Joyner from Gene targeting: a practical approach, ed. A.L. Joyner. The line was originally established on STO feeders, and the Rossant lab has since switched to either MEFs and media supplemented with LIF or media supplemented with LIF alone.

Our principal modification is growing the cells exclusively on gelatinized plastic in a slightly different media supplemented with LIF.

The cells came from the Rossant lab on feeders and were adapted to growth off of feeders. Following the instructions that came with the line for establishing a pool of R1's on feeders, 4x100mm plates were generated. Three of these were frozen and the fourth was passaged onto gelatinized plastic in medium supplemented with LIF. Most of these adapted cells were frozen to create a large pool of consumable vials at passage 11, but some were kept going for 4-5 more passages to establish a karyotype. Ultimate proof was provided by a number of targeting experiments. The rate in our hands has been roughly 50%.

Components:

Iscove's modified dulbecco's medium, Gibco #12440-020 for 500ml; 12440-046 for 1 L; aliquotted 80ml in small media bottles (125ml); store 4 degrees and protect from light. This comes in liter bottles which are aliquotted into smaller bottles: make sure it's a little orange in color. Discard any aliquots that have turned "kool-aid red".

Hyclone FBS tested for ES cells, heat inactivated, 40ml aliquots, stored -20.

To heat inactivate, thaw serum overnight in refrigerator. Put into a full (i.e. water up to the level of the serum in the bottle) 37 degree water bath and allow to thaw until only a large ice cube remains (approximately 1 hour). Mix the bottle gently and return it to the fridge. Heat the water bath up to 56 degrees (set the safety thermostat to maximum). This also takes about an hour. Make sure you can get to the serum promptly after heat inactivating. Incubate the serum at 56 degrees for 30 minutes, mixing once or twice during this half hour. Mix and immediately aliquot the serum into 40 ml aliquots and freeze. Some white particulate junk floating in the serum is normal.

100x 2-mercaptoethanol (Sigma M7522, 14.3 M stock): dilute 70 microliters into 100ml of sterile ddH2O and filter sterilize; 10ml aliquots; store 4 degrees.

100x MEM nonessential amino acids: Gibco #11140-019 for 100ml; 10ml aliquots; store 4 degrees.

100x Pen/strep: Gibco #15070-014 for 100ml; 10ml aliquots; store -20.

recombinant LIF protein, produced as per the V. Prideaux protocol; filter sterilized; put into 30 microliter aliquots; flash frozen in liquid nitrogen and stored -80; each batch tested for efficacy against an older batch.

Alternatively LIF can be purchased from Gibco (ESGro) following the Wurst and Joyner chapter (see above).

Gelatinized tissue culture dishes:
.1% gelatin (typeB from bovine skin, Sigma G9382); .3g in 300ml ddH2O autoclaved; cover dishes with solution and aspirate off; allow to air dry in hood--approximately 1/2 hour (alternatively can allow to air dry overnight): use within 24 hours.

ES cell culture medium/100 ml:
80ml Iscove's MDM (contains 4 mM L-glutamine and 1 mM sodium pyruvate)
To an aliquot of IMDM add in exactly the order listed:
20ml FBS (20% final)
1ml 100x (10 mM) 2-mercaptoethanol (.1mM final)
1ml 100x MEM nonessential amino acids (.1 mM final)
1ml 100x PEN/STREP (50 u or µg/ml final)
30 µl of LIF (one -80 aliquot)

I prefer not to feed cells with cold medium. The un-supplemented IMDM is therefore warmed up by sitting in the hood for 10-20 minutes with the light off (L-glutamine is light sensitive). Likewise fully supplemented media can be warmed from the refrigerator this way. I prefer not to warm media in the water bath.

R1's are relatively pokey in growth. Standard split is 1:6. Unless otherwise noted one vial should equal one confluent 35mm dish (i.e. 1/2 of a 60mm or 1/6 of a 100mm). To thaw, remove vial from liquid nitrogen and vent, then thaw ASAP in a 37 degree water bath. Gently mix thawed vial and transfer to 8ml fresh medium in a 15ml conical tube. Spin down setting 4 for five minutes and aspirate sup. Plate out one vial onto one fresh 60mm dish (1:2). The next day feed. The following day they should be ready to split 1:6 as usual.

Routine passaging from 35mm dishes and above

ES cells grow like weeds and quickly need more space. Generally cells are fed everyday and split every other day. Cells should be split once confluent. If you can't split them right away and the medium is yellow, then at least feed them. Get back to them as quickly as possible. To prevent differentiation colonies need to be vigorously broken up into single cells when passaged.

-To passage a plate of cells, aspirate medium and wash once with an equal volume (or greater) of PBS. Apply enough trypsin to cover: approximately 0.7-1.0 ml for a 35mm, 1.5-2.0 ml for a 60mm, or 3.0-4.0 ml for a 100mm.
We use a concentrated trypsin solution in Hank's balanced salt solution.

Trypsin-EDTA solution 1:250 .25% porcine trypsin, .02% EDTA-2Na	JRH Bioscience	59228-78P	500 ml	10.58

The 500 ml bottles are thawed at 37 degrees, then aliquotted 40ml per 125 ml bottle and refrozen. Once an aliquot is thawed, store in the refrigerator for one

week--this stuff is very cheap so don't be concerned with pitching some. I will note steps that require freshly thawed trypsin.

-Return plates to incubator for 5 min.

-Using a plugged pasteur pipet with a pasteur bulb, vigorously pipette each plate in order to break the colonies up. Swish all around the dish with a circular stream, avoiding bubbles as much as possible. The goal is an even suspension of single cells. ES cell colonies require vigorous pipetting in order to break them up--the high concentration trypsin and the pasteur pipets help this process, but ultimately it requires your patience and thoroughness. When first starting out examine the plates to see that the colonies are well broken up and mostly single cells. Usually the cells need to be drawn up at least 8 to 10 times to achieve this. When first starting out do only a few plates at a time. I do 6 plates at a time, maximum, since there are only 6 slots in the centrifuge.

-Transfer the suspension of cells to an equal volume of ES cell culture medium. Triturate (i.e. draw up and down) about three times to mix the suspension with the medium and stop the trypsinization. Cells can be left at this stage, for instance to que up more tubes for spinning down.

-Centrifuge the cells on setting 4 for four minutes, then turn the knob up to setting 5 for one last minute. Stop the spin and retrieve tubes promptly. Cells are very unhappy after being spun down so keep the time spent in a mass to an absolute minimum.

-Aspirate medium and promptly break up pellets. Be persistent: do not leave large clumps.

-For routine splitting into 60mm or 100mm dishes, I prefer to add a bolus of concentrated cells to one side of a dish that is covered with medium. Cells are therefore resuspended in a convenient volume of medium--say 0.5 or 1.0 ml for each plate they'll be put into. Since the cells are coming out of a stressful spin, be gentle. Mix cells by running them along the side of the tube. When thoroughly resuspended, apply the cells to one side of the receiving dish and gently rock the plate back and forth so that a wave front goes from side to side. When first starting out, do only one plate at a time and keep the dish down on the metal table--eventually you can do three or four plates by rocking them gently in the air. Whatever you do, do not swirl the dishes as this concentrates all the cells in the middle.

-35mm dishes (and below) are too small to rock back and forth--gently resuspend cells with their full volume of medium and apply to the dish

-The following table lists all the volumes you are likely to need:

dish	surface area,cm^2	volume of medium,ml
96-well	~0.2	0.200-0.300
24-well	2.0	0.500
35mm	9.5	1.5-2.0
60mm	21	3.5
100mm	56	10.0

-Incubate plates overnight and evaluate your technique the next morning. If there seem to be large numbers of clumps, or the dish is highly lopsided (cells weren't evenly distributed), then repeat the passaging onto fresh plates. If you were thorough when trypsinizing this is extremely rare. ES cells usually have a plating efficiency of ~20%. You can therefore expect a lot of floating material the next day.

-Feed the next day. Keep growth in culture to a minimum and freeze cells back.

Routine freezing of ES cells from 35mm dishes and above

For freezing, determine how many 35mm dishes worth of cells you have. Each vial contains the equivalent of one 35mm dish (i.e. 2 vials per 60mm or 6 vials per 100mm). Allow cells to grow highly confluent and feed four hours before freezing.

-To freeze, trypsinize using fresh trypsin just as if passaging (see above) and put into an equal volume of ES cell culture medium.

-Spin down as usual and break up pellets promptly

-Resuspend in 1.5ml of 1x CPM per vial. Gently resuspend cells and put into labeled vials. Put vials on ice until all the vials are ready to freeze.
{1xCPM is plain IMDM with 10% serum and 10% DMSO. Add serum to the IMDM first and swirl. Add the DMSO, swirl, then 0.2µ filter. 1xCPM can be frozen in 7.0 ml aliquots and thawed just before use (keep on ice). Always freeze the aliquots in a frost free freezer first, to insure that they all freeze completely. After that they can be stored in the Tissue culture -20 wrapped in foil to protect from light.}

-When all the vials are ready to freeze, put them in the isopropanol freezing box (kept under tissue culture sink when not in use--note number of uses and replace with fresh isopropanol after 5 uses). Promptly put the box in the -80. After 24 hours the vials can be put in a convenient freezer box and kept at -80 for at least another 24 hours before transferring to liquid nitrogen. They can stay in -80 longer than that (up to several months in fact) but need at least 48 hours to acclimate to extreme cold.

-The isopropanol box is normally stored under the tissue culture sink and takes several hours to thaw out. If you really need it, it can be thawed in the water bath or in a tub of water. It should be at room temperature before use. It sometimes happens that you have more than the 18 vials that fit into the isopropanol box

(for instance, when making feeders). While I wouldn't do this with your most precious stocks of ES cells, you can put the extra vials inside a pair of 15 ml falcon tube racks. Wrap the racks together with tape and put in the -80 as usual. I've never found anything wrong with this. It is in fact what many labs do routinely to achieve a gradual temperature drop (approximately 1 degree per minute) to minus 80 degrees.

-When ready to place vials in the dewer, keep vials on dry ice or inside the isopropanol box still frozen at -80. Since I top off the dewer on Fridays I often wait to put vials in until that day, then put in fresh liquid nitrogen.

Notes on Drugs

G418 (Geneticin)

Geneticin, G418-SO4 powder	Gibco	11811-049 or 11811-015 or 11811-023 or 10131-019	100 mg or 500 mg or 1 g or 20mlx50 mg/ml soln.	

Batches are purchased in bulk from the same lot #. We normally purchase the liquid stock and put into 1 mL aliquots--keeps in the fridge forever. Mock electroporated cells are plated out at electroporation density and subjected to daily G418 selection of varying concentrations for a week to ten days. Minimum concentration that guarantees 100% killing is chosen. Some differences between cell lines have been observed, e.g. 100 µg/mL for R1 cells versus 150 µg/mL for Cast #1-12, but not always.

Puromycin

Puromycin	Sigma	P-7255	10mg or 25mg	12.05 or 25.65

This is made up in 1mg/mL stock in PBS and put into 100 µL aliquots which are stored at -20°C. Requires gentle warming at 37°C to get completely into solution. Each new batch is tested for efficacy in kill curve experiments. So far no variation has been seen in several batches. Some variation between cell lines. R1 cells are markedly less sensitive to puromycin: while other lines typically die with .75 micrograms per ml, R1 cells require twice that amount for equivalent killing (i.e. 1.5 micrograms per ml). Very fast drug--death is complete in three days.

Gancyclovir (Cytovene)

Usually purchased as a prescription from an obliging M.D.
Stock is 22mM in PBS, distributed into 1 mL aliquots and frozen at -20°C. Large aliquots are then re-aliquotted at 10 µL and re-frozen without any problems. Tested for efficacy on mock electroporated Tk+ targeted cell lines. The stock is reported to be 10,000x. However, kill curves have shown repeatedly that 1/2x or even 1/4x is sufficient for complete killing in as little as three days in Tk+ cell lines. Lot of concern with bystander killing with this drug: best proof of this is in Jim Thomas' paper ((1998) *Proc Natl Acad Sci U S A* **95,** 1114-9) where he got decidedly fewer colonies with GANC than with FIAU.

FIAU(1-2'-deoxy-2'-fluoro-ß-D-arabinofuranosyl-5-iodouracil)

FIAU	Moravek Biochemicals	M-251	1 mg or 5 mg	65.00 or 205.00

Stock is 200 µM and requires an elaborate protocol to get into solution. Add 388 mg of FIAU to 9 mL PBS and add NaOH until the FIAU has dissolved. Make total volume to 10

mL. This solution is 100 mM and must be diluted 1:500 in PBS to give the 1000x stock solution. Store at -20°C, usually in 100 µL aliquots. (this recipe comes from the Bradley chapter in Methods in Enzymology Vol. 225). Scale down this recipe as appropriate for smaller amounts of FIAU. The published 1x concentration, 0.2 µM FIAU, has always proven correct in kill curve experiments (probably the only drug for which this is true). The drug of choice when selecting rare Tk- variants from a predominantly Tk+ population. Some concern about bystander killing when cells are on feeders (see:You, Y., Browning, V. L. & Schimenti, J. C. (1997) *Methods* **13,** 409-21). Most published accounts of selecting rare Tk- variants from a predominantly Tk+ population have in fact been done feeder free, but see Li, Z.W., *et al*. *Proc Natl Acad Sci U S A* **93**, 6158-62 (1996) for an exception.

David's Notes on preparing supercoiled DNA

Since most people have their own preferences with this task you are completely at your leisure to ignore this section. This procedure works for me.

-Most plasmids are kept in bacteria as glycerol stocks, restreak some out to achieve single colonies. If using a recently created plasmid, restreak some of your original miniprep culture to obtain single colonies. **I always go from single colonies on a plate.**

-Establish seeding cultures by picking 4 single colonies for each plasmid. Pick each colony into 3ml LB+amp. Allow to grow between 5-8 hours during the day. (The extra colonies will insure a good grower for seeding--should one of them prove non-amp resistant you can still proceed.)

-Before leaving for the day, seed 1 or 2 of the cultures 1:100 in LB+amp. (I normally do 250 ml large scale cultures. At 1.5-3 µg of plasmid per milliliter of culture this assures 400-500 µg of final purified plasmid.)

-Allow to grow overnight at least 12 and not more than 16 hours. Remove some for a miniprep and 0.85 ml for a glycerol stock. (I've had some plasmids that would not grow in large scale, so I prefer to check them by miniprep before I do a bunch of Qiagen preps)

-Spin down at 6000 rpm in the Beckman or Sorval. Dump sups and invert tubes over paper towel for a few minutes, then wipe out the inside of the tubes with clean kimwipes. Cultures can be stored -20 forever at this point. (I often que up several plasmids, even keeping back ups in the freezer in case something happens while preparing the DNA for transfection.)

-At this point I follow the Qiagen instructions for tip-500s using the maxi prep protocol. I prefer to use polycarbonate Oakridge tubes for the final DNA spin.(I reserve these tubes for my own exclusive use that I wash out myself--DNA is very sticky and you do not want to transfect someone else's DNA into your cells.)

-After the final spin I visualize the pellets, marking them with a pen on the tube. I then apply 1 ml of 70% ETOH and gently rinse the pellet, inverting the tube and allowing it to air dry for about 5 minutes. The pellets are large and sticky at this point so I apply 0.8 ml of regular TE to the pellets and keep them on their sides for 8 hours to overnight in the fridge to allow complete resuspension.

-Each preparation is split in half: 0.4 ml goes into two eppendorf tubes with 16.7 µl of 5 M NaCl to yield 0.2M final. Two milliliters of 95% ETOH is then applied to the sides of the Oak ridge tube. After rinsing the Oak ridge tube several times with the ETOH, I then put put 1 ml per eppendorf tube, mix (at which points a precipitate should be observable), and store the eppendorfs in the -20 for 20 min-1hour. Spin down and wash as usual, allowing to air dry for 5 min. Put each pellet in 100 µl of regular TE and store 8 hours-overnight in the fridge to allow complete resuspension. DNA can be stored at -20 forever at this point.

-Having milligram quantities of supercoiled DNA in the -20 is my minimum criteria for thawing ES cells and preparing them for transfection.

Preparing supercoiled DNA for transfection

-Normally targeting vectors need to be linearized before transfection. I prefer to linearize fresh before a transfection, and as the cells grow for 5 days there is plenty of time to do this (and even do it twice if need be).

-To each 1/2 of a prep, containing approximately 200-250 µg of supercoiled DNA, add the following:
40µl of 10x Enzyme buffer
50-100 units enzyme (I prefer a new tube of enzyme for this)
bring up to 400 µl with sterile ddH2O.
Digest 3 hours-overnight (with addition of more enzyme)
Remove a small aliquot to ensure complete digestion.

-Extract the 400µl of DNA with an equal volume of 1:1 phenol:Chloroform-IsoamylAlcohol (CIA: this is 24 parts chloroform and 1 part isoamyl alcohol).
-The sup is extracted once with CIA and the final supernatant is precipitated with NaCl and ETOH as usual.
-On the final wash with 70% ETOH take the tube into the hood and remove the sup with a P1000.
-Allow to air dry 5 min and apply 104µl of Low EDTA-TE (10 mM Tris-HCl pH 8.5; 0.1 mM EDTA).
-Store tube in the fridge for at least 8 hours to resuspend.
-Remove 4 µl and run some out on a gel and do the usual A260/280 nonsense.
-I prefer clean DNA, but I don't throw out dirty stuff in case I need it in a pinch. (remember this is the ONLY part of the process you actually control.)
-Keep linearized DNA in the fridge until consumed. You will need 40µg of linearized DNA per transfection so it quickly gets used up.

-Suprecoiled expression plasmids, like those for Cre recombinase, are prepared as follows:

-To the 100µl of DNA add 300µl of sterile ddH2O and extract twice with an equal volume of 1:1 phenol:Chloroform-IsoamylAlcohol (CIA: this is 24 parts chloroform and 1 part isoamyl alcohol).
-The sup is extracted once with CIA and the final supernatant is precipitated with NaCl and ETOH as usual.
-On the final wash with 70% ETOH take the tube into the hood and remove the sup with a P1000.
-Allow to air dry 5 min and apply 104µl of Low EDTA-TE (10 mM Tris-HCl pH 8.5; 0.1 mM EDTA).
-Store tube in the fridge for at least 8 hours.
-Remove 4 µl and run some out on a gel and do the usual A260/280 nonsense.

-I prefer clean DNA, but I don't throw out dirty stuff in case I need it in a pinch. (remember this is the ONLY part of the process you actually control.)
-Aliquot your final purified expression plasmid in convenient quantities--usually 25-50 µg per aliquot.
-Store -20 in a special "electroporation ready" box.

Notes on Transfection

We generally follow the Wurst and Joyner transfection protocol (chapter 2 from Gene Targeting: a practical approach) with slight modification.

-On Wednesday I thaw one vial of R1 p11s onto 1x60mm dish as usual. This is best done late before you leave to allow more lee-way in preparing the cells.

-On Thursday feed.

-On Friday split 1:6, usually onto 2x100mm dishes. (The ratio can be varied. For instance, if you won't be able to get to them till late on Saturday do 1:8 to allow more lee-way).

-On Saturday feed.

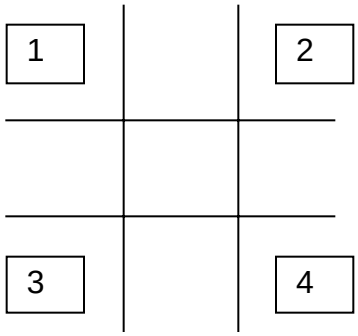
-On Sunday split cells 1:2 for transfection by the afternoon on Monday. Split them to a larger extent if you're planning to get to them later (if, for instance, you need more time to get the DNA resuspended).

-Early on Monday feed the cells 4 hours before electroporation. The procedure will take an hour to half a day to complete. (A dense plate of R1s will contain 15-20 million cells, enough for 2-3 electroporations per plate)

-Cells are trypsinized with 3-4 ml of fresh trypsin and spun down as usual. Gently resuspend cells in ~1.5 ml ice cold PBS per dish and pool them into one tube. Remove 0.1 ml for counting and store the rest on ice.

-The 0.1 ml of cells is put into 0.9 ml of erythrosin B (Sigma E-7379; 0.1 g dissolved in 100 ml PBS). Mix and apply a small volume to hemocytometer.

-Count the four 4x4 squares, usually the areas numbered 1-4 on the diagram below:



-Take the average of the four counts. Multiply this by 10^5 to yield the number of cells/ml. The final desired concentration is $7x10^6$ cells/ml. It's OK to re-spin if too dilute, but not preferred.

-Usually one does 6-12 electroporations at once. It's usually easier to make cocktails of cells+DNA and aliquot 0.8 ml into cuvettes. Each cuvette should contain:

0.8 ml of cells at $7x10^6$ cells/ml
40 µg linearized DNA of usually negligible volume

-When adding DNA and cells avoid bubbles (air is a resistor). Gently mix each cuvette. Allow to rest 5 min room temperature to allow cells and DNA to mix completely. Mix again half way through the 5 minutes.

-Remove the cuvette holder from the Tissue culture Freezer and put a cuvette into the holder. Slide into the electroporator and pulse at 0.240 kV and 500 µF. Repeat until all the cuvettes are electroporated. (Some familiarity with electroporation, and/or reading the manual is warranted for basic safety).

-Note the tau value of each electroporation--this will vary by tissue culturist and DNA. I normally average 6.8 when I do everything. (Each individual will establish their average as they go along. It's mostly irrelevant unless there are problems, in which case you'll want to be more meticulous and discard outliers)

-Each cuvette then goes on ice for 20 min. During this time you can warm up your media.

-Using a 1 ml pipette gently mix each cuvette, draw up and put into 1.5 ml of medium per cuvette. Wash each cuvette with 1.0 ml of medium and pool all the electroporations and washes.

-Remove several 0.050 ml aliquots and plate out on 6-well plates for quantification.

-Plate out 3.0 ml of the pooled electroporations into 7.0ml of medium on 1x100mm dish. Repeat for all the electroporations (the last plate is usually a little short)

-The next day observe plates to see if they have been evenly distributed. The expectation is that 50% of the cells died under the conditions of electroporation. (This was explicitly determined once when the electroporator was brand new with mock electroporated cells. If severe systemic problems are occurring with routine electroporations it may be worth re-verifying the conditions)

-Approximately twenty-four hours after the electroporations were plated out feed the cells with medium+G418+Gancyclovir (or whatever drugs are appropriate: see notes on drugs above). Also feed the quantification plates.

-Feed daily with media supplemented with fresh drugs. In G418 cells will take 3-4 days to start dying appreciably. After about 7-10 days of selection colonies will usually be visible.

-When the quantification plates have grown up they can be stained with methylene blue solution (0.33 g methylene blue, 0.11 g basic fuchsin in 100 ml of methanol). Aspirate medium from plates and wash once with PBS. Apply methylene blue to cover completely and incubate 5 min room temperature. Wash liberally with distilled water, dry the plates, and count colonies.

-Rates of transfection usually vary, especially between individuals. I would expect 500-1000 stable tranfectants per electroporation. Negative enrichment varies considerably as well. Using gancyclovir and single tk negative selection an average of 5-10 fold enrichment is expected. Enrichments that are much higher or much lower (that is 100 fold or 2 fold) are suggestive of problems and usually warrant repeating immediately, if for nothing else than to establish expectations for scaling up.

-Regardless of the numbers generated, at least 300 to 500 colonies need to be screened in total before concluding that a construct does not work. Often additional constructs are made in parallel as a back up against this possibility.

Notes on picking and growing colonies in 96-well plate format

The basic procedure follows closely the Bradley chapter from methods in enzymology volume 225: Guide to techniques in mouse development (pp. 855-877, chapter 51). This provides a basic guide but we have made several modifications to it. In particular we pick colonies in media into media. Before starting you must have a dedicated octapipetteman and Inotech octapette aspirator—if you don't then use one of the older 24-well techniques. Once trained it takes approximately one hour to pick a 96-well plate, so if you're going to do 5 plates plan accordingly.

-After 7-10 days of selection the colonies should be visible to the naked eye and most of the debris should have cleared away (feed every other day until ready to pick and discontinue any negative selection).

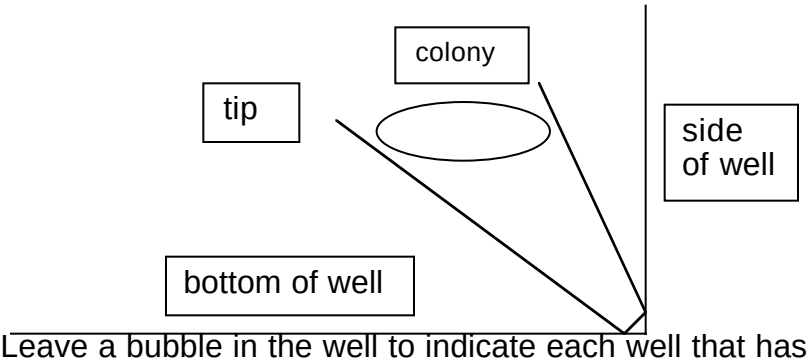
-Coat a 96-well plate as usual and fill with 180 µL of medium+G418. Warm up in incubator for at least 10-15 min.

-Remove 100mm dish and fresh 96-well from incubator. Draw a convenient grid on the bottom of the 100 mm dishes (I prefer to pick a different 100mm dish for each 96-well and rotate between the dishes and plates. That way no pair of plates stays out for an excessive amount of time).

-Use a P10 set to 10 µL and clean unbeveled yellow tips to pick colonies (I use pulled glass pipettes and a mouth pipetor, but everyone else has used yellow tips and it is much faster to learn and become proficient at it this way).

-Depress the plunger on the P10 and nudge the colony to dislodge it. Then rotate the P10 to a vertical position and release the plunger upward to suck up the colony. The colony should be visible in the tip. (this takes some practice but soon becomes second nature).

-Move to the 96-well and position the tip at the junction of the bottom of a well and its side (see diagram below). Draw up several times to break the colony up



into 3-5 pieces. Leave a bubble in the well to indicate each well that has received a colony.

-Examine the 96-well to see how you did. Usually this takes a while to become proficient, so you won't always have to check. If the colony is not well broken up it will be OK—3-5 pieces is a preferred goal.

-Continue picking for a couple of rows, using a clean tip each time. Then rotate to another pair of plates to give the current set a break.

-Once finished return the 96-wells to the incubator: ignore them the next day, except to check how they are settling down. Assuming this looks all-right you can now discard the primary selection plates.

Tryplating

The goal here is to achieve a nice even suspension of each cell line so they will spread out evenly. Avoid excessive jarring of recently tryplated colonies as this will tend to lump the cells to one side of a well.

-On the second day after picking colonies aspirate the medium and wash once with 180µL of warm PBS. When adding solutions to wells squirt against the side of each well, that way you won't need new tips for each row. (the chunks of colonies will often be distributed to one side of the wells. If so you can turn the plate around so that the aspirator touches the empty side of each well).

-Apply 50 µL of recently thawed trypsin (prefer fresh since this is an important step) and put the plates in the incubator for five minutes. (the trypsin will be stopped with medium next step—thus you can do up to 5 plates).

-Observe that the chunks of colonies are floating nicely and add 180µL fresh medium with G418. Do this to every plate to stop the trypsinization.

-Set the octapipetteman to 150 µL (to avoid sucking up solution into the barrel). Draw each row up vigorously 5-8 times until the suspension are single cells. Change tips each time for each row.

-Observe how well broken up they are after doing a few columns to evaluate your technique. If still not single cells then try more pipetting. If that doesn't work (if for instance the trypsin was bad) then do the best that you can. You'll want to repeat this whole procedure tomorrow after they've attached, rather than allowing a large proportion of your colonies to grow as clumps—checking early will make this highly unlikely.

-Place the plates back in the incubator in an out of the way spot. Try not to jar the plates much as this will tend to lump the cells to one side rather than settling down evenly across each well.

-Promptly change the medium the next day, preferably early the next morning so that they don't sit in diluted medium for a long time. Change medium daily hereafter for 3-5 days until they are highly confluent. Maintain G418 throughout these steps to weed out any false positives and assure clonality to your lines.

Freezing in 96-well plates

Realize from the outset that this is a high-stress freeze and colonies are not happy. Have all your probes already figured out and reagents ready for action so that you can retrieve your colonies from freezing as quickly as possible. Only one diagnostic southern is required to reduce the number of colonies to a manageable number—the other side of the recombination and other probes can wait until you have thawed your lines and know that they look OK.

-There are several items to prepare ahead on the day of freezing:

Feed the highly confluent plates 2-4 hours in advance (no drugs).

Gelatinize 2x96well for each original plate. These are for making DNA replicates.

1x96 well freezing plate for each original plate:
U-bottom polypropylene 96-well plates and matt caps from Marsh Bio Products; autoclaved. To each well is added 30µl of 2x freezing medium (This is plain Iscove's containing 20% serum and 20% DMSO (hybri-max)—freshly made and kept on ice). Overlay each well with 100µl of sterile mineral oil (embryo tested, 0.2 micron filtered). Put the labeled matt cap on loosely and place the plate on ice in dark.

Sufficient medium to put 105µl/well.

-When everything is ready, wash each plate once with PBS and apply 25µl of fresh trypsin.

-Place the plates in the incubator for 5-10 min. Gently agitate the plates and visualize that the colonies are floating freely.

-Apply 25µl of medium per well. At this point you can divide the plates into sub groups of 2 to process for freezing.

-Put the appropriate labeled freezing plates on a shallow tray with flat compacted ice.

-Set the P50 octapette at 30µl and vigorously break up a column of colonies. Transfer 30µl to the appropriate column of the freezing plate, stabbing under the oil and mixing gently 3 times.

-Repeat for each column using fresh tips until finished. Fully place the matt cap on the freezing plate and return it to ice. Process the second plate.

-When both freezing plates are finished transfer them to a stryrofoam box (NEB shipper or a pair of lids to a Sarstedt freezer tray taped together) and place the box in the -80.

-Repeat the processing steps until all the plates are in the -80.

-Now return to the original plates (which have 20µl/well remaining) and add 80µl/well. Return them to the incubator temporarily.

-Put approximately 100µl/well of medium in the 2x96well DNA replicate plates. Put these in the incubator as well.

-Remove an original plate and its 2x96well replicates (Do not attempt to do more than one original plate at a time).

-Gently mix the original plate and transfer 50µl of suspension to each replicate, mixing gently each time. The replicates should now have all the cells.

-Return the replicates to the incubator. Discard the original.

-The DNA plates can usually be ignored the next day, fed the second day and every day thereafter (no drugs are necessary because any false positives have already died).

-After the freezing plates have equilibrated at -80 for at least 48 hours, they can be transferred to liquid nitrogen. Wrap each plate in tin foil (so that the lid can't float off) and put on dry ice. Place two plates into a large dewer box and return to dewer (will be a tight fit but just jam it in there).

96 well Polypropylene U-bottom microplates **(Check autoclaved reserves)**	Marsh Bio Products	AB-0796	100/case	100.00

Autoclavable mat cap for deep well plates **(Check autoclaved reserves)**	Marsh Bio Products	AB-0674	50/case	106.00
Mineral oil, embryo tested **(Check 0.2µ filtered supply)**	Sigma	M8410	500 ml	15.45
DMSO, tissue culture tested, Hybri-max	Sigma	D2650	5x5ml	

Extracting DNA

Allow the DNA replicates to grow massively confluent. The protocol says they should turn the media yellow every day for 4-5 days (feeding every day as usual). I have gotten sufficient DNA from colonies grown only two days, so there is clearly some lee-way.

-Label all the dishes and lids to avoid confusion.

-Aspirate the medium and wash twice with 180 µL warm PBS.

-Apply 50 µL of freshly prepared lysis buffer to each well and cover the top with plastic-wrap. Put the lid back on and stack the dishes. Wrap the stack with plastic wrap and put into a closed, humid container, such as tupperware with wet paper towels. Put the humidified container at 50-60 degress overnight.

RECIPE FOR LYSIS BUFFER:

10 mM Tris-HCL, pH 7.5	0.1mL 1M Tris stock
10 mM EDTA	0.2 mL 0.5M EDTA stock
10mM NaCl	0.02mL 5M NaCl stock
0.5% sarkosyl	1.0 mL 5% stock of N-lauroylSarcosine sodium salt aka sarkosyl.(Sigma L-9150). Stock is prepared in sterile ddH2O with gentle mixing to dissolve—no other histrionics required.
1mg/mL proteinase K	0.5 mL 20mg/mL proteinase K stock. 8.18 mL sterile ddH2O 10 mL total (enough for 2 plates)

-The next day gently remove the plastic wrap. Do not concern yourself with small amounts of condensation on the wrap.

-Apply 100µL ethanol+ salt (10 mL of 95% ethanol+ 0.15 mL of 5M NaCl stock, put on ice) to each well. Put lid back on top. Do not mix vigorously—just squirt it on and go to the next row.

-Leave out on bench for 30-60 minutes, preferably on a dark piece of paper to visualize the DNA. Do not disturb the plates, knock them, or otherwise jar the solutions.

-DNA should be visible as a white filamentous network or a white sheet.

-Gently Invert the plates on a stack of paper towels.

-Wash three times with 150µL 70% ETOH, inverting gently each time.

-After the last wash invert once more to remove trace ETOH (you can be a little rougher here—visualize the DNA as you go to see how you are doing).

-Tilt plates slightly and allow to air dry for 20 minutes. Do not over-dry the plates as this makes resuspension harder.

-Apply 20 µL of low EDTA TE to each well (10 mM Tris/0.1mM EDTA) and cover the plate with plastic wrap. Put the lid back on and stack your dishes, then wrap again for safe keeping. Store overnight in the fridge (good forever).

Digestion and running the gels

After resuspending overnight the DNA is ready to digest. If the wells were dense then there is enough DNA for two gels, so you can load half of each digest and still have a nice bright smear. Freeze the remainder of each digestion as a back-up.

DNA	20µL
10x buffer	4 µL
BSA (100mg/ml)	0.4 µL (100µg/mL)
Spermidine (50mM)	0.8 µL (1mM final) {make concentrated spermidine in sterile ddH20, aliquot and store -20: no other preparation required}
RNase (10mg/mL)	0.2 µL (50µg/mL; this is standard RNAse ala Maniatis, i.e. heated to eliminate trace DNases)
Restriction enzyme	10-40 units (I prefer Boehinger[Roche] or new england biolabs enzymes. Always buy new tubes of enzyme for this so there is no chance of contamination by sloppy lab-mates and because you'll use a lot of it anyway. If the enzyme is not a reliable type, such as EcoRI, HindIII, BamHI, XbaI, etc., it is definitely worth trying it out first on some spare DNA prepared by this method to be sure it will work reliably).
Sterile ddH2O	q.v. 40µL total volume.

-Add cocktail to your DNA giving yourself enough for 2-3 extra rows. Mix gently by stirring the DNA with the cocktail and drawing up once or twice (DNA is very viscous and this will give you a sense of how thick your samples are--useful for comparing after they've digested overnight).

-Seal each plate with plastic wrap and put the lids back on. Stack several plates and wrap them together as well. Put in a humidified container and place in a 37 degree warm room or oven overnight.

-The next day check a few wells by drawing them up with a P200 and seeing that they drip smoothly down. If they seem too viscous add some more enzyme

(high concentration enzymes come in handy for this) up to 1/10 the total volume. Alternatively you can run a few microliters of some rows on a mini-gel to see that you get a nice smear.

-Assuming the digests are complete add 5µL 10x load dye (we usually use glycerol dye) and load 25µL of it into a 0.7% agarose/0.5X TBE gel. We have very large 12x8 inch gels which easily accommodate three rows of our thin 36 well combs. By loading 32 samples on each row of the gel you have room for a whole plate plus markers.

-SPECIAL NOTE ABOUT BRB: This building has terrible environmental controls, and it never fails that a brisk wind is always blowing in the bay in which you are loading a bunch of Southerns. Be aware that because some of the samples will still be a little viscous (they'll still work, they're just slightly thick) you'll want to be careful about the wind, since if a trail of sample reaches the top of the buffer surface, the wind will suck the sample out of the well. To counter this I always have the safety lid for the gel box resting on the box, just slightly below the row of wells that I am currently loading. If a sample is gooey and has a long trailer of goo floating up, as quickly as possible I cover the gel box with it's safety lid and wait for the trailer to sink down below the surface (believe me this works and can save your sample). As a bonus having the lid just below the row you are loading will guide you where to load as you robotically proceed with endless samples.

-There are two ways to keep the samples from running into each other. Run fast at 2 V/cm for about an hour until the samples have entered nicely, then separate the gel into three slabs with a clear space between them. Turn the juice down to 1 V/cm and run overnight. Alternatively you can start them running late at night and get back to the lab early the next day to separate the gel into three slabs.

-The gels are run overnight at 1 V/cm. I have easily detected large bands in the 22kb range after an overnight run. My usual practice for detecting 3 kb or higher is to run until the first dye (bpp) runs out and the xylene cyanol dye is about halfway down each slab.

-After running I always try to take a picture of each slab, since this provides a valuable back-up if you should mis-label any of the blots: the occasional blank wells from false positives can then be used to orient the autorads with the original picture.

-We use the basic Sleicher and Schuell Nytran plus membrane with alkaline transfer, but I see no reason to switch to this if you prefer something else and are confident that it works well.

Blotting, probe and hybridization: notes

I won't go over everything ad-nauseum, just a few pointers.

-Be aware that the gels are large: I keep the slabs within the casting tray during preparation and aspirate the solutions off with a 4L side arm flask hooked up to a sink vacuum.

-Don't even try to lift slabs out with your hands: always use the casting tray to lift them out and slide them off the tray onto your transfer rigs.

-I usually do two slabs per rig. Since they're big they need a large volume of buffer--at least a liter per rig.

-I generally block the probe with Cot1 DNA from Gibco-BRL (catalog number 188440-016). They have a clear protocol for using it for this purpose. Since blocking is so easy I don't think it's worth the risk of not doing it.

-A two day exposure is worth the extra day to see faint bands from slow growing wells.

Notes on thawing lines out

Once you've accumulated some long exposures to be sure of the entire plate, get the positives out of the 96-well freeze ASAP. After thawing them you'll quickly see why I said this was a high-stress freeze. You can count on being miserable during this anxious period: remember they usually pull through.

-As with any thaw you'll want to do this quickly. The fastest way I've found is to stack old yellow pipet tip racks in the 37 degree water bath until there is only a few millimeters of warm water above the rack (obviously you don't want the water to be so high that it seeps into the lid--practice with an old plate).

-Remove a frozen 96-well plate (keep any others in the box at -80) and remove the parafilm. Put the plate on the rack in the 37 degree water bath. To keep it in close contact with the water put a lead pig or spare beaker on top to weigh it down. Usually takes 8 to 10 minutes to thaw.

-Once you can see that the majority of clones have thawed (the wells will be translucent) remove the dish from the water. Place on top of 70% ETOH soaked kim-wipes and wipe all around the top and edges.

-Clearly mark all your positive clones. Also select one or two non-targeted lines to maintain as negative controls.

-Using a P200 set at 105 µl, stab under the oil and gently stir the well to mix. Draw up the entire volume plus a little oil. Place this in a 24-well with 0.5ml of warm culture medium. Rinse the tip to disperse cells evenly and remove 105 µl of the 24-well and put it back under the oil of the positive clone. Draw up repeatedly to mix and get most of the cells. Return this to the 24-well. Repeat--you can also examine the 96-well to see that most of the sample has been removed. Do not be concerned with little bits of oil--they can easily be skimmed off the 24-well later.

-Allow to grow overnight and replace with fresh culture medium promptly. At the earliest this can be done about 12 hours after plating. I usually remove and hold onto the 0.5 ml from each well until I'm sure there are cells attached to the 24-well.

-Since they are unhappy I refrain from all drugs. I feel that once picked and grown up for Southerns in the presence of G418 then a line is clonal and does not need any more selection.

-They will usually be refractory and look awful for a day or two. If the 24-well is vigorously growing, then you can passage to a fresh 35mm dish (that's a 1:5 split). If still looking poorly, passage to one or two fresh 24-wells. Sometimes this passaging gets them jump started. Don't leave them on the original 24 well for more than four days (feeding daily of course). Once up to the level of a 35mm dish you can passage them normally and freeze a vial or two back. Some derivatized lines act a little different in terms of growth dynamics--as always be flexible and use your best judgement.

-In addition to preparing a plate or two for freezing I also prepare 1x35mm for DNA. This is overkill but ensures a ton of DNA for additional characterization. It also requires a different protocol (see below).

-I also prepare 1x35mm for karyotyping, but stop at the first fixative step (step #7, below), leaving them in the fridge until I'm sure they are targeted and worth preparing spreads. This way I can leave my founder vials undisturbed until it is time to inject. When just starting out I wouldn't bother: instead relax and bask in the pure joy of having your lines safely frozen back.

Extracting DNA from 24-wells or larger

Original reference in Wurst and Joyner chapter 2.

-Allow plates to grow confluent. If there are slow growers just keep feeding the fast ones until they all que up.

-Aspirate medium and wash twice with PBS.

-Apply lysis buffer according to the following table:

dish	volume lysis buffer, ml	volume of TE to resuspend, ml
24-well	0.5	.050
35mm	2.0	.200
60mm	4.0	.400
100mm	10.0	1.0

Recipe for lysis buffer:

100mM Tris-HCl, pH 8.5	1.0 ml of 1 M Tris-HCl, pH 8.5
5mM EDTA	0.1 ml of .5M EDTA
0.2% SDS	0.2 ml of 10% SDS
200mM NaCl	0.4 ml of 5M NaCl
100µg/ml proteinase K	0.050 ml of 20mg/ml proteinase K

q.v. to 10.0 ml

-Wrap dishes or plates in parafilm and enclose inside a humidified container. Incubate 3 hours-overnight at 37 degrees.

-If in 24-wells the precipitation can be done inside the dish, otherwise transfer the lysate to either a 15ml Falcon (35 or 60mm) or a 50ml Falcon (100mm), using a cut blue tip or large bore pipet. Lysate is quite gooey.

-Add an equal volume of Isopropanol to the dish or tube: just dump on top: do not mix vigorously

-Put some plastic wrap over the 24-wells and put lid back on. Weigh dish down with several lead pigs. Spin on an orbital shaker, starting at very low speed and gradually turning up until going the fastest possible without spilling the wells. Tubes are done on a nutator.

-Allow to rotate for at least 20 min at room temperature. A large white jelly fish will begin to appear at the interface of the alcohol with the lysate. The longer you rotate the tighter and easier to manage these jelly fishes will become.

-Using a pasteur pipet, carefully remove the supernatant leaving the jelly fish behind. Apply an equal volume of 70% ETOH and rotate for another 20 minutes at room temperature.

-Lift the precipitate out with a clean yellow tip (or blue tip if really big). Put the white mass into a tube with the appropriate amount of TE. Sometimes the precipitate sticks to the tip. Carefully scrape off on side of tube.

-Incubate tubes at 50-60 degrees for 30 minutes with lids open to evaporate trace ethanol.

-Store tubes in fridge overnight (at least)--good forever.

-Cut approximately 15 µl of DNA in a digestion with identical conditions to that used with the initial Southern screen (see above), but skip the RNAse (not needed). The DNA is very viscous and takes some effort to remove 15 µl exactly (don't worry if it's a little short). I normally run the entire reaction out on a 0.7%agarose/0.5x TBE gel as usual.

Counting ES cell Chromosomes

(original reference: "tissue culture made easy" by Christian LaMantia from the Magnuson lab)

1) Plate cells onto gelatinized plates (35 or 60 mm) without feeders and culture 24 hours. A 1:4 split is best for most lines.

2) Culture for four hours in the presence of colcemid (3.125 µl of 10 µg/ml colcemid (Gibco # 15212-012) per ml of medium). 2ml per 35 mm and 4ml per 60 mm.

3) Remove media and place it in a 15 ml Falcon tube. Wash with trypsin, and trypsinize with 1.5 ml trypsin.

4) Centrifuge on 4 for 5 minutes. Aspirate supernatant and flick pellet.

5) Add 37°C KCl (0.559 g KCl in 100 ml water) drop by drop, flicking the pellet with each drop for the first 10-15 drops. Bring volume up to 7 ml, invert several times, and incubate in 37°C water bath for 10 minutes.

6) Centrifuge on setting 4 for 5 minutes.

7) Aspirate supernatant and flick pellet. In the next step, be careful to disperse the cells thoroughly in the fix. Add fresh fix (2 ml glacial acetic acid and 5 ml methanol) drop by drop, flicking the pellet with every drop for the first 10 to 15 drops. Cap and incubate at 4°C overnight. Can be kept in the fridge at this point forever, then processed for spreads when convenient.

8) Spin down and aspirate old fix with pasteur pipet. Resuspend cells in 0.5 to 0.75 ml of fresh fix and gently draw suspension up in a pasteur pitet(will make 4 to 5 slides).

9) Dip slides (plain, pre-cleaned slides (Fisher #12-549) labelled with etching tool) in water, leaving a puddle of water on the slide. Drop 3 drops of cell suspension per slide and air dry. Drops should make a gentle splat from a height of approximately one foot above the slide.

10) Stain for 10 minutes in Coplin jar with Giemsa (2.5 ml of Giemsa (Gibco #10092-013) in 47.5 ml of Gurr’s pH 6.8 buffer (Gibco #10582-013)).

11) Rinse with water 3-4 times until the excess stain is removed. Air dry.

Five spreads on 4 slides give a reasonable sampling. Scan slides with a 40X objective and count with 100X objective. Look at spreads in widely separated fields. Spreads with fewer than 40 chromosomes are ignored as probable splattered cells. Lines with 41 or 42 chromosomes are the usual problem.

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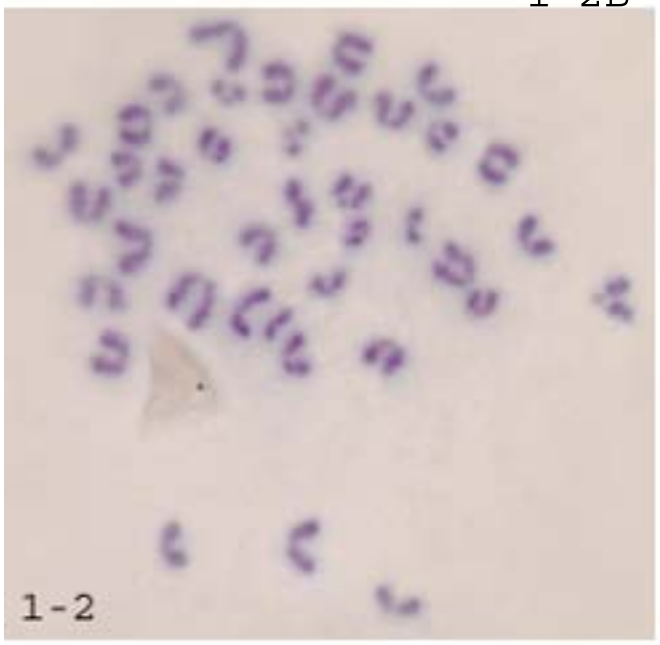

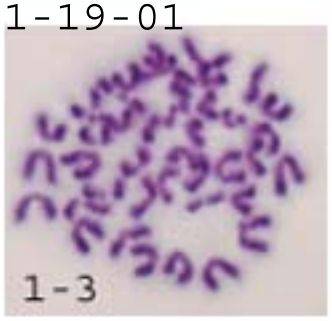

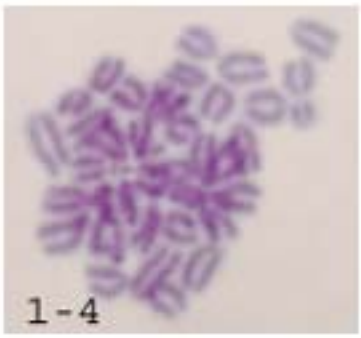

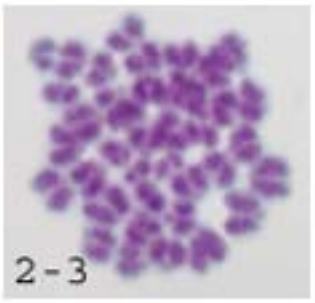

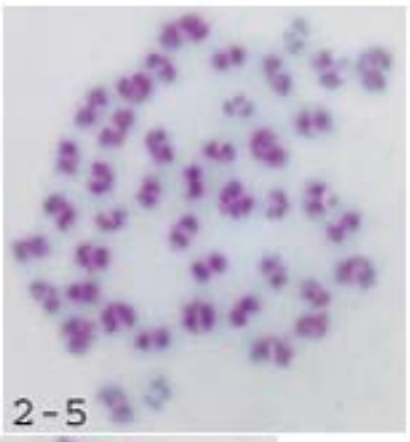

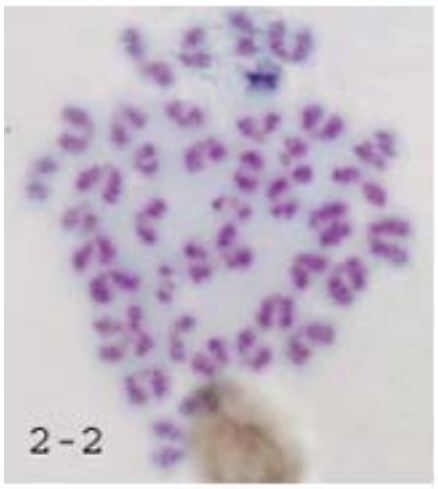

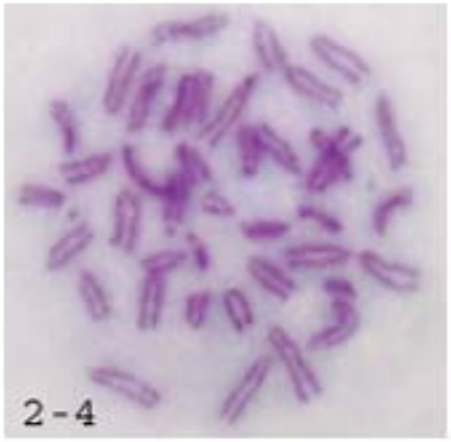

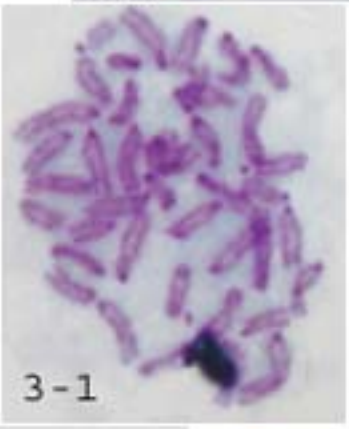

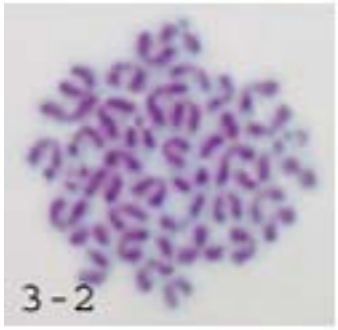

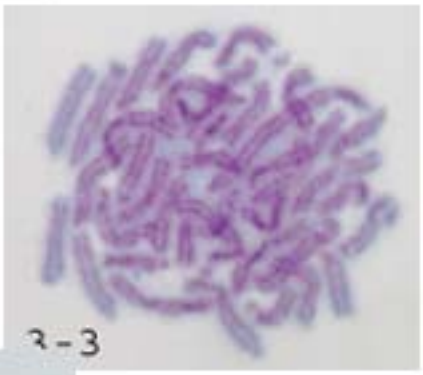

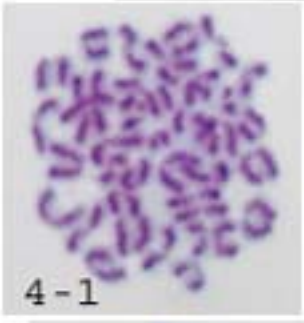

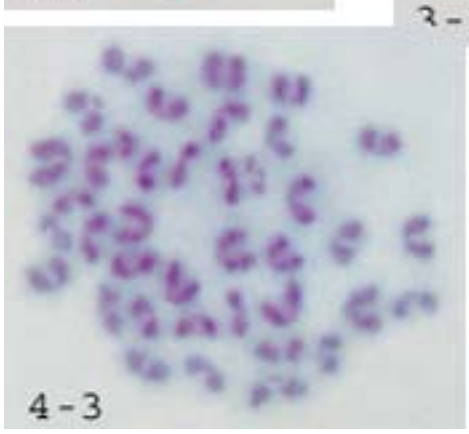

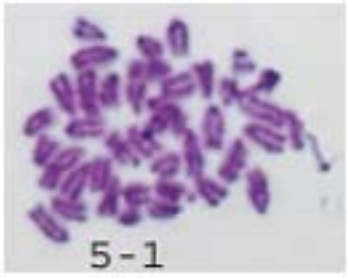

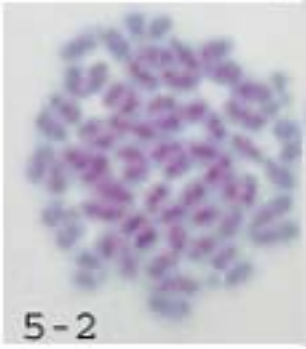

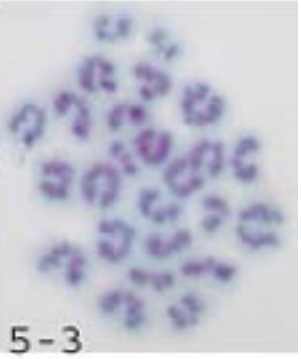

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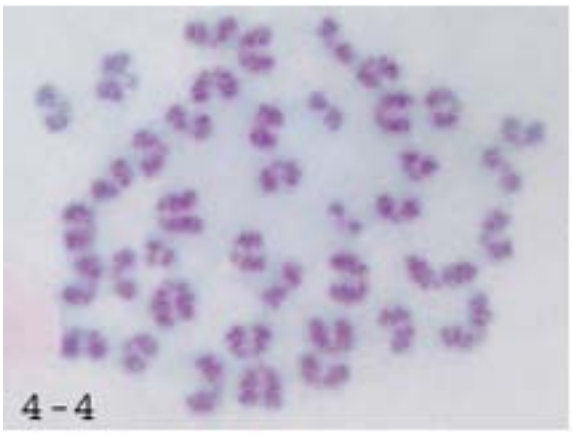

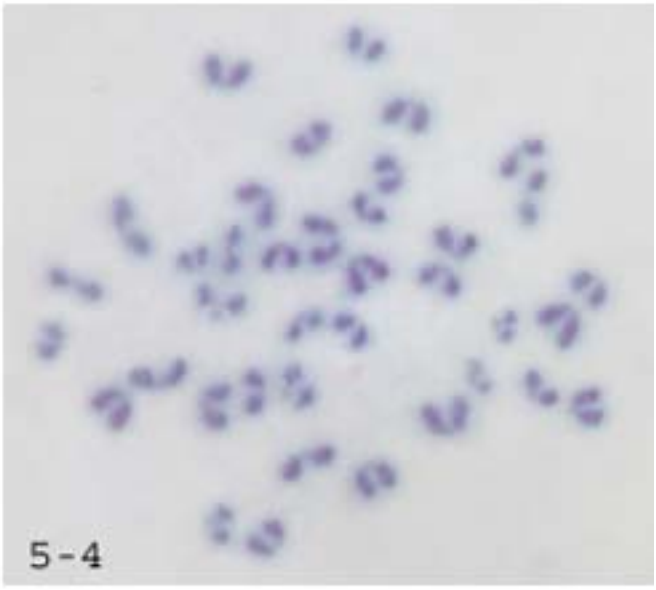

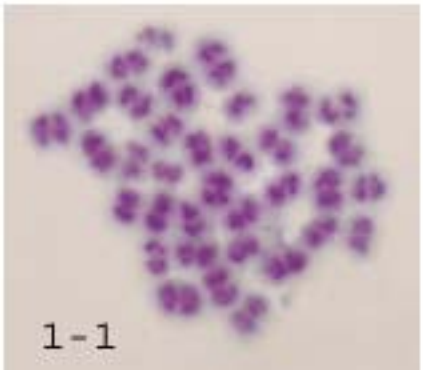

More detailed notes about counting chromosomes

I was recently asked to look at some ES cell chromosomes by Colleen Karlo in the Alzheimers center. The pictures can be opened up in photoshop and are called "Chromosomes Colleen Karlo 1 and 2". I thought this was a useful example of some problems that can be encountered with counting chromosomes. Here is the commentary on the pictures:

I was able to get a decent view of the spreads through photoshop. First some general impressions. A lot of the spreads are too small to count. What normally happens is the first couple of tries are rather lack luster, and then it becomes much easier with repetition. What I look for are well separated spreads: 1-2, 4-4, 5-4 are good examples. Generally the resolution using crude karyotyping is rather poor, so I don't put much stock in morphology, rather I concentrate on getting accurate numbers of chromosomes. Anything below 40 chromosomes is either nonviable or a splatter-punk and is therefore ignored. Anything over 40, especially 41 or 42 is a concern. When I get one of these I recount it and check in the same field for additional 41/2s. In my hands, when a line goes aneuploid these 41/2s quickly take over and this is very obvious on the slides--consistent 41/2s in every valid spread in every field. From counting your chromosomes I got a mixture: a few 40s and a few 42s (see below for breakdown). Unfortunately it's just not enough good spreads to tell.

My standard procedure is to scan at low magnification for well separated spreads within a field, then flip to an oil immersion high magnification lense to count. Anything below 40 is ignored. When I get a confident 40 I move to another field. Counting 4-5 fields per slide for 4-5 slides will usually generate at least 20 confident counts. If the line has gone 41/2 it's obvious.

Now the specific counts and scoring:

Field	#chromosomes	score
1-2	38	punk (ignore)
2-5	42	aneuploid
2-2	42	aneuploid
2-4	40	euploid
4-3	40	euploid
1-4	too small	punk
4-2	too small	punk
5-3	too small	punk
4-4	40	euploid
5-4	38	punk
1-1	36	punk

Assuming the naming is slide#-field#, then it looks like all the aneuploids were in two different fields on slide 2. The euploids were on 3 different fields on 2 different slides. As I said before, just not enough of a sample to tell. It does look like the procedure for making the spreads is working fine. It'll probably just come down to more hours logged on the microscope (sorry).

Preparing cells for injection

Original reference: "Tissue Culture made Easy" by Christian LaMantia from the Magnuson lab. Preplating protocol comes from the Doetschman chapter in Transgenic Animal Technology: a Laboratory Handbook, ed. Carl Pinkert (1994).

The most fundamental criteria for doing injections are having a cell line with an extensively characterized targeted event--both sides of the event evaluated with external probes or multiple overlapping internal probes. The cell line should be euploid. Some injection facilities also require that you test for mycoplasma. One derivatized cell-line from this facility was determined to be mycoplasma negative by ATCC (a copy of the results is in the protocol book).

The goal will be trysinized single cells (not clumps), but cells not trypsinized for so long that they've become sticky. This is achieved by doing the digestion at room temperature under the micro-scope for approximately 6 minutes. Have a back up plate in case of problems since injections are precious.

-Grow the cells in ES cell culture medium without antibiotics or selective agents like G418. These agents may be toxic to embryos.

-24 hours before the scheduled injections passage recently thawed targeted cell lines to generate a sub-confluent 35 mm dish. This will ensure that the cells are viable.

-Wash the plate a couple of times with culture medium to remove any floating cells, then cover dish with freshly thawed trypsin. Remove this trypsin and apply new trypsin.

-Put the dish on the microscope and observe the digestion periodically. When ready, almost all of the ES cells within each colony will look rounded. This is the most important step because if you under-trypsinze the cells they will be clumpy and if you over-trypsinize they will be sticky, therefore hard to inject. This normally takes about 6 minutes for cells grown exclusively on gelatinized plastic. Lines that grow on feeders may take a little longer. A good indicator is that the cells should rinse off easily when you go to break up the clumps.

-Break the colonies up with a plugged pasteur pipet (or other small bore pipet) and place the cells in an equal volume of ES cell culture medium.

-Spin down as usual and gently resuspend cells in 2 ml of ES cell medium. Feeders are removed by plating the cells onto a fresh gelatinized 35mm dish and incubating for 30 min. Gently remove the supernatant and plate again on a fresh gelatinized 35 mm dish. Incubate for 1 hour. The fibroblasts should now be largely attached. Gently remove supernatant and transfer to a convenient size tube. Place tube on ice and deliver to the injection facility.