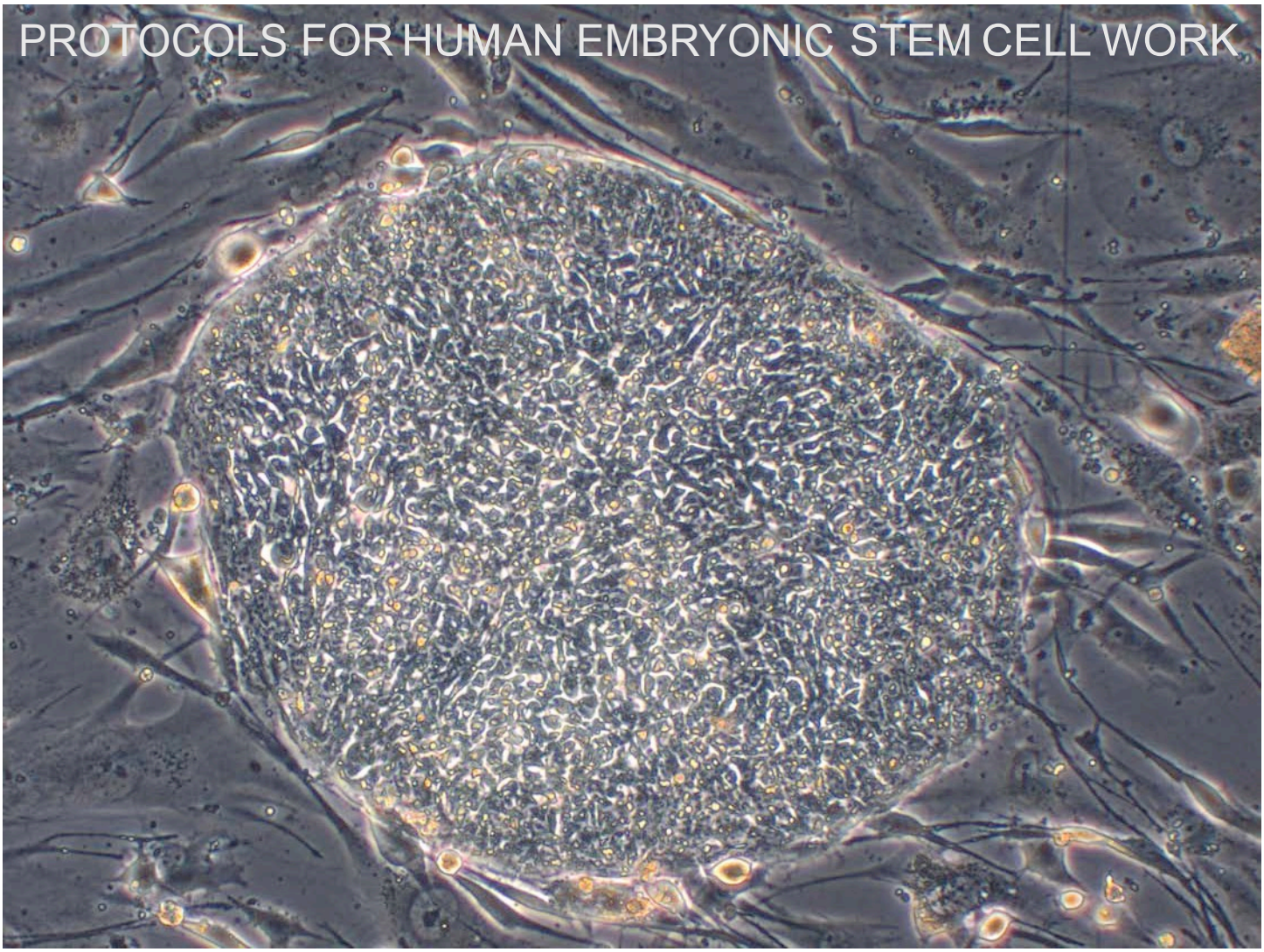
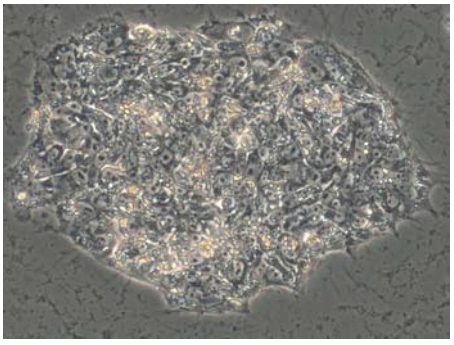
PROTOCOLS FOR HUMAN EMBRYONIC STEM CELL WORK

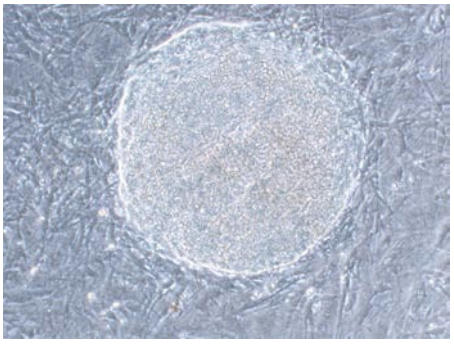


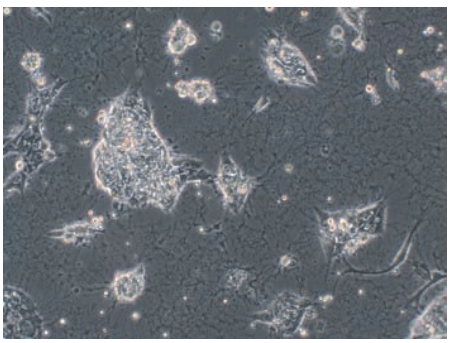
THE BRIVANLOU LABORATORY OF MOLECULAR EMBRYOLOGY
THE ROCKEFELLER UNIVERSITY
NEW YORK, NEW YORK

NYSTEM

DOCUMENT VERSION 7.0 SEPTEMBER 28, 2011







CONTENTS

PRODUCING MEF FEEDER CELLS

Scott Noggle, doc. Version 1.2 1-5-06

Mouse Embryonic Fibroblast (MEF) feeder cells from two different sources are used: MEFs prepared from E13 ICR embryos (strain CD-1 from Charles River Laboratory) and inactivated using Mitomycin-C or gamma irradiation or commercially available Mitomycin-C treated MEFs of the CF-1 strain (Specialty Media cat#PMEF-CF). Primary MEFs are used between 1 to 5 passages.

Medium: FM10: DMEM containing 10%FBS, 1X Lglutamine, 1X pen-strep, and 100μM 2-Mercaptoethanol (all from Gibco).

Isolation of p0 MEFs from embryos: Alternatively, primary Mouse Embryo Fibroblasts are isolated from E13 embryos essentially as described in Manipulating the Mouse Embyro1. Briefly, sacrifice a pregnant mouse by an institutionally approved method. Swab the mouse liberally with 70% ethanol. Using scissors make a cut across the belly and cut away the skin to expose the gut. With sterile forceps and scissors, dissect out the uterus and place it into a Petri dish in sterile PBS. Isolate the embryos from the uterus, and release the embryos from the embryonic membranes. Transfer embryos to a second Petri dish with sterile PBS. Using watchmaker forceps under a stereomicroscope, remove the embryo heads and liver, intestines, heart and all viscera with two pairs of watchmaker forceps leaving only the limbs and body cavity. Transfer the cleaned embryos into a sterile 10mL syringe with 5mls of 0.25%Trypsin/EDTA per 10 embryos. Pass the embryos and trypsin through an 18G needle slowly and gently, into a fresh Petri dish. Collect the partially dissociated embryos and trypsin with a serological pipette and pass through the needle a second time. Incubate the tissue for 15 minutes at 37oC, pipetting the tissue a few times through a 10ml pipette to dissociate the tissue. Allow the large pieces of cellular debris to settle (5 minutes 1g). Remove the supernatant into a fresh tube and add about an equal volume of fibroblast medium. Spin down cells and resuspend in medium. Discard the debris.

One embryo is plated on a T175 flask in a total of 30 mls of medium per flask. This density allows the cells to adhere but not become overly confluent before harvest at Day 3-4. Incubate at 37oC with 5% CO2. MEFs will attach and begin to divide overnight. Change the medium every other day. When the flasks are nearly confluent, usually in 3-4 days, the cultures are ready for freezing. Freeze cells in 10% DMSO/90%FBS at 6x106 or 12x106 per vial. This is considered passage p0.

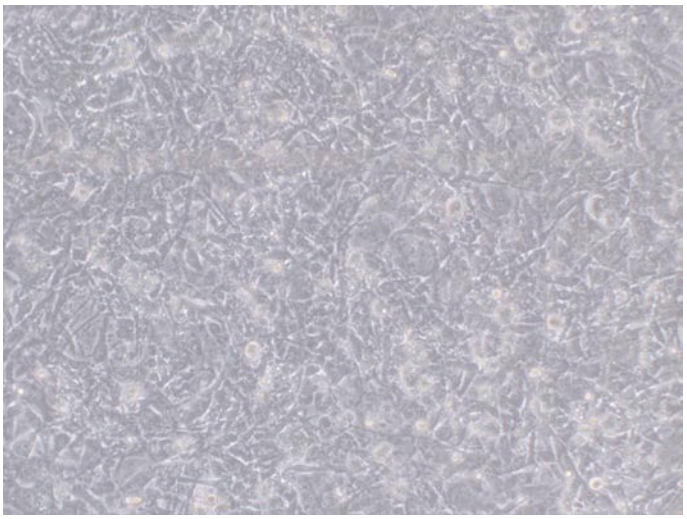
Inactivation by Mitomycin-C: Inactivated MEFs are prepared by thawing p0 MEFs. Cells are thawed quickly in a 37oC water bath with gentle shaking. The cells are gently transferred to a 50 ml conical tube with 20mls of fibroblast medium and centrifuged at 200xg for 4 minutes. The cells are resuspended in 30-50 mls medium and counted with trypan. Viability should be >95%. Cells in fibroblast medium are seeded into T175 flasks at 1.8X106 per flask with 25 mls medium per flask. Flasks are incubated at 37oC 5% CO2. This cell number should be optimized to give 75-90% confluency by day 3. On day 2, aspirate medium and add 35 mls fresh fibroblast medium to each T175. On day 3 after thawing, cells should be 75-90% confluent in T175 flasks. Check each flask to assure proper cell growth and sterility. Medium is aspirated and cells are treated with mitomycin-C at a final concentration of 10μg/ml in15 mls per T175 fibroblast medium for 2.5 hours at 37 oC and 5% CO2. To prepare mitomycin-C, dissolve 2 mgs per vial powdered mitomycin c (Sigma, Cat#M4287) in 200 mls fibroblast medium. This working stock is 10μg/ml. It can be stored at 4oC protected from light for up to 6 weeks or frozen at -20_ for longer storage. After use, add 15 ml bleach per 500 mls mitomycin-C soln to neutralize it.

After incubation, the mitomycin-C is aspirated and cells are washed with 20 mls PBS. Aspirate the PBS and add 15 mls fibroblast medium to each flask. It is convenient to only trypsinize five to six flasks at a time to minimize exposure to the

trypsin. Start by rinsing the flasks an additional 3 times by rinsing once with 20 mls PBS and twice with 15 mls each of Ca-Mg-free-PBS. After the last wash is aspirated add 2ml of 0.05% trypsin/EDTA. Disperse the trypsin by tilting the flask. Dissociation of the cell layer typically takes 1-2 minutes. When the cells detach from the flask 5 mls of fibroblast medium are added to each flask to stop the trypsin. Pool cell suspensions from the flasks in a 50 ml conical tubes and bring to 50 mls with fibroblast medium. Wash the cells once with 25ml of fibroblast medium. Count the cells and access viability with trypan blue. Plate as described below. Alternatively, the inactivated MEFs can be frozen as above and thawed for subsequent use. Freeze cells in 10% DMSO/90%FBS at $5x10^6$ per vial.

Inactivation by gamma-irradiation: MEFs to be inactivated are harvested as above, combined into one 50ml tube and irradiated with a dose of 8000RADs. The time of exposure must be calculated based on the activity of your gamma source. Count the cells and access viability with trypan blue. Plate as described below. Alternatively, the inactivated MEFs can be frozen as above and thawed for subsequent use. Freeze cells in 10% DMSO/90%FBS at $5x10^6$ per vial.

Plating inactivated MEFs for HESC culture: Dishes are pre-coated with 0.1% gelatin made with cell culture grade water for 20-30min in the incubator. Inactivated MEFs are plated fresh from the inactivation procedures outlined above or thawed from commercially available vials. If using previously frozen vials, MEFS are resuspended in the appropriate amount of medium and plated directly - they are not to be centrifuged. A density of $0.5X10^5/cm^2$ is a good starting density for MEF plating. This value should be optimized to give complete coverage without being too dense (see photo to right), as increased MEFs in the culture will deplete culture media components. Conversely, too few MEFs will not provide adequate conditioning of the media. Plating density is shown in the table below. Inactivated MEF feeder cells should be plated a day or two before additions of HESCs to allow for attachment and spreading of the MEF layer to completely cover the surface of the dish. It is important that the HESCs not come in contact with tissue culture plastic before the MEFs have laid down sufficient ECM to prevent premature differentiation. 35mm tissue culture treated dishes are most convenient, however larger dishes also work well. MEFS are fed every other day until used. MEF feeder layers should not be used after 4 days.



VESSEL	SURFACE AREA (PER WELL)	NUMBER OF MEFS (PER WELL)	OPTIMUM VOLUME
35mm dish	$10cm^2$	$0.5\text{-}1x10^6$	2ml
60mm dish	$20cm^2$	$1\text{-}2x10^6$	5ml
T25 flask	$25cm^2$	$1.2\text{-}3x10^5$	5ml
4-well chamber slide	$2cm^2$	$1\text{-}2x10^5$	1ml

HESC GROWTH MEDIA

Scott Noggle, doc. version 4, 9-28-07

Due to current uncertainties regarding ideal culture conditions for HESCs (for example, karyotype stability), we are currently attempting to improve on the standard formulations. HUESM is the current growth media that we use and differs from the standard H1 medium described by Thomson's group in the use of DMEM rather than DMEM/F12. In addition, our microarray analysis suggests that the HESCs express several genes involved in responses to oxidative stress. To reduce oxidative stress, I have tested a supplement (B27 without Vitamin A) that contains a number of anti-oxidants. It's addition has not resulted in altered pluripotency as accessed by derivation, markers, EB formation, or teratoma formation. The original formulation of H1 medium called for 4ng/ml of bFGF. Current studies suggest that MEFs respond optimally to 20ng/ml of bFGF. I have increased the bFGF concentration in HUESM (see table below) to reflect this. This dose of bFGF seems to compensate for some degree of variation between MEF batches.

HUESM	CATALOGUE NO.	FINAL CON.	FOR 500ML
Knockout-SR	10828	20%	100ml
GlutaMAX	35050	2mM	5ml
MEM non-essential amino acids	11140-050	0.1mM	5ml
Penicillin-streptomycin	15140-122	100U/ml-0.1mg/ml	5ml
2-Mercaptoethanol	21985-023	0.1mM	900ul
B27 Supplement - without Viatmin A	12587-010	1X	10ml
DMEM	11995-065	75%	375ml

KSR is thawed at 4oC, aliquoted into 50ml conical tubes and frozen at –20oC. A stock of growth medium is stored at 4^{o}C for no more than two weeks. Preheat only as much medium as is needed for ~ 20 to 40 min @37^{o}C. To prepare complete growth medium, bFGF is added just before feeding (see bottom table).

APPLICATION	BEFORE CONDITIONING ON MEFS	BEFORE FEEDING HESCS
Maintenance on MEF feeder layers	na	20ng/ml
Maintenance on Matrigel	20ng/ml	20ng/ml

Stocks of bFGF (Invitrogen cat 13256-029) are made to 100µg/ml in sterile 10mM Tris-HCl pH7.6/0.1%BSA. 20ul and 8ul aliquots are frozen at -20^{o}C and once thawed, are not refrozen.

DEFINED MEDIA: XVIVO-10 growth medium is an all-human sourced serum-free medium from BioWhittaker originally designed for human lymphocyte culture. According to the supplier, It contains no added growth factors. It can be used for HESC culture by adding high concentrations of bFGF (80ng/ml), 1XNEAA and 110µM 2-Mercaptoethanol to the XVIVO-10 base medium. Some suggest that adding TGFb1 (1ng/ml) is necessary. It already contains antibiotics and L-glutamine. It does not need to be conditioned by MEFs to maintain the HESCs. The use of this medium was recently described by Li et al (2005) Biotechnol Bioeng 91(6):688-98. The drawback of this mediums is that the formulation is proprietary and it has not been tested for long term maintenance capability. Some studies suggest that in may not be able to maintain cultures past 10 passages. However, it may be used for short term serum and mouse component-free work.

MEF-CONDITIONED MEDIUM (CM)

Scott Noggle, doc. Version 1.3 9-28-07

Mouse Embryonic Fibroblast (MEF) feeder cells from two different sources are used: MEFs prepared from E13 ICR embryos (strain CD-1 from Charles River Laboratory) and inactivated using Mitomycin-C or gamma irradiation or commercially available Mitomycin-C treated MEFs of the CF-1 strain (Specialty Media cat#PMEF-CF). Primary MEFs are used between 1 to 5 passages. See accompanying protocol for isolation and inactivation procedures. Passaged HESCs are plated in 2ml of CM per well of a 6-well plate. 5ml on a 60mm dish, or 8ml on a 10cm dish. They can be fed with the same volume for the first few days. When the colonies get bigger, increase the CM to 3ml/7/12ml, respectively. Cultures in CM on Matrigel can usually grow for 5 days before they need passaging.

Materials and Preparation of conditioned medium:

Dishes: Coat 10cm dishes in 0.1% gelatin (made in TC-grade distilled water) for at least 20min in the incubator.

Medium: FM10: DMEM containing 10%FBS, 1X L-glutamine, 1X pen-strep, and 100μM 2-Mercaptoethanol (all from Gibco).

Plating MEFs: Thaw one vial of Specialty Media Mitomycin-C inactivated MEFs (5x106 cells) or equivalent inactivated MEFs and resuspend in 12ml of FM10 medium. Aspirate the gelatin from the plates and immediately plate the 12ml of cells directly on one gelatin coated 10cm dish. Incubate overnight to attach.

Conditioning medium: The next day, rinse the MEFs with HUESM and replace with 12ml of HUESM with bFGF. Incubate overnight to condition the medium. After 24hrs, draw off the conditioned medium into a 50ml tube and replace with fresh HUESM. I try to keep to 24hrs of conditioning (+/- 2-4hrs is ok). The CM can be used immediately, stored at 4oC for a week or frozen at –80oC. When ready to use to feed Matrigel cultures, add fresh FGF2 before plating see table below for concentrations of bFGF.

APPLICATION	BEFORE CONDITIONING ON MEFS	BEFORE FEEDING HESCS
Maintenance on Matrigel	20ng/ml	20ng/ml

MATRIGEL PLATE COATING

Scott Noggle, doc. Version 1.2 9-28-07

Generating Matrigel stocks:

The Matrigel that we are currently using is qualified by Stem Cell Technologies to maintain hESCs. Thaw one 5ml vial of Matrigel (BD cat# 354277) at 4oC overnight. The original Matrigel stocks came at different stock concentrations and were diluted to a final of 0.333mg/ml to coat plates. This usually meant roughly a 1:30 final dilution. The current stocks do not come with a concentration listed, but instead come with dilution instructions. It is a good idea to check the Product specification sheet for the dilution factor for the current lot of Matrigel. If these are not available, call BD at the phone number listed on the vial and give them the lot number. They will tell you or email the sheet for that lot. It is important to keep the vial and all pipettes and tubes ice cold to prevent premature gelling of the matrix. Using the cold pipette, dispense the 10ml vial into 9-10 1ml aliquots in pre-chilled cryotubes on ice. These can be refrozen at –20oC.

Coating plates:

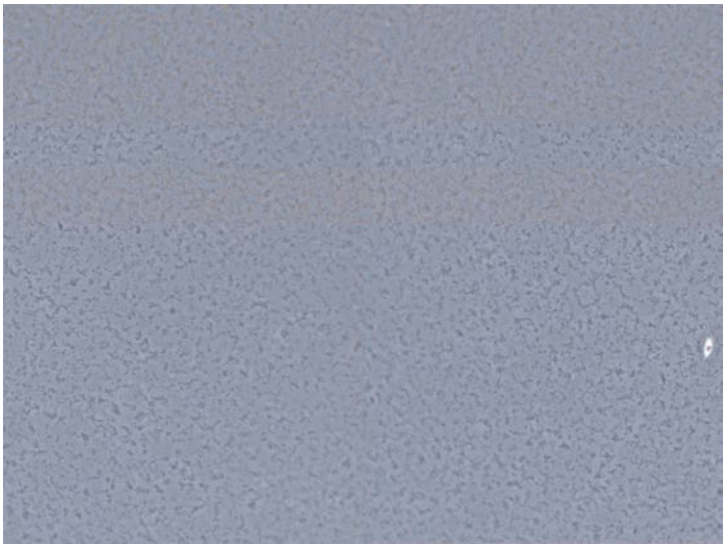
[Instructions for the current lot - you may need to change the dilution factors] In preparation, place a 512ul aliquot (from above or thawed slowly on ice, about 2hours) on ice. Prepare an ice bucket with a metal support tray for the plates/dishes. We use shallow rectangular ice buckets filled with ice and with small metal incubator trays on top of the ice. Pre-cool 6-well plates or dishes. Pre-cool p1000 filter-tips at –20oC for about 20min.

Dilute the 512ul aliquot of Matrigel with cold base medium (XVIVO-10, DMEM/F12, DMEM) to 40ml total in a pre-cooled 50ml conical tube. Mix well and dispense 2ml into each well of the 6-well plates or 8ml onto a 10cm dish (equivalent amounts per surface area of other dishes). Keep the plates on the ice cold platform at all times. If in a rush the plates can be kept at RT for about 4hrs in a tissue culture hood (covered with foil) to coat. Some lots of Matrigel will tolerate this and some will not. Alternatively, place the entire ice bucket with plates into the refrigerator to coat overnight. Sometimes, the plates will coat better after two days in the refrigerator.

Preparation of coated plates for HESCss:

30-2hr min before passaging, warm the plates with Matrigel in the incubator to allow the Matrigel to gel.

Before plating cells, check the coating on the microscope for a meshwork-like single layer matrix (see photo to right). When ready to plate HESCs, aspirate the Matrigel from the wells using a Pasteur pipette in the corner of the well. Get as much Matrigel off of the dish as possible leaving a thin coating on the surface of the dish. Do not scrape the bottom of the dish. Rinsing the Matrigel-coated plate is not necessary. Plate HESCs in conditioned medium or other desired conditions.



PASSAGING HESC LINES

MICRO-DISSECTION METHOD FOR PASSAGING HESCS

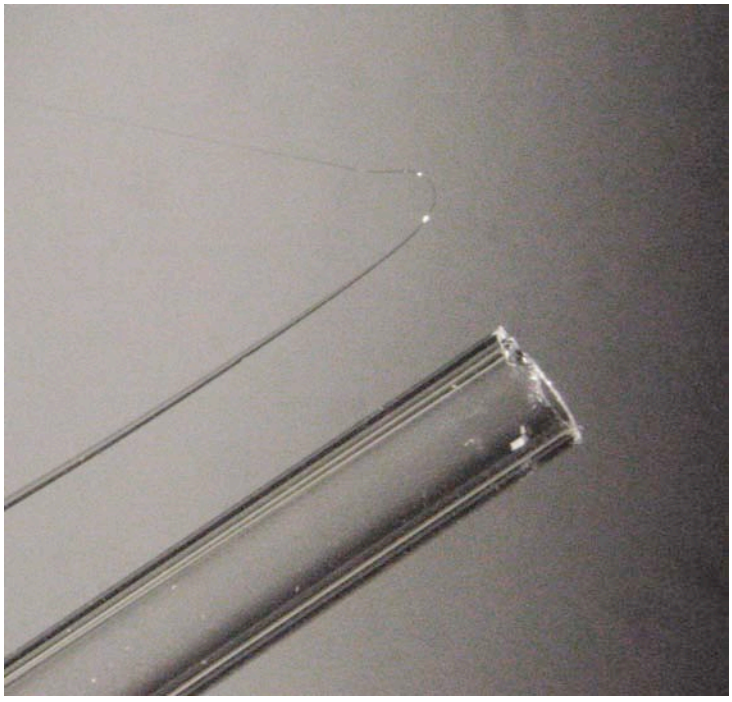
Scott Noggle, doc. version 1.4 9-11-07

Purpose: Used to maintain master stocks of HESCs. To minimize cell damage, karyotypic disturbance or cell fate changes, which might be produced by EDTA, enzymatic or chemical treatments.

Materials and Preparation:

Feeder cells: See accompanying protocol for generating MEF-feeder plates.

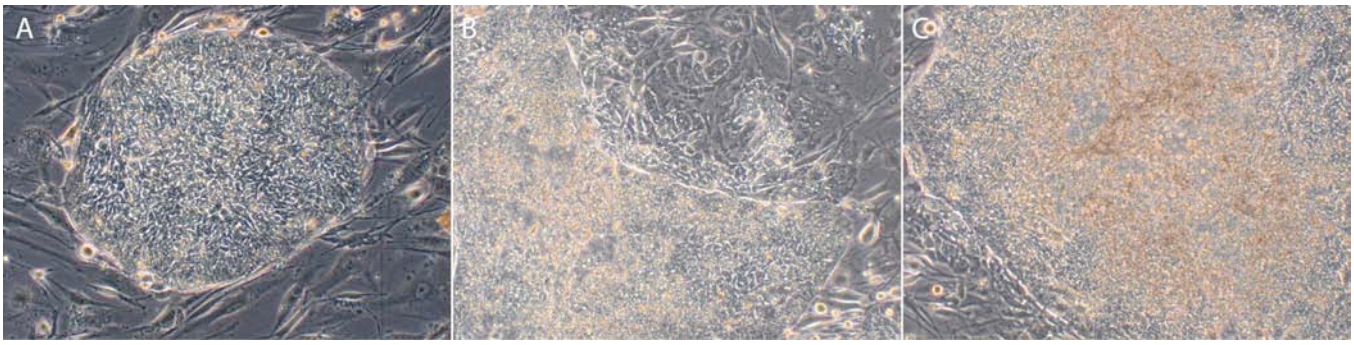
Tools: Glass tools, Tungsten needles, or laser dissection can be used for micro- dissection of HESC colonies for passaging. For glass tools, Pasteur pipettes are pulled hair thin. Fine glass needles with hooked ends are forged in two steps over a microburner assembled as described in Manipulating the Mouse Embyro[1] as follows. While holding the two ends of a long Pasteur pipette, place the thin end of the pipette at a distance of about half to two-thirds of the distance away from the tip into the orange part of the flame until the glass melts into a solid constriction. In a single motion upon removing the pipette from the flame, pull on each end of the pipette gently and quickly to draw out a thin filament before the glass hardens. This is done without breaking the connection between the two ends of the pipet. Second, beginning several inches above the flame and slowly moving the thin drawn part of the filament down towards the flame pull a very fine filament as previously described. The two ends of the pipette should separate this time forming a fine needle end on the tip of the pipette. If the tip remained straight after the second pull, pass the fine end a few inches quickly over the top of the flame. The force of the rising heat will curl the tip of the needle into a hook. The hooked end should be thin enough for the micro-dissection of the HESC colonies but thick enough to withstand some pressure during the dissection. Examples of the final product are shown in panel A of the figure below.



Medium: I have used both H1 medium and HUESM successfully. See section on growth media for recipes

Before passaging, examine the colonies under the microscope and choose colonies that are undifferentiated. Avoid colonies or parts of colonies that are showing signs of differentiation. Several types of differentiation can be morphologically identified

in spontaneously differentiating cultures. Avoid the center of colonies that show a depression or “crater” appearance. Areas of colonies that have begun forming cystic structures should be avoided. Also avoid the edges of colonies that do not have a tight border between the feeder layer and the colony. In these areas, the HESCs have started to flatten, polarize and migrate into the feeder layer. Some differentiation on the borders of the colonies can be tolerated, as these cells can be left behind with the micro-dissection technique. In some cases, it may be necessary to dissect a colony that has begun to differentiate. The region of differentiation can be avoided selecting only the undifferentiated parts of the colony to dissect. Leave the differentiated regions untouched. Ideal colonies are comprised of small, round, and randomly organized cells with a high nuclear to cytoplasmic ratio that have not begun forming structures within the colony (examples are shown in panel A in the figure below).

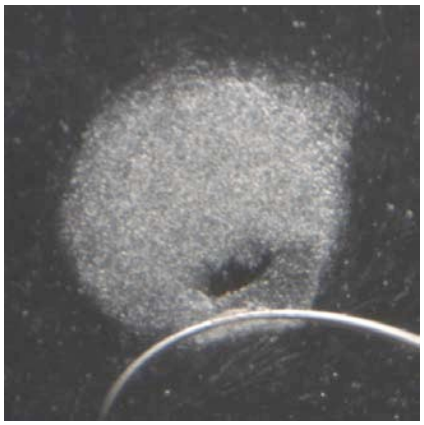


Transferring HESCs:

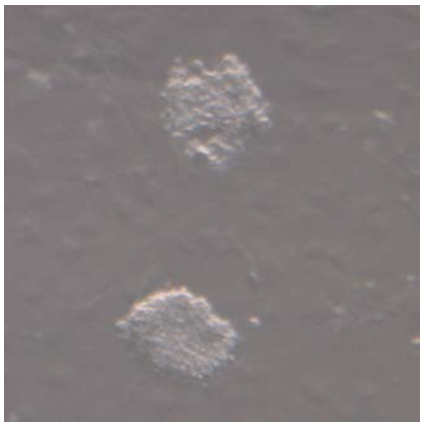
Prior to micro-dissection, medium is changed in the well to be passaged and on the feeders. The feeders are washed once and 2ml of fresh complete growth medium is placed on the new feeders on 35mm dishes. Care is taken to maintain temperature, pH and osmolarity of the media by working quickly. The feeders are kept in the incubator during the dissection. Alternatively, a humidified CO_2/O_2 mixed gas source and a warm plate are convenient to maintain an optimal environment while the cultures are out of the incubator. This is especially helpful after the colonies are transferred to the new feeders, as it is important to minimize handling of the dish. If the dishes are handled too much before the colonies have attached to the feeders, the chunks of HESCs will migrate to the center of the dish and attach too close together.

Passaging: Ideal colonies or parts of colonies are micro-dissected into chunks of about 100 cells using the glass hooks. The hook is used to gently pull apart pieces of the colony (see photo to right). This can also be accomplished by cutting a grid into

the colony with the back of the hook and pulling the pieces away from the colony one at a time. It is easier to pull out one piece at a time, as large pieces are more difficult to cut into smaller pieces. The size of the piece should be large enough to survive the cutting and adhere to the feeder layer (see photo on next page). A piece too large will tend to form an embryoid body-like structure on the feeder layer as it takes too long for the entirety of a large colony to come into contact with the feeders. The resulting colony will have an area of differentiation in the center arising from the embryoid body-like structure (see panel C in the figure above for an example).



After micro-dissection, the cell chunks are swirled into the center of the dish and 20 to 50 chunks are transferred to the new feeder wells using 1ml micro-pipets. Pre-coat the micro-pipet tip with the medium so that the cells do not stick (a regular sterile pipet or Pasteur pipet can be used also). Transfer no more than 500ul of medium to the new dish. In some cases, it may be necessary to transfer the entire well volume to the new feeder well or wells. Exchange medium 2.5 to 3ml/well. If possible, leave the dishes untouched on a warmed surface (preferably under O2/CO2 blood-gas mix) for 15-30min to allow the chunks to begin attaching to the dish before moving to an incubator. Excessive handling of the new dish will cause the chunks to migrate to the center of the dish rather than remaining evenly distributed across the dish. Good spacing between the colonies will allow proper growth of the colonies.



Maintenance: Complete growth medium is exchanged on the growing colonies every day as the feeder layer can use up nutrients quickly. In the example photos and movie BGN1 cells are used. The cell cycle for this line is about 24-36 hours. The lines should culture for no more than 6 days to a week. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center and edges of the colony (see figure above for examples of differentiation).

Cryopreservation and recovery: See accompanying protocols for vitrification of HESCs in cryo tubes or straws.

References:

1. Hogan, B. Manipulating the mouse embryo: a laboratory manual (Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1994).

ENZYMATIC PASSAGING OF HESCS

Scott Noggle, doc. Version 1.4 9-28-07

Purpose: For expansion of HESCs on Matrigel (or other matrix) coated plates in MEF-CM but can be modified for HESCs grown directly on MEFs.

Materials and Preparation:

Feeder cells for MEF-CM: See accompanying protocol for generating MEF-conditioned medium.

Matrix: Tissue culture plates are coated with Matrigel as described in the accompanying Matrigel plate coating protocol.

Medium: Growth medium is described in the section on Growth medium for HESCs and the section on generating Conditioned Medium. A stock of growth medium is stored at 4oC for no more than one week. Preheat only as much medium as is needed for ~ 20 to 40 min @37oC.

Enzyme: Dispase or Collagenase, type V (either at approximately 1mg/ml) dissolved in growth medium (and sterile filtered). We are currently buying Dispase from Stem Cell Technologies. Dilute these stocks 1:5 in DMEM or DMEM/F12.

Transferring HESCs:

Before passaging, examine the colonies under the microscope and look for any colonies that are differentiated. Spontaneously differentiating areas of the culture can be removed with a glass tool as described in the manual dissection protocol or aspirated using a pipette attached to a vacuum. Several types of differentiation can be morphologically identified in spontaneously differentiating cultures. Look for the center of colonies that show a depression or “crater” appearance. Areas of colonies that have begun forming cystic structures in the center of the colony should be removed. Also avoid the edges of colonies that do not have a tight border between the feeder layer and the colony. In these areas, the HESCs have started to flatten, polarize and migrate can also be removed. However, some differentiation on the borders of the colonies can be tolerated, as these cells will detach during the washing steps (see below). Ideal colonies are comprised of small, round, and randomly organized cells with a high nuclear to cytoplasmic ratio that have not begun forming structures within the colony (examples are similar to those shown in the manual dissection protocol).

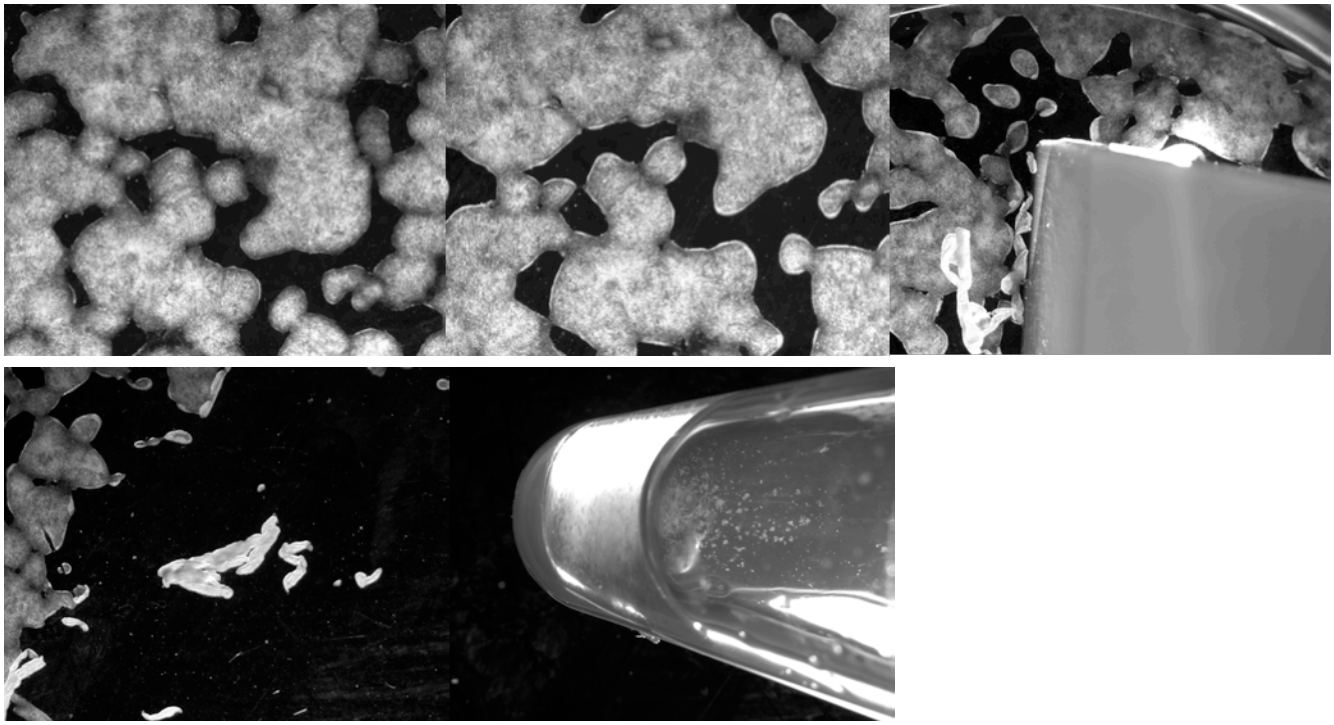
Passaging: Replace the growth medium with Dispase or Collagenase. Allow to incubate in the incubator for about 7 min. Check the progress of the matrix digestion, beginning at about 4 min. The colony borders will begin to peel away from the plate, while the center will remain attached. Ideally, gently wash the Dispase or Collagenase off of the plate with growth medium twice. The colonies should remain attached to the plate. If they have detached after the Dispase incubation transfer all of the colonies and Dispase solution to a conical tube and centrifuge and wash the colonies with growth medium. They should get two to three washes total – either on the plate or with centrifugation. If the colonies remained attached after washing, harvest the colonies with a cell lifter (Costar 3008 – NOTE: DO NOT USE THE SWIVEL-HEAD CELL SCRAPER (3010)). Transfer all of the colonies and growth medium to a conical tube and spin to pellet the colonies. Using the MEF-CM, resuspend the colonies using a p1000 pipette tip in about 500-700ul of medium. Triterate the colonies to clumps with an

average size of about 100 cells using the p1000 tip. Plate a proportion of the clumps (I currently use a 1:10 split ratio – but you will need to adjust this for the confluency of the starting population).

If possible, leave the dishes untouched on a warmed surface for 10 min. to allow the chunks to begin attaching to the dish before moving to an incubator. Excessive handling of the new dish will cause the chunks to migrate to the center of the dish rather than remaining evenly distributed across the dish. Good spacing between the colonies will allow proper growth of the colonies.

Maintenance: Complete conditioned growth medium is exchanged on the growing colonies every day from the MEF plates. The lines should be cultured for no more than 6 days before passage. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center or outer edges of the colony.

Cryopreservation and recovery: See accompanying protocol for cryopreservation in tubes or straws.



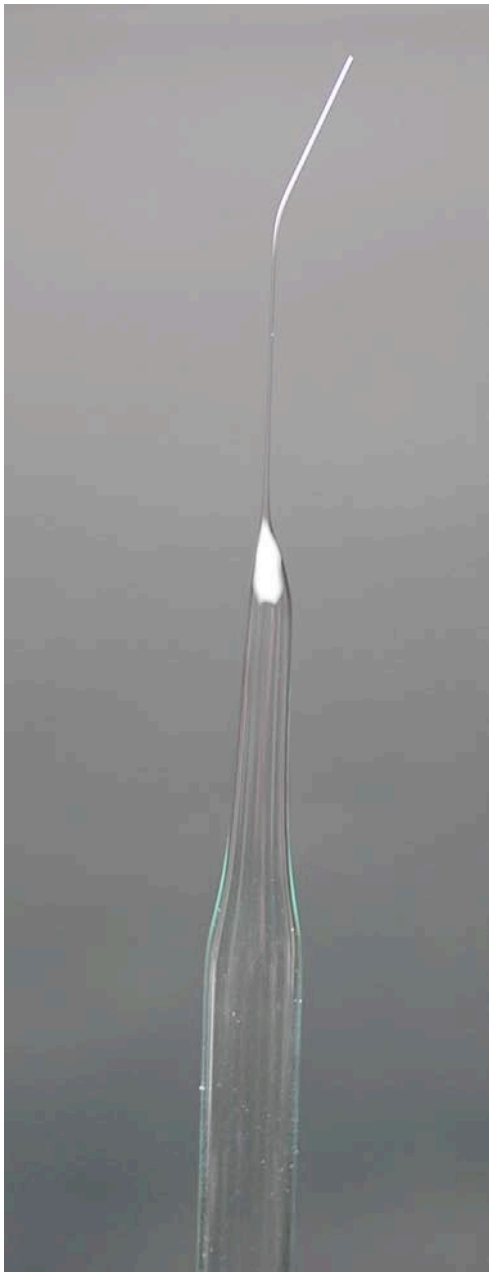
This sequence shows (from top left to bottom right) colonies before dispase treatment, after treatment, during scraping, after scraping and after triteration in a 15ml conical tube.

DERIVATION OF NEW HESC LINES

Scott Noggle, doc. version 1.1 9-28-07

PROTOCOL:

During thawing, recovery and blastocoel expansion it is important to reduce the stress on the embryos by maintaining temperature, pH and osmolarity. After the embryos are thawed by stepwise removal of the cryoprotectants, the embryos are maintained in equilibrated media optimized for their maintenance and growth prior to immunosurgery or plating on substrates for outgrowth. The embryos are moved from solution to solution by mouth-controlled pulled glass pipette. The pipette can be rinsed with a protein-containing solution to prevent sticking of the embryo to the inside of the pipette.



PIPETTE CONSTRUCTION FOR EMBRYO-HANDLING I

use a mouth-pipetting apparatus described in Manipulating the Mouse Embryo laboratory manual by Andras Nagy et al. (pg177 in the soft-cover third edition). We buy aspirator tube assemblies for calibrated microcapillary pipettes from Sigma (A5177-5EA), which provides the tubing, and mouthpiece. A syringe filter (Millex-GV, 0.22um from Millipore) is placed between two lengths of the tubing. A P1000 pipette tip with a filter is inserted into the end opposite the mouthpiece. This will hold the large end of a glass Pasteur pipette on which the small end has been pulled on a flame. The pulled end of the pipette should not have jagged edges - check the tip under a dissecting scope. The opening should be about 1.5 to 2 times the diameter of the embryos (or 2 to 2.5 times the diameter of a mouse blastocyst embryo).

To use the embryo-handling pipette, first aspirate a protein containing media to coat the inside to prevent sticking of the embryos. I use a 1:1 mix of M16 media and HSA. Expel this solution before aspirating the next media. It is helpful to have a 4-well dish with extra media from the microdrop cultures to use for washing the pipette between transfers. In general, I aspirate a little of the media that I will be moving the embryos into, then pick up the embryos a few at a time. Try to keep the embryos towards the end of the pipette tip. The embryos are washed few a couple of drops of the same media by picking them up and moving them around the drop and then to the next drop. Try to transfer as little media as possible between drops. When moving to the next solution expel the current solution in the middle of the 4-well dish and aspirate a little of the next media. When using the mouth pipette in the microdrop cultures, it is also important not to expel air bubbles into the microdrops. Keep and eye on the media column in the pipette tip and aspirate enough media between washes to prevent expelling all of the media into the microdrop.

PREPARING MEDIA

Make up the following media for thawing blastocyst stage embryos and should be set up several hours in advance of thawing:

1. M16 is from Specialty Media/chemicon and comes as a powdered media kit. Resuspend powder in diluent provided. You will need 30ml total (3 vials).
2. Dissolve 3.42g of Sucrose in 10ml of M16 media (add 8ml), heat to 37oc, and mix by inversion till dissolved.
3. Prepare thawing media:

SOLUTIONS	M16	PROH	1M SUCROSE	HSA SOLUTION
1) 1.5M PROH/0.2M Suc	3.2ml	0.55ml	1ml	0.25ml
2) 1.0M PROH/0.2M Suc	3.4ml	0.36ml	1ml	0.25ml
3) 0.5M PROH/0.2M Suc	3.6ml	0.18ml	1ml	0.25ml
4) 0.0M PROH/0.2M Suc	3.75ml	0ml	1ml	0.25ml
5) 0.0M PROH/0.0M Suc	4.75ml	0ml	0ml	0.25ml

4. The following medium is called M16complete and is used to culture the blastocysts for blastocoel reexpansion:

	M16	HSA	ESS AMINO ACIDS	NON-ESS AMINO ACIDS	GLUTAMAX
6) M16 complete	4.55ml	0.25	0.1ml	0.05ml	0.05ml

Solutions 1-4 are filter sterilized and aliquoted into 4-well dishes (0.5ml) and warmed to 37oC in CO_2 incubator for at least 4hrs to equilibrate the solutions.

SETTING UP MICRODROP DISHES

Use a P200 to make small (~10-20ul) drops of media with Solutions #5 and #6 (M16 complete medium) in 35mm dishes. It is helpful to draw a dividing line on the underside of the dish to visually separate the solutions into zones. Quickly but gently overlay the drops by flooding the dish with enough mineral oil (for embryo culture, Irvine Scientific) to cover the drops. These are allowed to equilibrate for at least 4 hours before use, but no more than 18hrs.

THAWING

1. Start by thawing the embryos by removing from the LN2, loosening the lid, swirling in 30oC water bath for 30 seconds, followed by 1min at RT, followed by another 30sec at 30oC to finish the thaw.
2. Using a flame polished glass pasteur pipette transfer the media/blastocysts into the center of a 60mm organ culture dish.
3. Quickly but gently rinse the vial again in solution #1 above (1.5M Propylene Glycol + 0.2M Sucrose in Base medium), and transfer the contents into a second organ culture dish
4. Search for the blastocysts under the dissecting scope, beginning with the first dish.
5. Move the embryos through the rehydration solutions #1-#4 stepwise with three min incubations in the incubator for each solution.
6. Transfer the embryos to the 1st drop of solution #5 in the 35mm plate for three min.
7. Wash the blastocyst 2x in the remaining drops of solution #5
8. Wash the blastocyst 2X in soln. #6 (M16complete) and leave in the third drop.

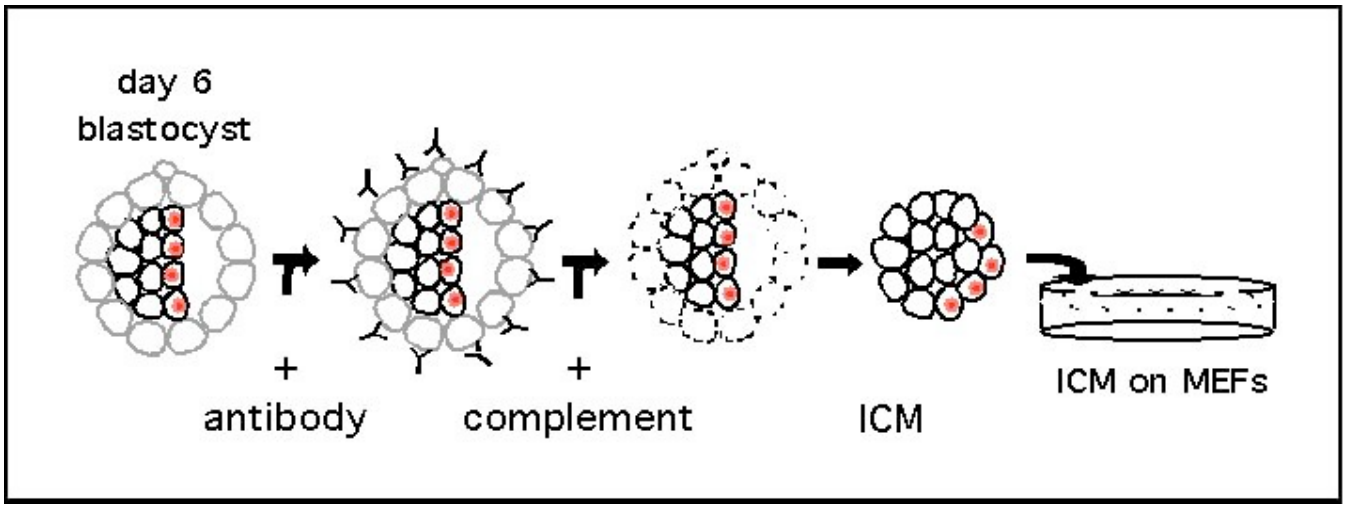
9. incubated for two hours before immunosurgery to allow for blastocoel expansion. Images are taken before this incubation, at 1hour, and at two hours (immediately before the next step). In my experience. the blastocoel should be expanded by two hours. However, others suggest it could take up to 6 hours.
10. Take several images at different focal planes under Hoffman or phase contrast to allow grading of the quality of the ICM, trophectoderm and degree of blastocoel expansion.
11. Embryos are graded as follows:
    1. Blastocoel expansion:
        1. Early blastocyst - blastocoel less than half of the embryo volume
        2. blastocyst - blastocoel equal to half or greater than half of the embryo volume
        3. full blast - blastocoel completly fille the embryo
        4. Expanded blast - blastocoel volume larger than that of the early blast and ZP is thinned
        5. Hatching blast - trophectoderm is beginning to protrude through the ZP
        6. Hatched blast - expanded blast is without ZP
    2. ICM grade:
        A. tightly packed ICM, with many cells
        B. loosely grouped, several cells
        C. very few cells
    3. Trophectoderm grade:
        A. many cells forming a cohesive epithelium
        B. few cells forming a loose epithelium
        C. very few cells
    4. For example, a high quality blast would be 4AA at day 6.

REMOVAL OF THE ZONA PELLUCIDA

Before immunosurgery or direct plating of the blastocyst on MEFs, the zona pellucida must be removed to allow access of the immunosurgery reagents to the trophectoderm or to allow attachment of the trophectoderm to the MEFs. There are two methods for ZP removal. The embryos can be incubated in pronase (2mg/ml) for several min to digest the ZP. Alternatively, the ZP can be dissolved in acidic Tyrodes solution (acidic PBS). The acid tyrodes method is provided below.

1. Begin by setting up microdrop cultures in 6cm dishes in rows as follows:
    (1) three 10ul drops of M16 complete medium, or 1 drop per embryo
    (2) six 10ul drops of acid tyrodes, or two drops per embryo
    (3) nine 10ul drops of M16 complete medium, or three drops per embryo
2. Cover with mineral oil and allow to equilibrate in the incubator for at least 1hr.
3. Transfer the embryos into one of the 1st three drops of M16 complete, one embryo per drop.
4. Process one embryo at a time by:
    (1) rinsing through the first drop of acid tyrodes and transfering into the second drop, then
    (2) watch for the ZP to disappear - immediately after the ZP disappears start
    (3) rinsing through the first two M16 drops in row 3 and into the third drop.
5. The embryo can be transferred into immunosurgery solutions or allowed to recover in the M16 complete drop before plating on MEFs.

IMMUNOSURGERY



If performing immunosurgery to isolate the ICM, use the following potocol:

1. Set up microdrop cultures for immunosurgery as follows:
   (1) three 10ul drops of anti-human placental alkaline phosphatase antibody (DAKO) diluted 1:10 in M16 complete
   (2) three 10ul drops of M16 complete
   (3) three 10ul drops of guinea pig complement (Zymed) diluted 1:10 in HUESM
   (4) three 10ul drops of HUESM
   (5) three 10ul drops of HUESM
2. Move the embryo through the solutions as follows:
   (1) Wash 2X in M16complete + 1:10 anti-hPLAP Ab, leaving in third drop 20min @37oC
   (2) Wash 3X in M16complete
   (3) Wash 2X in HESC medium + 1:10 gpComp., leaving in third wash 5-30min @37oC, image taken
   (4) Wash 3X in HESC medium
   (5) Remove lysed trophectoderm with pipetting
   (6) Wash 3X in HESC medium
3. Plate ICMs on MEFs plate

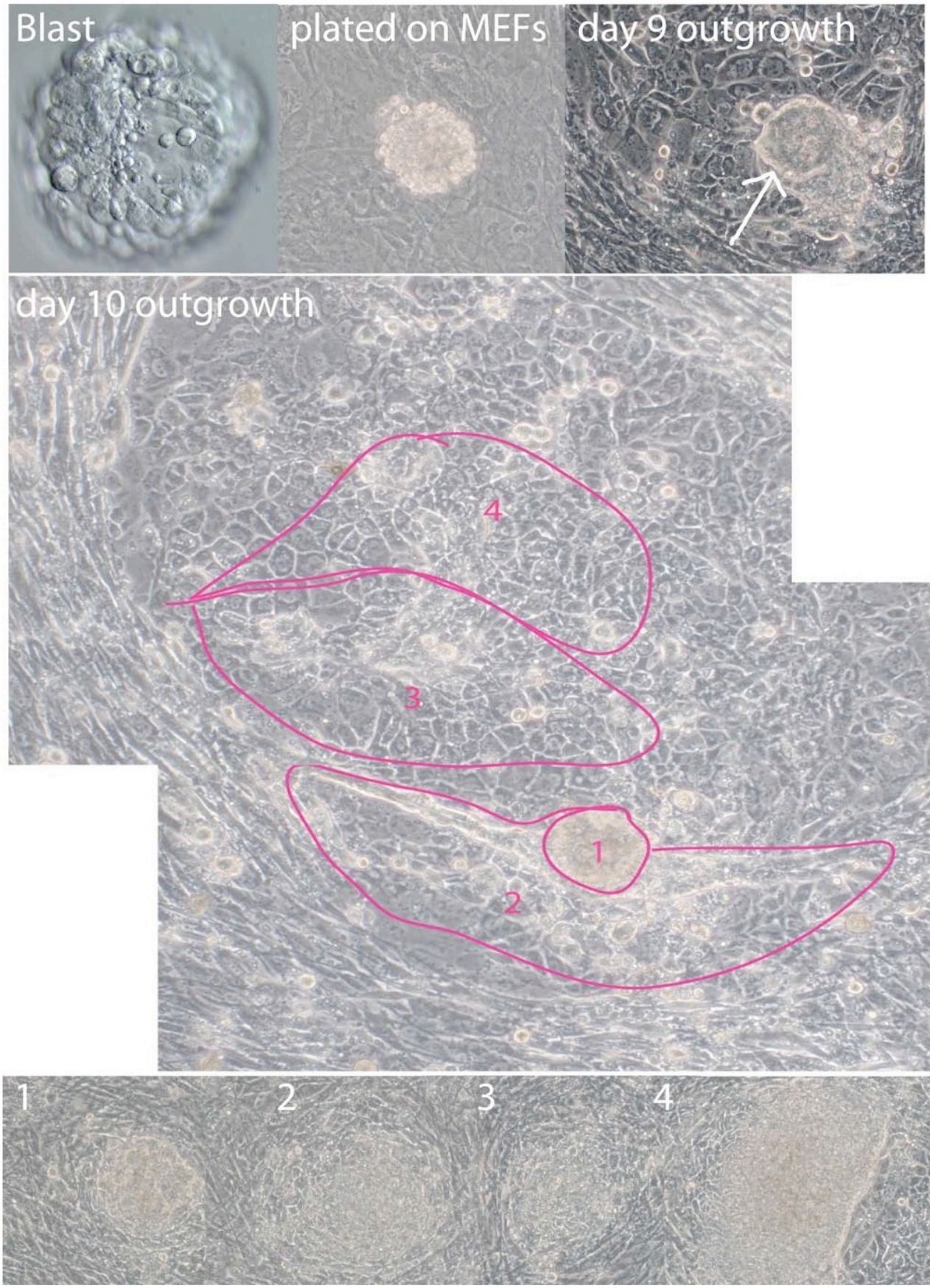
OUTGROWTH AND INITIAL PASSAGING

Before the blastocyst can outgrow to produce enough cells for passaging, the blastocyst or ICM must attach to the matrix or feeder layer. This usually happens within two to three days. The cultures are not fed during this period of attachment. I usually leave the blastocysts to attach without disturbing them for two days before making the initial observations. It is best to keep them in the mini-incubator and limit access to the incubator to minimize vibrations that might prevent attachment.

After two days, remove the plates from the incubator to the microscope to make the initial assessment of attachment an observe the morphology of the cells in the outgrowth. Take plenty of pictures to document the outgrowth.

I have noticed a range of morphologies of outgrowths that eventually produce a line. At this point it is difficult to describe a standard method for derivation of a line. Essentially, you will be looking for an outhgrowth of cells that morphologically

resemble HESCs. You will need to use your experience with HESC culture of established lines in order to notice the correct morphology. The outgrowth of the ICM may occur as a compact mass. This is pointed out by the arrow in the next figure. However, the ICM cells or cells with the potential to form an ES line may not have this morphology. Some embryos will give a cell line without having had a distinct ICM or a well defined outgrowth originating from the ICM. For instance, the outgrowth shown in the figure is from the embryo that gave the RUES2 HESC line. After 9-10 days of culture, the outgrowth was dissected into four pieces marked in the figure by red outlines. These were maintained separately and each gave rise to a cell line. At least one of the non-ICM outgrowths produced teratoma and exhibited all the characteristics of a HESC line. In addition, the Bresagen cell lines were derived from poor quality embryos without distinct ICMs. A successful strategy when you are unsure if there is an outgrowth of the ICM is to passage all of the primary outgrowth as individual pieces and track those that show the proper morphology. The tools needed for dissection and passaging of the outgrowths are similar to thosse described in the section on micro-dissection passaging of HESCs.


Blast
plated on MEFs
day 9 outgrowth
day 10 outgrowth
4
3
1
2
1
2
3
4

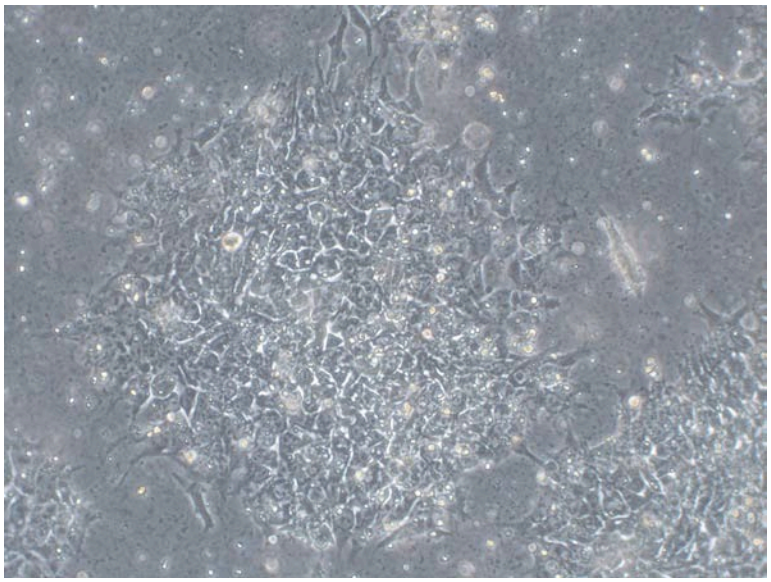
FREEZING HESCS BY VITRIFICATION IN CRYOVIALS

Scott Noggle, doc. Version 1.2 1-30-07

We have had difficulty cryopreserving RUES1 hES cells using traditional means of freezing hES cells. Survival is typically less than 1%. To improve recovery, we have optimized a protocol based on vitrification (Richards et al., 2004). This protocol uses cryovials instead of straws as originally described. This allows for higher through-put and faster processing times during the procedure. We have typically seen 40-50% recovery using this protocol. (The photo below is RUES2 on the day after thawing.)

Materials:

1. Ethylene glycol [Sigma cat E9129]
2. DMSO [Sigma cat. D2650]
3. Growth Medium (see section on Growth medium for HESCs)
4. Sucrose [Fisher cat S5-500]
5. 1M HEPES solution [Invitrogen, cat 15630-080]
6. Cryovials
7. liquid nitrogen in an ice bucket
8. Square floating microtube rack [Nalgene 5974-0404]



Prepare Media:

HM (Growth Medium with 20mM HEPES):
80% Growth medium (eg. HUESM)
20mM HEPES

HM+Sucrose:
3.42g Sucrose in 10ml HM

VS2:
30% HM
30% HM+Sucrose
20% ethylene glycol
20% DMSO

VS3:
40% HM+Sucrose
30% ethylene glycol
30% DMSO

WS3:
Growth medium +1M Sucrose

Prepare all fresh, sterile filter, and maintain on ice while working.

Freezing protocol:

Note: Work quickly. The HESC cannot be exposed to the cryoprotectants for very long or they will differentiate upon thawing. Viability may also be reduced if timing is not closely controlled. Steps 4-11 must be timed accurately.

1. Harvest HESCs in clumps by manual dissection or collagenase/dispase treatment. This protocol can also be used on collagenase/dispase-harvested HESC grown on Matrigel or MEFs.
2. Wash clumps well to remove collagenase/dispase if necessary.
3. Resuspend clumps into HM in about 0.5ml (depending on number of clumps). They can be kept at room temp in HM for up to 20min. Prolonged incubation will result in clumping and reduced attachment after thawing.
4. Transfer 40ul of the clumps into a sterile cryovial on ice in the microtube rack. Process 5 vials at a time.
5. Add 40ul of VS2 and mix by gentle pipetting let sit a 10-20 seconds
6. Add 160ul of VS3 and mix by gentle pipetting. Steps 5-6 should be completed in no more than 1 min. Handle only as many tubes as can be processed in this amount of time. Remember that handling and capping the tubes will take time.
7. Submerge the tubes quickly in liquid nitrogen and swirl while freezing. The frozen solution should have a pink glass-like appearance, while a thin layer at the top might be opaque. Be sure the caps are tightened and transfer vials to liquid nitrogen storage boxes. It is important to do this quickly to prevent the small solution volume from thawing. I usually place a storage box in 1-2 inches of LN2 in a large rectangular ice bucket while I am processing and transferring the tubes.

Thawing protocol:

Thawing is performed in the tubes and all solutions must be prepared in advance. Steps 1-3 must be performed quickly so that the cells are not exposed to the high concentration of cryoprotectants for too long. The incubation times in steps 4-7 remove the sucrose slowly and prevent osmotic shock and lysis of the cells.

1. Remove a tube from liquid nitrogen storage and quickly submerge bottom of tube in warm sterile water in a beaker.
2. quickly wipe with 70%ETOH-soaked kim-wipe.
3. Immediately add 800ul of cold WS3 (growth medium + sucrose), mix gently by stirring with the pipette tip and let sit for 30 seconds.
4. Add 1ml of growth medium, mix as above, let sit for 2min.
5. transfer to 15ml conical tube.
6. Rinse cryotube 2 times with 1ml each of growth medium and add to 15ml tube, mixing gently, let sit for 1min.
7. Add 6ml growth medium slowly dropwise to cells over about 2 min.
8. Spin 1000/4min
9. Resuspend gently in 1ml of growth medium with p1000.
11. Using a p1000 to transfer to a well of a 6-well plate with MEFs in growth medium. I have also thawed directly onto Matrigel with some success. The colonies should recover and show signs of growth within a week. Change medium daily.

ALTERNATE PROTOCOL: FREEZING HESCS BY VITRIFICATION IN STRAWS

Scott Noggle, doc. Version 1.1 1-30-07

Note: This protocol is based on a method outlined in a paper from the Bongso lab, Singapore:
An Efficient and Safe Xeno-Free Cryopreservation Method for the Storage of Human Embryonic Stem Cells.
Mark Richards, Chui-Yee Fong, Shawna Tan, Woon-Khiong Chan, and Ariff Bongso. Stem Cells 2004; 22:779-789.
It is useful for freezing small numbers of colonies. It was originally used to freeze RUES1 and RUES2 stocks and is therefore useful for thawing these early passage stocks. Use the alternate cryovial method found in this manual for bulk freezing of large numbers of colonies.

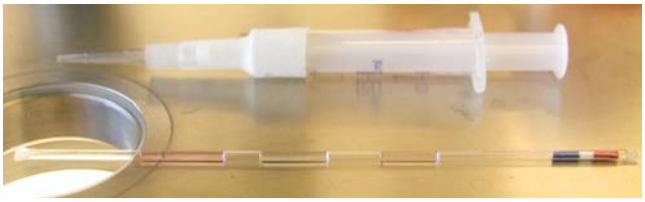
Materials:

1. Ethylene glycol [Sigma cat E9129]
2. DMSO [Sigma cat. D2650]
3. DMEM [Invitrogen cat. 11960-044]
4. FBS-ES certified [Hylcone] (can be replaced with Plasmanate or human serum albumin)
5. Sucrose [Fisher cat S5-500]
6. 1M HEPES solution [Invitrogen, cat 15630-080]
7. ZA475 Sterilized straws [IMV International Corporation cat 005592, in Pkg of 5]
8. 4-well Nunc plates
9. 3ml Syringe
10. Sterile 1.5ml eppendorf tubes
11. bag-sealer (guillotine-style)
12. benchtop LN2 container

Photo of straw with sealed ends. The liquid closest to the red/white/blue cotton plug is VS2, followed (right to left) by an air bubble, then the HESCs, then an air bubble, then a column of WS2, ending with an air column.

Straws can be purchased from:
IMV International Corporation
11725 – 95th Avenue North
Maple Grove, MN 55369
(763)-488-1881 or (763)-488-1888 (fax)

Prepare the aspiration syringe (top): The aspiration syringe is a 5ml syringe with a barrier filter p200 pipette tip attached using the rubber gasket that comes in the sterile straw package.



Prepare Media:

HM:
80% DMEM
20% FBS, HSA or Plasmanate
20mM HEPES

HM+Sucrose:
3.42g Sucrose in 10ml HM

VS2:
30% HM
30% HM+Sucrose
20% ethylene glycol
20% DMSO

VS3:
40% HM+Sucrose
30% ethylene glycol
30% DMSO

WS1:
80% HM
20% HM+Sucrose

WS2:
90% HM
10% HM+Sucrose

Prepare all fresh and maintain at room temp or cooler while working.

Freezing protocol:

Note: Work quickly. The HESC cannot be exposed to the cryoprotectants for very long or they will differentiate upon thawing. Viability may also be reduced if timing is not closely controlled. Steps 4-11 must be timed accurately and performed quickly.

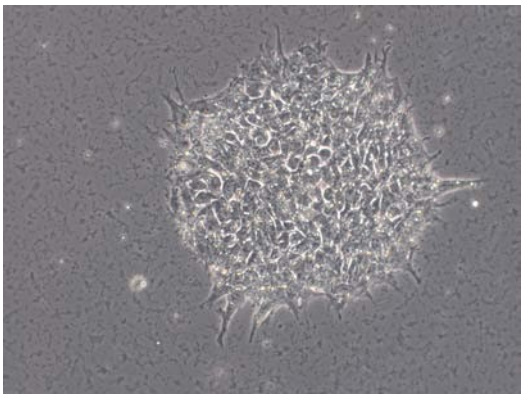
1. Harvest HESCs in clumps by manual dissection or collagenase/dispase treatment. Manual dissection from MEFs is recommended, however, this protocol can also be used on collagenase/dispase-harvested HESC grown on Matrigel.
2. Wash clumps well to remove collagenase/dispase in necessary.
3. Transfer clumps into HM in about 0.5ml into a well of a 4-well dish. They can be kept at room temp in HM for up to 20min. Prolonged incubation will result in clumping and reduced attachment after thawing.
3. Transfer 10ul of the clumps into a sterile 1.5ml eppendorf tube.
4. Aspirate a 5-10mm column of VS2 into a straw using the syringe
5. Add 10ul of VS2 and mix by stirring with end of pipette tip. Incubate for 30 seconds and no more than 1 min.
6. Add 20ul of VS3, mix, and incubate for 5 seconds
7. quickly pipette entire mix to a drop on a Petri dish lid.
8. quickly aspirate the drop into the straw from step 4 leaving an air-space between the column of VS2 in the straw and the column containing HESCs.
9. quickly aspirate a 5-10mm column of WS1 leaving an air-space between it and the HESC column.
10. quickly seal the straw ends while holding the straw with a pair of ice-cold forceps.
11. Submerge the straw quickly in liquid nitrogen. The VS2 column at the top and the HESC column should have a glass-like appearance, while the WS1 column should be opaque.

Thawing:

Thawing is performed in 4-well Nunc plates, transferring clumps from well to well with a p1000 or by mouth pipetting. It is possible to do this in the 1.5ml tubes as in the section on freezing in cryovials buy expelling the contents of the straw into the tube instead of the dish at step 6 below. Continue with step 3 in the thawing procedure for cryovials. Steps 3- must also be performed quickly.

1. set up a 4-well plate with WS1 in well 1, WS2 in well 2, HM in well 3, and 50%HM/%50 culture medium in well 4.
2. remove a straw from liquid nitrogen storage into a benchtop LN container.
3. quickly submerge straw in warm sterile water in a beaker.
4. quickly wipe with 70%ETOH-soaked kim-wipe.
5. cut the bottom end of the straw (closest to the WS1 column) with sterile scissors or blade.
6. While holding the straw tip in the WS2 in well 1 of the 4-well dish, cut the top end of the straw. The WS1 column and the HESC column should flow out of the straw. It may be necessary to use the aspiration syringe to force the entire HESC column out of the tube. The VS2 column does not have to be emptied into the well.
7. Incubate in WS1 for 1min.
8. Transfer the clumps to WS2 in well 2 for 5min using a p1000 or mouth pipette. Try to transfer in as small a volume as possible.
9. Transfer the clumps to HM in well 3 for 5min
10. Transfer the clumps to 50%HM/50% culture medium in well 4 for 5 min.
11. Using a p1000 to aspirate the clumps, transfer to a well of a 6-well plate with MEFs in growth medium. I have also thawed directly onto Matrigel with some success. The colonies should recover and show signs of growth within a week. Change medium as normally scheduled.

These photos are RUES1 colonies after thaw



KARYOTYPING HESCS

Scott Noggle, doc. versiton 1.1 9-28-07

This protocol was communicated to me by Maya Mitilipova, who used it to generate samples of BG01 and BG02 for karyotype analysis by G-banding. I have used it to generate samples of RUES1 and RUES2 for karyotype analysis by the cytogenetics service at Sloan Kettering.

PROTOCOL:

1. Feed the cells the day before karyotyping
2. Add colcemid for two hours (10ug/ml stock), 20ul per 1.5ml of medium.
3. Collect the supernatant into 15ml conical tubes and trypsinize cells, break into single cells and collect into the same tubes.
4. Centrigfuge cells at 1000rpm
5. Add about 2ml warm KCL (0.56% or 0.075M) and incubate at 37 for 20 min
6. Add 6-8 drops of fresh 3:1 (methanol:acetic acid) fixative and incubate for additional 15 min at room temp.
7. Centrifuge for 8min at 1000rpm
8. Remove supernatant and add 2ml of 3:1 fixative and incubate at RT for 10 min
9. Repeat step 7
10. Remove supernatant and add 2:1 fixative and refrigerate overnight at 4oC
11. Next morning change fixative and drop slides to check for proportion of metaphase spreads
12. Store at -20oC
13. send off for G-banding.

TERATOMA ASSAYS

Scott Noggle, Taken from our chapter in Human Embryonic Stem Cells; The Practical Handbook. 9-28-07

The ability for hES cells to generate teratomas (Keller G, 2005; Spagnoli FM and AH, 2006) in immuno-compromised mice is used as a diagnostic criteria for bona fide embryonic stem cells. In this in vivo assay, hES cells are engrafted into immuno-compromised adult mice in various tissues to generate teratomas. The resulting tumors are routinely analyzed by histology for the various derivatives of the three primary germ layers. With the exception of the host vasculature within the tumor, the teratomas are predominantly derived from the hES graft (Gertow et al., 2004). In the case of the vasculature, it was noted that both human graft-derived cells and host derived mouse cells can contribute to the vessel structures. Frequently, other differentiated and organized tissue can be found in the tumors. This can include, for example, neural tissue and retinal pigmented epithelium, muscle, cartilage, bone, and epithelial cells of the endoderm and ectoderm. However, many of these tissues may be immature and definitive identification of the mature tissue can be difficult. The assistance of a trained pathologist in evaluating the tissues is highly recommended.

Teratoma can be generated at various sites in adult SCID mice by subcutaneous, intraperitoneal or intramuscular injection, implantation under the kidney capsule or beneath the testis capsule (Pera et al., 2003; Przyborski, 2005). As the site of implantation may also influence the growth and differentiation of the teratoma (Przyborski, 2005; Cooke et al., 2006), it is recommended that several sites be tested to access the developmental potential of the hES cells. The strain of SCID mice may also make a difference in the success of engraftment (Przyborski, 2005). NOD-SCID mice are probably the best recipients, followed by the SCID-beige strain. Two protocols for implantation of hES cells into immuno-compromised mice are provided below. The protocols for subcutaneous, intraperitoneal, and intramuscular injection are similar and have the advantage of being technically simple to perform and do not require surgical manipulation of the mice.

PROCEDURES FOR ENGRAFTMENT OF HES CELLS IN SCID MICE:

1. hES cells are harvested as for passaging as described in the procedures for preparing cells for microinjection or aggregation with approximately 100-200 cells per clump. The hES cells are suspended in a small volume of media (100ul per injection) and mixed with an equal volume of thawed Matrigel and transfer to cold cryotubes. The mixture is held on ice until loaded into the syringe just before injecting.
2. The hES cells are loaded into syringes fitted with a large gage needle. Load the cells into the syringe by drawing in a small amount of media followed by the hES cell suspension before attaching the needle.
3. The suspension is injected at subcutaneous, intraperitoneal or intramuscular sites:
    - For subcutaneous injection, target the needle beneath the skin on the rear flank.
    - For intraperitoneal injections, target the abdomen.
    - For intramuscular injection, penetrate the muscle of a single rear leg to minimize discomfort and alteration of the mobility of the mouse.
4. Monitor the mice and the site of injection weekly for 6-22 weeks. The mice should be weighed weekly and watched for signs of infection during the incubation period.
5. Teratomas can be recovered by dissection with surrounding tissue and usually arise between 6-8 weeks after grafting. They are fixed in formalin and sent for histological examination by a pathology service. Alternatively, they can be embedded for cryosectioning and processed for immunohistochemical detection of germ layer markers.

EMBRYOID BODY ASSAYS

Scott Noggle, doc. versiton 1.1 9-28-07

Embryoid bodies are formed by aggregating or placing clumps of HESCs in suspension culture such that they differentiate into the three primary germ layers.

GENERAL PROTOCOL:

1. dispase treat cells as for 20 min until colonies are released from Matrix, whether on MEFs of MG.
2. Rinse colonies at least 2 times in media to remove dispase. Try not to break up colonies - they must remain in large chunks.
3. the cell clumps can be plated on bacterial dishes as a group or, alternatively, individually transferred to 96-well non-tissue culture treated V-bottom plates. If EBs attach to the bacterial dishes, the dishes can be coated in agarose.
    1. Make a 1% stock of agarose in PBS.
    2. Autoclave to sterilize
    3. while hot, pipette 10ml onto a bacterial dish to cover the bottom of the dish, then immediately aspirate as much of the agarose as possible to leave a thin coating on the dish.
    4. Allow agarose to set up for 5-10 min at room temperature.
    5. Rinse once in media
    6. plate EBs in media
4. Culture for desired number of days. Change media every 2-4 days depending on the density of the EBs in the dish. Media changes in bulk culture are performed as follows:
    1. Using a 25ml pipette, transfer EBs to a 50ml conical tube.
    2. allow EBs to settle by gravity (5-10min).
    3. remove all but 5ml of media, being sure not to aspirate any EBs.
    4. Add fresh media and transfer to a bacterial dish (preferably agarose coated). It is not necessary to change dishes at every media change - usually every other media change.

CHIMERA ASSAYS

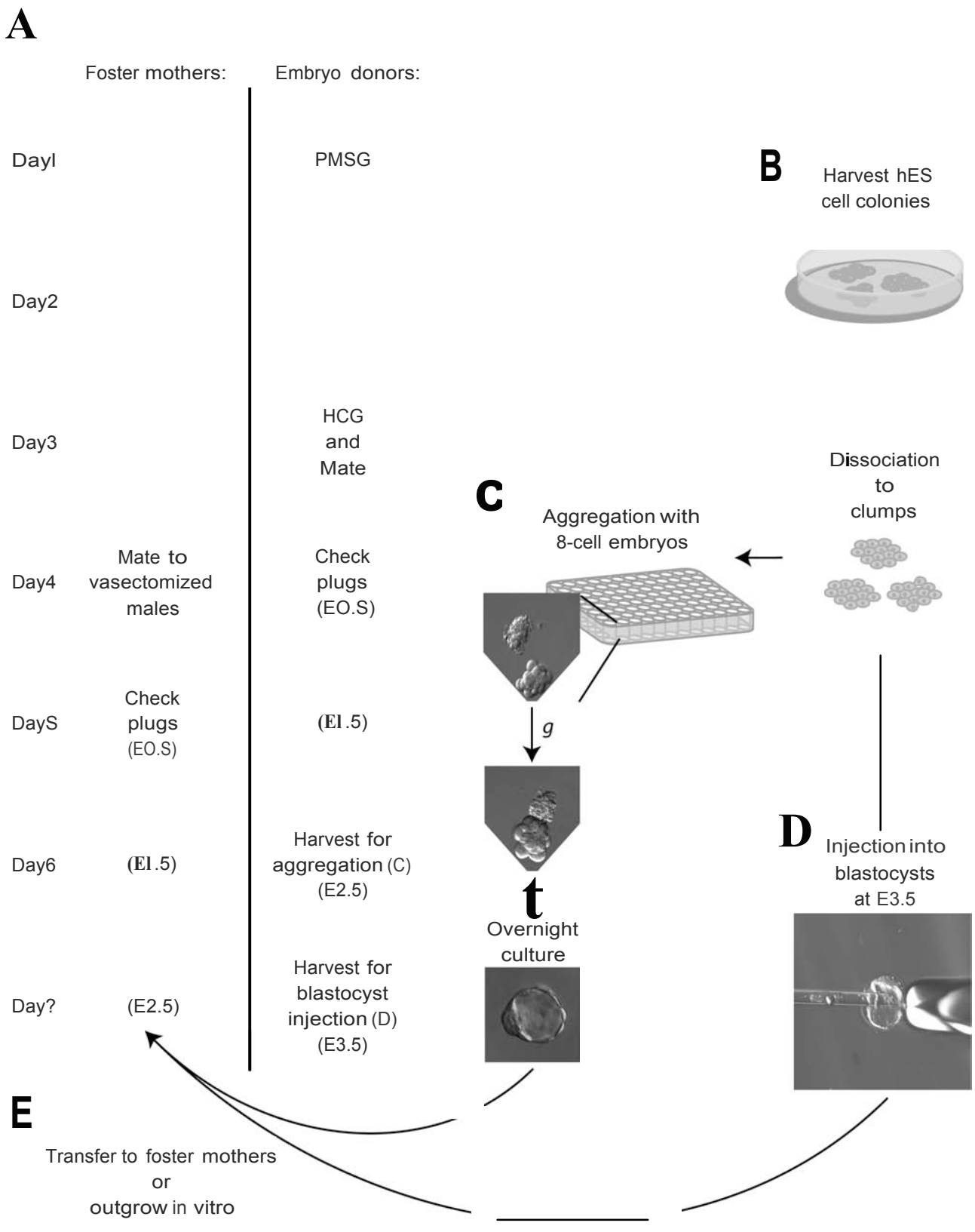
Scott Noggle, Modified from our chapter in Human Embryonic Stem Cells; The Practical Handbook. 9-28-07

These procedures are used for engrafting Human embryonic stem (hES) cells into blastocyst stage mouse embryos. In terms of developmental time in the mouse, the blastocyst represents a closely related in vivo embryonic environment for the hES cells. This tool can be used to study embryological properties of hES cells and, by using genetically modified or mutant hES cells as graft, can be extended to study the basis of disease. Protocols are presented include: (a) production of host embryos for hES cell incorporation, (b) preparation of pseudo-pregnant foster mothers for manipulated embryos, (c) preparation of hES cells for morula aggregation or blastocyst injection, (d) injection or aggregation of hES cells into appropriate stage embryos, and (e) growth in vitro as differentiating outgrowths or transfer to pseudo-pregnant foster mothers for in vivo development. The procedures are based on those described for the incorporation of mES cells into mouse embryos (Bradley, 1987; Nagy and Rossant, 1999). The pluripotent cells are incorporated directly into the mouse embryo at the blastocyst stage which, in developmental time, represents the closest related in vivo embryonic environment for the hES cells. This provides a tool for understanding the basic embryological properties of hES cells and can be extended in the future to study the bases of disease, by using genetically modified and/or diseased hES cells as graft.

Overview of Protocol:

Two protocols for the introduction of hES cells into pre-implantation mouse embryos are detailed in this chapter. A diagram of the procedure is shown in Figure 1. Both protocols require: (i) superovulation of female mice and timed matings to produce host embryos for injections or aggregations, (ii) preparation of pseudo-pregnant foster mothers to receive the manipulated embryos, (iii) preparation of hES cells for aggregation or blastocyst injection, and (iv) harvesting embryos after the appropriate number of days of gestation for aggregation or injection. For blastocyst injection, embryos are harvested at the early balstocyst stage in the morning of embryonic day 3.5. For aggregations, the embryos are harvested at the 8-cell stage in the morning of embryonic day 2.5. The embryos are injected or aggregated with hES cells and cultured in vitro to the expanded blastocyst stage on the afternoon corresponding to embryonic day 3.5. They are either transferred to the pseudo-pregnant foster mothers and allowed to develop in vivo or further grown in vitro as differentiating outgrowths. The resulting embryos can then be processed using common assays for immunofluoresence, RT-PCR, or in situ hybridization.

Figure 1


A
Foster mothers:
Embryo donors:
DayI
PMSG
Day2
Day3
HCG
and
Mate
Day4
Mate to
vasectomized
males
Check
plugs
(EO.S)
DayS
Check
plugs
(EO.S)
(El.5)
Day6
(El.5)
Harvest for
aggregation (C)
(E2.5)
Day?
(E2.5)
Harvest for
blastocyst
injection (D)
(E3.5)
B
Harvest hES
cell colonies
C
Aggregation with
8-cell embryos
Dissociation
to
clumps
g
t
Overnight
culture
D
Injection into
blastocysts
at E3.5
E
Transfer to foster mothers
or
outgrow in vitro

MATERIALS, REAGENTS, MEDIA AND EQUIPMENT

A. Mice, reagents and surgical tools for producing host embryos, recipient foster mothers and teratomas by kidney capsule surgery:

Pregnant mare serum gonadotrophin (PMSG), 50 IU/ml (Sigma #G4877)

Human chorionic gonadotrophin (HCG) 50 IU/ml (Sigma #C0684)

Blastocyst donor mice: C57Bl6/6J (The Jackson Laboratory, Strain #000664)

Morula donor mice: B6CBAF1/J (The Jackson Laboratory, Strain #100011)

Stud male mice of proven fertility for mating to produce host embryos: B6CBAF1/J (The Jackson Laboratory, Strain #100011)

Vasectomized male mice: B6CBAF1/J (The Jackson Laboratory, Strain #100011)

Female mice to produce pseudo-pregnant recipient fosters: B6CBAF1/J (The Jackson Laboratory, Strain #100011)

10g of 2,2,2-Tribromoethanol (Sigma #T48402)

10ml 2-methyl-2-butanol (tert-Amyl alcohol) (Sigma #240486)

Alcohol prep pad, sterile (Fisher #06-669-62)

Micro-dissecting forceps, Curved (Roboz #RS-5100)

Micro-dissecting forceps, Tip 0.10 x 0.06mm (Roboz #RS-4976

Fine scissors (Roboz #RS-6702)

Clamp (Roboz #RS-7440)

Aspirator tube assemblies (Sigma # A5177)

Reflex Autoclip (Roboz #RS9260)

Wound clips (Roboz #RS9962)

Calibrated glass capillary pipette, pulled over a flame (VWR #53432921)

Tween-20 (Sigma #P1379)

M2 medium (with Hepes, Sigma #M17167) or M2 medium (Specialty Media, MR-015P-5F)

35mm tissue culture dishes

4-0 gut suture attached to 3/8 circle C-13 needle

B. Manipulators, optics, cooling devices and injection chambers, injection apparatus, and pipettes for blastocyst injections:

Inverted microscope with Hoffman modulation optics and condensers

micromanipulation and injection apparatus suitable for microinjection of mouse ES cells into mouse embryos (such as Transfer-Man NK2 micromanipulation and microinjection systems from Eppendorf).

injection pipettes, 25 micron ID (CustomTip Type IV, Eppendorf #930 00 122-8 or similar). Note: The correct inner diameter of the injection pipette is critical and is larger than that typically used for microinjection of mouse ES cells.

holding pipettes (VacuTips, Eppendorf #930 00 101-5 or similar)

C. Reagents for aggregations:

60mm non-tissue culture treated dishes for embryo culture and manipulations

96-well cell culture plates with a conical bottom (Corning Costar #3894)

Acid Tyrodes Solution (Specialty Media ,MR-004-D)

Oli for Embryo Culture (Irvine Scientific, 9305)

MEDIA

	VENDOR	CAT. NO.	FINAL CONC.	VOLUMES
Embryo Culture Medium (G2v3)				for 10ml:
G2 version 3	Vitrolife	10092	to volume	9.4ml
HSA-solution	Vitrolife	10064	6mg/ml	0.6ml
Xvivo-10 Medium				for 50ml:
MEM Non-essential amino acids	Gibco	11140-050	0.1mM	0.5ml
2-mercaptoethanol	Gibco	21985-023	0.11mM	0.1ml
GlutaMAX	Gibco	35050	2mM	0.5
X-VIVO 10	BioWhittaker	04-380Q	to volume	49ml
Outgrowth Medium				For 500ml:
FBS, Certified	Gibco	16000-044	20%	100ml
Penicillin-Streptomycin	Gibco	15140-122	100U/ml	5ml
MEM Non-essential amino acids	Gibco	11140-050	0.1mM	5ml
GlutaMAX	Gibco	35050	2mM	5ml
DMEM, high glucose	Gibco	11995-065	to volume	385ml
Alternate Embryo Culture Medium (M16)				for 10ml:
M16	Specialty Media	MR-010P-5F	to volume	9.4ml
HSA-solution	Vitrolife	10064	6mg/ml	0.6ml

MOUSE STRAINS; GENERATION OF EMBRYOS AND FOSTERS MOTHERS.

If mouse-human hybrid embryos are to be transferred to foster mothers for further in vivo development, pseudo-pregnant foster mothers should be produced by mating to vasectomized males. This mating should occur one day after the mating to produce the embryo donor. Blastocysts at E3.5, whether produced by blastocyst injection or aggregation at E2.5 followed by in vitro culture to E3.5, are transferred to foster mothers at the equivalent of E2.5. A diagram of the necessary timeline is shown in Figure 1.

Procedure for timed matings to produce host embryos and pseudo-pregnant foster mothers:

Day1:

Embryo donors are intraperitoneally (IP) injected with 5 IU of PMSG using a 1ml syringe with a 26G needle. PMSG is diluted in 0.9% NaCl. In one experiment, 10 embryo donors and 10 stud males are used.

Day3:

42-48 hours after administration of PMSG, the same females are IP injected with 5 IU of hCG and mated to singly housed males of proven fertility.

Day4:

Copulation plugs are checked the in the morning. The copulation plug is a coagulation of seminal fluid and protein and is visible in the vagina of the female. Place all of the females into a cage and mark the day the animals were plugged. This day is considered 0.5day post-coitum (E0.5).

Females are checked for natural oestrus and are mated to singly housed vasectomized males. In order to ensure 10 pseudo-pregnant females, it will be necessary to examine approximately 100 animals for oestrus. An oestrus female may be identified by the degree of swelling, pink color and moistness in the vaginal area. Vasectomized males can be conveniently purchased or generated by surgical procedures described elsewhere (Bradley, 1987).

Day5:

Check for the presence of a copulation plug in the pairings with vasectomized males.

Place the females who have successfully mated, i.e. a copulation plug is clearly visible in a separate cage. Indicate on the cage card the date of the plug.

COLLECTION AND CULTURE OF BLASTOCYSTS AND PRE-COMPACTING MORULAE.

Procedure for collection of E2.5 8-cell stage host embryos:

1. Set up microdrop cultures:
   1.1. Micro-drop cultures are set up by arranging several 20µl drops of G2v3 culture media in 60mm dishes and quickly but gently overlaying with embryo culture grade mineral oil.
   1.2. The dishes are equilibrated in a tissue culture incubator at 5% CO2 for at least 30 min. before use.
2. Humanely sacrifice a superovulated embryo donor mouse in the morning of E2 and lay it on its back. Rinse with 70% Ethanol. Pinch the skin with micro-dissecting curved forceps and make a lateral incision in the midline area of the abdomen using fine scissors.
3. Holding firmly above and below the incision, pull firmly in opposite directions until the abdominal area is fully exposed.
4. To expose the reproductive tract, cut open the peritoneum to reveal the contents of the abdominal cavity. Push the intestines to one side to reveal the U-shaped uterine horns with the Ovary and oviduct at the top of each horn.
5. Cut away as a single unit the fat pad (above the ovary and attached to the kidney), ovary, and oviduct and just below the horn of the uterus and place into M2 medium (Specialty Media, MR-015P-5F).
6. In 3mL of M2 and 2mL of PBST (0.05% Tween-20 in PBS) cut away the fat pad, ovary and most of the oviduct using micro-dissecting scissors and micro-dissecting forceps.
7. Using a pair of micro-dissecting forceps, hold the uterus with one of the pair and extrude the content therein in the direction of the oviduct with a second of the pair.
8. Collect and move the precompacted 8-cell stage embryos into micro-drops using aspirator tube assembly and a pulled glass capillary pipette. Pulled Pasteur pipettes are used to move embryos between microdrops and are made by heating and pulling a calibrated glass capillary pipette over a flame of a micro-burner (see protocol for preparing glass tools)

9. The embryos can be used at this point for preparing aggregations with hES cells or cultured overnight for use in blastocyst injections.

Procedure for collection of E3.5 early blastocyst host embryos:

1. Set up micro-drop cultures of G2v3 or M16 medium as in the procedure for collection of E2.5 embryos.
2. To expose the reproductive tract, follow steps 2 through 4 in the procedure for collection of E2.5 embryos above.
3. Cut the horn of the uterus just above the cervix and above the fat pad above the ovary. Release the uterus by cutting through the mesenteries and place on a piece of absorbent paper towel. Grasp the uterus and cut away the mesenteries and blood vessels.
4. Move to a dish with M2 medium.
5. Insert a 25G needle and syringe loaded with 3mL of M2 and 2mL of PBST into the oviduct end of the uterus and hold the uterus on the needle with the curved micro-dissecting forceps.
6. Flush the blastocysts through the uterus towards the cervix end with about 1ml of media.
7. Collect and move the early blastocyst stage embryos into micro-drops of G2v3 or M16 using aspirator tube assembly and a pulled glass capillary pipette.
8. The embryos can be incubated at 5%CO2 until used for microinjection of hES cells.

PREPARATION OF HES CELLS FOR BLASTOCYST INJECTION.

One hES cell line derived in our lab, RUES1, has been recently described (James et al., 2006) and can be used with these protocols. A second cell line, RUES2, can also be used in these protocols. We are currently testing it's pluripotent properties. These lines are maintained under similar conditions to those used for most of the NIH registry cell lines. NIH approved cells lines, however, can currently not be used, as attached Material Transfer Agreements preclude their incorporation into mouse embryos. We have attempted to use the HUES6 cell line derived in the Melton lab and maintained as described (Cowan et al., 2004) without success. However, the treatment of the cells to be injected is critical to the success of incorporation into the embryos. The use of Trypsin/EDTA solutions for passaging as single cells, as described may be to harsh, limiting the ability of the cells to incorporate into the host blastocyst. As we have not adapted this line to our passaging protocols, it is still possible that this line, or similar lines, can be used.

Cells that have been maintained on MEF feeders or on Matrigel in MEF conditioned medium have been used for these protocols. We have included protocols for the passaging and harvesting methods that we used to produce cells for blastocyst injections and morula aggregations. Generally, cells grown on MEF feeders have been used for blastocyst injections and cells grown on Matrigel have been used for the morula aggregations. However, we have had success generating chimeras with both passaging schemes in the protocols.

Procedure:

1. Prepare micro-drop cultures as for embryos except use 50 µl drops HUESM supplemented with 20 U/ml of DNAse I.
2. Prepare MEF feeder plates if passaging hES cells. Prior to passaging the cells by micro-dissection, medium is changed in the well to be passaged and on a fresh feeder plate. MEF feeder plates can be prepared using a protocol such as that given in Chapter 5. We have also used a commercial source of Mitomycin-C inactivated MEFS.
3. Prepare media: We have used both H1 medium and HESM successfully. To prepare complete growth medium, bFGF is added just before feeding. To prepare complete growth medium, bFGF is added just before feeding (see table below). Stocks of bFGF are made to 100ug/ml in sterile 10mM Tris-HCl pH7.6 with 0.1%BSA. Aliquots of a convenient volume are frozen at -20oC. For maintenance on MEF feeder layers, bFGF is supplemented to 20ng/ml before feeding HES.

4. Prepare Glass tools for micro-dissection: For glass tools, Pasteur pipettes are pulled into hair-thin hooks. The hooked end of the glass tool should be thin enough for the micro-dissection of the hES cell colonies but thick enough to withstand some pressure during the dissection. Fine glass needles with hooked ends are forged in two steps over a microburner that can produce a very small flame.
   4.1. Establish a small candle-size flame with a micro-burner made from a Bunsen burner fitted with an 18guage needle.
   4.2. For glass hooks, hold a Pasteur pipette at both ends and melt the glass approximately one inch below the taper until the lumen is fused and the glass glows orange. (For pulled pipettes used in embryo manipulations, melt the glass, but do not fuse the lumen.)
   4.3. Quickly remove the glass from the flame while simultaneously pulling the ends away from each other. The glass should be drawn out to a filament without breaking. (For pulled pipettes used in embryo manipulations, stop here and break the filament with a inner diameter slightly larger than the embryos.)
   4.4. Again, while holding a Pasteur pipette at both ends several inches above the flame, slowly lower the drawn filament approximately one inch from the new taper towards the flame while gently pulling at each end. Before reaching the flame, the filament should melt in two while being drawn into a very fine filament.
   4.5. While holding the large end at approximately a 90^{o} angel to and several inches above the flame, lower the filament tip towards the flame. The rising heat should curl the filament tip up forming a “hook”.
   4.6. Using a no. 5 watchmakers forceps, trim the end of the filament to finish the end into a clean “hook”.
5. Preparing hES cells for injection or passaging: Ideal colonies or parts of colonies are micro-dissected into clumps of cells using the glass hooks. The hook is used to gently pull apart pieces of the colony. This can also be accomplished by cutting a grid into the colony with the back of the hook and pulling the pieces away from the colony one at a time. For routine passaging, a chunk size or 100-200 cells is optimal. For injection, move the clumps to a fresh micro-drop of media. The clumps are further dissected into clumps of 10-15 cells using glass needles (see Figure 2F). The back edge of the glass hook can be used to cut larger chunks into smaller pieces. Movies demonstrating this technique can be found on the Brivanlou lab web site (http://xenopus.rockefeller.edu).
6. For passaging, transfer clumps to fresh feeders: For routine passaging after micro-dissection, the cell clumps are swirled into the center of the dish and 20 to 50 clumps are transferred to the new feeder wells using p1000 micro-pipets. Pre-coat the micro-pipet tip with the medium so that the cells do not stick. If possible, leave the dishes untouched on a warmed surface (preferably under O2/CO2 blood-gas mix) for 15-30min to allow the clumps to begin attaching to the dish before moving to an incubator. Excessive handling of the new dish will cause the clumps to migrate to the center of the dish rather than remaining evenly distributed across the dish. Complete growth medium is exchanged on the growing colonies every day. The lines should culture for no more than 6 days to a week. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center of the colony. See also section on micro-dissection passaging of HESCs.

HARVESTING OF HES CELLS FOR MORULA AGGREGATION.

Human embryonic stem cells can also be grown in feeder-conditioned medium on a substrate of extracellular matrix (ECM). We routinely use Matrigel as a growth substrate for growing RUES1 hES cells in preparation for aggregation experiments. Here we describe the methods used to prepare these hES cells for aggregation and the passaging conditions used to maintain them. This protocol can also be used to prepare hES cells for blastocyst injections.

Before passaging, examine the colonies under the microscope and look for any colonies that are differentiated. Spontaneously differentiating areas of the culture can be removed with a glass tool as described in the manual dissection protocol or aspirated using a pipette attached to a vacuum. Several types of differentiation can be morphologically identified in

spontaneously differentiating cultures. Look for the center of colonies that show a depression or “crater” appearance. Areas of colonies that have begun forming cystic structures in the center of the colony should also be removed. Also avoid the edges of colonies that do not have a tight border between the feeder layer and the colony. Areas where the hES cells have started to flatten, polarize and migrate can also be removed. However, some differentiation on the borders of the colonies can be tolerated, as these cells will detach during the washing steps. Ideal colonies are comprised of small, round cells with a high nuclear to cytoplasmic ratio that are randomly organized and have not begun forming structures within the colony.

Procedure:

1. Prepare Matrigel coated plates for passaging and maintenance of cell lines: Matrigel-coated plates can be prepared as described in Chapter ? with the following modifications. Matrigel is diluted to 0.333mg/ml in cold XVIVO-10 medium. All plastics that come into contact with the undiluted Matrigel should be kept as cold as possible. We pre-cool 6-well plates, dishes and p1000 filter-tips at –20oC for about 20min. Keep the plates on the ice cold platform at all times. Place the entire ice bucket with plates into the refrigerator to coat overnight. Before plating cells, check the coating on the microscope for a meshwork-like single layer matrix. When ready to plate hES cells, aspirate the Matrigel from the wells using a Pasteur pipette in the corner of the well. Get as much Matrigel off of the dish as possible leaving a thin coating on the surface of the dish. Do not scrape the bottom of the dish. Rinsing the Matrigel-coated plate is not necessary.
2. Prepare Conditioned medium for passaging and maintenance of RUES1 cell line: MEFs for feeder plates can be prepared using a protocol such as that given in Chapter 5. We have also successfully used a commercial source of Mitomycin-C inactivated MEFS. Approximately 5 million cells from frozen vials or 3 million cells from freshly irradiated MEFs are plated on gelatin coated 10cm plates and allowed to attach overnight in FM10 media (see section on MEF production). HESM growth medium is exchanged and conditioned for 24 hours before use. For maintenance on Matrigel, bFGF is supplemented to 20ng/ml before conditioning on MEFs. The CM can be used immediately, stored at 4oC for a week or frozen at –80oC. The conditioned medium is further supplemented with 20ng/ml before feeding hES on Matrigel. Passaged hES cells are plated in 2ml of CM per well of a 6-well plate. Feeder plates can be used for up to 14 days to generate conditioned medium.
3. Passaging hES cells (See also section on enzymatic passaging HESC):
   3.1. Replace the growth medium with Dispase or Collagenase (type V) at 1mg/ml dissolved in growth medium and sterile filtered.
   3.2. Incubate for about 4-5 min in a tissue culture incubator. Check the progress of the matrix digestion, beginning at about 4 min. The colony borders will begin to peel away from the plate, while the center will remain attached (see Figure 2A, B).
   3.3. Ideally, gently wash the Dispase (or Collagenase) off of the plate with growth medium twice. The colonies should remain attached to the plate. If they have detached after the Dispase incubation transfer all of the colonies and Dispase solution to a conical tube and centrifuge at 150g for 4 min. and wash the colonies with growth medium. They should get two to three washes total – either on the plate or with centrifugation.
   3.4. If the colonies remained attached after washing, harvest the colonies with a cell lifter.
   3.5. Transfer all of the colonies and growth medium to a conical tube and spin to pellet the colonies at 150g for 4 min.
   3.6. Using the MEF-CM, resuspend the colonies using a p1000 pipette tip in about 500-700µl of medium.
   3.7. Triturate the colonies to clumps with an average size of about 100 cells using the p1000 tip.
   3.8. Plate a proportion of the clumps at a 1:10 split ratio. However, the ratio will need to be optimized for the confluence of the starting population. If possible, leave the dishes untouched on a warmed surface (preferably under O2/CO2 blood-gas mix) for 10 min to allow the clumps to begin attaching to the dish before moving to an incubator. Excessive

handling of the new dish will cause the clumps to migrate to the center of the dish rather than remaining evenly distributed across the dish. Good spacing between the colonies will allow proper growth of the colonies.

4. Maintenance: Complete conditioned growth medium is exchanged on the growing colonies every day from the MEF plates. Feeding can begin on the second day after passaging. When the colonies get bigger, increase the CM. Cultures in CM on Matrigel can usually grow for 4-5 days before they need passaging. The timing of passage is dependent upon the appearance of differentiation within the colonies—mainly from the center and edges of the colony.
5. Prepare cells for aggregation.
   5.1. Instead of breaking the clumps into ~100 cells per clump as for passaging, the clumps are further reduced to clumps of 10-15 cells by more vigorous pipetting with a p1000 pipette tip. Alternatively, colonies can be harvested before the dissociation step and micro-dissected with glass needles as described above for preparing cells for blastocyst injection.
   5.2. Spin to pellet the clumps at 150g for 4 min.
   5.3. Resuspend clumps in XVIVO-10 medium. The clumps should be at a density of about 100 clumps per micro liter.
   5.4. Proceed immediately to setting up aggregation plates as described below.

MORULA AGGREGATION

The protocol for aggregation of hES cells with mouse pre-compaction stage embryos is based on those described for aggregation of the embryos with mouse ES cells (for example, see Bradley, 1987; for example, see Nagy and Rossant, 1999). Mouse embryos prior to compaction in the early afternoon at E2.5 will aggregate and incorporate mouse ES cells with high efficiency if the zona pellucida is removed and the ES cells placed in close contact with the compacting embryo. Generally, mouse ES cells are dissociated to clumps of two to four cells and gently maneuvered into small depressions with compacting mouse embryos. These are incubated in vitro until the blastocyst stage before transfer to suitable fosters. This provides the advantage of pre-screening the embryos for incorporation if the ES cells are fluorescently labeled. Human ES cells will also aggregate with mouse embryos if healthy cells can make contact with the mouse blastomeres. Due to the low survival of hES cells after trypsinization, hES cells are dissagregated by pipetting or micro-dissection. However, the damaged cells on the edges of the resulting cell clumps can prevent the aggregation by limiting access of the health cells to the compacting blastomeres of the embryo. To overcome this limitation, we use centrifugation in conical bottom 96-well plates to bring the cells into contact with the embryos. Incorporation rates using this protocol average 38% and range from 5% to 50%. Mouse ES cells aggregated in parallel show incorporation rates of 95-100%.

Procedure for removal of the zona pellucida from 8-cell mouse embryos

1. set up 60mm plates with micro-drops of Acid Tyrodes and G2v3 medium or M16 medium.
2. select 8-cell mouse embryos and place them in one of the microdrops of G2v3 medium or M16 medium.
3. Using a mouth pipette filled with Acid Tyrodes solution transfer 10-20 embryos into a drop of Acid tyrodes under oil. Rinse by gently pipetting the embryos in and out of the micro-drop.
4. move the embryos to a fresh drop and watch for the zona pellucida to dissolve and disappear.
5. While the embryos incubate, quickly dispel the acid tyrodes from the pipette and refill with G2v3 medium or M16 medium.
6. Just as the zona pellucida disappears, move the embryos to a drop of G2v3 or M16 medium.
7. Rinse the embryos and transfer to a new drop.
8. continue processing the remaining embryos.
9. Let the embryos recover for 1 hour in the incubator in G2v3 medium or M16 medium.

Procedure for setting up the aggregation plates

1. While the embryos are recovering from the Acid Tyrodes treatment, harvest the hES cells (see above) for aggregation.
2. hES cells dissagregated to small clumps of 5-10 cells each are suspended in gassed XVIVO-10 medium at a density of about 100 clumps per microliter.
3. 5µl of hES cells are dispensed into the bottom of a 96-well conical bottom tissue culture plate. 12 wells are processed at a time to prevent evaporation.
4. Overlay the 5µl drop with 100µl of light mineral oil.
5. Incubate the assembled plate in a tissue culture incubator at 5% CO2 for 20 minutes to equilibrate the pH of the medium.
6. Retrieve the embryos from the incubator and transfer a single embryo into each well of the 96-well plate. The embryos are transferred in as small a volume as possible as the reduced volume improves the development of the embryo.
7. Briefly centrifuge the plate at 200g for 1 min to bring the embryos in contact with the hES cells.
8. Gently examine the plate under a stereo-microscope to ensure that the embryos have pelleted with the hES cells.
9. Return the plate to the incubator and leave undisturbed overnight. The embryos will have progressed to early blastocyst stage by mid-morning of the following day.
10. Embryos can be briefly examined for incorporation of hES cell if GFP labeled hES cell lines are used in the aggregation. Gently wash the unaggregated hES cells and debris from the embryos by pipetting and releasing them a few times in the well with a pulled Pasteur pipette that has an inner diameter slightly larger than the blastocyst. Transfer the embryos to fresh micro-drop of G2v3 medium under oil and examine under epifluoresence. Minimize exposure to UV to 30 seconds per embryo.
11. The manipulated embryos can be further cultured as outgrowths (see outgrowth protocol below) or transferred to foster mothers (see transfer protocol below) in the afternoon of E3.5 for further development in vivo.

BLASTOCYST INJECTION

The techniques used for microinjection of hES cells into mouse blastocysts are based on common protocols described for injecting mouse ES cells into mouse blastocysts (for example, seeBradley, 1987), with some notable differences. Current protocols for injection of mouse ES cells into blasts begin with mouse ES cells in a single cell suspension. Most hES cell lines, on the other hand, do not tolerate dissociation to single cells. They must instead be manipulated as aggregates of about 10 cells and micromanipultion strategies must accommodate this limitation. The technique we describe here uses the simplified micromanipulation set-up currently used for mouse ES cell injection, but uses larger diameter injection needles than those used for mouse ES cells. The aggregates of hES cells are gently drawn into the injection pipette. An embryo is aligned on a holding pipette with the embryonic pole oriented towards the bottom of the field. The sharp tip of the pipette is used to quickly, but precisely, puncture through the zona pellucida and trophoblast layers at the level of the equator and into the blastocoel cavity. The hES cells are then slowly expelled into the cavity and the tip withdrawn. The larger internal diameter of the injection pipette reduces, but does not eliminate, physical stress on the hES cells within the aggregates, while remaining small enough to inject the expanded mouse blastocyst without severe damage. Using these methods, we have seen an average incorporation rate of 14% when outgrowths are analyzed after 6 days of outgrowth (James et al., 2006). Though our experience with in vivo development of the hybrid embryos is limited, in one in vivo experiment, 3 of 28 implanted embryos contained hES cells at E8.5. However, only one of these embryos demonstrated normal development. At this time, the efficiency of incorporation is low and much work remains to optimize these protocols. It may also be possible to use other manipulation methods designed for the transfer of cell aggregates (Gardner, 1971; Gardner and Johnson, 1975).

Procedure for setting up the injection plates and pipettes

1. Prepare plates for injection with embryos in a micro-drop under oil in injection medium. The micro-drop plate should have two drops in the center of the dish. The top drop should have HUES media to hold hES cell clumps and the bottom drop should have G2v3 or M16 medium to hold the embryos. Also prepare additional micro-drop dishes with G2v3 medium or M16 medium.
2. Place the plate on the microscope stage.
3. Prepare micro-injection pipettes, being sure to purge air bubbles.
4. Position the pipettes in the injection plate with the coarse adjustment controls.
5. Focus on a group of embryos in a microdrop.
6. Without adjusting the focus, bring the ends of the pipettes into the focal plane with the coarse adjustment controls
7. Adjust the oil-medium interface in the injection and holding pipettes to a point near the end of the pipettes.

Procedure for injecting the blastocysts with hES cells

1. Pipette the hES cell aggregates into the reserved micro-drop in the injection plate.
2. Move the field to the the hES cell micro-drop
3. Aspirate a clump by carefully drawing the clump into the tip of the pipette.
4. Move the field to the micro-drop containing embryos.
5. Position a blastocyst on the holding pipette buy expelling medium from the holding pipette to rotate the embryo into position.
6. position the equator of the embryo in the focal plane.
7. move the injection pipette with the hES cell clump at the tip into the focal plane and with the sharp tip in line with the equator of the embryo.
8. Penetrate the zona pellucida and trophectoderm with the injection pipette in a single quick stabbing motion towards the holding pipette. This movement takes practice in order to prevent penetrating too far into the opposite trophectoderm and zona pellucida layers nearest the holding pipette. However, too slow of a motion will collapse the blastocoel and prevent full penetration into the cavity.
9. Once the injection pipette tip is in the blastocoel cavity, gently begin to expel the cell clump. Just as the clump leaves the pipette tip, pull the pipette out of the cavity with a quick motion opposite that performed in the previous step. With practice, this can be done without pulling the clump out of the cavity with the tip. As the sharp point of the injection pipette dulls or becomes clogged with hES cell debris, the tip will begin to hold onto the hES cell clumps. The injection pipette must be replaced.
10. Release the embryo from the holding pipette, segregating the injected blastocysts from the uninjected or failed injected embryos.
11. Continue procedure on remaining blastocysts, stopping every 30 min to remove successfully injected blastocysts for incubation in a micro-drop of G2v3 medium or M16 medium in a separate plate.
12. Embryos are transferred to day 2.5 pseudo-pregnant foster mothers within 4 hours of injection as detailed in the surgical procedures.

IN VITRO CULTURE AND OUTGROWTH OF HYBRID EMBRYOS

Mouse embryo-hES cell hybrids can be further cultured in vitro to assay for early germ layer formation and differentiation capability of the hES cells (Sherman, 1975). This can be done by plating the embryos from either the blastocyst injection procedure or aggregation procedure on adhesive substrates in serum containing medium that allows for attachment and

outgrowth. Though further germ layer differentiation of the mouse embryos is disorganized, this assay may be used to follow early cell fate choices.

Procedure for in vitro outhgrowth culture of hybrid embryos:

1. Coat 4-well plates with Matrigel as described in the enzymatic passaging protocol above.
2. Aspirate the Matrigel and add 0.5ml of Outgrowth medium (see Table 1) to each well.
3. Gently transfer the embryos individually to wells with a pulled transfer pipette.
4. Culture the embryos for 6 days at 5% CO2.
5. Check wells for outgrowth of the embryos.
6. Harvest the embryos by gently scraping the outgrowths from the matrix using glass hooks (see preparation of glass tools above).
7. Fix outgrowths in filtered 4% paraformaldehyde in PBS for immunofluoresence staining in suspension. The embryos can be processed using established protocols for immunofluorescent analysis of mouse embryos.

SURGICAL PROCEDURES; ANESTHESIA, TRANSFER OF MANIPULATED EMBRYOS

For in vivo analysis, the manipulated embryos are transferred at the blastocyst stage to pseudo-pregnant foster mothers to promote further organized embryonic development. This is accomplished by surgical procedures that allow access to the uterus, transfer of the manipulated embryos to the uterus, closing the wounds, and recovery of the foster mothers.

Procedure for embryo transfer to the foster mice:

1. Prepare 2.5pc pseudo-pregnant females for surgery. Administer 2.5% Avertin, 125-250mg/kg of body weight (~0.5mL/ adult female mouse) and wait several minutes for the anesthetic to take effect. Pinch the end of the tail of the animal to determine if it is fully anesthetized. If there is no response then proceed. Avertin is made as follows:
   1.1. To make a 100% stock solution, dissolve 10g of 2,2,2-Tribromoethanol in 10ml of 2-methyl-2-butanol (tert-Amyl alcohol) at 37oC. Store at 4oC in the dark.
   1.2. To make a 2.5% stock, add 0.625ml of 100% Avertin in 10ml of water dropwise while vortexing. Add 10ml of water to this and add another 0.625ml dropwise while vortexing. Bring volume to 50ml with water. The solution may need to be warmed to 37oC to dissolve. Store at 4oC in the dark.
2. Retrieve the embryos using the aspirator tube assembly-pulled glass capillary pipette from the micro-well by aspirating columns of media and air in the following order: Light Mineral Oil, air, M2 medium, air, embryos, air and medium. The length from the tip of the pipette until the first air bubble is approximately 3cm. We transfer 8-10 embryos per uterine horn. It is most convenient to load enough embryos for one transfer per pipette.
3. Surgical procedure to expose the uterus:
   3.1. Lay the animal on the stage of the dissecting microscope.
   3.2. Wipe with an alcohol prep pad.
   3.3. Pinch the skin with micro-dissecting curved forceps and make a lateral incision with fine scissors of approximately 1cm long just below the last rib.
   3.4. Wipe with an alcohol prep pad.
   3.5. Locate the ovary and cut the peritoneum.
   3.6. Retract the fat pad (attached to the kidney) with a clamp and lay it on the body.
4. Transfer of embryos:
   4.1. Gently hold the uterus with curved forceps and using a 25G needle make a hole through the uterine wall into the lumen in the area of the horn of the uterus, making sure to avoid the blood vessels.

4.2. Remove the needle and insert the capillary pipette containing the embryos. This should be done without loosing sight of the opening made with the needle.

4.3. Blow gently to release the embryos into the lumen and stop before the third air bubble enters the lumen.

5. Closing the wound:

5.1. Remove the capillary pipette and remove the clamp.

5.2. Return the uterus into the peritoneal cavity.

5.3. Clip the wound closed with Reflex Autoclip.

6. Recovery: Allow mice to recover from anesthesia in a warm cage or on a heating pad. They should recover within an hour.

HUSBANDRY PRECAUTIONS FOR HUMAN-MOUSE HYBRIDS GENERATED IN VIVO.

ES cells were first established from mouse blastocysts and have been extensively used as a tool for generating genetically modified mutant mice. This is accomplished by contribution of the genetically modified ES cell progeny to the germ line of the hybrid mice. We have not attempted term delivery of the hybrids at this point and have not found contribution of the hES cells to germ cell populations. However, considering the small number of mice we have used for in vivo transfers, it remains theoretically possible for hES cells to contribute germ cells in the hybrid mice. To prevent inadvertent mating of the hybrids and possible fertilization with hES-derived gametes, the United States National Academies of Science guidelines (http://www.nap.edu/catalog/11278.html) recommend strict separation of male and female hybrid mice if embryos are brought to term.

ANALYSIS OF CHIMERAS FROM IN VITRO AND IN VIVO PROCEDURES.

Embryos can be processed for immunofluoresence using procedures similar to those used for analysis of normal mouse embryos. Identifying incorporated human cells within the embryos is facilitated by an ubiquitous GFP label in the hES cells. Immunofluoresence for a human nuclei-specific marker (Chemicon, #MAB1281) can also be used. However, in our experience, this can sometimes result in ambiguous identification of the human cells due to cross-reactivity to antigens in the extra-embryonic tissues of the mouse. An alternative is to use FISH for human chromosomes or chromosomal features. We have followed the manufacturer's protocols with success. Handling the small preimplantation or peri-implantation embryos in these procedures is most easily accomplished by transferring the embryos through solutions in 4-well dishes using mouth pipettes. If BSA or another protein source is used in the solutions, sticking of the embryos to the mouth pipette is usually not a problem. Embryos from pre-implantation stages to E8.5 can be examined in whole mount by confocal imaging as live specimens or after fixation and staining by immunofluorescent techniques. Later stages can be fixed in 4% paraformaldehyde and sectioned with a vibratome for imaging. All solutions should be filtered to reduce staining artifacts and improve the quality of the confocal images.

LENTIVIRUS TRANSDUCTION OF HESCS

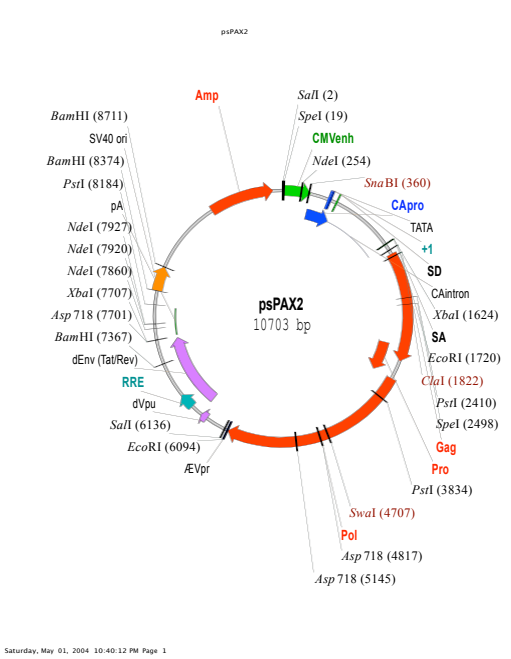
We generate Lentivector particles by co-expressing the virion packaging elements and the vector genome in 293FT human embryonic kidney cells. The packaging elements include the core and enzymatic components of the virion from HIV-1, while the envelope is derived from the vesicular stomatitis virus (VSV). We use a second generation LV packaging system that encompasses all HIV-1 genes with the exception of the envelope and all viral auxilliary genes, i.e. vpr, vif, vpu and nef.

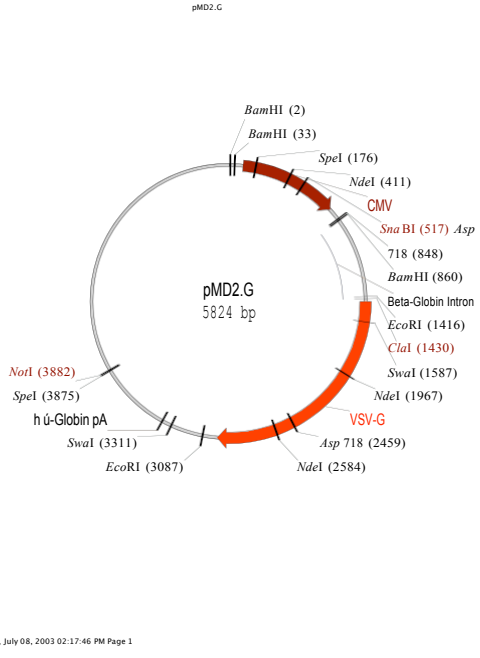
In order to produce lentivectors you need 3 components:

- vector, e.g. pCCL-PET-MCS or pCCL-PETNT-MCS, or other carrying your gene of interest
- packaging system :. psPAX2 (a 2nd generation packaging plasmid)
- envelope plasmid, e.g. pMD2G (also called VSV-G)

The vector is the only genetic material transferred to the target cells. It typically comprises the transgene cassette flanked by cis-acting elements necessary for its encapsidation, reverse transcription and integration. The vectors that we work with are self-inactivating (SIN) HIV-1-derived vectors, which lose the transcriptional capacity of the viral long terminal repeat (LTR) once transferred to target cells. This minimizes the risk of emergence of replication competent recombinant virus and avoids problems linked to promoter interference.

PLASMIDS





PLASMID PREPS

Grow up plasmids in DH5a or suitable bacteria:

From plasmid DNA stock:

1. Thaw 20ul aliquot on ice for 5 min
2. Add DNA (0.5 to 1ul) and mix by stirring (DO NOT VORTEX or PIPETTE UP AND DOWN)
3. Incubate 30 in on ice
4. Heat shock at 42oC for 30sec, then put immediately on ice for 2min.
5. Add 1ml of SOC
6. shake for 1hr at 37oC
7. Plate 50ul and 200ul on LB plate with antibiotic that has been pre-warmed.
8. Grow overnight
9. In the morning, pick a colony into 2ml liquid LB with antibiotic (AMP at 50ug/ml or 1:1000 dilution of stock)
10. inoculate cultures and shake overnitght for Qiagen Maxiprep
11. Perform Qiagen Maxipreps and quantitate DNA

293FT CELL CULTURE

Frozen stocks are available in liquid Nitrogen

From Frozen stocks:

1. Thaw 1 vail quickly in 37oC water bath
2. Resuspend in 293 media to 10ml
3. Spin at 1000rpm for 4min to pellet
4. Resuspend in 20ml of 293FT media and plate on two 10cm dishes
5. Check for density on the next day. They can be split if more than 50% confluent

From a confluent (90-100%) 10cm dish:

1. Aspirate media and add 5ml TrypLE Express
2. Remove TrypLE Express, and add 750-1000ul TrypLE Express
3. Incubate at 37oc for 3-5min to dissociate cells
4. Neutralize with 293 media, transfer to 50ml conical tube
5. Dilute to 50ml with 293 media
6. Plate 10ml per 10cm plate (1:5 split).
7. Incubate overnight, cells should be at the correct density for transfection 24hrs after split.

293FT MEDIUM	VENDOR	CAT. NO.	FINAL CONC.	FOR 500ML
FBS	Gibco		10%	50mL
GlutaMAX	Gibco	15140-122	2mM (1X)	5mL
MEM non-essential amino acids	Gibco	11140-050	0.1mM (1X)	5mL
DMEM, high glucose	Gibco	11995-065	to volume	440mL

CALCIUM PHOSPHATE TRANSFECTION

From a confluent plate of 293FT cells in 293 medium without antibiotics, split 1:5 onto 10 cm dishes and let attach overnight to dish.

1. Check that the 293FT cells are 60-80% confluent the next morning.
2. Set up transfection mixtures for 10cm dishes:

   Solution A: 36ul of 2M $CaCl_2$ and 20ug of DNA and bring to 300ul with sterile water.

vector name	10ug vector	7ug psPax2	3ug VSV-G	CaCl2	H2O to 300ul

   Solution B: 300ul of 2X HBS

3. Use one pasteur pipette and a pipetter to bubble air through the solution B and a second to add solution A drop-wise over 1-2 minutes.
4. Incubate mixture at rt for 30 min
5. add solution drop-wise to the 293FT cells
6. Incubate 6hrs (if set up in the morning) or overnight (if set up in afternoon)
7. Change media with 6ml of XVIVO (supplemented with 1XGlutaMax, 0.5XCholesterol, 1XNEAA)
8. estimate transfection efficiency the next morning, should be 70% or better.
9. Let incubate for 24 hrs, collect media and add 6ml of fresh media to incubate another 24h. PLEASE NOTE SPECIAL HANDLING PROCEDURES OUTLINED BELOW FOR TISSUE CULTURE SUPPLIES EXPOSED TO VIRUS!
10. Remove supernatant to new tube and centrifuge for 10min at 1400g(3000rmp) to remove dead cells.
11. Can repeat step 10 if there were a lot of floating cells. Can also filter with 0.45um filter.
12. It is also possible to further concentrate the virus by filtration on Amicon (100,000KDcut) columns or by ultracentrifugation.
13. Determine Titer on 293FT cells

LENTIVIRUS INFECTION OF HESCS

1. Supplement supernatant with bFGF to 100ng/ml before setting up infections
2. Add 12ul of ViroMag R/L to 1ml of viral supernatant and mix gently but thoroughly.
3. incubate for 15min at room temperature.
4. aspirate media from a one or two day old culture of HESCs growing on 4-well dishes on Matrigel in CM.
5. add 1ml of viral supernatant mix to the well.
6. place on magnet for at least 30 min (to ON).
7. The next day, gently remove the supernatant and replace with fresh conditioned medium.
8. Change media daily, reporter gene expression is usually noticeable 3-4 days after infection.

LENTIVIRUS HANDLING PRECAUTIONS:

While we use second generation packaging systems and self-inactivating vectors to greatly reduce the chance of producing replication competent vrius, the virus we produce is still capable of infecting human cells and integrating into the cell's genome. Therefore we use special handling procedures during tissue culture to prevent exposure to the virus. These precautions are outlined below.

IMMUNOFLUORESCENT PROCEDURES & MARKERS

These general protocols can be used for cells in culture on Matek coverslip plates or on mouse embryos and chimeras. If doing stains on embryos, filter the solutions to prevent particulates from sticking to the embryos. This will result in cleaner images. Embryos can be stained by transfering the embryos from solution to solution in 4-well or 24-well plates using a mouth pipetting apparatus. It is important to transfer only small volumes or rinse the embryo into the next solution. For the final step, embryos can be plated on Matek coverslips in the absence of protein to allow them to stick to the plate. This will prevent some migration of the embryos around the dish while imaging.

GENERAL PROTOCOL:

1. Wash plates once in 1X PBS
2. Fix in about 2ml of 4% PFA for 20 min at RTs
3. Wash two times in 1X PBS. The dishes can be stored at 4oC if sealed with parafilm
4. Block with 3% normal serum (species dictated by host of secondary antibody, usually Donkey or Goat for Alexa-conjugated secondaries) with 0.1% Triton-X in 1X PBS (without Ca/Mg) for 30 min at RT.
5. Remove Block and Add primary antibodies in Block
6. Incubate at 4oC overnight.
7. Wash three time for at least 30min each wash in PBST (PBS with 0.1% Tween-20) at RT. Washes can go overnight.
8. Add secondary diluted in Block
9. incubate at RT for 30min or overnight at 4oC
10. Wash twice in PBST for 30 min each wash
11. If counterstaining with SytoxOrange (or other nuclear counterstain), add in PBST. SytoxOrange is diluted at 1:25,000.
12. Wash twice in PBST and leave in PBST. They can be stored at 4oC sealed with parafilm or imaged. If photobleaching is a problem, the PBST can be removed and a drop of Vectashield mounting medium added. A coverslip can be placed on top to prevent evaporation.

MARKER ANTIBODIES:

	VENDOR	CAT. NO.	ISOTYPE	DILUTION	OTHER CONDITIONS
Pluripotency:					
Oct3	BD Transduction Laboratories	611203	Mouse IgG	1:500	0.1%Triton in Block
Oct4 (H134)	Santa Cruz	sc-9081	Rabbit poly	1:200	0.1%Triton in Block
Nanog	R&D Systems	AF1997	Goat poly	1:50	0.1%Triton in Block
Sox2	R&D Systems	245610	Mouse IgG	1:50	0.1%Triton in Block
SSEA4	Chemicon	MAB4304	Mouse IgG	1:100	no Triton
SSEA3	DSHB or Chemicon	MAB4303	Rat IgM	1:100	no Triton
Ectoderm:					
NFH	Sternberger	SMI32	Mouse, IgG	1:500	0.1% Triton

	VENDOR	CAT. NO.	ISOTYPE	DILUTION	OTHER CONDITIONS
beta III Tubulin (Tuj1)	R&D Systems	MAB1195	Mouse, IgG	1:75	0.1% Triton
Cytokeratin (Pan)	DAKO	Z0622	Rabbit poly	1:100	0.1% Triton
Cytokeratin 8	DAKO	M0631	Mouse IgM	1:50	0.1% Triton
Mesoderm:					
Muscle Actin (MF20)	DSHB	MF20	Mouse, IgG	1:10	0.1% Triton
Desmin	Abcam	ab8592-500	Rabbit Poly	1:100	0.1% Triton
CD31 (PECAM)	Abcam	ab9498-500	Mouse, IgG	1:100	0.1% Triton
Brachyury	Santa Cruz	sc-20109	Rabbit poly	1:50	0.1% Triton
Cardiac Actin	Fitzgerald	RD1-PRO61075	Mouse, IgG	1:10	0.1% Triton
Endoderm:					
AFP	DAKO	A0502	Rabbit poly	1:100	0.1% Triton
HNF3b	Santa Cruz	sc-6554	Goat poly	1:50	0.1% Triton
IFABP	Abcam	ab7805-500	Rabbit poly	1:100	0.1% Triton
GATA6	Santa Cruz	ssc-9055	rabbit poly	1:50	0.1% Triton
Trophectoderm:					
CDX2 (only early)	BioGenex/Novacastra	MU392-UC	Mouse, IgG	1:100	0.1% Triton
HCGbeta	Abcam	ab400-500	Mouse, IgG	1:100	0.1% Triton
Germ Cell:					
MVH (VASA)	Abcam	ab13840	Rabbit poly	1:100	0.1% Triton

Secondary Antibodies are from Molecular Probes and are conjugated to Alexa fluorophores. We use Alexa 488 (Green), Alexa 555 (orange-red), and Alexa 647 (far red). These match the best with our confocal using Multitrack settings.

Nuclear counterstains available for confocal work:

SytoxGreen (green)
SytoxOrange (orange-red)
TOPO-3 (far red)
(All are from Molecular Probes.)

REMEMBER - SytoxGreen and SytoxOrange will be detected in multiple channels under epifluoresence using the filters available on our scopes. However, these can be used with confocal detection without spill-over.

REAL-TIME RT-PCR PROTOCOLS & MARKERS FOR HESCS

Scott Noggle, doc. version 1.0 1-20-06

Experimental design considerations:

General experimental design considerations: The degree of regulation in gene expression expected determines the level of stringency in experimental design needed for proper interpretation of a real-time RT-PCT experiment. Generally, we are interested in changes in gene expression relative to a control conditions (ie undifferentiated vs. differentiated samples or drug treated vs. untreated samples) which allows us to use a relative quantification strategy rather than an absolute quantification. Absolute quantifications are difficult because of the need for a standard dilution series with known copy number of the desired target and the requirement of identical amplification efficiencies between the standards and unknowns. Relative quantification alleviates some of these worries. The next consideration is the use of a standard curve to derive quantities for unknowns from their Cp. We also have software available that does not need a standard curve to test for statistical differences between conditions. This software uses the raw Cps rather than quantities extrapolated from standard curves. This is nice for statistical analysis but is difficult to graph - or rather, generates graphs that are normalized to the control condition but that exclude this condition in the graph. This is a legitimate way of presenting the data, but confuses people that are used to actually seeing the control data. However, a standard curve is desirable for this software as it is used to calculate the actual PCR efficiency. Though, this probably does not need to be done for each run. If using the standard curve to generate quantitied from Cps, another consideration is wether or not to include a standard curve for every gene used in every run or generate a standard curve for use in multiple runs. The LightCycler software allows you to use an external standard curve but requires running one point from the original standard in each subsequent run. This is used to adjust the concentrations between runs. [relative vs. absolute, standard curves,]

Genes available for assays: We have, as of this writing, generated two publication describing microarray analysis of undifferentiated vs. differentiated HESCs. The first compared undifferentiated (+CM) and differentiated (-CM) samples in triplicate using the Affymetrix U133A chip (note: the U133B chip only has data available for the undifferentiated samples, therefore some genes of interest will not have been analyzed for regulation with CM withdrawal). This analysis resulted in a list of about 600 genes that were enriched in the undifferentiated HESCs. We followed up these experiments with a second analysis of three independent microarray studies (including our own) in which we determined a consistent set of 111 enriched and 95 depleted genes common and consistent among all three studies. About 106 of the 111 gene were verified by real-time RT-PCR and roughly 95% of these were found to be regulated in independent experiments on H1 and RUES1 HESC lines. The 95 depleted genes have not yet been analyzed. (I am working on verifying the nuclear factor set as the fraction showed the most stable expression levels in the enriched set of genes). The intention is to combine the nuclear factor sets from the enriched and depleted gene lists into an assay to be used for quality control of HESC cultures.

PRIMER DESIGN FOR REAL-TIME RT-PCR

High quality primers are needed quantification using real-time PCR and there are several considerations for good primer design.

Initial primer design strategies:

Begin by designing primers in regions of low homology to other closely related transcripts with a preference for intron-spanning primer pairs. Roche has a web application, called the Universal Probe Library, that will do this for you if you enter the Ensembl ID for your gene (preferred) or, alternatively, directly from entered sequence or gene bank identifiers. The Ensemble ID is preferred because it uses the intron/exon information in Ensembl rather than a computer algorithm to detect the junctions. In my experience, the Ensembl exon predictions are more accurate because they are based on database evidence. The limitation of the software comes from the fact that it is looking for primer and probe pairs for their Universal Probe Library, so it might miss some good sites. (The Universal Probes are essentially short TaqMan hydrolysis probes - not that they are bad - we have mainly used SYBRGreen instead because it's cheaper for screening large number of genes.) I have also used the PrimerQuest application at the IDT website with good success.

To verify specificity, perform a BLAST search using the "Search for short, nearly exact matches" option by entering the forward primer followed by a string of 11 "n's" followed by the reverse primer sequence. Blast against the "nr" (non-redundant) database and limit to your species (in our case, Homo sapiens) in the drop down menu under "Options". This performs a "virtual" PCR. In the results, scan for hits in which there are alignments of the full sequence of both primers in one gene. Be sure that your gene is the only one that meets these criteria. Some hits will have one primer or the other or sometimes multiple alignments with one primer. Also calculate the product length generated by the alignments to be sure the product size is the expected value and that products generated by genomic DNA derived products are large enough that they will not be amplified. Shorter products are more efficiently generated during fast real-time cycling.

Primer quality verification:

1. Check that the amplification gives a single product. This can be done using SYBRgreen reactions and melting curve analysis. Specific amplification should result in a single melting point peak, with no peak or a peak with a significantly lower melting point in the no-template controls. The low melting point peaks represent primer dimers. Gels and Southern's can also be run to identify multiple bands using probes for specificity. However, the melting point analysis is probably the most convenient method to quickly screen primer sets. Good sets can then be further verified by gel and Southern blots. TaqMan probes, instead of SYBRgreen, can be used to increase the specificity of the real-time PCR detection. However, the use of these probes increases the cost and can reduce the ability to identify the cause of poorly performing primer sets. If TaqMan probes are intended to be used in a multiplex reaction (for detection of more than one gene in a single tube), an effective strategy would be to design several primer and probe set together, but screen only the primer sets for effeciency, specificity and sensitivity as described here and below. After finding good primer pairs that are compatible, the probes can then be optimized.

2. Check the efficiency of the primer pair: PCR efficiency is critical for accurate results with real-time PCR. The efficiency can be measured from the slope of a standard curve. The standard curve should be at least 4 (preferably more) serial dilutions (I use 1:10 or 1:4) of sample cDNA. If sample amount is limiting, you can also use double stranded PCR product that has be cleaned up for this purpose. Every primer set should be tested for PCR efficiency prior to use. A standard curve with a slope

of -3.332 is near 100% efficient [E= ($10^{(-1/slope)}$) -1]. This can be done in Excel or in the LightCycler software by designating the dilution series as a standard curve.

3. The sensitivity of the primer set can be derived from a standard curve. This is the last point that is in the linear region of the standard dilution series that results in a high r^2 value. The dilution before the one that falls out of the linear range is the lowest quantifiable value for that primer set. It has bee recommended that the efficiency and sensitivity values for the primer sets be reported along with an identification of a database reference for the target sequence, primer sequences and length of the PCR product when an primer set is published.

RNA EXTRACTION AND RT REACTIONS

RNA extraction using Trizol or RNAbee:

RNA extraction:

1. Use 1ml Trizol/well of 6-well plate, minimize pipetting
2. Incubate at RT fro 10min, shake gently to mix
3. add 200 ul of Chloroform
4. shake for about 15 sec to mix phases.
5. spin at to speed for 5 min.
6. remove aqueous phase to new tube, add 600ul isopropanol, mix
7. spin 15 min in cold at top speed
8. wash pellet in 70% etoh
9. spin 5 min in cold at top speed
10. resuspend pellet in 20ul DEPC-water

Quantitate on fluorometer using RiboGreen reagent:

1. mix 1ml of DEPC-TE with 1ml of RiboGreen in a cuvette
2. Add 2ul of RNA sample
3. incubate in dark for 5min
4. take reading on fluorometer. Results are in ng/ul

DNAse treat using DNA-free kit from Ambion (for LESS THAN 10ug of RNA):

1. add 0.1volume of 10X Dnase I buffer and 1ul Dnase I
2. mix and incubate for 25min at 37˚ C.
3. add 5ul of Dnase Inactivation Reagent (make sure slurry is resuspended before pipetting)
4. Incubate for 2min at room temp
5. Centrifuge at top speed for 1min
6. Remove supernatant to new tube
7. quantitate on fluorometer as above

DNAse treat using DNA-free kit from Ambion (for MORE THAN 10ug of RNA):

1. dilute to 750ug/ml RNA (but try to keep volume low)

2. add 0.1volume of 10X Dnase I buffer and 2-3ul Dnase I (I also do 2ul for 30min followed by another 2ul for 30 min.)
3. mix and incubate for 1hr at 37˚ C.
4. add 0.2 vol (or 5ul whichever is greater) of Dnase Inactivation Reagent (make sure slurry is resuspended before pipetting)
5. Incubate for 5min at room temp
6. Centrifuge at top speed for 1min
7. Remove supernatant to new tube
8. quantitate on fluorometer as above

cDNA Synthesis:

cDNA synthesis using SuperScript III kit:

1. Set up RNA/OligodTVN/dNTP mix for each sample:
    - 2ug RNA
    - 2ul OligodTVN
    - 2ul 10mM dNTPs
    - H2O to 20ul

*Scale up appropriately for more RNA, but limit to 100ul/tube

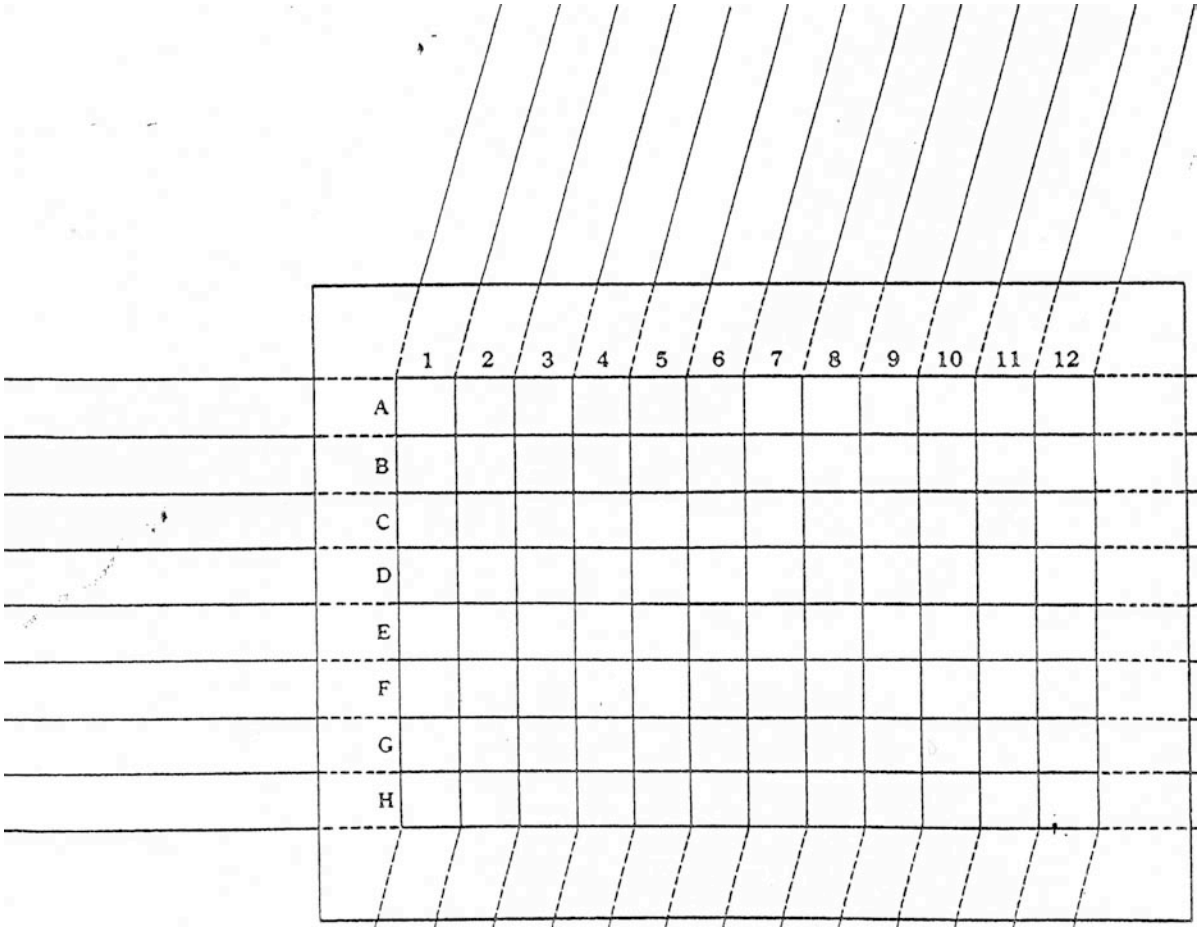
2. heat to 65˚C for 5min, immediately place tube on ice
3. Prepare RT-master mix (one mix with RT, one set without):
    - 10x RT-Buffer 2ul
    - 25mMMgCl2 4ul
    - RNAseOUT 1ul
    - SuperScript III 1ul (0ul for -RT)
    - H2O to 10ul/rxn 2ul (3ul for –RT)

(These volumes are per rxn or per 1ug RNA, scale up as needed)
†Also note there is NO DTT in the reaction – interferes with SYBR Green in PCR.

4. Mix 10ul from step 3 with 10ul of RNA/primer mix from step 2 in PCR tubes for a total of 20ul.
5. incubate 50 in at 50˚C followed by 85˚C for 5min on thermal cycler.
6. Add 1ul Rnase H to each rxn and inc for 20 min at 37˚C

This produces a final cDNA with a concentration of 50ng/ul. I use approximately 2.5-10ng per PCR reaction. So this means that I can run 100-400 reactions per microgram of RNA. However, standard curves also need to be generated - usually starting with 25ng/rxn followed by 1:10 dilutions.

REAL-TIME PCR SET-UP SHEET



	PER REACTION	NO. OF RXNSS	MASTER MIX
Template	5µL	X___	
Primers (2µM)	2µL		
H2O	3µL		
SYBRgreen Master Mix (2X)	10µL		

	PER REACTION	NO. OF RXNSS	MASTER MIX
Template	5µL	X___	
Primers (2µM)	2µL		
H2O	3µL		
SYBRgreen Master Mix (2X)	10µL		

MARKER	PRIMER NAME	FORWARD SEQ.	REVERSE SEQ.	SIZE	TM
Pluripotency					
Oct4	hPOU5F1rt-F/R	CAAGCTCCTGAAGCAGAAGAGGAT	CTCACTCGGTTCTCGATACTGGTT		
Nanog	hNanogrt-F/R	CCGGTCAAGAAACAGAAGACCAGA	CCATTGCTATTCTTCGGCCAGTTG		
Sox2 (Scott-microarr.)	SN_75_Sox2-F/R	TCAGGAGTTGTCAAGGCAGAGAAG	GCCGCCGCCGATGATTGTTATTAT		
Ectoderm					
Cytokeratin (Melton)	Keratin-F/R	AGGAAATCATCTCAGGAGGAAGGGC	AAAGCACAGATCTTCGGGAGCTACC		
Sox1	hSox1rt-F/R	GAGATTCATCTCAGGATTGAGATTCTA	GGCCTACTGTAATCTTTTCTCCACT		
NFH (Melton)	NFH	TGAACACAGACGCTATGCGCTCAG	CACCTTTATGTGAGTGGACACAGAG		
Mesoderm					
Brachyury	hBrachyury-rt-F/R	CACCTGCAAATCCTCATCCTCAGT	TGTCATGGGATTGCAGCATGGA		
Goosecoid	hGoosecoidrt-rt-F/R	CGCCTCGGCTACAACAACTACTTCTA	ACGTTCATGTAGGGCAGCATCT		
Chordin	hChordin-rt-F/R	TGTGAGCGGGATGACTGTTCACT	AAGAGCCTTCGGCTTCTTTCTCCA		
Cardiac actin	cACT	TCTATGAGGGCTACGCTTTG	CCTGACTGGAAGGTAGATGG		
Endoderm					
Gata6	hGATA6rt-F/R	TTTCCGGCAGAGCAGTAAGAGG	CCGTCAGTCAAGGCCATCCA		
Mix1					
Shirin					
IFABP (Pedersen)	IFABP	TGCCTAGAGGCTGACTCAACTGAAA	CCTTTTTAAAGATCCTTTTGGCTTC		
Sox17	hSox17rt	GGCGCAGCAGAATCCAGA	CCACGACTTGCCCAGCAT		

MARKER	PRIMER NAME	FORWARD SEQ.	REVERSE SEQ.	SIZE	TM
FoxA2	hFoxA2rt-F/R	CGT TCC GGG TCT GAA CTG	ACC GCT CCC AGC ATA CTT T		
CXCR4	hCXCR4rt-F/R	CAC CGC ATC TGG AGA ACC A	GCC CAT TTC CTC GGT GTA GTT		
Germ Cells					
Gdf9 (Elisa)	hGDF9-F/R	CTC TTC ACC CCC TGT ACC C	CAG TTC CAC TGA TGG AAG GAT		
Vasa (Elisa)	hVASA-F/R	AGG GCT TGG GAG AGC AAG	CAT AGG AAG ACA TAT GAG GGT TGA		
Trophectoderm					
hCG beta	hCGbrt-F/R	ATCACCGTCAACACCACC ATCTGTG	AGAGTGCACATTGACAGC TGAG		
House keeping					
Beta-2-microglobulin	hB2Mrt-HPLC-F/R	TTC TGG CCT GGA GGC TAT C	TCA GGA AAT TTG ACT TTC CAT TC		
TBP	hTBPrt-HPLC-F/R	GCT GGC CCA TAG TGA TCT TT	CTT CAC ACG CCA AGA AAC AGT		
ATP5O	hATP5Ort-HPLC-F/R	ACT CGG GTT TGA CCT ACA GC	GGT ACT GAA GCA TCG CAC CT		
UBC	hUBCrt-HPLC-F/R	ATT TGG GTC GCG GTT CTT G	TGC CTT GAC ATT CTC GAT GGT		
HPRT	hHPRTrt-HPLC-F/R	TGA CCT TGA TTT ATT TTG CAT ACC	CGA GCA AGA CGT TCA GTC CT		

SUPPLIES INVENTORY TABLE

ITEM:	CAT. NO.	VENDOR
0.22um Filter Millex-GV	SLGV013SL	Millipore
0.2um acrodisc Syringe Filter	PN 4612	Life Sciences
0.45um Filter MillexHV	SLHV033RS	Millipore
100mm TC Dishes	25382-166	Falcon
10ml pre-wrapped pipettes	4488	Costar
10ml Syringes for filtering solutions	D-78532	Henke Sass Wolf
12-well TC plates	353043	Falcon
14ml Falcon Tubes	21008-918	Falcon
150 ml Filter Systems	974101	Nalgene (Fisher)
150x20mm Nunc round TC dishes	157150	Nunc
1L Filter Systems	154-0020	Nalgene
1ml pre-wrapped pipettes	13-678-11B	Fisherbrand
24-well TC treated plates	353047	Falcon
250ml Filter Systems	974104	Nalgene
25ml pre-wrapped pipettes	4489	Costar
2-Mercaptoethanol, 100ml bottles	21985-023	Invitrogen
35 mm Glass Bottom Culture Dishes	P35GC-0-14-C	MatTek
35mm TC Dishes	25382-064	VWR
4-well TC plate	176740	Nunc
500ml Filter Systems	974105	Nalgene
50ml Falcon Tubes	21008-951	Falcon
5ml pre-wrapped pipettes	4487	Costar
60mm TC Dishes	25382-100	Falcon
6-well TC plate	353046	Falcon
96-well "U"-bottom with low evaporation lid	353077	Falcon
96-well "V"-bottom TC treated	3894	Costar

ITEM:	CAT. NO.	VENDOR
96-well plates, conical "V" bottom Untreated Polystyrene	222-8031-01V	Evergreen
96-well round-bottom with polystyrene lid	3790	Costar
96-well TC treated plates	62406-081	Falcon
Aqua Clean water bath solution	WAK-AQ-250-50L	WAK-Chemie Medical GMBH
Autoclave bags31x38 200/pk	1826D	-
B27 Supplement without vitaminA	12587-010	Invitrogen
Biocidal ZF	WAK-ZF-1 (1L spray)	WAK-Chemie Medical GMBH
Cell Lifter	7200364	Corning
Cell Strainer	352340	BD Falcon
Collagenase Type IV, 1g	17104-019	Invitrogen
Cryotubes, 1.8ml	377267	Nunc
Demecolcine Solution, 10ml	D1925	Sigma
Dispase (100ml bottle)	07913	Stem Cell Technologies
Dispase, lyophilized, 5g bottles	17105-041	Invitrogen
Distilled Water, 500ml bottles	15230-204	Invitrogen
DMEM High Glucose, 500ml bottles	11995-073	Invitrogen
DMEM/F12(1:1) with HEPES, 500ml bottles	11330-057	Invitrogen
DMEM/F12(1:1) without HEPES, 500ml bottles	11320-033	Invitrogen
DMSO Solution, 100ml bottles	D2650	Sigma
Dnase I solution	10776785001	Roche
Ethylene Glycol, 1L	102466-1L	Sigma
F-12K Medium, 500ml bottles	30-2004	ATCC
Fisherbrand Gloves (L)	19-050-550C	Fisher
Fisherbrand Gloves (M)	19-050-550B	Fisher
Fisherbrand Gloves (S)	19-050-550A	Fisher
Gelatin Solution(0.1% in water), 500ml bottles	ES-006-B	Specialty Media

ITEM:	CAT. NO.	VENDOR
Glutamax, 100ml bottles	35050-061	Invitrogen
Ham's F-10 medium without L-Glutamine, 500ml bottles	9056	Irvine Scientific
Hepa Filter 5.5diax1.50 for Forma Incubator	760175	-
HEPES buffer solution (1M)	15630-106	Invitrogen
Hygromycin B	H-9773	Sigma
Knockout DMEM, 500ml bottles	10829-018	Invitrogen
L-15 Medium Leibovitz, 500ml bottles	L1518	Sigma
L-Glutamine, 200mM, 100x, 100ml bottles	25030-081	Invitrogen
LIF-ESGRO	ESG1107	Chemicon
Lipofectamine 2000 Reagent	11668-019	Invitrogen
Matrigel hESC qualified matrix, 5ml bottles	354277	BD Biosciences
MatTek glass-bottom 24-well plates	P24GC-0-13F	MatTek
MEM Non-essential amino acid solution, 10mM, 100x, 100ml bottles	11140-050	Invitrogen
MEM Sodium Pyruvate Solution, 100mM, 100x, 100ml bottles	11360-070	Invitrogen
Mouse Embryonic Fibroblasts (Mefs)	PMEF-CF	Chemicon
mTeSR maintenance medium for hES cells	05850	Stem Cell Technologies
Mycoplasma-off (Mycoplasma disinfectant)	15-5000	WAK-Chemie Medical GMBH
Oil for Embryo Culture (light mineral oil), 100ml bottles	9305	Irvine Scientific
Opti-MEM I Reduced Serum Medium, 500ml bottles	31985-088	Invitrogen
Paraformaldehyde 4% in Phosphate Buffer Saline pH7.4	s2303-32oz	Poly Scientific
PBS without Ca and Mg, 500ml bottles	14190-250	Invitrogen
Pen/Strep, 100ml bottles	15140-122	Invitrogen

ITEM:	CAT. NO.	VENDOR
Poly-L-Ornithine (0.1% solution)	p4957 (50ml)	Sigma
Propylene Glycol, 500ml	p4347	Sigma
RPMI Medium1640 1x, 500ml bottles	21870-076	Invitrogen
Sucrose	S-7903, 1kg	Sigma
TrypLE Express, 100ml bottles	12605-010	Invitrogen
Trypsin-EDTA (0.5% Trypsin-EDTA), 100ml bottles	25300-054	Invitrogen
VenorGeM-Mycoplasma Detection Kit (50 tests)	-	Minerva Biolabs
ViroMag R/L Viral Gene Delivery Reagent,200ul	RL40200	Bocascientific
X-VIVO 10 medium, 1L	04-380Q	BioWhitaker

Protocol for generation of hiPS cells (Zeeshan Ozair)

Preparation of DNA

DNA purified by the Qiagen miniprep kit can be used for nucleofection. Fresh minipreps are preferred as DNA supercoiling is an important factor for nucleofection efficiency and is lost over time in frozen DNA stocks. To concentrate the DNA, I use the ammonium acetate-based ethanol precipitation to resuspend DNA to a concentration of about 1-2 µg/µl. I use the ammonium-acetate method because residual phenol from other DNA extraction protocols can reduce the viability of the nucleofected cells.

1. Ammonium acetate-based ethanol precipitation: Add 0.5 volumes of 7.5 M ammonium acetate and 0.7 volumes of isopropanolol to the DNA in solution and mix well.
2. Spin at full speed in a microcentrifuge for 30 minutes. Carefully remove the supernatant.
3. Rinse the pellet in a volume equal to the precipitate of ice cold 70% ethanol.
4. Spin at maximum speed for 15 minutes. Remove the supernatant.
5. Airdry the pellet on the 37°C heating block.
6. Resuspend precipitated DNA in TE prewarmed to 65°C.

Generally an assumption of about 75% recovery is good for determining the volume to resuspend. Then read A260 values on the spec to confirm.

For nucleofection, we'll be using 2µg of the ePiggybac transposase and 8µg of the ePB-TT-OSKM/PUΔTK reprogramming construct (Figure 1). This construct allows for high levels of OSKM expression only in the presence of DOX. The PUΔTK cassette allows for positive selection of cells carrying the reprogramming cassette with puromycin (for selecting iPS cells from MEFs after reprogramming). In addition, it also contains the thymidine kinase gene which allows for negative selection of iPS cells with ganciclovir once excision of the reprogramming cassette has been carried out with GAL4-transposase/UAS strategy.

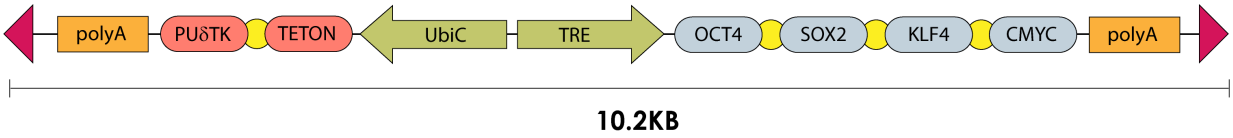


Figure 1: *Schematic of the ePB-TT-OSKM/PUΔTK bicistronic reprogramming construct. The promoter UbiC drives constitutive expression of the tetracycline transactivator and PUΔTK cassette (bridged by a 2A peptide), while the TRE/CMV hybrid promoter allows for OSKM expression only in the presence of DOX. This construct also allows for positive and negative selection of cells via the PUΔTK cassette. Note that OSKM transcription factors are also linked by 2A peptides.*

Nucleofection of NHDFs:

The day before nucleofection, plate one frozen vial of NHDFs on gelatin in a 6cm dish in fibroblast growth media.

1. On the day of nucleofection, mix 82µL of Nucleofection Solution NHDF-neo (or Solution L) and 18µL supplement per transfection and leave the mixture at room temperature for 10 minutes before use.
2. Add 1mL of fibroblast growth medium (with 20ng/mL bFGF) per 3cm dish and set aside at 37°C in the incubator. Place the remaining aliquot of medium in the water bath.

IMP: The medium needs to be either freshly prepared or thawed from stock to ensure good cell survival.

3. Wash the cultured NHDFs with PBS once and trypsinize with 5 mL TrypLE for 5-10 min at 37°C. Neutralize TrypLE with regular growth medium. Ensure dissociation into single cells by pipetting several times.
4. Spin down the cells at 300g for 5 minutes.
5. Resuspend the cells in 10mL of regular medium and count them in a hemocytometer (average of 4 squares, multiply by 10^4). This will give the number of cells/mL.
6. Spin down approximately 1 million cells per nucleofection.
7. Resuspend 1 million cells in 100 µL of nucleofection solution per nucleofection.

IMP: Steps 8-10 need to be carried out within 15 minutes to achieve good survival of cells.

8. Add the ePiggybac constructs at a ratio of 8µg: 2µg of ePB-TT-OSKM/PUΔTK: transposase and pipette gently several times.
9. Transfer to an Amaxa nucleofection cuvette. Nucleofect using program U-020 (B-016 for Solution L).
10. Immediately add 500µL of warm fibroblast growth medium to the cuvette.
11. Using the pipette provided with the Amaxa kit, transfer the nucleofected NHDFs into the 3cm dish set aside at the start and replace into 37°C for 4 days. On day three post-nucleofection, plate 50,000 MEFs/cm^2 on gelatin in a 3cm dish in reprogramming medium (Nutristem containing 100ng/mL of bFGF and 2µg/mL of DOX).
12. On the fourth day, trypsinize the nucleofected NHDFs and plate 50,000 cells onto the MEFs in reprogramming medium. This will be considered Day 0 of reprogramming. Continue to replace media everyday for six days.
13. On Day 5, plate 50,000 MEFs/cm^2 on gelatin per well of a six-well plate in reprogramming medium.
14. On Day 6 of induction, trypsinize the NHDF plate and split the cells at a ratio of 1:3 to 1:6, depending on the proliferative rate of the starting NHDF population. We generally use a ratio of 1:3. Replate the NHDFs onto MEFs plated the day before in reprogramming medium.
15. Change media everyday till colonies appear. Colonies start appearing on Day 14 and can be picked on Day 21-23 [Figure 2].
16. On picking, colonies should be plated onto MEFs in a 12-well plate in Nutristem media with 10µM ROCK-inhibitor and 100ng/mL of bFGF.

The iPS cells are further expanded and subjected to further characterization by IF, qRT-PCR (for ESC-specific and lineage-specific genes), as well as the teratoma assay. The number of ePiggybac integrations are determined by SplinkTA PCR as described [Lacoste et al., 2009]. The established lines can be subjected to excision for transgene free iPS cells and selected with ganciclovir, as described. The ratio of GAL4-transposase to pBS-UAS for excision is 1:2 [2µg of GAL4-transposase, 4µg of pBS-UAS].

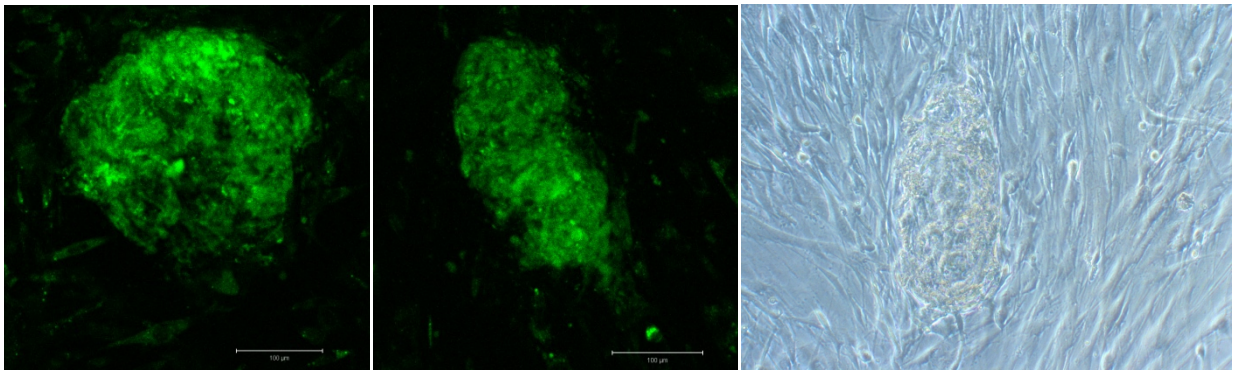


Figure 2: Live cell staining of iPS colonies on Day 21 of the ePiggybac reprogramming protocol. The colonies were stained with a conjugated TRA-1-81 antibody. The last panel shows a brightfield image of a typical colony surrounded by MEFs. Observe the ESC-like morphology of the colonies.

Picking colonies:

1. Detach about 6 colonies with two 10 µL pipette tips under an IVF microscope without pipettors. The colonies will now float on an acellular cleft left by the detached cells.
2. Break the floating colonies into smaller clumps by pipetting up and down with a P200.
3. Pipette cells into a 3cm MEF plate containing 2 mL of Nustristem medium supplemented with 100ng/mL of FGF and 10µM ROCK-inhibitor.
4. Change medium everyday.

Flow cytometry of hESCs

Preparation of samples

1. Wash cells with Ca^{2+}/Mg^{2+}-free PBS and detach with accutase for 20 minutes at 37°C. Pipet gently several times with a 1mL tip to ensure a single cell suspension and aliquot into a 1.5mL eppendorf. Addition of 5mM EDTA to accutase may promote dissociation.
2. Collect cells by centrifugation at 300g for 5 minutes and aspirate supernatant.
3. Resuspend cells thoroughly in 0.5 mL PBS. Add 0.5 mL of 4% PFA for a final concentration of 2% PFA.
4. Fix for 10 minutes at 37°C. **IMP:** Chill tubes on ice for 1 minute.

The fixed cells can be stored at this point in PBS with 0.1% Sodium Azide at 4°C. For intracellular staining, cells will need to be permeabilized with methanol. Antibodies that bind to extracellular antigens not require permeabilization, so we can proceed to staining directly.

5. Methanol permeabilization: Pellet the cells by centrifugation at 450g for 5 minutes at 4°C, aspirate PFA and resuspend the cells thoroughly in 100 µL PBS. Now add 900 µL **ice cold** methanol (in PBS) while vortexing gently. Incubate for 30 minutes on ice. Proceed with staining or store cells at -20°C.

6. Transfer the samples to FACS falcon tubes (12x75mm). Add 2 mL Incubation Buffer (0.5% BSA in Ca^{2+}/Mg^{2+}-free PBS) to each tube and rinse by centrifugation 450g for 5 minutes at 4°C. Flipping the tubes upside down to remove supernatant works fine. Repeat.
7. Resuspend cells in 100 µL Incubation Buffer per assay tube. Block for 10 minutes at room temperature.
8. Add the unconjugated or fluorochrome-conjugated primary antibody at the appropriate dilution to the assay tubes and incubate for 1 hour at room temperature. Rinse twice as before in 3 mL incubation buffer by centrifugation.
9. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 ml PBS and analyze on flow cytometer; for unconjugated primary antibodies, resuspend cells in Alexafluor-conjugated secondary antibody diluted in 100 µL incubation buffer (1:500).
10. Incubate for 30 minutes at room temperature. Rinse twice as before in incubation buffer by centrifugation.
11. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer. If DNA staining is required, resuspend cells in 0.5 ml of DNA dye (SYTOX). Incubate for 5 mins at room temperature and analyze cells on flow cytometer.

Required controls

At least two controls are essential for each antibody. In the first, only conjugated secondary antibodies or isotype control primary conjugated antibodies are added. In the second set, no antibodies are added. This will serve to set the gates for cell clumps and other debris. Addition of primary antibodies alone is not essential if the specificity of the antibodies has been established previously by immuno-cytochemistry.

The average lentiviral titers using this protocol is around 5 x 10^6 - 5 x 10^7 infection units per ml (IU/mL) when titered with 293T cells.

Day 0 Seed 6.0 × 10^6 (1.0 × 10^6) 293T cells in a 10-cm plate (6-well plate), so that the cell density will be around 1.0 × 10^7 (1.7 × 10^6) at the time of transduction. The plates must be coated with 5mL of 0.1% gelatin for 30 min to promote adhesion as the cells tend to detach after lentiviral production.
The 293FT cells should be grown in 500 µg/mL Geneticin after thawing. On the day of transfection, media must be replaced with 5.0 (1.0) mL DMEM/10% FBS WITHOUT antibiotics.

Day 1 Gently mix 45.0 (7.5) µL LF2000 and 1.5 (0.25) mL Opti-MEM medium and incubate at room temperature for 5 minutes. Meanwhile, mix 18.0 (3.0) µg of pCCL-cDNA vector and helper plasmid mixture into 1.5 (0.25) mL OptiMEM medium. Use polypropylene tubes to prevent LF2000 sticking to the walls.
Now gently combine the two mixtures and incubate at room temperature for 20 minutes to allow DNA-lipid complexes to form. In the meantime, replace the overnight culture medium with 5.0 (1.0) mL DMEM + 10% FBS without antibiotics. Add the 3.0 (0.5) mL DNA-LF2000 complexes to 293FT cells.

Day 2 Replace the media containing the DNA-LF2000 complexes with 10.0 (2.0) mL complete medium at 12-16 hours post-transfection.

Day 4 Collect supernatants at 48 hours post-transfection and transfer media to a polypropylene tube. Spin the virus-containing media at 1300 rpm for 5 minutes to pellet any 293T cells that were carried over during collection. Carefully transfer the supernatant to a sterile polypropylene eppendorfs.

IMP Lentiviral stock may be stored at 4 °C for up to 5 days, but should be aliquoted and frozen at -80 °C for long-term storage.
ALWAYS snap freeze viral aliqouts in liquid N_2 to prevent a severe loss in viral titers!

Complete medium

430mL	DMEM with glucose
50mL	Fetal calf serum, heat-inactivated
5mL	Non-Essential Amino Acids
5mL	Sodium pyruvate
5mL	GlutaMAX
5mL	Pen-strep

5mM Sodium butyrate

Number of lentiviral pools		***17***		
	10cm dish		6-well plate	
pCCL-cDNA	9 µg	*153 µg*	1.5 µg	*25.5 µg*
psPAX2	6 µg	*102 µg*	1 µg	*17 µg*
pMD2G	3 µg	*51 µg*	0.5 µg	*8.5 µg*
Lipofectamine 2000	45 µL	*765 µL*	7.5 µL	*119 µL*
Total OptiMEM	3 mL	*51 mL*	0.5 mL	*8.5 mL*
293FT cells / vol. of medium	1.0×10^7/ 5 mL	*85 mL*	1.7×10^6/ 1 mL	*17 mL*

Vector size 6910 **bp** **Lipofection amount** 9 **µg**

	Insert size	Insert + vector size	Ratio	DNA / 9µg	Conc.	Amount required
PAX6	1270	8180	1	572.8	400	1.4
LHX2	1220	8130	1	569.3	400	1.4
OTX2	870	7780	1	544.8	400	1.4
ZNF521	4300	11210	1	784.9	400	2
NR2F1	1660	8570	1	600.1	400	1.5
SOX2	970	7880	1	551.8	400	1.4
ZFHX1B	3645	10555	1	739.1	400	1.8
FOXG1	1470	8380	1	586.8	400	1.5
POU3F1	1360	8270	1	579.1	400	1.4
PLZF	2020	8930	1	625.3	400	1.6
HES5	580	7490	1	524.4	400	1.3
SIX3	999	7909	1	553.8	400	1.4
cMYC	1340	8250	1	577.7	400	1.4
BMI1	981	7891	1	552.5	400	1.4
GLI3R	2200	9110	1	637.9	400	1.6
					Total	22.5

Serial	Construct	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total amount (ng)
1	PAX6	8180		611.4	609.7	627.5	613.7	610.2	624	612.7	612.1	615.5	608.2	610.3	612	610.2	616.5	8594
2	LHX2	8130	608		605.9	623.7	609.9	606.4	620.2	609	608.4	611.8	604.5	606.6	608.3	606.5	612.7	8541.9
3	OTX2	7780	581.8	581.5		596.8	583.7	580.3	593.5	582.7	582.2	585.4	578.5	580.5	582.1	580.4	586.3	8175.7
4	ZNF521	11210	838.3	837.9	835.5		841	836.2	855.1	839.7	838.9	843.5	833.5	836.4	838.8	836.3	844.8	11755.9
5	NR2F1	8570	640.9	640.6	638.7	657.4		639.3	653.8	641.9	641.3	644.9	637.2	639.4	641.2	639.3	645.8	9001.7
6	SOX2	7880	589.3	589	587.3	604.5	591.2		601.1	590.2	589.7	593	585.9	587.9	589.6	587.8	593.8	8280.3
7	ZFHX1B	10555	789.3	789	786.7	809.7	791.9	787.3		790.6	789.9	794.2	784.8	787.5	789.7	787.4	795.4	11073.4
8	FOXG1	8380	626.6	626.4	624.6	642.8	628.7	625.1	639.3		627.1	630.6	623.1	625.2	627	625.1	631.5	8803.1
9	POU3F1	8270	618.4	618.2	616.4	634.4	620.4	616.9	630.9	619.4		622.3	614.9	617	618.8	616.9	623.2	8688.1
10	PLZF	8930	667.8	667.5	665.6	685	669.9	666.1	681.2	668.9	668.3		664	666.3	668.2	666.2	673	9378
11	HES5	7490	560.1	559.9	558.2	574.6	561.9	558.7	571.4	561	560.5	563.6		558.8	560.4	558.8	564.5	7872.4
12	SIX3	7909	591.4	591.2	589.5	606.7	593.3	590	603.3	592.4	591.9	595.1	588.1		591.8	590	596	8310.7
13	cMYC	8250	616.9	616.7	614.9	632.9	618.9	615.4	629.3	618	617.4	620.8	613.4	615.5		615.4	621.7	8667.2
14	BMI1	7891	590.1	589.8	588.1	605.3	592	588.6	602	591.1	590.5	593.8	586.7	588.8	590.4		594.7	8291.9
15	GLI3R	9110	681.2	681	679	698.8	683.4	679.5	694.9	682.4	681.7	685.5	677.4	679.7	681.6	679.6		9565.7
	Total		9000.1	9000.1	9000.1	9000.1	8999.9	9000	9000	9000	8999.9	9000	9000.2	8999.9	8999.9	8999.9	8999.9	
	DNA per pool	9000 ng																
	Number of plates	1																

Serial	Construct	Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total amount (µL)	DNA Conc.
1	PAX6	8180		1.5	1.5	1.6	1.5	1.5	1.6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	21.2	400
2	LHX2	8130	1.5		1.5	1.6	1.5	1.5	1.6	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	21.2	400
3	OTX2	7780	1.5	1.5		1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.4	1.5	1.5	1.5	1.5	20.9	400
4	ZNF521	11210	2.1	2.1	2.1		2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	29.4	400
5	NR2F1	8570	1.6	1.6	1.6	1.6		1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	22.4	400
6	SOX2	7880	1.5	1.5	1.5	1.5	1.5		1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	21	400
7	ZFHX1B	10555	2	2	2	2	2	2		2	2	2	2	2	2	2	2	28	400
8	FOXG1	8380	1.6	1.6	1.6	1.6	1.6	1.6	1.6		1.6	1.6	1.6	1.6	1.6	1.6	1.6	22.4	400
9	POU3F1	8270	1.5	1.5	1.5	1.6	1.6	1.5	1.6	1.5		1.6	1.5	1.5	1.5	1.5	1.6	21.5	400
10	PLZF	8930	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7		1.7	1.7	1.7	1.7	1.7	23.8	400
11	HES5	7490	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4		1.4	1.4	1.4	1.4	19.6	400
12	SIX3	7909	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		1.5	1.5	1.5	21	400
13	cMYC	8250	1.5	1.5	1.5	1.6	1.5	1.5	1.6	1.5	1.5	1.6	1.5	1.5		1.5	1.6	21.4	400
14	BMI1	7891	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		1.5	21	400
15	GLI3R	9110	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7		23.8	400
	Total		22.6	22.6	22.6	22.4	22.6	22.6	22.5	22.5	22.6	22.6	22.6	22.6	22.6	22.6	22.6		

1 Plate 500, 000 MEFs on gelatin per well of a six-well plate in fibroblast media.
2 The next day, plate 50, 000 human fibroblasts in Stemgent Nutristem media supplemented with 100ng/mL bFGF.
3 After two hours, add the reprogramming factor mRNAs at a ratio of 3:1:1:1:1. nlsRFP can also be added at a ratio of 1. Refer to "Calculations" for exact amounts and below for the transfection protocol. Media is replaced ***every*** day.
4 On Day 5 of induction, plate 500, 000 MEFs on gelatin per well of a six-well plate in Nutristem media supplemented with 100ng/mL bFGF.
5 On Day 6, split the NHDFs with TrypLE at a ratio of 1:6 and replate onto MEFs plated the day earlier. The NHDFs should attach within 2 hours, after which mRNA transfection for Day 6 can be carried out.
6 mRNA transfection should be continued for the next 12 days.
7 Colonies start appearing on Day 14 and can be picked on Day 21. On picking, colonies may be plated onto MEFs in a 12-well plate in Nutristem media with ROCK-inhibitor.

DO NOT ADD ANTIBIOTICS TO THE MEDIA.

1 Dilute RNA 5X in OptiMEM. For 12µL of RNA, add 48µL of OptiMEM.
2 Dilute 5µL per µg of RNAiMAX 10X in OptiMEM. For 1200ng of RNA, dilute 6µL RNAiMAX in 54µL of OptiMEM.
3 Pool the RNA/OptiMEM and Lipofectamine/OptiMEM mixture and incubate for 15 minutes at room temperature.
4 Dispense evenly into culture medium. Mix gently by rocking the plate back and forth.
5 Replace medium after 4 hours. For a 3cm dish, add 2.3mL of media. Use Stemgent Nutristem + 100ng/µL FGF for iPS reprogramming experiments. Add B18R interferon inhibitor to a final concentration of 200ng/mL (1:500 dilution).

DO NOT ADD ANTIBIOTICS TO THE MEDIA.

1 Dilute RNA in OptiMEM. For 48µL of RNA, add 192µL of OptiMEM and mix gently but thoroughly.
2 Dilute 24µL RNAiMAX in 216µL of OptiMEM and mix gently but thoroughly.
3 Pool the RNA/OptiMEM and Lipofectamine/OptiMEM mixture and incubate for 15 minutes at room temperature.
4 Dispense 120µL of the RNAiMAX/RNA mixture evenly into culture medium. Mix gently by rocking the plate back and forth. Use Stemgent Nutristem + 100ng/µL FGF for iPS reprogramming experiments. Add B18R interferon inhibitor to a final concentration of 200ng/mL (1:500 dilution).
5 Replace medium after 4 hours. For a 3cm dish, add 2.4mL of media.

mRNA	Size	Ratio	mRNA ng /1200ng	Conc.	Amount for 17 days (µL)	Amount for 4 wells (µL)	
OSKM	5350	0	0	100	0	0	
OCT4	1450	3	461.9	100	78.5	314	
LIN28	1000	1	106.2	100	18.1	72.4	
CMYC	1650	1	175.2	100	29.8	119.2	
KLF4	1800	1	191.2	100	32.5	130	
SOX2	1400	1	148.7	100	25.3	101.2	
nlsRED	1100	1	116.8	100	19.9	79.6	
							17 days
				Total	204.1	816.4	4 wells
				Per day	12µL	48µL	

DO NOT ADD ANTIBIOTICS TO THE MEDIA. Use 1.8mL of culture media per 3cm dish.

1. Dilute RNA in OptiMEM. For 22.5µL of RNA, add 67.5µL of OptiMEM.
2. Dilute 5µL per µg of RNAiMAX 10X in OptiMEM. For 1500ng of RNA, mix 7.5µL RNAiMAX in 82.5µL of OptiMEM.
3. Pool the RNA and Lipofectamine mixture and incubate for 15 minutes at room temperature.
4. Dispense evenly into culture medium. Mix gently by rocking the plate back and forth. Use N2B27 + 10ng/µL FGF for neuronal reprogramming experiments. Add B18R interferon inhibitor to a final concentration of 200ng/mL (1:500 dilution).
5. Replace medium after 4 hours. For a 3cm dish, add 1.8mL of media.

N2B27 medium
235mL DMEM/F12 with GlutaMAX
235mL Neurobasal w/o Vit. A
10mL B27 supplement
5mL N2 supplement
5mL NEAA
5mL Sodium pyruvate
5mL Penstrep
2.5mL GlutaMAX
900µL β-mercaptoethanol
10ng/mL FGF

1 Ligate insert into pIVT-KAN and do a miniprep.

2 Linearize 10μg of plasmid in a 100μL reaction with XbaI, SnaBI, SpeI, or SalI at 37°C for at least 6 hours. Inactivate RE at 75°C for 20 min.

3 PCR purify. Resuspend DNA in 31.5μL EB buffer. Measure the concentration on the nanodrop. Expected concentration = 300ng/μL.

4 Assemble the following reaction and mix well:

IMP: Thaw all components on ice except buffer, which should be at room temperature.

IMP: Use only the Megascript T7 kit. The mMessage mMachine kit has low yield.

IMP: Assemble the reaction at 25°C!

Scale		5		
Component	**Concentration**		**Mastermix**	
ATP	75	2	10	
GTP	75	0.4	2	
ARCA cap analog	100	1.2	6	
Pseudouridine TP	100	1.5	7.5	
Methylcytidine TP	100	1.5	7.5	
Reaction buffer	10X	2	10	
Enzyme Mix		2	10	
RNAsin	1U/μL	0.5	2.5	
ssDNA binding protein	5μg/mL	0.6	3	
Linearized DNA (1.0μg)		4		20
Nuclease free water		4.3	21.5	
Total (without DNA)		***16***	***80***	

5 Incubate reaction at 37°C for 4 hours. If transcription produces a truncated product due to GC-rich template, try incubating at 16°C.

6 Add 1 μL TURBO DNase, mix well and incubate for 15 minutes at 37°C.

Do NOT add EDTA to the reaction.

7 Add ~150bp of polyA tail using the Tailing Kit:

IMP: EPAP, $MnCl_2$ and ATP need to be on ice at all times!

Component	**Concentration**		**Mastermix**	
DNAse-treated reaction		21		105
E-PAP Buffer	5X	20	100	
$MnCl_2$	25mM	10	50	
ATP	10mM	10	50	
Nuclease free water		35	175	
Total (without RNA)		***75***	***375***	

Remove 0.5μL of the reaction mixture before adding the E-PAP enzyme.
Add 4μL of E-PAP and mix gently. Incubate at 37°C for 1 hour.
Store at -20°C.

8 Purify RNA with RNeasy Mini kit and elute in 50+40μL RNAse free water warmed to 95°C.

9 Treat with 1μL of Antarctic Phosphatase/25μg RNA for 30 min at 37°C to remove residual 5'-triphosphates.

10 Purify again with RNeasy MinElute kit and elute in 40+35μL RNAse free water warmed to 95°C.

RNA should always be thawed on ice!

1 Ligate insert into pIVT-KAN and do a miniprep.

2 Linearize 10μg of plasmid in a 100μL reaction with XbaI, SnaBI, SpeI, or SalI at 37°C for at least 6 hours. Inactivate RE at 75°C for 20 min.

3 PCR purify. Resuspend DNA in 31.5μL EB buffer. Measure the concentration on the nanodrop. Expected concentration = 300ng/μL.

4 Assemble the following reaction and mix well:

IMP: Thaw all components on ice except buffer, which should be at room temperature.

IMP: Use only the Megascript T7 kit. The mMessage mMachine kit has low yield.

IMP: Assemble the reaction at 25°C!

Scale		2		
Component	**Concentration**		**Mastermix**	
ATP	75	2	4	
GTP	75	2	4	
Pseudouridine TP	100	1.5	3	
Methylcytidine TP	100	1.5	3	
Reaction buffer	10X	2	4	
Enzyme Mix		2	4	
RNAsin	1U/μL	0.5	1	
ssDNA binding protein	5μg/mL	0.6	1.2	
Linearized DNA (1.0μg)		4		8
Nuclease free water		3.9	7.8	
Total (without DNA)		***16***	***32***	

5 Incubate reaction at 37°C for 4 hours. If transcription produces a truncated product due to GC-rich template, try incubating at 16°C.

6 Add 1 μL TURBO DNase, mix well and incubate for 15 minutes at 37°C.

Do NOT add EDTA to the reaction.

7 Purify RNA with RNeasy Mini kit and elute in 20μL RNAse free water warmed to 95°C.

IMP: Purification is essential as Mg^{2+} in reaction buffer degrades mRNA on heating.

8 Cap the mRNA using the ScriptCap m7G capping system

IMP: Do not add the capping enzyme to the mastermix!

Add 50.5μL of nuclease free water to bring up the reaction to 71.5μL.
Incubate at 65°C for 10 minutes, then transfer to ice.

Component			**Mastermix**	
ScriptCap Capping buffer	10X	10	20	
GTP	10mM	10	20	
SAM (diluted from stock)	2mM	5	10	
ScriptGuard RNAse inhibitor		2.5	5	
Total		***27.5***	***55***	

Now add 1μL Capping Enzyme per reaction individually.
Add 27.5μL of the mixture to the 71.5μL of reaction and mix gently.
Incubate at 37°C for 1 hour.

9 Add ~150bp of polyA tail using the Tailing Kit:

IMP: EPAP, $MnCl_2$ and ATP need to be on ice at all times!

Component			**Mastermix**	
DNAse-treated capped reaction		56		112
E-PAP Buffer	5X	20	40	
$MnCl_2$	25mM	10	20	
ATP	10mM	10	20	
Total (without RNA)		***40***	***80***	

Remove 0.5µL of the reaction mixture before adding the E-PAP enzyme.
Add 4µL of E-PAP and mix gently. Incubate at 37°C for 1 hour.
Store at -20°C.

10 Purify RNA with RNeasy Mini kit and elute in 50+40µL RNAse free water warmed to 95°C.

11 Treat with 1µL of Antarctic Phosphatase/25µg RNA for 30 min at 37°C to remove residual 5′-triphosphates.

12 Purify again with RNeasy MinElute kit and elute in 40+35µL RNAse free water warmed to 95°C.

RNA should always be thawed on ice!

DO NOT ADD ANTIBIOTICS TO THE MEDIA. Use 1.8mL of culture media per 3cm dish.

1. Dilute RNA in OptiMEM. For 22.5µL of RNA, add 67.5µL of OptiMEM.
2. Dilute 5µL per µg of RNAiMAX 10X in OptiMEM. For 1500ng of RNA, mix 7.5µL RNAiMAX in 82.5µL of OptiMEM.
3. Pool the RNA and Lipofectamine mixture and incubate for 15 minutes at room temperature.
4. Dispense evenly into culture medium. Mix gently by rocking the plate back and forth. Use N2B27 + 10ng/µL FGF for neuronal reprogramming experiments. Add B18R interferon inhibitor to a final concentration of 200ng/mL (1:500 dilution).
5. Replace medium after 4 hours. For a 3cm dish, add 1.8mL of media.

N2B27 medium
235mL DMEM/F12 with GlutaMAX
235mL Neurobasal w/o Vit. A
10mL B27 supplement
5mL N2 supplement
5mL NEAA
5mL Sodium pyruvate
5mL Penstrep
2.5mL GlutaMAX
900µL β-mercaptoethanol
10ng/mL FGF

N2B27 medium formulation for mESCs
234mL DMEM/F12 with GlutaMAX
234mL Neurobasal w/o Vit. A
10mL B27 supplement
5mL N2 supplement
5mL NEAA
5mL Sodium pyruvate
5mL Pen/Strep
2.5mL GlutaMAX
900μL β-mercaptoethanol
100μL 25% BSA fraction V
1mL 10mg/mL Insulin

Add fresh to 50mL media:
50μL LIF (final conc. 10ng/mL)
25μL PD0325901 (MEK-I; final conc. 0.5μM)
15μL CHIR (GSK3β inhibitor; final conc. 3μM)
5μL PD173074 (FGFR inhibitor; final conc. 100nM)

Component	Company	Quantity	Cat. No.
MEGAscript® T7 Kit w/Manual	Ambion	x1	AM1333M
Poly(A) Tailing Kit w/Manual	Ambion	x2	AM1350M
NorthernMax® Formaldehyde Load Dye	Ambion	x3	AM8552
BD Matrigel™, hESC-qualified Matrix	BD Biosciences	x2	354277
B18R Recombinant Protein	Ebioscience	x1	14-8185-62
0.5-10 Kb RNA Ladder	Invitrogen	x1	15623-200
Lipofectamine RNAiMAX	Invitrogen	x1	13778-150
Opti-MEM® I Reduced-Serum Medium	Invitrogen	x1	31985-062
FGF-basic Recombinant Human	Invitrogen	x5	13256-029
MOPS buffer (10X)	Millipore	x3	S4601
EmbryoMax® Primary MEFs, Mitomycin C treated	Millipore	x1	PMEF-CF
EmbryoMax® ES Cell Qualified 0.1% Gelatin Solution	Millipore	x1	ES-006-B
3´-O-Me-7mG(ppp)G RNA Cap Structure Analog	NEB	x1	S1411L
Antarctic phosphatase	NEB	x1	M0289S
SalI	NEB	x1	R0138S
RNeasy MinElute Cleanup Kit	Qiagen	x1	74204
Stemedia™ NutriStem™ XF/FF Culture Medium	Stemgent	x1	01-0005
StainAlive™ DyLight™ 488 MαH TRA-1-81 Antibody	Stemgent	x1	09-0069
5-Methylcytidine-5'-Triphosphate (100mM)	Trilink Biotech	5µmole	N-1014
Pseudouridine-5'-Triphosphate (100mM)	Trilink Biotech	5µmole	N-1019
Single-Stranded DNA Binding Protein (SSB)	USB via Affymetrix	x1	70032Y 100 UG

KAPA HiFi HotStart DNA polymerase

Scale Factor		1		
Components			***Mastermix***	
Sterile water	16.25		16.25	
5X KAPA HiFi GC Buffer	5		5	
dNTP (10mM)	0.75		0.75	
F primer (10µM)	0.75			0.75
R primer (10µM)	0.75			0.75
Template DNA (10 – 100ng)	1			1
$MgCl_2$ (25mM)	0		0	
Betaine (5M)	0		0	
KAPA HiFi DNA polymerase	0.5		0.5	
Total	***25***		***22.5***	***2.5***
				25

		Temp. (°C)	*Time*	
1	*Initial Denaturation*	95	2-5	min
2	*Denaturation*	98	20	sec
3	*Annealing*	60-75	15	sec
4	*Extension*	72	30	sec/kb
5	*Repeat steps 2-4*		15-35	times
6	*Final extension*	72	5	min
7	*Cooling*	4	∞	

GC-RICH PCR System

Scale Factor		2	
Components	**0.5M**	***Mastermix***	
Sterile water	22	44	
GC-RICH resolution solution	5	10	
dNTP	1	2	
F primer	1		2
R primer	1		2
Template DNA	5		10
Subtotal	***35***	***56***	***14***
Sterile water	3	6	
$MgCl_2$ (25mM)	1	2	
5X GC-RICH reaction buffer	10	20	
Polymerase mix	1	2	
Subtotal	***15***	***30***	
Total	***50***		***100***

		Temp. (°C)	*Time*	
1	*Initial Denaturation*	95	3	min
2	*Denaturation*	95	30	sec
3	*Annealing*	45-65	30	sec
4	*Extension*	68-72	45	sec/kb
5	*Repeat steps 2-4*		10	times
6	*Denaturation*	95	30	sec
7	*Annealing*	45-65	30	sec
8	*Extension*	68-72	45	sec/kb
			+5	sec/cycle
9	*Repeat steps 6-8*		20-25	times
10	*Final extension*	72	7	min
11	*Cooling*	4	∞	

For targets > 3kb, use extension temp. of 66°C

GC-RICH Reverse Transcription

This protocol is suitable only for Oligo(dT) and gene-specific primers, but not random hexamers.

1 Mix gently and centrifuge each component before use and assemble the RNA/primer mix on ice.

Scale Factor		2
Components		***Mastermix***
DEPC water	9	18
Oligo(dT)$_{20}$	1	2
10mM dNTP mix	2.5	5
Total RNA	12.5	
Subtotal	***25***	***25***

2 Incubate samples at 65°C for 5 min and immediately bring down to 55°C.

3 Prepare the cDNA synthesis mix in the following order:

DEPC water	3	6
10X RT buffer	5	10
25mM $MgCl_2$	10	20
0.1M DTT	5	10
RNaseOUT RNAse inhibitor	1	2
Superscript III RT	1	2
Subtotal	***25***	***50***
Total	***50***	

For minus RT control reaction, add DEPC water instead of Superscript RT.

4 Prewarm the cDNA synthesis mix to 55°C for 5 minutes. To each RNA/primer mix, add 25µL of this prewarmed mix. Mix gently and incubate at 55°C for 50 min.

5 Terminate the reactions at 85°C for 5 min. Cool down to 4°C.

6 Add 1µL of Rnase H to each tube and incubate at 37°C for 20 min. The cDNA's are ready for PCR.

25

25

100

Pfx Platinum polymerase protocol

Scale Factor		2	
Components		***Mastermix***	
Sterile water	14.3	28.6	
Buffer (5X)	5	10	
Q-sol	0	0	
dNTP	0.75	1.5	
Magnesium sulfate	0.75	1.5	
F primer	0.75		1.5
R primer	0.75		1.5
DNA	2.5		5
Pfx Polymerase	0.2	0.4	
Total	***25***	***42***	***8***
			50

		Temp. (°C)	*Time*	
1	*Initial Denaturation*	94	5	min
2	*Denaturation*	94	15	sec
3	*Annealing*	55-68	30	sec
4	*Extension*	68	1	min/kb
5	*Repeat steps 2-4*		25-35	times
6	*Final extension*	72	10	min
7	*Cooling*	4	∞	

Promega GoTaq Polymerase

Scale Factor		16		
Components			***Mastermix***	
5X Green GoTaq buffer	4		64	
dNTP mix	0.4		6.4	
GoTaq DNA polymerase	0.1		1.6	
F primer	2			32
R primer	2			32
Template DNA	2			32
Nuclease-free water	9.5		152	
Total	***20***		***224***	***96***
				320

		Temp. (°C)	*Time*	
1	*Initial Denaturation*	95	2	min
2	*Denaturation*	95	30	sec
3	*Annealing*	42-65	30	sec
4	*Extension*	72	1	min/kb
5	*Repeat steps 2-4*		25-35	times
6	*Final extension*	72	5	min
7	*Cooling*	4	∞	

Restriction digest			
Scale factor		2	
Components		***Mastermix***	
Sterile water	28.6	*57.2*	
Buffer (10X)	4	*8*	
BSA (100X)	0.4	*0.8*	
DNA	5	*10*	
Enzyme 1	1	*2*	
Enzyme 2	1	*2*	
Total	***40***	***80***	