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References

Wang GG, Calvo KR, Pasillas MP, Sykes DB, Häcker H, Kamps MP. Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods*. 2006 Apr;3(4):287-93. PMID: 16554834.

- This is the original methods paper describing the generation of the ER-Hoxb8 cell lines.

Sykes DB, Kamps, MP. Estrogen-dependent E2a/Pbx1 myeloid cell lines exhibit conditional differentiation that can be arrested by other leukemic oncoproteins. *Blood*. 2001 Oct 15;98(8):2308-18. PMID: 11588024.

- This is the original paper describing the generation of the ER-E2a/Pbx1 cell lines. These ECoM cell lines have not been as popular, mostly because of the higher efficiency of cell line generation using the ER-Hoxb8 system.

Sykes DB, Kamps, MP. Estrogen-regulated conditional oncoproteins: tools to address open questions in normal myeloid cell function, normal myeloid differentiation, and the genetic basis of differentiation arrest in myeloid leukemia. *Leuk Lymphoma*. 2003 Jul;44(7):1131-9. PMID: 12916864.

- This is a review we wrote back in 2003.

Shranjit S Lail, Corey R Arnold, Luiz G N de Almeida, Neil McKenna, Jose A Chiriboga, Antoine Dufour, Amy L Warren, Robin Michael Yates. Hox-driven conditional immortalization of myeloid and lymphoid progenitors: Uses, advantages, and future potential. *Traffic*. 2022 Nov;23(11):538-553. PMID: 36117140.

- This is a great 2022 review out of the University of Calgary.
- Be sure to download the **supplementary Table S2**!
- **"Table S2. Comprehensive table of research articles utilizing *Hoxb8*/*Hoxa9* immortalization.** This table list of all studies to the authors' knowledge that used the *Hox* immortalization system from the inception in 2006 until 2020, inclusive. It lists the article title, primary and corresponding authors, the method of immortalization, progenitor source, cytokines for maintenance and differentiation, genetic modifications, paper-specific nomenclature, and a brief description of what the cells were used for in the study."

Growth Medium

RPMI (containing or supplemented with L-glutamine)

10% fetal bovine serum

Penicillin/Streptomycin

[#] Stem Cell Factor or [##] GM-CSF

[###] ß-estradiol (0.5 □M)

[#] I only use **stem cell factor** from the conditioned media of the CHO-SCF cell line (see separate page on how to culture these cells, etc.). This is typically added to the RPMI at a concentration of 1-2%, and this 1-2% results in a final SCF concentration of **~100 ng/ml** if one compares the growth of the cells to recombinant SCF. This is a high concentration of SCF (!) and hence a good financial reason to use the conditioned media rather than recombinant protein.

[##] I only use **GM-CSF** from the conditioned media of the B16-GM-CSF cell line. This is typically added to the RPMI at a concentration of 1%, and this 1% results in a final GM-CSF concentration of **~10 ng/ml** if one compares the growth of the cells to recombinant GM-CSF. The GM-CSF cells require only 5-10 ng/ml for optimal growth so it is more feasible to use recombinant cytokine if you are so inclined.

[##] **ß-estradiol** is very stable in the culture media, at least for 6-weeks at 4-degrees. I generally prepare 2 bottles of growth medium at a time, one with estradiol (for maintenance) and one without (for differentiation). For differentiation of the cells, I pellet the cells, remove the supernatant, add PBS, and pellet the cells again to wash away traces of estradiol. Even at 0.5 □M the estradiol is at ~100-fold excess of what is required to maintain the ER-Hoxb8 protein in an active state and so these two centrifugation steps ensure that most of the estradiol is washed out of the system to permit efficient differentiation.

Growth Conditions, Maintenance, and Propagation

- These cells are non-adherent (SCF-derived) or slightly adherent (GM-CSF-derived lines).
- I use tissue-culture treated plates, for convenience, though I do not think that it makes any difference.
- I like the ease of 6-well plates or 12-well plates, especially when maintaining many clones.
- Most of culture is done in 6-well plates with 6 ml of media or 12-well plates with 3 ml of media.

- The cells must be passaged to new plastic with fresh media at least every 4 days.
- They can be passaged more frequently without problems.
- They should never be left for more than 4 days without fresh media.
- For reasons I do not understand, they simply crash on day 5 or 6, as though they have exhausted some critical part of the media.
- I typically passage the cells 1:10 – 1:20 every 3-4 days.
- This is easily done using a P1000, mixing the cells well within the 6-well plate, and transferring ~300 □l from one well to a fresh well with 6 ml of fresh media, for example.

- The cells do not seem to mind being kept at a low density.
- I do not think that there is a maximum density, but they will go through the media very rapidly as they get more dense so I typically try to keep them to <2 million cells/ml.

Cell Thawing

- These cells do not like to be frozen and thawed so one should expect a lot of death following thawing.
- I will typically thaw them into a 6 well plate containing 6 ml of media.
- The day after the thaw there will be many dead cells.
- These do not seem to affect the growth of the live cells, so I do not try to remove these.
- I will typically do a **half-media exchange** every 1-2 days for the first week until the cells recover or until the # of live cells gets to the point where the cells need to be split.
    - E.g., While the cells are non-adherent, they do settle to the bottom of the well, and it is easy to simply remove the top ~3 ml of media using a vacuum aspirator or P1000. Then one can mix the cells with the P1000 and then add ~3 ml of fresh media.

1. Thaw rapidly in a 37-degree water bath
2. Transfer the cells to a 15 ml conical with 10 ml of pre-warmed media
3. Pellet the cells 500g x 5 minutes
4. Mix the cells gently with a P1000 and transfer to a 6 well plate with 6 ml of media
5. The cells are quite sensitive to freeze/thaw so you will see 50-90% death at 24 hours
6. Every 1-2 days, do a half-media exchange and transfer to a fresh well with fresh plastic
7. Ultimately, you should see the growth of nice round and refractile cells in the background of dead cells

Cell Freezing

- I have tried several different commercial freezing medias but have not found a difference in terms of viability following freeze-thaw.

1. Prepare 'freezing media' – 80% FBS and 20% DMSO
   - I make 40 ml of FBS + 10 ml of DMSO and sterile-filter this and keep it for up to 3 months in the fridge.
   - Keep this on ice.
2. Resuspend the cells slowly and carefully with a P1000 in media
   - Creating a single-cell suspension is critical, clumps of cells do not freeze well.
   - Chill the cells on ice.
3. Add 1 volume of freezing media to the cells slowly while constantly agitating the cells
   - I like to do this using a vortex set on low in the hood, though this is not essential.
   - This brings the final FBS concentration up to ~50% and final DMSO concentration up to 10%.
   - For bone marrow cells, I like to freeze them at a final concentration of 10-20 million/ml.
4. Transfer the freezing tubes to a Mr. Frosty or similar freezing container
   - Freeze overnight at -80.
   - Transfer to liquid N2 the following day.

Expected Morphology

When cultured as undifferentiated cells in ß-estradiol, they should be:

1. Round, smooth edges.
2. Refractile
3. 80%+ viable
4. Non-adherent.

There will always be some dead cells (small, non-refractile, crenulated edges).

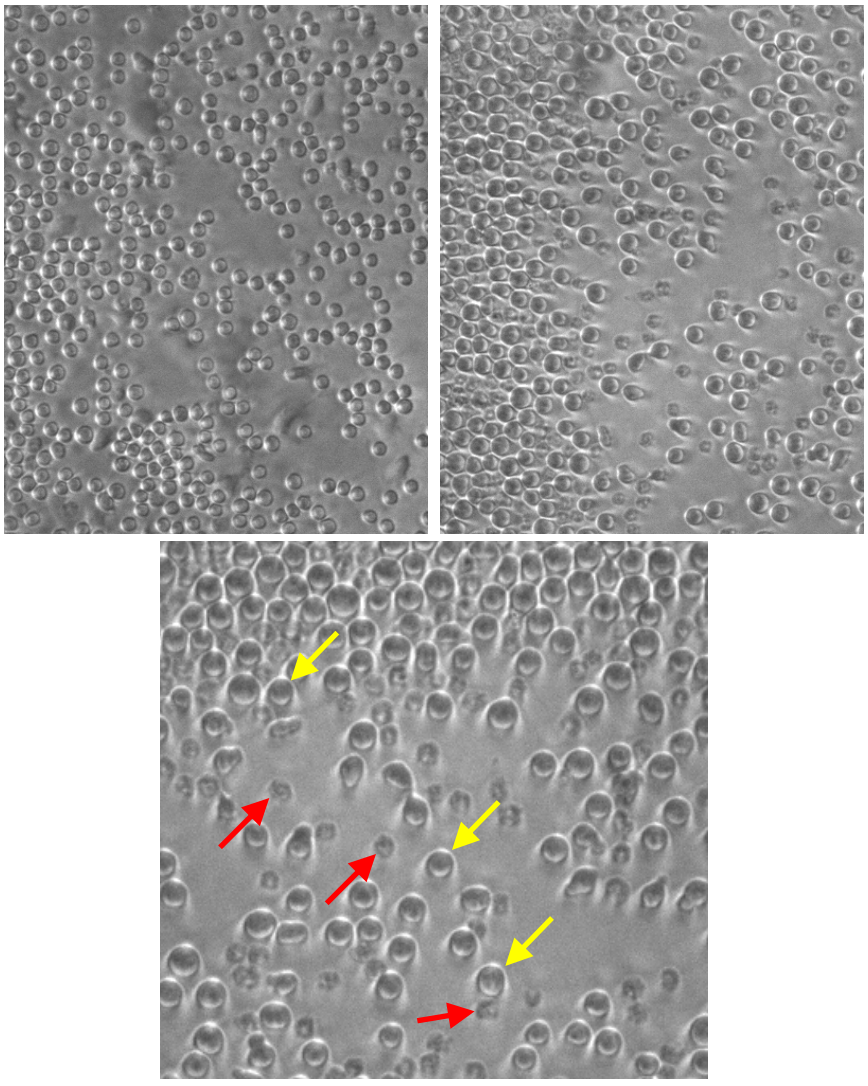
There will always be some adherent/differentiated cells.

There are many more adherent cells in the GM-CSF cultures as compared to the SCF cultures.

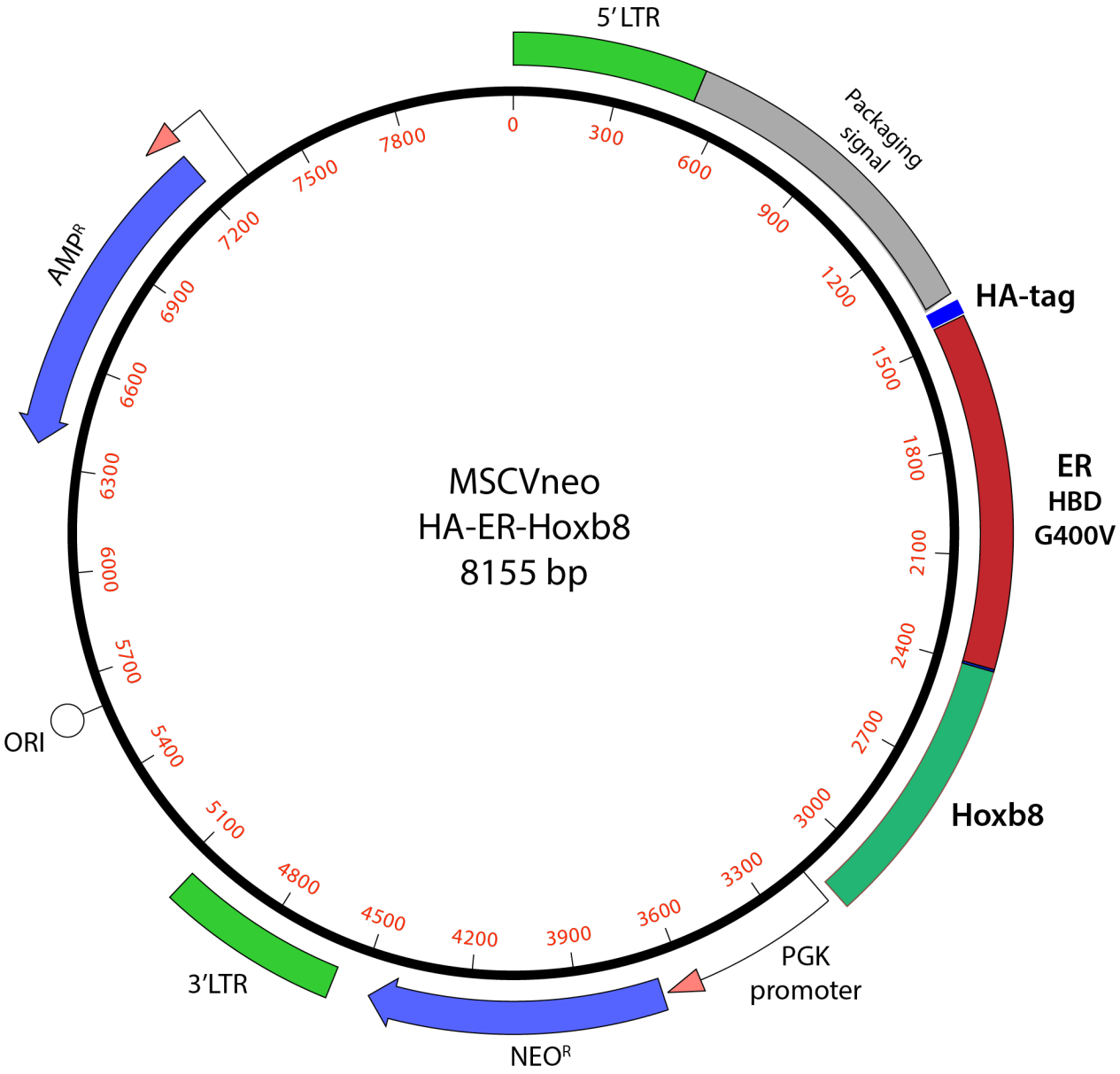
Below are light micrograph examples of a healthy culture of cells in SCF/estradiol.

Yellow arrows = LIVE cells.

Red arrows = DEAD cells.



Plasmid map


5′ LTR
Packaging signal
HA-tag
ER
HBD
G400V
Hoxb8
PGK promoter
NEO^R
3′LTR
ORI
AMP^R
MSCVneo
HA-ER-Hoxb8
8155 bp
0
300
600
900
1200
1500
1800
2100
2400
2700
3000
3300
3600
3900
4200
4500
4800
5100
5400
5700
6000
6300
6600
6900
7200
7500
7800

Materials & Reagents for generating your own ER-Hoxb8 cell lines

1. RPMI + 10% FBS + PEN/STREP + Glutamine
2. DMEM + 10% FBS + PEN/STREP + Glutamine
3. DMEM + 30% FBS + PEN/STREP + Glutamine
4. FACS Buffer: PBS + 2% FBS + 1 mM EDTA
5. Freezing media: 80% FBS + 2% DMSO
6. Ficoll-Paque Plus (Pharmacia) or Lympholyte-M (Cedarlane Labs)
7. Polybrene (10 mg/ml stock)
8. Beta-estradiol (Sigma E-275).
   - Prepare in 100% Ethanol at 10 mM (20,000x). Store at -20.
   - Final concentration in media 0.5 M.
9. Human fibronectin - supplied as a 1 mg/ml solution from Sigma (F-0895)
10. Recombinant murine cytokines
    a. I purchase these from *Peprotech* (IL-3, IL-6, SCF) or conditioned media
11. Lipofectamine 3000 or Fugene 6 (Promega; or other transfection reagent: CaPO4)
12. G418/Geneticin (50 mg/ml solution)
13. Puromycin (10 mg/ml solution)
14. 293T Cells grown in DMEM10% (for producing retrovirus)
15. NIH3T3 Cells grown in DMEM10% (for determining retroviral titer)
16. 40 micron filter, for filtering bone marrow
17. 0.45 micron syringe filter, low protein-binding

Harvesting Murine Bone Marrow

1. Sacrifice mouse
   - I have used Balb/c and C57Bl/6 and SJL mouse strains
   - I generally use mice that are 8-16 weeks old, though any age appears to work fine, even up to 1-year
   - There does not appear to be a gender issue, though make sure to record the gender of the mouse in case you are going to transplant the cells back into recipient mice in the future
   - One can also use **fetal liver cells** if one has an embryonic lethal phenotype
2. Remove intact femurs and tibia into sterile 10 cm dishes with 10 ml of FACS buffer
   - It is also fine to use RPMI + 10% FBS
   - The bones can be crushed, flushed, or spin-flushed to release the marrow
3. Flushing - Cut off the ends of the bones & use a 5 ml syringe (PBS, RPMI10%) and 25G needles to shoot the marrow into a conical. The bones should become translucent
4. Crushing - Crush the bones with the top of a sterile 50 ml conical. This requires a lot of pressure on a flat surface (usually in the tissue culture hood). The bones should be fragmented into little pieces.
   - One crush is fine to release ~80% of the marrow, though one can remove the marrow, add more buffer or media, and repeat the crushing if you want to get all the marrow out.
5. Spin-Flush – Get a 0.5 ml Eppendorf and poke a hole through the bottom of it with a 16G needle. Then put this inside a 1.5 ml Eppendorf. Add 200 ul of FACS buffer. Cut the ONE bone end off of a tibia and femur and put the two bones into the 0.5 ml Eppendorf (cut side down). Spin 10,000 g x 30-seconds. The bone marrow should all be in a nice pellet at the bottom of the 1.5 ml Eppendorf. Reuspend immediately and well with a P200 to avoid any clumping/clotting issues.
6. Now the marrow is released into FACS buffer
7. Set up a 50 ml conical with a 40 micron filter on the top
8. Using a transfer pipet or P1000, pass the marrow through the filter to remove fat and bone fragments
   a. Rinse well using FACS buffer to get all the marrow cells into the 50 ml conical and through the filter
9. Pellet the cells at 500 g for 10 minutes
10. Resuspend the cells in 4 ml of FACS buffer
11. Layer this filtered marrow over 4 ml of Ficoll-Paque-Plus (or Lympholyte-M) in a 15 ml conical
    - The protocol will say to use 3 ml of Ficoll – this is fine as well.
    - Typically a 15 ml conical gradient is good for up to 100 million cells (perfect for one mouse per gradient)
12. Centrifuge 400g at RT for 25-minutes
    - One should see a clear top layer (~4 ml buffer), the cellular interface, and then a pellet (RBC, granulocytes, debris)
13. Harvest cells from the interface
    - I like to use a transfer pipet for this. First discard the top ~2-3 ml of buffer. Then collect the interface and all the cells to within ~0.5 ml of the pellet, being careful not to disturb the pellet.
14. Transfer the cells to a fresh 50 ml conical and bring up to 25 ml with FACS buffer
15. Count the cells using Acridine Orange using an automated counter (e.g., Cellometer)
    - Of course, the traditional hemocytometer count will work just fine.
    - AO has the advantage of staining only nucleated cells, making them brightly green fluorescent.
    - This helps to avoid counting residual RBC.
    - AO is generally purchased as a 10 mg/ml stock solution.
    - This stock is diluted to 10 ug/ml working solution in PBS.

- Then one prepares a 1:1 mixture (20 ul of cells to 20 ul of AO).
- Count the cells (taking the 2x dilution factor into account).

16. Resuspend the cells at 2 million/ml in the **cytokine stimulation media**
    - I tend to use RPMI + 10% + SCF + IL3 + IL6 (all at 10 ng/ml).
    - The original protocol suggested IMDM, but I find that there is no difference.
    - Also, I suspect that the cytokine concentration is not critical, the point of these pre-stimulation is to get the progenitor cells cycling for the upcoming retroviral transduction step. I have even done this step in SCF only and it seems to work just fine!
17. Plate ~10 million cells in 5 ml of media in 1 well of a 6-well plate
    - At this point, one will usually have ~30-40 million cells from each mouse and can freeze the cells.
    - See the section on "Freezing" below.
18. Culture the cells for 24-36 hours prior to the retroviral transduction
    - The timing seems to be flexible.
    - ** However, if this initial culture goes on beyond ~3 days, the efficiency of immortalization goes way down. I imagine that must be because the cells are differentiating beyond the point at which they can be immortalized.
    - I have generally adopted a ~36-hour culture.
    - For an efficient retroviral infection, the cells must be actively cycling.
    - If this is the case, after 24 hours one will note that there are a lot of adherent cells (macrophages, etc.). So, using a P1000, I will gently mix all the cells and transfer the whole 5 ml culture to another well in the 6 well dish, basically transferring the non-adherent cells away from the adherent ones. I see this almost like a negative depletion step (leaving behind the more mature adherent cells and taking the progenitors to a fresh well).

Retroviral Infection

1. Coat a 12-well plate with Fibronectin
   - Human fibronectin is supplied as a 1 mg/ml solution from Sigma (F-0895)
   - Dilute the fibronectin 1:100 in PBS to a final 10 µg/ml solution
   - Aliquot ~500 ul into each well of a 12-well plate
   - Incubate at 37-degrees in the tissue culture incubator from 1 hour to 2 days
   - Fibronectin with increase the transduction efficiency by about 2-fold
2. Count the bone marrow cells
   - The cells that were pre-stimulated in 6-well plates will show a mixture of adherent and non-adherent cells.
   - Using a P1000, I remove the top half of the media and discard, leaving the cells at the bottom of the well
   - Using a P1000, I gently but methodically mix the cells and dislodge the non-adherent cells
   - Transfer all non-adherent cells to a 15 ml conical
   - Count the cells (using Acridine orange)
   - Dilute the cells to 500,000 per ml (total bone marrow)
   - This concentration is very flexible
3. Add polybrene from the 10 mg/ml stock
   - I tend to add polybrene to the cells to a final concentration of 24 ug/ml (3x)
   - Then, with the addition of 500 ul of cells + 1 ml virus the final polybrene becomes 8 ug/ml in a volume of 1.5 ml
   - Of interest, Lipofectamine (at a final concentration of 1:1000) also acts well as a polycationic compound.
4. Aspirate the fibronectin out of the wells
5. Add 500 ul of cells into each well (~250,000 cells)
   - Place the plate at 37°C while thawing the virus
6. Thaw the virus
   - Add 1 ml of virus to each well
   - Add 1 ml of DMEM to control wells
7. Spinfection (spinoculation)
   - Tape the plates edges with lab tape to decrease evaporation
   - Spin the plates: 1000g x 90 minutes at 22-degrees
8. Following the spinfection, add 3 ml of media to each well.
   - A variety of cytokine conditions will give rise to cell lines with different differentiation potentials
   - Neutrophil predominant lines – use SCF/E2 media
   - Macrophage predominant lines – use GM-CSF/E2 media
   - Mixed clones – use IL3/E2 media
   - Dendritic cell lines – use Flt3L/E2 media
   - The final volume of the well is now 4.5 ml and polybrene concentration 2.7 ug/ml

Cell Line Derivation

1. Day 2 – Half media exchange
   - Aspirate, using a P200 tip attached to the aspirating pipet, the supernatant down to ~ 2 ml.
   - The cells sit at the bottom of the well and so, if not disturbed, one can remove the supernatant without removing any of the cells.
   - This exchange is done to dilute the polybrene down to ~1.4 ug/ml where the cells are happy.
2. Day 3 – Optional G418 selection
   - Aspirate, using a P200 tip attached to the aspirating pipet, the supernatant down to ~ 2 ml
   - Add 2 ml of fresh media containing 2x G418 (2 mg/ml)
   - Antibiotic selection seems effective at a G418 concentration of 1 mg/ml
3. Day 5 – Passage 100% of cells to fresh plastic
   - Aspirate, using a P200 tip attached to the aspirating pipet, the supernatant down to about 1 ml
   - Using a P1000, slowly and gently mix the cells and resuspend the non-adherent cells from the well edges.
   - Transfer all of the non-adherent cells to a fresh plate with 3 ml of fresh media
   - Optional, one can maintain the G418 selection at this point, though it is not critical
4. Every 3-4 days – Passage the cells to fresh plastic
   - At this point, the control (uninfected) wells will continue to divide as will the ER-HoxB8 wells
   - However, over the next 1-2 weeks, it will be clear that the ER-HoxB8 wells will have many more round, non-adherent cells that are actively dividing
   - I will usually passage 25-50% of the cells to fresh media in a fresh plate every 3-4 days depending on their growth kinetics
   - By the end of week 3, most of the uninfected cells should have stopped dividing and you are left with the ER-HoxB8 lines!

Single cell cloning (Optional)

1. The single cell cloning can be done as early as day 7 following infection
2. Single cell cloning can be done by (a) FACS or (b) limiting dilution
3. Use 96 well **round bottom** plates

Fluorescence activated cell sorting (FACS)

1. Add 250 ul of media to each well
2. Add a viability dye to your cell culture
3. Using FACS, add 1 viable cell per well

Limiting dilution

- I generally do 2 plates of each cell line.
- Plate A at 1 cell/well + Plate B at 0.5 cells/well.
- (You can also do an additional Plate at 2 cells/well in populations with low cloning efficiency)

1. Mix your cells well and transfer 520 ul to the bottom of a 50 ml conical.
2. Take 20 ul for counting.
3. Fill the 50 ml conical with PBS (1:100 dilution).
4. Transfer 200 cells to 50 ml of media.
    a. Cells are now at 4 cells/ml = 1 cell/250 ul.
5. Mix well and transfer 20 ml of this cell mixture to 20 ml of media in a new conical
    a. Cells are now at 2 cells/ml = 0.5 cells/250 ul.
    b.
6. Plate 250 ul per well using a multi-channel pipet.
7. Leave the plates for ~10-12 days.
8. Visualize the clones by holding the plate up to the light to see the clones on the bottom of each well.
9. Usually the cloning efficiency is 10-30%, so you will get ~10-30 clones per plate.
10. Using a P200, remove the top half of the media and discard.
11. Mix the clone up and down and transfer all the cells to a 48-well plate with fresh media.
12. Every 3-4 days, move from 48 → 24 → 12 → 6 well plates during the expansion process.
    a. For unclear reasons, you will lose 1-2 clones early (96 to 48, 48 to 24) so start with a few more than you want to end up with.

Producing Helper-Free Ecotropic Retrovirus

Materials

- ☐ 293T HEK cells
- ☐ DMEM + 10% FBS + Pen/Strep + Glutamine (DMEM10)
- ☐ DMEM + 30% FBS + Pen/Strep + Glutamine (DMEM**30**)
  Apparently the higher protein concentration helps with virus recovery after freeze/thaw
- ☐ Fugene 6 (https://www.promega.com)
  Calcium phosphate & other lipid-based transfection (Lipofectamine) also work well
- ☐ Poly-D-Lysine (Corning #354210), dissolved at 1 mg/ml in water

1. Day 0 - Morning
   a. Dilute the poly-D-lysine 1:100 into PBS (final 10 ug/ml)
   b. Use ~5 ml of diluted poly-D-lysine to coat each 10 cm dish at 37-degrees for a few hours
2. Day 0 - Afternoon
   a. Trypsinize and gently resuspend the 293T cells as a single-cell suspension
   b. Aspirate the poly-D-lysine from the 10 cm dishes
   c. Count & seed ~3.5 x 10^6 cells into each 10 cm dish in DMEM10
3. Day 1 - Morning
   a. Cells should be at ~70% confluence
   b. Pre-warm 6 ml of DMEM10 per dish
   c. Aspirate media & replace with 6 ml of pre-warmed DMEM10
4. Day 1 – Afternoon
   a. Transfection per Fugene 6 protocol
   b. 30 µl Fugene 6 + 5 µg retroviral construct + 5 µg eco-pac construct (3:1 ratio)
5. Day 2
   a. Pre-warm 8 ml of DMEM**30** per dish
   b. Aspirate media & replace with 8 ml of pre-warmed DMEM**30**
6. Day 3
   a. Viral harvest #1
   b. Draw the virus up into a 10 cc syringe
   c. Filter through a 0.45µ filter (low protein binding) into 1 ml aliquots
   d. Freeze on dry ice and transfer to -80°C (or use fresh virus immediately)
   e. Add 8 ml of pre-warmed DMEM**30**
7. Day 4
   a. Viral harvest #2
   I usually freeze a small (~250 µl) aliquot for the purpose of determining the viral titer
   Depending on the size of the insert, we usually attain viral titers between 10^5 – 10^6/ml

Determining the Titer of the Retrovirus by Colony Counting

Notes

- If you are new to working with retrovirus, you should determine the titer by infecting an "easily-infectable" cell line such as NIH3T3 fibroblasts.
- Here the premise is that each infected fibroblast produces 1 colony.
- Having determined that your protocol generates a good titer of virus, it is rarely necessary to titer each additional batch.

Materials

- NIH3T3 cells
- DMEM + 10% FBS + Pen/Strep + Glutamine (DMEM10)
- Crystal Violet (0.5% crystal violet in final 20% methanol)

  Dissolve 0.5 grams of crystal violet in 80 ml of water (it dissolves slowly overnight w/ shaking)

  Then add 20 ml of 100% methanol to make the final solution and store in the fridge

1. Day 1
   a. Prepare a single-cell suspension of NIH3T3 fibroblasts
   b. Seed 50,000 NIH3T3 cells in ~2 ml of media (DMEM10) in each well of a 6-well plate
   c. I use 1 x 6-well plate per virus to titer
2. Day 2a
   a. The cells should be ~20-30% confluent
   b. Prepare DMEM10 with 8 ug/ml polybrene and pre-warm
   c. Aspirate the media from each well and replace with 2 ml of DMEM10+Polybrene
3. Day 2b
   a. For each virus to titer, set up Eppendorf tubes with 1 ml of DMEM10+Polybrene
   b. Thaw an aliquot of the virus(es) to titer
   c. Dilute 2 ul of virus into the 1 ml of DMEM (1:500 dilution) and mix well
   d. Add 100 ul, 50 ul, 20 ul, 10 ul, 5 ul, and 0 ul to each well of the 6-well plate
   e. Allow the infection to proceed 4 hours to overnight at 37°C
   f. Aspirate and replace the media with fresh DMEM10 without polybrene
4. Day 4
   a. Aspirate the media and replace with 6 ml of DMEM10 + antibiotic
   b. G418, e.g. Gibco's Geneticin (liquid, 50mg/ml, cat # 10131-035) use at final 1 mg/ml
   c. PURO, e.g. use at final 1 ug/ml
5. Day ~10-14
   a. Cells on the control plate should be completely dead and detached
   b. Individual colonies should be visible on the other plates
   c. If the virus is of good titer, the plates with the 50 and 100 ul of virus should be confluent
6. Colony counting
   a. Aspirate the media
   b. Rinse 2x with PBS

c. Add ~500-1000 ul of the crystal violet to each well, just enough to cover the bottom
d. The staining is almost immediate
e. Take the plates to the sink and flood the wells with water and rinse a few times

   Direct the stream of water onto the edge of the plate, and allow the wells to fill up indirectly a few times so as to avoid rinsing off the colonies! The background should become completely clear.

f. The colonies should be easily visible against a white background
g. Count the colonies

Example

Virus per well (ul)	# of colonies	Colonies per ml	Viral titer (500x dil)
0	0		
5	4	800	4×10^5
10	9	900	4.5×10^5
20	22	1100	5.5×10^5
50	Confluent		
100	Confluent		

Here, I would estimate ~500,000 virus particles per ml.

Editorial: What about starting with bone marrow progenitors rather than whole bone marrow?

1. Instead of whole bone marrow, purified marrow progenitors can be isolated from post-Ficoll mononuclear fraction by magnetic negative depletion of lineage-positive cells using the Stem Cell Technology murine progenitor isolation cocktail (or equivalent). Whether or not you wish to isolate progenitors depends mostly on the efficiency of the infection. If you are using low-titer virus or feel that the effect of the transduced cDNA might be subtle, then isolation of progenitors is a good idea. If you are using a high-titer virus of a very effective oncoprotein such as E2a/Pbx1, HoxA9, HoxB8, etc. then there is no need to isolate progenitors.
2. Using 5-Flurouracil? One can inject the mice with 5-FU 3-5 days prior to harvesting the bone marrow. Injections are done at 150 mg/kg I.P. The 5-FU will greatly reduce the total cellularity of the marrow with an increased % of progenitors (though we do not find a dramatic increase in total # of progenitors). The advantage of the 5-FU is that the marrow from more mice can be processed on the same Ficoll gradient and on the same StemCell Technology column (using less reagent). Of course, if one uses 5-FU, there is no real need for additional enrichment of progenitors. And as mentioned, if your retroviral infection/immortalization is of high efficiency, you do NOT need to treat with 5-FU or isolate marrow progenitors. Infections can be done on "crude" marrow simply by removing the bone marrow cells and passing them over a Ficoll gradient before plating them under cytokine pre-stimulating conditions.
   - No 5-FU → ~50 million cells per mouse; ~3% are progenitors as isolated over the StemCell Technologies column.
   - YES 5-FU → ~5-10 million cells per mouse; ~10% are progenitors

Editorial: Notes

1. With high-titer retrovirus ($2x10^5$-$2x10^6$ /ml), very few cells are required for each infection. We have gone with as few as 10,000 cells per infection with good success.
2. Fibronectin is a good substitute for the much more expensive commercially available Retronectin. Retronectin is merely the fragment of fibronectin which contains the viral binding domain.
3. During the spinoculation, the media will get quite basic. This does not seemed to affect the viability of the cells. However, media containing HEPES can be used to maintain a more physiological pH if necessary. HEPES-media can be purchased from Gibco.
4. The spinoculation can be done at 1000g up to 4500g without any loss of viability in my hands. The higher the G-force the better the transduction though this is a minimal effect. I have not seen any problem with temperatures from 20° up to 36° during centrifugation.
5. The most important predictor of good transduction efficiency is still the viral titer.
6. Lipofectamine (in place of polybrene) can be used as a poly-cationic compound at a final 1:1000 dilution. However, I do not believe that there is any significant advantage over using a final 8 µg/ml polybrene. Both of these compounds are designed to eliminate the repulsion between the negatively charged virus and the negatively charged cell membrane. Many protocols using fibronectin/retronectin do not also include a poly-cationic compound though I have continued to do so.
7. ß-estradiol can be purchased from Sigma (E-2758). It can be conveniently dissolved at 10mM in 100% ethanol (takes ~30 minutes of end over end at RT). This provides a 10,000x stock solution to be used at a final 0.5 µM in cell culture. When rinsing the cells of ß estradiol, it is a good idea to rinse 2x in 10ml PBS as the proteins are still reasonably active at concentrations down to 1nM.

Editorial: Monitoring the Infections – What to expect?

1. It is a good idea to keep a few wells of "uninfected" or "vector alone" infected cells maintained under the same conditions. Over the course of ~3-4 weeks, these cells will divide, mature, terminally differentiate to neutrophils and macrophages, and finally die.
2. Cells should be split to a new 12-well with fresh media every 3-4 days. Leaving them longer is a bad idea. All non-adherent cells can be transferred by pipetting the lightly adherent cells off the bottom of the dish. I tend to do this with a P1000 (using a filter tip) which gives me more control over the pipetting. The truly adherent macrophages will not come off. The cells should not be allowed to become too confluent. This is important as the differentiated monocytes stuck on the bottom of the dish seem to have a pro differentiative effect on all the other cells in the culture.
3. For example, if the cells are in 3 ml of media in a 12-well dish, then I will use the P1000 to remove the top 2ml of media and then pipet the cells up and down and transfer 50-100% of the non-adherent cells to a new well with 2-3 ml of fresh media. I almost never spin down the cells, and just try not to use more than 25% "old" media.
4. The emergence of immortalized cells will appear after about 3-4 weeks as clumps of round and refractile blasts, like little clusters of grapes. It will then become easier to transfer these cells off the differentiated layer of monocytes.
5. Interestingly, in the early stages of culture, the transfer of the immortalized cells to a new dish also seems to have a pro-differentiative effect, maybe due to the mechanical activation of the cells by the pipetting. Thus, the day after the transfer, one often notices many fewer blasts than one actually transferred! This is normal, and after 24hrs, the cells tend to recover.
6. Eventually, after 3-4 weeks, all that will be left are the immortalized myeloblasts. These grow very robustly and rapidly and can be cloned by limiting dilution in 96 well plates. Usually, I plate out one 96-well plate at 3 cells/well and one plate at 0.3 cells/well in 200µl of media. After 10-14 days, clones are easily identified and transferred to 24-well plates for characterization.

Editorial: Deriving the cells in different cytokines?

1. As mentioned, the ER-HoxB8 cells can be derived in a variety of single cytokines:
    a. Flt3L
    b. SCF
    c. IL3
    d. GMCSF
2. I have not done a formal side-by-side comparison of all of these conditions, but in general:
    a. **Flt3L**-derived cells: seem to be the most immature, but are slow growing
    b. **SCF**-derived cells: very reliable, grow rapidly, can identify neutrophil-committed clones (majority) as well as monocyte-committed clones (minority)
    c. **IL3**-derived cells: grow VERY rapidly, a little stickier at baseline, higher levels of baseline MAC1 expression
    d. **GM**-CSF-derived cells: grow VERY rapidly, quite sticky at baseline, highest level of baseline MAC1 expression, almost completely monocyte/macrophage committed

Editorial: Removing ß estradiol

1. As mentioned, the cells should be rinsed 2x in PBS or media to get rid of residual ß estradiol before plating in media without estrogen. Remember, because the "muted" G400V mutant version of the estrogen receptor was used, the estrogenic effects of phenol red or residual estrogens in the FBS do not have any effect.
2. In the absence of estrogen, the cells will undergo between 3-5 cell divisions and terminally differentiate to mature granulocytes and monocytes. This usually takes between 4-7 days, depending on the clone and also depending on the cell density. The less dense the culture, the faster the process of differentiation! The proportion of granulocytes to monocytes is also clone-dependent and clones can be selected that have a preferential bias one way or the other.

Preparing SCF or GM-CSF Conditioned Media from CHO-SCF or B16-GM-CSF cells

1. **CHO-SCF** cells are an adherent cell line that secretes SCF into the media
2. **B16-GM-CSF** cells are an adherent cell line that secretes GM-CSF into the media
   - I am not sure of the provenance of these cell lines
3. Growth media: IMDM + 10% FBS + Pen/Strep + Glutamine
   - I have added beta-mercaptoethanol (final 50 M) in the past, but this seems dispensable
4. Passaging: the cells are easily passaged using Trypsin/EDTA.
   - I wash 2x with PBS and aspirate
   - Add Trypsin/EDTA for ~2-3 minutes
   - The cells grow very rapidly, so they can be passaged 1:10 or 1:20 into a new vessel
5. Growth vessels: Corning 5-Layer flasks (CAT# 14-826-96)
   - These flasks are expensive, but very convenient for preparing conditioned media
   - They allow one to grow 5-layers of cells in 250 ml of media
   - It is easy to pour off the conditioned media and add fresh media to all 5-layers at once
   - In this manner, one can produced 500 ml to 1000 ml of conditioned supernatant daily using 2-4 flasks!
6. Seed the cells and allow them to become confluent in the flasks
   - The media will become very yellow/acidic as the cells reach confluence
7. Pour off the media into a 0.2 m filter cup & sterile filter.
   - Store DAY1 collection at 4-degrees
8. Replace the media with an additional 250 ml of pre-warmed media
9. Give the cells another 24-48-hours and collect DAY2 collection
   - I generally repeat this process for 2 to 3 collections
10. Sterile-filter daily, but then pool the collections at the end of the 4-5-day process
11. Aliquot the pooled media into 10 to 40 ml aliquots in 50 ml conicals
12. Store at -80-degrees
13. Freezing
    - I use the same freezing protocol as for the ER-HoxB8 cells
    - These cells are very hearty and freeze/thaw very well

Editorial: How much SCF (or GM-CSF) conditioned media is enough?

1. Usually, adding 5-20 ml (1% to 4%) of the conditioned media to 500 ml of media provides enough cytokine
   - This seems to equate functionally to 50 – 100 ng/ml of recombinant SCF
   - One can perform a cytokine (SCF) ELISA to quantify, though I tend to use a functional approach
2. I tend to use a functional approach to the SCF conditioned media
   - This approach takes advantage of the fact that the ER-HoxB8 or ER-HoxA9 cells are exquisitely SCF-dependent

Protocol

1. Prepare RPMI + 10%FBS + P/S + Gln + E2 media
   a. Make a 5 ml aliquot with 20% SCF-conditioned media (4 ml media + 1 ml CHO media)
   b. Make a 5 ml aliquot with 100 ng/ml recombinant SCF
2. Using 2 x 12-well plates, make 2-fold dilutions across the plate
   a. Use 2 ml of SCF media and 2 ml of media without SCF factor

Plate #1. CHO-SCF conditioned media

20%	10%	5%	2.5%
1.25%	0.625%	0.312%	0.156%
0%			

Plate #2. Recombinant-SCF conditioned media

100 ng/ml	50	25	12.5
6.25	3.12	1.56	0.78
0			

3. Wash the ER-HoxB8 cells
4. Resuspend in RPMI + 10%FBS + P/S + Gln + E2 media without SCF at 10^6/ml
   a. Add 100 ul of cells (100,000) to each well
   b. Allow the cells to grow for 4-days
5. At the end of day 4, take a look at the cells under the microscope
   - The no SCF wells should be completely dead
   - Also, the lower concentrations might be near-completely dead
   - Record and discard these wells
6. In the wells with nice live cells, mix the cells in each well with a P1000
7. Take 1 aliquot of cells for counting
8. Take 1 aliquot of cells for CD11b staining (to look for differentiation)
   - There seems to be a sweet spot between good proliferation and low background differentiation
   - Of interest, too high SCF concentrations lead to more cells, but higher background CD11b staining
9. As you can imagine, one well of the rSCF should look similar to one well of the CHO-SCF, giving a nice functional read-out of how much SCF is in the conditioned media