2i ES cell medium	stock	500ml	250ml	200ml
KO DMEM (Life Technologies # 10829018)		400ml	200ml	160ml
KO serum replacement (Life Technologies # 10828028)		75ml	37.5ml	30ml
Sodium Pyruvate (Life Technologies # 11360070)	100mM	5ml	2.5ml	2ml
NEAA (Life Technologies # 11140-050)	100mM	5ml	2.5ml	2ml
bMercaptoethanol		4.3ul	2.15ul	1.72ul
L-Glu (Life Technologies # 25030149)	200mM	5ml	2.5ml	2ml
Pen/Strep (Life Technologies # 15140-163)		5ml	2.5ml	2ml
LIF (Millipore # ESG1107)	10^7U/ml	100ul	50ul	40ul
PD0325901 (Selleck # S1036)	10mM	50ul	25ul	20ul
CHIR99021 (Selleck # S2924)	10mM	150ul	75ul	60ul

→ Combine everything and filter.

***For 2i+2% serum media, add 10ml FBS for ES cell and use 405ml KO DMEM**

FBS-ESC medium	stock	500ml	250ml	200ml
KO DMEM (Life Technologies # 10829018)		415ml	207.5ml	166ml
FBS (Pansera ES or defined FBS)		75ml	37.5ml	30ml
NEAA (Life Technologies # 11140-050)	100mM	5ml	2.5ml	2ml
bMercaptoethanol		3.5ul	1.75ul	1.4ul
Pen/Strep/L-Glu (Life Technologies # 15140-163)		5ml	2.5ml	2ml
LIF (Millipore # ESG1107)	10^6U/ml	500ul	250ul	200ul

→ Combine everything and filter.

ES cells – Standard Operating Procedures

ES cell media:

- Label with date, discard after 6 weeks.
- All media, PBS, trypsin/accutase has to be pre-warmed in 37C waterbath
- **Always change media in the morning, then work with ES cells in the afternoon (splitting, freezing, electroporation etc).**
- When plating ES cells on fresh feeder plate, change to ESC media in the morning, then plate ES cells in the afternoon.

Feeders:

- ES cells are grown on mouse embryonic fibroblast feeders. We use DR4 feeders that have 4 resistances (Puromycin, Neomycin/G418/Geneticin, Hygromycin, 6-Thioguanine).
- MEFs are derived from E13.5-14.5 DR4 embryos (DR4 homozygous parent crossed with wildtype parent). Stock feeders are cryopreserved at p0.
- One embryo is expanded to ~fifty 15cm plates (1plate -> 5 plates -> 15 plates -> 50 plates), mitotically inactivated with Mitomycin C (10ug/ml) for 2hrs at 37C, washed twice with PBS and frozen in approx. 100 cryovials (in 500ul Bambanker per vial).

Gelatin coating of plates:

- Cover surface of plate with Gelatin (0.1% (wt/vol) in water (filter sterilize)
- Leave on plate for 15 min in 37C CO2 incubator
- Dry in the hood for 5 min
- Add pre-warmed feeder media for each plate

Plate feeders:

- 1 day before plating ES cells, thaw feeders from the liquid nitrogen.
- Warm up the frozen vial thoroughly in water bath (only 3 min) and transfer content into 9.0 ml feeder media. Spin down 1,200 rpm for 3min.
- Resuspend cells into appropriate volume and plate onto appropriate surface area (pre-determined, usually 1vial per 10cm plate).
- Recommended to use 6cm or multiple wells of a 6-well dish.
- Change to ES media the next morning to plate ESC.
- Don't make feeder plates more than two days prior to thawing ES cells.

Thawing ES cells:

- In the morning, change feeder plate to pre-warmed ES cell media
- Thaw ES cell vial from dry ice immediately into 37C water bath – thaw completely (~3 minutes).
- Transfer ES cell carefully into 9.0 ml pre-warmed ES media with 1ml pipette.
- Spin 1,200 rpm for 3min.
- Aspirate media, break up the cell pellet thoroughly by tapping the bottom of the falcon tube.
- Resuspend ESC in 1.0-3.0 ml ES media and plate (6well plate or 6cm dish).
- Culture ES cells for 3 days before electroporation.

Expanding ES cells:

- Feed ES cells with fresh pre-warmed media every day.
- Observe the clones. ES cells should grow in compact round 3D colonies with distinct, bright clone borders.
    - Shouldn't be able to see distinct cell boundaries.
    - Differentiating ES cells become flattened and have more obvious edges, and colonies start to have jagged instead of round edges.
- Cells should be passaged every 3 days, record the passage

ES cell derivation

- Inject female with 5U pregnant mare serum gonadotropin (PMSG, Sigma Aldrich, Cat # G4877-2000IU) when approximately 28 days old. Injection is done around noon - I typically do this on a Tuesday.

- 48 hours later (noon on Thursday), inject female with 5U human chorionic gonadotropin (HCG, Sigma Aldrich, needs institutional approval) and pair with male.

- The next morning (Friday), separate male and plug-check female.

- Three days later (Monday morning), circle 4-5 spots on the bottom of a 6cm dish. Spot 100ul of EmbryoMax Advanced KSOM Embryo Medium (Millipore, Cat # MR-101-D) in the circles and overlay with Mineral Oil (Sigma Cat # M5904-500ML). Prepare 2-3 dishes and place in TC incubator.

- Euthanize plugged females and remove reproductive organs and place in 6cm dish. Avoid fat t issue as much as possible. Cut off ovaries and cut uterine horns at uterine fundus (i.e. just above where the two uterine horns connect). Collect uterine horns in fresh 6cm dish.

- Under a dissecting microscope, use 1ml of EmbryoMax Advanced KSOM Embryo Medium (Millipore, Cat # MR-101-D) and a 25G needle and 1ml syringe to flush blastocysts out of uterine horns. Flush from both sides of uterine horns. The needle may be blunted with a file to avoid pocking it through the uterine horns.

- Under the TC microscope, find blastocysts and late morulas and pick them up using the Stripper pipette (Origio, Stripper Micropipettor Cat # MXL3-STR-CGR, Stripper Tips Cat # MXL3-175). Collect all blastocysts/morulas in one 100ul drop of EmbryoMax to wash them in the media. Once all embryos have been collected, collect them with the Stripper pipette and transfer them to a different drop. Place the dish in the TC incubator for 4-6 hours. This will allow the morulas to develop to blastocysts.

- Gelatinize the appropriate number of 4-well dishes and plate arrested feeders in DMEM 10% FBS, P/S. After 4-6 hours of incubation, change media on feeders to 2i and pick up individual good-looking blastocysts and transfer to one well of a 4-well dish. Place in incubator and let grow for 48 hours. Do not move dish so that blastocysts can attach and ES cells grow out.

- After 2 days, start feeding ESC clones with 2i media until the colonies are big enough for picking, about 5-10 days.

- Pick ESC colonies in PBS with P20 pipette in a 20ul volume and transfer to a 96 well. Add 20ul of 0.05% Trypsin and incubate in TC incubator for ~5 minutes to obtain single cells. Transfer single cells one well of a gelatinized 24 well with feeders.

- Feed clones and freeze after 2-5 days in 1-2 freezing vial. Keep a small aliquot of the cells for i) genotyping, ii) to plate on gelatinized plate without feeders. This will be used to isolate ESC DNA without feeder contamination.

- Genotype using standard DNA isolation and genotyping PCR. Then isolate DNA from feederless plates of positive clones to determine sex of clone (real-time PCR to detect Y-linked Kdm5d [Mm00528628_cn, Applied Biosystems], Tfrc is used as internal control [4458366, Life Technologies]; males will be positives, females negative).

- Thaw and expand ESC clones with correct genotype and perform QC.

ES cells – Oct4 and Nanog staining

- plate cells on DR4 feeders in serum-containing ESC medium
- let cells grow for 2-3 days, change medium daily
- wash cells with PBS
- fix cells in 4% PFA for 20min
- permeabilize with TBS, 0.5% Triton for 10-15min
- block in 1% BSA in TBS, 0.1% Triton for 30min
- incubate with primary antibody (1:400) in blocking solution at 4C overnight
    mouse anti-Oct-3/4 (C-10; SCBT, sc-5279)
    rabbit anti-Nanog (Bethyl, A300-397A)
- wash 2-3 times with TBS, 0.1% Triton
- incubate with fluorescent secondary antibody in TBS, 0.1% Triton for 30min at room temperature
    Alexa Fluor 488 donkey anti-rabbit IgG (Life Tech, A-21202)
    Alexa Fluor 488 donkey anti-mouse IgG (Life Tech, A-21206)
- wash 2-3 times with TBS, 0.1% Triton
- take pictures under microscope (cells can be kept in TBS at 4C in the dark)

ES cell targeting

Homologous recombination

- Precipitate 25ug of targeting vector:
    - bring DNA to 100ul with ddH2O
    - add 200ul of 95% EtOH
    - add 10ul of 5M ammonium acetate
    - mix well, then centrifuge for 10min at full speed
    - wash DNA pellet with 70% EtOH
    - air dry and resuspend DNA in 10ul of ddH2O
    - add 82ul of P3 Primary Cell Solution and 18ul of Supplement 1 from P3 Primary Cell Nucleofector Kit to DNA

- Trypsinize ES cells
- count and spin down $3x10^6$ cells (add some PBS to tube before spin to dilute media)
- aspirate supernatant and carefully resuspend cells in DNA/P3 solution
- transfer to Nucleofector cuvette and electroporate with Nucleofector on “ES, mouse” setting
- transfer cells with Pasteur pipette from kit to 10cm dish with DR4 feeders in 2i media
- start selection after 24 hours

Flip-in into Collagen homing cassette (CHC)

- Combine and precipitate 10-20ug of targeting vector and 5-10ug of pCAGS-Flp:
    - bring DNA to a 100ul with ddH2O
    - add 200ul of 95% EtOH
    - add 10ul of 5M ammonium acetate
    - mix well, then centrifuge for 10min at full speed
    - wash DNA pellet with 70% EtOH
    - air dry and resuspend DNA in 10ul of ddH2O
    - add 82ul of P3 Primary Cell Solution and 18ul of Supplement 1 from P3 Primary Cell Nucleofector Kit to DNA

* The ratio of targeting vector to Flp should be 2:1. Too much DNA can cause increased cell death after nucleofection, increasing the cell number will alleviate this problem
** Targeting and Flp vector do not need to be precipitated if they are at a very high concentration after Maxi-prepping (>2ug/ul). It is highly recommended to use the Endotoxin-free Maxiprep kit from Qiagen (Cat# 12362)

- Trypsinize ES cells
- count and spin down $3x10^6$ cells (add some PBS to tube before spin to dilute media)
- aspirate supernatant and carefully resuspend cells in DNA/P3 solution
- transfer to Nucleofector cuvette and electroporate with Nucleofector on “ES, mouse” setting
- transfer cells with Pasteur pipette from kit to 6cm dish with DR4 feeders in 2i media
- split ES cells 24 hours after nucleofection either onto 1-2 6cm dish(es) or one 10cm dish, depending on how many cells survived nucleofection.
- start selection after 48 hours after nucleofection. For Flip-in into CHC, selection is with Hygromycin (100-125ug/ml)

Screening of ES cell clones

Colony PCR

- pick ES cell colony after 7-10 days on selection in 2ul PBS and place in sterile 96well PCR plate with 8ul Trypsin per well
- incubate for 5 minutes, dissociate colony by gentle pipetting
- plate 5 ul on 96 well plate with arrested feeders in 2i medium
- to the remainder of the ES cells add 50 ul tail lysis buffer and 5 ul proteinase K
- seal plate and incubate at 55C for 1-2 hours
- add 100ul of 95% EtO, mix, spin for 15minutes at 4000g at 4C
- invert plate carefully onto paper towel to discard supernatant, add 100ul 70% EtOH
- spin for 15minutes at 4000g at 4C, invert onto paper towel and air dry DNA
- resuspend in 20ul of H2O
- use 2ul of DNA for PCR reaction

Expanding ES cells and DNA isolation

(If colony PCRC is not possible, does not provide clear results, or DNA is needed for southern blots)

- Pick colonies after 7-10 days on selection in 20ul of PBS and transfer to gelatinized 96w plates with arrested feeders (1 colony per well).
- Expand cells for 2-4 days
- PBS wash, then trypsinize the entire 96 well plate
- resuspend trypsinized cells in media and split 1:3. One third is plated on a 96w plate without feeders, the other two thirds are each plated on a gelatinized 96w plate with feeders.
- freeze one feeder plate two days after splitting, and the other 3 days after splitting: trypsinize cells in 20ul of Trypsin. Add 30ul of media with a multichannel pipette and pipette up and down to make single cell suspension. Add 50ul of 2x freezing medium (20% DMSO in FBS) and mix. Overlay cell solution with 50ul of mineral oil. Seal plate and put in the -80C freezer in a Styrofoam box.
- The plate without feeders is used to isolate DNA after expanding for a few more days: add tail lysis buffer with proteinase K directly to the PBS-washed wells and incubate at 37C for a few hours. The DNA solution can either be precipitated directly in the 96w plate or transferred to Eppendorf tubes and precipitated there.

Screening of CHC targeting

- To confirm targeting of the expression cassette into the CHC, a simple PCR spanning the PGK promoter (in the targeting vector) and the Hygromycin resistance gene (in the CHC locus) is sufficient. We use the below PCR reaction, and p53 genotyping primers serve as the internal control. We have not observed any false positive clones with this targeting method, so the expected targeting rate is very high.

Primers	Sequence	Size
p53 common	5'-TATACTCAGAGCCGGCCT-3'	
P53 wt	5'-ACAGCGTGGTGGTACCTTAT-3'	~450bp
PGK F	5'-GAGCAGCTGAAGCTTATGGA-3'	
Hygro R	5′-CTGAATTCCCCAATGTCAAG-3′	~300bp

Primers used at a 0.5 : 0.5 : 1 : 1 ratio. Primer concentration is 25μM and we use GoTaq Green from Promega and follow the manufacturer's recommendations.

PCR program
1) 95°C for 2:00
2) 95°C for 0:30
3) 58°C for 0:30
4) 72°C for 1:00
35 cycles of steps2-4
5) 72°C for 5:00
6) hold at 4°C

- Targeting vectors may integrate into the CHC in tandem as concatamers. To validate single integration, one should perform a copy number qRT-PCR using Sybr green primers (or Taqman probes if available) that specifically detect the gene-of-interest in the targeting cassette.

- If ES cells harboring the LSL-BrafV600E allele are used to in combination with Flp recombinase to insert targeting cassettes into the CHC, an additional PCR has to be performed. The LSL-BrafV600E allele contains two FRT sites (one near the LSL, one near the V600E mutation in exon 15) that were used to remove a selection cassette and confirm targeting of the LSL and the V600E mutation to the same chromosome. During the RMCE-mediated targeting of the CHC, the LSL-BrafV600E allele may also recombine, resulting in a deletion of Braf exons 3-14.

Primers	Sequence	Size
X3-F (0.6ul)	CATGGCTTGAGTAAGTCTGC	
X3-R (0.6ul)	GATTCACATGGGACCTGAAC	400bp
X14-F2 (0.4ul)	CTACCTAGTGAGACCATATCTC	
X14-R2 (0.4ul)	CAACAGTTGGATCCGTTTAAACG	250bp

Primers used at a 1.5 : 1.5 : 1 : 1 ratio. Primer concentration is 25μM and we use GoTaq Green from Promega and follow the manufacturer's recommendations. **If LSL-BrafV600E is recombined, a 150bp band is detected and the 400bp and 250bp bands disappear.**

PCR program
1) 95°C for 2:00
2) 95°C for 0:30
3) 55°C for 0:30
4) 72°C for 1:00
35 cycles of steps2-4
5) 72°C for 5:00
6) hold at 4°C