

## Cre or Flp in vitro excision of targeted CSD KOMP

### (JM8-derived) ES cell clones

revised October 2, 2007

#### **REAGENTS:**

Item	Vendor	Catalog Number
DMEM	Gibco	11965
Knockout DMEM	Gibco	10829
Penicillin/Streptomycin, 100 u/ml	Gibco	15140-122
L-Glutamine	Gibco	25030-081
Sodium Pyruvate	Gibco	11360-070
Non-essential (NE) Amino Acids	Gibco	11140-050
Trypsin EDTA (1X, 0.25%) (supplement the 0.25% with 1% Chick Serum)	Gibco	25200-072 (0.25%)
LIF (ESGRO)	Gibco	13275-029
Fetal Bovine Serum (FBS), Defined	Hyclone	SH30070.03
PBS (1X without Ca or Mg)	Gibco	14190-144
DMSO, 100 ml	Sigma	D2650
Mitocycin C 10x2mg,	Sigma	M0503
2(β)-Mercaptoethanol, (1000x working soln: add 70μl 2-Mercaptoethanol to 19.93ml PBS. Store at 4°C, and make fresh every 2 weeks)	Sigma	M-7522
Gelatin, 2%, (0.1% working soln: add 25 ml of 2% solution to 475ml of PBS. Store at 4°C)	Sigma	G1393
Puromycin, 1μg/ml, (10μl of 10mg/ml stock in 100ml M-15 media, made fresh as needed)	Sigma	P9620
Zeocin, 5μg/ml, (5μl of 100mg/ml stock in 100ml M-15 media, made fresh as needed)	Gibco	R250-01
EDTA	Sigma	E-7889
D-glucose (1 kilogram)	Sigma	G7528-1KG
Chicken Serum	Gibco	16110-082

**CONSTRUCTS\*:**

- *Flp-e* electroporation:
  - pCAGGS-FLPe
  
- *Cre* electroporation:
  - pCMV-Zeocin
  - pMC1-Cre (contains nuclear localization signal[NLS])

**OR**

  - pCMV-Cre (also contains NLS) can be used instead of pMC1-Cre

***\*note:***

**Electroporations MUST be performed sequentially:**

- 1) Flp-e electroporation**
- 2) Cre electroporation**

**Before the Flp-e electroporation, the cell line is a functional KO due to the frt-flanked gene-trap cassette. Flp-e will delete this gene-trap cassette and convert the cell line to a conditional KO (which will have WT expression before Cre treatment).**

**Once we have this conditional KO (from the first Flp-e electroporation), the second Cre electroporation will now convert the WT to a deletional KO.**

**MEDIAS:**

**Mouse Embryonic Fibroblast (MEF) Feeder Medium** (sterilize by filtering final solution through a 0.2µM filter unit; may be stored at X C for Y days)

Reagent	Stock Conc.	Final Conc.	Volume
DMEM			435 ml
FBS	100%	10%	50 ml
L-Glutamine	200mM	2mM	5 ml
Sodium Pyruvate	100mM	1mM	5 ml
Pen/Strep	10,000U/ml	100U/ml	5 ml
<b>Total Volume</b>			<b>500 ml</b>

**M-15 Medium** (sterilize by filtering final solution through a 0.2µM filter unit; may be stored at X C for Y days)

Reagent	Stock Conc.	Final Conc.	Volume
Knockout DMEM			419 ml
FBS	100%	15%	75 ml
L-Glutamine	200mM	2mM	5 ml
LIF	10 <sup>6</sup> U/ml	1000U/ml	0.5 ml
1000x βME	5.5 x 10 <sup>-6</sup> M	1µM	0.5ml
<b>Total Volume</b>			<b>500 ml</b>

**Trypsin** (sterilize by filtering final solution through a 0.22µM filter unit; make 20 ml aliquots; may be stored at -20 C for Y days).

Reagent	Volume/ Mass
PBS (1X)	475 ml
EDTA	0.1g
D-glucose	0.5g
Chicken Serum	5 ml
Trypsin	20 ml
<b>Total Volume</b>	<b>500 ml</b>

Application:

For cell grown on feeders: 15 min @ 37 C

For cells grown on gelatin: 5 min @37

After trypsinization, pass cells @ 1:5 dilution (confluent in 2-3 days)

**MEF Inactivation Media** (sterilize by filtering final solution through a 0.2 $\mu$ M filter unit; may be stored at -20°C for up to 6 months)

<b>Reagent</b>	<b>Stock Conc.</b>	<b>Final Conc.</b>	<b>Volume</b>
MEF Feeder medium	1x	1x	200ml
Mitomycin C	2mg powder	10ug/ml	2mg
<b>Total Volume</b>			<b>200 ml</b>

**ES cell Freezing Media** (sterilize by filtering final solution through a 0.2 $\mu$ M filter unit; must be made up fresh for immediate use)

<b>Reagent</b>	<b>Stock Conc.</b>	<b>Final Conc.</b>	<b>Volume</b>
FBS	100%	80%	80ml
DMSO	100%	20%	20ml
<b>Total Volume</b>			<b>100 ml</b>

**ELECTROPORATION OF CSD KOMP CLONES (JM8-derived) WITH Cre OR Flp-e-:**

If the parental cell line is JM8.N4, use gelatinized dishes.

If the parental cell line is JM8.F6, use dishes with Mito-C treated feeder cells.

1. Day 1: Thaw 1 vial of KOMP clone (1/10 of a 10 cm plate) early in the morning onto a 6 cm plate with M-15 medium .
2. Day 2: Change M-15 medium. Closely monitor morphology, as it is vital that cells are in peak condition (*Differentiation is observed as loss of discreet ES cell colony border, the formation of cobblestone-like cells and stretched fibroblasts extending outward of the colony. Undifferentiated cells grow in small, smooth, round aggregates*).
3. Day 3: Change medium in the morning. The cells should be 70%-80% confluent around noon. Three hours after the medium change, pass cells to 2x 6cm plates (1:2 passing).
4. Day 4: Change medium of the cells 2-4 hours prior to electroporation (*Feeding will increase the likelihood of obtaining homologous recombinants. This is because homologous recombination occurs at a higher rate in cells that are actively undergoing mitosis i.e. still in their log phase of growth*). Electroporation should be performed 24 hours after the 1:2 passing.
5. As per culture protocol, wash cells with 10 ml PBS, trypsinize with 1ml of 0.25% Trypsin, inactivate the Trypsin with 3 ml M-15 medium, and pipette vigorously to ensure single cell suspension (we recommend 20-30 times).
6. Pellet the cells in a centrifuge at 1000 rpm for 5 min.
7. Aspirate the supernatant and re-suspend cells in 10 ml PBS.
8. Take 50 $\mu$ l of cell suspension, and determine the cell density using a hemocytometer.
9. Put  $1.1 \times 10^7$  cells in one centrifuge tube for electroporation, and the rest in another tube for analyses (via PCR, southern, northern, western, etc.) and mock electroporation.
10. Pellet the greater cell suspension in a centrifuge; at 1000 rpm for 5 min (*This PBS wash step removes media - particularly FBS traces that may hinder efficient electroporation*).
11. For the cells for electroporation, aspirate the supernatant and resuspend the cells in 0.9 ml of PBS ( $1.1 \times 10^7$  cells/0.9 ml).
12. For each electroporation: mix together 25-30  $\mu$ l of 15-40  $\mu$ g (approx. 1  $\mu$ g/ $\mu$ l) DNA; to 0.9 ml of the cell suspension (approx.  $1 \times 10^7$  cells each).
13. Transfer cell suspension to a 0.4 cm electroporation cuvette, and incubate for 5 minutes at room temperature.
14. Place the cuvette in the electroporator (*avoid touching the metal plates of the cuvette*). Pulse the cells once at 230 Volts and 500  $\mu$ F Capacitance (*the time constant [RC] should be between 6.9 – 7.9 [7.2 optimum]; this is an important indicator of the electroporation efficiency. It is important to note that a significant number of cells [around 2/3] are killed by the electroporation and viscous sticky DNA visibly released*).
15. Incubate cells at room temperature for 5 minutes, or place the cuvette on ice for 20 minutes (*ice is optional, but has been reported to improve cell recovery*).
16. Transfer the cell suspension in the cuvette to a sterile tube containing 9ml warmed M-15 medium, and pipette gently to mix (*avoiding the viscous DNA*).

- 17.** Plate 3.3 ml of the cell suspension onto a 10 cm feeder dish containing 7 ml warmed M-15 medium for drug selection, and plate another 3.3 ml of cell suspension onto another 10 cm dish for control electroporation (cells +DNA –drug selection). *The 'Control' is necessary to confirm the cytotoxicity level of the DNA, and may be stained with 1% Methylene Blue for record-keeping after 1-2 days culture.* Mix gently, and incubate in a 37°C humidified 5% CO<sub>2</sub> incubator overnight.
- 18.** The following day, aspirate off the medium, and refeed 1x 10 cm dish with drug selection medium (*cells should have attached well, be around 25-30% confluent, and have a good morphology. If less confluent, refeed with warmed M-15 medium and culture for an additional 24 hours*). Feed
- 19.** Change the drug selection medium daily for the first 2-3 days, then every other day for the duration of selection (*there will be massive cell death observed around day 2, and this will taper off around day 4; when small resistant colonies become apparent, and grow to a size that is amenable to picking*).
- 20.** For the cells for analyses and control, resuspend the cells in 6 ml of M-15 medium, and add 3ml to 10cm plate.
- 21.** Culture the cells for analysis at 37°C in 5% CO<sub>2</sub> until confluent. Wash once with PBS and store at -80 C until analysis.
- 22.** For the cells for mock electroporation, start selection with the KOMP cells, and stain the cells when selection is done. (*The 'Mock' is necessary to confirm the degree of cytotoxicity caused by drug selection; thereby indicating the duration of time necessary to kill 100% of wildtype cells. The 'Mock' must be drug selected alongside the main test dish, and similarly stained afterwards for record-keeping purposes*).
- 23.** Also perform additional control electroporations; using Dicer-C9 or FRAP cell line (to test *flp-mediated* excision of a frt-flanked cassette) and Dicer-C9 or ZAP cell line (to test *Cre-mediate* excision of a loxP-flanked cassette). One electroporation and one 10 cm plate with selection drug will be enough. Pick 24 clones.
- 24.** JM8 wild type cells should be thawed and expanded onto 2x 10 cm dishes for analytical controls.

**DRUG SELECTION:**

*Flp* Electroporation: Puromycin; for a maximum of 3-5 days at a concentration of 2  $\mu\text{g/ml}$ .

*Cre* Electroporation: Zeocin; for a maximum of 3-5 days at a concentration of 2  $\mu\text{g/ml}$ .

**PICKING CLONES AFTER DRUG SELECTION:**

1. Gently wash the electroporation test dish twice with 10 ml PBS.
2. Pick 48 colonies into separate wells of a 96-well plate (round-bottomed wells); containing 25 µl of 0.25% Trypsin each.
3. Incubate at 37°C for 4-5 minutes.
4. Inactivate the Trypsin with 200 µl warmed M-15 medium, and pipette to ensure single cell suspension (we recommend 15 times).
5. Transfer the cell suspension from each well to a flat-bottomed 96-well feeder plate (*consult feeder cell plating density guide in culture protocol*).
6. Incubate in a 37°C humidified 5% CO<sub>2</sub> incubator for 6 hours to ensure cell attachment.
7. After the 6 hours, refeed each well with 200 µl warmed M-15 medium (*This step is vital to remove damaging effects of Trypsin*).
8. Refeed daily with M-15 medium; until a majority of wells have reached 80% confluency (*around 2 days, and closely monitor morphologies*).
9. At confluency, rinse each well once with PBS, trypsinize with 25 µl of 0.25% Trypsin for 4-5 minutes, neutralize with 200 µl warmed M-15 medium, and pipette to ensure single cell suspension as before.
10. Split the cell suspension (1:3 ratio i.e. 75 µl), and plate onto triplicate labeled 96-well plates (*2 sets of plates are for PCR genotyping and therefore do not require feeders*).
11. As before, incubate in a 37°C humidified 5% CO<sub>2</sub> incubator for 6 hours to ensure cell attachment, then refeed each well with 200 µl warmed M-15 medium to remove Trypsin.
12. Refeed daily with M-15 medium; until a majority of wells have reached 80% confluency (*around 2 days, and closely monitor morphologies*).
13. At confluency, rinse the two feeder-free sets of plates twice with PBS, Parafilm-seal the lids and submit both sets to MGAL for PCR screening (*Plates may be stored at -20°C for up to 3 months*). Positives indicated on plate template (see below)
14. Freeze the remaining set; original plated on feeder cells (*Master Plates*).

<b>1<sup>st</sup> Electroporation</b>				
<b>ES cell</b>	<b>DNA</b>	<b>Selection drug, concentration</b>	<b>Purpose</b>	<b>No. clones to pick</b>
KOMP clones	pCAGGS-FLPe	puromycin, 2 µg/ml	flp excision	48
KOMP clones	pCAGGS-FLPe	-	Control	0
Dicer-C9/FRAP	pCAGGS-FLPe	puromycin, 2 µg/ml	+ Control	24
KOMP clones	-	puromycin, 2 µg/ml	mock	0
<b>2<sup>nd</sup> Electroporation</b>				
KOMP clones	pCMV-Zeocin and pMC1-Cre OR pCMV-Cre	Zeocin, 2 µg/ml	cre excision	48
KOMP clones	-	Zeocin, 2 µg/ml	Control	0
Dicer-C9/ZAP	pCMV-Zeocin and pMC1-Cre OR pCMV-Cre	Zeocin, 2 µg/ml	+ control	24
KOMP clones	-	Zeocin, 2 µg/ml	mock	0

**FREEZING MASTER PLATES (Slow Freeze):**

- 1.** At confluency, and one plate at a time; rinse wells once with PBS.
- 2.** Trypsinize with 25  $\mu$ l of 0.25% Trypsin for 4-5 minutes.
- 3.** Neutralize with 40  $\mu$ l warmed M-15 medium, and pipette to ensure single cell suspension.
- 4.** Add 65  $\mu$ l 2X Freezing Medium to each well, and pipette to ensure single cell suspension.
- 5.** Quickly Parafilm-seal the lids, and transfer to  $-20^{\circ}\text{C}$  for 30-45 minutes.
- 6.** Transfer plates to  $-80^{\circ}\text{C}$ , and store for up to 3 months (*The viability and quality of cells decline after longer periods*).

**RECOVERY AND EXPANSION OF PCR POSITIVE CLONES (Fast Thaw):**

1. One plate at a time, remove the Parafilm lid seal and thaw rapidly; utilizing either a slid warmer set at 37oC.
2. Once most wells are partially thawed (and ice crystals are still visible); quickly add 150 µl warm M-15 medium to each well and pipette gently to ensure mixing.
3. Incubate in a 37°C humidified 5% CO<sub>2</sub> incubator for 6 hours to ensure cell attachment.
4. After the 6 hours, refeed each well with 200 µl warmed M-15 medium to remove Trypsin and DMSO (*Important, or cells will die*).
5. Refeed daily with M-15 medium; until a majority of wells have reached 80% confluency (*roughly 3-5 days, and closely monitor morphologies*).
6. At confluency, passage to expand each clone to 24-w dish. At each passing, 80% confluency is normally reached within 2-3 days (*see guide below*).

Well size	Feeder density	Trypsin required when confluent	Media required to neutralize Trypsin
96-well	1.5 x 10 <sup>4</sup>	25 µl	200 µl
24-well	1 x 10 <sup>5</sup>	100 µl	500 µl
6-well	4 x 10 <sup>5</sup>	0.5 ml	2.5 ml
6 cm dish	1.5-2 x 10 <sup>6</sup>	1 ml	5 ml

**CONTINUED EXPANSION OF POSITIVE CLONES FOR FURTHER ANALYSIS:**

1. At 24-W dish confluency, passage to 6-W plate. Feed daily.
2. At 6-W dish confluency, split (1:6), freezing 5 parts as per culture protocol and re-plate 1 part onto a fresh 6-well gelatinized plate (*no feeders necessary*).
3. Refeed the 6-well plate daily, until 80% confluency is reached (*around 2 days*), rinse twice with PBS, begin extraction from cells.
4. Archive the 5 vials in liquid or vapor-phase Nitrogen (1) for *Cre* electroporation (2) further analysis and (3) backups.