EUCOMM Protocol for ES cells

1. SNL Feeder Cells

1.1. Preparing inactivated SNL Feeder Cells

1) Thaw one vial of SNL cells (approximately1.5-2 x 10^6 cells per vial) in a 37°C water bath and dilute into 10ml of pre-warmed SNL feeder cell medium
2) Pellet the cells by spinning for 3 minutes at 1000 rpm in a bench-top centrifuge
3) Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed SNL feeder cell medium
4) Transfer the cell suspension to a 6 cm gelatinized dish and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days)
5) When confluent, aspirate medium off and wash with 5 ml of pre-warmed PBS, pipetting it away from the cells. Rock dish gently and aspirate the PBS.
6) Cover cells with 1ml of Trypsin2x+G solution and incubate at 37°C for 2-4 minutes or until cells are uniformly dispersed into small clumps
7) Add 5 ml of SNL Feeder medium to inactivate the trypsin and pipette vigorously to produce a single cell suspension
8) Spin for 3 minutes at 1000rpm
9) Aspirate off medium and gently resuspend cells in 20ml of pre-warmed SNL Feeder cell medium
10) Split the cell suspension onto two gelatinized 10cm tissue culture dishes and grow at 37°C in a humidified 5% CO_2 incubator (should be confluent within 2-3 days)
11) To mitotically inactivate, replace medium with 10ml Inactivation Medium and incubate in 37°C in a humidified 5% CO_2 incubator for 2.5 hours. Aspirate inactivation medium, and rinse three times with pre-warmed PBS; aspirating completely between rinses
12) If you wish to freeze the cells for later usage, trypsinize and pellet the cells as before, and inactivate the trypsin with 5ml Medium
13) For each 10cm dish count cells and resuspend the cells in Freezing medium, to a density of 4 x 10^6 cells/vial (400µl per cryovial).
14) Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.
15) Freeze cryovials in a –80°C freezer. After 24 hours transfer cryovials to vapor phase nitrogen for longer term storage.

1.2. Plating inactivated SNL Feeder Cells

1) Coat tissue culture dishes of the desired size with 0.1% gelatin and aspirate off immediately before use
2) Thaw one vial of mitotically inactive SNL Feeder cells (approximately 4 x 10^6 cells per vial) in a 37°C water bath and dilute into 10ml of pre-warmed **M-SNL**
3) Pellet the cells by spinning for 3 minutes at 1000 rpm in a bench-top centrifuge
4) Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed **M-SNL**
5) Transfer the cell suspension in the prepared gelatinized dishes according to the following table and grow at 37°C in a humidified 5% CO_2 incubator
6) The feeders are ready for use after 7 to 21 days (change medium with **M-SNL** once a week)

Plate / Dish	Feeder Cell Density
24 well	1 – 2.5 x 10^5
6 well	5 - 8 x 10^5
6 cm dish	1.5 - 2 x 10^6
10 cm dish	3.5 – 4.5 x 10^6

The numbers in this table have been determined with the SNL cells inactivated in the WP9 ES cell lab and can be used as guideline for feeder cells received from us. Each lab should determine the optimal dilution of their own inactivated feeder cells; the feeders should be 70 to 80% confluent, non dividing but vital.

2. JM8 ES Cell Clones

2.1. Thawing gene targeting ES cell clones

1.) Aspirate off the medium for SNL cells from one well of a 6-well plate with prepared inactivated SNL feeder cells (incubated at 37°C for 7 to 21 days)
2.) Overlay the feeder cells with 2ml of **M15G + Lif**
3.) Thaw one vial of ES cells in a 37°C water bath and dilute into 5ml of pre-warmed **M15G + Lif**
4.) Pellet the cells by spinning for 3 minutes at 1200rpm in a benchtop centrifuge
5.) Aspirate off medium and gently resuspend the cells in **3ml** of **M15G + Lif**
[NOTE: 1 ml medium for a 6 well is not enough and will lead to uneven plating due to the meniscus effect – I recommend 3-4 ml medium]
6.) Transfer the resuspended JM8 cells onto the prepared one 6-well feeder dish and grow in a 37°C humidified 5% CO_2 incubator
7.) Change medium (**M15G + Lif**) daily until the ES cell clones have grown but not begun to differentiate (this should take 2 to 4 days)
8.) When confluent the cells may be split 1:5 (e.g. the 6-well dish may be split into one 10cm dish)
9.) The JM8 cells can be used for microinjection, further expansion, or freezing.

2.2. Expansion of gene targeting ES cell Clones:

We recommend that the user is growing ES cells in a 6well dish, split the cells onto two 6cm dishes. In one 6cm dish the ES cells should be grown on feeders (for freezing and further storage) and in the other 6cm dish the cells should be grown feeder-free (for microinjection). We therefore describe here the splitting of one 6well plate onto two 6cm dishes.
*We also recommend to use medium **without G418** when expanding cells for microinjection.*

1) At a confluency of 70 to 80% and an optimal morphology of the clones, the clones are ready to split (or (later) for the preparation of cells for microinjection); otherwise just exchange the medium
2) Add 2ml Gelatin solution to one 6cm dish
3) Take one 6cm dishes with prepared inactivated SNL feeder cells (incubated at 37°C for 7 to 21 days)
4) Aspirate off the **M-SNL** and overlay the SNL cells with 4ml of **M15G + Lif**
5) Aspirate off the **M15G + Lif** from the JM8 cells (from the 6 well dish)
6) Wash the JM8 cells once with 2ml PBS
7) Pipette **0.5 ml Trypsin 2x+G** to the JM8 cells
8) Incubate the cells with trypsin for **10-15** minutes in a 37°C humidified 5% CO_2 incubator (until the cells are uniformly dispersed)
9) Pipette **4.5 ml M15G + Lif** onto the trypsinzed ES cells in the 6well dish, in order to inactivate the trypsin (total volume of 5ml)
10) Pipette gently 3-4x up and down, in order to disperse the cells (if clumpy, allow the large clumps to settle for 2 minutes prior to plating)
11) Transfer one half of the suspension of ES cells onto two 6cm dishes (one with inactivated SNL feeder cells as described above and one just gelatinzed); the result should be a volume of 5ml per 6cm dish.

12) **For freezing** (for longtime storage) trypsinize the ES cells on the 6cm dish (with SNL feeders) by aspirating off the medium from the cells and adding **1ml Trypsin 2x+G** to the JM8 cells
13) Incubate the cells with trypsin for **10 to 15** minutes in a 37°C humidified 5% CO_2 incubator (until the cells are uniformly dispersed)
14) Pipette **4ml M15G + Lif** onto the trypsinzed ES cells in the 6well dish, in order to inactivate the trypsin (total volume of **5m**l)
15) Pipette gently 3-4X up and down, in order to disperse the cells
16) Pipette the trypsinized ES cells gently into 5ml of **M15G + Lif**
17) Pellet the cells by spinning for 3 minutes at 1200 rpm in a bench-top centrifuge and resuspend the cells in 800µl of **FM15G + Lif**
18) Aliquot 200µl per cryovial and immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel. Freeze vials in a -80°C freezer. After 24 hours transfer cryovials to liquid or vapor phase nitrogen for long term storage

19) **For Microinjection** trypsinize the ES cells on the 6cm dish (ES cells grown feeder-free on gelatin) as described above.

20) Pellet the cells by spinning for 3 minutes at 1200 rpm in a bench-top centrifuge and resuspend the cells in **Microinjection Medium**
21) Immediately place the ES cell suspension on ice and microinject within 1 to 2.5 hours

Reagents:

M15G + Lif (Medium for JM8 cells grown on feeders):

500 ml Medium (Knockout DMEM (GIBCO; cat #: 10829))
+ 90 ml FBS (GIBCO, Lot tested)
+ 5 ml 100x L-Glutamine (GIBCO; cat #: 25030)
+ 5 ml 100x beta-Mercaptoethanol (Sigma, cat #: M7522 (360µl/500ml PBS; filtered, stored at -20°C)
sterile filtered (e.g. Corning cat #: 431097)
+ 100µg (active)/ml **G418** (Gibco/ Invitrogen; cat #.: 10131-027)
Attention: concentration may vary throughout the protocol
+ **ESGRO** (LIF; Chemicon; cat #.: ESG1107) dilute as directed (1000 units/ml)

store at 4°C

M10G + Lif (Media for JM8 cells grown on gelatin):

500 ml Medium (Knockout DMEM (GIBCO; cat #: 10829))
+ 50 ml FBS (GIBCO, Lot tested)
+ 5 ml 100x L-Glutamine (GIBCO; cat #: 25030)
+ 5 ml 100x beta-Mercaptoethanol (Sigma, cat #: M7522 (360µl/500ml PBS; filtered, stored at -20°C)
sterile filtered (e.g. Corning cat #: 431097)
+ 100µg (active)/ml **G418** (Gibco/ Invitrogen; cat #.: 10131-027)
Attention: concentration may vary throughout the protocol
+ **ESGRO** (LIF; Chemicon; cat #.: ESG1107) dilute as directed (1000 units/ml)

Store at 4°C

FM15G + Lif (Freezing Medium for JM8 cells):

Prepare fresh every time before use (small volume)
M15G + Lif
+ 10% DMSO

M-SNL (Medium for SNL cells):

450 ml Medium (DMEM with high Glucose
+ L-Glutamine (GIBCO, cat #: 41965)
+ 50 ml Mycoplex FCS (PAA, cat #: A15-105)

FM-SNL (Freezing Medium for SNL cells):

Medium for SNL cells (see above)
+ 10% DMSO (Sigma, cat #: D-5879)
(prepare fresh and filter sterilize before use)

Inactivation Medium

200ml Medium for SNL cells (see above)
+ 2 mg of Mitomycin C (Sigma, cat #: M0503)
(prepare fresh before use)

Tryp2X+G (5-10 minutes for feeder-free; 10 minutes for feeders):

500ml PBS (GIBCO; Art.-Nr.: 14190)
+ 0.1g EDTA (Sigma; Art.-Nr.: E6511-100G)
+ 0.5g D-Glukose (Sigma; Art.-Nr.: G7528-1000G)
-> sterile filter (0.22µm)
+ 5ml Chicken Serum (Gibco; Art.-Nr.: 16110-082)
+ 20ml 2.5% Trypsin (Gibco; Art-Nr.: 151090-046)

Prepare aliquots (e.g. 10ml Aliquots) and store at -20°C

Gelatin Solution

0.1% gelatin in PBS **(2% gelatin, Sigma)**
Store at 4°C

Microinjection Medium

M10G + Lif **without** G418