

Experimental Protocols for Soriano's Gene Trap ES cell lines

Adapted from Dr. Soriano's paper and Philip Corrin's notes
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* Weisheng V. Chen and Philippe Soriano. *Gene Trap Mutagenesis in Embryonic Stem Cells. Methods in Enzymology, Vol. 365, 2003.*

Cell Line Information

Soriano Gene Trap cell lines are feeder-dependent.

Parental ES cells: AK7.1, isolated from 129S4/SvJaeSor (light bellied agouti). Injection of ES cells into C57BL/6 blastocysts will produce agouti chimeras. This cell line is mycoplasma free.

SNL Feeder cells: SNL 76/7, feeder cells express leukemia inhibitory factor (LIF) and neomycin phosphotransferase (*Neo*). This cell line is mycoplasma free.

Reagents and Supplies:

<u>Item</u>	<u>Vendor</u>	<u>Catalog Number</u>
DMEM, high glucose, with glutamine	Gibco	11965-092
DMEM, with 25mM Hepes	Gibco	12430-054
Penicillin/Streptomycin, 100 U/ml	Gibco	15140-122
L-Glutamine	Gibco	25030081
Trypsin EDTA (0.05%)	Gibco	25300-054
Trypsin EDTA (0.25%)	Gibco	25200-072
Fetal Bovine Serum, Defined	Hyclone	SH30070.03
PBS (1X without Ca or Mg)	Gibco	14190-144
DMSO, 100 ml (2X Freezing medium: FBS with 20% DMSO – make fresh as required)	Sigma	D2650
<u>Mitomycin C 10x2mg</u> (10µg/ml Inactivation media: add 2mg of Mitomycin C to 200 ml SNL feeder medium - may be stored at 20°C for up to 6 months)	Sigma	M0503
<u>2-Mercaptoethanol</u> (1000x working soln: add 70µl 2-Mercaptoethanol to 9.93ml PBS. Store at 4°C, and make fresh every 2 weeks)	Sigma	M-7522
<u>Gelatin, 2%</u> (0.1% working soln: add 25 ml of 2% solution to 475ml of PBS. Store at 4°C)	Sigma	G1393

Media

i) Soriano Gene Trap ES Cell Media (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume</u>
DMEM	1X	1X	417ml
FBS	100%	15%	75ml
100x Pen/Strep	100000 U/ml/ 100000ug/ml	50 U/ml 50ug/ml	2.5ml
2-Mercaptoethanol	1000x	1x, 0.1mM	0.5ml
100x L-Glutamine	200mM	2 mM	5 ml
Total Volume			500ml

* You may use alternative Feeder cells such as MEFs. However, we strongly recommend that you substitute the ES cell media with 1000X LIF *

ii) SNL Feeder Cell Media (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume</u>
DMEM	1X	1X	442.5ml
FBS	100%	10%	50ml
100x Pen/Strep	100000 U/ml 10000u/ml	50 U/ml 50u/ml	2.5ml
100x L-Glutamine	200 mM	2 mM	5ml
Total Volume			500ml

iii) SNL Inactivation Media (sterile filter through 0.2µM filter unit)

<u>Reagent</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Volume</u>
SNL feeder cell medium	1x	1x	200ml
Mitomycin C	2mg powder	10ug/ml	2mg
Total Volume			200 ml

Preparing SNL Feeder Cells

Thawing, Expanding and Treating Active SNL Cells

1. Thaw 1 vial of SNL cells (approximately $1.5-2 \times 10^6$ cells/vial) in a 37°C water bath and dilute into 10 ml of pre-warmed SNL feeder cell medium.
2. Pellet the cells by spinning for 4 minutes at 1000 rpm in a bench-top clinical centrifuge.
3. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed SNL feeder cell medium.
4. Transfer cell suspension to a 6 cm gelatinized dish, and grow at 37°C in a humidified 5% CO₂ 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 medium. Repeat.
6. Cover cells with 1 ml of 0.05% trypsin solution and incubate at 37°C for 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 make single cell suspension (we recommend 15 times).
8. Spin for 4 minutes at 1000 rpm.
9. Aspirate off medium and gently resuspend cells in 20 ml of pre-warmed SNL feeder cell medium.
10. Split the cell suspension onto two gelatinized 10 cm tissue culture dishes, and grow at 37°C in a humidified 5% CO₂ incubator (should be confluent within 2-3 days).
11. To mitotically inactivate, replace medium with 10 ml Inactivation medium (2mg of Mitomycin C to 200 ml SNL feeder medium), and incubate in a 37°C humidified 5% CO₂ incubator for 2.5 hours. Aspirate Inactivation medium, and rinse three times with pre-warmed PBS; aspirating completely between rinses. These dishes are now ready to use.
12. If you wish to freeze the cells for later usage, trypsinize and pellet the cells as before, but with 1.5 ml of 0.05% trypsin solution, and inactivate the trypsin with 5 ml medium.
13. For each 10 cm dish, count cells, and resuspend in an equal volume of SNL feeder cell medium and 2X Freezing medium; to a density of 1.5-2 x 10⁶ cells/0.5 ml. Decant 0.5 ml aliquots into labeled cryovials.
14. Immediately place cryovials in a Styrofoam container or temperature controlled freezing vessel.
15. Freeze vials in a -80°C freezer. After 24 hours, transfer cryovials to liquid or vapor-phase nitrogen for longer term storage.

Plating Mitotically Inactive SNL Feeder Cells

1. Coat a 6 cm tissue culture dish with 0.1% gelatin and aspirate off immediately before use.
2. Thaw 1 vial of mitotically inactive SNL feeder cells (approx. 1.5-2 x 10⁶ cells) in a 37°C water bath and dilute into 10 ml of pre-warmed SNL feeder cell medium.
3. Pellet the cells by spinning for 4 minutes at 1000 rpm.
4. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed SNL feeder cell medium.
5. Transfer cell suspension to the 6 cm gelatinized dish, and grow at 37°C in a humidified 5% CO₂ incubator.
6. The feeders are ready for use after 6-12 hours, or may be maintained in the incubator for a maximum of 8-10 days.

Thawing Soriano Gene Trap ES Cell Clones

1. Thaw 1 vial of ES cells (approximately 5 x 10⁶ cells/vial) in a 37°C water bath and dilute (drop wise) into 10 ml of pre-warmed Soriano Gene Trap ES cell medium.
2. Pellet the cells by spinning for 4 minutes at 1000 rpm.
3. Aspirate off medium and gently resuspend cells in 5 ml of pre-warmed Soriano Gene Trap ES cell medium.
4. Aspirate the old medium from your 6 cm mitotically inactive SNL feeder cell dish.
5. Transfer the ES cell suspension to the feeder dish, and grow in a 37°C humidified 5% CO₂ incubator.
6. Change medium the following day to remove dead cells and residual DMSO.
7. Change medium daily until 80% confluent (approx. 1.5-2 x 10⁷ cells); should take 2-4 days.
8. When confluent, the 6 cm dish may be split in two; half for microinjection and half to expand for freezing.

Expansion of Soriano Gene Trap ES Cell Clones for Microinjection and Future Use

1. 1 day prior, prepare one 10 cm SNL Treated Feeder dish, using the plating density guide below.
2. The next day, aspirate off the old medium prior to plating ES cells.
3. On the day, wash the confluent 6 cm ES cell dish once with 5 ml PBS.
4. Cover the cells with 1 ml of 0.25% trypsin solution and incubate at 37°C for 4-5 minutes or until cells are uniformly dispersed into small clumps.

5. Add 5 ml of Gene Trap ES cell medium; to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 10-15 times).
6. Split the cell suspension in half, placing 2.5 ml each into 15 ml centrifuge tubes (labeled 'Expansion' and 'Microinjection').
7. Spin both tubes for 4 minutes at 1000 rpm.
8. For the 'Expansion' cells; aspirate off the supernatant and resuspend the pellet in 10 ml Soriano Gene Trap ES cell medium. Transfer the cell suspension onto the 10 cm SNL Treated Feeder dish prepared the day before. Change medium daily until 80% confluent (should take 2-3 days).
9. For the 'Microinjection' cells; aspirate off the supernatant and resuspend the pellet in 150-400 μ l microinjection medium (Hepes Buffered DMEM with 5% FBS; filtered through 0.2 μ M filter unit, can be aliquoted and stored at -20°C for up to 12 months). Immediately place the cell suspension on ice, and microinject within 1-2.5 hours.

Freezing Expanded Soriano Gene Trap ES Cell Clones

1. Wash the confluent 10 cm ES cell dish once with 10 ml PBS each.
2. Cover the cells with 1.5 ml of 0.25% trypsin solution and incubate at 37°C for 4-5 minutes or until cells are uniformly dispersed into small clumps.
3. Add 5 ml Gene Trap ES cell medium to inactivate the trypsin, and pipette vigorously to make single cell suspension (we recommend 10-15 times).
4. Spin for 4 minutes at 1000 rpm.
5. Aspirate supernatant and resuspend the pellet in an equal volume of Soriano Gene Trap ES cell medium and 2X Freezing medium (we would recommend 8-10 vials containing 0.5 ml aliquots; per 10 cm dish). Decant into labeled cryovials.
6. 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 longer term storage.

SNL Treated Feeder Cells – Plating Density Guide

Plate/Dish	Feeder Cell Density
96 well	1.5×10^4
24 well	1×10^5
3.5 cm dish	8×10^5
6 cm dish	$1.5-2 \times 10^6$
100 mm dish	$3.5-4 \times 10^6$