Culturing Protocol for KOMP-Regeneron VGB6 ES Cell Clones

Revised July 2014

Cell Line Information

VGB6-derived KOMP clones from Regeneron Pharmaceuticals Inc. were isolated from C57BL/6NTac mouse strain (Taconic.) Injection of these cells (formerly B6A6) into Albino C57BL/6 blastocysts will produce black chimeras. Alternatively, chimeras can be produced by aggregation of ES cells with 8-cell stage embryos from ICR strain or by injection of ES cells into 8-cell embryos from either albino C57BL/6 or Swiss Webster strains. The parental cell line was derived at Regeneron and is certified *Mycoplasma Sp.* free.

VGB6-derived ES cells from Regeneron are **feeder-dependent.** These ES cells may be grown on the SNL 76/7 feeder cell line which expresses very small amounts of Leukemia Inhibitory Factor (LIF) and neomycin phosphotransferase (Neo) or on primary murine embryonic fibroblasts (MEF.) In addition, MEF feeder cells have been derived at the Mouse Biology Program (MBP) and Regeneron has approved the use of these feeders for growth of their ES cell clones. Please see the MBP Protocol for culturing JM8.F6 for details on using feeder cells.

This protocol is based on Regeneron procedures as adapted by the Mouse Biology Program from Poueymirou, W. T., et al, F0 Generation Mice Fully Derived from Gene-Targeted Embryonic Stem Cells Allowing Immediate Phenotypic Analyses. Nature Biotechnology 25, 91-99, 2007. Sanger procedures as adapted by the Mouse Biology Program are based on Pettitt, S. J., et al, Agouti C57BL/6N Embryonic Stem Cells for Mouse Genetic Resources. Nature Methods 6, 493-495, 2009. For procedures using 2i reagents, the protocol is adapted from Gertsenstein, M., et al, Efficient Generation of Germ Line Transmitting Chimeras from C57BL/6N ES Cells by Aggregation with Outbred Host Embryos. PLoS ONE 5, e11260, 2010.

Reagents and Supplies

Item	Vendor	Catalog Number
Knockout DMEM™, high glucose	Gibco	10829-018
Knockout Serum Replacement*	Gibco	10828-028
L-Glutamine (200 mM, 100X)	Gibco	35030-081
NE Amino Acids (100mM)	Gibco	11140-050
Sodium Pyruvate (100mM)	Gibco	11360-070
Insulin (bovine pancreas, 10 mg/ml)**	Sigma	10516
LIF***	Millipore	ESGRO (ESG 1107)
2(β)-Mercaptoethanol	Sigma	M-7522
Penicillin/Streptomycin****	Gibco	15140-122

PBS (1X without Ca or Mg)	Gibco	14190-144
Trypsin EDTA, 0.25%	Gibco	25200-072
Trypsin EDTA, 2.5%	Gibco	15090-046
Chicken serum	Gibco	16110-082
EDTA	Sigma	E6511
Hepes-Buffered D-MEM (for Microinjection media)	Gibco	12430-054
D-glucose	Sigma	G7528
DMSO, 100 ml	Sigma	D2650
Gelatin, 2%	Sigma	G1393

*Alternatively, ES Cell Grade FBS may be used, e.g. Gibco 10437028 or Hyclone FBS but serum should always be pre-tested to be ES Cell qualified prior to use.

**Because it may be back-ordered, alternate sources of Insulin could be Sigma I1882 or Gibco 51300.

***An alternate supplier of LIF is GlobalStem (GSR-7001) 100 ug/vial.

****It's generally preferable not to include antibiotics when culturing cells but we routinely include Pen/Strep because of our high volume and multiple sources of ES cells.

2i reagent: MEK inhibitor PD0325901	StemGent Also Cedarlane	04-0006 Axon 1408
2i reagent: GSK3 inhibitor CHIR99021	StemGent Also Cedarlane	04-0004 Axon 1386

1000x 2(β)-Mercaptoethanol

- To 10 ml PBS add 70 µl 2-Mercaptoethanol
- Store at 4°C and make fresh every 2 weeks

VGB6 ES Cell Medium (500 ml) Sterile filter through 0.2µm filter unit

Reagent	Stock Conc	Final Conc	Quantity
KO DMEM™		1X	399ml
Knockout Serum Replacement*		15%	75 ml

Na Pyruvate	100 mM	1 mM	5 ml
L-Glutamine	200 mM	4 mM	10 ml
NE Amino Acids	100 mM	1 mM	5 ml
LIF**	10^7 U/ml	2000 U/ml	100 ul
Insulin	10 mg/ml	5 ug/ml	250 ul
2(β)-ME	1000X	0.1 mM	1.0 ml
Pen/Strep	10,000 U-ug/ml	100 U-ug/ml	5 ml

***Or ES Cell Grade FBS may be used, 75 ml.

**For GlobalStem LIF, the 100 ug vial is reconstituted in 1 ml, used at 50 ul/500 ml media for a final concentration of 2000 units/ml.

Optional 2i Reagents (for 500 ml of media)

Reagent	Stock Conc.	Final Conc.	Quantity
2i reagent: MEK inhibitor PD0325901*	5 mM	1 uM	100 ul
2i reagent: GSK inhibitor CHIR99021**	3 mM	3 uM	500 ul

*To prepare StemGent MEK inhibitor, resuspend 2 mg vial in 830 ul of DMSO.

**To prepare StemGent GSK inhibitor, resuspend 2 mg vial in 1.4 ml of DMSO.

Chicken Serum Trypsin (0.1% Trypsin)

- To 475 ml PBS add 0.1 g EDTA and 0.5 g D-glucose
- Add 5 ml Chicken Serum
- Add 20 ml 2.5% Trypsin
- Filter sterilize (0.22 µm filter unit)
- Aliquot 20 ml into centrifuge tubes
- Store at -20°C

Note: We recommend the use of chicken serum trypsin which tends to be gentler on the cells but standard **0.25% trypsin-EDTA** (Gibco 15050-06) may also be used.

0.1% Gelatin (500 ml)

- Add 25 ml of 2% solution to 475 ml of PBS
- Filter sterilize (0.22 µm)
- Store at 4°C
- To prepare culture dish, add 0.1% gelatin to cover, remove after minimum of ~10 minutes

Alternatively, 0.1% Gelatin, ready to use, may be purchased from Millipore (ES-066-B.)

Microinjection Medium (500 ml)

- Add 25 ml of FBS to 475 ml of HEPES-buffered D-MEM
- Filter sterilize (0.22 um) and make 4 ml aliquots. May be stored at -20 to -80^{o}C for up to 1 year

2X Freezing Medium

- 60% JM8A ES Cell Medium (see above recipe)
- 20% FBS
- 20% DMSO

Note: Add FBS to Media before addition of DMSO

IMPORTANT SAFETY NOTE: When you remove the vial from a nitrogen tank, please loosen the lid immediately to release the pressure and thereby reduce the chance of an explosion. We recommend that you thaw tubes rapidly in a 37° water bath, submerging just the bottom half of the vial. Gently swirl until just a small ice crystal is left.

Manufacturer's Alert: **To prevent cryogenic vials from exploding** please review the following. Always use full face shields, heavy safety gloves and laboratory protective apparel when removing vials from cryogenic storage. Manufacturer recommends you review appropriate procedures outlined in the Nalge Nunc International Cryopreservation Manual located at in the FAQ. *We store vials in vapor phase nitrogen*, and recommend that you place the vials from the dry ice into your -80 freezer if you will be thawing within the next 3 weeks. If you will not be thawing within 3 weeks we recommend long term storage in a nitrogen vapor storage tank. If you choose to store in liquid nitrogen, **please use caution.**

Thawing ES cell Clones

1. Rapidly thaw 1 vial of ES cells (approximately 2 x 10^6 cells/vial) in a 37°C water bath and dilute (drop wise) into 3 ml of pre-warmed Medium.
2. Transfer the ES cell suspension to 1 well of a 6 well dish (or a 6 cm dish) which has been pre-plated with feeders and grow in a 37°C humidified 5% CO_2 incubator.
3. Change medium the following day to remove dead cells and residual DMSO.
4. Change medium daily until 80% confluent (approximately 1.5-2 x 10^7 cells); should take 2-3 days but some clones may be slower to become confluent.
5. When 80% confluent, the well or dish may be split in two; half for microinjection and half for expansion or freezing.
6. Media plus 2i (see above for formulation) is used routinely where any differentiation is seen or can be used in general for these cells. We've found it useful to pass the cells with media without 2i and resume using media plus 2i for daily feeding.

Expansion of Clones for Microinjection and Future Use

1. Wash the confluent ES cell well or dish once with 3 ml PBS.
2. Cover the cells with 0.5 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 14 minutes or until cells are uniformly dispersed into small clumps.
3. Add 3 ml of ES cell medium to inactivate the trypsin and pipette gently to make a single cell suspension (~7-10 times).
4. For '**Expansion**' half the cell suspension may be added to ~8 ml of pre-warmed media in a 10 cm dish containing pre-plated feeders for a final volume of ~10 ml/dish. Grow in a 37°C humidified 5% CO_2 incubator. Change medium daily until 80% confluent. Note: These lines are very sensitive to over-confluence. Cells should be passed or frozen between 75 and 85% confluence.
5. For the '**Microinjection**' cells, add 5 ml of media to the remaining trypsinized cells and centrifuge in a 15 ml tube for 5 minutes at 1000 rpm. Aspirate off the supernatant and re-suspend the pellet in ~200 ul of Microinjection Medium. Place the vial containing the cells on ice and microinject within 1-2.5 hours.

   In cases, where the cells are frozen and later thawed to expand for injection, the cells are usually passed 2-3 times as necessary up to 24 hours before injection at which point media without 2i reagents is used until the cells are injected. Accutase (Millipore SF006 or Invitrogen StemPro A1110501) may be used instead of trypsin when KOSR is used since there is no FBS in the media to inactivate the trypsin; Accutase does not require inactivation and may be spun out or diluted out.

Freezing ES Cell Clones

1. Wash the confluent 10 cm dish once with 10 ml PBS.
2. Cover the cells with 1.5-2.0 ml of 0.1% trypsin with chicken serum and incubate at 37°C for 14 minutes or until cells are uniformly dispersed into small clumps.
3. Add 10 ml ES cell medium to inactivate the trypsin and pipette gently to make single cell suspension (~7-10 times).
4. Centrifuge for 5 minutes at 1000 rpm.

5. Aspirate supernatant and re-suspend the pellet in ES cell medium. Add equal volume of 2X Freezing Medium (we would recommend 6-8 vials containing 0.5 ml aliquots from a 10 cm dish). So for 8 vials, re-suspend pellet in 2 ml of media and add 2 ml of 2X Freezing media for a total of 4 ml or 8 samples of 0.5 ml each. Decant into labeled cryo vials (we use Nunc cryotubes, 377267.)
6. Immediately place cryo vials in a Styrofoam container or temperature controlled freezing vessel.
7. Freeze vials in a -80ºC freezer. After minimum of 24 hours, transfer cryo vials to liquid or vapor phase nitrogen for longer term storage.

We use Nunc Cryo Tube vials, for which the manufacturer recommends that for storage in LIQUID N_2 you place them in Nunc Cryoflex Tubing (Catalogue # 343958). Please note that we have had reports of vials exploding if stored in liquid, therefore if you choose not to use Cryoflex tubing you may want to do the following to try and avoid injury: When you remove the vial from liquid nitrogen, unscrew the top immediately to release the pressure and thereby *reduce the chance of an explosion*. And again, always use proper personal protective equipment, such as full face shields, when handling items stored in liquid nitrogen.