**SOP: Propagation of E14 mouse embryonic stem cells**
**Date modified: 02/17/2012**
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## Ordering Information

E14 undifferentiated mouse embryonic stem cells were received as frozen ampoules from M. Ramalho-Santos Lab

## Materials List

**Reagent**

| | |
|---|---|
| DMEM | Cellgro Cat# 10-013-CV |
| Fetal Bovine Serum | HyClone Cat# SH30071 |
| Penicillin/Streptomycin | Cellgro Cat# 30-001-CI |
| Adenosine | Sigma-Aldrich Cat# A-4036 |
| Guanosine | Sigma-Aldrich Cat# G-6264 |
| Uridine | Sigma-Aldrich Cat# U-3003 |
| Cytidine | Sigma-Aldrich Cat# C-4654 |
| Thymidine | Sigma-Aldrich Cat# T-1895 |
| Non-essential Amino Acids | Invitrogen Cat# 11140-050 |
| L-Glutamine | Invitrogen Cat# 25030 |
| Beta-Mercaptoethanol | Sigma-Aldrich Cat# M6250 |
| LIF ($10^7$ U/ml) | Millipore Cat# ESG1107 |
| PD0325901 (4 μM) | Stemgent Cat# 04-0006 |
| CHIR99021 (GSK3β inhibitor) (3 mM) | Stemgent Cat# 04-0004 |
| Gelatine ` | Sigma-Aldrich Cat# G1890 |
| Accutase-Enzyme Cell Detachment Medium | EBioscience Cat# 00-4555 |
| DMSO, Hybri-Max | Sigma-Aldrich Cat# D2650 |
| PBS (1X) | Cellgro Cat# 21-040-CM |
| Doxycycline | Clontech Cat#631311 |
| PD0325901 | Stemgent Cat#04-0006 |
| CHIR99021 | Stemgent Cat#04-0004 |
| Percoll | GE Healthcare Cat#17-0891-01 |
| 1M HEPES | Cellgro Cat#25-060-CI |

**Materials**

10cm and 15cm culture dishes
Hemocytometer
Micropipet w/ tips (P20, P200, P1000)
Microscope
Cryovials
Graduated pipets (1, 5, 10, 25, 50 ml)
Cryofreezing container

**Growth Medium**

| | |
|---|---|
| DMEM | 80% |
| FBS | 15% |
| Pen/Strep | 2% |
| Nucleoside Mix | 2% |

| | |
|---|---|
| L-Glutamine | 1% |
| Non-essential Amino Acids | 1% |
| Beta-Mercaptoethanol | ~$10^{-4}$ M |
| LIF $10^7$ U/ml (10,000X) | $10^3$ U/ml (1x) |

***Filter sterilize***

Note:

Medium containing LIF should be used within 1 week. Therefore medium should initially be prepared without LIF and appropriate amounts of medium containing LIF should be prepared.

Additional factors were added directly to the gelatin-coated dish after plating the cells:
For regular maintenance of cell line the glycogen synthase kinase 3β inhibitor (CHIR99021) and MAPK/ERK kinase inhibitor (PD0325901) were added to a final concentration of 3μM and 0.2μM, respectively.

**Nucleoside Mix**

| | |
|---|---|
| Adenosine | 80 mg |
| Guanosine | 85 mg |
| Uridine | 73 mg |
| Cytidine | 73 mg |
| Thymidine | 24 mg |

1) Add to 100 ml distilled water and dissolve by warming to ~ 45°C.
2) Filter sterilize, aliquot, and store at -20°C.

**Freezing Medium**

| | |
|---|---|
| Growth Medium (w/o LIF) | 3 ml |
| FBS | 1.5 ml |
| DMSO | 0.5 ml |

**100% Percoll Solution**

| | |
|---|---|
| Percoll | 36ml |
| 10xPBS | 2.96ml |
| 1M HEPES | 0.4ml |

## Procedure

**A. Initiation of culture from cryopreserved cells**

mESC must be cultured on surfaces pre-coated with 0.1% gelatin.

1) Rapidly thaw cells by holding vial and gently rotating in a 37°C water bath.
2) As soon as ice crystals disappear, swab outside surface of the ampoule with 70% ethanol, then dispense contents of the vial into a tube with 7ml basic growth medium.
3) Spin cells down at 500 x g for 5 min (4°C).
4) Aspirate medium and resuspend cells in growth medium.
5) Add CHIR99021 and PD0325901 to medium to a final concentration of 3 μM and 0.4 μM, respectively.
5) Dispense cells onto a gelatin-coated 10 cm dish.
6) Change medium the next day.

**B. Sub-culture and Maintenance**

1) Propagate cells until density reaches 60-80% confluence.
2) Aspirate medium.
3) Wash cells with warm 1X PBS.

4) Add 4 ml of Accutase and return to incubator for 5-10 minutes, or until cells detach.
5) Pipet cell suspension gently, but well, to break up clumps and transfer to 15 ml tube, rinse plate with 1X PBS to collect residual cells, and pellet at 500 x g for 5 minutes ($4^oC$).
6) Gently re-suspend cell pellet in warm medium.
7) Split cells 1:3 on gelatin-coated dish.
8) Cells are grown in $37^oC$/5% $CO_2$ incubator with medium changes every 2 days. Cells should be passaged when ~60-80% confluent (2-3 days).

**C. Generation of Seed Stocks from a 10 cm dish**

1) Following second or third passage after initiation of culture, remove cells from plate according to protocol described above under 'Sub-culture and Maintenance'.
2) Resuspend cell pellet in 3 ml freezing medium.
3) Dispense into 3 cryovials and freeze in an $-80^oC$ isopropanol cryo-freezing container overnight.
4) Cryovials are transferred the next day to liquid $N_2$ freezer for long-term storage.

**D. Harvest**

1) Plate cells on multiple 10cm or one 15cm dish in mESC medium
2) Grow until confluent
3) Remove cells from plate according to protocol described above under 'Sub-culture and Maintenance'.
4) Examine viability using Trypan blue staining (SOP TP-7).

**E. Percoll Gradient**

1) Prepare 100% Percoll Solution
2) Make 60%, 50%, 40%, and 20% Percoll dilutions using 1xPBS
3) Built Percoll Gradient in 50ml Falcon Tube using 5ml of 60%, 50%, and 40% Percoll
4) Resuspend ESC pellet in 3ml 20% Percoll and add on top of gradient
5) Centrifuge for 30min at 2000 rcf at 0 break/0 acceleration on $4^0C$
6) Collect cells from the 20%/40% interphase
7) Wash cells with 1x PBS