SOP: **Propagation of ZHBTc4 mouse embryonic stem cells (ZHBTc4-mESC, Labs)**
Date modified: **03/01/2011**
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Ordering Information

ZHBTc4 undifferentiated mouse embryonic stem cells were received as frozen ampoules from D. Levasseur (University of Iowa).

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

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.

If Oct4 repression was desired Doxycycline was added to a final concentration of 100ng/ml.

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

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°C).

6) Gently re-suspend cell pellet in warm medium.
7) Split cells 1:10 on gelatin-coated dish.
8) Cells are grown in 37^{o}C/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 a -80^{o}C isopropanol cryo-freezing container overnight.
4) Cryovials are transferred the next day to liquid N_2 freezer for long-term storage.

D. Oct4 repression

1) Plate 2 to 4x10^{6} cells on a 15cm plate in mESC medium.
2) Add Doxycycline to a final concentration of 100ng/ml.
3) Culture cells for 6, 24, and 72 hours before harvest.

E. Harvest

1) Plate 2x10^{6} cells on a 15cm dish in mESC medium containing either PD0325901/CHIR9902 or Doxycycline (if Oct4 repression is desired).
2) Keep in culture for 6, 24, and 72 hours.
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).