

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 μ M)	Stemgent Cat# 04-0006
CHIR99021 (GSK3 β 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	$\sim 10^{-4}$ 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 3 β inhibitor (CHIR99021) and MAPK/ERK kinase inhibitor (PD0325901) were added to a final concentration of 3 μ M and 0.2 μ M, 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 $\sim 45^\circ\text{C}$.
- 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°C/5% CO₂ 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°C isopropanol cryo-freezing container overnight.
- 4) Cryovials are transferred the next day to liquid N₂ freezer for long-term storage.

D. Oct4 repression

- 1) Plate 2 to 4x10⁶ 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 2x10⁶ 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).