Propagation of H7 hESC

From: UW (John Stamatoyannopoulos) ENCODE group

Date: 12/17/2009

Prepared By: S. Paige/S. Hansen (UW)

Growth and Harvest Modifications Addendum to:

"Propagation of H7 hESC" from UW ENCODE group

From: Duke/UNC ENCODE Group

Date Modified: 12/20/2011

Modified by: Zhuzhu Zhang (UNC)

SOP: **Propagation of H7 hESC**
Date modified: **12/17/09**
Modified by: **S. Paige/S. Hansen (UW)**

Ordering Information

H7 undifferentiated human embryonic stem cells (H7-hESC) can be ordered as frozen ampoules from the WiCell Research Institute through the National Stem Cell Bank:

Name: WA07 (H7) – human embryonic stem cells

Materials List

Reagent	Vendor	Catalog Number
DMEM/F12 + glutamine	Gibco/Invitrogen	11320
DMEM + glutamine	Gibco/Invitrogen	11995
Knockout Serum Replacement	Gibco/Invitrogen	10828
Fetal Bovine Serum	Hyclone	SV30014
Sodium Pyruvate	Gibco/Invitrogen	11360
Non-essential amino acids	Gibco/Invitrogen	11140
Beta-Mercaptoethanol	Gibco/Invitrogen	21985-023
Penicillin/Streptomycin	Gibco/Invitrogen	15070
Matrigel (GFR)	BD	356230
DNase	Calbiochem	260913
Collagenase IV	Gibco/Invitrogen	17104-019
Trypsin (0.05%)-EDTA	Gibco/Invitrogen	25300
Basic fibroblast growth factor	Peprotech	AF-100-18B

Procedure

A. Subculturing and maintenance of undifferentiated H7-hESC cells on Matrigel (10cm plate format)

1) Aspirate media and add 5mL of Collagenase IV (200 U/mL)
2) Incubate for 5-7 min in 37°C incubator (watch for cells to start lifting off plate)
3) Aspirate collagenase
4) Add 5 mL of 0.05% Trypsin-EDTA
5) Incubate for 10 seconds at RT
   *Monitor cells on scope: want cells to round up but remain adherent
6) Aspirate Trypsin
7) Add 1mL hES Stop Solution (1:1 of FBS:DMEM/F12) plus 1:800 Matrigel and 200 U/mL DNase
8) Scrape cells gently and evenly with rubber scraper
9) Resuspend with 5 mL pipette
   **Hold pipette tip on bottom of dish to disperse clumps
10) Transfer cells to 14 mL snap cap tube(s)
11) Rinse with 5 mL hES Wash Medium (DMEM/F12 + P/S)
12) Pellet cells at 800 rpm x 5 min
13) Carefully aspirate supernatant down to pellet
14) Resuspend cells in MEF-CM supplemented with 8 ng/mL bFGF
15) Split cells 1:6 on Matrigel-coated plates
16) Cells are grown in 37°C/5% CO2 incubator with daily media changes. Cells should be passaged when ~70% confluent (4-6 days, depending on cell batch).

B. Production of mouse embryonic fibroblast conditioned medium (MEF-CM)

1) Plate 13 x 106 irradiated P4 MEFs on a T225 flask in MEF medium (DMEM with 10% FBS, 1% penicillin/streptomycin)
2) After cells have plated (~4 hours), add 60 mL pre-conditioned medium*
3) After 24 hours, collect conditioned medium and replace with fresh pre-conditioned medium
4) Collect conditioned medium for 7 consecutive days
5) Pool all collected conditioned medium and sterile-filter

*Pre-conditioned medium consists of DMEM/F12 + glutamine, 20% Knock-out serum replacement, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin/streptomycin and 0.1mM beta-mercapthoethanol. Just prior to conditioning, bFGF is added at a concentration of 4 ng/mL.

C. Harvest

1) Passage cells until the desired cell number is achieved
2) Remove cells from flasks according to protocol described above under 'subculturing'
3) Examine viability using Trypan blue staining (SOP TP-7)

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Date modified:	**12/20/2011**
Modified by:	**Zhuzhu Zhang (UNC)**

Material List Modification for H7 hESC

1. DMEM/F-12, HEPES (Invitrogen, Cat.# 11330-032)
2. mTeSR™1 (STEMCELL Technologies, Cat.# 05850)
3. BD Matrigel™ hESC-qualified Matrix (BD Biosciences, Cat.# 354277)
4. Phosphate-Buffered Saline (PBS) (Invitrogen, Cat.# 10010-023)
5. StemPro® Accutase® Cell Dissociation Reagent (Invitrogen, Cat.# A11105-01)
6. Recovery™ Cell Culture Freezing Medium (Invitrogen, Cat.# 12648-010)

Procedure Modifications

A. Thawing and starting cell culture

1. Day 1: Rapidly thaw the cells in 37°C water bath. When only an ice crystal remains, remove the vial from the water bath. Swirl the vial gently.
2. Add 1ml of pre-warmed mTeSR™1 Medium into thawed cells dropwise. Swirl gently to mix.
3. Add the cell suspension to 9ml pre-warmed mTeSR™1 Medium dropwise in a 15ml conical tube. While adding the medium, gently move the tube back and forth to mix the cells. This reduces osmotic shock to the ES cells.
4. Centrifuge at 700rpm for 5min.
5. Aspirate the media. Gently resuspend the cell pellet in 12ml pre-warmed mTeSR™1 Medium.
6. Slowly add the cell suspension dropwise to a 6-well plate coated with Matrigel™, 2ml per well.
7. Grow the cells in a 37°C, 5% CO2 humidified incubator.

Day 2-7: Examine the cells under a microscope and replace the medium daily (2ml pre-warmed mTeSR™1 Medium per well). Colonies may not be visible for up to a week. Propagate cells until the colonies are approximately 3-4 mm in diameter.

Note: Matrigel preparation
Matrigel is thawed on ice. The vial is swirled to ensure that Matrigel is evenly dispersed. Stocks of 1 mg are aliquoted and stored at -20. When ready to use, 1 mg of Matrigel is resuspended in 12 ml of DMEM/F12 (24 ml should be also fine) and added to plates (1 ml for 35 mm dishes/5 ml for 10 cm dishes). Plates should be coated for at least 1 hour at room temperature. Plates can be stored at 4°C for 2-3 days.

B. Sub-culturing and microdissection passaging

1. Cells need to be fed and examined under a microscope daily to assess colony size and differentiation. Spontaneous differentiation is often observed as visible structure or organization, or it exhibits a different shade, within a colony. The differentiated cells may reside at the colony perimeter or in the center of the colony. There can also be differentiated colonies containing large clumps of swirled tissue, or little balls of cells, like embryoid bodies. Remove the visible differentiated cells and their surrounding cells, or remove the entire colony

if a big proportion of the colony is undergoing differentiation, with a fine pointed tool (e.g. fire drawn Pasteur pipette needles) or a cell lifter.

2. Passage the cells every 4-5 days, when the colonies are ~3-4 mm in diameter. In general, passage cells when colonies are too dense or too large, or increased rate of differentiation occurs. We typically split one plate to 2-3 plates at passaging.

3. Microdissection passaging (under the built-in dissecting microscope in a biosafety cabinet):
    a. Examine the culture to remove all differentiated colonies or colony regions. Aspirate and discard those colonies with a P200 pipette.
    b. Use a fire drawn Pasteur pipette needle to grid the undifferentiated colonies: score 4 to 5 times across a colony, and perpendicular to that, another 4-5 times.
    c. Use the needle of a cell lifter to lift the subcolonies from the plate.
    d. Use a P200 or a mouth pipette to transfer the subcolonies to fresh Matrigel™ coated plates with pre-warmed mTeSR™1 medium. Plate 30-35 subcolonies on a 35mm plate containing 2ml media, or 250-300 subcolonies on a 100mm plate containing 10ml media.
    e. Gently swirl the plates to spread the subcolonies evenly over the dish.

Check the cells the next day without feeding or disturbing the cells to allow the subcolonies fully seeded. Feed, examine, and remove any differentiated cells daily there after.

C. Harvesting cells for DNase

1. Garden and grid the colonies as described above in microdissection passaging step a-b.
2. Wash cells gently with pre-warmed 1X PBS.
3. Add 2ml of warm Accutase to a 100mm plate, incubate for ~2 minutes while checking the cells under the microscope, and stop the reaction by adding 8ml of fresh medium when single cells start to round up.
4. Gently rinse to remove cells off of the plate's surface. Gently pipette up and down until cells are in a single cell suspension.
5. Transfer cell suspension to a 15ml conical tube.
6. Take 10 µL sample of the cell suspension to determine viable cell density.
7. Centrifuge at 700rpm for 5min.
8. Resuspend cell pellet in 1ml Recovery™ Cell Culture Freezing Media and transfter to a cryogenic vial.
9. Place the vial in an insulated container for slow cooling at -80°C overnight.