Human Pluripotent Stem Cell Starter Kit

Catalog Number SC029

Reagents for the expansion and characterization of human pluripotent stem cells.

This package insert must be read in its entirety before using this product.
For laboratory research use only. Not for diagnostic use.
The safety and efficacy of this product in diagnostic or
other clinical uses has not been established.

TABLE OF CONTENTS

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INTRODUCTION

Embryonic stem (ES) cells, derived from the inner cell mass of pre-implantation embryos, have been recognized as the most pluripotent stem cell population (1,2). More recently, it has been discovered that somatic cells are able to be reprogrammed to an ES cell-like fate. These induced pluripotent stem (iPS) cells are able to be cultured under similar conditions as ES cells and also have the ability to give rise to all three germ layers: ectoderm, mesoderm, and endoderm (3-5). Pluripotent stem cells hold great possibilities both for the understanding of developmental processes as well as for therapeutic purposes.

Traditional culture methods for pluripotent stem cells involve maintenance on irradiated mouse embryonic fibroblasts (MEFs) in media containing FGF basic (6). To eliminate the use of feeder cells, media can be conditioned on MEFs for use in stem cell culture. This method requires the use of basement membrane extracts as a culture substrate (7).

Pluripotent stem cells can be characterized by high expression of certain transcription factors, including Oct-3/4 and Nanog. These factors are required to sustain pluripotency and are downregulated during stem cell differentiation (8,9). Oct-4A is an isoform of Oct-3/4 that has been found to be specific to pluripotent stem cells (10). These cells can also be characterized through the expression of surface antigens such as SSEA-4 (6).

This kit provides the reagents required for the expansion of human pluripotent stem cells under feeder-free conditions. Additionally, antibodies to Oct-4A, Nanog, and SSEA-4 are included for characterization of the cultured cells by immunocytochemistry. Furthermore, SSEA-4 surface expression can also be assessed through flow cytometry.

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- This kit should not be used beyond the expiration date.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The quality of the pluripotent stem cells and any variation in this procedure can cause variation in expansion and maintenance of pluripotency.

PRECAUTION

The acute and chronic effects of overexposure to reagents in this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

MATERIALS PROVIDED

Store unopened kit at ≤ -70 °C in a manual defrost freezer.
This kit is stable for up to 3 months from the date of receipt.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Embryonic Fibroblast (MEF) Conditioned Media	390435	2 vials (100 mL/vial) of a 1X media.	Store under sterile conditions at 2-8 °C for up to 1 week, or aliquot and store at ≤ -20 °C in a manual defrost freezer until the expiration date of the kit. Avoid repeated freeze-thaw cycles.
Human FGF basic (5000X)	894643	1 µg of lyophilized recombinant human FGF basic; enough to make 50 µL of a 5000X stock.	Store under sterile conditions at 2-8 °C for up to 1 month, or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 3 months.* Avoid repeated freeze-thaw cycles.
Stem Cell Qualified RGF BME, PathClear®	894647	1 mL of frozen reduced growth factor basement membrane extract; enough to make 40 mL of a 1X coating solution.	Aliquot and store at ≤ -70 °C for up to 3 months.* Avoid repeated freeze-thaw cycles.
Mouse Anti-human Oct-4A (Mouse IgG_{2A}; Clone 653108)	894644	25 µg of lyophilized mouse anti-human Oct-4A monoclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.	Store under sterile conditions at 2-8 °C for up to 1 month, or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 3 months.* Avoid repeated freeze-thaw cycles.
Mouse Anti-human/mouse SSEA-4 (Mouse IgG_3; Clone MC-813-70)	894646	25 µg of lyophilized mouse anti-human/ mouse SSEA-4 monoclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.	
Goat Anti-human Nanog	894645	25 µg of lyophilized goat anti-human Nanog polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.	

*Provided this is within 3 months from the date of receipt.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 6-well culture plates
- 24-well culture plates
- 15 mL centrifuge tubes
- 12 mm cover slips (Carolina®, Catalog # 633009 or equivalent)
- 0.2 µm syringe filter
- 10 mL syringes
- Serological pipettes
- Pipettes and pipette tips
- Fine-pointed curved forceps
- Glass slides
- FACS™ tubes

Equipment

- 37 °C and 5% CO_2 incubator
- 37 °C water bath
- Clinical centrifuge
- Hemocytometer
- Inverted microscope
- Fluorescence microscope

Reagents

- Phosphate-Buffered Saline (PBS)
- DMEM/F-12
- Y-27632 dihydrochloride, p160ROCK inhibitor (R&D Systems, Catalog # 1254)
- Trypan Blue Stain
- Accutase™ (Innovative Cell Technologies, Catalog # AT-104 or equivalent)
- BSA (very low endotoxin)
- 4% Paraformaldehyde in PBS
- 1% BSA in PBS
- 0.3% Triton™ X-100, 1% BSA, 10% normal donkey serum in PBS (Blocking Buffer)
- Flow Cytometry Staining Buffer (R&D Systems, Catalog # FC001)
- Mounting Medium (R&D Systems, Catalog # CTS011)
- Secondary antibodies for Immunocytochemistry (R&D Systems, Catalog # NL001 and NL007)
- Secondary antibody for Flow Cytometry (R&D Systems, Catalog # F0102B)
- Isotype control for Flow Cytometry (R&D Systems, Catalog # MAB007)
- Deionized or distilled water
- 95% ethanol (EtOH)

REAGENT & MATERIAL PREPARATION

0.1% BSA in PBS - Dissolve 10 mg of BSA in 10 mL of PBS. Sterile filter the solution by syringe filter, and store at 2-8 °C for up to 3 months.

Human FGF basic (5000X) - Reconstitute with 50 µL of sterile 0.1% BSA in PBS. Mix gently.

Mouse Anti-human Oct-4A - Reconstitute with 250 µL of sterile PBS; this provides a 100 µg/mL stock solution. Mix gently.

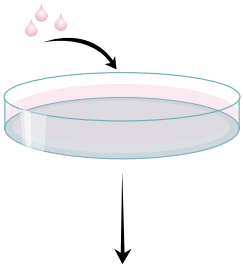
Mouse Anti-human/mouse SSEA-4 - Reconstitute with 250 µL of sterile PBS; this provides a 100 µg/mL stock solution. Mix gently.

Goat Anti-human Nanog - Reconstitute with 250 µL of sterile PBS; this provides a 100 µg/mL stock solution. Mix gently.

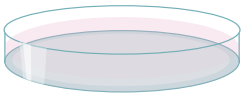
PROCEDURE OUTLINE

I. Coating Plates with Stem Cell Qualified RGF BME, PathClear®

Add BME in DMEM/F-12 (1:40).
Incubate 1–2 hours at room temperature.

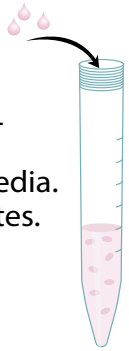


Remove media just prior to plating cells.

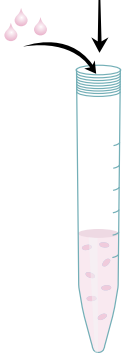


II. Preparation & Plating of Human Pluripotent Stem Cells

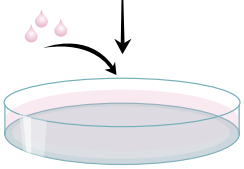
Transfer thawed human pluripotent stem cells to a 15 mL centrifuge tube containing prewarmed MEF Conditioned Media.
Centrifuge at 200 x g for 4 minutes.



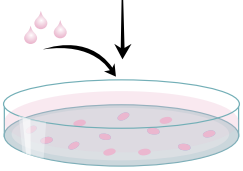
Resuspend the cell pellet in MEF Conditioned Media supplemented with FGF basic.



Add the human pluripotent stem cells to the BME-coated plate.

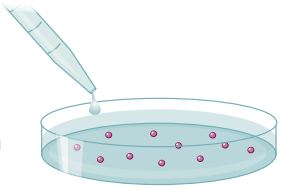


Culture cells at 37 °C and 5% CO_2 and change media daily.

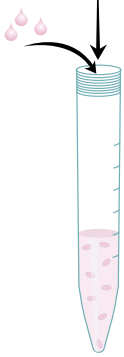


III/IV. Cell Dissociation & Cell Plating

Coat plates with BME.
Remove media from cells and add Accutase solution to each plate.
Pipette gently over plate until all of the cells have detached.



Transfer the cell suspension to a 15 mL centrifuge tube containing ≥ 5 mL of MEF Conditioned Media.
Centrifuge at 200 x g for 4 minutes.



Resuspend the pellet in MEF Conditioned Media.
Perform a cell count.

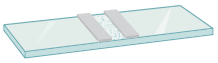
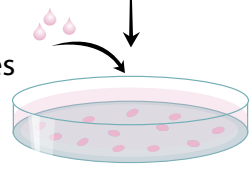
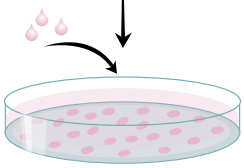


Plate the desired number of cells on BME-coated plates in MEF Conditioned Media containing FGF basic.



Culture the cells at 37 °C and 5% CO_2 and change media daily.



PROCEDURE

Notes: *This protocol is designed for use with BG01V human ES (hES) cells. If using different cell lines, the protocol may need to be modified.*

I. Coating Plates with Stem Cell Qualified RGF BME, PathClear

1. Thaw BME on ice at 2-8 °C overnight.
2. Aliquot thawed BME into pre-cooled tubes, and store at ≤ -70 °C. Keep the BME container and tubes for aliquoting on ice during this process to ensure that the BME does not gel. Avoid repeated freeze-thaw cycles as this will affect product integrity.
3. Thaw the aliquoted BME on ice at 2-8 °C overnight.
4. Dilute thawed BME 1:40 in cold DMEM/F-12 on ice. This can be stored at 2-8 °C for up to 2 weeks.
5. Coat the desired number of plates with diluted BME (approximately 1 mL per well of a 6-well plate or 0.5 mL per well of a 24-well plate). Incubate for 1-2 hours at room temperature.
6. Remove the BME solution immediately prior to plating the cells.

II. Preparation & Plating of Human Pluripotent Stem Cells

1. Warm the aliquoted MEF Conditioned Media to 37 °C.
2. Warm the vial of hES/iPS cells in a 37 °C water bath until just thawed, and immediately transfer to a 15 mL centrifuge tube containing at least 5 mL of pre-warmed MEF Conditioned Media. Rinse the cryovial with an additional 1 mL of media to ensure the removal of all the cells.
3. Spin in a clinical centrifuge at 200 x g for 4 minutes.
4. Remove the supernatant, and gently resuspend in an appropriate amount of MEF Conditioned Media supplemented with 1X human FGF basic. If using a single cell suspension, the addition of the ROCK inhibitor Y-27632 at a final concentration of 10 µM can enhance viability upon plating.
5. Add the hES/iPS cell suspension to the BME-coated plate. Gently shake the plate back and forth and side to side to ensure the even distribution of cells.
6. Grow in a 37 °C, 5% CO_2 incubator. Change the media daily with fresh, pre-warmed MEF Conditioned Media containing 1X human FGF basic, and monitor the cells. Passage the cells at approximately 70-80% confluency or when there is a noticeable increase in differentiation.

   Note: ***Do not add Y-27632 to growth media after the first 24 hours of culture as this may compromise the pluripotency of the cells.***

III. Cell Dissociation

1. Warm the aliquoted MEF Conditioned Media to 37 °C.
2. Remove the MEF Conditioned Media from the cells. Add 0.5-1.0 mL of Accutase to each well of a 6-well plate. Incubate at room temperature for 2-5 minutes or until cells begin to slough off the plate. If using cells from several plates, work in small batches (1-2 wells at a time) so the cells are not exposed to Accutase for an extended period of time.
3. Gently pipette Accutase repeatedly over the plate until the cells have become detached.
4. Gently pipette the cell suspension up and down to break up large cell clumps.
   Note: *Most cell lines have poor viability when dissociated to a single cell suspension. For greater viability, cells should be dissociated into small clumps for replating. If single cell suspensions are desired, ROCK inhibitor Y-27632 can be added to enhance viability as described in section II.4.*
5. Transfer the cell suspension to a 15 mL centrifuge tube containing ≤ 5 mL of MEF Conditioned Media, and centrifuge for 4 minutes at 200 x g.

IV. Cell Plating

1. Resuspend the cell pellet in MEF Conditioned Media containing 1X human FGF basic. If dissociating to a single cell suspension, count the viable cells using Trypan Blue and a hemocytometer. Cells in clumps cannot be accurately counted.
2. Plate the cells onto prepared BME-coated plates. The ratio of cells plated will depend on the confluency at dissociation and general speed of growth of the cell line of interest. It is recommended to use a ratio of 1:2 to 1:4. If dissociating cells to a single cell suspension, plate cells at a concentration of approximately 0.5×10^5 cells/cm^2. For example, plate ~0.5×10^6 cells/well of a 6-well plate. Use a final volume of 2.0-2.5 mL per well in a 6-well plate or 0.5-1.0 mL/well of a 24-well plate of MEF Conditioned Media containing 1X human FGF basic. Many cell lines have poor viability when put into a single cell suspension. Cell viability can be enhanced by the addition of the ROCK inhibitor as described in section II.4.
3. Incubate at 37 °C and 5% CO_2.
4. Feed and monitor the cells as described in section II.6. After 1-2 days in culture, cells should start to form colonies.

   Note: Do not add Y-27632 to growth media after the first 24 hours of culture as this may compromise the pluripotency of the cells.

V. Characterization of Cells by Immunocytochemistry

1. Insert a sterile coverslip (sterilized with 95% EtOH and flamed) into each well of a 24-well plate.
2. Coat wells with BME, and plate pluripotent stem cells as described in sections I and IV, respectively.
3. Culture cells to the desired confluency.
4. Remove the media from each well, and wash the cells twice with 1 mL of PBS.
5. Fix the cells with 0.5 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
6. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes each.
7. Permeabilize and block cells with 0.5 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS (Blocking Buffer) for 45 minutes at room temperature.
8. During step 7, when cells are being blocked, dilute the reconstituted goat anti-human Nanog, mouse anti-human Oct-4A, or mouse anti-human/mouse SSEA-4 antibody in block solution to a final concentration of 10 µg/mL.
9. After blocking, incubate the cells in 300 µL/well of 10 µg/mL goat anti-human Nanog, mouse anti-human Oct-4A, or mouse anti-human/mouse SSEA-4 for 3 hours at room temperature or overnight at 2-8 °C.
   Note: *A negative control should be performed using blocking buffer alone with no primary antibody.*
10. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes each.
11. Dilute the appropriate secondary antibody (e.g. NL557-conjugated donkey anti-goat IgG or donkey anti-mouse IgG secondary antibody) according to the manufacturer's instructions in PBS containing 1% BSA.
12. Incubate the cells with 300 µL/well of secondary antibody **in the dark** for 60 minutes at room temperature.
13. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes each.
14. Wash the cells once with 0.5 mL of PBS for 5 minutes. If desired, nuclei can be counterstained with DAPI.
15. Aspirate the PBS from the wells, and add 0.5 mL of deionized or distilled water. Carefully remove the coverslips with forceps and mount cell side down onto a drop of mounting media on a glass slide.
16. The slides are ready for microscopic observation. For data examples, see Figure 1.

VI. Characterization of Surface Marker Expression by Flow Cytometry

1. Harvest the cells as described in section III.
2. Resuspend the cells in Flow Cytometry Staining Buffer at a concentration of 1×10^6 cells/mL.
3. Transfer 90 µL of the cell suspension into a 5 mL FACS tube. Add 10 µL of mouse anti-human/mouse SSEA-4.
4. Incubate for 30 minutes at room temperature.
5. Following incubation, wash the cells twice in 2 mL of Flow Cytometry Staining Buffer. Spin the cells at 200 x g for 5 minutes after each wash.
6. Resuspend the cells in 200 µL of Flow Cytometry Staining Buffer, and add a secondary developing reagent such as goat anti-mouse IgG conjugated to a fluorochrome according to the manufacturer's instructions.
7. Incubate **in the dark** for 30 minutes at room temperature.
8. Following incubation, wash the cells twice; as described in step 5.
9. Resuspend the cells in 400 µL of Flow Cytometry Staining Buffer for flow cytometric analysis. For data examples, see Figure 1.
   Note: *As a control for analysis, cells in a separate tube should be treated with a flow cytometry isotype control.*

DATA EXAMPLES

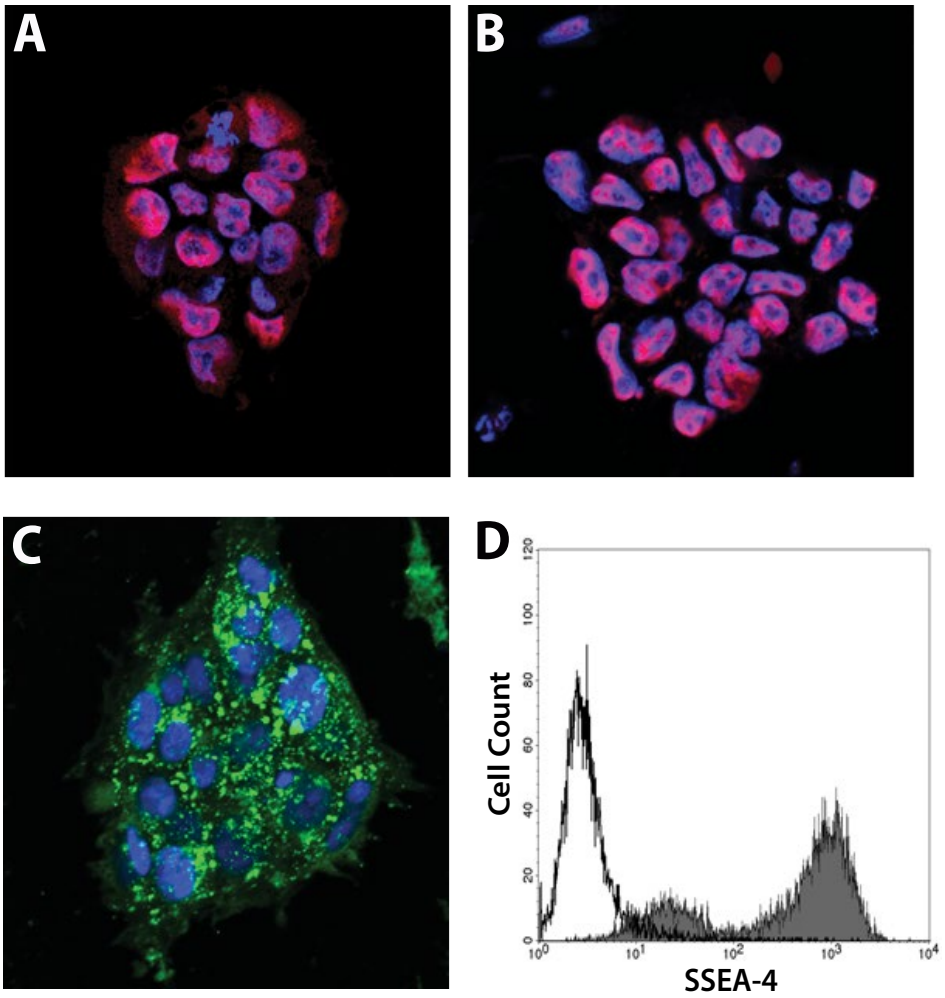


Figure 1: BG01V human embryonic stem cells were grown for a minimum of three passages prior to fixation and characterization by immunocytochemistry (A-C) or flow cytometry (D). Cells were characterized using Anti-human Nanog (A), Anti-human Oct-4A (B), or Anti-human/mouse SSEA-4 (C-D). For immunocytochemistry, cells were then stained with NorthernLights™ 557-conjugated donkey anti-goat (A; red; R&D Systems, Catalog # NL001), NorthernLights 557-conjugated donkey anti-mouse (B; red; R&D Systems, Catalog # NL007), or NorthernLights 493-conjugated donkey anti-mouse (C; green; R&D Systems, Catalog # NL009) and counterstained with DAPI (blue). For flow cytometry, cells were stained with PE-conjugated goat anti-mouse IgG (D; filled histogram; R&D Systems, Catalog # F0102B). As a control, cells for flow cytometry were also stained using a mouse IgG_3 isotype control (D; open histogram; R&D Systems, Catalog # MAB007).

RELATED REAGENTS

REAGENT	CATALOG NUMBER
Anti-Oct-4A Antibody	IC6344A, IC6344P, MAB17591, NL17591G, NL17591R, NL17591V
Anti-SSEA-4 Antibody	BAM1435, FAB1435A, FAB1435C, FAB1435F, FAB1435P, NL1435R, NL1435V, NLLC1435G, NLLC1435R, MAB1435
Anti-Nanog Antibody	AF1997, BAF1997, IC1997P, NL1997G, NL1997R
Mouse Embryonic Fibroblast (MEF) Conditioned Media	AR005
Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract, PathClear®	3434-005-02
Recombinant Human FGF basic (Tissue Culture Grade)	4114-TC
Recombinant Human FGF basic	233-FB
Y-27632 dihydrochloride	1254
Human Pluripotent Stem Cell 3-Color Immunocytochemistry Kit	SC021
Human Pluripotent Stem Cell Live Cell Imaging Kit	SC023
Human Pluripotent Stem Cell Functional Identification Kit	SC027
Human Three Germ Layer 3-Color Immunocytochemistry Kit	SC022
Human Embryonic Stem Cell Marker Antibody Panel	SC008
Human Embryonic Stem Cell Marker Antibody Panel Plus	SC009
Human Pluripotent Stem Cell Assessment Primer Pair Panel	SC012

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