

Human Pluripotent Stem Cell Functional Identification Kit

Catalog Number SC027

Reagents for the identification of human pluripotent stem cells by *in vitro* functional differentiation.

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.

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PRINCIPLE OF THE ASSAY

Pluripotent stem cells encompass both embryonic and induced pluripotent stem cells, which are non-pluripotent cells that have been reprogrammed to a pluripotent state. These cells provide much promise for the generation of sufficient quantities of specialized cells for use in regenerative medicine. Additionally, these cells are an important tool for understanding developmental and disease mechanisms.

The defining characteristics of pluripotent stem cells are their ability to self renew and to differentiate into each of the three germ layers. In order to determine if a cell is truly a pluripotent stem cell, it is important to assess these characteristics. The Human Pluripotent Stem Cell Functional Identification Kit contains specially formulated media supplements and growth factors that can be used to differentiate human pluripotent stem cells into endoderm, ectoderm, and mesoderm. An antibody to characterize each of the three cell types is also included: SOX17 (endoderm), Otx2 (ectoderm), and Brachyury (mesoderm). The quantity of each component in this kit is sufficient to make 200 mL of media for differentiation. This is enough media for the differentiation of one 24-well plate of each cell type.

LIMITATIONS OF THE PROCEDURE

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

PRECAUTION

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

MATERIALS PROVIDED

Store unopened kit at $\leq -20\text{ }^{\circ}\text{C}$ in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED REAGENTS
Differentiation Base Media Supplement (10X)	895090	20 mL of a 10X concentrated solution.	Store at 2-8 °C for up to 2 weeks or aliquot and store at $\leq -20\text{ }^{\circ}\text{C}$ in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Endoderm Differentiation Supplement I	390513	1 vial of lyophilized growth factors for Day 1 of endoderm differentiation; enough to make 50 μL of a 500X stock.	Store under sterile conditions at 2-8 °C for up to 1 month or aliquot and stored at $\leq -20\text{ }^{\circ}\text{C}$ in a manual defrost freezer for up to 3 months.* Avoid repeated freeze-thaw cycles.
Endoderm Differentiation Supplement II	390514	1 vial of lyophilized growth factors for Days 2 and 3 of endoderm differentiation; enough to make 100 μL of a 500X stock.	
Mesoderm Differentiation Supplement	390515	1 vial of lyophilized growth factors for mesoderm differentiation; enough to make 100 μL of a 500X stock.	
Ectoderm Differentiation Supplement	390516	1 vial of lyophilized growth factors for ectoderm differentiation; enough to make 150 μL of a 500X stock.	
Goat anti-human SOX17	967330	25 μg of lyophilized goat anti-human SOX17 polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 $\mu\text{g}/100\text{ }\mu\text{L}$.	Stored at 2-8 °C for up to 1 month or aliquot and stored at $\leq -20\text{ }^{\circ}\text{C}$ in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Goat anti-human Otx2	967331	25 μg of lyophilized goat anti-human Otx2 polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 $\mu\text{g}/100\text{ }\mu\text{L}$.	
Goat anti-human Brachyury	967332	25 μg of lyophilized goat anti-human Brachyury polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 $\mu\text{g}/100\text{ }\mu\text{L}$.	

*Provided this is within 6 months from the date of receipt.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 24-well culture plates
- 12 mm cover slips
- 15 mL and 50 mL centrifuge tubes
- 0.2 µm syringe filter
- 10 mL syringe
- Pipettes and pipette tips
- Serological pipettes
- Glass slides
- Fine pointed curved forceps

Reagents

- RPMI
- BSA, very low endotoxin
- D-MEM/F-12 (1X)
- GlutaMAX™ (Invitrogen, Catalog # 35050-079 or equivalent)
- Penicillin-Streptomycin (optional)
- Phosphate Buffered Saline (PBS)
- Cultrex® PathClear® BME Reduced Growth Factor Basement Membrane Extract (R&D Systems, Catalog # 3433-005-02)
- Recombinant human FGF basic (Tissue culture grade; R&D Systems, Catalog # 4114-TC)
- MEF Conditioned Media (R&D Systems, Catalog # AR005)
- Trypan Blue Solution
- Accutase®
- 95% Ethanol
- 4% Paraformaldehyde in PBS
- 1% BSA in PBS
- 0.3% Triton™ X-100, 1% BSA, 10% normal donkey serum in PBS
- 1% BSA, 10% normal donkey serum in PBS
- Mounting medium (R&D Systems, Catalog # CTS011)
- Secondary developing reagents (R&D Systems, Catalog # NL001)
- Deionized or distilled water

Equipment

- 37 °C and 5% CO₂ incubator
- Centrifuge
- Hemocytometer
- Inverted microscope
- 37 °C water bath
- Fluorescence microscope

REAGENT AND 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.

Endoderm Differentiation Supplement I (500X) - Reconstitute with 50 µL of sterile 0.1% BSA in PBS. Mix gently.

Endoderm Differentiation Supplement II (500X) - Reconstitute with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Mesoderm Differentiation Supplement (500X) - Reconstitute with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Ectoderm Differentiation Supplement (500X) - Reconstitute with 150 µL of sterile 0.1% BSA in PBS. Mix gently.

Differentiation Base Media - Combine 20 mL of 10X Differentiation Base Media Supplement with 180 mL of RPMI, 2 mL of Penicillin/Streptomycin (optional), and 2 mL of GlutaMAX.

Endoderm Differentiation Media I - Dilute the Endoderm Differentiation Supplement I stock solution 500-fold in Differentiation Base Media. Prepare fresh as needed.

Endoderm Differentiation Media II - Dilute the Endoderm Differentiation Supplement II stock solution 500-fold in Differentiation Base Media. Prepare fresh as needed.

Ectoderm Differentiation Media - Dilute the Ectoderm Differentiation Supplement stock solution 500-fold in Differentiation Base Media. Prepare fresh as needed.

Mesoderm Differentiation Media - Dilute the Mesoderm Differentiation Supplement stock solution 500-fold in Differentiation Base Media. Prepare fresh as needed.

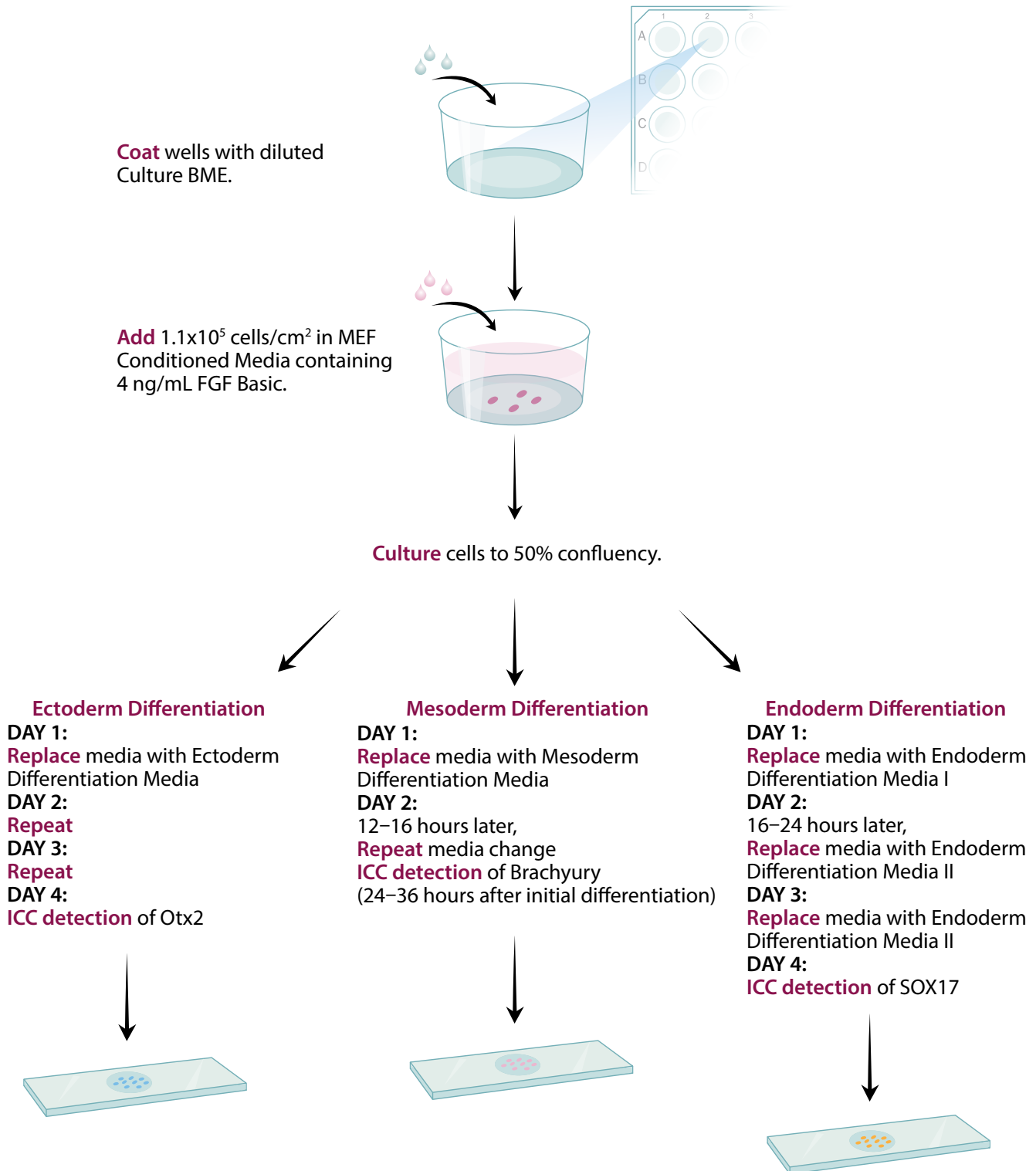
PREPARATION OF LYOPHILIZED ANTIBODIES

Goat anti-human SOX17 (10X) - Reconstitute with 250 µL of sterile PBS. Mix gently.

Goat anti-human Otx2 (10X) - Reconstitute with 250 µL of sterile PBS. Mix gently.

Goat anti-human Brachyury (10X) - Reconstitute with 250 µL of sterile PBS. Mix gently.

PROCEDURE OUTLINE



UNDIFFERENTIATED CELL PREPARATION PROCEDURES

Note: *This protocol is designed for BG01V human embryonic stem (hES) cells grown in MEF Conditioned Media. If using different cell lines or growth media, the protocol below may need to be modified.*

The quality of the human pluripotent cells used in the differentiation is imperative. Use of suboptimal quality or very high passage pluripotent cells can result in decreased differentiation efficiency and/or increased cell death.

Coating Plates with Cultrex BME

1. Thaw Cultrex PathClear BME on ice at 2-8 °C overnight.
2. Aliquot thawed Cultrex PathClear BME into pre-cooled tubes and store at ≤ -20 °C.
3. Thaw the aliquot on ice at 2-8 °C overnight.
4. Dilute Cultrex PathClear BME 1:40 in D-MEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
5. Place a sterile coverslip (sterilized with 95% ethanol and flamed) in each well of a 24-well plate.
6. Coat the desired number of wells with diluted Cultrex PathClear BME (0.5 mL/well of a 24-well plate) and incubate for 1-2 hours at room temperature.
7. Remove the Cultrex PathClear BME solution immediately prior to plating the cells.

Cell Dissociation

1. Warm the MEF Conditioned Media to 37 °C.
2. Remove the existing media from the cells. Add 1 mL of Accutase solution to each 60 mm plate or 3 mL to each 100 mm plate. Incubate at room temperature for 2-5 minutes or until the cells begin to slough off the plate. If using cells from several plates, work in small batches (1-2 plates at a time) so the cells are not exposed to the Accutase for an extended period of time.
3. Pipette gently over the plate until the cells become detached.
4. Gently pipette the cell suspension up and down to break up large cell clumps.
5. Remove the cell suspension to a 15 mL centrifuge tube containing 5 mL of MEF Conditioned Media (or 12 mL if using a 100 mm plate) and spin at 200 x g for 4 minutes.

Cell Plating

1. Resuspend the pellet in MEF Conditioned Media containing 4 ng/mL of FGF basic and count the viable cells using Trypan Blue and a hemocytometer.
2. Plate cells onto prepared Cultrex PathClear BME-coated plates at a concentration of 1.1×10^5 cells/cm². For example, plate 4.5×10^6 cells divided among all wells of a 24-well plate. If your cells routinely grow slowly, the initial plating density can be increased.
3. Grow overnight at 37 °C and 5% CO₂. The next day each plate should be approximately 50% confluent. If cells are not 50% confluent, replace the media with fresh media and culture until they reach 50% confluency.
4. Proceed to differentiation.

ECTODERM DIFFERENTIATION PROCEDURE

1. Warm the Differentiation Base Media to 37 °C.
2. Prepare the required amount of Ectoderm Differentiation Media. Use 1 mL of media/well of a 24-well plate.
3. Remove the MEF Conditioned Media from each plate/well.
4. Add the prepared Ectoderm Differentiation Media to each plate and incubate overnight at 37 °C and 5% CO₂.
5. Repeat steps 1-4 on Days 2 and 3.
6. On Day 4, the cells are ready for analysis by immunocytochemistry. Proceed to the Fixing and Staining Procedure.

MESODERM DIFFERENTIATION PROCEDURE

1. Warm the Differentiation Base Media to 37 °C.
2. Prepare the required amount of Mesoderm Differentiation Media. Use 1 mL of media/well of a 24-well plate.
3. Remove the MEF Conditioned Media from each plate/well.
4. Add the prepared Mesoderm Differentiation Media to each plate and incubate overnight at 37 °C and 5% CO₂.
5. Repeat steps 1-4 after 12-16 hours.
6. Approximately 24-36 hours after the initial differentiation, the cells are ready for analysis by immunocytochemistry. Proceed to the Fixing and Staining Procedure.

ENDODERM DIFFERENTIATION PROCEDURES

Endoderm Differentiation (Day 1)

1. Warm the Differentiation Base Media to 37 °C.
2. Prepare the required amount of Endoderm Differentiation Media I. Use 1 mL of media/well of a 24-well plate.
3. Remove the MEF Conditioned Media from each plate/well.
4. Add the prepared Endoderm Differentiation Media I to each plate and incubate overnight at 37 °C and 5% CO₂.

Endoderm Differentiation (Days 2 and 3)

1. Approximately 16-24 hours after adding the Endoderm Differentiation Media I, warm the Differentiation Base Media to 37 °C.
2. Prepare the required amount of Endoderm Differentiation Media II. Use 1 mL per well of a 24-well plate.
3. Remove the Endoderm Differentiation Media I and replace it with prepared Endoderm Differentiation Media II.
4. Repeat steps 1-3 on Day 3.
5. On Day 4, the cells are ready for analysis by immunocytochemistry. Proceed to the Fixing and Staining Procedure.

FIXING AND STAINING PROCEDURE

1. Wash the cells twice with PBS (1 mL/well of a 24-well plate).
2. Fix the cells with 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
4. Permeabilize and block the cells with 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes (0.5 mL/well of a 24-well plate).
5. During the blocking, dilute the appropriate reconstituted primary antibody in PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum to a final concentration of 10 µg/mL.

ANTIBODY	GERM LAYER
Goat anti-human Otx2	Ectoderm
Goat anti-human Brachyury	Mesoderm
Goat anti-human SOX17	Endoderm

Note: A negative control should be performed using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with no primary antibody.

6. After blocking, incubate the cells with diluted primary antibody (300 µL/well of a 24-well plate) for 3 hours at room temperature or overnight at 2-8 °C.
7. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
8. Dilute the secondary antibody [e.g. NL557-conjugated donkey anti-goat secondary antibody (R&D Systems, Catalog # NL001)] at 1:200 in PBS containing 1% BSA.
9. Incubate the cells with diluted secondary antibody in the dark for 60 minutes at room temperature (300 µL/well of a 24-well plate).
10. Wash the cells 3 times with 1% BSA in PBS for 5 minutes (0.5 mL/well of a 24-well plate).
11. Cover the cells with PBS (1 mL/well of a 24-well plate) and visualize with a fluorescence microscope.
12. Alternatively, aspirate the PBS and add distilled or deionized water (0.5 mL/well of a 24-well plate). Carefully remove each coverslip with forceps and mount cell-side down onto a drop of mounting medium on a glass slide.
13. Slides are ready for microscopic observation.

