Human Pluripotent Stem Cell Functional Identification Kit

Catalog Number SC027B

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 ≤ -20 °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 (50X)	896193	5.5 mL of a 50X concentrated solution.	Store at 2-8 °C for up to 2 weeks or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Endoderm Differentiation Supplement I	390513	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 ≤ -20 °C in a manual defrost freezer for up to 3 months.* Avoid repeated freeze-thaw cycles.
Endoderm Differentiation Supplement II	390514	Lyophilized growth factors for Days 2 and 3 of endoderm differentiation; enough to make 100 µL of a 500X stock.	
Mesoderm Differentiation Supplement	390515	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	963121	100 µg of lyophilized goat anti-human SOX17 polyclonal antibody; enough to make 10 mL of staining solution when used at the suggested concentration of 10 µg/mL.	Stored at 2-8 °C for up to 1 month or aliquot and stored at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Goat anti-human Otx2	963273	100 µg of lyophilized goat anti-human Otx2 polyclonal antibody; enough to make 10 mL of staining solution when used at the suggested concentration of 10 µg/mL.	
Goat anti-human Brachyury	963427	100 µg of lyophilized goat anti-human Brachyury polyclonal antibody; enough to make 10 mL of staining solution when used at the suggested concentration of 10 µg/mL.	

*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

- Flow Cytometry Fixation Buffer (R&D Systems®, Catalog # FC004)
- Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems®, Catalog # FC005)
- 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® Reduced Growth Factor Basement Membrane Extract, PathClear® (R&D Systems®, Catalog # 3433-005-01)
- 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% CO_2 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 4 mL of 50X Differentiation Base Media Supplement with 196 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

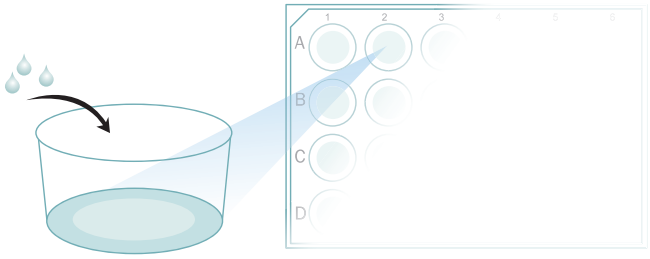
Goat anti-human SOX17 - Reconstitute with 1.0 mL of sterile PBS. Mix gently. Results in a 100 µg/mL stock solution.

Goat anti-human Otx2 - Reconstitute with 1.0 mL of sterile PBS. Mix gently. Results in a 100 µg/mL stock solution.

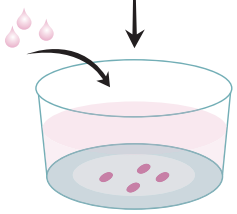
Goat anti-human Brachyury - Reconstitute with 1.0 mL of sterile PBS. Mix gently. Results in a 100 µg/mL stock solution.

PROCEDURE OUTLINE

Coat wells with diluted RGF/BME.



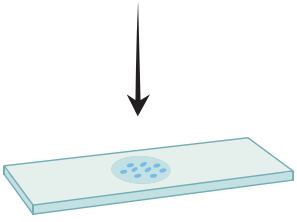
Add 1.1x10^5 cells/cm^2 in MEF Conditioned Media containing 4 ng/mL FGF Basic.



Culture cells to 50% confluency.

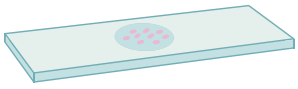
Ectoderm Differentiation

DAY 1:
Replace media with Ectoderm Differentiation Media
DAY 2:
Repeat
DAY 3:
Repeat
DAY 4:
ICC detection of Otx2



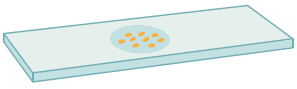
Mesoderm Differentiation

DAY 1:
Replace media with Mesoderm Differentiation Media
DAY 2:
12–16 hours later,
Repeat media change
ICC detection of Brachyury
(24–36 hours after initial differentiation)



Endoderm Differentiation

DAY 1:
Replace media with Endoderm Differentiation Media I
DAY 2:
16–24 hours later,
Replace media with Endoderm Differentiation Media II
DAY 3:
Replace media with Endoderm Differentiation Media II
DAY 4:
ICC detection of SOX17



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

1. Thaw Cultrex® Reduced Growth Factor Basement Membrane Extract, PathClear® (RGF/BME) on ice at 2-8 °C overnight.
2. Aliquot thawed RGF/BME into pre-cooled tubes and store at ≤ -20 °C.
3. Thaw the aliquot on ice at 2-8 °C overnight.
4. Dilute RGF/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 RGF/BME (0.5 mL/well of a 24-well plate) and incubate for 1-2 hours at room temperature.
7. Remove the RGF/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 1.0 mL of Accutase solution to each 60 mm plate or 3.0 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 5.0 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 4.0 ng/mL of FGF basic and count the viable cells using Trypan Blue and a hemocytometer.
2. Plate cells onto prepared RGF/BME-coated plates at a concentration of 1.1×10^5 cells/cm^2. 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% CO_2. 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 1.0 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% CO_2.
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 1.0 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% CO_2.
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 PROCEDURE

Endoderm Differentiation (Day 1)

1. Warm the Differentiation Base Media to 37 °C.
2. Prepare the required amount of Endoderm Differentiation Media I. Use 1.0 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% CO_2.

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 1.0 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 (1.0 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 (1.0 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.

FLOW CYTOMETRY STAINING PROTOCOL

1. Remove the existing media from the cells. Add 0.5 mL of Accutase® to each well. Incubate at 37 °C for 4-5 minutes or until the cells begin to slough off the plate.
2. Transfer cells into microcentrifuge tubes and centrifuge at 300 x g for 5 minutes. Decant liquid.
3. Wash the cells with 2.0 mL of PBS. Centrifuge at 300 x g for 5 minutes and then decant buffer from pelleted cells. Repeat wash one more time.
4. Aliquot up to 1 x 10^6 cells/100 µL into FACS tubes. Add 0.5 mL of cold Flow Cytometry Fixation Buffer (R&D Systems®, Catalog # FC004, or an equivalent solution containing 1-4% paraformaldehyde).
5. Mix the solution and incubate at room temperature for 10 minutes. Mix the cells intermittently in order to maintain a single cell suspension.
6. Centrifuge cells at 300 x g for 5 minutes and decant the Flow Cytometry Fixation Buffer.
7. Wash the cells with 2.0 mL of PBS. Centrifuge at 300 x g for 5 minutes and then decant buffer from pelleted cells. Repeat wash one more time.
8. Resuspend the cell pellet in 100–200 µL of Flow Cytometry Permeabilization/Wash Buffer I (R&D Systems®, Catalog # FC005, or an equivalent solution containing saponin).
9. Add 10 µL of primary antibody and mix. Incubate cells for 30 minutes at room temperature in the dark.
   Note: *Because saponin-mediated cell permeabilization is a reversible process, it is important to keep the cells in the presence of Permeabilization Buffer/Wash Buffer I during intracellular staining.*
10. Wash cells 2 times with Flow Cytometry Permeabilization/Wash Buffer I, as in Step 3.
11. Resuspend the cell pellet in 100–200 µL of Flow Cytometry Permeabilization/Wash Buffer I.
12. Dilute the desired secondary antibody (e.g. PE-conjugated Donkey Anti-Goat IgG, R&D Systems®, Catalog # F0107) in Flow Cytometry Permeabilization/Wash Buffer I. It is recommended to start with the concentration suggested in the secondary antibody product datasheet.
13. Incubate for 20-30 minutes at room temperature **in the dark**.
14. Wash cells 2 times with Flow Cytometry Permeabilization/Wash Buffer I, as in Step 3.
15. Resuspend the cells in 200–400 µL PBS buffer for flow cytometric analysis.

- Stain undifferentiated pluripotent stem cells with each of the lineage-specific antibodies.
- Stain a separate set of differentiated cells with an isotype control secondary antibody.
  Note: *The following should be considered for negative controls:*

SAMPLE DATA

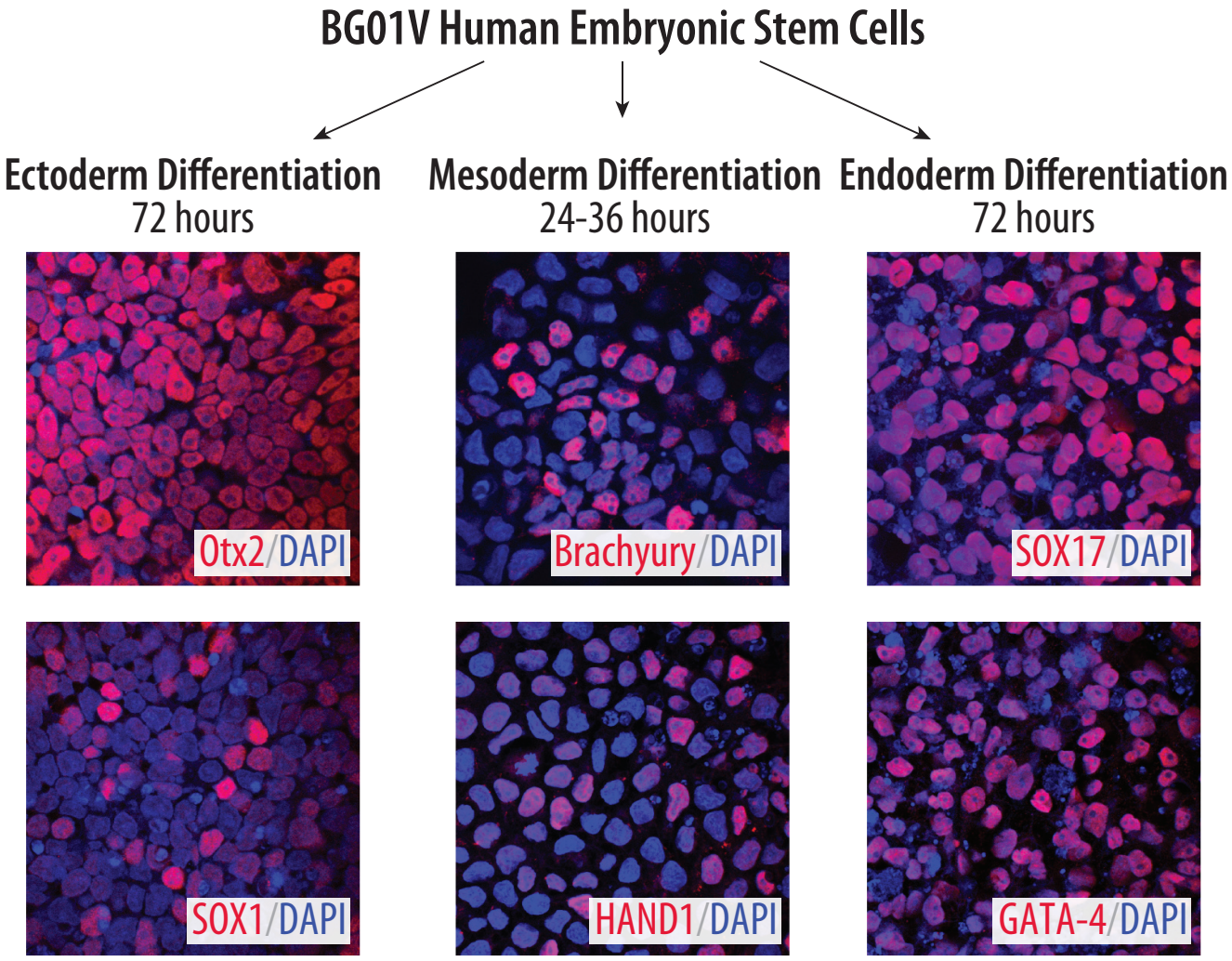


Figure 1: Verification of Human Embryonic Stem Cell Pluripotency. BG01V human embryonic stem cells were differentiated to ectoderm, mesoderm, and endoderm using the media supplements included in this kit. To further evaluate lineage commitment, cells were stained with Goat Anti-Human SOX1 Antibody (R&D Systems®, Catalog # AF3369), Goat Anti-Human HAND1 Antibody (R&D Systems®, Catalog # AF3168), and Goat Anti-Human GATA-4 (R&D Systems®, Catalog # AF2606). The cells were stained using NorthernLights™ 557-conjugated Donkey Anti-Goat IgG Secondary Antibody (R&D Systems®, Catalog # NL001; red) and the nuclei were counterstained with DAPI (blue).

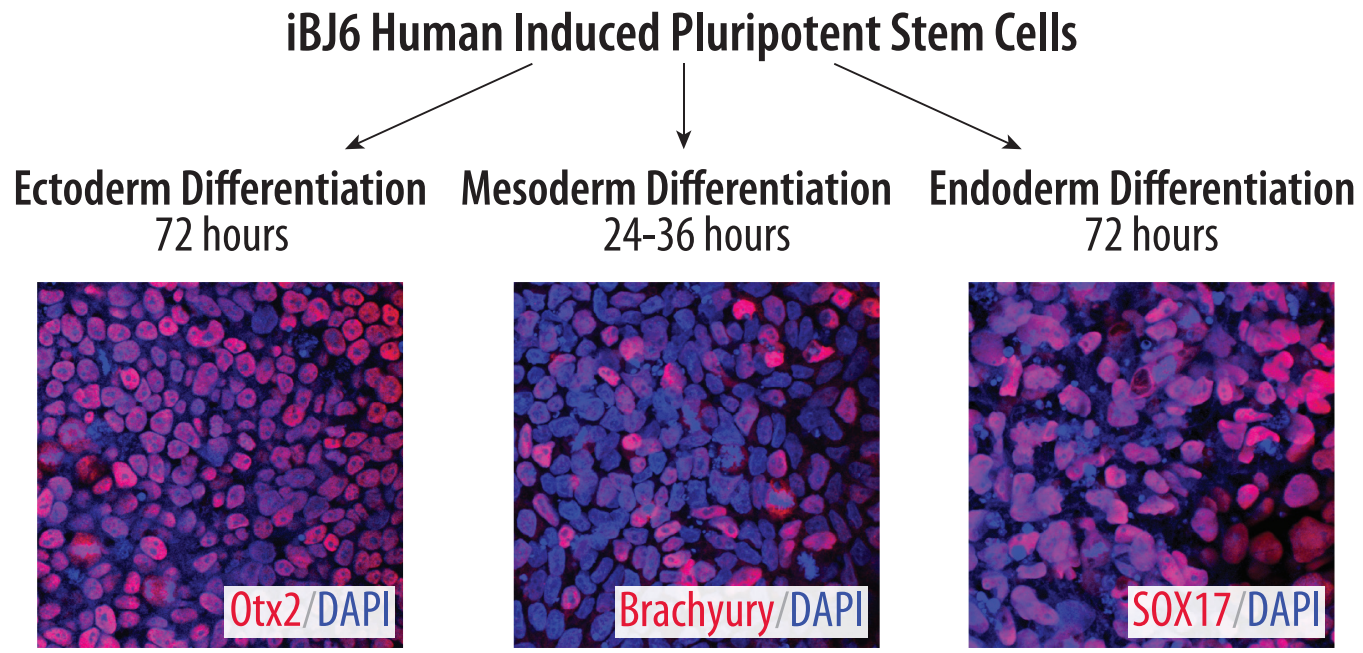


Figure 2: Verification of Human Induced Pluripotent Stem Cell Pluripotency. iBJ6 human induced pluripotent stem cells were differentiated to ectoderm, mesoderm, and endoderm using the media supplements included in this kit. The cells were stained using NorthernLights™ 557-conjugated Donkey Anti-Goat IgG Secondary Antibody (R&D Systems®, Catalog # NL001; red) and the nuclei were counterstained with DAPI (blue).

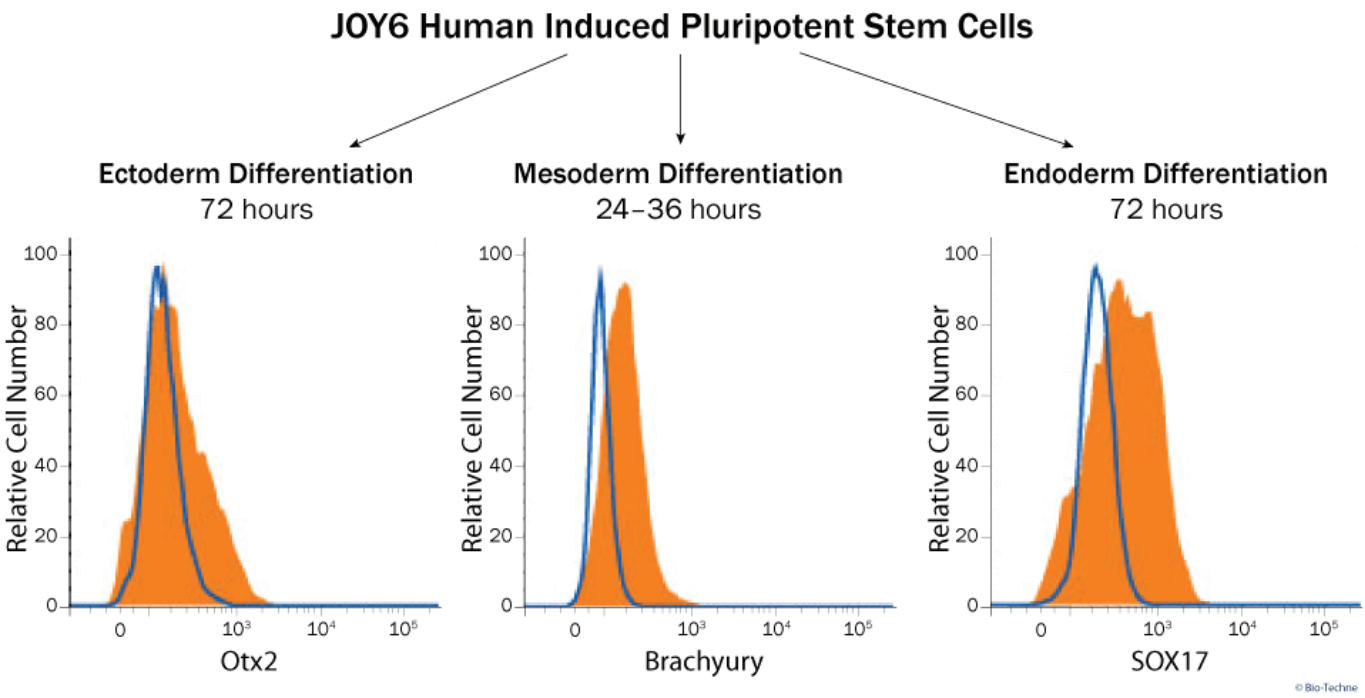


Figure 3: Analysis of Functional Tri-lineage Differentiation using Flow Cytometry. JOY6 human induced pluripotent stem cells were differentiated to ectoderm, mesoderm, and endoderm using the Human Pluripotent Stem Cell Functional Identification Kit. Differentiation was analyzed by flow cytometry using the protocol and antibodies provided with this kit. Differentiated cells (orange) show increased expression of their respective linage specific markers (ectoderm - Otx2, mesoderm - Bachyury, endoderm - SOX17 compared to differentiated JOY6 pluripotent stem cells (blue).

NOTES

NOTES

BG01V human embryonic cells are licensed from ViaCyte, Inc.