StemXVivo®

Endoderm Kit

Catalog Number SC019B

Reagents for the differentiation of human pluripotent stem cells into definitive endoderm.

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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INTRODUCTION

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.

Definitive endoderm forms during gastrulation of the vertebrate embryo and gives rise to the lining of the gut as well as multiple organ systems including lungs, liver, and pancreas (1-2). The ability of pluripotent stem cells to differentiate into definitive endoderm is one of the required characteristics to define these cells. Endoderm differentiation is also an essential first step for the formation of other important downstream derivative cell types which can be used in regenerative medicine as well as toxicological and developmental studies (3-5).

PRINCIPLE OF THE ASSAY

The StemXVivo® Endoderm Kit contains specially formulated media supplements and growth factors for the differentiation of human pluripotent stem cells into definitive endoderm. An antibody against SOX17 is also included to characterize differentiation status. The quantity of each component is sufficient to make 250 mL of media for differentiation, which is enough for the differentiation of six 60 mm plates or two 24-well plates.

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.
- The 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 the efficiency of endoderm differentiation.

PRECAUTIONS

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

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at ≤ -20 °C in a manual defrost freezer. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF RECONSTITUTED MATERIAL
Endoderm Base Media Supplement (50X)	896204	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 3 months.* Avoid repeated freeze-thaw cycles.
Human FGF basic (1000X)	965985	1 vial of lyophilized recombinant human FGF basic; enough to make 350 µL of a 1000X 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.
Human Activin A (1000X)	965989	1 vial of lyophilized recombinant human Activin A; enough to make 250 µL of a 1000X stock.	
Human Wnt-3a (1000X)	965986	1 vial of lyophilized recombinant human Wnt-3a; enough to make 100 µL of a 1000X stock.	
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 µg/100 µL.	Store at 2-8 °C for up to 1 month or aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.

*Provided this is within the expiration of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 60 mm culture plates
- 24-well culture plates
- 15 mL centrifuge tubes
- 50 mL centrifuge tubes
- 0.2 µm syringe filter
- 0.2 µm, 500 mL filter units
- 10 mL syringes
- Serological pipettes
- Pipettes and pipette tips

Reagents

- RPMI
- DMEM/F-12
- GlutaMAX™
- Penicillin-Streptomycin (optional)
- Sterile Phosphate-Buffered Saline (PBS)
- Cultrex® PathClear® Basement Membrane Extract Reduced Growth Factor (R&D Systems, Catalog # 3433-005-01 or equivalent)
- MEF Conditioned Media (R&D Systems, Catalog # AR005 or equivalent)
- Trypan Blue stain
- Accutase®
- BSA (very low endotoxin)
- Sterile, deionized water
- 95% Ethanol
- 4% Paraformaldehyde in PBS
- 1% BSA in PBS
- 0.3% Triton™ X-100, 1% BSA, 10% normal donkey serum in PBS
- Mounting medium (R&D Systems, Catalog # CTS011)
- Secondary developing reagent (R&D Systems, Catalog # NL001, NL002, or NL003)

Equipment

- 37 °C and 5% CO_2 incubator
- 37 °C water bath
- Centrifuge
- Hemocytometer
- Inverted microscope

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 (1000X) - Reconstitute with 350 µL of sterile 0.1% BSA in PBS. Mix gently.

Human Activin A (1000X) - Reconstitute with 250 µL of sterile 0.1% BSA in PBS. Mix gently.

Human Wnt-3a (1000X) - Reconstitute with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Anti-Human SOX17 - Reconstitute with 250 µL of sterile PBS. Mix gently.

Differentiation Base Media - Add 5 mL of 50X Endoderm Base Media Supplement to 245 mL of RPMI, 2.5 mL of Penicillin/Streptomycin (optional), and 2.5 mL of GlutaMAX.

Differentiation Media I - Dilute the FGF basic, Activin A, and Wnt-3a stock solutions 1000-fold in pre-warmed Differentiation Base Media. Prepare fresh as needed.

Differentiation Media II - Dilute the FGF basic and Activin A stock solutions 1000-fold in pre-warmed Differentiation Base Media. Prepare fresh as needed.

PROCEDURE OUTLINE

Coat wells with Cultrex® Basement Membrane Extract (BME).
Incubate at room temperature for 1-2 hours.

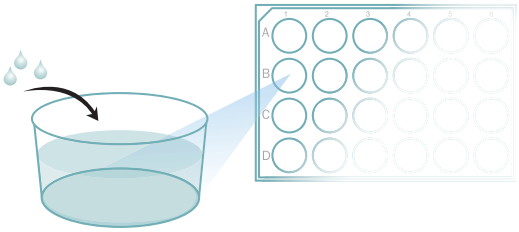
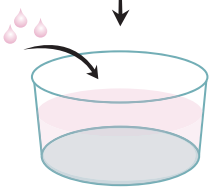
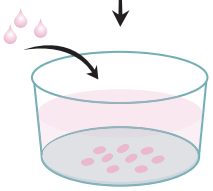


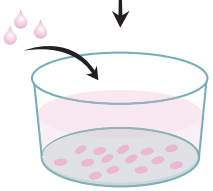
Plate BG01V human embryonic stem cells onto the coated plates at $3.3x10^5$ cells/cm^2 in MEF Conditioned Media containing FGF basic.



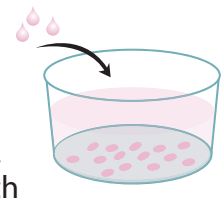
Culture cells overnight at 37 ˚C and 5% CO_2. The next day each well should contain a tightly packed monolayer of cells.



Day 1 of Differentiation
Remove the MEF Conditioned Media from the wells 12-24 hours after initial plating.
Add fresh MEF Conditioned Media containing FGF basic.

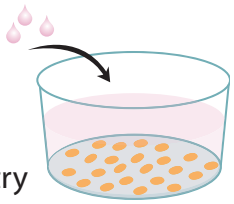


Incubate at 37 ˚C and 5% CO_2 for a minimum of 2-4 hours prior to adding Differentiation Media I.
Remove the MEF Conditioned Media from each well.
Wash each well once with 1X PBS.
Add Differentiation Media I to each well and incubate overnight at 37 ˚C and 5% CO_2.



Day 2 and 3 of Differentiation
Approximately 12-16 hours after adding the Differentiation Media I, **replace** the media with Differentiation Media II.
Continue to replace Differentiation Media II every 8-12 hours.

Day 4 of Differentiation
On Day 4, the cells are ready for **further differentiation** to downstream cell types or analysis by immunocytochemistry and/or flow cytometry.



STAGE I: UNDIFFERENTIATED CELL PREPARATION

This protocol is designed for use with BG01V human embryonic stem 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 critical. 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 BASEMENT MEMBRANE EXTRACT (BME)

1. Thaw Cultrex® PathClear® Reduced Growth Factor Basement Membrane Extract (RGF BME) on ice at 2-8 °C overnight.
2. Aliquot the 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 the RGF BME 1:40 in DMEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
5. Coat the desired number of plates with diluted RGF BME (approximately 2.5 mL per 60 mm plate and 0.5 mL per well of a 24-well plate). If desired, sterile coverslips (sterilized with 95% ethanol and flame) can be added to each 24-well plate for staining purposes.
6. Incubate for 1-2 hours at room temperature.

CELL DISSOCIATION

1. Warm the MEF Conditioned Media to 37 °C.
2. Remove the MEF Conditioned Media from the cells. Add 1 mL of Accutase solution to each 60 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 that the cells are not exposed to the Accutase solution beyond the time it takes for the cells to slough off the plate.
3. Gently pipette over the plate until the cells have become detached.
4. Gently pipette the cell suspension up and down to break up large cell clumps.
5. Transfer the cell suspension to a 15 mL centrifuge tube containing 5 mL of conditioned media, and centrifuge for 4 minutes at 200 x g.

CELL PLATING

1. Resuspend the pellet in MEF Conditioned Media containing human FGF basic (1X) and count the viable cells using Trypan Blue and a hemocytometer.
2. Plate the cells onto prepared RGF BME-coated plates at a concentration of approximately 3.3 x 10^5 cells/cm^2. For example, plate 7 x 10^6 cells/60 mm plate. Because the cells are plated so densely, add extra media for growth overnight to ensure that the cultures do not become too acidic. Use 7-8 mL of MEF Conditioned Media containing human FGF basic (1X) per 60 mm plate and 1.0-1.5 mL/well of a 24-well plate.
3. Grow overnight at 37 °C and 5% CO_2. The next day each plate should contain a tightly packed monolayer of cells.

   Note: *As an alternative to plating cells at a high density and initiating differentiation the following day as described above, cells can be plated at a lower density and be grown to confluency prior to differentiation. This method may introduce some variability during differentiation.*

STAGE II: DAY 1 OF DIFFERENTIATION

1. Warm the MEF Conditioned Media to 37 °C.
2. Remove the media from the plates 12-24 hours after initial plating, and replace with fresh MEF Conditioned Media containing human FGF basic (1X). Use 5-6 mL of MEF Conditioned Media containing human FGF basic (1X) per 60 mm plate and 1.0 mL/well of a 24-well plate.
3. Incubate the cells at 37 °C and 5% CO_2 for a minimum of 2-4 hours prior to adding Differentiation Media I.
4. Warm Differentiation Base Media to 37 °C.
5. Prepare the required amount of Differentiation Media I. Due to the large quantity of cells in each plate, large volumes of media are required to ensure plates do not become too acidic. Use 7 mL of media per 60 mm plate and 1.0 mL per well of a 24-well plate.
6. Remove the MEF Conditioned Media from each plate or well.
7. Wash each plate or well once with sterile 1X PBS. Use approximately 3-4 mL per 60 mm plate and 0.5-1.0 mL/well of a 24-well plate.
8. Add prepared Differentiation Media I to each plate, and incubate overnight at 37 °C and 5% CO_2.

STAGE III: DAYS 2 & 3 OF DIFFERENTIATION

1. Approximately 12-16 hours after adding Differentiation Media I, warm Differentiation Base Media to 37 °C.
2. Prepare the required amount of Differentiation Media II. Due to the large quantity of cells in each plate, large volumes of media are required to ensure that the cultures do not become too acidic. Use 7 mL of media per 60 mm plate and 1.0 mL per well of a 24-well plate.
3. Remove Differentiation Media I, and replace it with Differentiation Media II.
4. Repeat steps 2-4 within 8-12 hours (the media should be replaced twice daily).
5. Repeat steps 1-4 on Day 3.
6. On Day 4, the cells are ready for further differentiation to downstream cell types or analysis by immunocytochemistry and/or flow cytometry. For immunocytochemistry, proceed to the Fixing & Staining Procedure.

FIXING & 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 Anti-Human SOX17 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.

   Note: *A negative control should be performed using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in the absence of a primary antibody.*
6. After blocking, incubate the cells with diluted Anti-Human SOX17 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. NorthernLights™ (NL)557-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. 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.
12. Slides are ready for microscopic observation.

DATA EXAMPLE

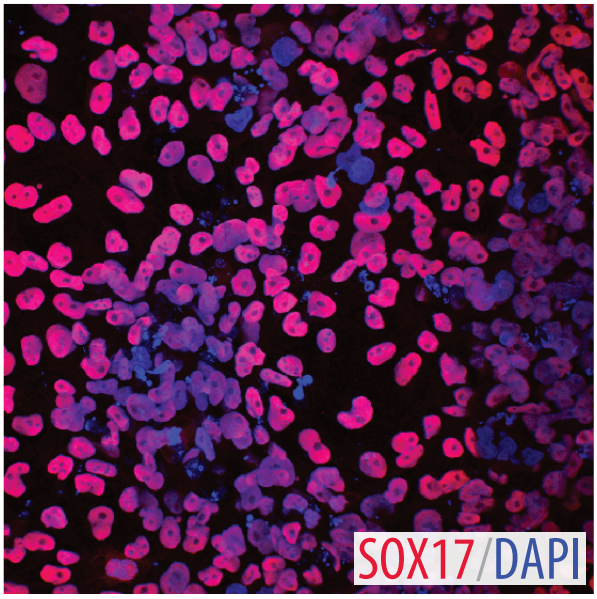


Figure 1: Definitive Endoderm Differentiation of BG01V Human Embryonic Stem Cells. BG01V cells were differentiated into definitive endoderm using the media supplements included in this kit. To evaluate lineage commitment, the cells were stained with the Anti-Human SOX17 antibody included in this kit. The cells were stained with NorthernLights™ 557-Conjugated Donkey Anti-Goat IgG Secondary Antibody (R&D Systems, Catalog # NL001; red) and the nuclei were counterstained with DAPI (blue).

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