StemXVivo™

Mesoderm Kit

Catalog Number SC030

Reagents for the differentiation of human pluripotent stem cells into mesoderm.

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.

Mesoderm is one of three primary germ layers formed during the process of gastrulation in the early embryo. Embryonic mesoderm gives rise to several important organs and tissues including the heart, muscle, bone, and cartilage. The ability of pluripotent stem cells to differentiate into mesoderm is one of the defining characteristics of these cells. Mesoderm 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 (1-3).

PRINCIPLE OF THE ASSAY

The StemXVivo™ Mesoderm Kit contains specially formulated media supplements and growth factors for the differentiation of human pluripotent stem cells into mesoderm. An antibody against Brachyury is also included to verify differentiation status. The quantity of each component is sufficient to make 100 mL of media for differentiation. This is enough media 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 mesoderm 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
Mesoderm Base Media Supplement (10X)	895183	10 mL of a 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.
Mesoderm Differentiation Supplement	390515	2 vials of lyophilized growth factors for ectoderm differentiation; enough to make 100 µL of a 500X 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.
Anti-Human Brachyury	967332	25 µg of lyophilized goat anti-human Brachury 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
- 24-well culture plates
- 60 mm culture plates
- 12 mm cover slips
- 15 mL and 50 mL centrifuge tubes
- 0.2 µm syringe filter
- 10 mL syringe
- Serological pipettes
- Glass slides
- Fine pointed curved forceps

Reagents

- RPMI
- BSA, very low endotoxin
- D-MEM/F-12
- GlutaMAX™ (Invitrogen, Catalog # 35050-079 or equivalent)
- Penicillin-Streptomycin (optional)
- Sterile 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
- Mounting medium (R&D Systems, Catalog # CTS011)
- Secondary developing reagents (R&D Systems, Catalog # NL001, NL002, or NL003)
- Deionized or distilled water

Equipment

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

Mesoderm Differentiation Supplement - Reconstitute each vial with 100 µL of sterile 0.1% BSA in PBS. Mix gently.

Mesoderm Base Media - Combine 10 mL of 10X Mesoderm Base Media Supplement with 90 mL of RPMI, 1 mL of Penicillin/Streptomycin (optional), and 1 mL of GlutaMAX.

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

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

PROCEDURE OUTLINE

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

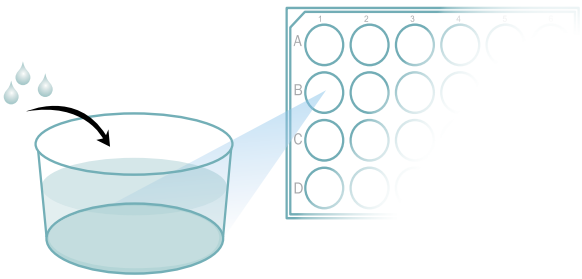
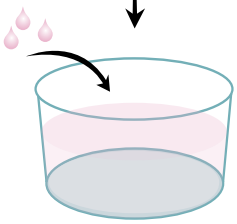
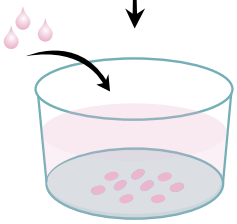


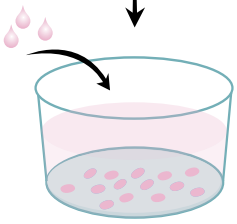
Plate BG01V human embryonic stem cells onto the coated plates at $1.1x10^5$ cells/cm^2 in MEF Conditioned Media containing FGF basic.



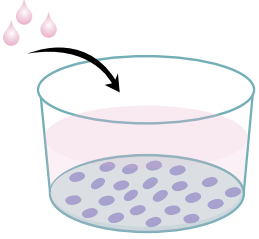
Culture cells overnight at 37 ˚C and 5% CO_2. The next day each plate should be approximately 50% confluent.



Day 1 of Differentiation
Replace the MEF Conditioned Media with Mesoderm Differentiation Media.
Incubate at 37 ˚C and 5% CO_2 for 12-16 hours.



Day 2 of Differentiation
Repeat media change.
After 12-20 hours, the cells are ready for **further differentiation** to downstream cell types or analysis by immunocytochemistry and/or flow cytometry.



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 BME on ice at 2-8 °C overnight.
2. Aliquot the 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 the Cultrex PathClear BME 1:40 in D-MEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
5. Coat the desired number of plates with diluted Cultrex PathClear BME (approximately 2.5 mL per 60 mm plate and 0.5 mL per well of a 24-well plate) and incubate for 1-2 hours at room temperature. If desired, sterile coverslips (sterilize with 95% ethanol and flame) can be added to each 24-well plate for staining purposes.
6. 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 beyond the time it takes the cells to slough off the plate.
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 x 10^5 cells/cm^2. For example, plate 4.5 x 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.

MESODERM DIFFERENTIATION PROCEDURE

1. Warm the Mesoderm Base Media to 37 °C.
2. Prepare the required amount of Mesoderm Differentiation Media. Use 5 mL per 60 mm plate or 1 mL of media per well of a 24-well plate.
3. Remove the MEF Conditioned Media from each plate or 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 initial differentiation, cells are ready for further differentiation to downstream cell types or analysis by immunocytochemistry and/or flow cytometry. For immunocytochemistry, proceed to the Fixing and 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 Brachyury 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 Brachyury 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. 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 EXAMPLES

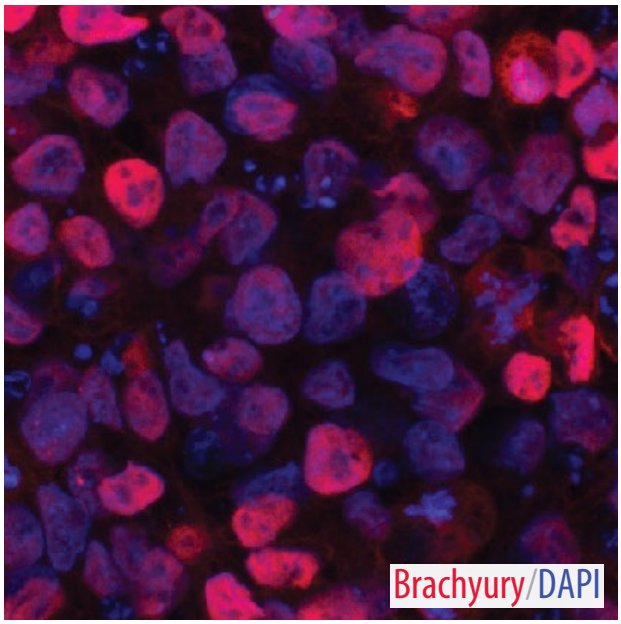


Figure 1: Differentiation of Pluripotent Stem Cells into Mesoderm. BG01V human embryonic stem cells were differentiated into mesoderm using the media supplements included in this kit. To evaluate lineage commitment, the cells were stained with the Anti-Human Brachyury antibody included. The cells were 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).

REFERENCES

1. Robinton, D.A. and G.Q. Daley (2012) Nature **481**: 295.
2. Dambrot C. *et al.* (2011) Biochem. J. **434**: 25.
3. Mercola M. *et al.* (2013) Circ. Res. **112**: 534.

NOTES

BG01V cells are licensed from Viacyte, Inc.