StemXVivo™

Hepatocyte Differentiation Kit

Catalog Number SC033

Reagents for the directed differentiation of human pluripotent stem cells to the hepatocyte lineage.

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

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Pluripotent stem cells, including both embryonic and induced pluripotent stem 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 ability of pluripotent stem cells to differentiate into functional hepatocytes holds tremendous promise for applications in therapeutics, pharmaceuticals, and developmental research. The potentially unlimited supply of hepatocytes generated from pluripotent cell sources can provide a high throughput platform for drug toxicity screening as well as drug candidate screening for the elimination of harmful drugs before moving into clinical trials. Fully characterized differentiated hepatocytes may eventually be transplanted as a therapy for end-stage liver disease. Additionally, studying the process of hepatocyte differentiation from pluripotent stem cells will increase our understanding of the mechanisms and processes involved in embryonic development and organogenesis (1, 2).

PRINCIPLE OF THE ASSAY

The StemXVivo™ Hepatocyte Differentiation Kit contains specially formulated media supplements and cocktails that can be used for the directed differentiation of pluripotent stem cells toward a hepatocyte-like cell fate. An antibody to serum albumin is included to characterize the extent of differentiation. The quantity of each component in the kit is sufficient to differentiate two 24-well plates, or an equivalent surface area, of pluripotent stem cells.

TECHNICAL HINTS & LIMITATIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix or substitute reagents with those from other lots or sources.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The quality and differentiation potential of human pluripotent stem cells at the onset of the differentiation protocol are of paramount importance to the efficiency of differentiation.
- If little to none of the cells are positive for serum albumin at day 19, it is recommended to test your pluripotent stem cell starting population for their ability to differentiate into endoderm. This can be done using the StemXVivo™ Endoderm Kit (R&D Systems, Catalog # SC019) or by staining the cells with Goat Anti-Human SOX17 Affinity-purified Polyclonal Antibody (R&D Systems, Catalog # AF1924) after the first 3 days of differentiation using this kit.
- If differentiation is observed, but with low efficiency, the quality of the starting population should be verified prior to differentiation. Cell quality can be determined morphologically by staining with pluripotency markers such as Oct-4A (R&D Systems, Catalog # MAB17591), Nanog (R&D Systems, Catalog # AF1997), SSEA-4 (R&D Systems, Catalog # MAB1435), or others. Alternatively, use the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems, Catalog # SC027) to determine the ability of the starting cell population to functionally differentiate into each of the three germ layers.

PRECAUTION

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.

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 OPENED/ RECONSTITUTED MATERIAL
Hepatocyte Differentiation Base Media Supplement I (50X)	390232	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.
Hepatocyte Differentiation Base Media Supplement II (50X)	390233	7 mL of a 50X concentrated solution.	
Hepatocyte Differentiation Cocktail I	390543	1 vial of lyophilized factors; enough to make 250 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.
Hepatocyte Differentiation Cocktail II	390547	1 vial of lyophilized factors; enough to make 200 µL of a 500X stock.	
Hepatocyte Differentiation Cocktail III	390548	1 vial of lyophilized factors; enough to make 200 µL of a 500X stock.	
Hepatocyte Differentiation Cocktail IV	390549	1 vial of lyophilized factors; enough to make 300 µL of a 500X stock.	
Anti-Human Serum Albumin	967976	25 µg of lyophilized mouse anti-human Serum Albumin monoclonal antibody. Enough to make 2.5 mL of staining solution when used at the suggested concentration of 10 µg/mL.	

*Provided this is within the expiration of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Human pluripotent stem cells
- 24-well culture plates (or other, as needed)
- 12 mm coverslips (optional)
- 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

- MEF Conditioned Media (R&D Systems, Catalog # AR005)
- Cultrex® Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract, PathClear® (R&D Systems, Catalog # 3434-001-02)
- Accutase®
- RPMI 1640 Media
- BSA, very low endotoxin
- Recombinant Human FGF basic (146 aa) (R&D Systems, Catalog # 233-FB)
- D-MEM/F-12 (1X)
- GlutaMAX™ (Invitrogen, Catalog # 35050-079 or equivalent)
- Penicillin-Streptomycin (optional)
- Phosphate Buffered Saline (PBS)
- 95% Ethanol
- 4% Paraformaldehyde
- 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 # NL007)
- Deionized or distilled water

Equipment

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

REAGENT & MATERIAL PREPARATION

Cultrex® Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract, Pathclear® (RGF BME) - Store tubes of RGF BME at ≤ -70 °C until ready to use. As needed, thaw RGF BME overnight on ice at 2-8 °C. Dilute 1:40 in ice cold stem cell growth media for coating the tissue culture dish prior to seeding cells for differentiation.

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.

Hepatocyte Differentiation Cocktail I (500X) - Reconstitute with 250 µL of sterile 0.1% BSA in PBS. Mix gently.

Hepatocyte Differentiation Cocktail II (500X) - Reconstitute with 200 µL of sterile 0.1% BSA in PBS. Mix gently.

Hepatocyte Differentiation Cocktail III (500X) - Reconstitute with 200 µL of sterile 0.1% BSA in PBS. Mix gently.

Hepatocyte Differentiation Cocktail IV (500X) - Reconstitute with 300 µL of sterile 0.1% BSA in PBS. Mix gently.

Hepatocyte Differentiation Base Media I - Dilute Hepatocyte Differentiation Base Media Supplement I 1:50 with RPMI 1640, 1X Penicillin/Streptomycin (optional), and 1X GlutaMAX™. Prepare fresh as needed.

Hepatocyte Differentiation Base Media II - Dilute Hepatocyte Differentiation Base Media Supplement II 1:50 with RPMI 1640, 1X Penicillin/Streptomycin (optional) and 1X GlutaMAX™. Prepare fresh as needed.

Anti-Human Serum Albumin - Reconstitute with 250 µL of sterile PBS to obtain a 100 µg/mL stock solution. Mix gently.

PROCEDURE OUTLINE

Coat wells with Stem Cell Qualified PathClear RGF BME (RGF BME).
Incubate at room temperature for 1-2 hours.

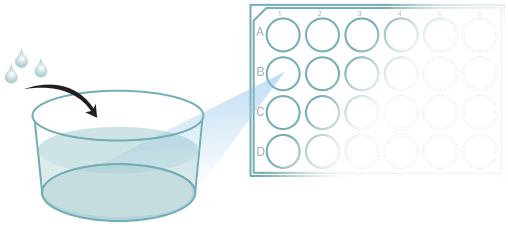
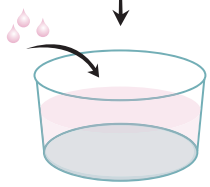
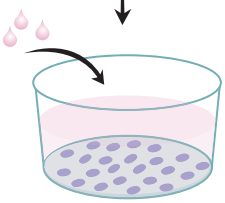


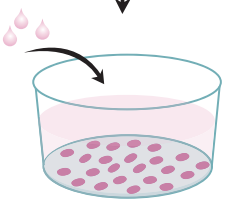
Plate human pluripotent stem cells onto the coated plates at 1.10-1.25 x10^5 cells/cm^2 in MEF Conditioned Media containing FGF basic.
Culture cells to 80-90% confluency.



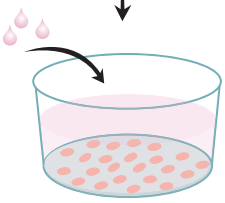
Stage 1 of Differentiation
Replace the stem cell culture media with Stage 1 Hepatocyte Differentiation Media.
Incubate at 37 ˚C and 5% CO_2 for 24 hours.
Exchange media daily for an additional 4 days.



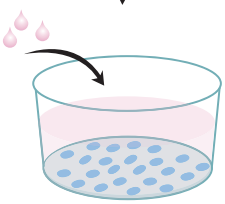
Stage 2 of Differentiation
Replace the media with Stage 2 Hepatocyte Differentiation Media.
Incubate at 37 ˚C and 5% CO_2.
Exchange media every other day for an additional 3 days.



Stage 3 of Differentiation
Replace the media with Stage 3 Hepatocyte Differentiation Media.
Incubate at 37 ˚C and 5% CO_2.
Exchange media every other day for an additional 3 days.



Stage 4 of Differentiation
Replace the media with Stage 4 Hepatocyte Differentiation Media.
Incubate at 37 ˚C and 5% CO_2.
Exchange media every other day for an additional 5 days.



UNDIFFERENTIATED CELL PREPARATION

Note: *This protocol is designed for BG01V human embryonic stem (hES) cells grown in MEF Conditioned Media (Catalog # AR005) and differentiated in 24-well culture dishes on coverslips. If using different cell lines or growth media, the protocol below may need to be modified. If using different culture vessels, additional optimization may be required to determine appropriate volumes of media.*

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® Stem Cell Qualified RGF BME, Pathclear® (RGF BME) on ice at 2-8 °C overnight.
2. Dilute RGF BME 1:40 in ice cold DMEM/F-12. This can be stored at 2-8 °C for up to 2 weeks.
3. If desired, place a sterile coverslip (sterilized with 95% ethanol and flamed) in each well of a 24-well plate for downstream immunocytochemical analysis.
4. Coat the desired number of wells or plates with diluted RGF BME (0.5 mL/well of a 24-well plate) and incubate for 1-2 hours at room temperature.

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. Transfer 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 the cells onto prepared RGF BME-coated plates at a concentration of 1.1-1.25 x 10^5 cells/cm^2, or an appropriate seeding density to reach 80-90% confluence within 1-2 days. **Note:** *If using cells cultured on a fibroblast feeder layer, it may be necessary to include feeder depletion prior to seeding.*
3. Allow the cells to attach and grow at 37 °C and 5% CO_2 with daily media exchanges until the monolayer reaches approximately 80-90% confluence.
4. Proceed to differentiation. **Note:** *Efficiency of differentiation is highest when the differentiation is started the day after plating the cells.*

HEPATOCYTE DIFFERENTIATION PROCEDURE

Differentiation Initiation, Stage 1: Day 0 through Day 4

1. Pluripotent cells should be grown to approximately 80% confluence on RGF BME prior to the initiation of differentiation.
2. Prepare Stage 1 Hepatocyte Differentiation Media as follows:
    a. Determine the appropriate volume of total media needed. For 24-well plates, use 0.5 mL/well.
    b. Pipette the needed volume of Hepatocyte Differentiation Base Media I into a sterile centrifuge tube and warm to 37 °C.
    c. Dilute the Hepatocyte Differentiation Cocktail I 1:500 into the pre-warmed Hepatocyte Differentiation Base Media I.
3. Remove and discard spent media and add the Stage 1 Hepatocyte Differentiation Media to each well.
4. Culture the cells overnight at 37°C and 5% CO_2.
5. Repeat steps 2-4 daily for 4 additional days (days 1-4).

Differentiation, Stage 2: Day 5 through Day 8

1. Prepare Stage 2 Hepatocyte Differentiation Media as follows:
    a. Determine the appropriate volume of total media needed. For 24-well plates, use 1.0 mL/well.
    b. Pipette the needed volume of Hepatocyte Differentiation Base Media I into a sterile centrifuge tube and warm to 37 °C.
    c. Dilute the Hepatocyte Differentiation Cocktail II 1:500 into the pre-warmed Hepatocyte Differentiation Base Media I.
2. Remove and discard spent media and add the Stage 2 Hepatocyte Differentiation Media to each well.
3. Culture the cells overnight at 37 °C and 5% CO_2.
4. Repeat steps 1-3 on day 7.

HEPATOCYTE DIFFERENTIATION PROCEDURE *CONTINUED*

Differentiation, Stage 3: Day 9 through Day 12

1. Prepare Stage 3 Hepatocyte Differentiation Media as follows:
    a. Determine the appropriate volume of total media needed. For 24-well plates, use 1.0 mL/well.
    b. Pipette the needed volume of Hepatocyte Differentiation Base Media II into a sterile centrifuge tube and warm to 37 °C.
    c. Dilute the Hepatocyte Differentiation Cocktail III 1:500 into the pre-warmed Hepatocyte Differentiation Base Media II.
2. Remove and discard spent media and add the Stage 3 Hepatocyte Differentiation Media to each well.
3. Culture the cells overnight at 37 °C and 5% CO_2.
4. Repeat steps 1-3 on day 11.

Differentiation, Stage 4: Day 13 through Day 19

1. Prepare Stage 4 Hepatocyte Differentiation Media as follows:
    a. Determine the appropriate volume of total media needed. For 24-well plates, use 1.0 mL/well.
    b. Pipette the needed volume of Hepatocyte Differentiation Base Media II into a sterile centrifuge tube and warm to 37 °C.
    c. Dilute the Hepatocyte Differentiation Cocktail IV 1:500 into the pre-warmed Hepatocyte Differentiation Base Media II.
2. Remove and discard spent media and add the Stage 4 Hepatocyte Differentiation Media to each well.
3. Culture the cells overnight at 37 °C and 5% CO_2.
4. Repeat steps 1-3 on days 15 and 17.
5. At Day 19, cells are ready for immunocytochemical analysis or other downstream experimentation.

FIXING & STAINING PROCEDURES

Immunocytochemistry

1. Wash the cells twice with PBS (1 mL/well for 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 for 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 for a 24-well plate).
5. During the blocking, dilute the reconstituted Anti-Human Serum Albumin Primary Antibody to a final concentration of 10 µg/mL in PBS containing 0.3% Triton™ X-100, 1% BSA, and 10% normal donkey serum.

   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 Serum Albumin 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-Mouse Secondary Antibody (R&D Systems, Catalog #NL007)] 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

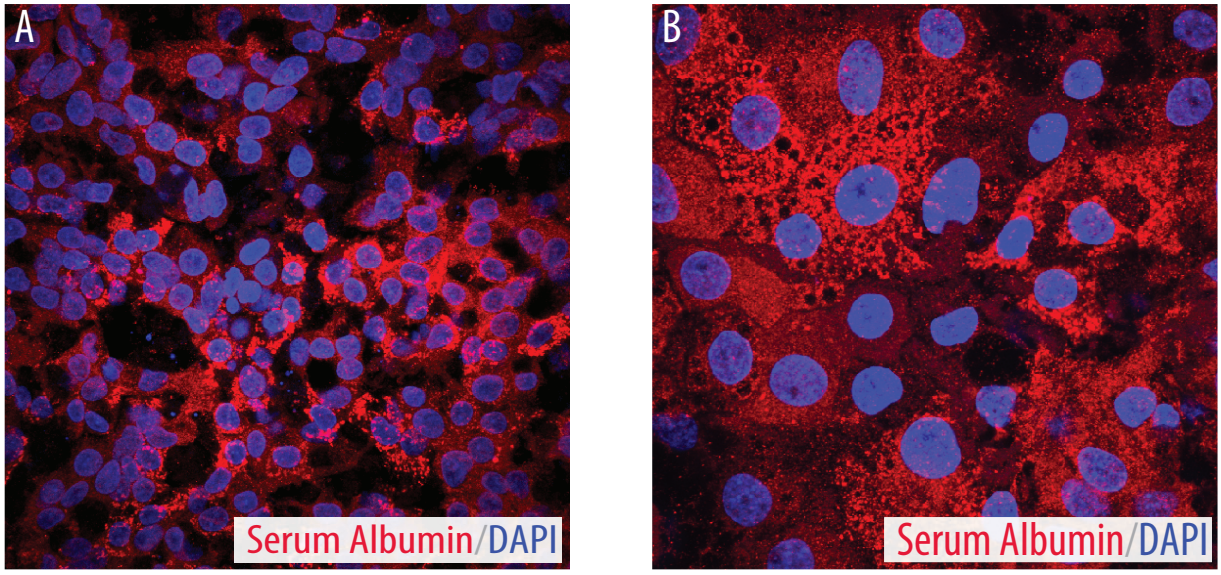


Figure 1: Differentiation of Pluripotent Stem Cells into Hepatocyte-like Cells. BG01V human embryonic stem cells **(A)** and iPS2 human induced pluripotent stem cells **(B)** were differentiated into hepatocyte-like cells using the media supplements included in this kit. Commitment to the hepatocyte fate was evaluated by immunocytochemical staining with the kit-included Anti-Human Serum Albumin antibody. Cellular serum albumin was visualized using NorthernLights™ (NL)557-conjugated Donkey Anti-Mouse Secondary Antibody (R&D Systems, Catalog # NL007; red). The nuclei were counterstained with DAPI (blue).

REFERENCES

1. Gerbal-Chaloin, S. *et al.* (2014) Am. J. Path. **184**: 332.
2. Schwartz R. *et al.* (2014) Biotechnol. Adv. **32**: 504.

NOTES

NOTES

NOTES

BG01V human embryonic stem cells are licensed from Viacyte, Inc.
All trademarks and registered trademarks are the property of their respective owners.