

Human Mesenchymal Stem Cell Functional Identification Kit

Catalog Number SC006

Reagents for the identification of human bone marrow-derived stem cells (BMSCs)/mesenchymal stem cells (MSCs) 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.

TABLE OF CONTENTS

SECTION	PAGE
PRINCIPLE OF THE ASSAY	1
LIMITATIONS OF THE PROCEDURE	1
PRECAUTIONS.....	1
MATERIALS PROVIDED & STORAGE CONDITIONS	2
OTHER SUPPLIES REQUIRED	3
REAGENT & MATERIAL PREPARATION.....	4
PREPARATION OF LYOPHILIZED ANTIBODIES.....	4
PROCEDURE OUTLINE	5
ADIPOGENIC DIFFERENTIATION PROTOCOL	6
OSTEOGENIC DIFFERENTIATION PROTOCOL.....	8
CHONDROGENIC DIFFERENTIATION PROTOCOL.....	10
DATA EXAMPLES	12
REFERENCES	13

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

PRINCIPLE OF THE ASSAY

Stem cells are functionally defined by their capacity to self renew and their ability to generate a large number of differentiated progenitor cells, which commit to further maturation along specific lineages. Multiple stem cell populations have been discovered from various adult tissues, including bone marrow-derived stem cells (BMSCs) and mesenchymal stem cells (MSCs). BMSCs/MSCs are capable of differentiating into multiple cell types including adipocytes, chondrocytes, osteocytes, hepatocytes, cardiomyocytes, and neurons (1-6). With the availability of cell selection technologies and recombinant growth factors, many attempts to isolate and expand stem cells *in vitro* are ongoing (7, 8). The ability of a cell to differentiate into adipocytes, chondrocytes, and osteocytes is included as one of the minimal criteria to define an MSC as set by the International Society for Cellular Therapy (9).

The Human Mesenchymal Stem Cell Functional Identification Kit is designed for the identification of human BMSCs/MSCs based on their ability to differentiate into multiple mesenchymal lineages. This kit contains specially formulated Adipogenesis, Chondrogenesis, and Osteogenesis Media Supplements, which can be used to effectively differentiate BMSCs/MSCs into adipogenic, chondrogenic, or osteogenic lineages. A panel of antibodies, consisting of anti-mFABP4, anti-hAggrecan, and anti-hOsteocalcin, are included to define the mature phenotypes of adipocytes, chondrocytes, and osteocytes, respectively.

LIMITATIONS OF THE PROCEDURE

- 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.
- The kit should not be used beyond the expiration date on the kit label.
- The quality of the mesenchymal stem cells and any variation in the procedure can cause variation in the efficiency of cell differentiation.
- The supplements may contain a precipitate. Mix well before use.

PRECAUTIONS

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.

The Adipogenic Supplement contains 95% ethanol and is highly flammable. Keep the container tightly closed, and keep it away from sources of ignition.

The ITS Supplement contains human transferrin. The transferrin was tested at the donor level using an FDA licensed method and found to be non-reactive for anti-HIV-1/2, anti-HCV, and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, this reagent should be handled as if capable of transmitting infection.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at $\leq -20^{\circ}\text{C}$ in a manual defrost freezer.

Do not use past the expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Adipogenic Supplement	390415	0.5 mL of a 100X concentrated solution containing hydrocortisone, isobutylmethylxanthine, and indomethacin in 95% ethanol; enough to supplement 50 mL of medium.	Store tightly sealed at 2-8 °C for up to 6 months.*
Osteogenic Supplement	390416	2.5 mL of a 20X concentrated solution containing dexamethasone, ascorbate-phosphate, and β -glycerolphosphate; enough to supplement 50 mL of medium.	Aliquot and store at $\leq -20^{\circ}\text{C}$ in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
Chondrogenic Supplement	390417	0.5 mL of a 100X concentrated solution containing dexamethasone, ascorbate-phosphate, proline, pyruvate, and recombinant TGF- β 3; enough to supplement 50 mL of medium.	
ITS Supplement	390418	0.5 mL of a 100X concentrated solution containing insulin, transferrin, selenious acid, bovine serum albumin, and linoleic acid; enough to supplement 50 mL of medium.	
anti-mFABP4	967799	50 μg of lyophilized goat anti-mouse FABP4 polyclonal antibody; enough to make 5 mL of staining solution when used at the suggested concentration of 10 $\mu\text{g}/\text{mL}$.	Store at 2-8 °C for up to 1 month or aliquot and store at $\leq -20^{\circ}\text{C}$ in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.
anti-hAggrecan	967800	50 μg of lyophilized goat anti-human Aggrecan polyclonal antibody; enough to make 5 mL of staining solution when used at the suggested concentration of 10 $\mu\text{g}/\text{mL}$.	
anti-hOsteocalcin	967801	50 μg of lyophilized mouse anti-human Osteocalcin monoclonal antibody; enough to make 5 mL of staining solution when used at the suggested concentration of 10 $\mu\text{g}/\text{mL}$.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

Materials

- Human mesenchymal stem cells
- 24-well culture plates
- 12 mm coverslips
- 15 mL centrifuge tubes
- Pipettes and pipette tips
- Serological pipettes
- Fine pointed curved forceps
- Glass slides
- Slide box
- Liquid barrier pen

Reagents

- Basal media (R&D Systems, Catalog # CCM007) or (α Minimum Essential Medium (α MEM))
- D-MEM/F-12 (1X)
- Fetal Bovine Serum
- Phosphate-Buffered Saline (PBS)
- Penicillin-Streptomycin-Glutamine (100X)
- 4% Paraformaldehyde in PBS
- 95% Ethanol
- 1% BSA in PBS
- Triton™ X-100
- Normal donkey serum
- Fibronectin [optional; Catalog # 1030-FN (bovine) or 1918-FN (human)]
- Mounting medium (R&D Systems, Catalog # CTS011)
- Secondary developing reagents (R&D Systems, Catalog # NL001, NL002, NL003, NL007, NL008, and NL009)
- Deionized or distilled water

Equipment

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

All trademarks and registered trademarks are the property of their respective owners.

REAGENT & MATERIAL PREPARATION

αMEM Basal Media - For use with Adipogenic Supplement and Osteogenic Supplement.

Mix the following sterile ingredients to make 101 mL of media. Store at 2-8 °C for up to 1 month. Alternatively, basal medium that has been prequalified for Adipogenic and Osteogenic differentiation may be used (R&D Systems, Catalog # CCM007).

ITEM	AMOUNT	FINAL CONCENTRATION
α MEM	90 mL	90%
Fetal Bovine Serum	10 mL	10%
100X Penicillin-Streptomycin-Glutamine	1 mL	100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine

D-MEM/F-12 Basal Media - For use with Chondrogenic Supplement. Mix the following sterile ingredients to make 50 mL of media. Store **in the dark** at 2-8 °C for up to 1 month.

ITEM	AMOUNT	FINAL CONCENTRATION
D-MEM/F-12	49 mL	99%
ITS Supplement	500 µL	1%
100X Penicillin-Streptomycin-Glutamine	500 µL	100 U/mL Penicillin, 100 µg/mL Streptomycin, 2 mM L-Glutamine

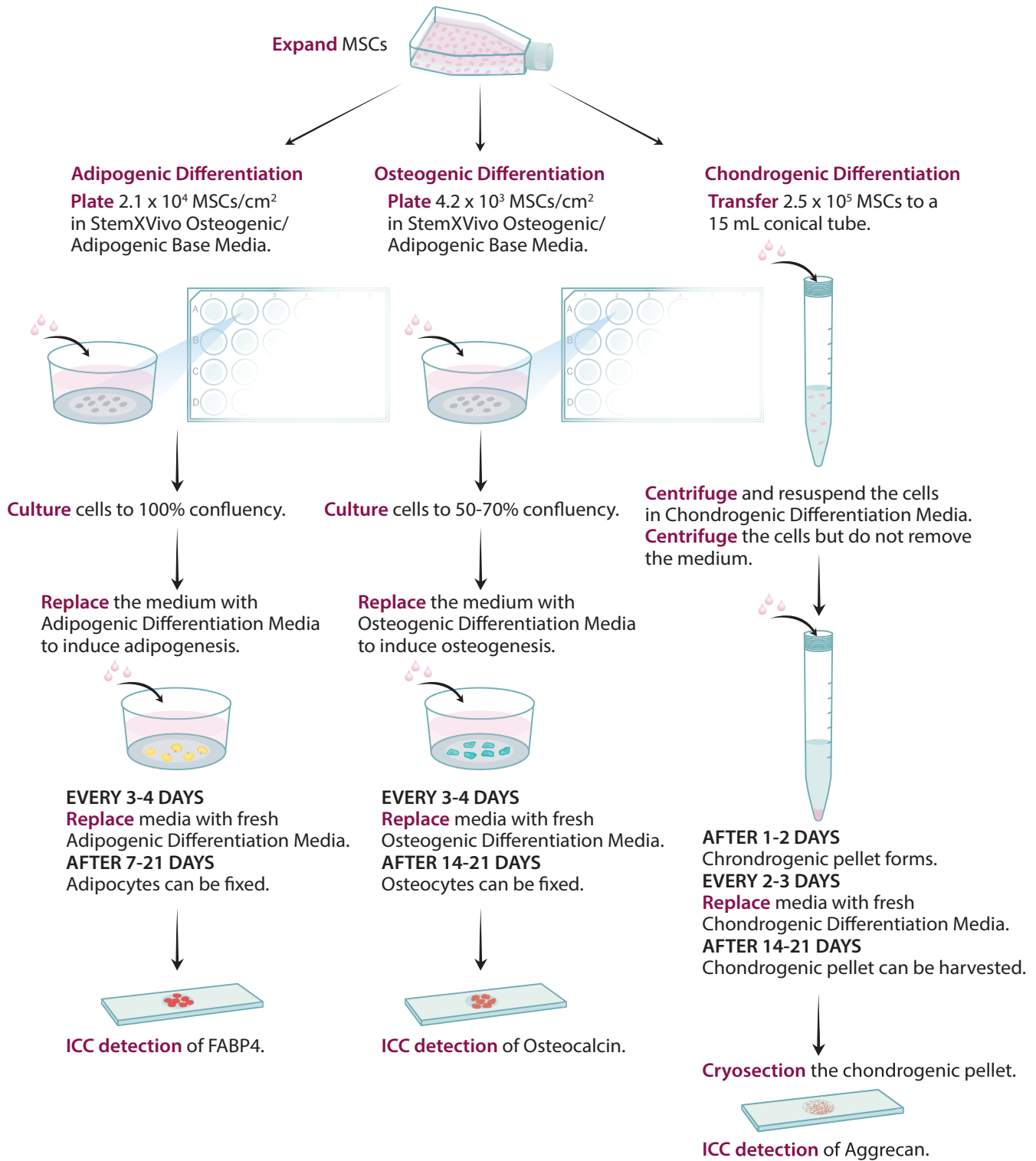
PREPARATION OF LYOPHILIZED ANTIBODIES

Anti-mFABP4 - Reconstitute with 500 µL of sterile PBS. Mix Gently. Results in a 100 µg/mL stock solution.

Anti-hAggrecan - Reconstitute with 500 µL of sterile PBS. Mix Gently. Results in a 100 µg/mL stock solution.

Anti-hOsteocalcin - Reconstitute with 500 µL of sterile PBS. Mix Gently. Results in a 100 µg/mL stock solution.

PROTOCOL OUTLINE



ADIPOGENIC DIFFERENTIATION PROTOCOL

Fresh, supplemented media should be made for each usage or media change. The recommended amount of media for a 24-well plate is 0.5 mL/well. Make 5 mL of medium for 10 wells.

PREPARATION OF ADIPOGENIC DIFFERENTIATION MEDIA

1. If a precipitate forms, warm the Adipogenic Supplement vial in a 37 °C water bath for 5 minutes. Vortex until the precipitate dissolves.
2. Add 50 µL of the Adipogenic Supplement to 5 mL of α MEM Basal Media. Mix gently.

PREPARATION OF CULTURE PLATES FOR ADIPOGENIC DIFFERENTIATION

1. Insert a sterile coverslip (sterilized with 95% Ethanol and flamed) into each well of a 24-well plate.
2. Add 0.5 mL of sterile PBS to each well. Gently sink the floating coverslips with a sterile pipette tip.
3. Store in a 37 °C incubator until needed.
4. Remove the PBS from the wells before beginning the Adipogenesis Culture Protocol.

ADIPOGENESIS CULTURE PROTOCOL

Note: 50 mL of Adipogenic Differentiation Media is sufficient to culture and differentiate 16 wells for 21 days with 6 media changes. The culturing of 10 wells will provide enough coverslips for weekly monitoring of cells; 1 coverslip for oil red staining and 2 coverslips for immunostaining.

1. Seed cells at a density of 2.1×10^4 cells/cm². Each well is approximately 1.76 cm² requiring 3.7×10^4 cells/well.
2. Prepare 3.7×10^5 cells in 5 mL of α MEM Basal Media.
3. Dispense 0.5 mL of the cell suspension into each of the 10 wells. Incubate overnight in a 37 °C and 5% CO₂ incubator. **Note:** Cells should be 100% confluent after overnight incubation. If they are not confluent, replace the media every 2-3 days with a MEM Basal Media until 100% confluency is reached.
4. When the cells are 100% confluent, replace the media in each well with 0.5 mL of Adipogenic Differentiation Media to induce adipogenesis.
5. Replace with fresh Adipogenic Differentiation Media (0.5 mL/well) every 3-4 days. After 5-7 days, lipid vacuoles will start to appear in the induced cells. **Note:** The adipogenic cells are fragile; media replacement should be performed gently so as not to disturb the lipid vacuoles. The appearance of vacuoles can be monitored by microscopic examination. Coverslips may be removed for oil red staining (refer to the Procedure Outline on page 5). For a staining protocol, please see reference 8.
6. After 7-21 days, adipocytes can be fixed and saved for immunostaining (refer to the Fixing and Staining Protocol on page 7).

FIXING & STAINING PROTOCOL-IMMUNOCYTOCHEMISTRY OF ADIPOCYTES

1. Wash the cells twice with 1 mL of PBS.
2. Fix the cells with 0.5 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
4. Permeabilize and block the cells with 0.5 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, dilute the reconstituted anti-mFABP4 antibody in PBS containing 0.03% Triton X-100, 1% BSA, and 10% normal donkey serum to a final concentration of 10 µg/mL. **Note:** A negative control should be run 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 300 µL/well of anti-mFABP4 antibody working solution for 3 hours at room temperature or overnight at 2-8 °C.
7. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
8. Dilute the secondary antibody (e.g., NL557-conjugated donkey anti-goat secondary antibody, Catalog # NL001) 1:200 in 1% BSA in PBS.
9. Incubate the cells with secondary antibody working solution at 300 µL/well **in the dark** for 60 minutes at room temperature.
10. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
11. Cover the cells with 1 mL of PBS, and visualize with a fluorescence microscope.
12. Alternatively, aspirate the PBS from the wells and add 0.5 mL of distilled water. Carefully remove the coverslips with forceps and mount cell side down onto a drop of mounting medium on a glass slide.
13. The slides are ready for microscopic observation (refer to the images in the Procedure Outline on page 5).

OSTEOGENIC DIFFERENTIATION PROTOCOL

Fresh supplemented media should be made for each usage or media change. The recommended amount of media for a 24-well plate is 0.5 mL/well. Make 5 mL of media for 10 wells.

PREPARATION OF OSTEOGENIC DIFFERENTIATION MEDIA

1. Warm the Osteogenic Supplement vial in a 37 °C water bath for 5 minutes.
2. Add 250 µL of the Osteogenic Supplement to 5 mL of α MEM Basal Media. Mix gently.
3. Divide the unused supplement into 250 µL aliquots.

PREPARATION OF CULTURE PLATES FOR OSTEOGENIC DIFFERENTIATION

1. Insert a sterile coverslip (sterilized with 95% Ethanol and flamed) into each well of a 24-well plate.
2. Add 0.5 mL of sterile PBS to each well. Gently sink the floating coverslips with a sterile pipette tip.
3. Store in a 37 °C incubator until needed.
4. Remove the PBS from the wells before beginning the Osteogenesis Culture Protocol.

OSTEOGENESIS CULTURE PROTOCOL

Note: 50 mL of Osteogenic Differentiation Media will provide adequate media to culture and differentiate 16 wells for 21 days with 6 media changes. The culturing of 10 wells will provide enough coverslips for weekly monitoring of cells; 1 coverslip for Alizarin red staining and 2 coverslips for immunostaining.

Cell detachment can occur during osteogenic differentiation. Coating the coverslips with fibronectin can be used to delay cell detachment. Add 0.5 mL of a fibronectin solution at a concentration of 1 µg/mL to each well. Incubate at 37 °C for 3-30 hours. Refer to R&D Systems Catalog # 1918-FN for Human Fibronectin.

1. Seed cells at a density of 4.2×10^3 cells/cm². Each well is approximately 1.76 cm² requiring 7.4×10^3 cells/well.
2. Prepare 7.4×10^4 cells in 5 mL of α MEM Basal Media.
3. Dispense 0.5 mL of the cell suspension into each of the 10 wells. Incubate overnight in a 37 °C and 5% CO₂ incubator. **Note:** The cells should be about 50-70% confluent in 1-2 days.
4. At 50-70% confluency, replace the media in each well with 0.5 mL of Osteogenic Differentiation Media to induce osteogenesis.
5. Replace with 0.5 mL of fresh Osteogenic Differentiation Media (0.5 mL/well) every 3-4 days.
6. After 14-21 days (or when cells start to detach), osteocytes can be fixed and saved for immunostaining (refer to the Fixing and Staining Protocol on page 9). Cells may also be ethanol fixed and stained with Alizarin Red. For a staining protocol, please see reference 8.

FIXING & STAINING PROTOCOL-IMMUNOCYTOCHEMISTRY OF OSTEOCYTES

1. Wash the cells twice with 1 mL of PBS.
2. Fix the cells with 0.5 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
4. Permeabilize and block the cells with 0.5 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes.
5. During the blocking, dilute the reconstituted anti-hOsteocalcin 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 run 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 300 µL/well of anti-hOsteocalcin antibody working solution for 3 hours at room temperature or overnight at 2-8 °C.
7. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
8. Dilute the secondary antibody (e.g., NL557-conjugated donkey anti-mouse secondary antibody, Catalog # NL007) 1:200 in 1% BSA in PBS.
9. Incubate the cells with secondary antibody working solution at 300 µL/well **in the dark** for 60 minutes at room temperature.
10. Wash the cells three times with 0.5 mL of 1% BSA in PBS for 5 minutes.
11. Cover the cells with 1 mL of PBS, and visualize with a fluorescence microscope.
12. Alternatively, aspirate the PBS from the wells and add 0.5 mL of distilled water. Carefully remove the coverslips with forceps and mount cell side down onto a drop of mounting medium on a glass slide.
13. The slides are ready for microscopic observation (refer to the images in the Procedure Outline on page 5).

CHONDROGENIC DIFFERENTIATION PROTOCOL

This kit contains adequate media to culture 10 pellets for 3 weeks with media changes 3 times per week. Fresh supplemented media should be made for each use or media change. Make 2.5 mL of media for 5 tubes. Culture the cells in 15 mL conical tubes (each 15 mL conical tube requires 0.5 mL of media).

PREPARATION OF CHONDROGENIC DIFFERENTIATION MEDIA

1. Warm the Chondrogenic Supplement vial in a 37 °C water bath for 5 minutes.
2. Add 25 µL of the Chondrogenic Supplement to 2.5 mL of D-MEM/F-12 Basal Media. Mix gently.
3. Divide the unused supplement into 25 µL aliquots.

CHONDROGENESIS CULTURE PROTOCOL

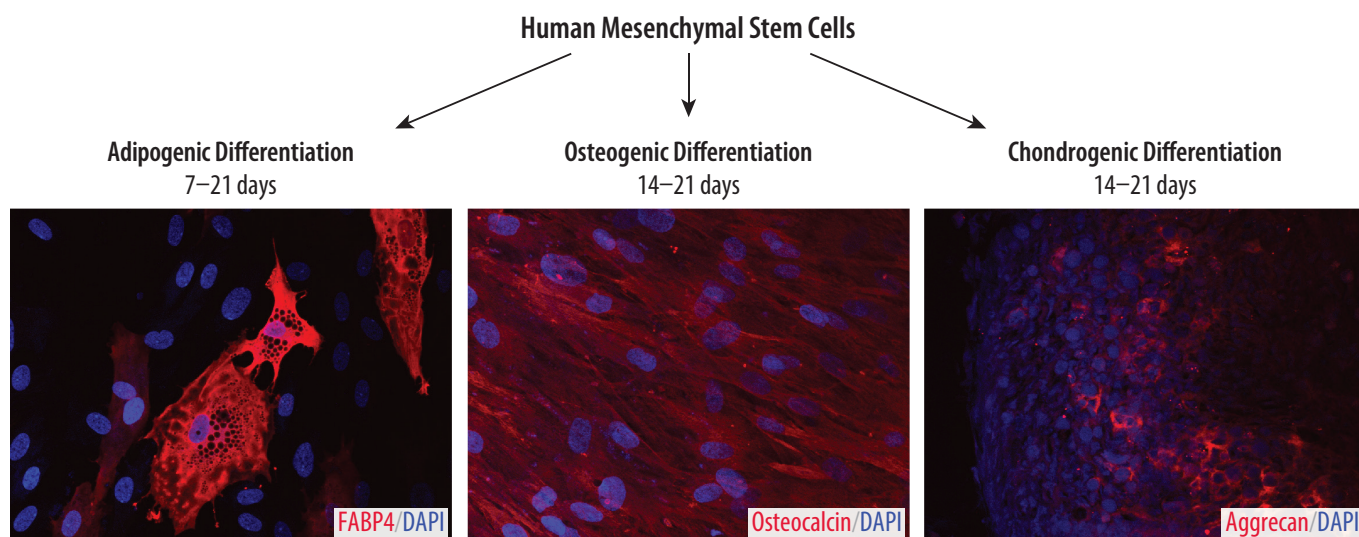
1. Transfer 2.5×10^5 cells in their existing culture media to a 15 mL conical tube.
2. Centrifuge the cells at 200 x g for 5 minutes at room temperature. Remove the media and resuspend the cells with 1.0 mL of D-MEM/F-12 Basal Media.
3. Centrifuge the cells at 200 x g for 5 minutes. Aspirate and discard the media.
4. Resuspend the cells in 0.5 mL of Chondrogenic Differentiation Media, and centrifuge the cells at 200 x g for 5 minutes at room temperature. Do not remove the media.
5. Loosen the cap(s) of the tube(s) to allow gas exchange, and incubate upright at 37 °C and 5% CO₂.
6. Replace the media with 0.5 mL of fresh Chondrogenic Differentiation Media every 2-3 days. The pellet should not be attached to the tube. **Note:** *Use caution when replacing the media to avoid aspirating the pellet.*
7. After 14-21 days, the chondrocyte pellet can be fixed and prepared for frozen sectioning (refer to the Fixing and Staining Protocol on page 11).

FIXING & STAINING PROTOCOL-IMMUNOCYTOCHEMISTRY OF CHONDROCYTES

Note: Staining is done on glass slides. To contain the solutions, use a liquid barrier pen to circle the tissue sections. Amounts of solutions needed to cover the tissue will vary depending on the size of the circle drawn. The amounts listed in the following procedure will be more than adequate.

1. Wash the pellet twice with 1 mL of PBS.
2. Fix the pellet with 0.3 mL of 4% paraformaldehyde in PBS for 20 minutes at room temperature.
3. Wash the pellet twice with 1 mL of PBS for 5 minutes.
4. Freeze and section the pellet using standard cryosectioning methods. Cut the sections at a nominal thickness of 5-10 μm .
5. Draw a hydrophobic barrier around each section on the slide using a liquid barrier pen.
6. Permeabilize and block the mounted pellet sections with 0.15 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 45 minutes.
7. During blocking, dilute the reconstituted anti-hAggrecan antibody in PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum at 1:10 to a final concentration of 10 $\mu\text{g/mL}$. **Note:** A negative control should be run using PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum with no primary antibody.
8. After blocking, incubate the sections with the anti-hAggrecan antibody working solution overnight at 2-8 °C. Keep in a covered container with adequate moisture.
9. Wash the sections three times with PBS containing 1% BSA for 5 minutes.
10. Dilute the secondary antibody (e.g., NL557-conjugated anti-goat secondary antibody, Catalog # NL001) 1:200 in PBS containing 1% BSA.
11. Incubate the sections with the secondary antibody working solution **in the dark** for 60 minutes at room temperature.
12. Wash the sections three times with PBS containing 1% BSA for 5 minutes.
13. Wash the sections once with distilled water, and remove the excess water.
14. Place a drop of mounting medium on the section, and cover with a glass coverslip.
15. The slides are ready for microscopic observation (refer to the images in the Procedure Outline on page 5).

DATA EXAMPLES



Verification of Multipotency using the Human Mesenchymal Stem Cell Functional Identification Kit. Human mesenchymal stem cells were cultured in StemXVivo™ Mesenchymal Stem Cell Expansion Media (R&D Systems, Catalog # CCM004) and differentiation was induced as indicated using the media supplements included in this kit. The cells were stained using the NorthernLights™ 557-conjugated Donkey Anti-Goat (R&D Systems, Catalog # NL001; red) or Anti-Mouse (R&D Systems, Catalog # NL007; red) IgG Secondary Antibodies, and the nuclei were counterstained with DAPI (blue).

REFERENCES

1. Gronthos, S. *et al.* (1995) *Blood* **85**:929.
2. Pittenger, M.F *et al.* (1999) *Science* **284**:143.
3. Liechty, K.W. *et al.* (2000) *Nature Med.* **6**:1282.
4. Orlic, D. *et al.* (2001) *Nature* **410**:701.
5. Lagasse, E. *et al.* (2000) *Nature Med.* **6**:1229.
6. Mezey, E. *et al.* (2000) *Science* **290**:1779.
7. Simmons, P.J. *et al.* (1991) *Blood* **78**:55.
8. Colter, D.C. *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98**:7841.
9. Dominici, M. *et al.* (2006) *Cytotherapy* **8**:315.

