Mouse Mesenchymal Stem Cell Functional Identification Kit

Catalog Number SC010

Reagents for the identification of mouse bone marrow-derived stem cells (BMSC)/mesenchymal stem cells (MSC) 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

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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 labs undertake isolation and expansion of stem cells *in vitro* (7-10). During the isolation and expansion of BMSCs/MSCs, the status of stem cells is best evaluated by measuring their ability to differentiate into multiple mesenchymal lineages.

The Mouse Mesenchymal Stem Cell Functional Identification Kit is designed for the identification of mouse 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 Goat Anti-mouse FABP4, Sheep Anti-mouse Collagen II, and Goat Anti-mouse Osteopontin, are included to define the mature phenotypes of adipocytes, chondrocytes, and osteocytes.

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

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 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

PART	PART #	DESCRIPTION
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.
Mouse/Rat Osteogenic Supplement	390441	2.5 mL of a 20X concentrated solution containing ascorbate-phosphate, β-glycerolphosphate, and recombinant human BMP-2; enough to supplement 50 mL of medium.
Chondrogenic Supplement	390417	0.5 mL of a 100X concentrated solution containing dexamethasone, ascorbate-phosphate, proline, pyruvate, and recombinant human TGF-β3; 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.
Goat Anti-mouse FABP4	962643	25 µg of lyophilized Goat Anti-mouse FABP4 polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.
Sheep Anti-mouse Collagen II	965490	25 µg of lyophilized Sheep Anti-mouse Collagen II polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.
Goat Anti-mouse Osteopontin	964400	25 µg of lyophilized Goat Anti-mouse Osteopontin polyclonal antibody; enough to make 2.5 mL of staining solution when used at the suggested concentration of 1 µg/100 µL.

STORAGE CONDITIONS

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at ≤ -20 °C in a manual defrost freezer. Do not use past the expiration date.</td></tr>
<tr><td rowspan="5">Opened Reagents</td><td>Adipogenic Supplement</td><td>Store tightly sealed at 2-8 °C for up to 6 months.*</td></tr>
<tr><td>Mouse/Rat Osteogenic Supplement</td><td rowspan="3">Aliquot and store at ≤ -20 °C in a manual defrost freezer for up to 6 months.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Chondrogenic Supplement</td></tr>
<tr><td>ITS Supplement</td></tr>
<tr><td>Reconstituted Antibodies</td><td>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.</td></tr>
</table>

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

OTHER SUPPLIES REQUIRED

Materials

- Mouse mesenchymal stem cells
- 24-well culture plates
- 12 mm coverslips (Carolina Biologicals, Catalog # 633009 or equivalent)
- 15 mL centrifuge tubes
- Pipettes and pipette tips
- Serological pipettes
- Fine pointed curved forceps
- Glass slides
- Slide box
- Liquid barrier pen

Reagents

- α Minimum Essential Medium (α MEM)
- D-MEM/F-12 (1X)
- Fetal Bovine Serum
- Phosphate-Buffered Saline (PBS)
- Penicillin-Streptomycin-Glutamine (100X)
- Zinc Formalin
- 4% Paraformaldehyde in PBS
- 1% BSA in PBS
- 0.05% Tween® 20 in PBS
- 0.3% Triton™ X-100, 1% BSA, 10% normal donkey serum in PBS
- 1% BSA, 10% normal donkey serum in PBS
- Fibronectin [optional; Catalog # 1030-FN (bovine) or 1918-FN (human)]
- Mounting medium (Catalog # CTS011)
- Secondary developing reagents (Catalog # NL001 and NL010)
- Universal Antigen Retrieval Reagent (Catalog # CTS015)
- Deionized or distilled water

Equipment

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

REAGENT & MATERIAL PREPARATION

Use serological pipettes to transfer and remove solutions.

Preparation of α MEM Basal Medium (*for use with Adipogenic Supplement and Mouse/Rat Osteogenic Supplement*).

Mix the following sterile ingredients to make 101 mL of medium. Store **in the dark** 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	1.0 mL	100 U/mL Penicillin 100 µg/mL Streptomycin 2 mM L-Glutamine

Preparation of D-MEM/F-12 Basal Medium (*for use with Chondrogenic Supplement*).

Mix the following sterile ingredients to make 50 mL of medium. Store **in the dark** at 2-8 °C for up to 1 month. Alternatively, basal medium that has been prequalified for Chondrogenic differentiation may be used (R&D Systems, Catalog # CCM005).

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

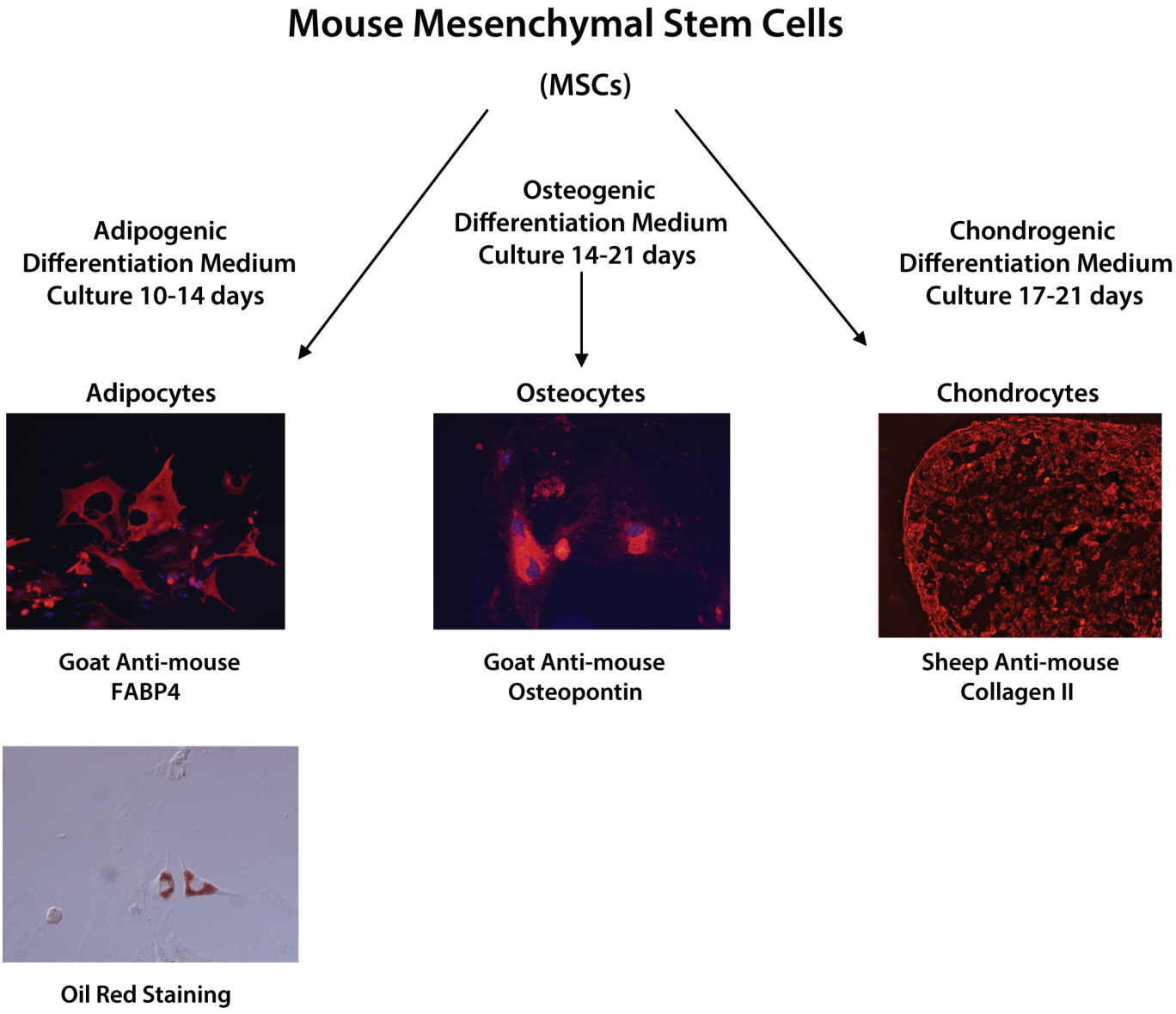
Preparation of Lyophilized Antibodies

Goat Anti-mouse FABP4 - Reconstitute with 250 µL of sterile PBS. Mix gently.

Sheep Anti-mouse Collagen II - Reconstitute with 250 µL of sterile PBS. Mix gently.

Goat Anti-mouse Osteopontin - Reconstitute with 250 µL of sterile PBS. Mix gently.

PROCEDURE OUTLINE



Please refer to the website for full color images (www.RnDSystems.com/pdf/SC010.pdf)

PROCEDURES

I. Adipogenic Differentiation

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

Preparation of Adipogenic Differentiation Medium

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 Medium. Mix gently.
3. Store the unused supplement tightly sealed at 2-8 °C.

Preparation of Culture Plates for Adipogenic Differentiation

1. Insert a sterile coverslip (sterilized with 95% EtOH 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 Medium is sufficient to culture and differentiate 12 wells for 14 days with 6 medium changes. The culturing of 10 wells will provide enough coverslips for 2 monitor stainings and 1 final staining of cells; 1 coverslip for oil red staining and 2 coverslips for immunstaining.*

1. Seed cells at a density of 2.1 x 10^4 cells/cm^2. Each well is approximately 1.76 cm^2 requiring 3.7 x 10^4 cells/well.
2. Prepare 3.7 x 10^5 cells in 5 mL of α MEM Basal Medium.
3. Dispense 0.5 mL of the cell suspension into each of the 10 wells. Incubate overnight in a 37 °C and 5% CO_2 incubator. **Note:** *Cells should be 100% confluent after overnight incubation. If they are not confluent, replace the medium every 2-3 days with α MEM Basal Medium until 100% confluency is reached.*
4. When the cells are 100% confluent, replace the medium in each well with 0.5 mL of Adipogenic Differentiation Medium to induce adipogenesis.
5. Replace with fresh Adipogenic Differentiation Medium (0.5 mL/well) every 3-4 days. After 10 days, lipid vacuoles will start to appear in the induced cells. **Note:** *The adipogenic cells are fragile; medium 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 10-14 days, adipocytes can be fixed and saved for immunostaining (refer to the Fixing and Staining Procedure on page 8).

Fixing & Staining Procedure - 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 PBS containing 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum at room temperature for 45 minutes.
5. During the blocking, dilute the reconstituted Goat Anti-mouse FABP4 Antibody in PBS containing 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 1% BSA and 10% normal donkey serum with no primary antibody.*
6. After blocking, incubate the cells with 300 µL/well of Goat Anti-mouse FABP4 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 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).

II. Osteogenic Differentiation

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

Preparation of Osteogenic Differentiation Medium

1. Warm the Mouse/Rat Osteogenic Supplement vial in a 37 °C water bath for 5 minutes.
2. Add 250 µL of the Mouse/Rat Osteogenic Supplement to 5 mL of α MEM Basal Medium. Mix gently.
3. Divide the unused supplement into 250 µL aliquots and store at ≤ -20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.

Preparation of Culture Plates for Osteogenic Differentiation

1. Insert a sterile coverslip (sterilized with 95% EtOH 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 Medium will provide adequate medium to culture and differentiate 16 wells for 21 days with 6 medium changes. The culturing of 10 wells will provide enough coverslips for weekly monitoring of cells. Additional coverslips can be used for Alizarin red staining if desired.*

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 x 10^3 cells/cm^2. Each well is approximately 1.76 cm^2 requiring 7.4 x 10^3 cells/well.
2. Prepare 7.4 x 10^4 cells in 5 mL of α MEM Basal Medium.
3. Dispense 0.5 mL of the cell suspension into each of the 10 wells. Incubate overnight in a 37 °C and 5% CO_2 incubator. **Note:** *The cells should be about 50-70% confluent in 1-2 days.*
4. At 50-70% confluency, replace the medium in each well with 0.5 mL of Osteogenic Differentiation Medium to induce osteogenesis.
5. Replace with 0.5 mL of fresh Osteogenic Differentiation Medium (0.5 mL/well) every 2-3 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 Procedure on page 11). Cells may also be ethanol fixed and stained with Alizarin Red. For a staining protocol, please see reference 8.

Fixing & Staining Procedure - 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 Goat Anti-mouse Osteopontin Antibody in PBS containing 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 1% BSA and 10% normal donkey serum with no primary antibody.*
6. After blocking, incubate the cells with 300 µL/well of Goat Anti-mouse Osteopontin 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 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).

III. Chondrogenic Differentiation

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

Preparation of Chondrogenic Differentiation Medium

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 Medium. Mix gently.
3. Divide the unused supplement into 25 µL aliquots and store at ≤ -20 °C in a manual defrost freezer. Avoid repeated freeze-thaw cycles.

Chondrogenesis Culture Protocol

1. Transfer 250,000 cells in their existing culture medium to a 15 mL conical tube.
2. Centrifuge the cells at 200 x g for 5 minutes at room temperature. Remove the medium and resuspend the cells with 1.0 mL of D-MEM/F-12 Basal Medium.
3. Centrifuge the cells at 200 x g for 5 minutes. Aspirate and discard the medium.
4. Resuspend the cells in 0.5 mL of Chondrogenic Differentiation Medium, and centrifuge the cells at 200 x g for 5 minutes at room temperature. Do not remove the medium.
5. Loosen the cap(s) of the tubes to allow gas exchange, and incubate upright at 37 °C and 5% CO_2.
6. Replace the medium with 0.5 mL of fresh Chondrogenic Differentiation Medium every 2-3 days. The pellet should not be attached to the tube. **Note:** *Use caution when replacing the medium to avoid aspirating the pellet.*
7. After 17-21 days, the chondrocyte pellet can be fixed and prepared for frozen sectioning (refer to the Fixing and Staining Procedure on page 13).

Fixing & Staining Procedure - 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 0.5 mL of Zinc formalin solution overnight at 2-8 °C.
3. After the overnight incubation, 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. Perform antigen retrieval using the Universal Antigen Retrieval Reagent from R&D Systems (Catalog # CTS015) according to the instructions in the product insert.
6. Permeabilize and block the mounted pellet sections with 0.3 mL of 0.3% Triton X-100, 1% BSA, and 10% normal donkey serum in PBS at room temperature for 30 minutes.
7. During blocking, dilute the reconstituted Sheep Anti-mouse Collagen II Antibody in PBS containing 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 1% BSA and 10% normal donkey serum with no primary antibody.*
8. After blocking, incubate sections with 300 µL/well of Sheep Anti-mouse Collagen II Antibody working solution for 1 hour at room temperature. Keep in a covered container with adequate moisture.
9. Wash the slides three times with 0.3 mL of PBS containing 0.05% Tween 20 for 3 minutes.
10. Dilute the secondary antibody [e.g., NL557-conjugated Donkey Anti-sheep Secondary Antibody (Catalog # NL010)] 1:200 in PBS containing 1% BSA.
11. Incubate the sections with secondary antibody at 300 µL per section **in the dark** for 30 minutes at room temperature.
12. Wash the slides three times with 0.3 mL of PBS containing 0.05% Tween 20 for 3 minutes.
13. Wash the slides once with distilled water, and remove 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).

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. Sun, S. *et al.* (2003) Stem Cells **21**:527.
10. Phinney, D.G. *et al.* (1999) J. Cell. Biochem. **72**:570.