TECHNICAL MANUAL

VERSION 2.2.0

Enumeration, Expansion, and Differentiation of Human Mesenchymal Progenitor Cells Using MesenCult®

STEMCELL TECHNOLOGIES

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1.0 Introduction

The bone marrow stroma was originally thought to function mainly as a structural framework for hematopoietic stem and progenitor cells in the bone marrow. It has now been established that the stroma consists of a heterogeneous population of cells including endothelial cells, fibroblasts, adipocytes, and osteogenic cells, a subset of which exerts both positive and negative regulatory effects on the proliferation and differentiation of hematopoietic cells.[1,2] The adherent stromal cell population is also believed to contain other non-hematopoietic cells that are capable of both self-renewal and differentiation into bone, cartilage, muscle, tendon, and fat.[3-5] Characterization of the stromal cells was initiated in the early 1980s when the morphological and cytochemical properties of the cultured cells were described (Sudan Black$^+$, alkaline phosphatase$^+$, esterase$^-$, collagen IV$^+$, fibronectin$^+$).[6,7] In 1991, Simmons and Torok-Storb described the first antibody (STRO-1) that targeted the stromal precursor in human bone marrow.[8]

The colony-forming unit-fibroblast (CFU-F) assay is used by many investigators as a functional method to quantify stromal progenitor cells. There appears to be a strong correlation between age and proliferative potential, with decreasing progenitor cell proliferation associated with increasing age.[9,10] Abnormal function of stromal precursor cells has been implicated in several diseases.[11,12] Transplantation of unprocessed bone marrow cells can restore micro-environmental function, suggesting that unprocessed bone marrow contains both stromal precursor cells and hematopoietic precursor cells. Studies by Gallatto *et al.* demonstrate that these microenvironmental precursor cells, as measured by the CFU-F assay, are susceptible to damage following chemotherapy or radiation and remain at a significantly reduced frequency for a considerable time following transplantation.[13] Acknowledging that stromal cells provide regulatory stimuli to other cells may explain in part why there is a slow and skewed recovery of many immune cell populations following transplantation.[14]

In recent years there has been increased interest in stromal cells and their function in both the tissue engineering and stem cell plasticity fields. This increase in interest has been fueled by the observation that cultured stromal cell populations are capable of both self-renewal and differentiation, characteristics typically associated with stem cells. These traits have led many researchers to refer to cultured stromal cells as mesenchymal stem cells (MSC).

Horwitz and colleagues attempted to clarify and standardize the terminology for mesenchymal stem cells (MSC) when they proposed the terminology "multipotent mesenchymal stromal cells" to describe all cultured fibroblast-like plastic-adherent cells, reserving the term "mesenchymal stem cells" for a subset of cells that clearly demonstrate stem cell properties.[15] Horwitz and colleagues further stated that for both cell populations the acronym MSC may be used with detailed description of the characteristics of the cell population studied by each investigator. This distinction is important in the mesenchymal research field because mesenchymal cells exhibit different phenotypes depending on various factors such as culture method, medium composition, plating density of the cells, and source of isolated cells.

Cultured mesenchymal cells have been characterized using panels of antibodies and are defined as CD45$^-$CD34$^-$ SH2(CD105)$^+$SH3$^+$CD90(THY-1)$^+$ cells.[5] Other markers used by researchers to identify these cultured mesenchymal cells include STRO-1, CD49a, SB10, D7-FIB, LNGFR, CD106, HOP-26, CD144, CD166, CD115, CD29 and HLA-ABC.[16-20] However, the exact phenotype of the stromal (mesenchymal) precursor cell in human bone marrow (i.e. the cell phenotype prior to culture) is still debated. The isolation and enrichment of human mesenchymal cells have utilized some of the simple characteristics of the human MSC, such as adherence of the cell when cultured, as well as cell separation strategies using cocktails of antibodies that deplete the bone marrow of specific cell populations.[21,22]

Cultured mesenchymal cells have been shown to exhibit some unique properties that challenge the dogma that stem cells derived from adult tissue produce only the cell lineages characteristic of tissues in which they reside. Studies by Verfaillie's group have demonstrated the ability of cultured MAPC, a subset of cells copurified with MSC, to differentiate into neural cells, skeletal cells, cardiomyocytes, endothelial cells and smooth muscle cells.[23] The expanding knowledge of the biology of specific cell populations may be the foundation for future therapies in many areas outside of hematology and oncology.

There is a great deal of clinical interest in mesenchymal cells and the following clinical applications are presently being evaluated:

- expansion and reinfusion of MSC into patients in an attempt to reconstitute the microenvironment and provide optimal conditions to support hematopoiesis[24]
- gene transfer into MSC[25]
- repair of mesenchymal tissues[26,27]
- ability of mesenchymal cells to support hematopoietic cell expansion[28,29]
- treatment of graft versus host disease (GVHD)[30,31]

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2.0 Materials

2.1 Product Description

Product Description	Volume	Catalog #
MesenCult® MSC Basal Medium (Human)	450 mL	05401
Mesenchymal Stem Cell Stimulatory Supplements (Human)	50 mL	05402
MesenCult® Adipogenic Stimulatory Supplements (Human)	50 mL	05403
MesenCult® Osteogenic Stimulatory Kit (Human)	To make 500 mL	05404

2.2 Related Products

Product Description	Volume	Catalog #
Frozen Marrow Stromal Cells	7.5 x 10^5 cells	MSC-001F
Fetal Bovine Serum for Human Mesenchymal Stem Cells	100 mL 500 mL	06471 06472
Fetal Bovine Serum for Human Osteogenic Precursors	100 mL 500 mL	06473 06474
RosetteSep® Mesenchymal Stem Cell Enrichment Cocktail	2 mL 10 mL	15128 15168

2.3 Storage Conditions

MesenCult® MSC Basal Medium (Human; Catalog #05401) is stable at 2 - 8°C for 1 year from date of manufacture.

Mesenchymal Stem Cell Stimulatory Supplements (Human; Catalog #05402) and Adipogenic Stimulatory Supplements (Human; Catalog #05403) are stable at -20°C for 2 years from date of manufacture. Storage at 2 - 8°C is not recommended. If the entire volume of supplements is not needed at one time, they can be aliquoted and refrozen. Do not freeze-thaw aliquots more than twice.

Storage Conditions for MesenCult® Osteogenic Stimulatory Kit (Catalog #05404) Components:

Catalog #	Description	Unit Size	Stability and Storage Conditions
05401	MesenCult® MSC Basal Medium (Human)	450 mL	Store at 2 - 8°C. Stable for 1 year from date of manufacture.
05405	Osteogenic Stimulatory Supplements (Human)	80 mL	Store at -20°C. Stable for at least 1 year.
05406	ß-Glycerophosphate 1.0 M	10 mL	Store at -20°C. Stable for at least 2 years.
05407	Dexamethasone	1 mg	Store at 2 - 8°C. Stable for at least 2 years.
07157	Ascorbic Acid	100 mg	Store at room temperature. Stable for at least 2 years.

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2.4 Equipment, Supplies, and Reagents Required

To perform all assays, the following equipment and reagents are required, in addition to MesenCult® MSC Basal Medium and Supplements:

- Biohazard safety cabinet certified for level II handling of biological materials (e.g. Canadian Cabinets)
- 37°C incubator with humidity and gas control to maintain >95% humidity and an atmosphere of 5% CO_2 in air (e.g. Forma 3326)
- Laboratory centrifuge
- Standard light microscope (for cell counting)
- Inverted microscope
- Hemacytometer
- Good quality tissue culture-treated dishes, flasks, or plates: 100 mm culture dishes (Catalog #27125/27127), T-25 cm^2 tissue culture flasks (Falcon Catalog #353109), or 6-well tissue culture-treated plates (Falcon Catalog #353502 or Corning Catalog #3506)

  Always use tissue culture-treated dishes, flasks, or plates to culture mesenchymal stem cells.
- 1 mL and 10 mL sterile pipettes
- Pipette-aid (e.g. Drummond Scientific)
- 14 mL polypropylene tubes (Falcon Catalog #352001)
- PBS with 2% Fetal Bovine Serum (Catalog #07905)
- Ammonium Chloride Solution (Catalog #07800)
- PBS (Catalog #37350)
- Trypsin-EDTA (Catalog #07901)
- Ficoll-Paque™ PLUS (Catalog #07957)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Trypan Blue (Catalog #07050)

To stain CFU-F colonies, the following materials are required:

- PBS (Catalog #37350)
- Methanol ACS (BDH Catalog #ACS531)
- Giemsa Staining Solution (EMD Chemicals Catalog #R03055)
- Distilled water

The Osteogenic Assay requires:

- 70 µm cell strainer (Falcon Catalog #352350)

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3.0 Human Colony-Forming Unit - Fibroblast (CFU-F) Assay

Fresh samples of human bone marrow can be processed and plated in limiting cell densities to assess the number of mesenchymal stem and progenitor cells using the CFU-F assay.

3.1 Culture Set-Up

3.1.1 Preparation of Complete MesenCult® Medium (Human)

Thaw Mesenchymal Stem Cell Stimulatory Supplements (Human; Catalog #05402) at room temperature or 2 - 8°C overnight. Add the entire contents of the Mesenchymal Stimulatory Supplements (Human) to MesenCult® MSC Basal Medium (Human; Catalog #05401) and mix thoroughly. This is now referred to as **Complete MesenCult® Medium (Human)**.

Complete MesenCult® Medium (Human) is stored at 2 - 8°C and should be prepared in volumes that can be used within 1 month. If less than 500 mL will be required, smaller volumes can be prepared. Prepare Complete MesenCult® Medium (Human) by diluting Mesenchymal Stem Cell Stimulatory Supplements (Human) 1/10 with MesenCult® MSC Basal Medium (Human). For example, prepare 100 mL of Complete MesenCult® Medium (Human) by adding 10 mL of Mesenchymal Stem Cell Stimulatory Supplements (Human) to 90 mL of MesenCult® MSC Basal Medium (Human).

It is not necessary to add additional fetal bovine serum to Complete MesenCult® Medium (Human). Fetal bovine serum is already a component of the Mesenchymal Stem Cell Stimulatory Supplements (Human).

For long-term storage of Complete MesenCult® Medium (Human) refer to Section 5.1: Helpful Hints.

3.1.2 Processing of Cells and the CFU-F Assay

1. When working with a fresh bone marrow (BM) sample, the cells need to be processed to remove the red blood cells or enrich desired cells prior to culture. Choose one of the following methods:
   - Ammonium chloride lysis (to remove the red blood cells, Catalog #07800)
   - Isolation of the mononuclear cells by Ficoll-Paque™ PLUS (Catalog #07907/07957) density gradient separation
   - Enrichment of mesenchymal stem cells using the RosetteSep® Human Mesenchymal Stem Cell Enrichment Kit (Catalog #15128/15168, refer to Appendix 1 for more information)

   The CFU-F assay cannot be performed with previously frozen bone marrow mononuclear cells or culture-expanded mesenchymal stem cells.
2. After processing, wash the cells by adding 10 mL of PBS with 2% FBS (Catalog #07905) to the cell pellet. Centrifuge the cells at 300 x *g* (~1200 rpm) for 10 minutes at 20°C. Remove the supernatant and resuspend the cells in 1 - 2 mL of Complete MesenCult® Medium (Human).
3. If working with BM cells processed with ammonium chloride or Ficoll-Paque™ PLUS, perform a nucleated cell count (using 3% Acetic Acid with Methylene Blue, Catalog #07060) and dilute the cells to a stock cell concentration of 2 x 10^6 cells/mL in Complete MesenCult® Medium (Human). If working with enriched mesenchymal cells isolated using the RosetteSep® Enrichment Kit for Human Mesenchymal Stem Cells (Catalog #15128/15168), perform a cell count and dilute cells to a stock cell concentration of 5 x 10^5 cells/mL in Complete MesenCult® Medium (Human).
4. Plate three different cell densities by adding 1.0 mL, 0.5 mL, and 0.25 mL of the cells at stock concentration to separate 100 mm tissue culture-treated dishes (or T-25 cm^2 tissue culture flasks) prefilled with Complete MesenCult® Medium (Human) to a total volume of 10 mL. For Ficolled or lysed BM cells, this will yield final cell count of 2 x 10^6 cells, 1 x 10^6 cells and 0.5 x 10^6 cells in 10 mL of medium. For RosetteSep®-enriched cells this will yield final cell count of 5 x 10^5 cells, 2.5 x 10^5 cells and 1.25 x 10^5 cells in 10 mL of medium.

   Plating three concentrations will ensure that the resulting numbers of colonies can be scored, as there are differences in the proliferative potential of CFU-F from various bone marrow samples.
5. Place the 100 mm dishes (or T-25 cm^2 tissue culture flasks) into a 37°C humidified incubator with 5% CO_2 in air and >95% humidity for 14 days.

   Maximum colony size and numbers are typically observed at 14 days.

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3.2 Suggested Procedure for Staining and Enumeration of CFU-F-Derived Colonies

3.2.1 Staining

1. Remove the medium from cultures of CFU-F grown in 100 mm tissue culture dishes or T-25 cm^2 tissue culture flasks and discard into the biohazardous waste. The adherent colonies will remain attached to the plate.
2. Wash the culture dishes or flasks twice using PBS (Catalog #37350) to remove any remaining medium. Discard the PBS from the two washes into the biohazardous waste.
3. Add 5 mL of methanol to each culture dish or flask for 5 minutes at room temperature.

   Addition of methanol fixes the cells to the tissue culture dishes or flasks.
4. Remove the methanol and discard into the biohazardous waste. Let the culture dishes or flasks air dry at room temperature.
5. Add 5 mL of Giemsa Staining Solution (EMD Chemicals Catalog #R03055) to each culture dish or flask and leave for 5 minutes.
6. Remove the Giemsa Staining Solution and rinse the culture dishes or flasks with distilled water to remove non-bound stain. Rinse until water remains clear.
7. Discard the distilled water into the biohazardous waste and allow the tissue culture dishes or flasks to dry at room temperature.

3.2.2 Enumeration

CFU-F colonies from human cells are typically between 1 - 8 mm in diameter and may be scored macroscopically. Photographs of representative CFU-F-derived colonies are shown in Section 3.3. Ensure that there is a linear relationship between the cell numbers plated and the resulting colony numbers, by confirming that there are twice as many colonies when 2 x 10^6 cells are plated as compared to 1.0 x 10^6 cells. Likewise, there should be twice as many colonies when 1.0 x 10^6 cells are plated as compared to 0.5 x 10^6 cells. Ideally there should be 10 - 40 colonies per 100 mm dish or T-25 cm^2 flask. Linearity may not be observed outside of this range as the cells would have been under or overplated.

Each bone marrow sample is unique for that donor and the number of CFU-F may depend on a number of factors including age, presence of disease and previous treatments given to the patient.

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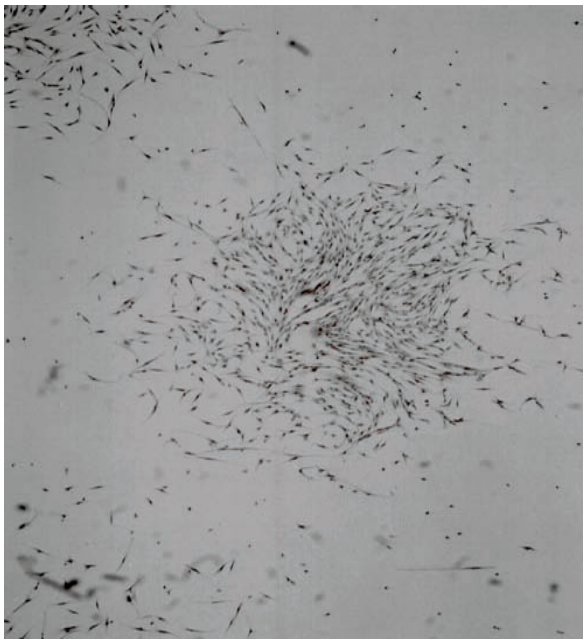
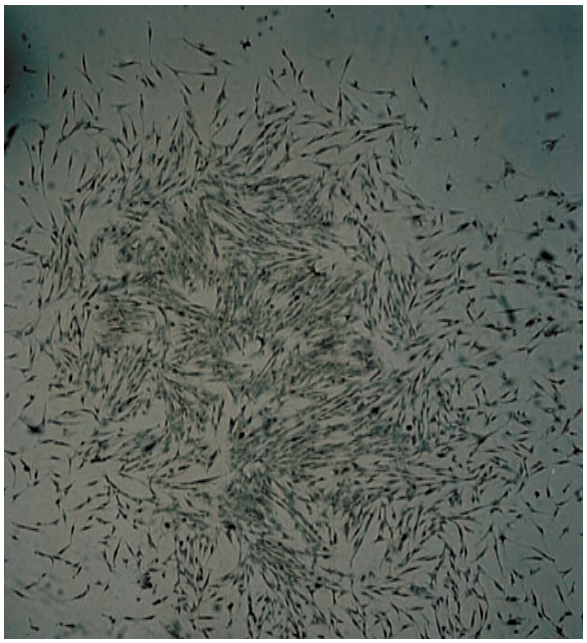
3.3 Photographs of Human CFU-F-Derived Colonies

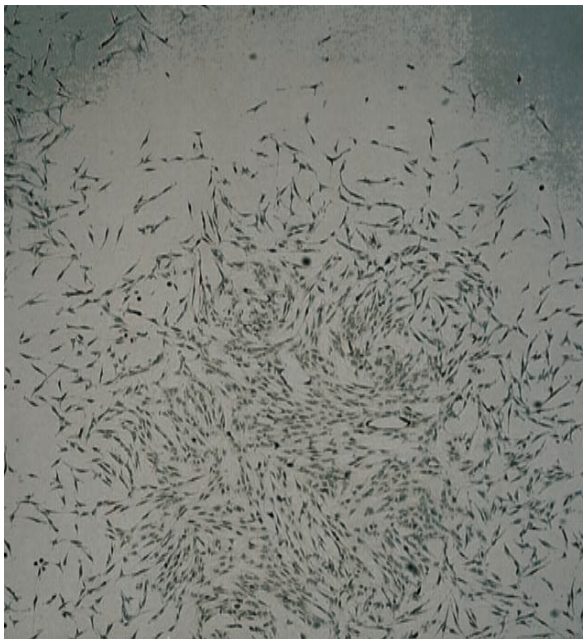


Medium colony stained with Giemsa



Large colony stained with Giemsa

Large colony stained with Giemsa



Large colony stained with Giemsa

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4.0 Expansion of Cultured Mesenchymal Cells

Confluent mesenchymal cell cultures can be produced when cells from bone marrow are plated at relatively high densities on tissue culture-treated flasks or dishes in Complete MesenCult® Medium (Human). Mesenchymal cell numbers can then be expanded by passaging when the cells become 80% confluent in the flask or dish. If cells remain in a confluent state for a significant time (days) it may reduce their longevity and their potential to differentiate. Culture-expanded mesenchymal cells can be used for a number of applications including plasticity studies, assessment of differentiation or expansion potential, and the evaluation of phenotype.

4.1 Culture Set-up

1. Use the following table for recommended plating concentrations for processed human bone marrow cells. Plate the recommended cell number in a T-25 cm^2 flask in 10 mL Complete MesenCult® Medium (Human).

   Cells must be plated using tissue culture-treated flasks.

Cell Source	Cells Plated
Fresh BM treated with NH_4Cl	1.0 - 1.5 x 10^7 mononuclear cells
Fresh BM purified with Ficoll-Paque™ PLUS	1.0 - 1.5 x 10^7 mononuclear cells
Fresh BM enriched using the RosetteSep® Human Mesenchymal Stem Cell Enrichment Kit	1.0 - 2.0 x 10^6 enriched cells
Frozen Marrow Stromal Cells (Catalog #MSC-001F)	1.25 - 2.5 x 10^5 cells

4.2 Passaging Cultured Mesenchymal Cells

1. Check mesenchymal cells under a microscope to ensure that the cells are at an adequate stage for passaging (~80% confluence). This should take approximately 7 - 10 days for primary bone marrow cells but less time for culture-expanded cells. If the medium in the flask or dish appears acidic (more yellow in color than orange/red) prior to reaching 80% confluency, a half-medium change can be done by removing one half of the acidic medium and replacing it with fresh Complete MesenCult® Medium (Human) prewarmed to 37°C.

   The proliferative ability of each marrow is donor-dependent and can be affected by a number of factors including age, disease or whether the sample comes from a transplant recipient. Therefore not all bone marrow samples may be confluent in a week and a half-medium change may help cells to proliferate in some samples.

2. Remove Complete MesenCult® Medium (Human) from cultures. Adherent mesenchymal cells will remain behind. Wash the cells with PBS (Catalog #37350) to remove residual FBS-containing medium.
3. Add 5 mL Trypsin-EDTA (Catalog #07901) to cover cells and incubate at 37°C for 3 - 7 minutes.
4. Check under microscope to ensure that the mesenchymal cells have detached. Add 1 mL FBS (Catalog #06471 or quality cell culture tested equivalent) to neutralize the action of trypsin or alternatively add 5 mL of Complete MesenCult® Medium (Human).
5. Collect trypsinized cells into a 14 mL tube and centrifuge the cells at 300 x *g* (~1200 rpm) for 8 minutes at room temperature with the brake on. Remove supernatant and resuspend pelleted cells in Complete MesenCult® Medium (Human).
6. The cells can now be divided into new tissue culture-treated flasks. The recommended dilution is 1/4 (e.g. one T-25 cm^2 tissue culture-treated flask containing 80% confluent mesenchymal cells can be passaged into four T-25 cm^2 tissue culture-treated flasks).

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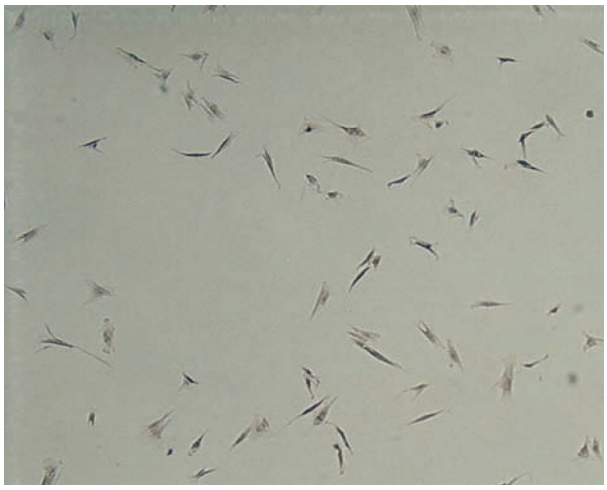
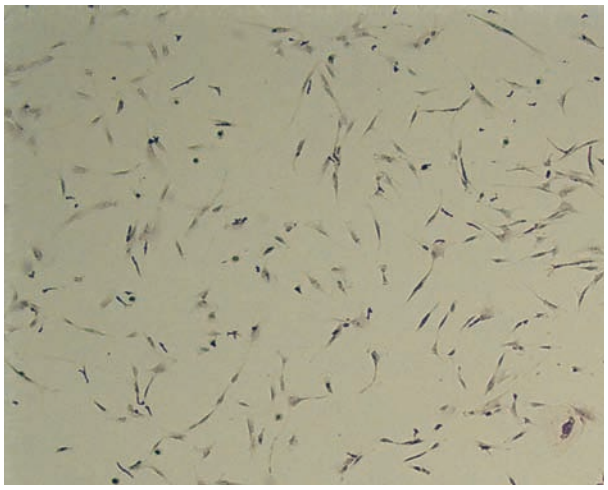
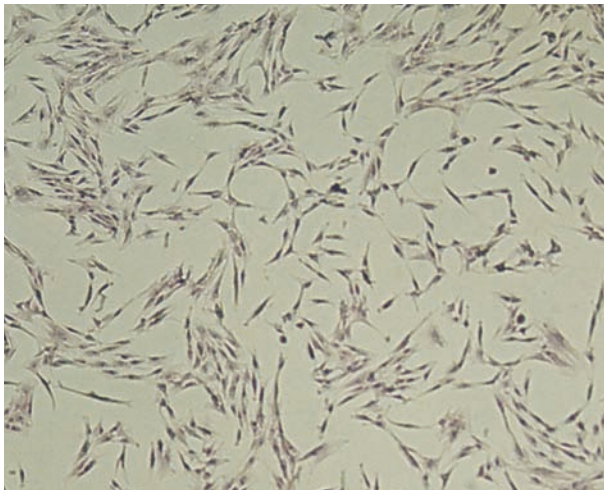
4.3 Photographs of Cultured Mesenchymal Cells



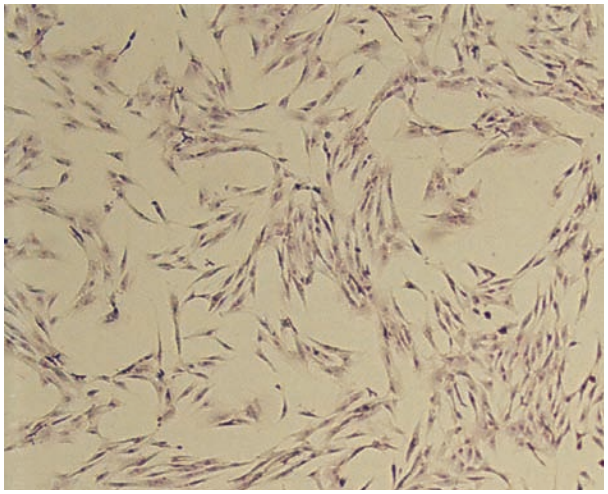
Mesenchymal cell culture at ~20% confluency.



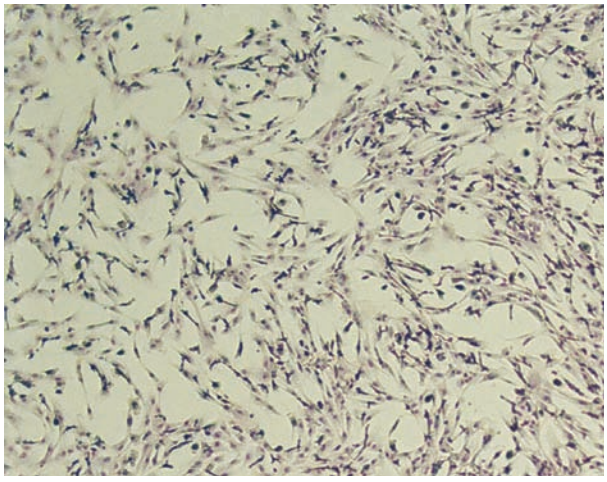
Mesenchymal cell culture at ~40% confluency.



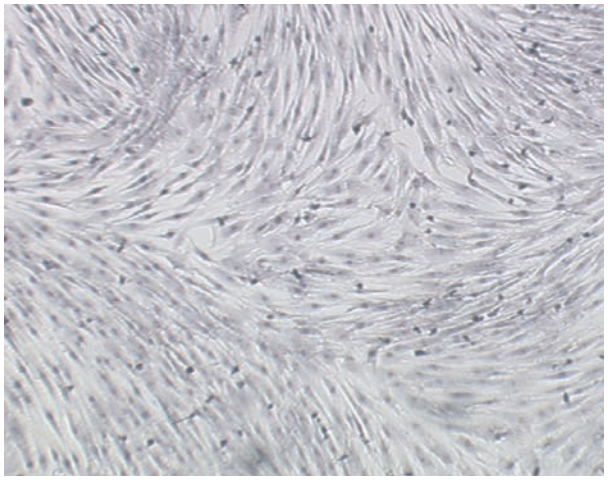
Mesenchymal cell culture at ~60% confluency.



Mesenchymal cell culture at ~70% confluency.



Mesenchymal cell culture at ~80% confluency (ideal for passaging).



Confluent mesenchymal cell culture (past optimal time for passaging).

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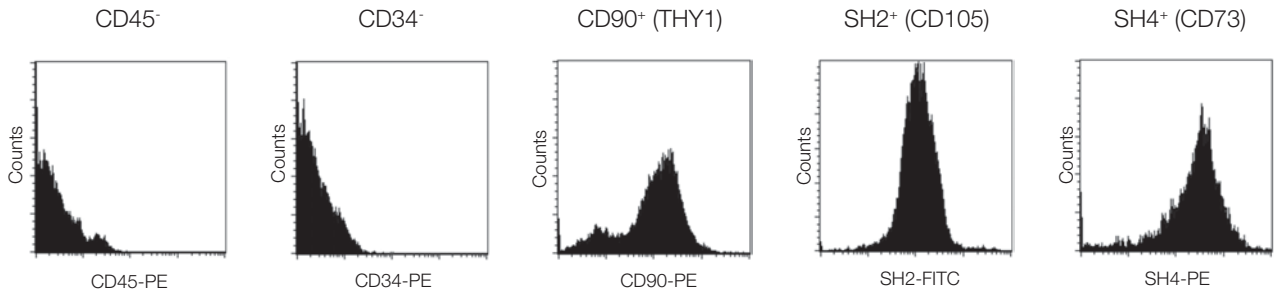
4.4 Phenotype of Cultured Cells

StemCell Technologies classifies cultured human mesenchymal stem cells as being negative for the expression of CD45 and CD34 and positive for CD90 (THY-1), SH2 (CD105), and SH4 (CD73). The phenotype of cultured MSC is therefore defined as: $CD45^-CD34^-CD90(THY\text{-}1)^+SH2(CD105)^+SH4(CD73)^+$.



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5.0 Helpful Hints and Frequently Asked Questions

5.1 Helpful Hints

1. Thawing of Mesenchymal Stem Cell Stimulatory Supplements should preferably be performed overnight under refrigeration (2 - 8°C). If this is not possible, thawing of supplements in a 37°C water bath is permissible.

   Do not thaw supplement in a 56°C water bath.

2. Once prepared, the complete medium is stable at 2 - 8°C for one month. If the volume of the complete medium exceeds your monthly requirements, it is possible to aliquot the supplements and store at -20°C. Therefore, smaller volumes of complete medium can be prepared ensuring the supplements represent one-tenth of the total volume (i.e. 10 mL of supplements to 90 mL of basal medium). Do not freeze-thaw supplements more than twice.

3. Cell counts should be performed in 3% Acetic Acid with Methylene Blue (Catalog #07060) to obtain an accurate white cell count.

4. Tissue culture-treated dishes must be used to support the growth of CFU-F and expansion of cultured mesenchymal cells. *StemCell Technologies* recommends 100 mm culture dishes (Catalog #27125/27127) or T-25 cm^2 tissue culture flasks (Falcon Catalog #353109) or 6-well tissue culture treated plates (Falcon Catalog #353502 or Corning Catalog #3506).

5.2 Frequently Asked Questions

1. Why don't my cultured cells look like the photographs?

There are a number of potential reasons for abnormal morphology of cultured mesenchymal cells:

- Cells reached 100% confluency and were not trypsinized in a timely fashion
- Bone marrow sample was not fresh or stored properly
- Bone marrow sample was from a patient post transplantation (often not able to make confluent layer due to low proliferative potential)
- Bone marrow sample was treated with specific drugs such as 5FU

2. How long can I expect cultured mesenchymal cells to maintain their potential?

Usually culture-expanded mesenchymal cells can be maintained for 6 - 8 passages depending on the marrow. We have expanded cultures of mesenchymal cells for longer than 7 weeks and confirmed that phenotypically the cells were no different at passage 8 than they were at passage 2.

Expansion of mesenchymal cells from 4 different bone marrow samples grown with Complete MesenCult® Medium (Human):

Bone Marrow Sample	Fold Expansion	Number of Passages	Days in Culture
A	3730X	5	40
B	6970X	8	58
C	1770X	6	41
D	230X	4	28

3. Can I grow or isolate mesenchymal stem cells from cord blood?

There are groups that have shown that MSCs can be isolated and cultured from cord blood samples when the cord blood is very fresh (i.e. less than 6 hours old).[32-34] We have not been successful when working with samples that are processed and plated more than 6 hours from collection.

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4. Can I cryopreserve cultured mesenchymal stem cells?

Mesenchymal stem cells can be frozen at any passage. Studies in our laboratory have shown that cryopreserved cells from various passage numbers 2 - 7 maintain their phenotype and differentiation potential.

Recommended Protocol for Freezing Mesenchymal Cells

Before beginning have all reagents COLD (2 - 8°C) and label sterile cryovials using an indelible marker.

1. Make up 20% Dimethyl Sulfoxide (DMSO) in Fetal Bovine Serum (FBS; Catalog #06471 or quality cell culture tested equivalent) and filter sterilize using a 0.2 µm filter. Keep on ice.
2. Harvest cells from the tissue culture surface (refer to Section 4.2 for a suggested protocol). Centrifuge cells and resuspend in FBS to give a maximum concentration of 2 x 10^6 cells per mL. Place this cell suspension on ice.
3. Mix cells gently with 20% DMSO in FBS at a ratio of 1:1 (the final cell suspension will be 90% FBS/10% DMSO). Transfer 1 mL of cells in freezing medium to each cryovial. Final cell concentration will be ~1 x 10^6 cells per vial.
4. Place cryovials immediately into thawed 70% isopropanol freezing container. Place container in -135°C freezer overnight.

   Do not let cells sit in freezing medium at room temperature. Keep on ice and transfer within 5 minutes to the freezing container.
5. On the next day, remove frozen vials from the freezing container and store at -135°C or colder or in liquid nitrogen.

Recommended Procedure for Thawing Cells

1. Thaw cells quickly in a 37°C water bath or beaker of warm water in a tissue culture hood. Wipe the cryovial with 70% ethanol.

   Do not vortex cells at any time.
2. Gently transfer cells into a 50 mL centrifuge tube.
3. Slowly add 15 mL IMDM with 2% FBS (Catalog #07700) dropwise while holding tube and gently swirling.
4. Fill tube to 50 mL with IMDM containing 2% FBS. Gently invert tube to mix.
5. Centrifuge cells at 300 x *g* (~1200 rpm) for 8 minutes.
6. Discard supernatant and 'flick' tube gently to resuspend the pellet.

   If cells are clumpy, add 0.25 - 0.5 mL of 1 mg/mL DNAse I (Catalog #07900) to the resuspended cells and repeat Steps 3 - 6.
7. Resuspend cells at desired concentration in Complete MesenCult® Medium (Human).

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6.0 Mesenchymal Adipogenic Assay using MesenCult®

6.1 Culture Set-up

1. Thaw MesenCult® Adipogenic Stimulatory Supplements (Catalog #05403) at room temperature or 2 - 8°C overnight.
2. Add the entire contents of the Adipogenic Supplements to MesenCult® MSC Basal Medium (Human; Catalog #05401) and mix thoroughly. This is now referred to as Complete MesenCult® Adipogenic Medium. If less than 500 mL will be required in one month, smaller volumes can be prepared. Aliquot supplements and prepare Complete Mesencult® Adipogenic Medium by using the same method described in Section 3.1.1. Complete MesenCult® Adipogenic Medium should be stored at 2 - 8°C and used within one month.
3. Use the following table for recommended plating concentrations for processed human bone marrow cells. Plate the recommended cell numbers in a T-25 cm^2 flask in 10 mL of Complete MesenCult® Adipogenic Medium.

Cell Source	Cells Plated
Fresh BM treated with NH_4Cl	1.0 - 1.5 x 10^7 mononuclear cells
Fresh BM purified with Ficoll-Paque™ PLUS	1.0 - 1.5 x 10^7 mononuclear cells
Fresh BM enriched using the RosetteSep® Human Mesenchymal Stem Cell Enrichment Kit	1.0 x 10^6 enriched cells
Frozen Marrow Stromal Cells (Catalog #MSC-001F)	1.25 - 2.5 x 10^5 cells

4. Culture cells for 1 - 2 weeks. No medium changes are required unless medium become yellow/orange in color, in which case a half-medium change should be performed. Adipogenic development will be visible as fat globules in cells in specific areas throughout the culture. Refer to Section 6.2 for representative photographs.

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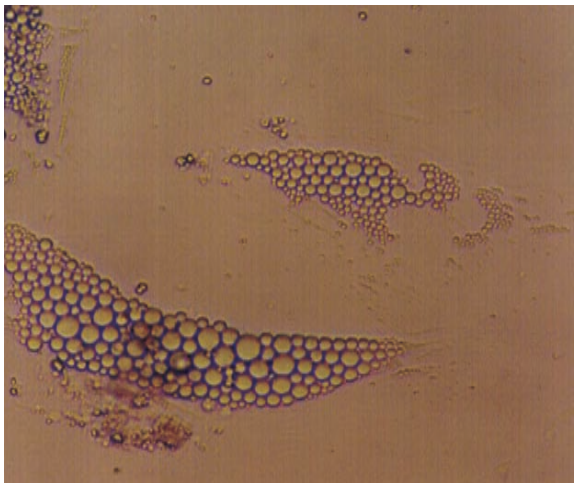
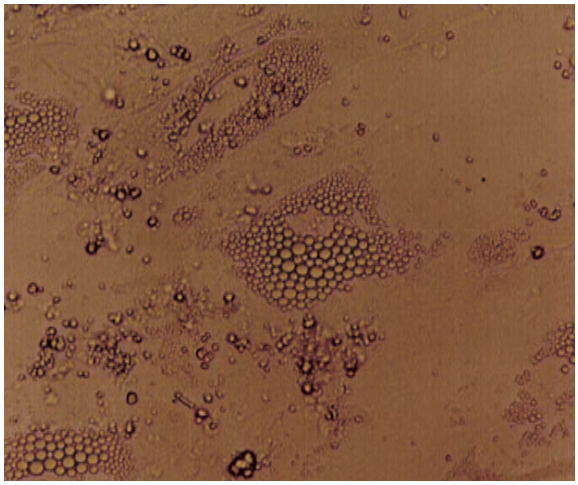
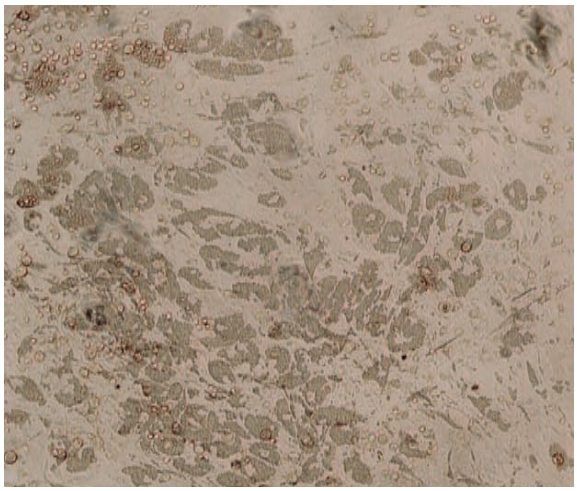
6.2 Photographs of Human Adipocytes



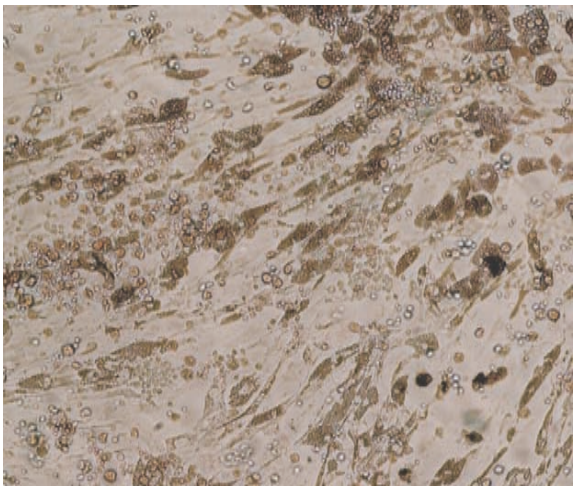
Human adipocytes generated from confluent cultured mesenchymal cells.



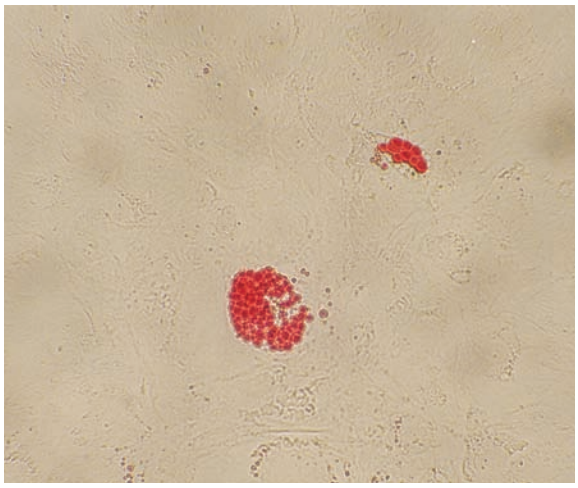
Human adipocytes generated from confluent cultured mesenchymal cells.



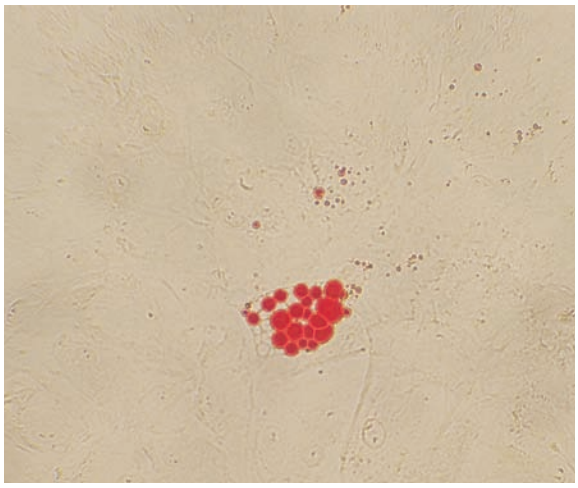
Human adipocytes generated from ficolled bone marrow.



Human adipocytes generated from ficolled bone marrow.



Human adipocytes generated from ficolled bone marrow, stained with Oil Red-O.



Human adipocytes generated from ficolled bone marrow, stained with Oil Red-O.

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7.0 Mesenchymal Osteogenic Assay using MesenCult®

7.1 Preparation of Complete MesenCult® Osteogenic Medium

1. Fresh Complete MesenCult® Osteogenic Medium should be prepared weekly. The amount of medium to be prepared should be based on the numbers of cultures that need medium. Instructions are provided for the preparation of 50 mL of Complete MesenCult® Osteogenic Medium. To facilitate this, reagents should be aliquoted and stored upon arrival as described below:
   - **MesenCult® MSC Basal Medium** (Human; Catalog #05401, 450 mL).
     - Aliquot into 10 x 45 mL and store at 2 - 8°C.
   - **Osteogenic Stimulatory Supplements** (Human; Catalog #05405, 80 mL) used at 15% final volume.
     - Aliquot into 10 x 8 mL and store at -20°C.
   - **ß-Glycerophosphate** (Catalog #05406, 10 mL, 1 M), used at a final concentration of 3.5 mM in human assays and 5.0 mM in rat assays.
     - Aliquot into 10 x 1 mL vials and store at -20°C.
   - **Dexamethasone** (Catalog #05407, 1 mg), used at a final concentration of 10^{-8} M.
     - Dissolve the powder in a small volume of absolute ethanol and then add ethanol to a final volume of 25.5 mL to make a stock concentration of 10^{-4} M.
     - Aliquot into multiple 500 µL vials and store at -20°C.
   - **Ascorbic Acid** (Catalog #07157, 100 mg), used at a final concentration of 50 µg/mL.
     - Dissolve the powder in 10 mL of MesenCult® MSC Basal Medium (Human) to obtain a stock solution of 10 mg/mL.
     - Aliquot into 10 x 1 mL vials and store at -20°C.

2. To prepare Complete MesenCult® Osteogenic Medium, pipette 42.5 mL of MesenCult® MSC Basal Medium (Human) into a 50 mL conical tube and add the following:
   - 7.5 mL Osteogenic Stimulatory Supplements
   - 5 µL Dexamethasone (10^{-4} M stock solution)
   - 250 µL Ascorbic Acid (10 mg/mL stock solution)
   - 175 µL ß-Glycerophosphate (stock solution at 1.0 M) (**if required, see Note below**)

   Note: ß-Glycerophosphate is not added to the complete medium at initiation of the assay. Typically ß-Glycerophosphate is added only after there is evidence, by phase microscopy, of cell multilayering.

   Antibiotics and antimycotics may be added at the researcher's discretion.

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7.2 Culture Set-up

There are many protocols in the literature that describe the development of osteogenic cells from either bone marrow or cultured mesenchymal cells[5,35-38] which vary slightly in the concentrations of reagents used. The protocol below is an example of a method that supports the growth of osteogenic cells from human bone marrow.

Complete MesenCult® Osteogenic Medium also supports the proliferation of rat osteogenic cells. The optimal concentration of ß-Glycerophosphate is 5 mM for rat cells.

1. Prepare cancellous bone fragments by mincing the bone into very small pieces (1 - 3 mm in size).
2. Flush fragments with 20 - 30 mL of PBS (Catalog #37350) and then vortex the fragments with another 20 - 30 mL of PBS.

   The fragments should appear almost white at this stage.
3. Pass the cell suspension through a 70 µm cell strainer (BD Catalog #352350) to remove bone fragments.
4. Centrifuge cells at 400 x *g* (~1320 rpm) for 15 minutes.
5. Discard the supernatant and resuspend cells in PBS.
6. Place cells on Ficoll-Paque™ PLUS and centrifuge at 400 x *g* (~1320 rpm) for 25 minutes with the brake set to the "off" position.

   If unprocessed bone marrow cells are available, dilute the bone marrow 1/3 with PBS + 2% FBS and start at this step.
7. Remove the cells at the interface and resuspend the cells in Complete MesenCult® Osteogenic Medium **without ß-Glycerophosphate**.
8. Seed cells in tissue culture-treated flasks or plates at a concentration of 1 - 2 x 10^5 cells per cm^2.
9. Replenish the culture medium after 5 days by removing the medium (and non-adherent cells). These cells and the medium can be discarded. The cultures are replenished with fresh Complete MesenCult® Osteogenic Medium, again without ß-Glycerophosphate unless cell multilayering has been noted.

   Multilayering is the layering of cells on top of each other, forming a matrix as opposed to growing in a planar manner. Multilayering is indicative of the beginning of bone generation.
10. Once multilayering has been observed, add ß-Glycerophosphate to Complete MesenCult® Osteogenic Medium as directed in Section 7.1. Continue to replenish cultures with ß-glycerophosphate-containing medium every 2 - 3 days for a minimum of three weeks (for rat cultures) or 5 weeks (for human cultures).
11. Osteogenic cells may be detected by tetracycline labeling[35] or von Kossa staining. Cultures may be maintained for extended time periods (>8 weeks) for other types of studies.

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8.0 Appendix 1: RosetteSep® Human Mesenchymal Stem Cell Enrichment Cocktail

Complete information concerning the enrichment of mesenchymal stem cells from unprocessed human bone marrow using RosetteSep® can be obtained at: *http://www.stemcell.com/product_catalog/hmesenchymal.asp.*

8.1 RosetteSep® Procedure (Catalog #15128/15168)

Ensure that bone marrow sample, PBS + 2% FBS (Catalog #07905), Ficoll-Paque™ PLUS (Catalog #07957), EDTA, and centrifuge are all at room temperature. Perform a nucleated cell count on fresh unprocessed bone marrow using 3% Acetic Acid with Methylene Blue (Catalog #07060). A 1/50 or 1/100 dilution is recommended.

1. Aliquot a known volume of bone marrow into a 14 mL tube. Add 50 µL RosetteSep® Human Mesenchymal Stem Cell Enrichment Cocktail per mL of bone marrow and mix well.
2. Incubate 20 minutes at room temperature.
3. Dilute sample with twice the volume of PBS + 2% FBS and 1 mM EDTA. Mix gently.
4. Layer the diluted sample on top of the Ficoll-Paque™ PLUS. Be careful to minimize mixing of Ficoll-Paque™ PLUS and sample. See table below for volume recommendations. With 50 mL centrifuge tubes, we suggest using a minimum of 15 mL Ficoll-Paque™ PLUS to make it easier to remove the enriched cell layer.

Recommended Volume and Tube Sizes			
Unprocessed Bone Marrow	PBS + 2% FBS + 1 mM EDTA	Ficoll-Paque™ PLUS	Tube Size
1 mL	2 mL	5 mL	14 mL
2 mL	4 mL	5 mL	14 mL
5 mL	10 mL	15 mL	50 mL
10 mL	20 mL	15 mL	50 mL

5. Centrifuge for 25 minutes at 300 x *g* (~1200 rpm) at room temperature, with the brake off.
6. Remove the enriched cells from the Ficoll-Paque™ PLUS : plasma interface.

   Sometimes it is difficult to see the cells at the interface, especially when very rare cells are enriched. It is advisable to remove some of the Ficoll-Paque™ PLUS along with the enriched cells in order to ensure their complete recovery.
7. Wash enriched cells with PBS + 2% FBS and 1 mM EDTA.
8. We recommend that enriched samples are lysed with ammonium chloride to remove residual red blood cells prior to flow cytometric analysis (this can be done as one of the wash steps) or if residual red blood cells will interfere with subsequent assays. Resuspend cells in Complete MesenCult® Medium (Human) for culture or appropriate medium for flow cytometric analysis.

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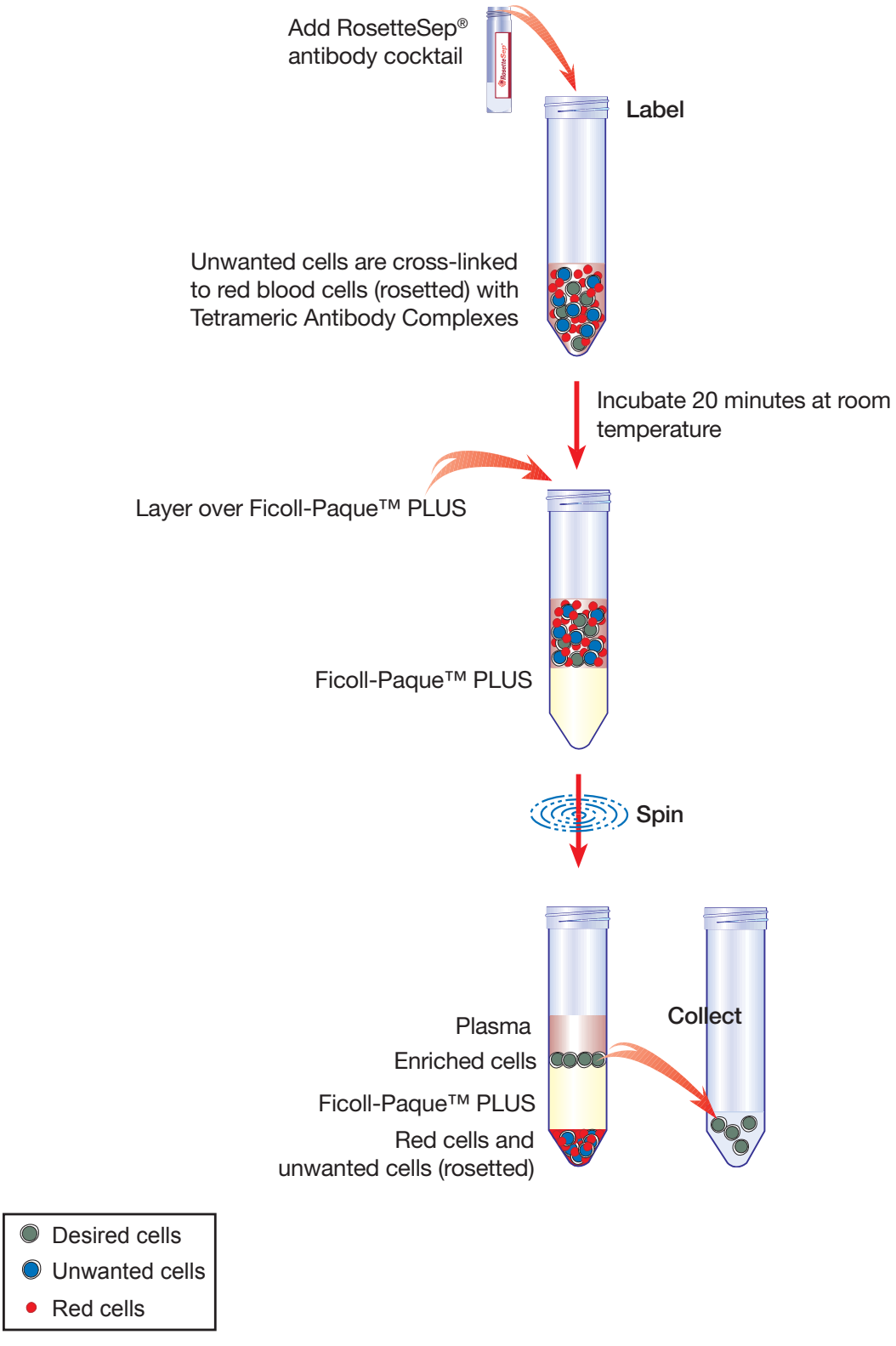
8.2 RosetteSep® Procedure Summary



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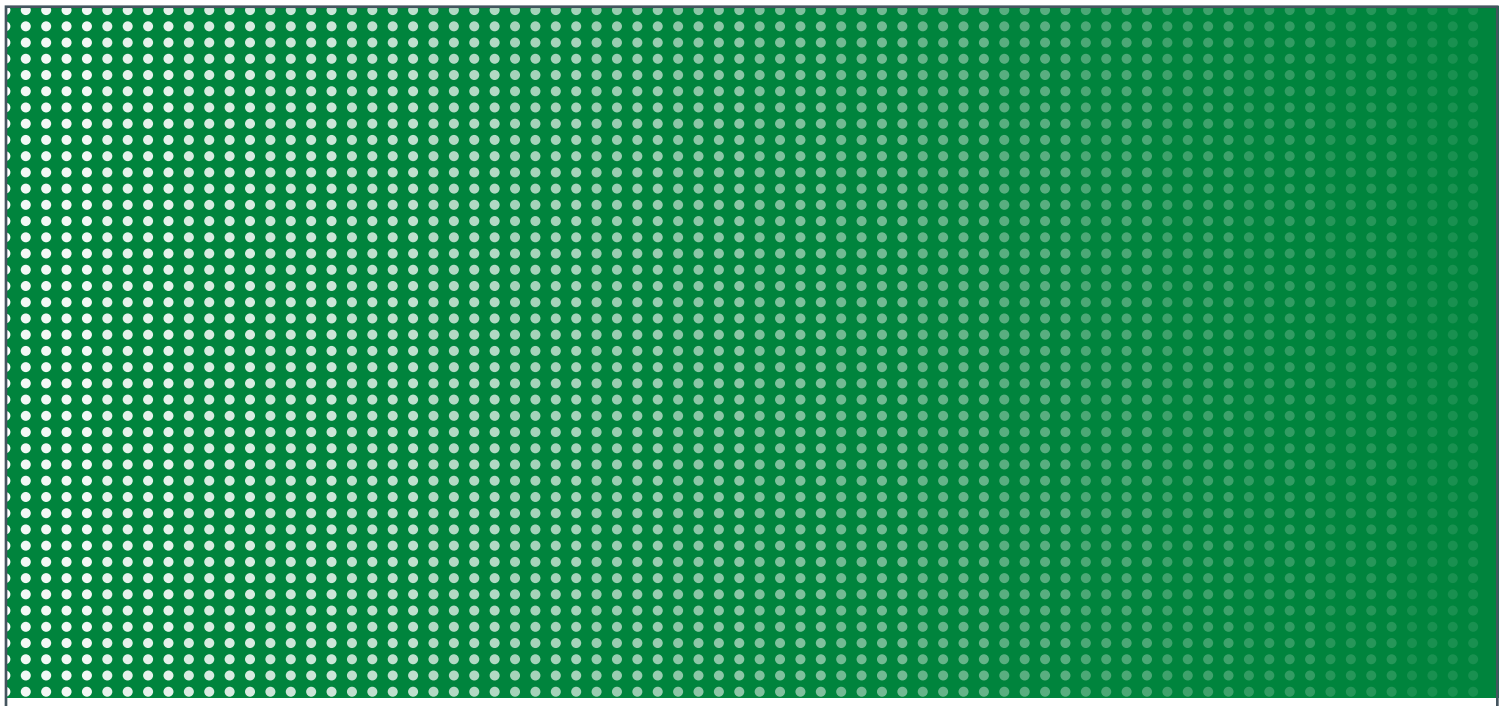

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