TECHNICAL MANUAL

Culture of Human Mesenchymal Stem Cells Using MesenCult™-XF Medium

STEMCELL™ TECHNOLOGIES

Table of Contents

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1.0 Materials

1.1 MesenCult™-XF Medium and Required Products

MesenCult™-XF Medium is a standardized, xeno-free, serum-free medium for the culture of human mesenchymal stem cells (MSCs). MesenCult™-XF Medium is optimized for the expansion of bone marrow (BM)-derived human MSCs in vitro as well as for use in the colony-forming unit - fibroblast (CFU-F) assay. MesenCult™-XF Medium supports long-term growth of MSCs and cells maintain multi-lineage differentiation potential.

MesenCult™-XF Medium must be used in conjunction with the MesenCult™-SF Attachment Substrate and the MesenCult™-ACF Dissociation Kit; components are tested for optimal cell adherence when MSCs are cultured with MesenCult™-XF Medium. Refer to Table 1 for product information. For product storage and stability information, refer to the Product Information Sheet (PIS) for each product, available on our website at www.stemcell.com or contact us to request a copy.

Table 1. MesenCult™-XF Medium, Kits, and Required Products

NOTE: MesenCult™-SF Attachment Substrate and MesenCult™-ACF Dissociation Kit are required for optimal cell adherence when MSCs are cultured in MesenCult™-XF Medium.

PRODUCT NAME	CATALOG #	SIZE
MesenCult™-XF Medium† • MesenCult™-XF Basal Medium (Catalog #05421) • MesenCult™-XF Supplement (5X; Catalog #05422)	05420	500 mL
MesenCult™-SF Attachment Substrate*	05424	5 mg
MesenCult™-SF Culture Kit • MesenCult™-XF Medium (Catalog #05420) • MesenCult™-SF Attachment Substrate (Catalog #05424)	05429	1 Kit
MesenCult™-ACF Dissociation Kit† • MesenCult™-ACF Enzymatic Dissociation Solution (Catalog #05427) • MesenCult™-ACF Enzyme Inhibition Solution (Catalog #05428)	05426	1 Kit

*MesenCult™-SF Attachment Substrate must be resuspended and diluted prior to use (see section 2.1).

†Components are not available for individual sale.

1.2 Additional Required Reagents

- L-Glutamine (Catalog #07100)
- Dulbecco's-PBS Without Ca++ and Mg++ (D-PBS; Catalog #37350)
- Lymphoprep™ (Catalog #07801)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Trypan Blue (Catalog #07050)
- 0.5 M EDTA (ethylenediaminetetraacetic acid)
- Sterile distilled water
- Alpha MEM without Nucleosides (Catalog #36453; optional)

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

- Methanol, ACS Grade (BDH Catalog #ACS531)
- Giemsa Stain Solution (EMD Chemicals Catalog #R03055)

1.3 Required Equipment

- Biohazard Safety Cabinet certified for Level II handling of biological materials
- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Benchtop centrifuge with swinging bucket rotor
- Inverted microscope
- Standard light microscope (for cell counting)
- Hemacytometer
- Pipette-aid
- Sterile pipettes: 1 mL, 5 mL and 10 mL
- Micropipette with 20 µL, 200 µL and 1 mL sterile tips
- 14 mL polystyrene tubes (Corning Catalog #352057)
- 50 mL conical tubes (Corning Catalog # 352070)
- Parafilm® (Sigma Catalog #P7793)
- Recommended tissue culture plates/flasks:

TISSUE CULTURE VESSEL	RECOMMENDED PRODUCT
6-well plate	Corning Catalog #3516
T-25 cm^2	Corning Catalog #353109
T-75 cm^2	Corning Catalog #353136

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2.0 Preparation of Reagents

2.1 Reconstitution of MesenCult™-SF Attachment Substrate

1. Dissolve the lyophilizate with 5 mL sterile tissue grade water to obtain a final concentration of 1 mg/mL. Do not agitate or pipette vigorously.
2. Incubate at 37°C for 30 - 60 minutes to ensure the lyophilizate is fully in solution. Swirl gently.
3. Following reconstitution, store the solution at -20°C. It is recommended to aliquot the reconstituted solution into multiple vials of 100 - 500 µL. Avoid additional freeze-thaw cycles.

2.2 Coating Plates with MesenCult™-SF Attachment Substrate

1. Coat plates one day prior to usage (i.e: coat overnight at 2 - 8°C), or if time is limited, coat for 2 hours at room temperature (15 - 25°C). **It is recommended to coat plates overnight.**
2. Plates must be coated with different dilutions of MesenCult™-SF Attachment Substrate when performing CFU-F assays and when expanding cells in culture.

   Coating plates for CFU-F assay:
   Dilute MesenCult™-SF Attachment Substrate to a **1 in 30 final dilution** in sterile D-PBS. Gently mix by inverting the tube twice. Prepare an amount slightly more than required to account for pipetting variability. For example, to coat one 6-well plate (e.g. Corning Catalog #3516), dilute 160 µL MesenCult™-SF Attachment Substrate in 4.8 mL D-PBS.

   For each well of a 6-well plate, add 800 µL of diluted MesenCult™-SF Attachment Substrate.

   Coating plates for cell expansion:
   When culturing cells obtained from primary tissue: Dilute MesenCult™-SF Attachment Substrate to a **1 in 20 final dilution** in sterile D-PBS. Gently mix by inverting the tube twice. Prepare an amount slightly more than required to account for pipetting variability. For example, to coat one T-75 cm^2 flask, dilute 250 µL MesenCult™-SF Attachment Substrate in 5 mL D-PBS.

   When culturing previously cultured cells: Dilute MesenCult™-SF Attachment Substrate to a **1 in 28 final dilution** in sterile D-PBS. Gently mix by inverting the tube twice. Prepare an amount slightly more than required to account for pipetting variability. For example, to coat one T-75 cm^2 flask, dilute 185 µL MesenCult™-SF Attachment Substrate in 5 mL D-PBS.

   Add diluted MesenCult™-SF Attachment Substrate as follows:

TISSUE CULTURE VESSEL	VOLUME OF ATTACHMENT SUBSTRATE	RECOMMENDED PRODUCT
6-well plate	800 µL/well	Corning Catalog #3516
T-25 cm^2 flask	2 mL	Corning Catalog #353109
T-75 cm^2 flask	5 mL	Corning Catalog #353136

3. Wrap plates with Parafilm®, sealing the junction between the base and lid and incubate at 2 - 8°C (in the refrigerator) overnight or for 2 hours at room temperature (15 - 25°C). For flasks, seal the vent on the cap with Parafilm® and incubate as described.
4. If plates/flasks were incubated overnight at 2 - 8°C, bring to room temperature (15 - 25°C) (approximately 20 minutes) prior to washing. Gently pipette off remaining MesenCult™-SF Attachment Substrate without touching the newly-coated surface.

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5. Wash plates/flasks once with sterile distilled water by slowly pipetting water down the side of the well/flask, being careful not to scrape the newly-coated surface. Swirl gently to rinse the entire surface and then carefully aspirate off water.
6. Allow to dry at room temperature (15 - 25°C) for at least 15 minutes prior to use.

2.3 Preparation of Complete MesenCult™-XF Medium

Use sterile techniques to prepare Complete MesenCult™-XF Medium (MesenCult™-XF Basal Medium + MesenCult™-XF Supplement (5X) + L-Glutamine). The following example is for preparing 500 mL of Complete MesenCult™-XF Medium. For other volumes, adjust accordingly. Prepare Complete MesenCult™-XF Medium in volumes that can be used within 5 days.

1. Thaw MesenCult™-XF Supplement (5X) at 2 - 8°C overnight.

   Note: If not used immediately, aliquot Supplement into smaller working volumes and store at -20°C. Do not exceed the expiry date as indicated on the label. Avoid repeated freeze-thaw cycles.
2. Add 100 mL MesenCult™-XF Supplement (5X) to 400 mL of MesenCult™-XF Basal Medium.
3. Add L-Glutamine to a final concentration of 2 mM. This is now referred to as **Complete MesenCult™-XF Medium**.

   Note: If not used immediately, store Complete MesenCult™-XF Medium at 2 - 8°C for up to 5 days.

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3.0 Preparation of Human MSCs for Culture with MesenCult™-XF Medium

3.1 Preparation of Mononuclear Cell Suspension from Fresh Human Bone Marrow

When working with a fresh bone marrow (BM) sample, the cells need to be processed to remove red blood cells prior to culture.

1. Prepare 500 mL Isolation Buffer (D-PBS + 2 mM EDTA) by adding 2 mL EDTA (0.5 M stock solution) to 498 mL 1X D-PBS.

   Note: If any of the components are not sterile (i.e. EDTA), filter sterilize the individual components or the complete buffer with a 0.2 µm filter. If not used immediately, store the Isolation Buffer at 2 - 8°C.

2. Count the total number of nucleated cells in the BM sample by diluting 10 µL BM 1 in 50 to 1 in 100 with 3% Acetic Acid with Methylene Blue*. Count cells using a hemacytometer.
3. Warm 50 mL Isolation Buffer at room temperature (15 - 25°C) for 20 minutes prior to use. Dilute BM to a **5 in 14 final dilution** with room temperature (15 - 25°C) Isolation Buffer (e.g. dilute 25 mL BM with 45 mL Isolation Buffer for a total volume of 70 mL).
4. In 3 x 50 mL conical tubes, pipette 17 mL Lymphoprep™ into each tube. Carefully layer 23 mL diluted BM on top of the Lymphoprep™ in each tube.
5. Centrifuge at 300 x *g* for 30 minutes at room temperature (15 - 25°C) in a benchtop centrifuge with the **brake off**.
6. Remove and discard the upper plasma layer without disturbing the plasma:Lymphoprep™ interface. Carefully remove and retain the mononuclear cells located at the interface layer and place in a new 50 mL conical tube. Resuspend the mononuclear cells with 40 mL cold (2 - 8°C) Isolation Buffer. Mix gently by pipetting.
7. Centrifuge the cells at 300 x *g* for 10 minutes at room temperature (15 - 25°C) in a benchtop centrifuge with the **brake on**. Remove the supernatant and resuspend cells in 1 - 2 mL cold Isolation Buffer.
8. Dilute cells 1 in 50 in 3% Acetic Acid with Methylene Blue* and count the total number of nucleated cells using a hemacytometer.
9. Dilute cells in Complete MesenCult™-XF Medium at a final concentration of 1 x 10^6 cells/mL.

*3% Acetic Acid with Methylene Blue will lyse red blood cell and white blood cell membranes. The remaining white blood cell nuclei will stain lightly with Methylene Blue. For more information on counting cells using a hemacytometer, refer to the Product Information Sheet for 3% Acetic Acid with Methylene Blue (Document #29604) available at www.stemcell.com or contact us to request a copy.

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4.0 Colony Forming Unit - Fibroblast (CFU-F) Assay

4.1 Plating Cells for the CFU-F Assay

A fresh bone marrow (BM) sample or culture-expanded MSCs are required when performing CFU-F assays. Do not use previously frozen BM mononuclear cells.

1. CFU-F assays must be performed using tissue culture-treated plates that have been coated with MesenCult™-SF Attachment Substrate as described in section 2.1.
2. Using fresh BM-derived MSCs processed as described in section 3.0, seed cells at 3 different densities (between 1.5 - 5 x 10^4 cells/cm^2) in Complete MesenCult™-XF Medium. Refer to Table 2 for recommended plating densities and volumes for a 6-well plate.

   Note: At this point you should have a stock solution of cells at 1 x 10^6 cells/mL.

Table 2. Recommended Plating Volumes for the CFU-F Assay

TISSUE CULTURE VESSEL	VOLUME OF COMPLETE MESENCULT™-XF MEDIUM	RECOMMENDED PLATING DENSITY	VOLUME OF CELL STOCK SOLUTION (1 X 10^6 CELLS/mL)
6-well plate	2.5 mL/well	1.5 x 10^5 cells/well	150 µL
		2.5 x10^5 cells/well	250 µL
		5.0 x 10^5 cells/well	500 µL

OR

Using culture-expanded MSCs, seed between 25 - 250 cells/well of a 6-well plate at 5 different densities in Complete MesenCult™-XF Medium.

Note: Plating different cell densities will ensure that the resulting colonies can be counted. The proliferative potential of CFU-F from various BM samples is widely variable. If too few cells are plated, CFU-F may be undetectable or the number of colonies counted may be too low to give a reliable estimation of CFU-F. If too many cells are plated, too many CFU-F may grow such that individual colonies cannot be determined. Refer to section 5.0 for representative images.

3. Incubate at 37°C with 5% CO_2 in air and 95% humidity for 9 - 12 days. After day 7, monitor the growth of colony-forming cells daily. Colonies are ready to be stained before colonies start touching the neighboring colonies.

   Note: Monitor CFU-F colony size. MSCs cultured in MesenCult™-XF Medium proliferate faster than cells cultured in a traditional serum-based medium and therefore CFU-F assays must be stopped earlier when cells are cultured in MesenCult™-XF Medium.

4.2 Giemsa Staining of CFU-F Colonies

1. Gently remove MesenCult™-XF Medium from CFU-F cultures and discard. Adherent CFU-F colonies will remain attached.
2. Gently wash colonies once with D-PBS to remove any residual culture medium.

3. Fix cells by adding 2 mL methanol, ACS grade to each well of a 6-well plate. Incubate at room temperature (15 - 25°C) for 5 minutes.
4. Remove methanol and discard. Let the culture dishes air dry at room temperature (15 - 25°C) (~5 minutes).
5. Add 2 mL Giemsa Stain Solution to each well of a 6-well plate. Incubate at room temperature (15 - 25°C) for 5 - 10 minutes.
6. Remove Giemsa Stain Solution and rinse with distilled water to remove unbound stain. Rinse until water is clear.
7. Discard the distilled water and allow the tissue culture dishes to dry at room temperature (15 - 25°C) with the lid open.

   Note: 6-well plates typically take 2 - 3 hours to dry.

4.3 Counting CFU-F Colonies

1. Human BM-derived CFU-F colonies are large enough to see with the naked eye following staining with Giemsa. Mark each CFU-F colony with a felt-tip pen on the bottom of the well when counted. This prevents counting colonies more than once.

   Note: Some colonies do not take up enough stain to be easily visible macroscopically, and therefore it is important to verify the number of colonies by counting colonies microscopically.

   Note: CFU-F colonies cultured in MesenCult™-XF Medium have a slightly different morphology than CFU-F colonies typically obtained when performing the CFU-F assay with the MesenCult™ Proliferation Kit (Human; Catalog #05411). Refer to section 5.0 for representative images.
2. Ensure that there is a linear relationship between the cell numbers plated in a given well and the resulting colony numbers. For example, there should be twice as many CFU-F colonies when 5.0×10^5 cells are plated than when 2.5×10^5 cells are plated. It is important that the plates do not contain too many CFU-F colonies such that individual CFU-F colonies cannot be determined, as this will lead to inaccurate estimations. Refer to section 5.0 for representative images.

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

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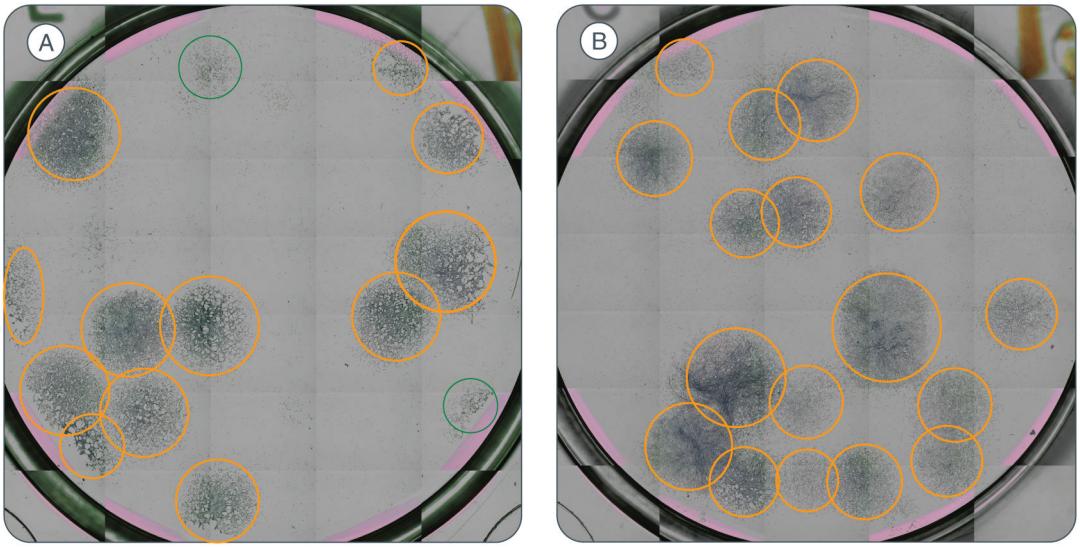
5.0 Representative Images of Human CFU-F Colonies

It is important that CFU-F colonies are distinct, so that an accurate assessment of CFU-F colony frequency can be determined. The images below show a reasonable number of CFU-F colonies for counting purposes.

The colonies circled in orange are easily visible macroscopically. It is important to look at the CFU-F cultures under a microscope for confirmation because some colonies may not take up enough stain and could be missed when scored macroscopically (e.g. the colonies circled in green are only truly distinguishable as CFU-F under a microscope).



The CFU-F assay was performed in a 6-well plate (note the edges of the well in each image). A Lumenera INFINITY2-3C camera was used to capture the images using Image Pro 6.2 software.

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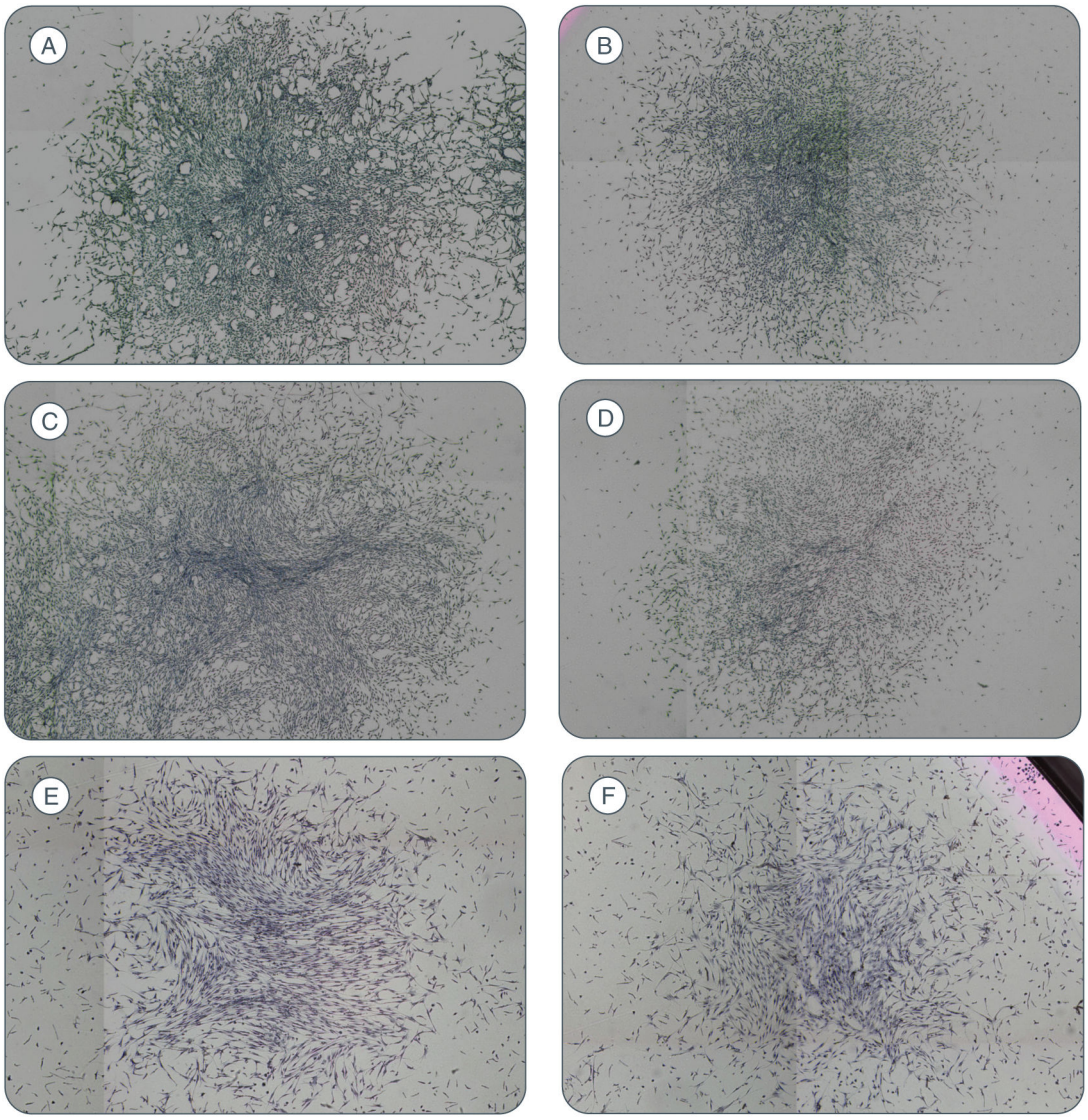
5.1 Comparison of CFU-F Colonies Cultured with MesenCult™-XF Medium or the MesenCult™ Proliferation Kit

The morphology of CFU-F colonies generated when MSCs are cultured in MesenCult™-XF Medium (A-D) differs from the morphology of CFU-F colonies generated when MSCs are cultured with the MesenCult™ Proliferation Kit (Human; Catalog #05411) (E,F).



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6.0 Expansion of Cultured MSCs

Note: The proliferation potential of cells obtained from different bone marrow (BM) donors is highly variable. To ensure that cultures contain an optimal number of cells for expansion, it is recommended to seed 2 - 3 different cell densities. If too few cells are plated, cells grow too slowly and reach the recommended splitting density too late (cells start to detach from the surface). If too many cells are plated, the cells will reach confluence too fast and will become senescent and lose pluripotency.

6.1 Initial Plating of MSCs for Expansion

1. When initially plating BM mononuclear cells in MesenCult™-XF Medium for expansion, plate between 3.0 - 7.0 x 10^4 cells/cm^2 in Complete MesenCult™-XF Medium into tissue culture-treated plates/flasks that have been coated with MesenCult™-SF Attachment Substrate, as described in section 2.1. If plating two cell densities, plate one flask at the lower end of the range and the other flask at the upper end of the range.

   Table 3. Recommended Plating Densities for Expansion of MSCs

TISSUE CULTURE VESSEL	VOLUME OF COMPLETE MESENCULT™-XF MEDIUM	SURFACE AREA	RECOMMENDED PLATING DENSITIES
6-well plate	2.5 mL/well	9.5 cm^2/well	3 x 10^5 cells/well
			4.5 x 10^5 cells/well
			6.0 x 10^5 cells/well
T-25 cm^2 flask	10 mL	25 cm^2	8.0 x 10^5 cells/flask
			10 x 10^5 cells/flask
			12.5 x 10^5 cells/flask
T-75 cm^2 flask	15 mL	75 cm^2	2.5 x 10^6 cells/flask
			4.0 x 10^6 cells/flask
			5.0 x 10^6 cells/flask

2. Incubate at 37°C with 5% CO_2 in air and 95% humidity for 9 - 13 days.[†]
3. At 7 days post-plating, observe primary MSCs under a microscope to determine if they are ready for passaging.

   The cells are ready to be passaged when they reach 80% confluence. Normally, cells reach 70 - 80% confluence between 9 - 13 days after initial plating of primary BM mononuclear cells, but this depends on the donor and initial plating density. Usually by day 10 - 11 the cells will be ready to passage. Refer to section 7.2 for images of cells at different densities.

   Monitor the color of the medium after day 7. If the medium appears acidic (yellowish in color) prior to reaching 80% confluence, perform a half-medium change by removing 1/2 of the medium and replacing with fresh Complete MesenCult™-XF Medium warmed to 37°C.

[†] At this point, the cells are considered to be at passage 0.

6.2 Passaging Cultured MSCs

1. Warm MesenCult™-ACF Enzymatic Dissociation Solution and MesenCult™-ACF Enzyme Inhibition Solution to room temperature (15 - 25°C). Do not warm at 37°C.
2. To passage cells, slowly remove medium from cultures. The adherent cells will remain attached to the culture dish.
3. Wash cells with sterile D-PBS to remove residual culture medium. Remove D-PBS.
4. Add 1 mL MesenCult™-ACF Enzymatic Dissociation Solution to each well of a 6-well plate, 3 mL to a T-25 cm^2 flask, or 6 mL to a T-75 cm^2 flask. Incubate at 37°C for 2 - 5 minutes.
5. After 2 minutes, observe cells under the microscope to ensure that all cells have detached. Gently tap plate/flask to detach remaining cells.
6. Add 1 mL MesenCult™-ACF Enzyme Inhibition Solution to each well of a 6-well plate, 3 mL to a T-25 cm^2 flask, or 6 mL to a T-75 cm^2 flask.
7. **6-well plate:**
   Collect cells into a 14 mL polystyrene tube and wash each well with 3 mL Alpha MEM without Nucleosides* to recover remaining cells. Add Alpha MEM without Nucleosides* to bring the total volume to 8 mL.

   T-25 cm^2 flask:
   Collect cells into a 14 mL polystyrene tube and wash each flask with 5 mL Alpha MEM without Nucleosides* to recover remaining cells. Add Alpha MEM without Nucleosides* to bring the total volume to 12 mL.

   T-75 cm^2 flask:
   Collect cells into a 50 mL conical tube and wash each flask with 6 mL Alpha MEM without Nucleosides* to recover remaining cells. Add Alpha MEM without Nucleosides* to bring the total volume to 30 mL.
8. Centrifuge cells at 300 x *g* for 8 minutes at room temperature (15 - 25°C) with the **brake on**.
9. Remove supernatant and resuspend cell pellet in 0.5 - 1 mL Complete MesenCult™-XF Medium.
10. Dilute cells with Trypan Blue† and perform a viable cell count using a hemacytometer.

*The cell washes can also be performed with Complete MesenCult™-XF Medium. It is important to add the additional medium when washing the cells, so that the MesenCult™-ACF Enzymatic Dissociation Solution is sufficiently washed from the cells.

†Only non-viable cells will stain with Trypan Blue dye; viable cells remain unstained. Note: If cells are incubated for greater than 15 minutes in Trypan Blue, toxicity effects may occur and the viable cell count will be inaccurate. For more information on how to count cells using a hemacytometer, refer to the Product Information Sheet for Trypan Blue (Document #29603) available at www.stemcell.com or contact us to request a copy.

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11. Plate cells in Complete MesenCult™-XF Medium into new tissue culture-treated plates or flasks that have been coated with MesenCult™-SF Attachment Substrate, as described in section 2.2. The recommended plating density for passaged cells is between 1.5 - 4.0 x 10^3 cells/cm^2. Refer to Table 4 for recommended plating densities for different tissue culture vessels.

Table 4. Recommended Plating Densities for Culture-Expanded MSCs

TISSUE CULTURE VESSEL	VOLUME OF COMPLETE MESENCULT™-XF MEDIUM	SURFACE AREA	RECOMMENDED PLATING DENSITIES
6-well plate	2.5 mL/well	9.5 cm^2/well	1.5 x 10^4 cells/well
			3.0 x 10^4 cells/well
T-25 cm^2 flask	10 mL	25 cm^2	7.5 x 10^4 cells/flask
			12.5 x 10^4 cells/flask
T-75 cm^2 flask	15 mL	75 cm^2	15 x 10^4 cells/flask
			25 x 10^4 cells/flask

12. Incubate at 37°C with 5% CO_2 in air and 95% humidity until the cells reach 80% confluence. When cells reach 80% confluence and are ready to be passaged, repeat steps 1 - 12 of section 6.2. A half-medium change is only necessary if the medium appears acidic (yellowish in color) prior to reaching 80% confluence.

*Note: For culture-expanded MSCs (passage 1 onward) it takes approximately **3 - 6 days** for the culture to reach 80% confluence (i.e. are ready for passaging), although this depends on the proliferative ability of cells from the particular BM donor and the initial plating density.*

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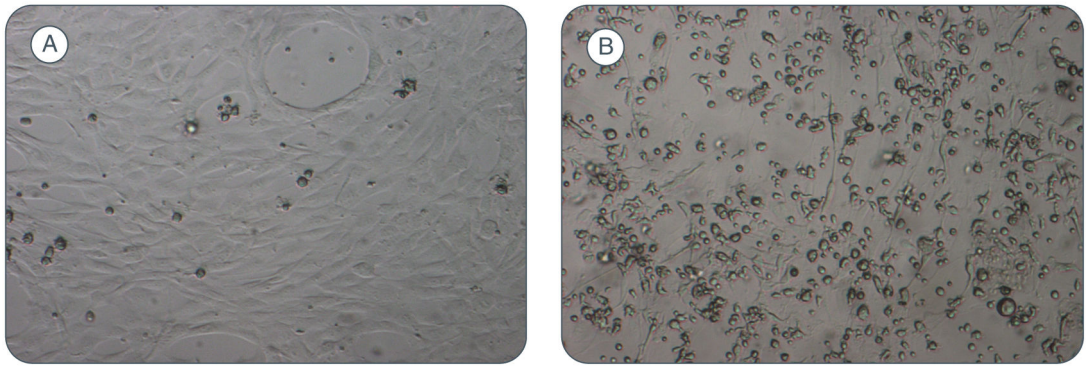
7.0 Representative Images of Human MSCs

7.1 Comparison of Primary Human Bone Marrow-Derived MSCs Cultured in MesenCult™-XF Medium or Traditional Serum-Based Medium

Passage 0 human bone marrow (BM)-derived MSCs show less hematopoietic cell contamination when cultured in MesenCult™-XF Medium (A) compared to serum-based medium (B).



Magnification: 5X

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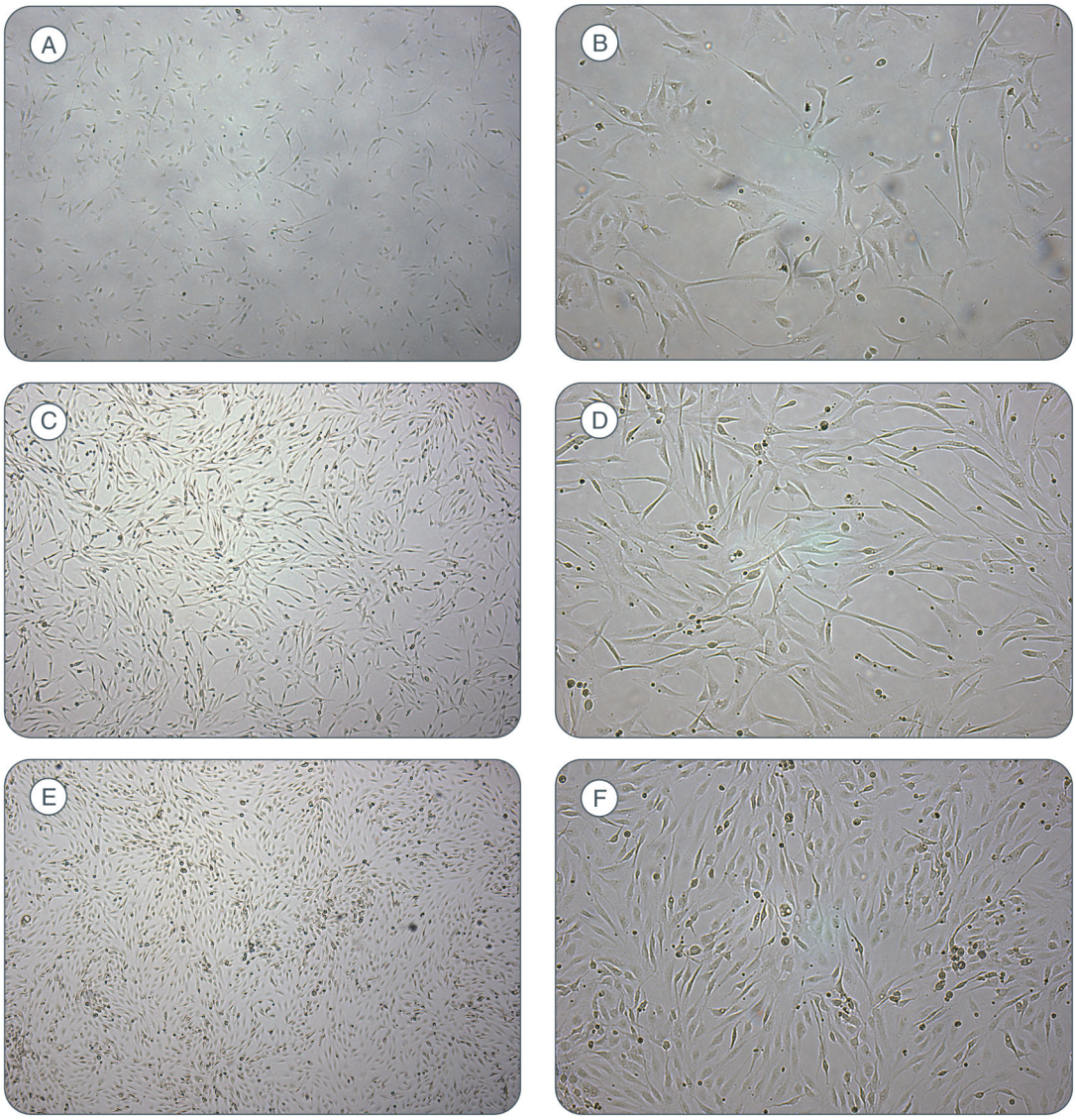
7.2 Cell Densities of MSCs Cultured in MesenCult™-XF Medium

It is important that cells are passaged when they reach 80% confluence. Figures C and D depict cells at an optimal density for passaging. Figures A and B are what cells should look like the day before passaging and in Figures E and F the cells are too confluent (i.e. they should not be used).



Magnification: 5X (A, C, E); 10X (B, D, F).

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