invitrogen™

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells

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User Manual

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Contents and Storage

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Type of cells: GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells

Amount supplied: One vial containing ≥1 × 10^6 viable cells.

Composition: 1 mL of cells in freezing medium.*

***Freezing medium:** 60% D-MEM, 30% MSC-Qualified FBS, and 10% DMSO.

Shipping and Storage

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells are shipped on dry ice. Upon receipt, store the cells in **liquid nitrogen**.

Handle GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. This product contains Dimethyl Sulfoxide (DMSO), a hazardous material. Review the Safety Data Sheet (SDS) before handling. Safety Data Sheets (SDSs) are available at www.invitrogen.com/sds.

Intended Use

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells are for research use only. They are not intended for any animal or human therapeutic or diagnostic use.

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs)

Mesenchymal Stem Cells (MSCs) are multipotent stem cells that have a large capacity for self-renewal while maintaining their multipotency. They can differentiate into multiple mature cell phenotypes *in vitro*, including adipocytes, osteocytes, and chondrocytes (De Ugarte *et al.*, 2003; Meirelles Lda & Nardi, 2003; Pittenger *et al.*, 1999; Wu *et al.*, 2002). *In vitro* differentiation into non-mesenchymal cell types, such as neuronal and myogenic cells have also been described (Anjos-Afonso *et al.*, 2004; Deng *et al.*, 2001; Han *et al.*, 2002; Han *et al.*, 2004; Moscoso *et al.*, 2005; Phinney *et al.*, 1999; Wakitani *et al.*, 1995). In addition, MSCs are shown to be involved in certain types of cancers (Houghton *et al.*, 2004; Singh *et al.*, 2004), and are known to secrete immunomodulatory, anti-angiogenic, anti-inflammatory, pro-cardiovasculogenic, and pro-arteriogenic factors (Djouad *et al.*, 2003; Gojo *et al.*, 2003; Houghton *et al.*, 2004; Kinnaird *et al.*, 2004; Krampera *et al.*, 2003; Oh *et al.*, 2008; Olivares *et al.*, 2004; Orlic *et al.*, 2001).

Source of GIBCO® Mouse (C57BL/6) MSCs

GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells (MSCs) are produced from bone marrow isolated from C57BL/6 mice at ≤ 8 weeks of gestation through mechanical and enzymatic digestion. The cells were isolated under sterile conditions, expanded in D-MEM/F-12 medium containing 10% MSC-Qualified FBS, and cryopreserved at passage 8 (P8) in cryopreservation medium consisting of 60% D-MEM/F-12, 30% FBS, and 10% DMSO.

Uses of GIBCO® Mouse (C57BL/6) MSCs

GIBCO® Mouse (C57BL/6) MSCs can be used for studies of adult stem cell differentiation, tissue engineering, cell and gene therapy, and potential future clinical applications. Mouse is a preferred animal model for performing genetic manipulations and tracking cells, and GIBCO® Mouse (C57BL/6) MSCs can be used in testing and evaluating MSCs in the host animal as the cells differentiate into mature phenotypes.

We recommend using D-MEM/F-12 medium with GlutaMAX™-I and MSC-Qualified FBS (see page 23) for optimal growth and expansion.

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In vitro Growth Capacity

The *in vitro* growth capacity of MSCs has not been definitely established and can vary greatly depending on the culture conditions such as seeding density and growth factors used, but the cells can be expected to expand for at least 10 to 11 population doublings before their growth rate decreases significantly (Bruder *et al.*, 1997; Meirelles Lda & Nardi, 2003). GIBCO® Mouse (C57BL/6) MSCs exhibit a population doubling time of ~21 to 23 hours when cultured in D-MEM/F-12 with GlutaMAX™-I and MSC-Qualified FBS.

Differentiation Potential

Multiple investigators have demonstrated that MSCs can be differentiated towards multiple mature cell phenotypes. In addition to traditional mesenchymal lineages, MSCs have been differentiated towards cardiomyocytic and neuronal phenotypes using specialized media. The *in vitro* differentiation potential of MSCs has not been definitely established, but long-term culture and high cell density are implicated in the loss of differentiation potential (Meirelles Lda & Nardi, 2003).

Characteristics of GIBCO® Mouse (C57BL/6) MSCs

- Prepared from low-passage (passage 8) adherent mouse primary cell cultures
- Express a flow-cytometry cell-surface protein profile positive for CD29, CD34, CD44, and Sca-1 (> 70%), and negative for CD117 (< 5%)
- Exhibit a population doubling time of ~21 to 23 hours
- Demonstrate at least tri-potential differentiation (i.e., can differentiate into osteogenic, adipogenic, and chondrogenic lineages)

Methods

Handling GIBCO® Mouse (C57BL/6) MSCs

As with other mammalian cell lines, handle GIBCO® Mouse (C57BL/6) MSCs as potentially biohazardous material under at least Biosafety Level 1 (BL-1) containment. For more information on BL-1 guidelines, refer to *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed., published by the Centers for Disease Control, or see the following website:
www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm

Guidelines for GIBCO® Mouse (C57BL/6) MSC Culture

Follow the general guidelines below to grow and maintain GIBCO® Mouse (C57BL/6) MSCs.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper aseptic technique and work in a laminar flow hood.
- Before starting experiments, ensure cells have been established (at least 1 passage), and also have some frozen stocks on hand.
- For differentiation studies and other experiments, we recommend using cells below passage 10.
- For general maintenance of cells, cell confluency should be 60–80%, cell viability should be at least 90%, and the growth rate should be in mid-logarithmic phase prior to subculturing.
- When thawing or subculturing cells, transfer cells into **pre-warmed** medium.
- Antibiotic-antimycotic containing penicillin, streptomycin, and amphotericin B may be used if required (see page 23 for ordering information).

Important

It is very important to strictly follow the guidelines for culturing GIBCO® Mouse (C57BL/6) MSCs in this manual to keep the cells undifferentiated.

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Handling GIBCO® Mouse (C57BL/6) MSCs, continued

Media Requirements

We recommend culturing and expanding GIBCO® Mouse (C57BL/6) MSCs in D-MEM/F-12 medium with GlutaMAX™-I supplemented with 10% MSC-Qualified Fetal Bovine Serum (FBS) for optimal growth performance, and to keep the MSCs undifferentiated (see page 23 for ordering information).

Note: For the appropriate basal medium used in differentiation studies, refer to the specific differentiation protocol (pages 14–19).

- Prepare your growth medium prior to use.
- When thawing or subculturing MSCs, transfer the cells into **pre-warmed** medium at 37°C.
- You may store the complete growth medium **in the dark** at 4°C for up to four weeks.
- Avoid repeated freeze-thaw cycles of MSC-Qualified FBS.

Important

We have observed that a small percentage of GIBCO® Mouse (C57BL/6) MSCs adhere poorly after their initial thaw; however, the cells recover and adhere well after their first passage. We recommend that you treat your cells gently (i.e., do not vortex, bang the flasks to dislodge the cells, or centrifuge the cells at high speeds).

Thawing and Establishing Cells

Materials Needed

- GIBCO® Mouse (C57BL/6) MSCs, stored in liquid nitrogen
- Ethanol or 70% isopropanol
- Mouse MSC growth medium (see below); pre-warmed to 37°C
- Disposable, sterile 15-mL and 50-mL tubes
- 37°C water bath
- 37°C incubator with a humidified atmosphere of 5% CO_2
- Microcentrifuge
- Tissue-culture treated flasks, plates or dishes
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

Mouse MSC Growth Medium

Mouse MSC growth medium consists of D-MEM/F-12 medium with GlutaMAX™-I, 10% MSC-Qualified FBS, and 5 µg/mL gentamycin reagent solution. To prepare 500 mL of Mouse MSC growth medium, aseptically mix the following (see page 23 for ordering information):

Component	Final Conc.	For 500 mL
D-MEM/F-12 medium with GlutaMAX™-I	1X	450 mL
FBS, **MSC-Qualified**	10%	50 mL
Gentamicin (10 mg/mL)	5 µg/mL	250 µL

Note: For convenience, you may substitute α-MEM with GlutaMAX™-I for D-MEM/F-12 medium with a negligible difference in proliferation efficiency.

Note

Invitrogen's Countess™ Automated Cell Counter is a benchtop counter designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue technique (see page 23 for ordering information).

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Thawing Procedure

1. Pre-warm the prepared mouse MSC growth medium (page 5) to 37°C.
2. Remove the cells from liquid nitrogen storage, and wipe the cryovial with ethanol or 70% isopropanol before opening. In an aseptic field, briefly twist the cap a quarter turn to relieve the pressure and then re-tighten it. **Do not** expose the cells to air before thawing.
3. Quickly thaw the vial of cells by swirling it in a 37°C water bath, and remove it when the last bit of ice has melted, typically < 2 minutes. **Do not** submerge the vial completely. **Do not** thaw the cells for longer than 2 minutes.
4. When thawed, wipe the cryovial with ethanol or 70% isopropanol to sterilize it. Immediately transfer the cells into a 50-mL sterile tube and slowly add pre-warmed mouse MSC growth medium **dropwise** up to 10 mL while swirling the tube to mix.
5. Centrifuge the cells for 5 minutes at 300 × *g*.
6. Aspirate the supernatant and resuspend the cells in 2 mL of mouse MSC growth medium.
7. Take a 50 µL aliquot of the cells and determine the viable cell count using your method of choice.
8. Calculate the total number of viable cells, and add enough mouse MSC growth medium to the cells to generate a cell solution at 1×10^6 cells/mL.
9. Plate the resuspended cells at a seeding density of **5,000 cells per cm²**.

   Note: A seeding density of 5×10^3 viable cells/cm² is equivalent to 1.25×10^5 cells for a T25 flask, 3.75×10^5 cells for a T75 flask, and 1.125×10^6 cells for a T225 flask. Each vial of GIBCO® Mouse (C57BL/6) MSCs contains roughly 1×10^6 viable cells.
10. Following seeding, swirl the medium in the flasks to evenly distribute the cells.
11. Incubate the cells at 37°C, 5% CO_2 and 90% humidity, and allow the cells to adhere for at least 24 hours.
12. The next day, replace the medium with an equal volume of fresh, pre-warmed mouse MSC growth medium.
13. Change the medium every 2–3 days until the culture is 70–80% confluent.

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Expected Results

The bright field image (100X) below shows GIBCO® Mouse (C57BL/6) MSCs two days after thaw.

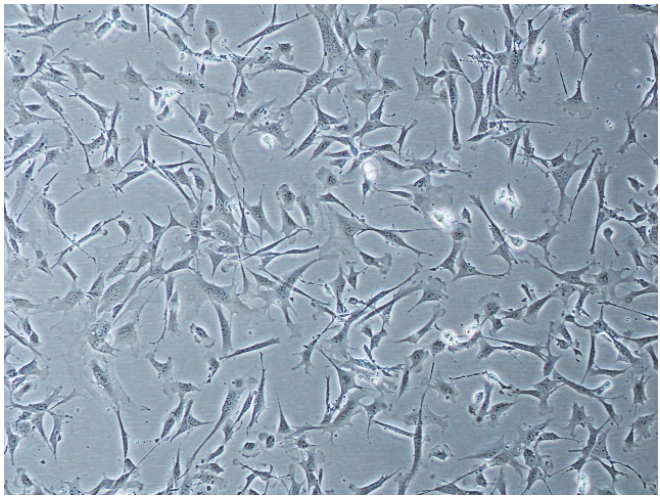

Figure 1. GIBCO® Mouse (C57BL/6) MSCs at passage 1 (P1) post-thaw were expanded for two days in D-MEM/F-12 medium with GlutaMAX™-I supplemented with 10% MSC-qualified FBS. The seeding density was 5×10^3 cells/cm^2 in a T75 culture vessel.

Subculturing Cells

When to Subculture

Subculture GIBCO® Mouse (C57BL/6) MSCs when they are near confluency, typically every 4 to 5 days.

Materials Needed

- Culture vessels containing GIBCO® Mouse (C57BL/6) MSCs
- Tissue-culture treated flasks, plates or dishes
- Mouse MSC growth medium (page 5), **pre-warmed** to 37°C
- Disposable, sterile 15-mL and 50-mL tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red
- TrypLE™ Express Dissociation Reagent, **pre-warmed** to 37°C
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

Passaging Cells

1. Aspirate the spent mouse MSC growth medium from the culture vessel.
2. Rinse the surface of the cell layer with D-PBS without Ca^{2+} and Mg^{2+} (approximately 1–2 mL of D-PBS per 10 cm^2 culture surface area) by adding the D-PBS to the side of the vessel opposite the attached cell layer, and rocking the vessel back and forth several times.
3. Aspirate and discard the D-PBS.
4. Add a sufficient volume of pre-warmed TrypLE™ Express to cover the cell layer (5 mL for T75 or 10 mL for T225).
5. Incubate the cells at 37°C for 3–6 minutes.

Procedure continued on next page

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Passaging Cells, continued

Procedure continued from previous page

6. Observe the cells under a microscope. If the cells are less than 90% detached, continue incubating the cells and observe within 2 minutes for complete detachment. You may tap the vessel **gently** to expedite the cell detachment.
7. Once the cells are detached, pipet the cell solution up and down a few times to generate a homogenous suspension. Transfer the cell suspension to a sterile 15-mL tube.
8. Take a 50 µL aliquot of the cell suspension and determine the total number of viable cells using your method of choice.
9. During the cell count, centrifuge the rest of the cells at 300 × *g* for 5 minutes at room temperature. Aspirate and discard the medium without disturbing the cell pellet.
10. Calculate the total number of vessels to inoculate by using the following equation:

    Number of vessels = Number of viable cells ÷ (growth area of vessel in cm^2 × 5,000 cells per cm^2 recommended seeding density)
11. Add mouse MSC growth medium to each vessel so that the final culture volume is 0.2–0.5 mL per cm^2.
12. Add the appropriate volume of cells to each vessel and incubate the cells at 37°C, 5% CO_2 and 90% humidity.
13. 2–3 days after seeding, completely remove the spent medium and replace with an equal volume of pre-warmed mouse MSC growth medium.

Freezing Cells

Materials Needed

- Culture vessels of GIBCO® Mouse (C57BL/6) MSCs
- Mouse MSC growth medium
- Fetal Bovine Serum, MSC-Qualified
- DMSO (use a bottle set aside for cell culture; open only in a laminar flow hood)
- Disposable, sterile 15-mL conical tubes.
- D-PBS, containing no calcium, magnesium, or phenol red
- TrypLE™ Express
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter
- Sterile freezing vials

Guidelines

When freezing MSCs, we recommend the following:

- Freeze cells at a density of 1×10^6–2×10^6 viable cells/mL.
- Use a freezing medium composed of final concentrations of 60% D-MEM/F-12 medium with GlutaMAX™-I, 30% **MSC-Qualified** FBS, and 10% DMSO.
- Bring the cells into freezing medium in two steps.

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Freezing Cells, continued

Preparing Freezing Media

Prepare the Freezing Media A and B immediately before use. You will need enough of each freezing medium to resuspend cells at a density of 1×10^6–2×10^6 cells/mL (see the freezing procedure below).

1. In a sterile 15-mL tube, mix together the following reagents for every 1 mL of **Freezing Medium A** needed:

D-MEM/F-12 medium with GlutaMAX™-I	0.4 mL
FBS, MSC-Qualified	0.6 mL

2. In another sterile 15-mL tube, mix together the following reagents for every 1 mL of **Freezing Medium B** needed:

D-MEM/F-12 medium with GlutaMAX™-I	0.8 mL
DMSO	0.2 mL

3. Place the tube with Freezing Medium B on ice until use (leave Freezing Medium A at room temperature).

   Note: Discard any remaining freezing medium after use.

Procedure for Freezing Cells

1. Aspirate the mouse MSC growth medium from the culture vessel.
2. Follow the **Passaging Cells** protocol, steps 2–9 (pages 8–9).
3. After the centrifugation step, esuspend the MSCs to a concentration of 2×10^6–4×10^6 cells/mL in Freezing Medium A.
4. Add the same volume of Freezing Medium B to the cells in a **dropwise** manner to bring the final cell concentration to 1×10^6–2×10^6 cells/mL.
5. Aliquot 1 mL of the cell suspension into each freezing vial. Store vials at –80°C overnight in an isopropanol chamber.
6. The next day, transfer the frozen vials to a liquid nitrogen tank (vapor phase) for long-term storage.

   Note: You may check the viability and recovery of frozen cells 24 hours after storing the cryovials in liquid nitrogen by following the procedure outlined in **Thawing and Establishing Cells**, page 6.

Differentiation Media

Introduction

One critical hallmark of MSCs is their ability to differentiate into three or more mature cell types. Traditional and modern bioassays are used to demonstrate the multipotency of MSCs to differentiate along the osteogenic, adipogenic, and chondrogenic lineages. This section provides guidelines for preparing media that are used for inducing GIBCO® Mouse (C57BL/6) MSCs to differentiate into osteogenic, adipogenic and chondrogenic cell types.

Mesenchymal Stem Cell Basal Medium

The MSC basal medium is used as a cell attachment medium and as a negative control during differentiation experiments. The medium consists of α-MEM medium with GlutaMAX™-I containing 10% **MSC-Qualified** FBS and 5 µL/mL gentamicin (see page 23).

Component	Final Conc.	For 500 mL
α-MEM medium with GlutaMAX™-I	1X	450 mL
FBS, MSC-Qualified	10%	50 mL
Gentamicin (10 mg/mL)	5 µg/mL	250 µL

Osteogenic Differentiation Medium

To prepare the osteogenic differentiation (OD) medium, combine the following in a sterile flask. You may store the OD medium at 4°C **in the dark** for up to four weeks.

Component	Final Conc.	For 100 mL
StemPro® Osteocyte/Chondrocyte Differentiation Basal Medium	1X	90 mL
StemPro® Osteogenesis Supplement	1X	10 mL
Gentamicin (10 mg/mL)	5 µg/mL	50 µL

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Differentiation Media, continued

Adipogenic Differentiation Medium

To prepare the adipogenic differentiation (AD) medium, combine the following in a sterile flask. You may store the AD medium at 4°C **in the dark** for up to four weeks.

Component	Final Conc.	For 100 mL
StemPro® Adipocyte Differentiation Basal Medium	1X	90 mL
StemPro® Adipogenesis Supplement	1X	10 mL
Gentamicin (10 mg/mL)	5 µg/mL	50 µL

Chondrogenic Differentiation Medium

To prepare the chondrogenic differentiation (CD) medium, combine the following in a sterile flask. You may store the CD medium at 4°C **in the dark** for up to four weeks.

Component	Final Conc.	For 100 mL
StemPro® Osteocyte/Chondrocyte Differentiation Basal Medium	1X	90 mL
StemPro® Chondrogenesis Supplement	1X	10 mL
Gentamicin (10 mg/mL)	5 µg/mL	50 µL

StemPro® MSC Differentiation Kits

StemPro® MSC differentiation kits contain the appropriate basal media and the differentiation supplements necessary for efficiently inducing your mesenchymal stem cells along the osteogenic, adipogenic, and chondrogenic lineages. For ordering information, refer to page 23.

Differentiating GIBCO® Mouse (C57BL/6) MSCs

Materials Needed

- Culture vessels containing GIBCO® Mouse (C57BL/6) MSCs
- Tissue-culture treated flasks, plates, or dishes
- MSC Basal Medium, pre-warmed to 37°C (see page 12)
- Appropriate Differentiation Medium, pre-warmed to 37°C (see pages 12–13)
- Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red
- Disposable, sterile 50-mL tubes
- 37°C incubator with humidified atmosphere of 5% CO_2
- TrypLE™ Express, pre-warmed to 37°C
- Hemacytometer, cell counter and Trypan Blue, LIVE/DEAD® Cell Vitality Assay Kit, or the Countess™ Automated Cell Counter

Harvesting MSCs

Follow the protocol below to harvest the GIBCO® Mouse (C57BL/6) MSCs for your differentiation experiments. We recommend that you expand your cells to ≤ 70% confluency in a tissue-culture treated T225 flask, and prepare the appropriate differentiation medium ahead of time.

1. Aspirate the spent growth medium from the flask.
2. Follow the **Passaging Cells** protocol, steps 2–9 (pages 8–9).
3. Calculate the required amount of MSC basal medium to obtain the appropriate seeding concentration of MSCs (see differentiation protocols, pages 15–19).
4. Resuspend the cells in the appropriate amount of MSC basal medium.
5. Dispense the cell solution according to the differentiation condition being tested (see differentiation protocols, pages 15–19).

Osteogenic Differentiation

Osteogenic Differentiation Protocol

1. Seed the MSCs into culture vessels at 0.5×10^4 cells/cm^2. For classical stain differentiation assays, seed the cells into a 12-well plate. For gene-expression profile studies, seed the cells into a T75 flask. For immunocytochemistry studies, seed the cells into a 16-well CultureWell™ chambered coverglass or a 96-well plate.
2. To six wells of a 12-well plate, add 1 mL of cell solution per well, and allow the cells to attach in the 37°C, 5% CO_2 incubator for a minimum of two hours.

   Note: Culturing the cells for up to four days in MSC basal medium before switching to OD medium has been shown to enhance osteogenic differentiation.
3. Replace three of the wells with MSC basal medium as negative controls, and the other three wells with fresh OD medium. Incubate the cultures at 37°C with 5% CO_2.
4. Refeed the cultures every 2–3 days with the medium prepared at the initiation of differentiation. The MSCs will continue to expand as they differentiate under the osteogenic conditions.
5. After specific periods of cultivation, osteogenic cultures can be processed for alkaline phosphatase staining (7–14 days) or Alizarin Red S staining (>21 days), gene expression analysis, or protein detection. For long term culture (>21 days), we recommend that you reduce the seeding density by half to prevent overgrowth.

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Expected Results

The bright field images below show GIBCO® Mouse (C57BL/6) MSCs at passage 3 (P3) post-thaw that were induced to differentiate along the osteogenic lineage.

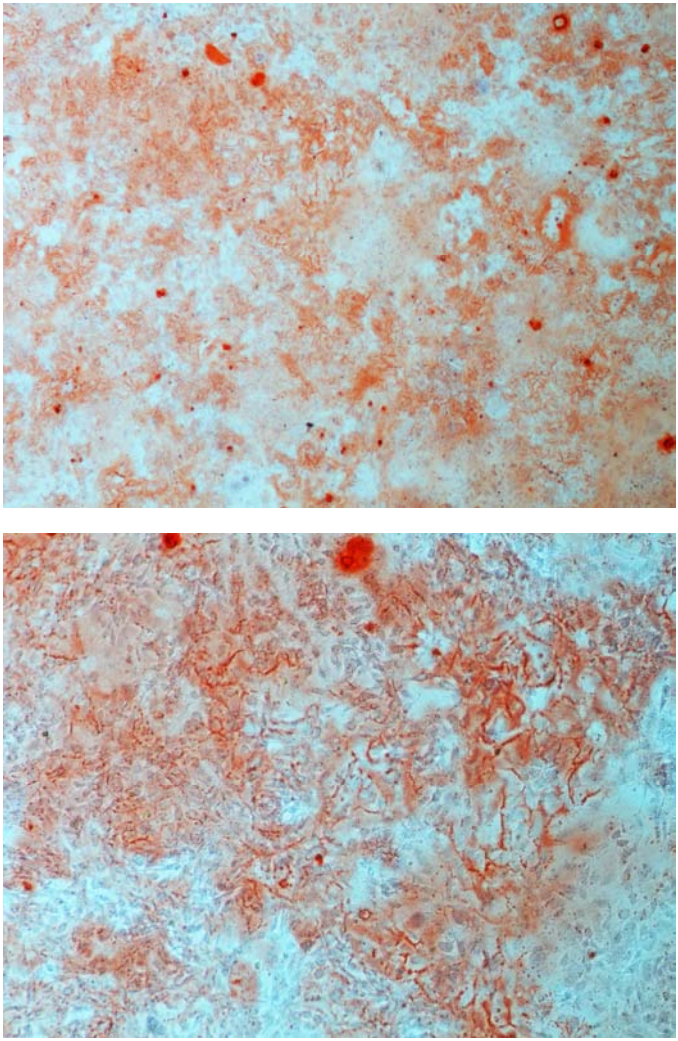

Figure 2. GIBCO® Mouse (C57BL/6) MSCs at P3 post-thaw were differentiated in OD medium (page 12) for 28 days, and stained with Alizarin Red S. The images were obtained using 4X (top) and 10X (bottom) objectives.

Adipogenic Differentiation

Adipogenic Differentiation Protocol

1. Seed the MSCs into culture vessels at 2.0×10^4 cells/cm^2. For classical stain differentiation assays, seed the cells into a 12-well plate. For gene-expression profile studies, seed the cells into a T75 flask. For immunocytochemistry studies, seed the cells into a 16-well CultureWell™ chambered coverglass or 96-well plate.
2. To six wells of a 12-well plate, add 1 mL of cell solution per well, and allow the cells attach in the 37°C, 5% CO_2 incubator for a minimum of two hours.

   Note: Culturing the cells for up to four days in MSC basal medium before switching to OD medium has been shown to enhance osteogenic differentiation.
3. Replace medium in three of the wells with MSC basal medium as negative controls, and other three wells with fresh AD medium. Incubate the cultures at 37°C and 5% CO_2.
4. Refeed the cultures every 3–4 days with the media prepared at the initiation of differentiation. The MSCs will continue to undergo limited expansion as they differentiate under the adipogenic conditions.
5. After specific periods of cultivation, adipogenic cultures can be processed for Oil Red O or LipidTOX™ staining (beginning at 7–14 days), gene expression analysis, or protein detection.

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Expected Results

The bright field images below show GIBCO® Mouse (C57BL/6) MSCs at passage 3 (P3) post-thaw that were induced to differentiate along the adipogenic lineage.

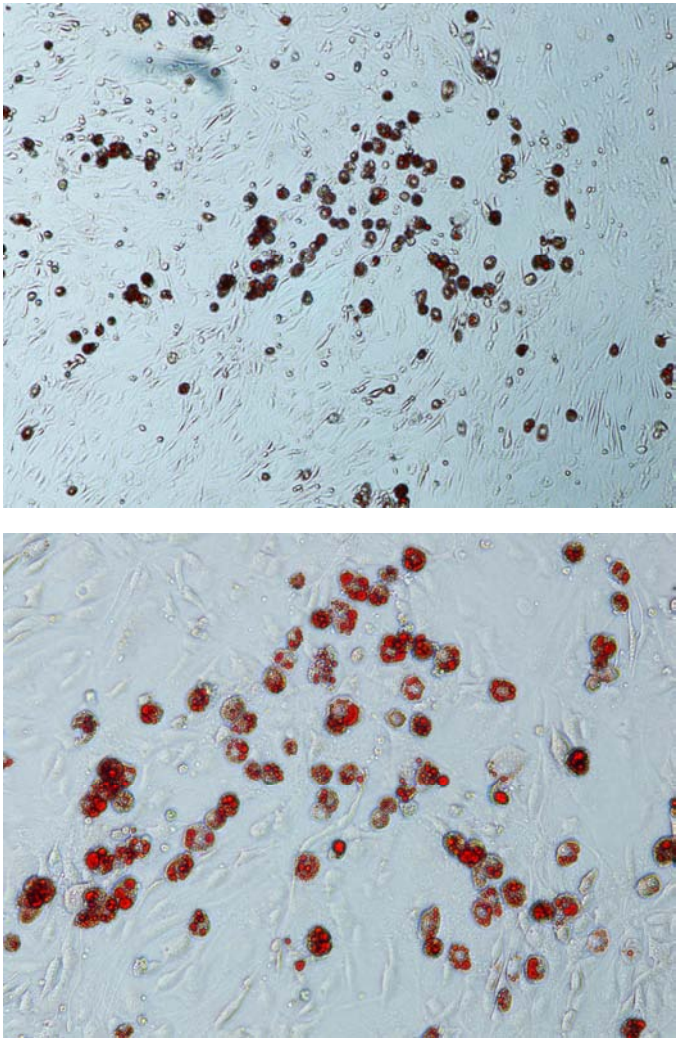

Figure 3. GIBCO® Mouse (C57BL/6) MSCs at P3 post-thaw were differentiated into adipocytes in AD medium (page 13) for 15 days, and stained with Oil Red O. The images were obtained using 10X (top) and 20X (bottom) objectives.

Chondrogenic Differentiation

Chondrogenic Differentiation Protocol

1. Detach the MSCs using TrypLE™ Express and perform a cell count as described in **Harvesting MSCs**, page 14 (through Step 3).
2. Resuspend the cells in MSC basal medium to a concentration of 8×10^6 cells/mL.
3. To each of the six wells of a 12-well tissue-culture dish, spot 10 µL of cells.
4. Incubate the cells for two hours at 37°C, 5% CO_2, and **90% humidity**.

   Note: If this step is not performed under high humidity conditions, the spots may dehydrate, inhibiting the formation of chondrogenic pellets.
5. To three of the spotted wells, add 1 mL of MSC basal medium as a negative control. To the other three wells, add 1 mL of CD medium.
6. Incubate the cultures at 37°C, 5% CO_2, and 90% humidity. Refeed the cultures every 2–3 days with same media, prepared at the initiation of differentiation.
7. Check for chondrogenesis after a set period of cultivation. You may perform alcian blue staining on the pellets (to detect glycosaminoglycans) after 14 days, or paraffin section of pellets for collagen 2a immunohistological staining after ~21 days.

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Expected Results

The bright field images below show GIBCO® Mouse (C57BL/6) MSCs at passage 3 (P3) post-thaw that were induced to differentiate into chondrocytes.

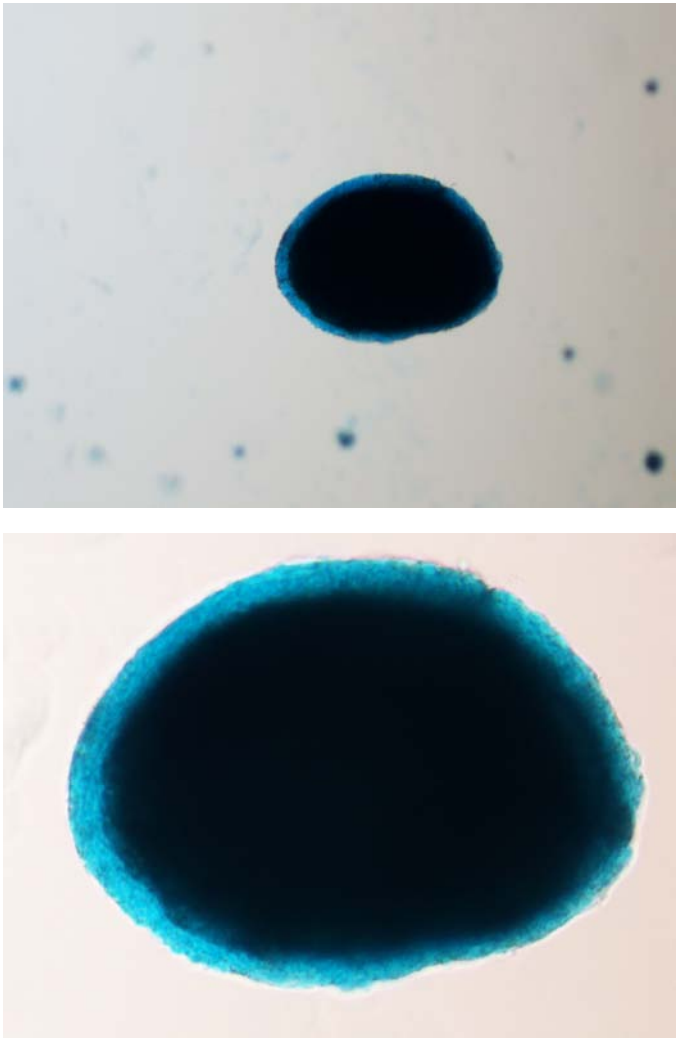

Figure 4. GIBCO® Mouse (C57BL/6) MSCs at P3 post-thaw were differentiated in CD medium (page 13) for 29 days, and stained with Alcian Blue. The images were obtained using 4X (top) and 10X (bottom) objectives.

Appendix

Troubleshooting

Culturing Cells

The table below lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Problem	Cause	Solution
No viable cells after thawing stock	Stock not stored correctly	Order new stock and store in liquid nitrogen. Keep in liquid nitrogen until thawing.
	Home-made stock not viable	Freeze cells at a density of 1×10^6 –2×10^6 viable cells/mL.
		Use low-passage cells to make your own stocks.
		Follow the procedures in **Freezing Cells** (pages 10–11) exactly. Slow freezing and fast thawing is the key. Add Freezing Medium B in a **dropwise** manner (slowly). At the time of thawing, quickly transfer the cells from the liquid nitrogen tank to the 37°C water bath, thaw the cells quickly, and do not expose them to the air.
		Obtain new GIBCO® Mouse MSCs.
	Thawing medium not correct	Use pre-warmed mouse MSC growth medium, prepared as described on page 5. Be sure to use **MSC-Qualified** FBS.
	Cells too diluted	Generally we recommend inoculating culture vessels at a density of 5,000 cells/cm^2.
	Cell not handled gently.	GIBCO® Mouse MSCs are fragile; treat your cells gently, do not vortex, bang the flasks to dislodge the cells, or centrifuge the cells at high speeds.
Cells grow slowly	Growth medium not correct	Use pre-warmed mouse MSC growth medium.
	Cells too old	Use healthy MSCs, under passage 11; do **not** overgrow or passage the MSCs more than 11 times.

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Culturing Cells, continued

The table below lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Problem	Cause	Solution
Cells differentiated	Culture conditions not correct	Thaw and culture a fresh vial of GIBCO® Mouse (C57BL/6) MSCs. Follow the thawing instructions (page 6) and subculture procedures (pages 8–9) exactly.
	Cells too old	MSCs above passage 11 may lose multipotency and become more differentiated.
Cells not adherent after initial thaw	Used serum other than MSC-Qualified FBS	Be sure to prepare your culture medium using **MSC-Qualified** FBS (see page 23 for ordering information).

Differentiating Cells

The table below lists some potential problems and solutions that help you troubleshoot your cell culture problems.

Problem	Cause	Solution
Cells fail to differentiate	Initial spotting step not performed under high humidity (if differentiating into chondrocytes)	If this step is not performed under high humidity conditions, the spots may dehydrate and the formation of chondrogenic plates inhibited. Repeat the initial spotting step at 37°C, 5% CO_2, and 90% humidity, and incubate the culture in a humidified box with loose-fitting cover or aluminum foil perforated with small holes.
Cells have overgrown the culture plates and have detached	Initial seeding density too high	For long term culture (>21 days), we recommend that you seed at a lower cell density of 3×10^3 cells/cm^2 to prevent overgrowth and cell detachment.

Additional Products

Additional Products

The products listed in this section may be used with GIBCO® Mouse (C57BL/6) Mesenchymal Stem Cells. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (see page 24).

Item	Quantity	Cat. no.
D-MEM/F-12 (1X) with GlutaMAX™-I, liquid, 1:1	500 mL	10565-018
Minimum Essential Medium (MEM) α Medium (1X) with GlutaMAX™-I, ribonucleosides and deoxyribonucleosides	500 mL	32571-036
GlutaMAX™-I Supplement	100 mL	35050-061
Fetal Bovine Serum (FBS), MSC-Qualified	100 mL 500 mL	12662-011 12662-029
StemPro® Adipogenesis Differentiation Kit	100 mL	A10070-01
StemPro® Chondrogenesis Differentiation Kit	100 mL	A10071-01
StemPro® Osteogenesis Differentiation Kit	100 mL	A10072-01
Gentamicin (10 mg/mL)	10 mL	15710-064
Dulbecco's Phosphate Buffered Saline (D-PBS), containing no calcium, magnesium, or phenol red	500 mL	14190-144
TrypLE™ Express Dissociation Enzyme	100 mL	12604-013
Antibiotic-Antimycotic (100X), liquid	100 mL	15240-062
Gentamycin Reagent Solution (10 mg/mL), liquid	10 mL	15710-064
Gentamycin Reagent Solution (50 mg/mL), liquid	10 mL	15750-060
Trypan Blue Stain	100 mL	15250-061
HCS LipidTOX™ Green neutral lipid stain	1 each	H34475
LIVE/DEAD® Cell Vitality Assay Kit	1000 assays	L34951
Countess™ Automated Cell Counter (includes 50 Countess™ cell counting chamber slides and 2 mL of Trypan Blue Stain)	1 unit	C10227
CultureWell™ chambered coverglass (16 wells per coverglass, set of 8)	1 set	C37000

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete Technical Support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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Material Safety Data Sheets (SDSs)

Safety Data Sheets (SDSs) are available on our website at www.invitrogen.com/sds.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Purchaser Notification

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