Human Bone Marrow Derived Mesenchymal Stem Cell (MSC) Care Manual

INSTRUCTION MANUAL ZBM0101.02

SHIPPING CONDITIONS

Human Bone Marrow Derived MSC (HBMMSC-F)

All US and Canada orders are shipped via Federal Express Priority service and are usually received the next day. International orders are shipped via FedEx or DHL service using dry ice or a dry vapor shipper if transit time will exceed 3 days. Primary human cells are very sensitive to extended times (> 3 days) transported using dry ice. Please inquire for dry vapor shipper availability if your transit time will exceed 3 days. Cells should always be stored in liquid nitrogen vapor phase immediately upon arrival.

STORAGE CONDITIONS

BMSC-1 Media: 4°C: 30 days from ship date -20°C **3** months from ship date

Other Media: 4°C: 30 days from ship date -20°C **6** months from ship date

Cells: Store in vapor phase nitrogen (-150°C to -190°C) IMMEDIATELY UPON RECEIPT.

All Zen-Bio Inc. products are for research use only. Not approved for human or veterinary use or for use in diagnostic or clinical procedures.

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THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

HBMMSC-F	CRYOPRESERVED HUMAN BONE MARROW DERIVED MESENCHYMAL STEMS CELLS, 1 MILLION CELLS/VIAL

PRECAUTIONS

This product is for research use only. *It is not intended for human, veterinary, or in vitro diagnostic use*. Proper precautions and biological containment should be taken when handling cells of human origin, due to their potential biohazardous nature. **Always wear gloves and work behind a protective screen when handling primary human cells.** All media, supplements, and tissue culture ware used in this protocol should be sterile.

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

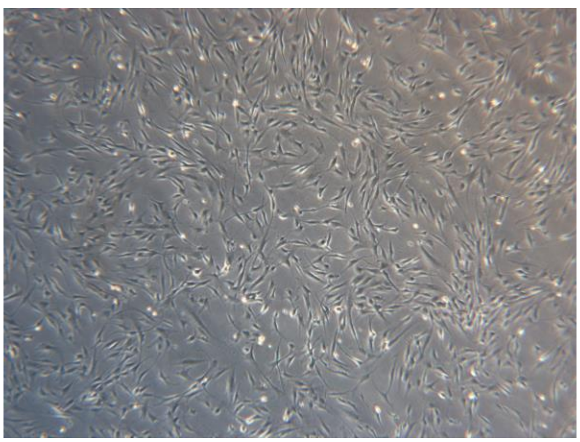
Zen-Bio, Inc. warrants its cells only if Zen-Bio media are used and the recommended protocols are followed without amendment or substitution. Human Bone Marrow Derived Mesenchymal Stem Cells depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed cell responsiveness in assays may be lower than expected.

Contact ZenBio, Inc. within no more than 24 hours after receipt of products for all claims regarding shipment damage, incorrect ordering or other delivery issues. Delivery claims received after 7 days of receipt of products are not subject to replacement or refund.

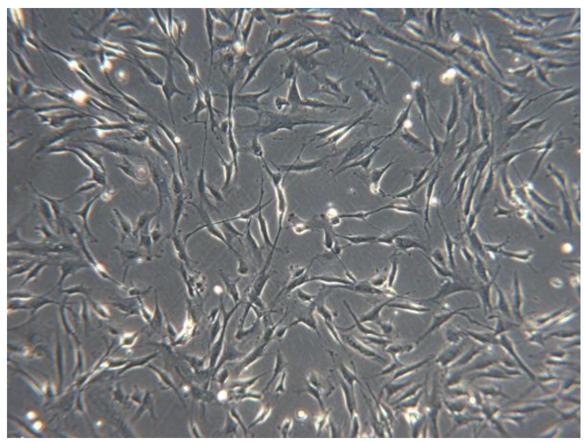
INTRODUCTION

Human Bone Marrow Derived Mesenchymal Stem Cells (HBMMSC) are isolated from bone marrow aspirate obtained from competent consented adult donors undergoing elective procedures in the United States. The donor has signed an ethical review board validated donor consent form that specifically lists both the intended uses for the donation for non-clinical research and confirms the procedures for processing the samples are Standard Operating Procedure (SOP) managed Good Laboratory Practices (GLP) protocols in compliance with ethical regulations. All samples are collected and processed in the United States.

HBMMSC can be differentiated into mature cells that produce cartilage, fat, bone, tendons, and muscle. HBMMSCs have potent immunomodulatory effects and are capable of limiting immune cell growth and producing anti-inflammatory cytokines. In addition, these cells are compatible for the 3D fabrication of human in vitro tissue for use in drug screening. During culture, these stem cells can be differentiated into various lineages using Zen-Bio media formulations and protocols. This instruction manual describes procedures to induce human bone marrow derived mesenchymal stem cells to differentiate into 1) mature adipocytes, 2) osteoblasts, and 3) chondrocytes.



Subconfluent Bone Marrow Derived MSCs in BMSC-1 (P3)
5X



Subconfluent Bone Marrow Derived MSCs in BMSC-1 (P3)
10X

MATERIALS PROVIDED FOR EACH CATALOG ITEM

❖ **Cryopreserved Human Bone Marrow Derived Mesenchymal Stem Cells (Catalog # HBMMSC-F)**

- Frozen vial containing a minimum of 1 million viable Human Bone Marrow Derived Mesenchymal Stem cells.
- Store cells in vapor phase nitrogen (-150°C to -190°C) immediately upon receipt

MEDIA COMPOSTIONS

Bone Marrow Stem Cell Growth Medium (catalog #BMSC-1)	**Storage and Expiration Date**
Alpha Minimal Essential Medium (α-MEM) Fetal Bovine Serum (FBS) Fibroblast Growth Factor-basic (bFGF) human Epidermal Growth Factor (hEGF), human Insulin-like Growth Factor-I (IGF-1), human Insulin, human Apo-transferrin Sodium Selenite Bovine Serum Albumin (BSA) 2-Mercaptoethanol Hydrocortisone Gentamicin	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, the media is stable for **3 months.** Add fresh gentamicin at 1% when you are ready to use. The media will expire 30 days after the thaw date.
Adipocyte Differentiation Medium Cat # DM-2	**Storage and Expiration Date**
DMEM / Ham's F-12 (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Isobutylmethylxanthine PPARγ agonist Penicillin Streptomycin Amphotericin B	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, the media is stable for **6 months**. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.
Adipocyte Maintenance Medium Cat # AM-1	**Storage and Expiration Date**
DMEM / Ham's F-12 (1:1, v/v) HEPES pH 7.4 Fetal Bovine Serum (FBS) Biotin Pantothenate Human insulin Dexamethasone Penicillin Streptomycin Amphotericin B	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, the media is stable for **6 months**. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.

MEDIA COMPOSTIONS (continued)

Chondrocyte Differentiation Medium Cat# CM-1; CM-1-100	**Storage and Expiration Date**
DMEM-high glucose Fetal Bovine Serum (FBS) Transforming growth factor β_1(TGF- β1) Ascorbate-2-phosphate Dexamethasone Insulin Transferrin Selenium Penicillin Streptomycin Amphotericin B	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, the media is stable for **6 months**. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.
Human Osteoblast Differentiation Medium for Bone Marrow MSCs Cat# HBMOB-1	**Storage and Expiration Date**
Alpha Minimal Essential Medium (α-MEM) Fetal Bovine Serum (FBS) β-glycerophosphate L-ascorbic acid-2-phosphate Dexamethasone Penicillin Streptomycin	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label. If stored at -20°C upon arrival, the media is stable for **6 months**. Add fresh antibiotics at 1% when you are ready to use. The media will expire 30 days after the thaw date.

THAWING AND PLATING PROCEDURES for Cryopreserved Human Bone Marrow Derived MSC ___________________

NOTE: Primary cells can be very sensitive to brands of cultureware. Zen-Bio does not currently recommend the use of Corning Falcon brand plates or flasks. Our scientists are using Nunc, Corning Costar, or Greiner Bio-One tissue culture treated plates and flasks. Please contact us if you have any questions.

1. Warm Bone Marrow Stem Cell Growth Medium (BMSC-1) in a 37°C water bath prior to thawing cells.
2. Wipe the vial of cells with 70% alcohol. Transfer to a sterile environment, briefly open the vial to release any pressure, and then retighten the cap.
3. Transfer the vial to a 37°C water bath. Do not submerge the vial past the bottom of the cap. Thawing requires at most 90 seconds. Do not keep the vial in the water bath past this time as it may result in poor cell viability.
4. When a single small piece of ice remains, remove vial from water bath, wipe with 70% alcohol, and place in sterile environment.
5. Add 0.5 ml of BMSC-1 growth medium to the vial, mix gently by pipetting up and down. The total volume should now be 1 ml.
6. Transfer the cell suspension into 5 ml of growth (BMSC-1) medium.
7. Centrifuge the cells at 400 x g for 5 minutes to pellet the cells.
8. Aspirate off the supernatant and resuspend the cells in growth (BMSC-1) medium by gently pipetting up and down.
9. Plate cells in cell culture flasks. We recommend plating at a cell density of 3,000-5,000 cells/cm^2.
10. Aspirate and re-feed the cells with growth (BMSC-1) medium every 4-5 days.

SUBCULTURING BONE MARROW DERIVED MSC______________

NOTE: Human Bone Marrow Derived MSC are frozen at the end of passage 2-3, we recommend using the cells by the end of passage 6.

1. Bone Marrow derived mesenchymal stem cells should be passaged once they reach 60-80% confluency.
2. Aspirate the medium from the culture vessels containing cells
3. Wash cells by adding appropriate amount of phosphate buffered saline (PBS) without calcium and magnesium. Add the PBS along the side of the dish as to not disturb the cell layer, aspirate off the PBS. growth
4. Add appropriate amount of 0.25% Trypsin/EDTA per culture vessel.

5. Incubate cells with Trypsin 5-8 minutes, wash cells, and collect the cells into an appropriate size centrifuge tube with an equal volume of pre-warmed medium (BMSC-1) to neutralize the cells.
6. Centrifuge cells at 500 x g for 5 minutes to pellet and resuspend the pellet with growth medium
7. Count and plate at seeding density of 3,000 cells/cm^2
8. Aspirate and re-feed the cells with growth (BMSC-1) medium every 4-5 days.

ADIPOGENESIS PROCEDURE

Differentiation of Human Bone Marrow Derived Mesenchymal Stem Cells into Adipocytes

Adipocyte Differentiation Medium (DM-2) and Adipocyte Maintenance Medium (AM-1) are BOTH necessary to differentiate the cells to mature adipocytes

1. Seed 10,000 cells/cm^2 into a tissue culture plate in 0.2 ml per cm^2 of pre-warmed growth medium (BMSC-1).
2. Incubate at 37°C for 2-3 days until the cells reach 90% confluence.
3. Aspirate and re-feed the cells with pre-warmed Adipocyte Differentiation Medium (DM-2).
4. Allow cells to remain in Differentiation Medium (DM-2) for 2-3 days and the medium should then be aspirated and replaced with Adipocyte Maintenance Medium (AM-1)
5. Change the medium with fresh AM-1 every 3-4 days.
   Note: When cells start to appear rounded with large lipid droplets, prevent lifting of cells by using a micropipette to slowly remove the medium.
6. Stain with Oil Red O to detect the presence of lipid droplets 21 days after the initiation of differentiation. ZenBio does offer a Lipid Staining Kit based on Oil Red O staining (cat# ST-R100)

CHONDROGENESIS PROCEDURE

Differentiation of Human Bone Marrow Derived Mesenchymal Stem Cells into Chondrocytes

Chondrocyte Medium (CM-1) is necessary in culturing chondrocytes

1. Prepare a cell suspension of 16 million cells/ml.
2. Seed a 5 µl droplet (80,000 cells) into a tissue culture plate.
3. In 3 hours, check that cells have adhered and fill each well with 0.2 ml per cm^2 of pre-warmed Chondrocyte Medium.
4. Change the medium with fresh pre-warmed Chondrocyte Medium every 3-4 days.
5. Stain for chondrocyte presence with Alcian Blue or Toluidine Blue 21 days after the initiation of differentiation.

OSTEOGENESIS PROCEDURE

Differentiation of Human Bone Marrow Derived Mesenchymal Stem Cells into Osteoblasts

Human Osteoblast Differentiation Medium for Bone Marrow MSCs (HBMOB-1) is necessary in culturing chondrocytes

1. Seed 5,000 cells/cm^2 into a tissue culture plate in 0.2 ml per cm^2 of pre-warmed growth medium (BMSC-1).
2. Incubate at 37°C for 2-3 days until the cells reach 50% confluence.
3. Aspirate and re-feed the cells with pre-warmed (37°C) Osteoblast Differentiation Medium for Bone Marrow MSC.
4. Change the medium with fresh pre-warmed Osteoblast Medium (HBMOB-1) every 3-4 days.
5. Stain for osteoblast presence with Alizarin Red S or alkaline phosphatase 21 days after the initiation of differentiation.

FREQUENTLY ASKED QUESTIONS

1. **Can I passage these cells? If yes, what is the maximum passage?**
   Yes, these human bone marrow derived stem cells can be trypsinized and replated
   Until passage 6. Human Bone Marrow Derived MSC are shipped at passage 2-3.

2. **What is the average doubling time of these cells?**
   The average doubling time is 24-36 hours. However, keep in mind that the replication rate for these stem cells varies slightly from donor to donor.

3. **Are antibiotics included in the medium?**
   Yes. Antibiotics and/or anti-fungal agents are always recommended since the cells are primary cells.

4. **What quality control testing is performed on the cells?**
   We do confirm the presence of several cell surface markers indicative of stem cells via flow cytometry.
   - The adult stem cells :
     - Cell surface marker staining >90% positive for CD105 and CD44
     - Cell surface marker staining negative for CD31 and CD45.
   - Adipocytes
     - lipid staining, total triglyceride content, functional lipolysis
   - Osteoblasts
     - measurement of degree of mineralization as assessed by Alizarin Red staining

- Chondrocytes
    - Positive collagen staining

5. **Where are the cells obtained?**
   Human Bone Marrow Derived Mesenchymal Stem Cells are isolated from bone marrow aspirate obtained from competent consented adult donors undergoing elective procedures in the United States. All samples are collected and processed in the United States.

6. **Do you test for pathogens? Which ones?**
   Yes. All tissue donor samples are screened and found to be negative for HIV-1, HIV-2, and Hepatitis B, and Hepatitis C using US Food and Drug Administration (FDA) licensed tests.

7. **What is the concentration of ingredients in your media?**

   We do not disclose the concentrations of the components of our media. We are happy to prepare custom media to your specifications.

8. **Can I re-freeze the cells?**
   At this time we do not recommend re-freezing the cells and make no guarantees as to future performance.

PATHOGEN TESTING

All tissue donor samples are screened and found to be non-reactive to HIV, Hepatitis B and Hepatitis C using United States Food & Drug Administration (FDA) licensed testing procedures. However, no known test can offer complete assurance that viruses are not present. Since we cannot test all pathogens, always treat the culture as a potentially infectious reagent. We recommend using the US Centers for Disease Control (CDC) Universal Precautions for prevention of blood-borne pathogens as a minimum guideline for standards of practice at Biosafety Level 1 (BLS-1) or higher.