Human Skeletal Muscle Myoblast Care Manual: Maintenance and Differentiation from Myoblasts to Myocytes

INSTRUCTION MANUAL ZBM0044.09

SHIPPING CONDITIONS

Human Skeletal Muscle Myoblast Cells

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

STORAGE CONDITIONS

- **Cryopreserved cells:** Vials of cryopreserved cells are to be stored in vapor phase nitrogen (-150°C to -190°C) IMMEDIATELY UPON RECEIPT.
- **Media** : +4°C Expires 1 month from ship date
  :-20°C Expires 6 months from ship date

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

ORDERING INFORMATION AND TECHNICAL SERVICES

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THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

SKB-F, SKB-F-1, SKB-F-SL	HUMAN SKELETAL MYOBLASTS, SINGLE DONOR, LEAN DONOR (BMI <24.9), MIXED DONOR LOT (POOLED)
SKM-D-F	HUMAN SKELETAL MYOBLASTS, TYPE 2 DIABETIC DONOR

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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 product storage, handling, media and protocols are followed without amendment or substitutions. Human myoblast viability depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed, limited viability, attachment and differentiation may occur and cell growth may be slow. Cryopreserved myoblasts are assured to be viable when stored, thawed and maintained according to Zen-Bio reagents and protocols.

PRECAUTIONS

This product is for research uses 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 cultureware used in this protocol should be sterile.

INTRODUCTION

Cultured human skeletal muscle myoblasts

Skeletal muscle is an important site of insulin-stimulated glucose disposal and often the site of insulin resistance in obesity. Human primary cultured skeletal muscle cells can directly reflect a patient's metabolic phenotype, because many of the signaling pathways are maintained intact. Zen-Bio offers human primary skeletal muscle cells from a variety of donors, including obese donors with Type 2 diabetes.

Cryopreserved human skeletal muscle myoblasts are isolated from skeletal muscle tissue from competent consented adult donors in the United States undergoing elective surgery. Each competent adult volunteer donor has signed an Institutional Review Board (IRB) 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 protocols in compliance with ethical regulations. All samples are collected and processed in the United States.

MATERIALS PROVIDED FOR EACH CATALOG ITEM_____

Cryopreserved Human Skeletal Muscle Myoblasts

Store in vapor phase liquid nitrogen IMMEDIATELY upon receipt. *Any other storage negates the warranty*

1. **Catalog # SKB-F**
   - Cryopreserved Human Skeletal Myoblasts 500,000 viable cells/vial
2. **Catalog # SKB-F-1**
   - Cryopreserved Human Skeletal Myoblasts, 500,000 cells/vial, BMI <25 (LEAN)
3. **Catalog # SKM-D-F**
   - Cryopreserved Human Skeletal Myoblasts, 500,000 cells /vial- DIABETIC DONOR
4. **Catalog # SKB-F-SL**
   - Cryopreserved Human Skeletal Myoblasts, 500,000 cells /vial- pooled (mixed donors) lot

MEDIA COMPOSTIONS _____

Skeletal Muscle Cell Growth Medium catalog # SKM-M)	**Storage and Expiration Date**
DMEM (1g/L D-glucose) Fetal Bovine Serum (US origin) Bovine Serum Albumin Fetuin Human Epidermal Growth Factor (hEGF) Dexamethasone Human Insulin 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% volume when you are ready to use. The media will expire 30 days after the thaw date.
Skeletal Muscle Cell Differentiation Medium (catalog# SKM-D)	**Storage and Expiration Date**
DMEM (1g/L D-glucose) Horse serum (US Origin) Bovine Serum Albumin Fetuin 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% volume when you are ready to use. The media will expire 30 days after the thaw date
Skeletal Muscle Cryopreservation Medium (catalog# SKM-100)	**Storage and Expiration Date**
DMSO DMEM (1g/L D-glucose) Fetal Bovine Serum (US origin) Bovine Serum Albumin Fetuin Human Epidermal Growth Factor (hEGF) Dexamethasone Human Insulin	• 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.

Plating Procedure for Cryopreserved Human Skeletal Muscle Myoblasts

Cryopreserved Human Skeletal Muscle Myoblasts (Catalog # SKB-F)

Please note: Primary human skeletal myoblast cells require use of sterile tissue culture treated cultureware. No extracellular matrix coatings are required.

1. Remove cells from liquid nitrogen and place immediately into a 37° C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.
2. Upon thawing, transfer the cells to a sterile conical bottom centrifuge tube containing 10 ml of Skeletal Muscle Growth Medium (cat # SKM-M). Centrifuge: 1,200 rpm (282 X g) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.
3. The cell vial contains a minimum of 500,000 viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 0.5 ml Skeletal Muscle Growth Medium, dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 µl of cells and mixing with 100 µl of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemacytometer.
4. Plate approximately 5,000-15,000 cells / cm^2 using the media volumes from the table below. Refer to the manufacturer's specifications for the specific cultureware brand you are using.

FORMAT	VOLUME PER WELL	TOTAL VOLUME PER FORMAT*
96 well plate	150 µl	14.4 ml
48 well plate	500 µl	24.0 ml
24 well plate	1 ml	24.0 ml
12 well plate	2 ml	24.0 ml
6 well plate	3 ml	18.0 ml
10 cm dish	15 ml	15.0 ml
T-75 flask	20 ml	20.0 ml
T25 flask	7 ml	7.0 ml

***We recommend preparing slightly larger volumes to allow for loss due to foam and pipet error.**

5. Plate cells in desired format and place in a humidified 37ºC incubator with 5% CO_2. Do not agitate the plate, as cells will not plate evenly.
   To differentiate the cells please see the protocol on page 6 starting at step 1.

DIFFERENTIATION OF MYOBLASTS INTO MYOCYTES

1. Plated myoblasts in Skeletal Muscle Growth Medium (cat # SKM-M) can undergo differentiation using Skeletal Muscle Cell Differentiation Medium (cat # SKM-D). **Differentiation should be initiated when the plated myoblasts reach 80-90% confluence**. The exact number of days necessary to reach 80-90% confluence will depend on your initial seeding density (typically 1-3 days).
2. To start the process, aspirate the entire volume of Skeletal Muscle Growth Medium from all wells. Add the appropriate volume of Skeletal Muscle Cell Differentiation Medium (catalog # SKM-D) to the wells (see Table 1. Feeding Volumes). Incubate the plate at 37^0C and 5% CO_2. Fresh Differentiation Medium will need to be added every 2-3 days. Remove all of the medium and replace with fresh medium.
3. After 6 days the cells should have fused to form myotubes. These are elongated, multinucleated cells. They will appear to be lined up when viewed under a microscope.
4. The myocytes may be used for assays 6-8 days after the initiation of differentiation and are suitable for most assays.

Table 1. Feeding Volumes

Format	Plating	Change SKM-M to SKM-D		Change SKM-D to SKM-D		Change SKM-D to SKM-D	
	IN	OUT	IN	OUT	IN	OUT	IN
96 well plate	150 µl/ well	150 µl/ well	150µl/ well	150µl/ well	150µl/ well	150µl/ well	150µl/ well
48 well plate	500 µl/ well	500µl/ well	500µl/ well	500µl/ well	500µl/ well	500µl/ well	500µl/ well
24 well plate	1.0 ml/well	1.0 ml/well	1.0 ml/well	1.0 ml/well	1.0ml/well	1.0ml/well	1.0ml/well
12 well plate	2.0 ml/well	2.0 ml/well	2.0 ml/well	2.0 ml/well	2.0 ml/well	2.0 ml/well	2.0 ml/well
6 well plate	3.0 ml/well	3.0 ml/well	3.0 ml/well	3.0 ml/well	3.0 ml/well	3.0 ml/well	3.0 ml/well
T-75 flask	20 ml/flask	20 ml/flask	20 ml/flask	20 ml/flask	20 ml/flask	20 ml/flask	20 ml/flask
T-25 flask	7 ml/flask	7 ml/flask	7 ml/flask	7 ml/flask	7 ml/flask	7 ml/flask	7 ml/flask

A. 80% Confluent Myoblasts **B. 3 day old myocytes** (3 days post-differentiation) **C. 1-week-old myocytes** (1 wk post-differentiation)

MYOBLAST ⟶ MATURE SKELETAL MYOCYTE

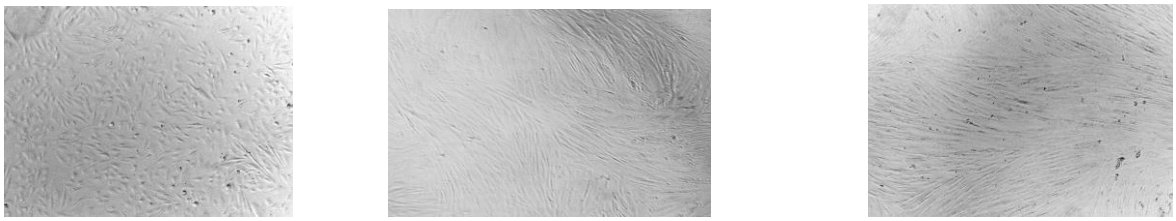

Figure 1: Photographs of 80% confluent Myoblasts (A), 3 day-old (post-differentiation) cultured myocytes (B) and mature (1 week post-differentiation) cultured Myocytes (C). These are unstained photographs of human myocyte morphology (20X). The cells should appear comparable in appearance to these pictures. The myocytes should be 80% confluent after plating for differentiation. If they are not 80% confluent, the cells will not differentiate well. Please see the Troubleshooting guide for any problems.

EXPANSION PROCEDURE

Cryopreserved Human Skeletal Muscle Myoblasts (Catalog # SKB-F)

1. Remove cells from liquid nitrogen and place immediately into a 37°C water bath with agitation. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.
2. Upon the thawing, add the cells to a sterile conical bottom centrifuge tube, containing 10 ml of Skeletal Muscle Growth Medium (SKM-M).
3. Centrifuge at 280 x g, 20°C, 5 minutes. Aspirate the medium and resuspend cells in a volume of SKM-M appropriate for counting the cells. Count using a hemacytometer.
4. Place approximately 500,000 cells in T-75 culture flasks using Skeletal Muscle Growth Medium (cat# SKM-M).
5. Incubate cells until they are 70% confluent (in about 3-5 days). **Do not let the cells become 100% confluent**. Cells will need to be fed every 2-3 days with SKM-M. Replace all medium with fresh SKM-M.
6. Aspirate medium and wash myoblasts 4-5 times using sterile Phosphate Buffered Saline (PBS) without Calcium or magnesium to remove all traces of serum (until there is no foaming of the medium). Remove the PBS and release the cells from the flask bottom by adding 2 mL/T-75

flask of 0.25% trypsin/ 2.21mM EDTA solution. Allow cells to trypsinize for 5 minutes at 37°C. GENTLY tap the side of the flask to softly loosen the cells.

7. Neutralize the trypsin using 7 ml Skeletal Muscle Cell Growth Medium (cat# SKM-M) per T-75 flask. Check the flask under a microscope to ensure all cells are free of the flask bottom.
8. Count the cells and plate in desired format (see page 5 for plating protocol) or freeze (see Cell Freezing Protocol below).
    a. Ensure cells are evenly suspended when plating large numbers of plates or flasks.
    b. Do not agitate plates and flasks after plating.
    c. Place in a humidified incubator at 37°C and 5% CO_2, making sure the surface is level for even cell distribution.
9. Follow the differentiation protocol as outlined on page 6 or split the cells 1:4 for further expansion.
10. We DO NOT recommend differentiating myoblasts that are older than Passage 6. Cells will arrive at Passage 3 or 4.

SKELETAL MYOBLAST CELL CRYOPRESERVATION PROTOCOL_

1. Incubate cells until they are 70% confluent (in about 3-5 days). **Do not let the cells become 100% confluent**.
2. Aspirate medium and wash myoblasts 4-5 times using sterile Phosphate Buffered Saline (PBS) to remove all traces of serum (until there is no foaming of the medium).
3. Remove the PBS and release the cells from the flask bottom by adding 2 mL/T-75 flask of 0.25% trypsin/ 2.21mM EDTA solution.
4. Allow cells to trypsinize for 5 minutes at 37°C. gently tap the side of the flask to loosen the cells.
5. Neutralize the trypsin using 7 ml Skeletal Muscle Cell Growth Medium (cat# SKM-M) per T-75 flask. Check the flask under a microscope to ensure all cells are free of the flask bottom.
6. Centrifuge the cells at 1200 rpm for 5 min, 20°C.
7. Resuspend the cells in appropriate volume of cold Skeletal Myoblast Cryopreservation Medium (SKM-100).

Cell Yield	Volume SKM-100	Cryovial Size
0-11 million cells	1.0ml	2.0ml
12-13 million cells	1.5ml	2.0 ml
13-40 million cells	4.0	5.0ml

8. Immediately place vials of cells at -80°C overnight using controlled rate system to allow cooling of -1°C/minute.
9. Store cells in vapor phase liquid nitrogen less than 24 hours after freezing the cells.

TROUBLESHOOTING GUIDE

Observation	Possible causes	Suggestions
Myoblasts do not differentiate	1. Cells have been passaged too many times 2. Differentiation conditions not optimal 3. Cells were plated at a low density 4. Cultureware used not optimal for human primary myoblasts	1. Use cells of a lower passage number 2. Use our defined differentiation media. Make sure that wells are 80% confluent BEFORE initiating differentiation. 3. Use the cell density recommended in our manual 4. Verify the surface area for the cultureware brand you are using.
Myoblasts do not grow	1. Cells have been passaged too many times 2. Cells expanded too high	1. Use cells of a lower passage number 2. Do not exceed 1:4 expansion ratio
Edge effects	1. Medium in outside wells evaporated	1. Ensure a saturated humidity in the incubator and feed the cells no less than every 3 days. Make sure multiple plates are stacked no more than 3 plates high.

FREQUENTLY ASKED QUESTIONS

1. Can I passage (expand) the cells?
   -ZenBio Human Myocytes cannot be passed because they are terminally differentiated.
   -ZenBio Human Myoblasts can be trypsinized and replated. Please note myoblasts grow slower with each passage and differentiate poorly after passage 6.
   -ZenBio Myoblasts are shipped at Passage 3-4.
2. When do the cells differentiate?
   Cells should begin to fuse and line up within 3 days after differentiation is induced. [See Figure 1]
3. How fast do the cells replicate?
   The average doubling time is 24-36 hours. However, keep in mind that the replication rate for human myoblasts varies slightly from donor to donor.
4. How long do the cells last in culture?
   Myocytes retain similar morphology and express myocyte specific genes for at least 1 week after induction of differentiation.
5. Should antibiotics be included in the medium?
   Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells. All Zen-Bio media contain antibiotics and anti-fungal agents.
6. From where are the myoblast cells sourced?
   The myoblasts are isolated from human skeletal muscle tissue from competent consented adult donors undergoing elective surgery in the United States. Each competent adult volunteer donor

has signed an Institutional Review Board (IRB) 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 protocols in compliance with ethical regulations. All samples are collected and processed in the United States.

7. How are the cells shipped?
   Cryopreserved cells will be packaged on dry ice or a dry vapor shipper (if transit time will exceed 3 days) and sent to customers via Federal Express overnight delivery or international priority courier service.

8. How long do I have to wait before receiving the cells?
   We do not ship cells to domestic locations on Fridays. In general, myoblasts can be shipped the second day after the purchase order is confirmed.

9. Do you test for pathogens? Which ones?
   Yes. Samples from each donor are tested via PCR to confirm non-reactivity for HIV-1, HIV-2, hepatitis B and hepatitis C. However, since we cannot test all pathogens, please treat the culture as a potentially infectious agent. All human based products should be handled at a BSL-1 (Biosafety Level 1) or higher.

10. What donor information do I receive?
    The donor's age, gender, race, diabetes status, and Body mass index (BMI) will be provided.

11. Are the cells from one donor?
    Yes. We can also provide lot numbers containing cells mixed donors to get average responses. Please inquire about availability of single donor (cat# SKB-F), single donor with low Body Mass Index (BMI) less than 24.9 (cat# SKB-F-1) and mixed donor (Cat# SKB-F-SL) lots at time order is placed.

PATHOGEN TESTING

Samples from each donor are tested via PCR and found non-reactive to viral DNA from Hepatitis B and viral RNA from HIV 1, HIV-2 and Hepatitis C. Hepatitis B Surface antigen (HBsAg) and HIV antibody (Ab), and STS (Syphilis) are also found non-reactive by US Food and Drug Administration (FDA) licensed tests. However, no known test can offer complete assurance that the cells are pathogen free. 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 (BSL-1) or higher. 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. Mycoplasma contamination is not detected in our labs.