



Human Melanocyte Care Manual

INSTRUCTION MANUAL ZBM0058.03

SHIPPING CONDITIONS

Human Melanocyte Cells

Cells are shipped using dry ice or dry vapor shipper. Orders are delivered via Federal Express or DHL courier. All US and Canada orders are shipped via Federal Express Priority service and are usually received the within 1-2 days. International orders are usually received in 2-4 days. Alternate couriers and dry vapor shippers are available if you expect your delivery time to exceed 3 days. Please inquire for details.

STORAGE CONDITIONS

Media: Store at 4°C. Expiration date 60 days 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.

THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

MEL-F	HUMAN ADULT MELANOCYTES, CRYOPRESERVED
MEL-F-NEO	NEONATAL HUMAN MELANOCYTES, CRYOPRESERVED

LIMITED PRODUCT WARRANTY

This warranty limits our liability for 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 the performance of cells only if Zen-Bio media are used and the recommended protocols and storage conditions are followed. Cryopreserved cells are assured to be viable when thawed according to Zen-Bio protocols and using the recommended cultureware.

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.

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.

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INTRODUCTION

Melanocytes are dendritic cells that are derived from the neural crest cell population in the developing embryo. They are located in the basal layer of the epidermis where they connect with their numerous processes to the surrounding keratinocytes. They represent between 5% and 10% of the total epidermis. Melanocytes synthesize a specific pigment, Melanin in organelles called melanosomes and transfer it to surrounding keratinocytes. It is melanin that determines skin, eye and hair color.

Because of their role in skin pigmentation, skin protection and aging there is a great need for cellular studies that use Human Adult Melanocytes in cosmetic and skin biology studies. Melanocytes are also responsible for malignant melanoma formation. As such cultured melanocytes are an excellent tool for medical research.

ZenBio's human melanocytes are isolated from the epidermis of healthy consented donors who have undergone elective surgery in the United States. Each donor has signed an 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 GLP protocols in compliance with all legal and ethical regulations. The cells are isolated by trypsin/versene (1:1) digestion of the epidermal sheet and collected by centrifugal force. This instruction manual describes procedures to passage and culture ZenBio's adult human melanocytes.

The purity of ZenBio's adult melanocytes is routinely verified by Mel-5 (a melanocyte pigment-associated glycoprotein marker) immunofluorescence staining and cell morphology observation. In addition the ability to produce melanin is assessed by L-DOPA conversion assay. ZenBio's Melanocytes lots are >95% Mel-5 positive. Donor matched dermal fibroblasts and keratinocytes are also available.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

Cryopreserved Human Adult Melanocytes

- Cat # MEL-F
- Frozen vial containing 0.5×10^6 viable human melanocytes (store in liquid nitrogen upon receipt)

Cryopreserved Neonatal Human Melanocytes

- Cat# MEL-F-NEO
- Frozen vial containing 0.5×10^6 viable human melanocytes (store in liquid nitrogen upon receipt)

MEDIA COMPOSTIONS

Melanocyte Growth Medium Cat# MEL-2	<u>Storage and Expiration Date</u>
Dermal Base Medium Insulin FGF Bovine Pituitary Extract Fetal Bovine Serum (FBS) Endothelin Apo transferrin Hydrocortisone Phorbol myristate acetate Penicillin Streptomycin Amphotericin B	If placed at 4°C upon arrival, the media is stable until the expiration date on the bottle label, which is 60 days from the ship date. <u>Do Not Freeze</u>
Melanocyte Cryopreservation Medium Cat# MEL-100	<u>Storage and Expiration Date</u>
Fetal Bovine Serum (FBS) DMSO	Store at -20°C upon arrival until ready for use or expiration date on bottle. The media will expire 45 days after the thaw date.

MELANOCYTE PLATING AND EXPANSION PROCEDURES

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

THAWING AND CULTURING

1. Pre-warm the Melanocyte Medium (cat# MEL-2) at 37°C, and prepare all pipets and vessels.
2. Transfer 4 ml of warm MEL-2 Melanocyte Medium to a sterile 15 ml conical tube.
3. Remove cells from liquid nitrogen and place **immediately** into a 37°C water bath with mild agitation. Be careful not to submerge the cap of the vial into water. For best results, the

thawing step should not take more than 2 minutes and should be stopped when there is still some ice in the vial. Rinse the vial with 70% ethanol before opening.

4. Transfer the cells to the sterile conical bottom centrifuge tube containing 4 ml of warm Melanocyte Medium prepared in step 2.
5. Centrifuge at 280 x g, 20°C, 5 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in a volume of Melanocyte Medium appropriate for counting the cells. Count cells using a hemocytometer or automated cell counter.

NOTE: Step 6 should not take more than 30 minutes. If melanocytes are kept too long in suspension they will not recover after plating. If several vials need to be plated, thaw, count and plate no more than 2 vials at the same time.

7. Seed the cells in a T25 flask at 10,000 cells/cm² in 10 ml MEL-2 Melanocyte Medium. Place in a humidified incubator at 37°C and 5% CO₂, making sure the surface is level for even cell distribution.
8. Change the medium after 24 hours in culture.
9. Medium should be changed every 4 days until the cells reach 70% confluence (see Figure 2.).

MELANOCYTE SUBCULTURE

Human melanocytes should be passaged for subculture or cryopreservation when they are no more than 70% confluent (in about 10-15 days in culture).

1. Pre-warm MEL-2 Melanocyte Medium, HBSS Ca²⁺/Mg²⁺ free and soybean trypsin inhibitor in a 37°C water bath.
2. Aspirate medium on the cells and wash the cells 2 times with sterile HBSS Ca²⁺/ Mg²⁺ free.
3. Remove the HBSS and add 0.5mL/T-25 flask (or 1 ml/T-75 flask) of cold 0.25% trypsin/ 2.21mM EDTA solution. Incubate the cells at room temperature for 30-60 seconds monitoring cell detachment under the microscope. A longer incubation in trypsin can damage the melanocytes.
4. Neutralize the trypsin using an equal volume of 0.5mg/ml soybean trypsin inhibitor. Collect the cells in a conical tube containing 4 ml of melanocyte medium.
5. Centrifuge at 280 x g, for 5 minutes at 20°C.
6. Aspirate the medium and resuspend the cell pellet in a desired volume of melanocyte medium for cell counting.
7. Seed cells at 10,000 cells/cm² using MEL-2 Melanocyte medium. Ensure cells are evenly suspended when plating large numbers of plates or flasks. Do not agitate plates and flasks

after plating. Place in a humidified incubator at 37°C and 5% CO₂, making sure the surface is level for even cell distribution.

- Replace the medium 24 hours after plating and every 4 days until the melanocytes are 70% confluent (see Figure 2).

Figure 1. Melanocytes day 2-3

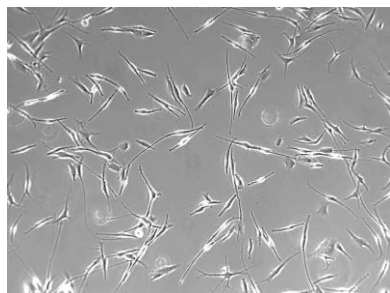
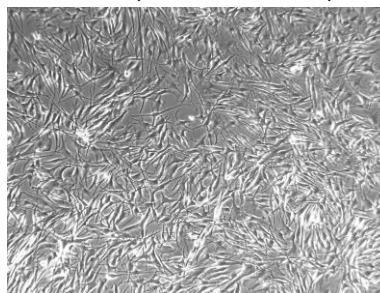


Figure 2. Melanocytes Day 8-12 (70% confluent)



TROUBLESHOOTING GUIDE

Observation	Possible causes	Suggestions
Melanocytes do not grow	<ol style="list-style-type: none"> Cells have been passaged too many times Cells expanded too high Cells not stored properly 	<ol style="list-style-type: none"> Use cells of a lower passage number Do not seed the cells lower than 10,000 cells/cm² Store cells in vapor phase liquid nitrogen upon arrival

FREQUENTLY ASKED QUESTIONS

Can I pass the cells?

All cells are shipped after establishing a primary culture and cryopreserved at passage 3. Cryopreserved melanocytes can be passaged at least 1 time using ZenBio medium and protocols.

How fast do the cells replicate?

The replication rate for human melanocytes varies from donor to donor.

Should antibiotics be included in the medium?

Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells.

Where are the cells obtained?

The melanocytes are isolated from human epidermal tissue consented donors undergoing elective surgery in the United States. Each donor has signed an 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 GLP protocols in compliance with all legal and ethical regulations.

Do you test for pathogens? Which ones?

Yes. Each lot of primary cells is tested via PCR and found non-reactive to viral DNA from HIV and hepatitis B and viral RNA from Hepatitis C. However, no known test can offer complete assurance that these viruses are not present. Since we cannot test all pathogens, always treat the culture as a potentially infectious reagent at Biosafety Level 1 or higher. 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.

What donor information do I receive?

The donor's age, gender, and BMI are provided in the certificate of analysis that accompanies each lot of cells.

PATHOGEN TESTING

Each lot of primary cells is tested via PCR and found non-reactive to viral DNA from HIV and hepatitis B and viral RNA from Hepatitis C. However, no known test can offer complete assurance that these 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 (BSL-1) or higher. Our cells are tested for mycoplasma contamination via direct plating and DNA fluorochrome staining; mycoplasma contamination is not detected.

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