Human Keratinocyte Manual

INSTRUCTION MANUAL ZBM0032.11

SHIPPING CONDITIONS

Human Keratinocyte Cells

Orders are delivered via Federal Express courier. All USA and Canada orders are shipped via Federal Express Priority service and are usually received the next day. Non North American International orders are usually received in 2-4 days. Primary human cells can be sensitive to extended times at dry ice temperatures. If your transit time will exceed 3 days, please inquire about dry vapor shipper options. Please inquire if alternate couriers are needed. All orders should be processed immediately upon shipment receipt.

STORAGE CONDITIONS

- KM-2 Medium (adult): +4°C Expires 30 days from ship date -20°C Expires 6 months from ship date
- KM-3 Medium (neonatal) +4°C Expires 30 days from ship date -20°C Expires 6 months from ship date
- KB-1 Basal Medium +4°C Expires 30 days from ship date -20°C Expires 6 months from ship date
- Cryopreserved cells: Vials of frozen preadipocytes are to be stored in vapor phase nitrogen (-150°C to -190°C) immediately upon arrival

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

ORDERING INFORMATION AND TECHNICAL SERVICES

ZenBio, Inc.
3920 South Alston Avenue
Durham, NC 27713 U.S.A.

Telephone	+1 (919) 547-0692
Toll free (continental US only)	1-866-ADIPOSE 1-(866)-234-7673
Electronic mail (e-mail)	information@zen-bio.com
World Wide Web	http://www.zen-bio.com

THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

KR-F	HUMAN ADULT KERATINOCYTES, 500,000 VIABLE CELLS/VIAL
KR-F-SL	HUMAN ADULT KERATINOCYTES, SUPERLOT (MIXED DONOR LOT), VIABLE CELLS/VIAL
KRD-F	HUMAN ADULT KERATINOCYTES- DIABETIC DONOR, VIABLE CELLS/VIAL
KRN-F	HUMAN NEONATAL KERATINOCYTES, VIABLE CELLS/VIAL
KRNP-F	HUMAN NEONATAL KERATINOCYTES, POOLED LOT (MIXED DONOR LOT), VIABLE CELLS/VIAL

CONTENTS PAGE

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 the performance of cells only if Zen-Bio media are used and the recommended storage conditions and protocols are followed without amendment or substitution. ZenBio, Inc cryopreserved cells are assured to be viable when stored as recommended and thawed according to Zen-Bio protocols and using the recommended protocol.

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 cultureware used in this protocol should be sterile.

Human keratinocyte viability depends greatly on the use of suitable media, reagents, and sterile plastic wear. If these parameters are not carefully observed, cell growth may be slower than expected.

INTRODUCTION

ZenBio, Inc. cryopreserved primary human adult keratinocytes are isolated from the epidermis of donated skin from a consented adult donor undergoing elective surgery in the United States. Each competent volunteer adult 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.
ZenBio, Inc. cryopreserved primary neonatal keratinocytes are isolated from foreskin of healthy males aged newborn to infant from elective circumcisions. Each donor sample has documented parental consent for use in research uses.

The cells are isolated by trypsin digestion of the epidermal sheet and collected by centrifugal force. This instruction manual describes procedures to passage and culture the adult and neonatal human keratinocytes. For the adult keratinocytes, donor matched dermal fibroblasts and preadipocytes are also available for many samples.

1.
2. All samples are tested non-reactive/negative for pathogens HIV-1, HIV-2, Hepatitis B and Hepatitis C. All keratinocytes exhibit the cobblestone morphology and highly express the basal keratinocyte markers keratin-5 and keratin-14 as assessed by immunostaining.

MATERIALS PROVIDED FOR EACH CATALOG ITEM

- **Cryopreserved Human Adult Keratinocytes**
  - Cat # KR-F
  - Cryopreserved vial containing 500,000 viable human keratinocytes (store in vapor phase liquid nitrogen upon receipt- any other storage negates the warranty)

- **Cryopreserved Human Neonatal Keratinocytes**
  - Cat # KRN-F
  - Cryopreserved vial containing 500,000 viable human neonatal keratinocytes (store in vapor phase liquid nitrogen upon receipt- any other storage negates the warranty)

MEDIA COMPOSTIONS

Adult Keratinocyte Growth Medium Cat# KM-2	**Expiration Dates**
• MCDB153 • Human Epidermal growth factor (rEGF) • Human Insulin • Human apo transferrin • Human Epidermal growth factor (hEGF) • Bovine serum albumin, fatty acid free • *O*-Phosphorylethanolamine • Ethanolamine • Hydrocortisone • Calcium Chloride • Epinephrine • Bovine Pituitary Extract (BPE) • Penicillin • Streptomycin • Amphotericin B	• If placed at +4°C upon arrival, the media is stable 30 days from the ship date Use the +4°C expiration date listed on the label. • If stored at -20°C upon arrival, it is stable 6 months after the ship date • Use the 3 month -20°C expiration date listed on the label. • Upon thawing, add fresh antibiotics at 1% volume when you are ready to use. • The media will now expire 30 days after the thawing date.
Neonatal Keratinocyte Growth Medium Cat# KM-3	**Expiration Dates**
• MCDB153 • Human Epidermal growth factor (rEGF) • Human Insulin • Human apo transferrin • Human Epidermal growth factor (hEGF) • *O*-Phosphorylethanolamine • Ethanolamine • Hydrocortisone • Calcium Chloride • Epinephrine • Bovine Pituitary Extract (BPE) • Penicillin • Streptomycin • Amphotericin B	• If placed at +4°C upon arrival, the media is stable 30 days from the ship date Use the +4°C expiration date listed on the label. • If stored at -20°C upon arrival, it is stable 6 months after the ship date • Use the 3 month -20°C expiration date listed on the label. • Upon thawing, add fresh antibiotics at 1% volume when you are ready to use. • The media will now expire 30 days after the thawing date.
Keratinocyte Basal Medium [suitable for both neonatal and adult keratinocytes] Cat# KB-1	**Expiration Dates**
• MCDB153 • Penicillin • Streptomycin • Amphotericin B	• If placed at 4°C upon arrival, the media is stable 30 days from the ship date • Use the +4°C expiration date listed on the label. • If stored at -20°C upon arrival, it is stable 6 months after the ship date • Use the -20°C expiration date listed on the label • Upon thawing, add fresh antibiotics at 1% volume when you are ready to use. • The media will expire 30 days after the thaw date.

MEDIA COMPOSTIONS

Keratinocyte Cryopreservation Medium [suitable for both neonatal and adult keratinocytes] Cat# KF-1-100	Expiration Date
• MCDB153 • Human Epidermal Growth Factor (rEGF) • Bovine Pituitary Extract (BPE) • DMSO	Expiration date will be 9-12 months from the ship date stored at -20°C.

PLATING AND EXPANSION PROCEDURES: Cryopreserved Adult Keratinocytes

THAWING AND CULTURING: Human Adult Keratinocytes

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

1. Pre-warm the Keratinocyte Medium (cat# KM-2) medium, at 37°C. Prepare all your pipets and vessels.
2. 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. For best results, the thawing step should not take more than 2 minutes. Stop thawing when there is still some ice in the vials. Rinse the vials with 70% ethanol before opening.
3. Transfer the cells to a sterile conical bottom centrifuge tube, containing 9 ml of Keratinocyte Growth Medium (KM-2).
4. Centrifuge at 300 x g, 20°C, for 5 minutes.
5. Aspirate the medium and resuspend the cell pellet in a volume of KM-2 appropriate for counting the cells. Count cells using a hemacytometer.
6. Seed the cells 5,000-10,000 cells/cm^2 in KM-2 medium.
7. Incubate at 37°C in a humidified incubator with 5% CO_2. Change the medium after 24 h in culture.
8. Medium needs to be changed every 2-3 days until the cells reach 70-80% confluent (see Figure 1). **Do not allow the cells to reach 100% confluency**.

SUBCULTURE: Human Adult Keratinocytes

Human adult keratinocytes should be passaged for subculture or cryopreservation when they are no more than 70-80% confluent (in about 5-6 days in culture).

1. Pre-warm, KM-2 medium, 0.25% trypsin/ 2.21mM EDTA solution and sterile Phosphate Buffered Saline (PBS) Ca^{2}+/Mg^{2+} free, in a water bath at 37°C.
2. Aspirate medium and wash the cells 2-3 times with sterile (PBS) Ca^{2+}/ Mg^{2}+ free, to remove all traces of medium.
3. Remove the PBS and add 2mL/T-75 flask (or 6 ml/T-225 flask) of pre-warmed 0.25% trypsin/ 2.21mM EDTA solution.
4. Incubate the cells at 37°C. Monitor cell detachment, under the microscope, after 2 minutes. Tap the flask gently to loosen the cells. If the cells are still attached, place them at 37 °C for another 1-3 minutes. A longer incubation in trypsin can damage the keratinocytes.
5. Neutralize the trypsin using an equal volume of 0.5 mg/ml soybean trypsin inhibitor. Collect all the cells in a conical tube containing 4ml of KM-2.
6. Centrifuge at 300xg, for 5 minutes at 20°C.
7. Aspirate the medium, and resuspend the cell pellet in a desired volume of KM-2 and proceed to cell counting.
8. Seed cells at 5,000-10,000 cells/cm^{2}, in KM-2. 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% CO_2, making sure the surface is level for even cell distribution.
9. Replace the medium 24 hours after plating and then every 2-3 days until they are 70-80% confluent (see Figure 1).

PLATING AND EXPANSION PROCEDURES:
Cryopreserved Neonatal Keratinocytes _____

THAWING AND CULTURING: Human Neonatal Keratinocytes

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

1. Pre-warm the KM-3 medium, at 37°C. Prepare all your pipets and vessels.
2. 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. For best results, the thawing step should not take more than 2 minutes. Stop thawing when there is still some ice in the vials. Rinse the vials with 70% ethanol before opening.
3. Transfer the cells to a sterile conical bottom centrifuge tube, containing 9 ml of Neonatal Keratinocyte Growth Medium (KM-3).
4. Centrifuge at 300 x g, 20°C, for 5 minutes.
5. Aspirate the medium and resuspend the cell pellet in a volume of KM-3 appropriate for counting the cells. Count cells using a hemacytometer.
6. Seed the cells at 5,000-10,000 cells/cm^2 in KM-3.
7. Incubate at 37°C in a humidified incubator with 5% CO_2. Change the medium after 24 h in culture.
8. Medium needs to be changed every 2-3 days until the cells reach 70-80% confluency (see Figure 1). **Do not allow the cells to reach 100% confluency**.

SUBCULTURE: Human Neonatal Keratinocytes

1. Human neonatal keratinocytes should be passaged for subculture or cryopreservation when they are no more than 70-80% confluent (in about 5-6 days in culture).
2. Pre-warm, KM-3 medium, 0.25% trypsin/ 2.21mM EDTA solution and sterile Phosphate Buffered Saline (PBS) Ca^{2+}/Mg^{2+} free, in a water bath at 37°C.
3. Aspirate medium and wash the cells 2-3 times with sterile PBS (Ca^{2+}/ Mg^{2+} free), to remove all traces of medium.
4. Remove the PBS and add 2mL/T-75 flask (or 6 ml/T-225 flask) of pre-warmed 0.25% trypsin/ 2.21mM EDTA solution.

1. Incubate the cells at 37°C. Monitor cell detachment, under the microscope, after 2 minutes. Tap the flask gently to loosen the cells. If the cells are still attached, place them at 37 °C for another 1-3 minutes. A longer incubation in trypsin can damage the keratinocytes.
2. Neutralize the trypsin using an equal volume of 0.5 mg/ml soybean trypsin inhibitor. Collect all the cells in a conical tube containing 4ml of KM-3.
3. Centrifuge at 300xg, for 5 minutes at 20°C.
4. Aspirate the medium, and resuspend the cell pellet in a desired volume of KM-3 and proceed to cell counting.
5. Seed cells at 5,000-10,000 cells/cm^2 in KM-3. 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% CO_2, making sure the surface is level for even cell distribution.
6. Replace the medium 24 hours after plating and then every 2-3 days until they are 70-80% confluent (see Figure 1).

CRYOPRESERVATION Procedure for Human Keratinocytes

1. Cryopreserve adult or neonatal human keratinocytes after counting the number of viable cells.
2. Centrifuge at 300 x g, 20°C, 5 minutes.
3. Suspend in cold Keratinocyte Cryopreservation medium (Cat# KF-100) at a concentration of $0.5X10^6$ cells/ml. Do not exceed a 6:1 ratio of cells (per million): volume freeze medium (per ml). If using a controlled-rate freezer: Freeze by reducing the temperature 1°C per minute until the temperature reaches -80° C. If using a cell cryopreservation container, prepare according to the manufacturer's instructions.
4. For best results we recommend transferring the vials to the vapor phase of a liquid nitrogen storage facility 1-4 days after the cells have reached -80°C.

HUMAN KERATINOCYTES Morphology:

Figure 1. 70% confluent

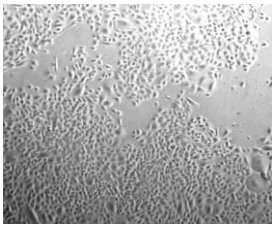
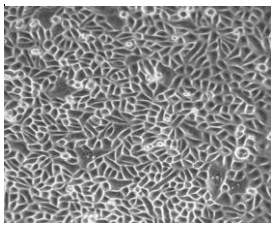


Figure 2. 100% confluent



KERATINOCYTE TROUBLESHOOTING GUIDE

Observation	Possible causes	Suggestions
Keratinocytes do not attach	Cultureware should be tissue culture treated	Ensure you are using tissue culture treated cultureware and using ZenBio recommended medium
Keratinocyte cells do not grow	Cells have been passaged too many times	Use cells of a lower passage number
Edge effects	Medium in outside wells evaporated	Ensure a saturated humidity in the incubator and feed the cells no less than every 2-3 days. Make sure multiple plates are stacked no more than 3 plates high. If the wells are not all used, fill the empty wells with medium.

FREQUENTLY ASKED QUESTIONS

1. Can I expand/passage the cells?
    a. Keratinocytes can be trypsinized and replated up to passage 4 or 5. All keratinocytes are shipped at passage 2 or 3 after establishing a primary culture.
2. How fast do the cells replicate?
    a. The average doubling time is 48-84 hours. However, keep in mind that the replication rate for human keratinocytes varies from donor to donor.
3. Should antibiotics be included in the medium?
    a. Yes. Antibiotics and anti-fungal agents are always recommended since the cells are primary cells.

4. Where are the cells obtained?
    a. The adult keratinocytes are isolated from human epidermal tissue obtained from consented adult volunteer donors undergoing elective surgery. All cells are collected and processed in the United States.
    b. The neonatal keratinocytes are isolated from human male foreskin from elective circumcisions with parental consent documentation. All cells are collected and processed in the United States.
5. Do you test for pathogens? Which ones?
    a. 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.
6. What quality control tests are performed on the keratinocytes?
    a. All samples are assessed for viability and test NON-REACTIVE/NEGATIVE for common pathogens HIV-1, HIV-2, Hepatitis B and Hepatitis C. All keratinocytes exhibit the cobblestone morphology and highly express the basal keratinocyte markers keratin-5 and keratin-14 as assessed by immunostaining.
7. What donor information do I receive?
    a. The donor's age, gender, race and body mass index (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 using US Food and Drug Administration (FDA) tests. 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.

Always wear gloves and work behind a protective screen when handling primary human cells.