



Human Keratinocyte Manual

INSTRUCTION MANUAL ZBM0032.09

SHIPPING CONDITIONS

Human Keratinocyte Cells

Orders are delivered via Federal Express courier. All US and Canada orders are shipped via Federal Express Priority service and are usually received the next day. International orders are usually received in 2-4 days. Please inquire if alternate couriers are needed.

Must be processed upon shipment receipt.

STORAGE CONDITIONS

Media: 3 weeks from ship date 4°C

Cells: Vapor phase liquid nitrogen (-150°C to -190°C).

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.

ORDERING INFORMATION AND TECHNICAL SERVICES

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THIS MANUAL IS SUITABLE FOR USE WITH THE FOLLOWING PRODUCTS:

KR-F	HUMAN ADULT KERATINOCYTES
KR-F-SL	HUMAN ADULT KERATINOCYTES, SUPERLOT (MIXED DONOR LOT)
KRD-F	HUMAN ADULT KERATINOCYTES- DIABETIC DONOR
KRN-F	HUMAN NEONATAL KERATINOCYTES

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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 the performance of cells only if Zen-Bio media are used and the recommended protocols 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 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, limited differentiation may occur and cell growth may be slow.

INTRODUCTION

ZenBio's adult human keratinocytes are isolated from the epidermis of consented adult volunteer donors who have undergone elective surgery. ZenBio neonatal keratinocytes are isolated from the foreskin of healthy males aged newborn to infant from elective circumcisions. 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.

All samples are negative for common pathogens HIV, 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
- Frozen vial containing 0.5×10^6 viable human keratinocytes (store in vapor phase liquid nitrogen upon receipt)
 - **Effective February 1, 2016, we will no longer provide 50ml support medium with each cryopreserved vial ordered. Please order media according to your needs.**

- **Cryopreserved Human Neonatal Keratinocytes**

- Cat # KRN-F
- Frozen vial containing 0.5×10^6 viable human neonatal keratinocytes (store in vapor phase liquid nitrogen upon receipt)
 - **Effective February 1, 2016, we will no longer provide 50ml support medium with each cryopreserved vial ordered. Please order media according to your needs.**

MEDIA COMPOSTIONS

Adult Keratinocyte Growth Medium Cat# KM-2

- MCDB153
- Human Epidermal growth factor (rEGF)
- Human Insulin
- Human apo transferrin
- Bovine serum albumin, fatty acid free, low endotoxin
- Phosphoethanolamine / Ethanolamine
- Hydrocortisone
- Calcium Chloride
- Epinephrine
- Bovine Pituitary Extract (BPE)
- Penicillin
- Streptomycin
- Amphotericin B

Neonatal Keratinocyte Growth Medium Cat# KM-3

- MCDB153
- Human Epidermal growth factor (rEGF)
- Human Insulin
- Human apo transferrin
- Phosphoethanolamine / Ethanolamine
- Hydrocortisone
- Calcium Chloride
- Epinephrine
- Bovine Pituitary Extract (BPE)
- Penicillin
- Streptomycin
- Amphotericin B

Keratinocyte Basal Medium

[suitable for both neonatal and adult keratinocytes]

Cat# KB-1

- MCDB153
- Penicillin
- Streptomycin
- Amphotericin B

Keratinocyte Cryopreservation Medium

[suitable for both neonatal and adult keratinocytes]

Cat# KF-1-100

- MCDB153
- Human Epidermal Growth Factor (rEGF)
- Bovine Pituitary Extract (BPE)
- DMSO

NOTE:

KM-2 and KM-3 are prepared fresh prior to shipment and expires approximately 3 weeks from the medium ship date. Please call ZenBio to coordinate media shipments with your experiment schedule

Please inquire for custom media requests.

PLATING AND EXPANSION PROCEDURES: Cryopreserved Adult Keratinocytes

THAWING AND CULTURING: Human Adult Keratinocytes

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.

1. Pre-warm the 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 at 0.37-0.75 X 10⁶ cells per T-75 culture flask (5,000-10,000 cells/cm²) in 20 ml KM-2.
7. Incubate at 37°C in a humidified incubator with 5% CO₂. 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

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) Ca²⁺/Mg²⁺ free, in a water bath at 37°C.
2. Aspirate medium and wash the cells 2-3 times with sterile (PBS) Ca²⁺/ Mg²⁺ 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/cm², (0.37-0.75x10⁶ cells per T75 flask) in 20 ml of 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% CO₂, 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

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 0.37-0.75 X 10⁶ cells per T-75 culture flask (5,000-10,000 cells/cm²) in 20 ml KM-3.
7. Incubate at 37°C in a humidified incubator with 5% CO₂. 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) Ca²⁺/Mg²⁺ free, in a water bath at 37°C.
3. Aspirate medium and wash the cells 2-3 times with sterile PBS (Ca²⁺/ Mg²⁺ 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/cm², (0.37-0.75x10⁶ cells per T75 flask) in 20 ml of 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% CO₂, 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 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 0.5X10⁶ 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.

- 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

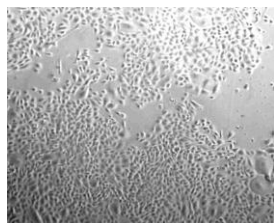
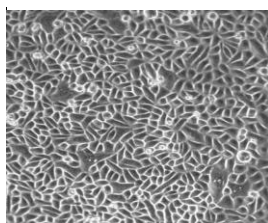


Figure 2. 100% confluent



KERATINOCYTE TROUBLESHOOTING GUIDE _____

Observation	Possible causes	Suggestions
keratinocyte cells do not grow	1. Cells have been passaged too many times	1. Use cells of a lower passage number
Edge effects	1. Medium in outside wells evaporated	1. 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

Can I pass the cells?

Keratinocytes can be trypsinized and replated up to passage 4 or 5. All cells are shipped at passage 2 or 3 after establishing a primary culture.

How fast do the cells replicate?

The average doubling time is 48-84 hours. However, keep in mind that the replication rate for human keratinocytes 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 adult keratinocytes are isolated from human epidermal tissue obtained from consented adult volunteer donors undergoing elective surgery. The neonatal keratinocytes are isolated from human male foreskin from elective circumcisions.

Do you test for pathogens? Which ones?

Yes. Samples from each donor are tested via PCR to confirm non-reactivity for HIV, hepatitis B and hepatitis C. However, since we cannot test all pathogens, please treat the culture as a potentially infectious agent.

What quality control tests are performed on the keratinocytes?

All samples are negative for common pathogens HIV, 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.

What donor information do I receive?

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

PATHOGEN TESTING

Samples from each donor are tested via PCR to confirm non-reactivity for HIV, hepatitis B and hepatitis C. However, no known test can offer complete assurance that the cells are pathogen free. Our products are tested and are free from mycoplasma contamination. Proper precautions and biological containment should be taken when handling cells of human origin, due to their potential biohazardous nature. All human based products should be handled at a BSL-2 (Biosafety Level 2) or higher. Always wear gloves and work behind a protective screen when handling primary human cells.