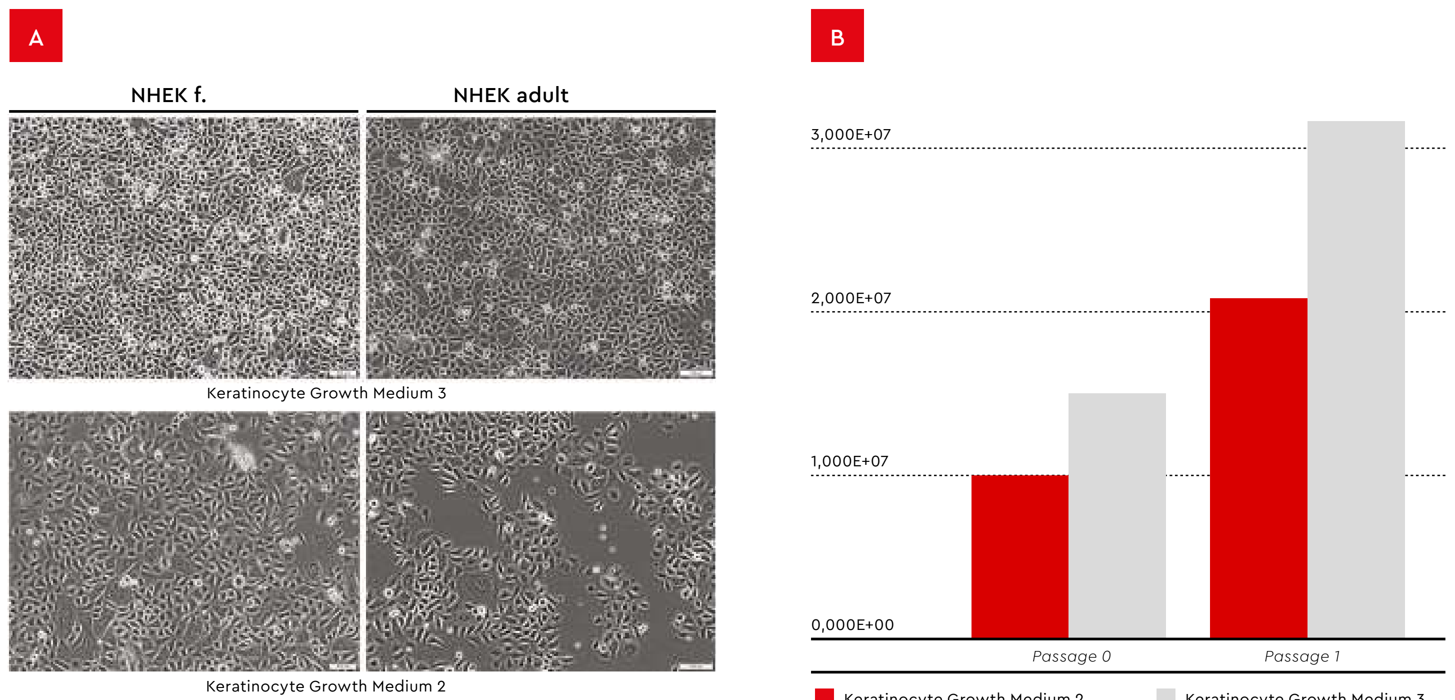
PromoCell®

Isolation of Fast Proliferating and Homogeneous Keratinocyte Populations

Application Note

PromoCell's Keratinocyte Growth Medium 3 is the improved, bovine pituitary extract (BPE)-free version of our popular and well-established Keratinocyte Growth Medium 2. Both culture media enable the isolation and expansion of keratinocytes with at least 15 population doublings. Compared to other commercial bovine pituitary extract (BPE)-free keratinocyte media the Keratinocyte Growth Medium 3 does not require extracellular matrix coatings of the cell culture vessel and allows proper adherence to cell culture treated plastics. A comparison of both PromoCell media show that isolated primary keratinocytes in passage 0 have a significant smaller cell size and a homogenous morphology in Keratinocyte Growth Medium 3. This small and homogenous cell population could be isolated succesfully from juvenile and adult donor skin. Lastly, overall isolation efficiency was ~50% increased when using Keratinocyte Growth Medium 3 (Figure 1).



Morphology and growth performance of Normal Human Epidermal Keratinocytes (NHEK) cultured in PromoCell's Keratinocyte Growth Media.

A- *Phase microscopy images taken from isolated normal human epidermal keratinocytes (NHEK) in passage 0. NHEK were isolated from juvenile foreskin (NHEK f.) or adult donor (NHEK adult) using PromoCell's standard protocol. Cells were cultivated in Keratinocyte Growth Medium 3 (upper panel) or Keratinocyte Growth Medium 2 (lower panel). Scale bar 100µm.*

B- *Quantitative analysis of cell growth in Passage 0 and Passage 1 of NHEK f. in BPE-free Keratinocyte Growth Medium 3 or Keratinocyte Growth Medium 2. Cells were counted using an automated standard procedure (Viacount Assay, MUSE Cell Analyzer, Millipore®).*

Along with the right balance of calcium ions, growth factors, amino acids, vitamins or hormones in the cell culture medium formulation, keratinocyte proliferation can also be regulated by confluence. Keratinocytes can be kept in a highly proliferative state by avoiding hyperconfluence [3]. This application note describes the isolation of primary human keratinocytes and the recommended cell culture technique of confluence sensitive epidermal cells to enrich a small and quickly proliferating cell population.

Background

Our skin, subdivided into epidermis and dermis, is the first defense line against environmental stress, pathogens, body temperature and hydration loss. Epidermal tissue maintenance and repair is carried out by keratinocytes which are highly specialized cells. Basal cells with little differentiation are located on the basal membrane close to the dermis, these cells have high proliferative potential. Highly differentiated cells with low proliferation potential can be find in the upper layer of the epidermis. The most differentiated cells, so-called cornified cells, are located at the top of the cell layer and are not capable of further division [4].

Keratinocyte basal cells have a high turnover and their self-renewal can be regulated by different factors like adhesion receptors (e.g. integrins), growth factor receptor (e.g. EGFR) or signal transduction pathways (e.g. Notch) [5]. They can also be involved in severe skin disorders, for example when reduced adherence of keratinocytes leads to cell cycle arrest and limited cell proliferation [6]. Patients with this disorder suffer blistering of the skin. Those genetically changed keratinocytes without integrin expression are not able to proliferate even in the presence of stimulating growth factors [7],[6]. Researchers can use primary human keratinocytes for dermatological cell research or as a model for cosmetic testing, removing the need for animal experiments.

The first keratinocyte isolation protocol was established by Rheinwald and Green in 1975 [8] and involved coculture of a NIH-3T3 fibroblast feeder layer. Since then, a lot has improved. The isolation and cultivation has progressed from the fibroblast feeder-layer model to a serum-free culture medium to choleratoxin-free and now to animal extract (e.g., BPE) free medium. There are several advantages of animal extract-free culture medium. Animal extracts are produced by pooling of several animal donors that vary in their physiological status. This introduces batch-to-batch variations and means the composition is unknown. The use of animal BPE can also contaminate the culture system with infectious agents.

Keratinocyte Growth Medium 3 is free from poorly defined animal extracts like bovine pituitary extract and brings a safe culture environment. The advanced formulation of the medium displays less batch-to-batch variations compared to animal extract-containing medium, enabling controlled and reproducible conditions. Animal extract free media have highly selective potential during the cell isolation process and select the rapidly adhering and proliferating cells and less contamination with unwanted cells. PromoCell's Normal Human Epidermal Keratinocytes (NHEK) are characterized by ≥80% cytokeratin positive cell populations.

Protocol for isolation and expansion of NHEK

This application note describes the isolation and expansion of primary human keratinocytes in Keratinocyte Growth Medium 3 from juvenile foreskin or skin from adult donors. The isolation efficiency of NHEK from juvenile donors is typically higher than for adult donors.

I. Isolation protocol

Materials

- 70% EtOH
- Scalpel, centrifuge tube
- Tissue culture treated cell culture vessels (e.g. Falcon® 150mm TC-treated cell culture dish, article number 353025 Corning®)
- Physiological buffer (Dubecco's PBS- C-40230/C-40232, HepesBSS- C-40000 or similar)
- Dispase solution
- Trypsin/ EDTA (C-4100)
- Keratinocyte Growth Medium 3 (C-20021)

1

Prepare Keratinocyte Growth Medium 3.

Thaw the SupplementMix at 15 – 25°C. Aseptically mix the supplement solution by carefully pipetting up and down. Then transfer the entire contents of the supplement to the 500ml of Growth Medium.

2

Adjust the medium to room temperature.

Calculate the amount of PromoCell's Keratinocyte Growth Medium 3 needed for isolation. Prewarm only an aliquot of the complete medium protected from light and keep the remaining medium refrigerated at 4 – 8°C.

3

Rinse the skin tissue with physiological buffer.

4

Place the tissue in a container containing 1 x Dispase Solution.

Incubate the skin tissue in Dispase Solution overnight at 4°C.

5

Separate the epidermis from the dermis.

Next morning transfer the tissue from the container in a culture dish. Seperate the epidermis from the dermis using forceps. Throw away the dermis and continue with the epidermis.

6

Digest the epidermis to yield a single-cell-solution.

Digest the epidermis using trypsin solution. Stop the enzyme reaction by adding the same amount of TrypsinNeutralizationSolution.

7

Collect the single-cell-solution.

Filter the cell suspension and collect the cells by centrifugation. Resuspend the cell pellet in Keratinocyte Growth Medium 3 and count the cells using your standard method.

8

Plate the cells.

Plate the cells at a seeding density of 10.000 cells per cm² in Keratinocyte Growth Medium 3 (~180 µl medium per cm²). We recommend culture vessels from Falcon® by Corning® e.g. article nr. 353025. Incubate the culture vessel in an humified incubator (37°C, 5 % CO_2).

II. Culture protocol

Materials

- Keratinocyte Growth Medium 3 (C-21080)
- Culture vessel (e.g. Falcon® 150mm TC-treated cell culture dish, article number 353025 Corning®)
- DetachKit (C-41210; 125ml) contains the following products: Hepes BSS Solution, 0.04% Trypsin/0.03% EDTA and TrypsinNeutralizationSolution (0.05% Trypsin Inhibitor in 0.1% BSA)

9

Let the cells grow.

Change the medium every 2–3 days.

10

Detach the cells by reaching ≥50% confluence.

Once the cells have reached ≥50% confluence they can be passaged. Carefully aspirate the culture medium. Add 100µl Hepes BSS Solution per cm² of vessel surface to wash the cells and agitate the vessel carefully for 15 seconds. Aspirate the Hepes BSS Solution and cover the cells with 100µl Trypsin/EDTA Solution per cm². Transfer the vessel in an incubator for 3 minutes.

Note: Try to avoid hyperconfluence since the cells can become contact inhibited and growth rate will decrease.

11

Harvest the detached cells.

Monitor the round-up process of the cells under a microscope. Tap the side of the culture vessel to enhance cell dissolution from the plastic. Do not over-trypsinize the cells, which occurs at ≥6 minutes. When cells are detached add 100µl TrypsinNeutralizationSolution per cm^2 and gently agitate. Resuspend the cells and pipet the cell suspension in a centrifuge tube with appropriate volume of Keratinocyte Growth Medium 3. Rinse the culture vessel with Hepes BSS and pool the cell suspension into the centrifuge tube. Place the culture vessel under a microscope to check if all the keratinocytes are harvested. Centrifuge the cell suspension at 300 x g for 3 minutes.

12

Resuspend the cell pellet.

Discard the supernatant and resuspend the cell pellet carefully by carefully pipetting up and down in an appropriate volume of Keratinocyte Growth Medium 3.

13

Count the cells using your standard method.

14

Reseed the cells in a culture vessel.

Use a seeding density of 5.000 cells per cm^2.

15

Change medium every two or three days.

Use Keratinocyte Growth Medium 3 for serial expansion of the cells. We recommend using the cells in early passages for experiments.

Products

Product	Size	Catalog Number
Keratinocyte Growth Medium 3 (ready to use)	500 ml	C-20021
HEPES Buffered Saline Solution	250 ml	C-40020
Dulbecco's PBS, without Ca^{2+}/ Mg^{2+}	500 ml	C-40232
DetachKit	125 ml	C-41210

Related Products

Product	Size	Catalog Number
NHEK.f-c GM3 single donor	500,000 cryopreserved cells 500,000 proliferating cells	C-12011 C-12012
NHEK-c adult GM3 single donor	500,000 cryopreserved cells 500,000 proliferating cells	C-12013 C-12014
NHEK.f-c GM3 pooled donors	500,000 cryopreserved cells 500,000 proliferating cells	C-12015 C-12017
NHEK-c adult GM3 pooled donors	500,000 cryopreserved cells 500,000 proliferating cells	C-12016 C-12018

References

1. Chapman, S., et al., The effect of Rho kinase inhibition on long-term keratinocyte proliferation is rapid and conditional. Stem cell research & therapy, 2014. 5(2): p. 60.
2. Menon, G.K., S. Grayson, and P.M. Elias, Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. Journal of Investigative Dermatology, 1985. 84(6): p. 508–512.
3. Roshan, A., et al., Human keratinocytes have two interconvertible modes of proliferation. Nature cell biology, 2016. 18(2): p. 145–156.
4. Candi, E., R. Schmidt, and G. Melino, The cornified envelope: a model of cell death in the skin. Nature reviews Molecular cell biology, 2005. 6(4): p. 328–340.
5. Suter, M.M., et al., The keratinocyte in epidermal renewal and defence. Veterinary dermatology, 2009. 20(5–6): p. 515–532.
6. Moreno-Layseca, P. and C.H. Streuli, Signalling pathways linking integrins with cell cycle progression. Matrix Biology, 2014. 34: p. 144–153.
7. Nikolopoulos, S.N., et al., Targeted deletion of the integrin beta4 signaling domain suppresses laminin-5-dependent nuclear entry of mitogen-activated protein kinases and NF-kappaB, causing defects in epidermal growth and migration. Mol Cell Biol, 2005. 25(14): p. 6090–102.
8. Rheinwatd, J.G. and H. Green, Seria cultivation of strains of human epidemal keratinocytes: the formation keratinizin colonies from single cell is. Cell, 1975. 6(3): p. 331–343.

PromoCell GmbH
Sickingenstr. 63/65
69126 Heidelberg
Germany

info@promocell.com
www.promocell.com

USA/Canada
Phone: 1 – 866 – 251 – 2860 (toll free)
Fax: 1 – 866 – 827 – 9219 (toll free)

Deutschland
Telefon: 0800 – 776 66 23 (gebührenfrei)
Fax: 0800 – 100 83 06 (gebührenfrei)

France
Téléphone: 0800 – 90 93 32 (ligne verte)
Téléfax: 0800 – 90 27 36 (ligne verte)

United Kingdom
Phone: 0800 96 03 33 (toll free)
Fax: 0800 169 85 54 (toll free)

Other Countries
Phone: +49 6221 – 649 34 0
Fax: +49 6221 – 649 34 40

© PromoCell GmbH