

## Clonetics™ human epidermal keratinocyte cell systems

### Instructions for use

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#### Safety statements

**THESE PRODUCTS ARE FOR RESEARCH USE ONLY.** Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* procedures.

**WARNING: CLONETICS™ AND POIETICS™ PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS.** Each donor is tested and found non-reactive by an FDA approved method for the presence of HIV-1, hepatitis B virus and hepatitis C virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, hepatitis B virus, and hepatitis C virus. Testing can not offer complete assurance that HIV-1, hepatitis B virus, and hepatitis C virus are absent. All human sourced products should be handled at the biological safety level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH manual, [Biosafety in Microbiological and Biomedical Laboratories](#), 5<sup>th</sup> edition. If you require further information, please contact your site safety officer or scientific support.

#### Unpacking and storage instructions

1. Check all containers for leakage or breakage.
2. For cryopreserved cells – remove cryovials from the dry ice packaging and immediately place into liquid nitrogen storage. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact customer service.
3. For proliferating cells – swab down the flask of proliferating cells with 70% ethanol or isopropanol, then place the flask in 37°C, 5% CO<sub>2</sub>, humidified incubator and allow to equilibrate for 3 to 4 hours. After cells have equilibrated, remove shipping medium from the flask and replace with fresh medium.
4. BulletKit™ instructions: upon arrival, store basal medium at 4-8°C and SingleQuots™ at –20°C in a freezer that is not self-defrosting. If thawed upon arrival, growth factors can be stored at 4°C and added to basal media within 72 hours of receipt. After SingleQuots™ are added to basal medium, use within 1 month. Do not re-freeze.
5. ReagentPack™ subculture reagents are sterile-filtered and then stored at –20°C until shipment. Subculture reagents may thaw during transport.

They may be refrozen once. If you plan to use within 3 days, store at 4°C. Trypsin/EDTA solution has a limited shelf life or activation at 4°C. If, upon arrival, trypsin/EDTA is thawed, immediately aliquot and refreeze at –20°C. We recommend that the HEPES-BSS and the trypsin neutralizing solution be stored at 4°C for no more than 1 month.

**NOTE:** To keep trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at –20°C.

Using media or reagents other than what's recommended will void the cell warranty. Please contact scientific support if you need help selecting media and/or reagents.

#### Preparation of media

**NOTE:** Lonza offers a variety of media for the cultivation of keratinocytes. Some media systems are formulated with calcium and others are calcium-free. Calcium is needed for successful plating and proliferation of cells.

Perform the following steps in a sterile field.

**For a bottle of fully supplemented media, do the following:**

1. Decontaminate the vial of BPE and medium bottle with ethanol or isopropanol.
2. Add the entire contents of the vial (approximately 2 ml) to the media with a pipette. Rinse the BPE vial with medium and pipette the contents back into the 500 ml bottle.
3. Replace the cap and swirl the medium gently a few times to mix.
4. Record the date the BPE was added on the medium label.

**For a BulletKit™, perform the following steps:**

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
2. Aseptically open each supplement vial and add the entire amount to the basal medium with a pipette.

3. Rinse each cryovial with the medium. It may not be possible to recover the entire volume listed for each cryovial. Small losses, even up to 10%, should not affect the cell growth characteristics of the supplemented medium.
4. Transfer the label provided with each kit to the basal medium bottle being supplemented. Use it to record the date and amount of each supplement added. We recommend that you place the completed label over the basal medium label (avoid covering the basal medium lot # and expiration date) to avoid confusion or possible double supplementation.
5. Record the new expiration date on the label based on the shelf life.

**NOTE:** If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be refiltered with a 0.2 µm filter to assure sterility. Routine refiltration is not recommended.

### Thawing of cells / initiation of culture process

1. The recommended seeding density for NHEK and D-HEK is 3,500 cells/cm<sup>2</sup>.
2. To set up cultures calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used. Do not seed cells into a well plate directly out of cryopreservation. Add the appropriate amount of medium to the vessels (1 ml/5 cm<sup>2</sup>) and allow the vessels to equilibrate in a 37°C, 5% CO<sub>2</sub>, humidified incubator for at least 30 minutes.
3. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then retighten. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Watch your cryovial closely; when the last sliver of ice melts remove it. Do not submerge it completely. Thawing the cells for longer than 2 minutes results in less than optimal results.
4. Resuspend the cells in the cryovial and using a micropipette, dispense cells into the culture vessels set up earlier. Gently rock the culture vessel to evenly distribute the cells and return to the incubator.
5. Centrifugation should not be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture.

### Subculturing

The following instructions are for a 25 cm<sup>2</sup> flask. Adjust all volumes accordingly for other size flasks.

**NOTE:** Lonza warrants its Clonetics™ cells only if Lonza subculturing reagents are used. The recommended subculturing reagents for these cells are trypsin/EDTA (CC-5012), trypsin neutralizing solution (CC-5002), and HEPES buffered saline solution (CC-5022). These reagents can be purchased individually or together as part of the ReagentPack™ subculture reagents (CC-5034).

### Preparation for subculturing the first flask:

1. Subculture the cells when they are 70%-80% confluent and contain many mitotic figures throughout the flask.
2. For each 25 cm<sup>2</sup> of cells to be subcultured:
  - a. Thaw 2 ml of trypsin/EDTA and allow to come to room temperature.
  - b. Allow 7-10 ml of HEPES buffered saline solution (HEPES-BSS) to come to room temperature.
  - c. Allow 4 ml of trypsin neutralizing solution (TNS) to come to room temperature.
  - d. Remove growth medium from 4°C storage and allow to start warming to room temperature.
3. Prepare new culture vessels.
4. Subculture 1 flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol based on calculated cell count, cell viability, and seeding density.

### In a sterile field:

1. Aspirate the medium from 1 culture vessel.
2. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the HEPES-BSS from the flask.
4. Cover the cells with 2 ml of trypsin/EDTA solution.
5. Examine the cell layer microscopically.
6. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 2-6 minutes, depending on cell type.
7. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and rap again. If cells

still do not detach, wait and rap every 30 seconds thereafter.

8. After cells are released, neutralize the trypsin in the flask with 4 ml of room temperature TNS. If the majority of cells do not detach within 7 minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described above, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with TNS and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available.
9. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
10. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
11. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
12. Centrifuge the harvested cells at 220 x g for 5 minutes to pellet the cells.
  - a. Aspirate most of the supernatant, except for 100-200 µl.
  - b. Flick the cryovial with your finger to loosen the pellet.
13. Dilute the cells in 2-3 ml of growth medium and note the total volume of the diluted cell suspension.
14. Determine cell count and viability using a hemacytometer and trypan blue. Make a note of your cell yield for later use.
15. If necessary, dilute the suspension with the HEPES-BSS to achieve the desired "cells/ml" and re-count the cells.
16. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

17. Determine the total number of flasks to inoculate by using the following equation. The number of flasks needed depends upon cell yield and seeding density. If seeding into well plates at this time, the recommended density is 10,000 cells/cm<sup>2</sup>.

$$\text{Total \# of Flasks to inoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Density}}$$

18. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}$$

19. Prepare flasks by labeling each flask with the passage number, strain number, cell type and date.
20. Carefully transfer growth medium to new culture vessels by adding 1 ml growth medium for every 5 cm<sup>2</sup> surface area of the flask (1 ml/5 cm<sup>2</sup>).
21. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks.
22. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 37°C humidified incubator with 5% CO<sub>2</sub>.

## Maintenance

1. Change the growth medium the day after seeding and every other day thereafter. As the cells become more confluent, increase the volume of media as follows: under 25% confluence then feed cells 1 ml per 5 cm<sup>2</sup>, 25-45% confluence then feed cells 1.5 ml per cm<sup>2</sup>, over 45% confluence then feed cells 2 ml per 5 cm<sup>2</sup>.
2. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer and warm only the required volume to a sterile secondary container.
3. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container.

## Ordering information

Cryopreserved cells		
00192907	NHEK-neo in KGM-Gold™	≥ 500,000 cells
00192906	NHEK-neo pooled in KGM-Gold™	≥ 500,000 cells
00192627	NHEK adult in KGM-Gold™	≥ 500,000 cells
CC-2926	D-HEK adult diabetic type II in KGM-Gold™	≥ 500,000 cells

For additional proliferating formats, please call scientific support.

## Related products

Keratinocyte media (must be purchased separately):

00192060	KGM-Gold™ BulletKit™	Kit which contains a 500 ml bottle of KBM-Gold™ and KGM-Gold™ SingleQuots™.
00192151	KBM-Gold™	Keratinocyte basal medium-gold (no growth factors) (500 ml)
00192152	KGM-Gold™ SingleQuots™	Supplements and growth factors (BPE, hEGF, insulin, hydrocortisone, epinephrine, transferrin and gentamicin/ amphotericin-B)
00195769	KGM-Gold™ w/o Ca <sup>++</sup> , phenol red free, BulletKit™	Kit which contains a 500 ml bottle of KBM-Gold™ w/o Ca <sup>++</sup> , phenol red free (00195130) and KGM-Gold™ SingleQuots™ (00192152)
00195130	KBM- Gold™ w/o Ca <sup>++</sup> , phenol red free	Keratinocyte basal medium- gold w/o calcium, phenol red free (500 ml)
CC-4455	KGM™-CD BulletKit™	Kit which contains a 500 ml bottle of KBM™-CD, (CC-3255) and KGM™-CD SingleQuots™ (CC-4456).
CC-3255	KBM™-CD	Keratinocyte basal medium-chemically defined (500 ml)
CC-4456	KGM™-CD SingleQuots™	Supplements and growth factors (growth supplement and recombinant human insulin)
CC-5034	ReagentPack™	Contains 100 ml each of Trypsin/EDTA, HEPES buffered saline solution, and trypsin neutralizing solution

**NOTE:** Antibiotics are not required for culturing primary keratinocytes and are not included with the KGM™-CD BulletKit™. For added flexibility, a variety of cell culture antibiotic/antimycotic solutions are available separately from Lonza. Please consult our Lonza catalog, website or contact scientific support for assistance.

**NOTE:** Clonetics™ KGM™ BulletKit™ and KGM™-2 BulletKit™ are also currently available. Please contact scientific support for more information.

\* See "Preparation of medium" section. Calcium is required for successful plating and proliferation of cells.

## Product warranty

CULTURES HAVE A FINITE LIFESPAN *IN VITRO*. Lonza warrants its cells in the following manner only if Clonetics™ media and reagents are used.

1. Clonetics™ NHEK-neo cryopreserved cultures are assured for experimental use for 20 population doublings. Clonetics™ NHEK-adult cryopreserved cultures are assured for experimental use for 18 population doublings. Clonetics™ D-HEK cryopreserved cultures are tested for three passages for population doublings FIO (For information only).
2. Clonetics™ NHEK proliferating cultures are assured for experimental use for 10 population doublings. Clonetics™ D-HEK proliferating cultures are assured for experimental use for one passage upon receipt.
3. Additional population doublings and subcultures are possible, but growth rate, biological responsiveness and function deteriorate with subsequent passage.
4. Epidermal keratinocytes can become irreversibly contact-inhibited if allowed to reach confluence. To avoid the loss of your cells and forfeiture of your warranty, subculture cells before they reach 80% confluence.

## Quality control

HIV-1, hepatitis B and hepatitis C are not detected for all donors and/or cell lots. NHEK and D-HEK are characterized by morphological observation throughout serial passage. For detailed information concerning QC testing, please refer to the certificate of analysis.