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Clonetics™ Normal Human Melanocytes

Instructions for Use

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-I, 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, 5th 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℃, 5% CO_2, humidified incubator and allow to equilibrate for three to four 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℃ and SingleQuots™ (including ET-3, sold separately, if culturing NHEM-Ad) at -20℃ in a freezer that is not self-defrosting. If thawed upon arrival, growth factors can be stored at 4℃ and added to the basal medium within 72 hours of receipt. After SingleQuots™ are added to basal medium, use within one month. Do not re-freeze.
5. Subculture reagents include:

Phosphate Buffered Saline (PBS)	500 ml	17-516F
Trypsin/EDTA	100 ml	CC-5012
Versene® (EDTA) 0.02%	100 ml	17-711E
Trypsin Neutralizing Solution (TNS)	100 ml	CC-5002

6. Subculture reagents may thaw during transport. They may be refrozen once.
   - Trypsin/EDTA Solution has a limited shelf life or activation at 4℃. If, upon arrival, Trypsin/EDTA is thawed, immediately aliquot and refreeze at -20℃.
   - To keep Trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at -20℃.
   - We recommend that the TNS be stored at 4℃ for no more than one month.

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 Medium

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. For the NHEM-Ad only, add the full vial of lyophilized Endothelin-3 (sold separately).
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 number 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 NHEM is 10,000 cells/cm^2.
2. To establish cultures, calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used. Add the appropriate amount of medium to the vessels (1 ml/5 cm^2) and allow the vessels to equilibrate in a 37°C, 5% CO_2 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.
6. Replace the growth medium 72 hours after initial seeding. (1 ml medium/5 cm^2 surface area)

Subculturing

Note: Cells are only evaluated and guaranteed for one passage out of cryopreservation. The melanocytes are loosely adherent. Please read these instructions carefully to avoid damage to the cells.

The following instructions are for a 25 cm^2 flask. Adjust all volumes accordingly for other size flasks.

Preparation for subculturing the first flask:

1. Subculture the cells when they are 70 to 80% confluent and contain many mitotic figures throughout the flask.
2. Label new culture vessels, then add 5 ml of growth medium for every 25 cm^2 surface area of the flask (5 ml/25 cm^2). Place the flasks into a 37°C, 5% CO_2, humidified incubator to equilibrate for 30 minutes.
3. For each 25 cm^2 of cells to be subcultured:

- Thaw 1 ml of Trypsin/EDTA (CC-5012) and allow to come to room temperature.
- 1 ml of Versene® (EDTA) will be needed to dilute Trypsin/EDTA.
- 5 ml of Phosphate Buffered Saline Solution (PBS) will be needed for the subculture procedure.
- Allow 2 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
- Remove the volume of growth medium required from 4°C storage and allow the medium to warm to 37°C.

4. Subculture one 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. In a size appropriate vessel add 1 ml of room temperature Trypsin/EDTA (CC-5012) with 1 ml 0.02% Versene® (EDTA). Mix gently.
2. Aspirate the medium from one culture vessel.
3. Rinse the cells with 5 ml of room temperature PBS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin. Pipette the PBS down the cell-free surface of the flask; NHEM may be disassociated from the flask surface.
4. Aspirate the PBS from the flask.
5. Cover the cells with 2 ml of the diluted Trypsin/EDTA solution prepared in step 1. Exposure to greater concentrations of Trypsin/EDTA will damage the melanocytes.
6. Examine the cell layer microscopically.
7. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 30 to 60 seconds. Extended exposure to Trypsin/EDTA will damage the melanocytes.

8. 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, a slightly longer trypsinization time may be required. Wait 30 seconds and rap again. If cells still do not detach within 2 minutes remove the lifted cells and neutralize the Trypsin/EDTA.
9. After cells are released, neutralize the trypsin in the flask with 2 ml of room temperature Trypsin Neutralizing Solution. If the majority of cells do not detach within two 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 diluted Trypsin/EDTA solution prepared in step 1 or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available.
10. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
11. Rinse the flask with a final 2 ml of MGM™-4 to collect residual cells, and add this rinse to the centrifuge tube.
12. Examine the harvested flask under the microscope to make sure the harvest was successful. Less than 5% of cells should remain attached to the culture vessel.
13. Do not centrifuge the cells!

Counting the cells:

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 MGM™-4 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.

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

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. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks (Subculturing Step 3).
20. If not using vented cap flasks, loosen caps of flasks. Place the new culture vessels into a 37°C humidified incubator with 5% CO_2.

Maintenance

1. Change the growth medium the day after subculture and every other day thereafter. As the cells become more confluent, increase the volume of medium as follows: under 25% confluence then feed cells 1 ml per 5 cm^2, 25-45% confluence then feed cells 1.5 ml per 5 cm^2, over 45% confluence then feed cells 2 ml per 5 cm^2.
2. Warm an appropriate amount of medium to 37℃ in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator. Pipette the medium down the cell-free surface of the flask.
3. 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.

Ordering Information

Cryopreserved Cells (Single donor)

CC-2504	NHEM-neo	≥500,000 cells
CC-2586	NHEM-Ad	≥500,000 cells

Proliferating formats are available. Contact Scientific Support or refer to the Lonza website for details.

Related Products

Melanocyte Medium (Must be purchased separately):

CC-3249	MGM™-4 BulletKit™	Kit which contains a 500 ml bottle of MBM™-4 (CC-3250) and MGM™-4 SingleQuots™ (CC-4435).
CC-3250	MBM™-4	Melanocyte Cell Basal Medium (no growth factors) (500 ml)
CC-4435	MGM™-4 SingleQuots™	Supplements and growth factors ($CaCl_2$, hFGF-B, PMA, rh-Insulin, Hydrocortisone, BPE, FBS and Gentamicin/ Amphotericin-B)
CC-4510	ET-3	Endothelin-3 (NHEM-Ad only), 130 µg

Subculturing Reagents

(Must be purchased separately):

17-516F	Phosphate Buffered Saline (PBS)	500 ml
CC-5012	Trypsin/EDTA Solution	100 ml
17-711E	Versene® (EDTA) 0.02%	100 ml
CC-5002	Trypsin Neutralizing Solution (TNS)	100 ml

Product Warranty

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

Quality Control

Melanocyte Cell Systems undergo extensive Quality Control testing*.

Melanocyte testing routinely includes:

- HIV-1, Hepatitis B and Hepatitis C are not detected for all donors and/or cell lots..
- Immunofluorescent labeling of Mel-5 (gp75/TRP-1) to ensure purity.
- Verification of melanocyte conversion of L-dopa into dopa-melanin to ensure functional activity.
- Morphology and proliferative capacity throughout serial passage after recovery from cryopreservation (FIO).

Clonetics™ MGM™-4 is formulated for optimal growth of melanocytes. MGM™-4 SingleQuots™ testing routinely includes:

- Verification of melanocyte conversion of L-dopa into dopa-melanin to ensure support of functional activity.
- Promotion of melanocyte morphology and proliferative capacity throughout serial passage after recovery from cryopreservation.

* For detailed information concerning QC testing and release specifications, please refer to the Certificate of Analysis (COA). COAs for each cell lot are shipped with each order. COAs for all other products are available upon request.

When placing an order or for technical service, please refer to the product numbers and descriptions listed above. For a complete listing of all Clonetics™ Products, refer to the Lonza website or our current catalog. To obtain a catalog, additional information or technical support you may contact Lonza by web, e-mail, telephone, fax or mail.