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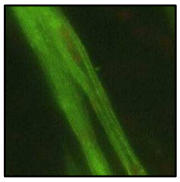

Clonetics™ Skeletal Muscle Cell System

SkMC– Instructions for Use

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_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™ Medium instructions: store basal medium at 2°-8°C and SingleQuots ™ Kit at ≤20°C in a freezer that is not self-defrosting. Once thawed, SingleQuots™ Kit should be stored at 2°-8°C and added to basal medium within 72 hours. After SingleQuots™ Kit is added to basal medium, use within 1 month. Do not re-freeze. Using medium or reagents other than what is recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.
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 one 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 is recommended will void the cell warranty. Please contact Scientific Support if you need help selecting media and/or reagents.

Preparation of Media

1. Decontaminate external surfaces of all vials and the medium bottle with ethanol or isopropanol.
2. To formulate Skeletal Muscle Growth Media (SkGM™ Medium), transfer the contents of the SkGM™ SingleQuots™ Kit (Catalog No. CC-4139 containing human Epidermal Growth Factor [hEGF], Fetuib, Bovine Serum Albumin [BSA], Dexamethasone, Insulin, and Gentamicin/Amphotericin-B [GA]) to SkBM™ Basal Medium with a pipette, and rinse each vial with medium.

3. When preparing these BulletKit™ Media, it may not be possible to recover the entire volume listed for each vial. Small losses (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(s) being supplemented (avoid covering the basal medium lot # and expiration date). Use it to record the date and amount of each supplement added.

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

Thawing of Cells / Initiation of Culture Process

1. The recommended seeding density for SkMC is 3,500 cells/cm^2..
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 directly into a well plate directly out of cryopreservation. Add the appropriate amount of medium to the vessels (1 ml/5 cm^2) and allow the vessels to equilibrate in a 37℃, 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, and then retighten. Quickly thaw the cryovial in a 37℃ 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

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 Reagent Pack™ Subculture Reagents (CC-5034).

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 50%-70% confluent and contain many mitotic figures throughout the flask.
2. For each 25 cm^2 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 warming to room temperature.
   e. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol (explained later in this procedure), based on calculated cell count, cell viability, and seeding density.

In a Sterile Field:

1. Aspirate the medium from one 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 two to six minutes, depending on cell type.
7. At this point, tap 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 tap again. If cells still do not detach, wait and tap every 30 seconds thereafter.

8. After cells are released, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. If the majority of cells do not detach within seven 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 Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel. 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 five 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 to a final volume of 2 to 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 Buffered Saline Solution (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^2.

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

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. 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^2 surface area of the flask (1 ml/5 cm^2).
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_2.

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^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.
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.

Quality Control

All cells are performance assayed and test negative for HIV-1, mycoplasma, Hepatitis-B, Hepatitis-C, bacteria, yeast and fungi. Cell viability, morphology, cell number, and proliferative capacity are measured after recovery from cryopreservation. Clonetics™ Media are formulated for optimal growth of specific types of normal human cells. Certificates of Analysis (COA) for each cell strain are shipped with each

order. COAs for all other products are available upon request.

Ordering Information

Cryopreserved Skeletal Muscle Cells (Single Donor):

Cat. No.	Product	Description
CC-2561	SkMC	≥500,000 cells

Proliferating Skeletal Muscle Cells (Single Donor):

Cat. No.	Product	Description
CC-2661	SkMC (T-25)	Proliferating SkMC in a T-25 flask
CC-0231	SkMC (T-75)	Proliferating SkMC in a T-75 flask
CC-0144	SkMC (96-well plate)	Proliferating SkMC in a 96-well plate
Other proliferating formats are available. Refer to the Lonza website or contact Scientific Support for details.		

Skeletal Muscle Myoblast Growth Media (Sold Separately):

Cat. No.	Product	Description
CC-3160	SkGM™ BulletKit™ Medium	500 ml SkBM™ Basal Medium plus CC-4139 SingleQuots**™** Kit to formulate SkGM™ Medium (growth medium)
CC-3161	SkBM**™** Basal Medium	Skeletal muscle myoblast basal medium (500 ml)
CC-4139	SkGM™ SingleQuots**™** Kit	Formulates 500 ml of SkBM™ Basal Medium to SkGM™ Growth Medium; contains hEGF, 0.5 ml; Fetuin, 5.0 ml; BSA, 5.0 ml; Dexamethasone, 0.5 ml; Insulin, 5.0 ml; GA, 0.5 ml.

Subculturing Reagents (Sold Separately):

Cat. No.	Product	Description
CC-5034	ReagentPack™	Provides necessary components for subculture of SkMC; contains Trypsin/EDTA Solution, 100 ml; Trypsin Neutralizing Solution (TNS), 100 ml; HEPES Buffered Saline Solution, 100 ml

Product Warranty

Cultures have a finite lifespan *in vitro.*

Lonza guarantees the performance of its cells in the following manner only if Clonetics™ Media and Reagents are used exclusively, and the recommend protocols are followed. The performance of cells is not guaranteed if any modifications are made to the complete cell system.

1. Clonetics™ SkMC Cryopreserved Cultures are assured for experimental use for 15 population doublings.
2. Clonetics™ SkMC Proliferating Cultures are assured for experimental use for 10 population doublings.
3. Additional population doublings and subcultures are possible, but growth rate, biological responsiveness and function deteriorate with subsequent passage.
4. SkMC 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.

When placing an order or for Scientific Support, please refer to the product numbers and descriptions listed above. For a complete listing of all Clonetics™ Products, refer to the Lonza website or the current Lonza catalog. To obtain a catalog, additional information or want to speak with Scientific Support, you may contact Lonza by web, e-mail, telephone, fax or mail
(See page 1 for details).

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 cannot 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 ed. If you require further information, please contact your site safety officer or Scientific Support.