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Clonetics™ normal human osteoblasts (NHOst)

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°C, 5% CO_2, 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 medium 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

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 NHOst is 5,000 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 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°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.

Subculturing

The following instructions are for a 25 cm^2 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 80% 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 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 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 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^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 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°C 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.

Bone mineralization using differentiation SingleQuots™

Differentiation SingleQuots™ (CC-4194)

- Hydrocortisone-21-hemisuccinate, 200 µM (CC-4333) 0.5 ml
- β-Glycerophosphate, 1.0 M (CC-4399) 5.0 ml

Differentiation SingleQuots™ are made available for bone mineralization experiments. The volumes supplied are enough to supplement at least 500 ml of osteoblast growth medium (OGM™). It is likely that a smaller volume of medium may be required for the completion of an experiment, in which case, the supplements can be added to a smaller volume of OGM™ as in the example below. It is recommended that the hydrocortisone and β-glycerophosphate be titrated or used at two different volumes for initial experiments as cell strains differ in tolerance to this supplement.

Example: Using completely supplemented OGM™

1. Transfer 100 ml of OGM™ to a separate container.
2. Add the following volumes of differentiation SingleQuots™:
   a. Hydrocortisone-21-hemisuccinate 0.1 ml (200 nM final concentration)

      or

   b. Hydrocortisone-21-hemisuccinate 0.2 ml (400 nM final concentration)
   c. β-Glycerophosphate 1.0 ml (10 mM final concentration)

      or

   d. β-Glycerophosphate 0.75 ml (7.5 mM final concentration)

Once thawed, unused portions of supplements may be re-frozen only once and then stored at -20°C for up to 1 year.

General procedure for bone mineralization

1. Thaw the frozen amp of NHOst and grow up the cells to about 90% confluence.

2. Prepare differentiation medium using two levels of β-Glycerophosphate, and transfer 2 ml each into a well of a 6-well culture plate or other appropriate culture vessel. Transfer 2 ml of OGM™ into a third well/vessel to serve as a negative control. Equilibrate plate/vessel for a least 30 minutes in a humidified 37°C CO_2 incubator.
3. Harvest cells and seed wells/vessels with 10,000 cells per cm^2, and return plate to the incubator. **Note**: to minimize peeling during differentiation, coat wells with collagen before seeding the cells.
4. Change medium every 3 to 4 days or twice weekly with the appropriate medium. Examine culture at each media change to be sure that the monolayer is intact.
5. Stain culture after 3 to 4 weeks incubation for bone mineralization using von Kossa stain or appropriate method. Some lots may peel before this time and should be assayed at that time.
6. NHOst generally produce more mineralization with increasing passages.

Ordering information

Cryopreserved cells (single donor)		
CC-2538	NHOst	≥500,000 cells
Proliferating cells		
CC-2538T25	NHOst	T-25 flask
CC-2538T75	NHOst	T-75 flask
CC2538T150	NHOst	T-150 flask
CC2538T225	NHOst	T-225 flask
Proliferating cells in plates		
CC-2538W6	NHOst	6-well plate
CC-2538W12	NHOst	12-well plate
CC-2538W24	NHOst	24-well plate
CC-2538W48	NHOst	48-well plate
CC-2538W96	NHOst	96-well plate

Related products

Osteoblast growth media (must be purchased separately):

CC-3207	OGM™ BulletKit™	Kit which contains a 500 ml bottle of OBM™, (CC-3208) and OGM™ SingleQuots™ (CC-4193).
CC-3208	OBM™	Osteoblast basal medium (no growth factors) (500 ml)
CC-4193	OGM™ SingleQuots™	Supplements and growth factors (FBS, ascorbic acid and gentamicin/ amphotericin-B)
CC-4194	OGM™ differentiation SingleQuots™	Hydrocortisone-21-hemisuccinate and beta-glycerophosphate

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™ NHOst cryopreserved cultures are assured for experimental use for 10 population doublings.
2. Clonetics™ NHOst proliferating cultures are assured for experimental use for 5 population doublings.
3. Additional population doublings and subcultures are possible, but growth rate, biological responsiveness and function deteriorate with subsequent passage.
4. NHOst 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. Routine characterization of NHOst includes positive immunofluorescent staining for alkaline phosphatase and bone mineralization (von Kossa stain). For detailed information concerning QC testing, please refer to the certificate of analysis.