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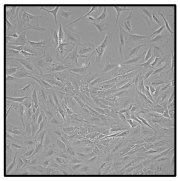

Clonetics™ Sertoli Cell System

HSEC – 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. Medium instructions: store basal medium at 2°-8°C and Fetal Bovine Serum at –20°C in a freezer that is not self-defrosting. Store penicillin/streptomycin (optional) at –20°C in a freezer that is not self-defrosting. Once thawed, Fetal Bovine Serum and penicillin/streptomycin may be immediately aliquoted and refrozen at –20°C. After supplements are 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.
4. Trypsin/EDTA and Trypsin Neutralizing Solution are sterile-filtered and then stored at –20°C until shipment. Trypsin/EDTA and Trypsin Neutralizing Solution 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 Trypsin Neutralizing Solution be stored at 4°C for no more than one month.
5. Store Versene® and PBS at room temperature (15°-30°C).

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

1. Decontaminate external surfaces of all vials and the medium bottle with ethanol or isopropanol.
2. To formulate Sertoli Cell Growth Media, transfer 25 ml of Fetal Bovine Serum to 500 ml of SeBM™ Basal Medium with a pipette for a final concentration of 5% FBS. To prevent bacterial contamination, 5 ml of penicillin/streptomycin (Lonza no. 17-602E or equivalent) may also be added to 500 ml of SABM™ Basal Medium with a pipette for a final concentration of 100 units/ml potassium penicillin and 100 µg/ml streptomycin sulfate. It is not recommended to use gentamicin/amphotericin-B to prevent bacterial contamination, as these reagents have been shown to effect cell growth.

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 HSEC is 450-500 cells/cm^2. A vial containing ≥500,000 cells is sufficient to seed five T-225 flasks.
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/3.75 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 Versene® [EDTA] 0.02% (17-711E), Trypsin/EDTA (CC-5012), Trypsin Neutralizing Solution (CC-5002), PBS (17-516F).

The following instructions are for a 225 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%-80% confluent and contain many mitotic figures throughout the flask.
2. For each 225 cm^2 of cells to be subcultured:
   a. Allow 18 ml of Versene® [EDTA] to come to room temperature.
   b. Thaw 12 ml of Trypsin/EDTA and allow to come to room temperature.
   c. Allow 45 ml of phosphate buffered saline (PBS) to come to room temperature.
   d. Allow 24 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
   e. Remove growth medium from 4°C storage and allow warming to room temperature.
   f. 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 30 ml of room temperature PBS (without calcium or magnesium). DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the PBS from the flask.
4. Cover the cells with 18 ml of Versene® [EDTA] solution and incubate at 37℃ for three to five minutes.
5. Examine the cell layer microscopically.
6. Maintain the Versene® [EDTA] solution on the cells until approximately 60-80% of the cells are liting off the surface of the flask. This entire process takes about three to five minutes.
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, add 12 ml of trypsin-EDTA to the flask and return to the incubator (37℃) for an additional two to three minutes. During the three minute incubation, take out the flask and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.
8. If trypsin/EDTA is being used, after cells are released, neutralize the trypsin in the flask with 24 ml of Trypsin Neutralizing Solution at room temperature. If the majority of cells does 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 50 ml centrifuge tube. Evenly distribute amongst multiple tubes if necessary.
10. Rinse the flask with a final 15 ml of PBS to collect residual cells, and add this rinse to the centrifuge tube(s).
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 growth medium 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 flasks or culture dishes at this time, the recommended density is 450-500 cells/cm^2. 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 3.75 cm^2 surface area of the flask (1 ml/3.75 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 three to four days thereafter using 1 ml of fresh media per 3.75 cm^2 of culture area.
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, Hepatitis B & C, mycoplasma, bacteria, yeast and fungi. Cell viability and morphology are measured after recovery from cryopreservation. 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 Sertoli Cells (Single Donor):

Cat. No.	Product	Description
MM-HSE-2305	HSEC – Human Sertoli Cells	≥500,000 cells

Sertoli Cell Growth Media (Sold Separately):

Cat. No.	Product	Description
00191051	SeBM™ Basal Medium	Sertoli cell basal medium (500 ml)
17-602E	Penicillin-Streptomycin Mixture	Contains 10,000 units potassium penicillin and 10,000 µg streptomycin sulfate per ml in 0.85% saline (100 ml)

Fetal Bovine Serum (FBS) is also necessary to create complete sertoli cell growth media and must be purchased separately.

Subculturing Reagents (Sold Separately):

Cat. No.	Product	Description
17-711E	Versene® [EDTA] 0.02%	0.2 g/L Ethylenediaminetetraacetic acid (0.53 mM) in DPBS, without calcium or magnesium (100 ml)
CC-5012	Trypsin/EDTA Solution	Trypsin/EDTA Solution (100 ml)
CC-5002	TNS	Trypsin Neutralizing Solution (TNS) (100 ml)
17-516F	PBS	Phosphate Buffered Saline (1 x); 6.7 mM (PO_4) without calcium or magnesium (500 ml)

Limited Use License

All Sertoli cells are produced for Lonza by MandalMed Inc. and are subject to the following limited use license:

The included biological material, including progeny and derivatives (collectively referred to as "Material"), is licensed to you under specific terms. You are responsible for ensuring that the terms of the license agreement are met.

1. **GRANTS OF LICENSE:** Lonza grants you a nontransferable, nonexclusive license to use the Material for research.
2. **NOT FOR HUMAN USE:** The Material may not be used: a) in humans; b) in conjunction with human clinical trials; c) in association with human diagnostics.
3. **MATERIAL NOT TRANSFERABLE:** You may not transfer the Material to any other person or organization.
4. **PATENT NOTICE:** Material is under license from MandalMed Inc. Material is covered by US Patent 2009/0028833 A1.

Product Warranty

Cultures have a finite lifespan *in vitro.*

Lonza guarantees the performance of its cells in the following manner only if the recommended 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™ HSEC Cryopreserved Cultures are assured to be viable and functional when thawed and maintained properly.
2. HSEC 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.