ChondroLife™ Complete Chondrogenesis Medium Instruction Sheet

Safety and Use Statement

This product is for Research Use Only. This product is not approved for human or veterinary use or for use in *in vitro* diagnostics or clinical procedures.

Lifeline recommends storing cryopreserved vials in liquid nitrogen vapor phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures. Aseptically vent any nitrogen from cryopreserved vials by carefully loosening the vial cap in a biosafety cabinet prior to thawing the vials in a water bath. If vials must be stored in liquid phase, the vials should be transferred to vapor phase storage or -80°C for up to 24 hours prior to being thawed.

ChondroLife Chondrogenesis Medium is optimized for the differentiation of Human Mesenchymal Stem Cells into chondrocytes.

Medium Storage

ChondroLife Complete Chondrogenesis Medium should be stored at -20°C until ready to use. For long-term storage, DifFactors should be stored at -70°C or lower. Once the medium is thawed, it should be stored at 2 to 8°C for up to three weeks. Do not use product beyond expiration date. Multiple freeze/thaw cycles are not recommended. Users should take care to protect medium from extended exposure to light.

Product	Part No.	Volume	Final Concentration in Supplemented Medium	Storage
ChondroLife Complete Chondrogenesis Medium	LM-0022	100 mL	Proprietary	-20°C or 2-8°C once thawed

Other Recommended Products	Part No.	Unit	Components	Storage
Human Mesenchymal Stem Cells-Wharton's Jelly	FC-0020	0.5 x 10^6 Cells		-150°C
Human Mesenchymal Stem Cells-Adipose	FC-0034	10^6 Cells		-150°C
StemLife™ MSC Medium Complete Kit	LL-0034	Kit	StemLife Basal Medium StemLife MSC LifeFactors® Kit	2-8°C when prepared
Human Mesenchymal Stem Cells-Bone Marrow	FC-0057	10^6 Cells		-150°C
StemLife MSC-BM Medium Complete Kit	LL-0062	Kit	StemLife Basal Medium StemLife MSC-BM LifeFactors Kit	2-8°C when prepared
Human Pre-Adipocyte Cells, Adult	FC-0062	10^6 Cells		-150°C
StemLife PA Medium Complete Kit	LL-0058	Kit	StemLife PA Basal Medium StemLife PA LifeFactors Kit	2-8°C when prepared
Alcian Blue Staining Kit	LL-0051	Kit	Fixative Stabilizer Solution Alcian Blue Stain Wash Solution	-20°C RT RT RT
TrypKit™	LL-0013	Kit	PBS 0.05% Trypsin/0.02% EDTA Trypsin Neutralizing Solution	RT -20°C -20°C

Optional Supplements	Part No.	Volume	Concentrations of Supplement	Storage
Phenol Red Supplement	LS-1009	1 mL	33 mM	RT
Antimicrobial Supplement: Gentamicin and Amphotericin B *(Provided with purchase of LL-0034, LL-0058, or LL-0062)*	LS-1104	0.5 mL	Gentamicin 30 mg/mL Amphotericin B 15 µg/mL	-20°C

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Basic Aseptic Technique

Medium, LifeFactors®, and cells should only be used in an aseptic environment, a Class II biological safety cabinet with front access and filtered laminar airflow, or an equivalent device. Always wear gloves and eye protection when working with these materials. Wipe or spray all bottles and vials with 70% ethanol or isopropanol, especially around the area of the cap, before placing them in the biological safety cabinet. Allow these surfaces to dry completely before opening the bottle or vials. Transfer cells, medium, or LifeFactors with disposable sterile pipettes. Do not mouth pipette! Take up the volume needed into the pipette, being careful not to touch the sterile tip to the rim of the container or any other surface. Close the container and open the container into which the transfer is being made, again being careful not to touch any surfaces with the sterile tip. Transfer the material and close the container. Wash your hands before and after working with cell cultures. Do not block airflow in a laminar flow hood as this may compromise sterility. Ensure that biological safety cabinets are certified routinely and that the HEPA filters are replaced regularly.

Pre-warming Medium

If using less than 100 mL of medium, Lifeline® recommends warming only the volume needed in a sterile conical tube. Repeated warming of the entire bottle over extended periods will cause degradation and reduced shelf life of the medium. When warming the entire bottle of medium, Lifeline recommends using a Lifeline water bath sleeve (included with medium) to help protect the medium from contaminants in the 37°C water bath. Medium will warm to 37°C in 10 to 30 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

Thawing and Plating Cryopreserved Cells

Pre-warm fully supplemented StemLife™ Medium, respective to cell type. See the StemLife Instruction Sheets, for details on how to prepare each medium.

Remove vial from dewar and check the cap to be sure that the vial is securely sealed. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet. Allow it to dry thoroughly and carefully loosen the cap to vent any liquid nitrogen that may have entered the vial. Recap the vial and hold only the bottom half of the vial in a 37°C water bath for approximately one minute or until vial is almost completely thawed—a small amount of ice should still be visible. To avoid potential contamination, do not allow the vial cap to make contact with the water. Do not over thaw as this may damage the cells. Dry the vial, spray the exterior of the vial with 70% ethanol or isopropanol and place the vial in a biological safety cabinet and allow it to dry. Carefully remove the cap to avoid contamination or splatter. Gently resuspend the cells in the vial using a 1 or 2 mL sterile pipette. Do not centrifuge; the cells may be directly plated from the vial. Plate cells into pre-warmed fully supplemented medium (respective to cell type) in the desired culture vessel at a density of 5,000 cells per cm^2. Flasks with vented caps, commonly referred to as filter caps, are strongly recommended. Gently rock the culture vessel from side to side and front to back to evenly distribute cells within the vessel. Place seeded culture vessel in a 37°C, 5% CO_2 incubator. Re-feed the cells after they have attached (approximately 4 to 36 hours after inoculation) to remove cryopreservation reagents.

Recommended Feeding Guidelines for Undifferentiated Expansion of HMSC:

Guidelines for a T-75 Flask. Adjust volumes according to culture surface area.
Every other day, remove medium and feed with 15 mL of fresh supplemented medium.
Most cultures which are 50% confluent will be ready for passage within 2 to 4 days and should be fed with 15 to 20 mL of supplemented medium.
Do not use more than 10 mL of medium per 25 cm^2 of culture surface to ensure the depth of the medium is at a level where gas diffusion will be sufficient to support the cells' requirements for oxygen.

Gas diffusion gradients through the culture medium to the cells are affected by the depth of the medium. The volumes of medium recommended in this table result in a range of depths between 2 mm and 5 mm, which is compatible with general recommendations, 30 mL being at the maximum depth allowable (5 mm) for a T-75 flask.

Passaging Cells

HMSC may be passaged when the culture is 70 to 80% confluent and actively proliferating. Lifeline® recommends passaging HMSC before reaching confluence since post-confluent cells exhibit morphological changes, slower growth and differentiation.

Lifeline recommends using the TrypKit™ Subculture Reagent Kit (LL-0013). Aspirate the medium from the culture vessel. Rinse the flasks with Lifeline's Phosphate Buffered Saline (PBS; CM-0001) by adding at least 1 mL of PBS per each 5 cm^2 and gently tilting the flask to cover the surface with PBS. Aspirate the PBS from the culture vessel, repeat the rinse if desired. Add at least 2 mL of Lifeline's 0.05% Trypsin/0.02% EDTA (CM-0017) per each 25 cm^2 to the vessel. Swirl gently to ensure all cells are coated with the Trypsin/EDTA. Observe the cells carefully under the microscope. When the cells round up they are ready to be released. This normally takes from 2 to 3 minutes depending on the confluence of the cells. Do not over trypsinize as this may damage the cells. Detach the cells by gently striking the culture vessel against your hand several times. Observe the cells under the microscope to be sure they have become detached. Once the cells are fully detached, add Lifeline's Trypsin Neutralizing Solution (TNS; CM-0018) using a volume equal to the amount of Trypsin/EDTA that was originally used. Gently swirl to ensure all of the trypsin solution is neutralized. Using aseptic laboratory techniques, pipette the cells into a sterile centrifuge tube. Collect the remaining cells by rinsing the culture vessel with at least 1 mL of PBS per each 5 cm^2 and pipetting the cells into the sterile centrifuge tube. Check culture vessel under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel. All steps must be completed under aseptic conditions in a biological safety cabinet.

Centrifuge the cells at 250 x *g** for 3 to 5 minutes. For best results, calculate speed for individual centrifuge type. Time may also be centrifuge dependent. Do not over centrifuge cells as this will cause cell damage. After centrifugation, the cells should form a clean loose pellet. Please consult Lifeline's technical service department if issues arise from trypsinization or centrifugation.

Aspirate the neutralized trypsin solution from the centrifuge tube and resuspend the cell pellet in pre-warmed culture medium (respective to cell type) by gently pipetting up and down with a 2 or 5 mL pipette. Count cells using a hemacytometer. If expansion of cells is desired, re-plate at 5,000 cells per cm^2 **(or use a split ratio of 1:6 to 1:10)** in vessel containing the respective pre-warmed culture medium. If differentiation of cells is desired, see instructions on page 4.

***To calculate RCF ('x *g*')** **$RCF = 0.00001118 \times (rpm)^2 \times r$**
r = rotational radius in centimeters
rpm = rotations or revolutions per minute

Standard Calculation for Plating of Cells

Gently re-suspend the cells evenly in the respective pre-warmed complete StemLife™ Culture Medium. Using a clean hemacytometer and aseptic technique, remove 25 µL of the cell suspension to a separate tube, such as a microcentrifuge tube. Add 75 µL of 0.4% Trypan Blue solution to the cell suspension in the microcentrifuge tube and allow it to sit for up to 1 to 5 minutes. Place 10 µL of the cell suspension into each chamber of the hemacytometer. Count a minimum of 4 quadrants on the hemacytometer (see diagram below). Dead and dying cells are permeable to Trypan Blue, viable cells will not be blue. For accurate cell counts, optimal number of cells per quadrant should be 25 to 75 cells. After counting the cells, calculate the average of the number of quadrants counted. Take the cell count average and multiply by the dilution factor and by 10^4 to get the number of cells per mL. Multiply the desired inoculation density (5,000 viable cells per cm^2) by the surface area of the well(s) to be inoculated. This will give you the total number of cells to inoculate one well. Divide the number of cells needed to inoculate the well(s) by the total number of cells in the cell suspension. This will give you the volume of cell suspension with which to inoculate the well(s). Inoculate the cells into the well(s) of the culture vessel(s) prepared with pre-warmed culture medium. Mix gently to evenly distribute the cells and place culture vessel(s) into the incubator at 37°C, 5% CO_2. (See TrypKit Instructions for more detailed cell counting instructions.)

Sample calculation:

Average viable cells per quadrant = 31

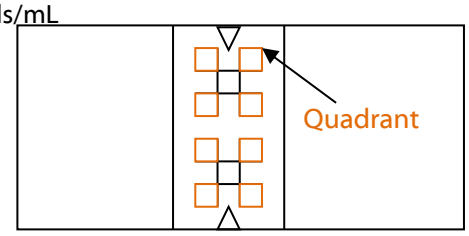
31 cells/quadrant x 10,000 quadrants/mL x 4 (dilution factor) = 1,240,000 cells/mL

Inoculating a T-75 flask at 5,000 cells/cm^2:

5,000 cells/cm^2 x 75 cm^2/well = 375,000 cells/flask

Calculate volume of cell suspension required to inoculate each flask with:

375,000 cells/flask divided by 1,240,000 cells/mL = 0.302 mL/well



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Alginate Encapsulation and Initiating Chondrogenesis

Chondrogenesis requires a three-dimensional aggregate cell culture. Micromass culture can be used but for the best results, Lifeline® recommends the use of scaffolding or matrix, such as alginate, to provide a structure for deposition of proteoglycans. The following items are recommended to prepare alginate encapsulated cells for chondrogenesis, but are not supplied in this kit. All steps must be performed using aseptic technique in a biosafety cabinet.

Additional Materials Recommended but Not Provided by Lifeline Cell Technology	
• Sodium Alginate	• 150 mM Sodium Chloride solution (sterile)
• Syringe (e.g. 3 mL)	• 100 mM Calcium Chloride solution (sterile)
• Fine gauge needle (e.g. 27-gauge)	• Small sterile magnetic stir bar (e.g. 25 mm)
• Wide-bore pipette tip	• Sterile 250 mL beaker
• Vacuum-driven 50 mL filtration system (e.g. Steriflip®)	• Magnetic stir plate
(Steriflip is a registered trademark of Millipore Corporation.)	• Sterile forceps or stir bar extractor

Preparation of 1.5% (w/v) Alginate Solution in 150 mM Sodium Chloride (NaCl) Solution

Add 0.15 g of alginate to 10 mL of 150 mM NaCl solution while stirring rapidly or vortexing to minimize clumping. Agitate the solution on a rocker at room temperature for at least two hours to overnight to completely solubilize the alginate. Filter sterilize (0.22 µm) the solution and store at 4°C for up to one week.

ChondroLife™ Complete Chondrogenesis Medium Preparation

ChondroLife Complete Chondrogenesis Medium contains no antimicrobials and no phenol red. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures. Antimicrobials and phenol red may be purchased separately from Lifeline.

ChondroLife Complete Chondrogenesis Medium contains all the growth factors necessary to support the differentiation of HMSC into chondrocytes. ChondroLife Medium is prepared by thawing and warming to 37°C. ChondroLife Medium contains no antimicrobials or phenol red. Antimicrobials and phenol red are not required for chondrogenesis, but may be purchased separately.

Preparation of Alginate Encapsulated Cells (Chondrogenic Microbeads)

Lifeline® recommends using a minimum of 2.5 x 10^7 HMSC (approximately 9 to 15 T-75 flasks) to create 1 mL of alginate encapsulated cells (Chondrogenic Microbeads). The 1.5% (w/v) alginate solution must not be diluted lower than 1.2% (w/v) by the addition of the cells. Therefore Lifeline recommends that the cells are harvested and counted as described on page 3 and that the resulting cell pellet (containing a minimum of 2.5 x 10^7 cells) is resuspended in 800 µL of the 1.5% (w/v) alginate solution. (The quantities may be adjusted as long as the aforementioned ratios are maintained.) Gently mix the alginate-cell suspension, taking care not to introduce air bubbles in to the solution.

Transfer 75 mL of sterile 100 mM Calcium Chloride ($CaCl_2$) solution and a sterile stir bar into a sterile 250 mL beaker. Create a gentle funnel in the $CaCl_2$ solution on a stir plate. Transfer the alginate-cell suspension to a sterile syringe that has a fine gauge (e.g. 27 gauge) needle attached. Rapidly dispense the alginate-cell solution (in a single fast stream) into the $CaCl_2$ solution to form the Chondrogenic Microbeads. Allow the $CaCl_2$ solution containing the newly formed Chondrogenic Microbeads to stir for an additional 10 minutes to solidify the alginate.

Remove the beaker from the stir plate and allow the Chondrogenic Microbeads to settle. Transfer the Chondrogenic Microbeads solution into a conical tube and attach to a vacuum-driven 50 mL filter device (e.g. Steriflip® from Millipore). Immediately break the vacuum as soon as the liquid is removed to prevent damage to the beads.

Resuspend Chondrogenic Microbeads in 2 mL of ChondroLife™ Complete Chondrogenesis Medium. Aseptically transfer enough Chondrogenic Microbeads to cover the bottom surface of a single well of a 48-well plate. The method described above yields enough Chondrogenic Microbeads to seed approximately 4 wells of a 48-well plate.

After the Chondrogenic Microbeads settle to the bottom of the well, remove and replace the fluid in each well twice with 0.5 mL ChondroLife Complete Chondrogenesis Medium to remove residual $CaCl_2$. Incubate the cells in a 37°C, 5% CO_2 incubator.

Culturing of the Chondrogenic Microbeads for Chondrogenesis

Every 2 to 3 days carefully remove the spent medium from each well, so as to not disturb or aspirate the Chondrogenic Microbeads. Add 0.5 mL of pre-warmed ChondroLife Complete Chondrogenesis Medium to each well containing Chondrogenic Microbeads. Return the plate to a 37°C, 5% CO_2 incubator.

After 21 days of differentiation, chondrogenesis is complete and the Chondrogenic Microbeads can be fixed and stained for proteoglycan deposition or other analysis. Lifeline recommends the use of the Alcian Blue Staining Kit to assay for sulfated proteoglycans.

Quick Steps for Differentiation of Chondrocytes from HMSC Using Alginate Encapsulation

Pre-Differentiation

1. **Always wash hands before and after working with cell cultures.**
2. **Always wear eye protection and gloves when working with cell cultures.**
3. **When working with cells or medium, always use a certified biological safety cabinet.**
4. **Store cryopreserved cells in liquid nitrogen, vapor phase.**
5. **Handle cryopreserved vials with caution. Aseptically vent any nitrogen from cryopreserved vials in a biosafety cabinet prior to thawing in a water bath.**
6. Feed cells using pre-warmed Lifeline® culture medium according to feeding guidelines.
7. Expand HMSC in the respective StemLife™ Medium as detailed on page 3.

Differentiation

Day 0

1. When cells are 70 to 80% confluent and actively proliferating, passage cells using Lifeline subculture reagents (as detailed on page 3).
2. Resuspend pellet of 2.5 x 10^7 cells in 1.5% alginate solution.
3. Push cell-alginate solution into actively stirring calcium chloride solution to form Chondrogenic Microbeads.
4. Remove calcium chloride solution and transfer Chondrogenic Microbeads to 48 well plate.
5. Replace medium on the Chondrogenic Microbeads twice with 0.5 mL ChondroLife™ Complete Chondrogenesis Medium.
6. Incubate multi-well plate(s) at 37°C, 5% CO_2.

Day 2

7. Replace medium on the Chondrogenic Microbeads with 0.5 mL of ChondroLife Complete Chondrogenesis Medium. Do not aspirate the Chondrogenic Microbeads from well(s).
8. Incubate multi-well plate(s) at 37°C, 5% CO_2.

Day 4 or 5 Through Day 21

9. Replace medium on the Chondrogenic Microbeads every 2 to 3 days with 0.5 mL of ChondroLife Complete Chondrogenesis Medium. Do not aspirate the Chondrogenic Microbeads from the well(s).
10. Incubate multi-well plate(s) at 37°C, 5% CO_2.

Day 21

11. Differentiation is complete.
12. Fix the cells then slice and stain for proteoglycan deposition with Alcian Blue Stain.

For any question on cell handling, differentiation, or staining;
please contact technical service at 877.845.7787.
We are here to help.

Notes: