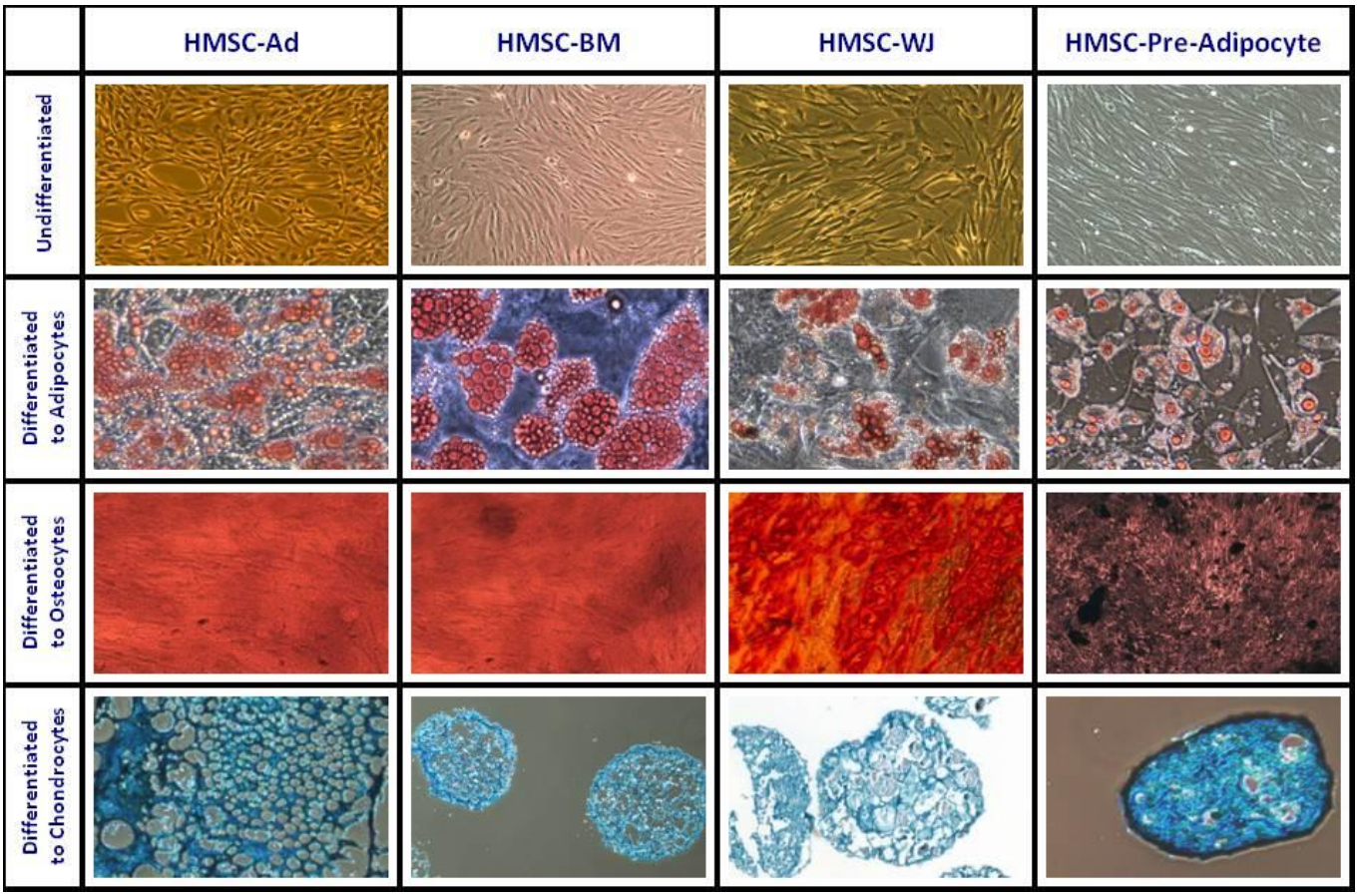
Human Mesenchymal Stem Cells
Instruction Manual

Human Mesenchymal Stem Cells – Adipose-Derived (HMSC-Ad)
Human Mesenchymal Stem Cells – Bone Marrow-Derived (HMSC-BM)
Human Mesenchymal Stem Cells – Wharton's Jelly-Derived (HMSC-WJ)
Human Mesenchymal Stem Cells – Pre-Adipocyte (HMSC-Pre-Adipocyte)



To place an order, please visit lifelinecelltech.com or call customer service at 877.845.7787.

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General Information

Shipping

Cells, frozen media, and LifeFactors® Kits are shipped on dry ice. Basal media are shipped at ambient temperature.

Safety and Use Statement

Lifeline® products are For Research Use Only. They are not approved for human or veterinary use, for use in *in vitro* diagnostics, or clinical procedures.

Lifeline recommends storing cryopreserved vials in liquid nitrogen vapor phase, NOT in liquid phase. Handle cryopreserved vials with caution. Always wear eye protection and gloves when working with cell cultures. Aseptically vent any liquid 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 liquid nitrogen vapor phase storage or in a -80°C freezer for up to 24 hours prior to being thawed.

Safe Handling of Cryopreserved Vials

Human Mesenchymal Stem Cells are sold as cryopreserved vials and are shipped in insulated packages containing dry ice to ensure the cells remain in a cryopreserved state. To maintain the cells' integrity, unpack the products immediately upon receipt and store at a temperature lower than -150°C or in the vapor phase of a liquid nitrogen dewar. If the cells are to be thawed and plated within 24 hours, they may be stored at -80°C. Do not store the vial for more than 24 hours at -80°C as the cells will slowly degrade at this temperature.

Basic Aseptic Technique

Cells, medium, and LifeFactors should only be used in an aseptic environment, such as 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 cell culture 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.

Lifeline Technical Note

There are different and often contradictory terminologies used by cell culture companies to define the passage number of cells. Lifeline's designation of 'primary cells' are cells that have been isolated from tissue, plated onto culture vessel(s), expanded, harvested, and cryopreserved. The term 'secondary' indicates that the cells have been isolated, plated, and expanded in culture vessel(s) twice before being harvested for cryopreservation.

Product Ordering Information

Mesenchymal Stem Cells

Part Number	Cell Type
FC-0020	HMSC-WJ – Derived from the Wharton's Jelly of the umbilical cord
FC-0034	HMSC-Ad – Derived from adipose tissue
FC-0057	HMSC-BM – Derived from bone marrow
FC-0062	HMSC-Pre-Adipocyte – Derived from mature adipocytes that have been dedifferentiated

Expansion Media

Part Number	Components	For Use With Cell Part Number(s)
LL-0034	StemLife™ MSC Medium Complete Kit (StemLife Basal Medium, StemLife MSC LifeFactors® Kit)	FC-0020 FC-0034
LL-0062	StemLife MSC-BM Medium Complete Kit (StemLife Basal Medium, StemLife MSC-BM LifeFactors Kit)	FC-0057
LL-0011	FibroLife® S2 Medium Complete Kit (FibroLife Basal Medium + FibroLife S2 LifeFactors Kit)	FC-0062

Differentiation Media and Staining Reagents

Part Number	Components	For Use With Cell Part Number(s)
LL-0050	AdipoLife™ DfKt™-1 (AdipoLife Basal Medium + DifFactor™ 1 + DifFactor 2)	FC-0034 FC-0062
LL-0059	AdipoLife DfKt-2 (AdipoLife Basal Medium + DifFactor 3)	FC-0020 FC-0057
LL-0052	Oil Red O Staining Kit (4% Paraformaldehyde Fixative Solution + 100% 1,2-Propanediol Dehydration Solution + 0.5% Oil Red O Stain Solution + 85% 1,2-Propanediol Stain Differential Solution)	LL-0050 LL-0059
LM-0022	ChondroLife™ Complete Chondrogenesis Medium, 100 mL	FC-0020 FC-0034 FC-0057 FC-0062
LL-0051	Alcian Blue Staining Kit (4% Paraformaldehyde Fixative Solution + 20% Sucrose Stabilizer + 3% Acetic Acid Wash + 1% Alcian Blue Stain Solution)	LM-0022
LM-0023	OsteoLife™ Complete Osteogenesis Medium, 100 mL	FC-0020 FC-0034 FC-0057 FC-0062
CM-0058	2% Alizarin Red Stain, 100 mL	LM-0023

Additional Products

Part Number	Components	Part Number	Components
LS-1009	Phenol Red Supplement [33 mM], 1 mL	LL-0013	TrypKit™ Subculture Reagent Kit
LS-1104	GA Antimicrobial Supplement, 0.5 mL (Gentamicin 30 mg/mL, Amphotericin B 15 µg/mL); provided with purchase of LL-0011, LL-0034, or LL-0062	LM-0015	FrostaLife™ Cryopreservation Solution, 100 mL

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Thawing and Expanding Undifferentiated HMSC

Materials and Equipment Needed

1. StemLife™ Basal Medium or FibroLife® Basal Medium
2. StemLife MSC (Ad or WJ), StemLife MSC-BM, or FibroLife S2 LifeFactors® Kit
3. Tissue culture treated vessels
4. 70% ethanol or isopropanol for disinfecting surfaces
5. 37°C water bath
6. Cell culture incubator (37°C, 5% CO_2, humidified)
7. Biosafety cabinet

Preparing Culture Medium (see individual media instructions for more detail)

1. Thaw frozen supplements at room temperature or in a 37°C water bath.
2. Aseptically add the labeled volume of each LifeFactor to the respective bottle of basal medium using a pipette.
3. A vial of Gentamicin and Amphotericin B (GA; LS-1104) is provided with the purchase of StemLife MSC (LL-0034), StemLife MSC-BM (LL-0062), or FibroLife S2 (LL-0011) Medium Complete Kits for your convenience. The use of GA is recommended to inhibit potential fungal or bacterial contamination of eukaryotic cell cultures.
4. Cap the bottle securely and mix supplemented medium by gently swirling or inverting bottle.
5. Do not shake or froth the medium.
6. The supplemented medium may be stored at 2 to 8°C for up to two weeks.

Pre-warming Medium

1. If using less than 100 mL of complete 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 of the medium and reduced shelf life.
2. 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.
3. Medium will warm to 37°C in 10 to 20 minutes, depending on the volume. Do not leave medium in water bath for extended periods.

Thawing and Plating Cryopreserved Cells

1. Remove the vial from storage in the vapor phase of a liquid nitrogen dewar and check the cap to be sure that the vial is securely sealed.
2. Spray the vial with 70% ethanol or isopropanol and transfer it to a biosafety cabinet.
3. Allow it to dry thoroughly and carefully loosen the cap a quarter turn to vent any liquid nitrogen that may have entered the vial.
4. Recap the vial and hold only the bottom half of the vial in a 37°C water bath for approximately one minute, or until the vial is almost completely thawed—a small amount of ice should still be visible.
5. Dry the thawed 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.
6. 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.
7. Plate the cells into pre-warmed culture medium in the desired culture vessel(s) at a density of 5,000 cells per cm^2.
8. Gently rock the culture vessel(s) from side to side and front to back to evenly distribute cells within the vessel(s).
9. Place seeded culture vessel(s) in a 37°C, 5% CO_2 incubator.
10. Replace the culture medium after the cells have attached, approximately 4 to 36 hours after inoculation to remove cryopreservation reagents.
11. Change the culture medium every 2 to 3 days until the cells reach 70% to 90% confluence.

Passaging/Subculturing Cells

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see expansion media chart on page 2)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. Sterile, disposable conical-bottom centrifuge tube(s)
5. Tissue culture treated vessels
6. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
7. 70% ethanol or isopropanol for disinfecting surfaces
8. 37°C water bath
9. Cell culture incubator (37°C, 5% CO_2, humidified)
10. Biosafety cabinet

Passaging Cells

1. All steps must be completed under aseptic conditions in a biological safety cabinet.
2. HMSC may be passaged when the culture is 70 to 90% confluent and actively proliferating.
3. Lifeline® recommends using TrypKit™ Subculture Reagent Kit (LL-0013).
4. Aspirate the medium from the culture vessel(s).
5. Rinse the vessel(s) with PBS (CM-0001) by adding at least 1.0 mL of PBS per 5 cm^2.
6. Aspirate the PBS from the culture vessel(s), repeat the rinse if desired.
7. Trypsinize the cells with Lifeline's 0.05% Trypsin/0.02% EDTA (CM-0017) by adding at least 2 mL per 25 cm^2 to the vessel(s). Make sure all cells are coated with the Trypsin/EDTA.
8. Observe the cells carefully under the microscope. When the cells contract, they are ready to be released. This normally takes at least 2 to 3 minutes depending on the confluence of the cells and the temperature. Do not trypsinize longer than needed to detach the cells as this may damage the cells.
9. 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.
10. Using aseptic laboratory techniques pipette the cells into a sterile centrifuge tube.
11. Collect the remaining cells by rinsing the culture vessel(s) with PBS (1.0 mL per 5 cm^2) and pipetting the cells into the sterile centrifuge tube.
12. Check culture vessel(s) under the microscope for cells still attached and repeat steps if necessary to retrieve all the cells from the vessel(s).
13. Centrifuge cells at 150 to 250 x *g** for 3 to 5 minutes. For best results, calculate speed for individual centrifuge type. Do not centrifuge cells longer or at higher speeds than necessary as this will cause cell damage.
14. After centrifugation, the cells should form a clean loose pellet.
15. Aspirate neutralized trypsin from the centrifuge tube and gently resuspend the cell pellet in pre-warmed culture medium.
16. Inoculate new culture vessels at 5,000 cells/cm^2.
17. Please consult Lifeline's technical service department if issues arise from trypsinization or centrifugation.

***To calculate Relative Centrifugal Force (x *g*)**

$$\mathbf{RCF = 0.00001118 \times (rpm)^2 \times r}$$

r = rotational radius in centimeters
rpm = rotations or revolutions per minute

Cryopreserving Cells

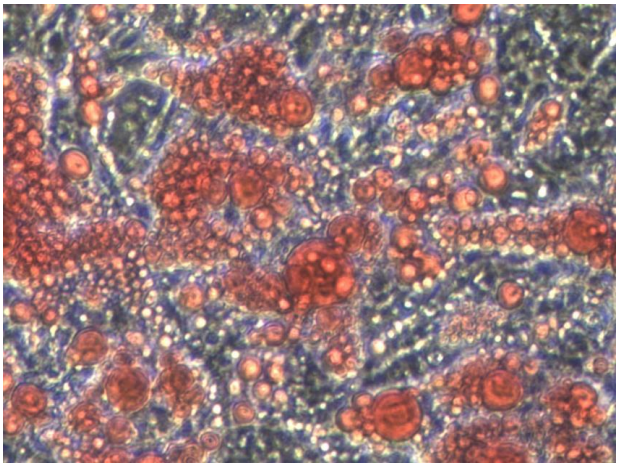
Materials and Equipment Needed

1. HMSC culture(s)
2. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
3. FrostaLife™ Cryopreservation Solution (LM-0015)
4. Or to make your own cryopreservation medium, combine:
    a. 80% (v/v) fully supplemented expansion medium (see expansion media chart on page 2)
    b. 10% (v/v) Fetal Bovine Serum (FBS), sterile
    c. 10% (v/v) Dimethyl Sulfoxide (DMSO), sterile
5. Sterile, disposable 50 mL conical-bottom tube(s)
6. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
7. 70% ethanol or isopropanol for disinfecting surfaces
8. Isopropanol freezing chamber
9. Ultralow freezer (-70°C to -80°C)
10. Biosafety cabinet

Cryopreservation of Cells

1. Detach cells following the Passaging/Subculturing procedure beginning on the previous page.
2. After centrifugation, resuspend the cells in a minimal volume of expansion medium (approximately 100 µL per 25 cm^2) and perform a cell count.
3. Dilute the cells to 1 x 10^6 cells/mL using FrostaLife Cryopreservation Solution or other cryopreservation medium.
4. Immediately dispense the cells into sterile cryovials and freeze using a controlled rate freezer or an isopropanol chamber at -80°C overnight.
5. Transfer the vials to the vapor phase of a liquid nitrogen storage dewar at ≤ -130°C.

Adipogenesis with AdipoLife™ DfKt™-1



HMSC-Ad differentiated into Adipocytes using AdipoLife DfKt-1 and stained with Lifeline's Oil Red O Lipid Staining Kit, post 3 weeks differentiation, (200X).

Seeding Density for Adipogenesis by Cell Type

Part Number	Cell Type	Seeding Density (cells/cm^2)	Expansion Medium	Part Number
FC-0034	HMSC-Ad	15,000 to 20,000	StemLife™ MSC Medium	LL-0034
FC-0062	HMSC-Pre-Adipocyte	15,000 to 20,000	FibroLife® S2 Medium	LL-0011

Cell Density and Media Volume for Standard Well Plates

Format	Cells per well HMSC-Ad and HMSC-Pre-Adipocyte	Media Volume	Feeding
6-well	142,000 to 190,000	2 mL	Every 3 to 4 days for 3 to 4 weeks
12-well	71,000 to 95,000	1 mL	Every 3 to 4 days for 3 to 4 weeks
24-well	30,000 to 40,000	0.5 mL	Every 3 to 4 days for 3 to 4 weeks
48-well	15,000 to 20,000	0.3 mL	Every 3 to 4 days for 3 to 4 weeks

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see chart above)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. AdipoLife DfKt-1 Medium (LL-0050)
    a. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures.
    b. Antimicrobials may be purchased separately from Lifeline®.
5. A fibronectin coating solution (optional, but highly recommended)
6. Tissue culture treated 6-well plates
7. Sterile, disposable 50 mL conical-bottom tube(s)
8. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
9. 70% ethanol or isopropanol for disinfecting surfaces
10. Oil Red O Staining Kit (LL-0052)
11. Cell culture incubator (37°C, 5% CO_2, humidified)
12. Biosafety cabinet

AdipoLife™ DfKt™-1 Medium Preparation

	AdipoLife Initiation Medium	AdipoLife Maintenance Medium
AdipoLife Basal Medium (LM-0021)	15 mL	85 mL
DifFactor™ 1 (LS-1074)	1 mL	
DifFactor 2 (LS-1075)		5 mL
Store at 2 to 8°C for up to	4 weeks	6 weeks

Adipogenesis Differentiation Procedure 1

Day 0

1. Coat 6-well plate(s) with a fibronectin coating solution (optional, but highly recommended).
2. When cells are 70 to 90% confluent and actively proliferating, passage cells using Lifeline® subculture reagents (as detailed on page 4).
3. Resuspend pellet in expansion medium (see expansion medium chart on pages 2 or 6).
4. Perform a cell count.
5. Dispense 2 to 3 mL of respective expansion medium per well.
6. Inoculate cells into 6-well plate(s) at 15,000 to 20,000 cells/cm^2.
7. Incubate plate(s) at 37°C, 5% CO_2.

Day 2

8. Gently aspirate the expansion medium from each well and rinse with PBS.
9. Replace the PBS with AdipoLife Initiation Medium (AdipoLife Basal + DifFactor 1).
10. Incubate at 37°C, 5% CO_2.

Day 4

11. Gently replace the AdipoLife Initiation Medium with 2 mL of fresh AdipoLife Initiation Medium.
12. Incubate plate at 37°C, 5% CO_2.

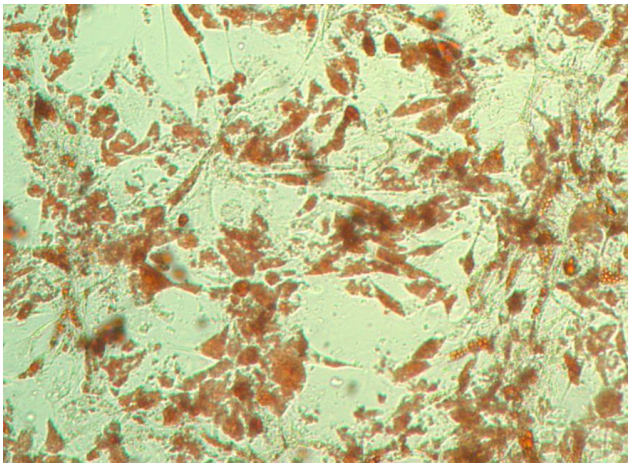
Day 6 Through Day 21

13. Gently replace the medium in the wells every 3 to 4 days with AdipoLife Maintenance Medium (AdipoLife Basal + DifFactor 2) by dispensing the medium slowly down the side wall of the well.
14. Incubate at 37°C, 5% CO_2.

Day 21

15. Differentiation is complete.
16. Fix the cells and stain for lipid accumulation with Oil Red O Staining Kit (LL-0052). See instructions on page 10.

Adipogenesis with AdipoLife™ DfKt™-2



HMSC-WJ differentiated into Adipocytes using AdipoLife DfKt-2 and stained with Lifeline's Oil Red O Lipid Staining Kit, post 4 weeks differentiation, (100X).

Seeding Density for Adipogenesis by Cell Type

Part Number	Cell type	Seeding Density (cells/cm²)	Expansion Medium	Part Number
FC-0020	HMSC-WJ	40,000 to 60,000	StemLife™ MSC Medium	LL-0034
FC-0057	HMSC-BM	20,000 to 40,000	StemLife BM Medium	LL-0062

Cell Density and Media Volume for Standard Well Plates

Format	Cells per well HMSC-WJ	Format	Cells per well HMSC-BM	Media volume	Feeding
6-well	380,000 to 570,000	6-well	190,000 to 380,000	2 mL	Every 3 to 4 days for 4 to 5 weeks
12-well	190,000 to 285,000	12-well	95,000 to 190,000	1 mL	Every 3 to 4 days for 4 to 5 weeks
24-well	80,000 to 120,000	24-well	40,000 to 80,000	0.5 mL	Every 3 to 4 days for 4 to 5 weeks
48-well	40,000 to 60,000	48-well	20,000 to 40,000	0.3 mL	Every 3 to 4 days for 4 to 5 weeks

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see chart above)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. AdipoLife DfKt-2 Medium (LL-0059)
    a. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures.
    b. Antimicrobials may be purchased separately from Lifeline®.
5. A fibronectin coating solution (optional, but highly recommended)
6. Tissue culture treated 6-well plates
7. Sterile, disposable 50 mL conical-bottom tube(s)
8. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
9. 70% ethanol or isopropanol for disinfecting surfaces
10. Oil Red O Staining Kit (LL-0052)
11. Cell culture incubator (37°C, 5% CO_2, humidified)
12. Biosafety cabinet

AdipoLife™ DfKt™-2 Medium Preparation

	AdipoLife Differentiation Medium-2
AdipoLife Basal Medium (LM-0021)	100 mL
DifFactor™ 3 (LS-1083)	10 mL
Store at 2 to 8°C for up to	6 weeks

Adipogenesis Differentiation Procedure 2

Day 0

1. Coat 6-well plate(s) with a fibronectin coating solution (optional, but highly recommended).
2. When cells are 70 to 90% confluent and actively proliferating, passage cells using Lifeline® subculture reagents (as detailed on page 4).
3. Resuspend pellet in expansion medium (see expansion medium chart on pages 2 or 8).
4. Perform a cell count.
5. Dispense 2 to 3 mL of respective expansion medium per well.
6. Inoculate cells into multi-well plates according to the chart on page 8.
7. Incubate plate at 37°C, 5% CO_2.

Day 1 to 2

8. Gently aspirate the expansion medium from each well and rinse with PBS.
9. Replace the PBS with AdipoLife DfKt-2 Medium (AdipoLife Basal + DifFactor 3)
10. Incubate at 37°C, 5% CO_2.

Day 5 Through Day 21 to 35

11. Gently replace the medium in the wells every 3 to 4 days with AdipoLife DfKt-2 Medium by dispensing the medium slowly down the side wall of the well.
12. Incubate at 37°C, 5% CO_2.

Day 21 to 35

13. Differentiation is complete.
14. Fix the cells and stain for lipid accumulation with Oil Red O Staining Kit (LL-0052). See instructions page 10.

Staining with Oil Red O

Materials and Equipment Needed

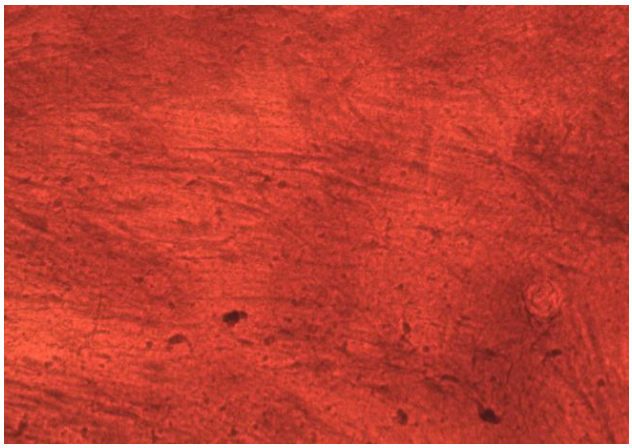
1. Fully differentiated cell cultures
2. PBS (CM-0001)
3. Oil Red O Staining Kit (LL-0052)
    a. 4% Paraformaldehyde Fixative Solution (CM-0055)
    b. 100% 1,2-Propanediol Dehydration Solution (CM-0056)
    c. 0.5% Oil Red O Stain Solution (CM-0054)
    d. 85% 1,2-Propanediol Stain Differential Solution (CM-0057)
4. Deionized water
5. 50 mL conical-bottom tubes
6. Whatman® coarse filter paper, or paper towel
7. Chemical fume hood
8. Oven and/or waterbath, set at 37°C or 60°C
9. Cell culture incubator (37°C, 5% CO_2, humidified)
10. Biosafety cabinet

(Whatman is a registered trademark of GE Healthcare.)

Fixing and Oil Red O Staining Procedure

1. Always wear eye protection and gloves when working with staining reagents.
2. Use 4% Paraformaldehyde Fixative Solution in a chemical fume hood.
3. Adipocytes may be stained with Oil Red O at either 37°C or 60°C.
4. Pre-heat an oven to 60°C or a water bath to 37°C.
5. For each well (6-well plate) to be stained, pre-warm 2.5 mL of Oil Red O (37°C or 60°C) in a 50 mL conical tube.
6. Once Oil Red O has been warmed, filter through paper towel or coarse Whatman® filter paper into a fresh tube to remove small particulates. Return Oil Red O to warming at 37°C or 60°C.
7. Gently wash the adipocyte-differentiated cells by aspirating the medium and adding 2 mL of Lifeline® PBS (CM-0001) to each well.
8. Repeat the PBS wash two more times.
9. Aspirate all the PBS from the wells. **Immediately** add 2 mL of 4% Paraformaldehyde Fixative Solution (CM-0055) to each well.
10. Fix for at least 20 minutes at room temperature.
11. Partially aspirate fixative solution.
12. Rinse well twice with deionized water.
13. Add 1 mL of 100% 1,2-Propanediol Dehydration Solution (CM-0056) to each well and incubate for 5 minutes at room temperature. Gently tilt the plate 2 to 3 times to mix.
14. Remove dehydration solution. Add another 1 mL of 100% 1,2-Propanediol Dehydration Solution to each well and incubate for 5 minutes at room temperature. Gently tilt the plate 2 to 3 times to mix.
15. Remove dehydration solution.
16. Add 2 mL Oil Red O Stain Solution (CM-0054) to each well.
17. Incubate the plate in a 37°C incubator for 30 minutes or in a 60°C oven for 8 minutes. Tilt the plate 2 to 3 times during the incubation.
18. Remove Oil Red O.
19. Add 2 mL of 85% 1,2-Propanediol Stain Differential Solution (CM-0057) for 1 minute to differentiate stain. Do not mix the stain differential solution in the well(s)!
20. Rinse wells carefully twice with 2 mL deionized water per well.
21. Add 2 mL of deionized water to each well to keep them submerged to prevent the lipid vesicles from bursting.

Osteogenesis



HMSC-Ad differentiated into Osteocytes using OsteoLife and stained with Lifeline's Alizarin Red Stain to visualize the calcium deposition, post 3 weeks differentiation, (1X).

Seeding Density for Osteogenesis by Cell Type

Part Number	Cell type	Seeding Density (cells/cm^2)	Expansion Medium	Part Number
FC-0020	HMSC-WJ	15,000 to 20,000	StemLife™ MSC Medium	LL-0034
FC-0034	HMSC-Ad	15,000 to 20,000	StemLife MSC Medium	LL-0034
FC-0057	HMSC-BM	5,000 to 10,000	StemLife MSC-BM Medium	LL-0062
FC-0062	HMSC-Pre-Adipocyte	15,000 to 20,000	FibroLife® S2 Medium	LL-0011

Cell Density and Media Volume for Standard Well Plates

Format	Cells per well HMSC-WJ, Ad, and Pre-Adipocyte	Format	Cells per well HMSC-BM	Media Volume	Feeding
6-well	142,000 to 190,000	6-well	48,000 to 95,000	2 mL	Every 3 to 4 days for 3 to 4 weeks
12-well	71,000 to 95,000	12-well	25,000 to 48,000	1 mL	Every 3 to 4 days for 3 to 4 weeks
24-well	30,000 to 40,000	24-well	10,000 to 20,000	0.5 mL	Every 3 to 4 days for 3 to 4 weeks
48-well	15,000 to 20,000	48-well	5,000 to 10,000	0.3 mL	Every 3 to 4 days for 3 to 4 weeks

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see chart above)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. OsteoLife™ Complete Osteogenesis Medium (LM-0023)
    a. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures.
    b. Antimicrobials may be purchased separately from Lifeline®.
5. A fibronectin coating solution (optional, but **highly recommended**)
6. Tissue culture treated 6-well plates
7. Sterile, disposable 50 mL conical-bottom tube(s)
8. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
9. 70% ethanol or isopropanol for disinfecting surfaces
10. 2% Alizarin Red Staining Kit (CM-0058)
11. Absolute ethanol
12. Cell culture incubator (37°C, 5% CO_2, humidified)
13. Biosafety cabinet

OsteoLife™ Complete Osteogenesis Medium Preparation

1. Thaw at 37°C.
2. Store at 2 to 8°C for up to 6 weeks.

Osteogenesis Differentiation Procedure

Day 0

1. Coat 6-well plates with a fibronectin coating solution (optional, but **highly recommended**).
2. When cells are 70 to 80% confluent and actively proliferating, passage cells using Lifeline® subculture reagents (as detailed on page 4).
3. Resuspend pellet in expansion medium (see expansion medium chart on pages 2 or 11).
4. Perform a cell count.
5. Dispense 2 to 3 mL of respective expansion medium per well.
6. Inoculate cells into 6-well plate(s) at the respective seeding density listed in the chart on page 11.
7. Incubate plate(s) at 37°C, 5% CO_2.

Day 1 to 2

8. Gently aspirate the medium from each well and rinse with PBS.
9. Replace the PBS with 3 mL per well of OsteoLife Complete Osteogenesis Medium
10. Incubate at 37°C, 5% CO_2.

Day 4 to 6 Through Day 21

11. Gently replace‡ the medium in the wells every 3 to 4 days with OsteoLife Complete Osteogenesis Medium; carefully remove and replace 2 mL of medium per well.
12. Incubate at 37°C, 5% CO_2.

Day 21

13. Differentiation is complete.
14. Fix the cells then stain for calcium deposition with Alizarin Red (CM-0058). See instructions on page 13.

‡The monolayer of cells is fragile, under tension, and must be handled with extreme care or it may detach from the plate: Do not tilt plate during aspiration. Once differentiation has been initiated, never completely aspirate a well and expose the monolayer to air. When adding OsteoLife Complete Osteogenesis Medium, pipette the medium gently down the side of the well. This will also prevent disturbance of the accumulating calcium crystals.

Staining with 2% Alizarin Red

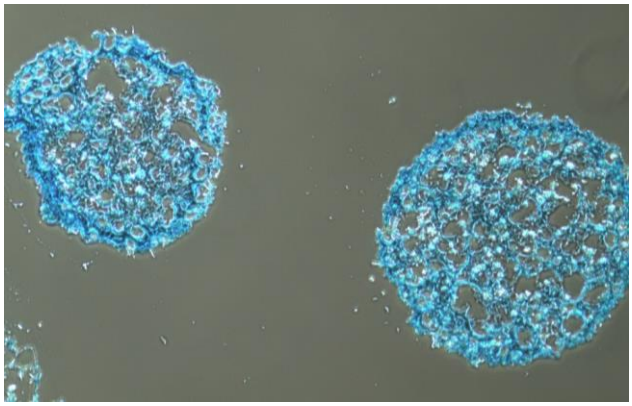
Materials Needed

1. Fully differentiated cell cultures
2. PBS (CM-0001)
3. Absolute ethanol
4. 2% Alizarin Red Stain Solution (CM-0058)
5. Deionized water

Fixing and Alizarin Red Staining Procedure

1. Always wear eye protection and gloves when working with staining reagents.
2. Remove medium completely from well(s).
3. Gently add 1 mL PBS.
4. Aspirate PBS.
5. Add 3 mL of absolute ethanol and fix for 30 minutes.
6. Remove ethanol and allow well(s) to dry completely.
7. Add 1.0 mL of 2% Alizarin Red Stain Solution (CM-0058) per well and gently tilt side-to-side until solution completely covers well.
8. Incubate for 15 minutes at room temperature.
9. Remove Alizarin Red from the well(s).
10. Using care, rinse well(s) three times with 1 mL deionized water and allow to dry. Take extreme care when rinsing well or calcium crystals may be dislodged and rinsed away.

Chondrogenesis (Alginate Encapsulation)



HMSC-Ad differentiated into Chondrocytes using ChondroLife and stained with Lifeline's Alcian Blue Staining Kit to visualize the proteoglycan deposition, post 3 weeks differentiation, (100X).

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see chart on page 2)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. ChondroLife™ Complete Chondrogenesis Medium (LM-0022)
    a. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures.
    b. Antimicrobials may be purchased separately from Lifeline®.
5. Alcian Blue Staining Kit (LL-0051)
6. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
7. 70% ethanol or isopropanol for disinfecting surfaces
8. Sodium alginate
9. 150 mM Sodium Chloride (NaCl) Solution, sterile
10. 100 mM Calcium Chloride ($CaCl_2$) Solution, sterile
11. Tissue culture treated 48-well plates
12. Sterile, disposable 50 mL conical-bottom tube(s)
13. Syringe (e.g. 3 mL)
14. Fine gauge needle (e.g. 27G)
15. Vacuum-driven 50 mL filtration system (e.g. Steriflip®)
16. Wide-bore pipette tip
17. Small sterile magnetic stir bar (e.g. 25 mm)
18. Sterile forceps or stir bar extractor
19. Sterile glass beaker (e.g. 250 mL)
20. Magnetic stir plate
21. Cell culture incubator (37°C, 5% CO_2, humidified)
22. Biosafety cabinet

(Steriflip is a registered trademark of Millipore Corporation.)

ChondroLife Complete Chondrogenesis Medium Preparation

1. Thaw at 37°C.
2. Store at 2 to 8°C for up to 6 weeks.

Preparation of 1.5% (w/v) Alginate Solution

1. Add 0.15 g of alginate to 10 mL of 150 mM NaCl solution and vortex to minimize clumping.
2. Agitate the alginate solution on a rocker or orbital shaker at room temperature for at least two hours to overnight, to completely solubilize the alginate.
3. Filter sterilize (0.22 µm) the solution and store at 2 to 8°C for up to one week.

Chondrogenesis Differentiation Procedure 1

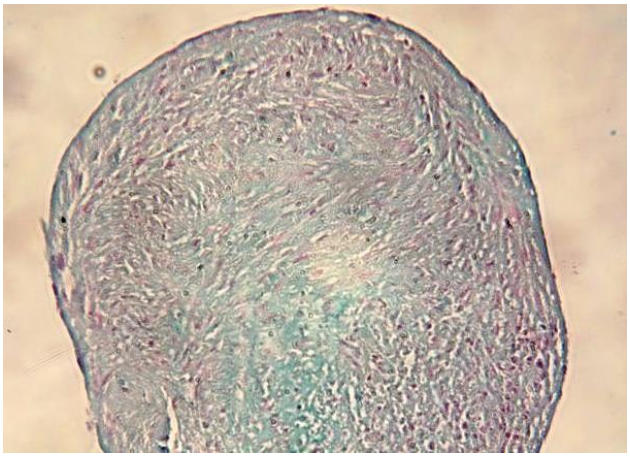
Day 0

1. Lifeline® recommends using a minimum of 2.5 x 10^7 HMSC cells (approximately 10 to 20 T75 flasks) to create 1 mL of alginate encapsulated cells (Chondrogenic Microbeads).
2. When cells are 70 to 90% confluent and actively proliferating, passage cells using Lifeline subculture reagents (as detailed on page 4).
3. Perform a cell count prior to centrifugation.
4. Resuspend the cell pellet (containing a minimum of 2.5 x 10^7 HMSC cells) in 800 µL of the 1.5% (w/v) alginate solution.
    a. The quantities may be adjusted as long as the aforementioned ratios are maintained.
    b. The 1.5% (w/v) alginate solution must not be diluted lower than 1.2% (w/v) by the addition of the HMSCs.
5. Gently mix the alginate-cell suspension, taking care not to introduce air bubbles in to the solution.
6. Transfer 75 mL of sterile 100 mM $CaCl_2$ solution and a sterile stir bar into a sterile 250 mL beaker.
7. Create a gentle funnel in the $CaCl_2$ solution on a stir plate.
8. Transfer the alginate-cell suspension to a sterile syringe.
9. Attach a fine gauge (e.g. 27G) needle to the syringe.
10. Rapidly dispense the alginate-cell solution (in a single fast stream) into the $CaCl_2$ solution to form the Chondrogenic Microbeads.
11. Allow the 100 mM $CaCl_2$ solution containing the newly formed Chondrogenic Microbeads to stir for an additional 10 minutes to solidify the alginate.
12. Remove the beaker from the stir plate and allow the Chondrogenic Microbeads to settle.
13. Transfer the Chondrogenic Microbeads solution into a sterile conical tube and attach to a vacuum-driven 50 mL filter device (e.g. Steriflip® from Millipore).
14. Immediately break the vacuum as soon as the liquid is removed to prevent damage to the beads.
15. Resuspend Chondrogenic Microbeads in 2 mL of ChondroLife™ Complete Chondrogenesis Medium.
16. Aseptically transfer enough Chondrogenic Microbeads to cover the bottom surface of a single well of a 48-well plate.
17. The method described above yields enough Chondrogenic Microbeads to seed approximately 4 wells of a 48-well plate.
18. 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 $CaCl_2$.
19. Incubate the cells in a 37°C, 5% CO_2 incubator.

Day 2 Through Day 21

20. Every 2 to 3 days carefully remove the spent medium from each well, so as to not disturb or aspirate the Chondrogenic Microbeads.
21. 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.
22. After 21 days of differentiation, chondrogenesis is complete and the Chondrogenic Microbeads can be fixed and stained for proteoglycan deposition using Lifeline's Alcian Blue Staining Kit (LL-0051) or other analysis. See instructions page 17.

Chondrogenesis (Micromass)



HMSC-WJ differentiated into Chondrocytes using ChondroLife and stained with Lifeline's Alcian Blue Staining Kit to visualize the proteoglycan deposition, post 25 day differentiation, (100X).

Materials and Equipment Needed

1. HMSC culture(s)
2. Warm expansion medium (see chart on page 2)
3. TrypKit™ Subculture Reagent Kit (LL-0013)
    a. PBS (CM-0001)
    b. 0.05% Trypsin/0.02% EDTA (CM-0017)
    c. Trypsin Neutralizing Solution (CM-0018)
4. ChondroLife™ Complete Chondrogenesis Medium (LM-0022)
    a. Antimicrobials are not required, but are recommended during differentiation due to the duration of culture and the frequency of handling of the cultures.
    b. Antimicrobials may be purchased separately from Lifeline®.
5. Alcian Blue Staining Kit (LL-0051)
6. Supplies for counting viable cells (e.g. Trypan Blue, hemacytometer)
7. 70% ethanol or isopropanol for disinfecting surfaces
8. Tissue culture treated plates (e.g. 96-well, 24-well)
9. Cell culture incubator (37°C, 5% CO_2, humidified)
10. Biosafety cabinet

ChondroLife Complete Chondrogenesis Medium Preparation

1. Thaw at 37°C.
2. Store at 2 to 8°C for up to 6 weeks.

Chondrogenesis Differentiation Procedure 2

Day 0

1. When cells are 70 to 90% confluent and actively proliferating, passage cells using Lifeline subculture reagents (as detailed on page 4).
2. Count the cells prior to centrifugation.
3. Resuspend pellet in expansion medium to a concentration of 1.6 x 10^7 cells/mL (see expansion media chart on page 2).
4. Inoculate a 5 µL drop of cells into multi-well plates (no more than 1 drop per 0.33 cm^2).
5. Incubate plate at 37°C, 5% CO_2 for 2 hours.
6. Cover the cell masses with at least 0.5 mL of warm ChondroLife Complete Chondrogenesis Medium per cm^2.
7. Return the plates to the incubator.

Day 2 Through Day 21

8. Gently replace the medium in the wells every 2 to 3 days with ChondroLife Complete Chondrogenesis Medium.
9. Incubate at 37°C, 5% CO_2.
10. On day 21, the differentiation is complete.
11. Fix the cells then stain for sulfated proteoglycans with Lifeline's Alcian Blue Staining Kit (LL-0051). See instructions on page 17.

Staining with Alcian Blue

Materials and Equipment Needed

1. Fully differentiated chondrogenic microbeads or micromass cultures
2. PBS (CM-0001)
3. Alcian Blue Staining Kit (LL-0051)
    a. 4% Paraformaldehyde Fixative Solution (CM-0055)
    b. 20% Sucrose Stabilizer (CM-0051)
    c. 3% Acetic Acid Wash (CM-0053)
    d. 1% Alcian Blue Stain Solution (CM-0052)
4. 50 mL conical-bottom tubes
5. Embedding material such as paraffin or OCT™ (Optimal Cutting Temperature) Compound
6. Dry ice/ethanol bath for snap freezing
7. Absolute ethanol

(OTC is a trademark of Tissue-Tek®, Andwin Scientific.)

Alcian Blue Staining Procedure

1. Always wear eye protection and gloves when working with staining reagents.
2. Use 4% Paraformaldehyde Fixative Solution in a chemical fume hood.
3. Remove medium completely from well(s).
4. Add 0.5 mL per well (if using a 48-well plate) of 4% Paraformaldehyde Fixative Solution (CM-0055) and fix for 3 hours. Shield from light.
5. Remove fixative and add 0.5 mL of 20% Sucrose Stabilizer (CM-0051) per well.
6. Incubate overnight at room temperature.
7. Store at 4°C until ready to embed the Chondrogenic Microbeads or micromasses in a material such as OCT (Optimal Cutting Temperature) Compound for slicing on a microtome or cryostat.
8. Transfer Chondrogenic Microbeads or micromasses to a plastic cryomold.
9. Remove residual sucrose solution.
10. Layer embedding material over Chondrogenic Microbeads or micromasses and allow them to settle to the bottom of the mold.
11. Snap freeze the cryomold in a dry ice/ethanol bath. Store at -80°C until ready to cut.
12. Cut 5 µm sections from block and mount on glass slide.
13. Place slide in 3% Acetic Acid Wash (CM-0053) for 3 minutes.
14. Place in 1% Alcian Blue Stain Solution (CM-0052) for 30 minutes.
15. Wash in running tap water for 1 minute.
16. Dip briefly in alcohol to dehydrate and air dry.

Troubleshooting

Problem	Likely cause	Solution
Low or no viable cells when thawing a vial of HMSC	Vial was not stored correctly	• Order a new vial of Lifeline's HMSC and store in vapor phase liquid nitrogen until ready to use. • Keep cells in a -80°C freezer or on dry ice the day of use and when ready to thaw immediately transfer them to a 37°C water bath. • Vials of frozen cells should not be kept above -80°C for more than 1 minute.
	Home-made cryopreserved HMSC	• Cryopreserve cells between 0.5 x 10^6 to 2 x 10^6 cells/mL.
		• Cryopreserve cells at low passage.
		• Follow procedures for cryopreservation exactly. (See page 5.)
		• Obtain a new vial of HMSC.
Poor cell attachment	Incorrect thawing medium	• Use pre-warmed, fully supplemented medium that is correct for the type of HMSC being cultured. (See page 2 for media recommendations.)
	Cells were not inoculated at the correct density	• Lifeline recommends inoculating HMSC at a density of 5,000 to 10,000 cells/cm^2. This means inoculating 1 vial of HMSC into 1 to 3 T75 flasks.
Cells grow slowly	Incorrect growth medium	• Use pre-warmed, fully supplemented medium that is correct for the type of HMSC being cultured. (See page 2 for media recommendations.)
	Cells are too old	• Lifeline recommends using healthy HMSC prior to passage 6; do not allow the cells to become over confluent.
Cells appear to be differentiating	Improper culture conditions	• Thaw a fresh vial of HMSC. Follow thawing and culturing instructions exactly. (See pages 3 through 4.)
	Cells are too old	• Lifeline recommends using healthy HMSC prior to passage 6; do not allow the cells to become over confluent.
Adipogenesis efficiency is low	Inoculation density is too low or too high	• Follow the inoculation density guidelines on pages 10 or 12. Utilize the lower inoculation density if cells will be in expansion medium for the longer time period provided in the instructions. • Utilize the higher density if the cells will be changed to adipogenesis medium the day after inoculation.
Cell monolayer peels off the culture surface (adipogenesis or osteogenesis)	High differentiation efficiency	• Coating the culture surface with a fibronectin coating solution prior to inoculating the cells will help to prevent this.
Alginate encapsulated cells form threads instead of beads	Incorrect gauge needle	• Use a 27G needle.
	Calcium chloride solution is not stirring fast enough or with enough volume	• Use a 250 mL beaker and create a gentle funnel that does not contact the stir bar. • Rapidly dispense the alginate/cell suspension into the calcium chloride solution, but not directly into the funnel.