Recommended Protocol for Fed-Batch hMSC Expansion in Spinner Flasks

Protocol Summary

To expand one vial of xeno-free (XF), human bone marrow (hBM), adipose (hAD), or umbilical cord-derived (hUC) Mesenchymal Stem/Stromal Cells (RoosterVial™-hBM/hAD/hUC-1M-XF) using 4x 125 mL spinner flasks, you will need the following reagents, materials, and equipment:

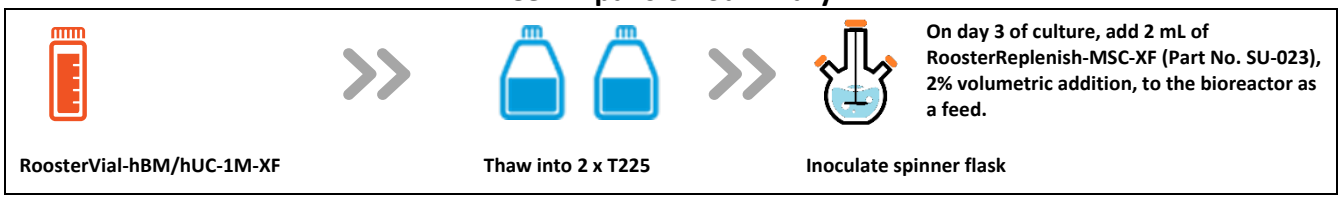
(The protocol may be scaled proportionally to accommodate multiple vessels.)

Materials & Equipment

Item	Quantity	Vendor	Part Number*
RoosterVial-hBM/hAD/hUC-1M-XF	1 Vial	RoosterBio	MSC-031/C46001AD/C43001UC
RoosterNourish™-MSC-XF	2 Kits	RoosterBio	K82016
RoosterReplenish™-MSC-XF	1 Bottle	RoosterBio	SU-023
T225 CellBIND flasks	2 Flasks	Corning	3293
Low Concentration Synthemax™ II Microcarriers	5.0 g	Corning	3781
125 mL Corning® Glass Spinner Flask	2 unit	Corning	4500-125
Sigmacote	1 unit	Millipore Sigma	SL2-25mL
TrypLE™ Select Enzyme	1 Bottle	Life Technologies	12563029
DPBS (without Ca^{++}, Mg^{++})	1 Bottle	Life Technologies	14190144
10 mL Costar Wide Tip Serological Pipettes	1 Pack	Corning	4492
100 µm Cell Strainer	5 Units	BD Falcon	352360
150 mL Sterile Bottle	1 Bottle	Corning	431175
500 mL Centrifuge Bottle	1 Bottle	Corning	431123
Slow-Speed Stir Plate			
Biosafety Cabinet			
Centrifuge			
Incubator			
Water Bath (or ThawSTAR®)			

Process Overview

Cell Expansion Summary



**RoosterBio strongly recommends the use of Corning CellBIND surfaces for expansion of the Xeno-Free product line.*
***Please refer to protocol for full process instructions.*

Recommended Protocol

1. Expansion Options

Vessel	Surface Area (cm^2)	Number of Vessels Needed	Total Surface Area (cm^2)	Seeding Density (cells/cm^2)	Approx. Yield at Harvest	Days of Culture
T225	225	2	450	2,200	≥ 12M	3 to 6

2. Media Preparation

2.1. Bring RoosterNourish-MSC-XF components to room temperature, protected from light, for up to four hours.
2.2. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster™-MSC-XF (Part No. SU-016) to 1 bottle of RoosterBasal™2.0-CC (Part No. M22520).
2.3. Mix well by capping and gently mixing the bottle.

3. Cell Thawing & Seeding

3.1. Aseptically transfer 10 mL of prepared medium into a 15 mL centrifuge tube
3.2. Thaw RoosterVial in an automated thawing device (e.g., ThawStar), or manually in a 37°C water bath. When thawing in a water bath, monitor the vial closely and remove from water bath once only a small bit of ice is remaining (2-3 min).
3.3. Aseptically transfer vial into a Biosafety Cabinet (BSC).
3.4. Transfer vial contents into the 15 mL centrifuge tube containing prepared medium and mix cell suspension well.
3.5. Centrifuge at 300 x g for 6 min.
3.6. Aspirate the supernatant and resuspend cells in 10 mL of RoosterNourish-MSC-XF medium.
3.7. Mix well and seed cells equally into two T225 vessels, and add medium to bring volume up to final volume according to table below:

Type of Culture Vessel	Total Volume of Cell Suspension per Vessel	Final Volume per Vessel
T225	5 mL	45 mL

3.8. Transfer vessels into an incubator (37°C, 5% CO2) and ensure surfaces are covered evenly with media.

4. Cell Expansion

4.1. Microscopically monitor cell confluency starting on day 3 of culture.
4.2. When culture is >80% confluent, cells are ready to harvest.

Day	3	4	5	6
Cell Confluency				

Note: For best expansion and functional performance, it is recommended to passage the cultures before reaching 90% confluence. If the cultures reach over confluence, this may result in increased aggregation, decreased cell viability, growth inhibition and loss of differentiation potential.

5. Spinner Flask Preparation: Silanization

Note: Silanization is required to prevent cell attachment to spinner flask surfaces.

5.1. Sterilize spinner flask and transfer applicable materials to the BSC.
5.2. Allow for flask surfaces to completely dry.
5.3. Cover or immerse the glass surface in Sigmacote (undiluted).
5.4. Excess solution can be collected for reuse.
5.5. Allow the treated glass surface to air dry in a hood. No heating is required.
5.6. Rinse the siliconized articles with water to remove the HCl byproducts before use.
5.7. Sterilize spinner flask prior to use.

Note: Steps **5.7-5.8** should be completed one day before Section **6. Flask Harvest & Bioreactor Inoculation**

The following instruction reflects the steps required for each vessel and can be performed in parallel for additional vessels.

5.8. In a BSC, weigh 1.25 g of sterile microcarriers into a sterile 50ml conical.
5.9. Add 10 mL of RoosterNourish™-MSC-XF to the microcarriers and pipette to wet the microcarriers and equilibrate them for culture as per manufacturer's recommendation.
    5.9.1. If no manufacturer recommendations are provided, store the bottle containing microcarriers and complete medium overnight at 4°C.

6. Flask Harvest & Bioreactor Inoculation

The following instruction reflects the steps required for each vessel and can be performed in parallel for additional vessels.

6.1. Transfer the 10ml and as many microcarriers as possible to a sterile spinner flask.
6.2. Wash the 50ml conical 2x with 10 mL RoosterNourish™-MSC-XF and add to spinner flask for a total of 30ml medium and microcarriers.
6.3. Place the spinner in a 37°C, 5%CO_2, 95% RH incubator and leave static.
6.4. Transfer the T225 flask into a BSC and remove spent media.
6.5. Add 10 mL TrypLE per T225 flask.
6.6. Distribute TrypLE evenly to cover all the cells and place flasks in 37°C incubator. Check culture every 5 minutes until cells are detached from surface (typically 10-15 minutes).
6.7. Gently tap to dislodge remaining cells from surface.

Total Time Required for Cell Detachment

6.8. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
    6.8.1. If the freshly harvested cell solution contains significant cell aggregates or extracellular matrix clumps due to over confluence, filter solution through a 100 µm cell strainer to remove.
6.9. Transfer the cell suspension into a sterile 50 mL centrifuge tube.

6.10. Centrifuge at 300 x g for 6 min.
6.11. Aspirate the supernatant.
6.12. Resuspend cells in ~10 mL medium. Measure the total volume of cell suspension:

Total Volume of Cell Suspension (=A)

6.13. Transfer 0.5 mL of cell suspension into microcentrifuge tubes for cell counts.
6.14. Count cells with a cell counting device, performing a dilution if required to get within its acceptable range:

Raw Data		Adjusted Data	
Dilution Factor (=B)	Viable Cell Concentration (=C)	Cell Concentration (D)=B*C	Total Cells at Harvest (E)=D*A

Add 2.1 x 10^6 cells to each spinner that contains the microcarriers and 30 mL of expansion media by transferring the calculated volume of cell suspension to each vessel.
6.15. Place spinner in a 37°C, 5%CO^2, 95% RH incubator and leave static.
6.16. Incubate for 20 minutes, then swirl the culture to redistribute the cells.
6.17. Repeat previous step 4-5 times.
6.18. After the last 20min incubation remove the spinner from the incubator and place in BSC.
6.19. Add RoosterNourish™-MSC-XF to a final volume of 100ml.
6.20. Place spinner on stir platform set on 50-60 RPM in a 37°C, 5%CO_2, 95% RH incubator. The stir speed should be fast enough to keep microcarriers suspended.

7. Bioreactor Feeding: Addition of RoosterReplenish™-MSC-XF

7.1. On day 3 of culture, add 2 mL of RoosterReplenish-MSC-XF (Part No. SU-023), 2% volumetric addition, to the bioreactor as a feed.
7.2. Return to 37°C, 5%CO^2, 95% RH incubator.
  7.2.1. As cell cultures become more confluent, adjust stir speed should be fast enough to keep microcarriers suspended.

8. Bioreactor Sampling

8.1. Borrow the vessels and transfer into a biosafety cabinet.
8.2. Open the vessel side arm cap and using a wide tip serological pipette aspirate 2 mL of uniform cell/microcarrier suspension into a 15 mL tube. (Additionally, 1 mL of cell/microcarrier suspension can be sampled to monitor cell attachment, growth, and aggregation via microscopy). Ensure cell / microcarrier suspension are homogeneous when sample is drawn by pipetting the cell-microcarrier suspension up and down first and sampling when the cell-microcarriers are suspended.
8.3. Return the bioreactor vessels to the stir plate and continue agitation.
8.4. For the 2 mL cell count sample, allow cells and microcarriers to settle into bottom of tube by gravity, typically 5-10 minutes.
8.5. Once the sample of cells and microcarriers settle, carefully remove as much spent medium/supernatant from the tube, without disturbing the cell/microcarrier suspension.
8.6. Add ~2 mL (equivalent to the volume of media removed from the sample; the volume of cells / microcarrier is typically 0.3 mL) of TrypLE solution to conical tube with cells/microcarriers and gently mix, then incubate for 15 minutes at 37˚C for cell

dissociation from microcarriers. If large cell clumps remain, mix gently with pipette, and return to 37˚C for an additional 10 minutes.

8.6.1. For Day 1 and Day 2 Sampling, it may be necessary to concentrate the sample by reducing the volume of TrypLE so that the cell count falls within the optimal range of the cell counting device.

8.7. When cells are dissociated from microcarriers, separate cells from the microcarriers using a 100 µm cell strainer. Sample the cell suspension to perform a cell count to determine cell density in bioreactor.

Day	Raw Data		Adjusted Data	
	Dilution Factor (=B)	Viable Cell Concentration (=C)	Cell Concentration (D)=B*C	Total Cells at Harvest (E)=D*500 mL
3				
4				
5				
6				

Note: Cells are typically ready to harvest on day 5-6 of culture. Cultures should be harvested during exponential growth phase (i.e., not when the cells have reached a growth plateau).

9. Bioreactor Harvest

9.1. Remove the bioreactor vessels from the stir plate for transfer into a BSC.
9.2. Allow the cells/microcarriers to settle to the bottom of the bioreactor vessels.
9.3. Remove the bioreactor cap and aspirate as much spent media as possible from the culture without removing the cells/microcarriers (100 mL).
9.4. Add 100 mL DPBS into the bioreactor and swirl gently to wash the cells/microcarriers.
9.5. Allow the cells/microcarriers to settle to the bottom of the bioreactor vessel.
9.6. Aspirate as much DPBS as possible from the culture without removing the cells/microcarriers.
9.7. Add 100mL of TrypLE into the bioreactor.
9.8. Return the vessels to the modular units and continue agitation at 100-120 RPM for 30 minutes at 37°C.
9.9. Transfer the bioreactor into the biological safety cabinet and measure the total volume of cells by transferring the cells/microcarrier solution into a sterile 500 mL bottle:

Total Volume of Cell Suspension (=A)

9.10. Pipette to break up remaining cell/microcarrier clusters.
9.10.1. If aggregates still remain, return the vessel to the incubator checking every 5-10 mins.
9.11. Filter solution through a 100 µm cell strainer.
9.12. Quench TrypLE with equivalent volume of fresh media, passing through cell strainer to rinse microcarriers.
9.13. Mix cell solution and obtain final cell count for the bioreactor harvest.

Raw Data		Adjusted Data	
Dilution Factor (=B)	Viable Cell Concentration (=C)	Cell Concentration (D)=B*C	Total Cells at Harvest (E)=D*2A

9.14. Cells are ready for your application.