Recommended Protocol for Fed-Batch hMSC Expansion and Extracellular Vesicle Production in PBS Mini

Protocol Summary

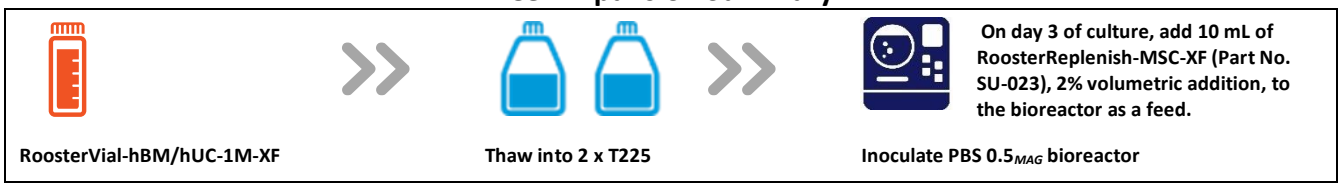
To expand one vial of xeno-free, human bone marrow or umbilical cord-derived Mesenchymal Stem/Stromal Cells (RoosterVial-hBM/hAD/hUC-1M) using a PBS 0.5$_{MAG}$ bioreactor and collect conditioned medium, you will need the following reagents, materials, and equipment:

Materials & Equipment

Item	Quantity	Vendor	Part Number*
RoosterVial-hBM/hAD/hUC-1M	1 Vial	RoosterBio	MSC-031/C46001AD/C43001UC
RoosterNourish™-MSC-XF	2 Bottles	RoosterBio	KT-016
RoosterReplenish™-MSC-XF	1 Bottle	RoosterBio	SU-023
RoosterCollect ™-EV	2 Bottles	RoosterBio	M2001
T225 CellBIND flasks	2 Flasks	Corning	3293
Low Concentration Synthemax™ II Microcarriers	6.25 g	Corning	3781
PBS-MINI MagDrive Base Unit	1 unit	PBS Biotech	IA-UNI-B-501
PBS 0.5 Single-Use Vessel	1 unit	PBS Biotech	IA-0.5-D-001 (Pack of 4)
TrypLE Select Enzyme	1 Bottle	Life Technologies	12563029
DPBS (without Ca^{++}, Mg^{++})	1 Bottle	Life Technologies	14190144
D-(+)-glucose	Varies	Millipore Sigma	G8644
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
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™-MSC (Part No. SU-005/SU-022).
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 350 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% CO_2) 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. Media Preparation

5.1. Bring RoosterNourish-MSC-XF components to room temperature, protected from light, for up to four hours.
5.2. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster™-MSC-XF (Part No. SU-016) to 1 bottle of RoosterBasal™-MSC (Part No. SU-005/SU-022).
5.3. Mix well by capping and gently mixing the bottle.

6. Cell Harvest & Bioreactor Inoculation

Note: Steps **6.1-6.2** may have to be completed up to one day before Step **6.3**, depending on the supplier (microcarrier) recommendations.

6.1. In a BSC, weigh out 6.25 g of microcarriers into a sterile 150 mL bottle that allows for complete collection of liquid/microcarrier suspension.
6.2. Add 50 mL of complete medium (RoosterBasal-MSC + RoosterBooster-MSC-XF) to the microcarriers in each bottle and swirlto wet the microcarriers and equilibrate them for culture as per manufacturer's recommendation.
6.2.1. If no manufacturer recommendations are provided, store the bottle containing microcarriers and complete medium overnight at 4°C.
6.3. Transfer the microcarrier suspension to a 500 mL PBS bioreactor. Use an additional 100 mL of growth media to rinse and transfer any remaining microcarriers from the 150 mL bottle.
6.4. Place the bioreactor in a 37°C incubator to equilibrate temperature/gas of the growth media until it is ready to be inoculated.
6.5. Transfer vessels into biosafety cabinet and remove spent media.
6.6. Add 10 mL TrypLE to each T225 flask.
6.7. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO_2) incubator. Check culture every 5 min until cells are detached from surface (typically 10-15 minutes). 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 overconfluence, filter solution through a 100 µm cell strainer to remove.
6.9. Transfer the cell suspension into a 50 mL centrifuge tube.
6.10. Centrifuge at 350 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

6.15. Adjust cell concentration in fresh media to achieve a density of 1 M cells/mL.
6.16. Remove the bioreactor from the incubator and place in the BSC. Add 10.5 x 10^6 cells to the bioreactor that now contains the microcarriers and 150 mL of expansion media.
6.17. Swirl gently to mix and incubate at 37˚C for 20 min to allow cell attachment to microcarriers.
6.18. Remove the bioreactor from the incubator and gently swirl once more to redistribute the cells that have not adhered and the microcarriers to allow for contact to occur.
6.19. Incubate at 37˚C for an additional 20 min.
6.20. In the BSC, add additional ~300 mL of growth media to bring the final volume to 450 mL.
6.21. Place the bioreactor on the magnetic base unit in 37°C incubator, and initiate agitation at 25 rpm.

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

7.1. On day 3 of culture, transfer the bioreactor from the base unit into the biosafety cabinet.
7.2. Add 9 mL of RoosterReplenish-MSC-XF (Part No. SU-023), 2% volumetric addition, to the bioreactor as a feed.
7.3. Place the bioreactor back on the magnetic base unit in 37°C incubator.
7.4. Increase agitation to 30 rpm to ensure microcarriers remain in suspension.

8. Bioreactor Sampling

8.1. Transfer the bioreactor from the base unit in the incubator to a base unit in a biosafety cabinet.
8.2. Set agitation (30-35 rpm) to the bioreactor to achieve a uniform suspension.
8.3. Remove the bioreactor cap and collect 3mL of uniform cell/microcarrier suspension into a 15 mL tube using a wide tip serological pipette.
8.4. Return the bioreactor/base unit to the incubator and continue agitation at 30 rpm.
8.5. Allow cells and microcarriers from the 3 mL cell count sample to settle into bottom of tube, typically 5-10 minutes.
8.6. Carefully remove as much spent media/ supernatant from the tube, without disturbing the cell/microcarrier suspension.
8.7. Add ~2.7 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.
8.7.1. 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.
8.8. Filter sample solution through a 100 µm cell strainer.
8.9. Count cells with a cell counting device, performing a dilution if required to get within its acceptable range:

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 reach a growth plateau).

9. Media Preparation

9.1. Allow RoosterCollect-EV to warm to room temperature away from light for up to 4 hours.

10. EV Collection and Harvest

10.1. Transfer cell culture vessels, room temperature RoosterCollect-EV, and other necessary materials to biosafety cabinet.
10.2. Allow cells/microcarriers to settle to the bottom of the bioreactor.
10.3. Wash cultures to remove impurities and residuals from RoosterNourish.
  10.3.1. Open the bioreactor cap and aspirate as much spent medium from the culture as possible, without removing the cells/microcarriers.
  10.3.2. Add half the working volume of RoosterCollect-EV, or equivalent wash solution, (e.g. 200-250 mL for a 0.5L bioreactor) to the bioreactor and swirl to wet the microcarriers.
  10.3.3. Allow cells/microcarriers to settle to the bottom of the bioreactor.
  10.3.4. Aspirate as much wash medium from the culture as possible, without removing the cells/microcarriers.
  10.3.5. Repeat steps **10.3.1-10.3.4** for a second wash. If not needed, proceed to step **10.4**.
10.4. Add complete working volume of RoosterCollect-EV.
10.5. Return bioreactor to incubation (37°C, 5% CO_2) and standard agitation for up to 72 hours.
  10.5.1. Agitation may be slightly increased if aggregation is observed (+5 rpm every 24 hours).
10.6. Monitor glucose concentration daily.

Collection Day	Concentration (g/L)
0	
1	
2	
3	

  10.6.1. If concentration falls below 1.0 g/L, adjust to 2.5g/L with D-(+)-glucose (Millipore Sigma G8644).
10.7. After culture time, allow cells/microcarriers to settle and harvest conditioned media for particle collection.