

## Recommended Collection Protocol for Fed-Batch Culture Regimen

### Protocol Summary

To expand one vial of xeno-free, human bone marrow, adipose, and umbilical cord-derived Mesenchymal Stem/Stromal Cells or human neonatal foreskin derived Dermal Fibroblasts (RoosterVial™-hBM/hAD/hUC/hDF-1M) using a PBS 0.1<sub>MAG</sub> bioreactor, you will need the following reagents, materials, and equipment.

The following process recommendations account for up to 4x total PBS 0.1<sub>MAG</sub> bioreactors.

### 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.*

### Materials & Equipment

Item	Quantity	Vendor	Part Number*
RoosterVial-hBM/hAD/hUC/hDF-1M	1 Vial	RoosterBio	MSC-031/C46001AD/C43001UC/C55001DF
RoosterNourish™-MSC-XF	2 Bottles	RoosterBio	K82016
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	5 g	Corning	3781
PBS-MINI MagDrive Base Unit	4 units	PBS Biotech	IA-UNI-B-501
PBS 0.1 Single-Use Vessel	4 units	PBS Biotech	FA-0.1-D-001 (Pack of 4)
TrypLE Select Enzyme	1 Bottle	Life Technologies	12563029
DPBS (without Ca <sup>++</sup> , Mg <sup>++</sup> )	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	15 Units	BD Falcon	352360
150 mL Sterile Bottle	5 Bottles	Corning	431175
250 mL Centrifuge Tube	4 Units	Corning	430776

*Note: This is not an exhaustive material list. Common laboratory equipment, reagents, and consumables may be required.*

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## 1. Media Preparation

- 1.1. Bring RoosterNourish-MSX components to room temperature, protected from light, for up to four hours.
  - 1.1.1. RoosterBooster™-MSC-XF may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
- 1.2. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster-MSX (Part No. SU-016) to 1 bottle of RoosterBasal™2.0-CC (Part No. M22520).
- 1.3. Mix well by capping and gently mixing the bottle.

## 2. Cell Thawing & Seeding

- 2.1. Aseptically transfer 10 mL of prepared medium into a 15 mL centrifuge tube.
- 2.2. Thaw RoosterVial-1M-XF vial 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).
- 2.3. Aseptically transfer vials into a Biosafety Cabinet (BSC).
- 2.4. Transfer vial contents into the 15 mL centrifuge tube containing prepared medium and mix cell suspension well.
- 2.5. Wash inside of cryovial with 1 mL of RoosterNourish-MSX and transfer remaining volume.
- 2.6. Centrifuge at 360 x g for 6 min on low to medium brake at room temperature.
- 2.7. Aspirate the supernatant and resuspend cells in 10 mL of RoosterNourish-MSX medium.
  - 2.7.1. NOTE: Cell concentration may be too dilute to count on certain devices. If necessary, a sample should be taken prior to full volume suspension.
  - 2.7.2. Remove supernatant ensuring not to disrupt pellet.
  - 2.7.3. Gently tap conical to dislodge pellet and create cells suspended in remaining solution.
  - 2.7.4. Add RoosterNourish-MSX to resuspend pellet.
- 2.8. Measure total volume of suspension:

Total Volume of Cell Suspension (=A)

- 2.9. Transfer <0.5 mL of cells into microcentrifuge tubes for cell counts.
- 2.10. 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)	NC-200 Viable Cell Concentration (=C)	Viable Cell Concentration (D)=B*C	Total Viable Cells at Harvest (E)=D*A
	cells/mL	cells/mL	

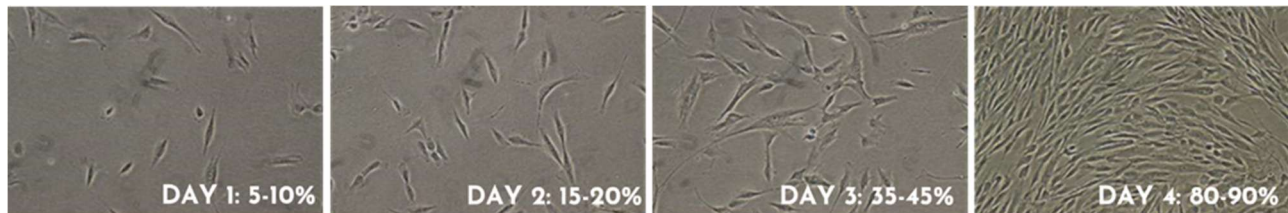
- 2.11. Mix well and seed cells equally into two T225 vessels.
- 2.12. Add additional volume to each flask to achieve a total of 45 mLs per T225.
- 2.13. Transfer vessels into an incubator (37°C, 5% CO<sub>2</sub>) and ensure surfaces are covered evenly and leveled with media.
- 2.14. Transfer unused RoosterNourish-MSX to 2-8°C, away from direct light for up to two weeks.

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### 3. Cell Expansion

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

Day	3	4	5	6
Cell Confluency				



*\*Representative confluency image of hBM-MSCs.*

**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.

### 4. Microcarrier Preparation

**Note:** Steps 4.1-4.2 should be completed up to one day before Section 5. **Flask Harvest & Bioreactor Inoculation.**

- 4.1. In a BSC, weigh out 1.25 g of microcarriers into 4x sterile 150 mL bottles that allows for complete collection of liquid/microcarrier suspension.
- 4.2. Add 30 mL of complete medium (RoosterBasal2.0-CC + RoosterBooster-MSX-XF) to the microcarriers in each bottle and swirl to wet the microcarriers and equilibrate them for culture as per manufacturer's recommendation.
  - 4.2.1. If no manufacturer recommendations are provided, store the bottle containing microcarriers and complete medium overnight at 2-8°C.

### 5. Flask Harvest & Bioreactor Inoculation

- 5.1. Bring RoosterNourish-MSX-XF components to room temperature, protected from light, for up to four hours.
  - 5.1.1. RoosterBooster-MSX-XF may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
- 5.2. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster-MSX-XF (Part No. SU-016) to 1 bottle of RoosterBasal2.0-CC (Part No. M22520).
- 5.3. Mix well by capping and gently mixing the bottle.
- 5.4. Transfer the microcarrier suspension to each 100 mL PBS bioreactor.
- 5.5. Use an additional 40 mL of growth media to rinse and transfer any remaining microcarriers from the 150 mL bottle.
- 5.6. Place the bioreactor in a 37°C incubator to equilibrate temperature/gas of the growth media until it is ready to be inoculated.
- 5.7. Transfer vessels into biosafety cabinet and remove spent media.
- 5.8. Add 10 mL TrypLE to each T225 flask.
- 5.9. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO<sub>2</sub>) incubator. Check culture every 5 min until cells are detached from surface (typically 10-15 minutes). Gently tap to dislodge remaining cells from surface.

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**Total Time Required for Cell Detachment**

- 5.10. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
- 5.10.1. 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.
- 5.11. Transfer the cell suspension into a 50 mL centrifuge tube.
- 5.12. Centrifuge at 360 x g for 6 min on low to medium brake at room temperature.
- 5.13. Aspirate the supernatant.
- 5.14. Resuspend cells in ~10 mL medium. Measure the total volume of cell suspension:

**Total Volume of Cell Suspension (=A)**

- 5.15. Transfer 0.5 mL of cell suspension into microcentrifuge tubes for cell counts.
- 5.16. 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

- 5.17. Remove the bioreactor from the incubator and place in the BSC.
- 5.18. Add  $2.1 \times 10^6$  cells to each bioreactor that now contains the microcarriers and 70 mL of expansion media.
- 5.19. Swirl gently to mix and incubate at 37°C for 20 min to allow cell attachment to microcarriers.
- 5.20. 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.
- 5.21. Incubate at 37°C for an additional 20 min.
- 5.22. In the BSC, add additional ~20 mL of growth media to each bioreactor to bring the final volume to 90 mL.
- 5.23. Place the bioreactor on the magnetic base unit in 37°C incubator, and initiate agitation at 25 rpm.

**6. Bioreactor Feeding: Addition of RoosterReplenish™-MSC-XF**

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

**7. Bioreactor Sampling**

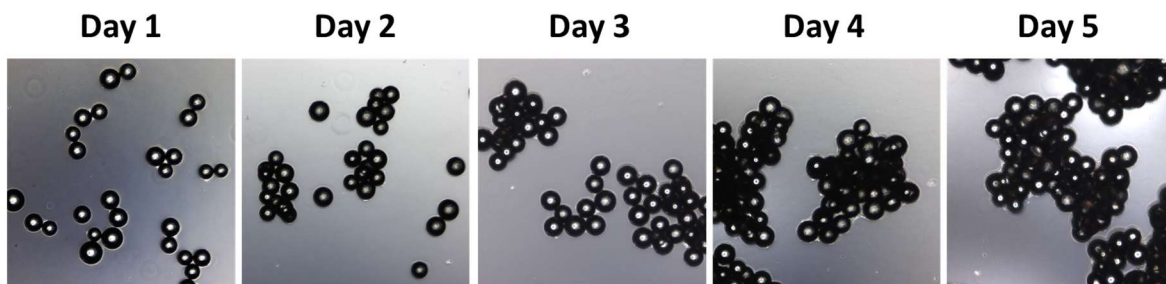
- 7.1. Transfer the bioreactor from the base unit in the incubator to a base unit in a biosafety cabinet.
- 7.2. Set agitation (30-35 rpm) to the bioreactor to achieve a uniform suspension.
- 7.3. Remove the bioreactor cap and collect 3mL of uniform cell/microcarrier suspension into a 15 mL tube using a wide tip serological pipette.
- 7.4. Return the bioreactor/base unit to the incubator and continue agitation at 30 rpm.
- 7.5. Allow cells and microcarriers from the 3 mL cell count sample to settle into bottom of tube, typically 5-10 minutes.
- 7.6. Carefully remove as much spent media/ supernatant from the tube, without disturbing the cell/microcarrier suspension.

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## PBS 0.1<sub>MAG</sub> Collection

- 7.7. Add ~2.8 mL (equivalent to the volume of media removed from the sample; the volume of cells / microcarrier is typically 0.2 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.
- 7.7.1. 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.
- 7.8. Filter sample solution through a 100 µm cell strainer.
- 7.9. 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				



\*Representative confluency image of hBM-MSCs.

- 7.10. Monitor glucose concentration daily.

Collection Day	Concentration (g/L)
0	
1	
2	
3	
4	
5	

- 7.10.1. If concentration falls below 1.0 g/L, adjust to 2.5g/L with D-(+)-glucose solution (Millipore Sigma G7021).

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**8. Media Preparation**

- 8.1. When cultures reach desired cell density (cells/mL), proceed with the following steps.
- 8.2. Allow RoosterCollect-EV to warm to room temperature away from light for up to 4 hours.

**9. EV Collection and Harvest**

- 9.1. Transfer cell culture vessels, room temperature RoosterCollect-EV, and other necessary materials to biosafety cabinet.
- 9.2. Allow cells/microcarriers to settle to the bottom of the bioreactor.
- 9.3. Wash cultures to remove impurities and residuals from RoosterNourish.
  - 9.3.1. Open the bioreactor cap and aspirate as much spent medium from the culture as possible (~70%), without removing the cells/microcarriers.

Total Volume Aspirated

- 9.3.2. Add half the working volume of RoosterCollect-EV, or equivalent wash solution, to the bioreactor and swirl to wash microcarriers.

- 9.3.3. Allow cells/microcarriers to settle to the bottom of the bioreactor.

- 9.3.4. Aspirate as much wash medium from the culture as possible, without removing the cells/microcarriers.

Total Volume Aspirated: Wash 1

- 9.3.5. Repeat steps 9.3.1-9.3.4 for a second wash.

Total Volume Aspirated: Wash 2

- 9.4. Add the remaining working volume of RoosterCollect-EV to each bioreactor vessel.

Total Volume Aspirated		Total Volume Aspirated: Wash 1		Total Volume Aspirated: Wash 2		Target Volume		Addition Volume of RoosterCollect-EV
	+		+		-	90mL	=	

- 9.5. Return bioreactor to incubation (37°C, 5% CO<sub>2</sub>) and return agitation to 30-40 rpm for up to 72 hours.

- 9.5.1. Agitation may be slightly increased if aggregation is observed (+5 rpm every 24 hours).

- 9.6. Monitor glucose concentration daily.

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

- 9.6.1. If concentration falls below 1.0 g/L, adjust to 2.5g/L with D-(+)-glucose solution (Millipore Sigma G7021).

- 9.7. Monitor particle concentration.

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Collection Day	Yield Concentration (particles/mL)
0	
1	
2	
3	
4	
5	

9.8. After culture time, allow cells/microcarriers to settle and harvest conditioned media for particle collection.

*Caution to Users: RoosterBio products contain human sourced materials and should be treated as potentially infectious. Employ universal safety precautions and wear protective clothing and eyewear while handling. Practice appropriate disposal techniques per CDC guidelines for biohazardous material.*

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