

Recommended Collection Protocol for Fed-Batch Culture Regimen

Protocol Summary

To expand one vial of xeno-free, human bone marrow, adipose, or umbilical cord-derived Mesenchymal Stem/Stromal Cells or human neonatal foreskin derived Dermal Fibroblasts (RoosterVial™-hBM/hAD/hUC/hDF-1M) using Ambr[®] 250 Modular, multi-parallel bioreactors, and collect conditioned medium you will need the following reagents, materials, and equipment:

(The protocol may be scaled proportionally to accommodate up to 8 Ambr[®] 250 bioreactor vessels.)

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-XF	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	21.0 g	Corning	3781
Ambr [®] Vessel, Single Impeller, Unbaffled	2 unit	Sartorius	001-2A33
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	2 Bottles	Corning	431175
500 mL Centrifuge Bottle	2 Bottles	Corning	431123

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

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

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	

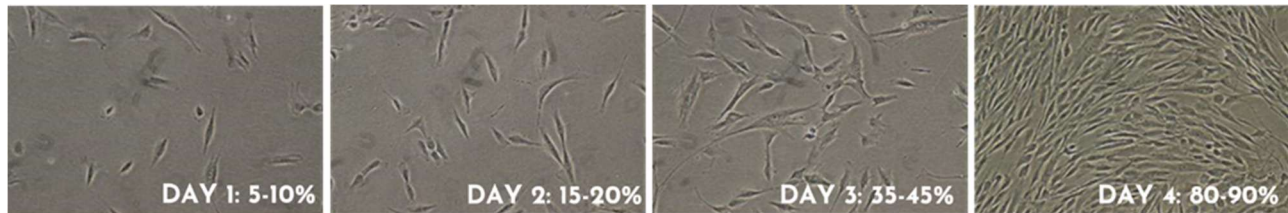
- 2.11. Mix well and seed cells equally into two T225 vessels.
- 2.12. Add additional volume to each flask to achieve a total of 45 mLs per T225.
- 2.13. Transfer vessels into an incubator (37°C, 5% CO₂) and ensure surfaces are covered evenly and leveled with media.
- 2.14. Transfer unused RoosterNourish-MSX to 2-8°C, away from direct light for up to two weeks.

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

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. Ambr® 250 Modular Setup & Media Conditioning

Note: Steps 4.1-4.8 should be completed one day before Section 5. **Flask Harvest & Bioreactor Inoculation.**

- 4.1. Write a recipe in the Ambr® definition software. Considerations for the recipe are based on the following recommended parameters for hMSCs (RoosterVial-hBM-XF, RoosterVial-hAD-XF, or RoosterVial-hUC-XF) in the Ambr® 250 bioreactor system:
 - 4.1.1 Agitation direction: Down
 - 4.1.2 Agitation speed: To facilitate cell attachment, 6 – 10 cycles of static (0 rpm for 45 min) and dynamic (100 rpm for 2 min) after bioreactor inoculation. The agitation speed should be maintained at 100 rpm for the proceeding 18 hours, and then increased by 25 rpm every 12 hours.
 - 4.1.3 CO2 Flow: 5% of headspace gas flow (Initiated after DO spot calibration)
 - 4.1.4 DO(%) Saturation: 100% (Initiated after DO spot calibration)
 - 4.1.5 Headspace gas flow (Air / mix): 24 mL / min
 - 4.1.6 pH: pH control is only monitored for this process
 - 4.1.7 Temperature: 37°C
- 4.2. Open the pre-written recipe in the Ambr® runtime software. The user interface will prompt the following:
 - Check process definition
 - Confirm connected gases
 - Enter spot calibration data
 - Clean reservoir slots
- 4.3. In a BSC, unpackage bioreactor vessels and aseptically add 150 mL of RoosterBasal2.0-CC medium into each of the 2 bioreactor vessels.
- 4.4. Transfer vessels out of the BSC and place on the Ambr® 250 Modular units. Continue the recipe to start the run and initiate media conditioning at the set parameters.

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

AMBR® 250 Collection

- 4.5. Perform a DO spot calibration to 100% and confirm initiation of DO controller at 100% saturation and headspace CO₂ flow at 5% of headspace gas flow.
- 4.6. After 4 hours of media conditioning, perform pH calibration:
 - 4.6.1 Take 1 mL medium sample through the sampling port.
 - 4.6.2 Take an offline pH reading.
 - 4.6.3 Input offset pH data into the Ambr® software.
- 4.7. In a BSC, weigh 10.5 g of sterile microcarriers into two sterile bottles that allow for complete collection of liquid/microcarrier suspension for each bioreactor vessel.
- 4.8. Add 60 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.
 - 4.8.1 If no manufacturer recommendations are provided, store the bottle containing microcarriers and complete medium overnight at 4°C.

5. Flask Harvest & Bioreactor Inoculation

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

- 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. Warm bottles containing microcarriers to room temperature.
- 5.3. Add 3ml of RoosterBooster-MSX-XF to each bioreactor unit containing 150 mL of RoosterBasal2.0-CC.
 - 5.3.1 Borrow the vessels from the modular units and transfer to a BSC. Open the septum cap and aseptically add 3 ml of RoosterBooster-MSX-XF to each vessel.
 - 5.3.2 Return the vessels to the modular unit.
- 5.4. Transfer the T225 flask into a BSC and remove spent media.
- 5.5. Add 10 mL TrypLE per T225 flask.
- 5.6. 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).
- 5.7. Gently tap to dislodge remaining cells from surface.

Total Time Required for Cell Detachment

- 5.8. Add equivalent volume of RoosterNourish™-MSX-XF to each vessel to stop the TrypLE activity.
 - 5.8.1 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.
- 5.7. Transfer the cell suspension into a sterile 50 mL centrifuge tube.
- 5.8. Centrifuge at 360 x g for 6 min on low to medium brake at room temperature.
- 5.9. Aspirate the supernatant.
- 5.10. Resuspend cells in ~10 mL medium. Measure the total volume of cell suspension:

Total Volume of Cell Suspension (=A)

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

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

5.12. 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

5.13. For each Ambr250 bioreactor, proceed with the following steps, working quickly:

- 5.13.1 Add 5.8×10^6 cells to each bottle that now contains the microcarriers and expansion media.
- 5.13.2 Mix contents well.
- 5.13.3 Add bottle contents to the AMBR250 vessel.
- 5.13.4 Wash microcarrier bottle with remaining RoosterNourish-MSX volume required to bring vessel to 250 mL.

Target:		Volume of Microcarriers & Nourish (mL)		Volume of Booster (mL)		Volume of inoculum (mL)		Volume of additional Nourish to add to Bioreactor (mL)
250 mL	-	220.2	-	3	-		=	

5.13.5 Engage vessel in the modular unit.

5.14. Proceed with recipe to facilitate cell attachment to microcarriers using the intermittent seeding method: 8 cycles of static culture for 45 minutes, with 2 minutes of agitation at 100 RPM in between cycles to redistribute the cells and microcarriers.

5.14.1 Turn off the DO controller during the static cycles to ensure no inaccurate control is initiated when the sensor is covered by the settled microcarriers.

5.15. Following the cell attachment phase, continue recipe with agitation speed at 100 RPM for 24 hours following inoculation, followed by a 25 RPM speed increase every 12 hours.

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

- 6.1. On day 3 of culture, add 5 mL of RoosterReplenish-MSX (Part No. SU-023), 2% volumetric addition, to the bioreactor as a feed.
 - 6.1.1 Borrow the vessels from the modular units. In a BSC, open the septum cap and aseptically add 5 ml of RoosterReplenish to each vessel.
 - 6.1.2 Return the vessel to the modular unit.

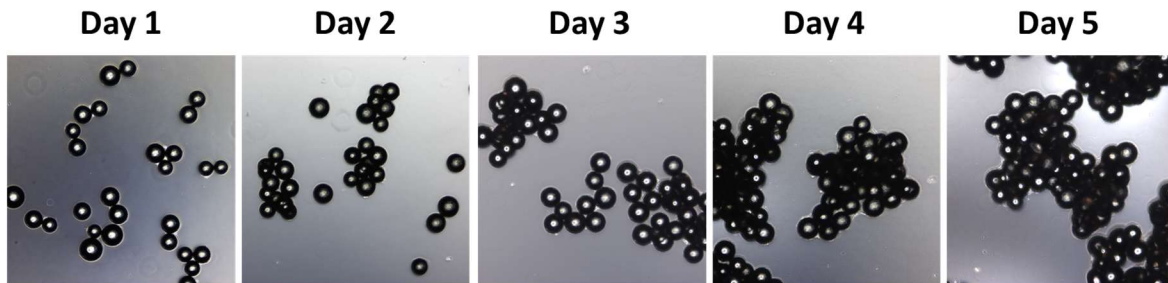
7. Bioreactor Sampling

- 7.1. Borrow the vessels from the modular units and transfer into a biosafety cabinet.
- 7.2. Open the bioreactor septum cap and using a wide tip serological pipette aspirate 3 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.
- 7.3. Return the bioreactor vessels to the modular unit and continue recipe.
- 7.4. For the 3 mL cell count sample, allow cells and microcarriers to settle into bottom of tube by gravity, typically 5-10 minutes.
- 7.5. Once the sample of cells and microcarriers settle, carefully remove as much spent medium/ supernatant from the tube, without disturbing the cell/microcarrier suspension.

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

- 7.6. 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 15 minutes.
- 7.6.1 For Day 1-3 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.
- 7.7. 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				



*Representative confluency image of hBM-MSCs.

- 7.8. Monitor glucose concentration daily.

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

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

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

8. Media Preparation

- 8.2. When cultures reach desired cell density (cells/mL) typically on day 5, proceed with the following steps.
- 8.3. Allow RoosterCollect-EV to warm to room temperature away from light for up to 4 hours.

9. EV Collection and Harvest

- 9.4. Transfer cell culture vessels, room temperature RoosterCollect-EV, and other necessary materials to biosafety cabinet.
- 9.5. Allow cells/microcarriers to settle to the bottom of the bioreactor.
- 9.6. Wash cultures to remove impurities and residuals from RoosterNourish.
 - 9.6.1 Open the bioreactor cap and aspirate as much spent medium from the culture as possible, without removing the cells/microcarriers.

Total Volume Aspirated

- 9.6.2 Add half the working volume of RoosterCollect-EV, or equivalent wash solution, (e.g. 125 mL for a 250 mL bioreactor) to the bioreactor and swirl to wet the microcarriers.
- 9.6.3 Allow cells/microcarriers to settle to the bottom of the bioreactor.
- 9.6.4 Aspirate as much wash medium from the culture as possible, without removing the cells/microcarriers.

Total Volume Aspirated: Wash 1

- 9.6.5 Repeat steps 9.6.1-9.6.4 for a second wash.

Total Volume Aspirated: Wash 2

- 9.7. 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
	+		+		-	250mL	=	

- 9.8. Return bioreactor to incubation (37°C, 5% CO₂) and return agitation to 300 rpm for up to 120 hours.
 - 9.8.1 Agitation may be slightly increased if aggregation is observed (+25 rpm as needed).
- 9.9. Monitor glucose concentration.
 - 9.9.2 If concentration falls below 1.0 g/L, adjust to 2.5g/L with D-(+)-glucose (Millipore Sigma G8644).

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

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.

5	
---	--

9.10. Monitor particle concentration.

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

4.1. 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.

Provision of Seller Product subject to Seller Standard Terms and Conditions. Any technical advice furnished, or recommendation made concerning any use or application of any Seller Product is believed to be reliable, but Seller makes no warranty, either express or implied, as to its accuracy or completeness or of the results to be obtained. Purchaser assumes full responsibility for quality control, testing and determination of suitability of Seller Product for its intended application or use.

CONFIDENTIAL. FOR SOLE USE OF RECIPIENT. ALL PRODUCTS ARE FOR RESEARCH USE ONLY.