

Recommended Collection Protocol for Fed-Batch Culture Regimen

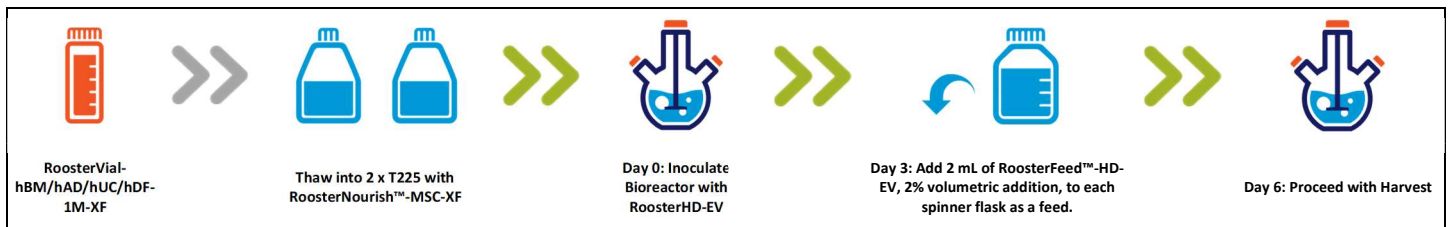
Protocol Summary

To expand one vial of xeno-free (XF) 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 125 mL spinner flasks, you will need the following reagents, materials, and equipment:

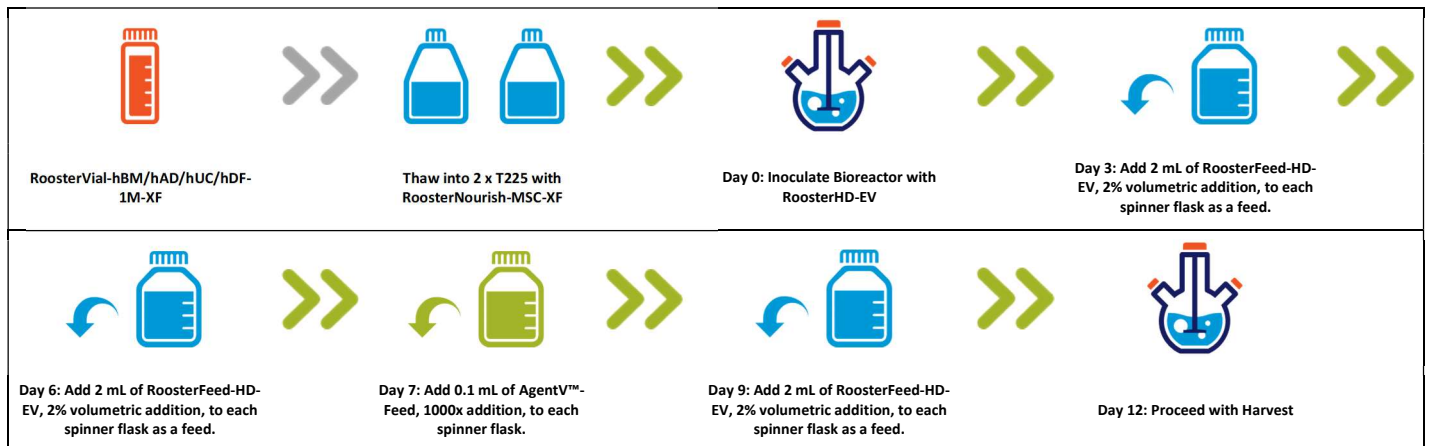
The protocol may be scaled to accommodate up to 4 x 125 mL spinner flasks (additional materials required).

Process Overview

Process Summary: Day 6 Harvest



Process Summary: Day 12 Harvest



**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.*

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Materials & Equipment

Item	Quantity	Vendor	Part/SKU Number*
RoosterVial-hBM/hAD/hUC/hDF-1M	1 Vial	RoosterBio	MSC-031/C46001AD/C43001UC/C55001DF
RoosterNourish-MSX-XF	1 Kit	RoosterBio	K82016
RoosterHD-EV	1 Kit	RoosterBio	6-Day: K87306 12-Day: K87312
T225 CellBIND flasks	2 Flasks	Corning	3293
Low Concentration Synthemax™ II Microcarriers	8.4 g	Corning	3781
125 mL Corning® Disposable Spinner Flask	2 unit	Corning	3152
TrypLE™ Select Enzyme	1 Bottle	Life Technologies	12563029
DPBS (without Ca ⁺⁺ , Mg ⁺⁺)	1 Bottle	Life Technologies	14190144
D-(+)-glucose	Varies	Millipore Sigma	G8644
L-Glutamine	Varies	Life Technologies	25030081
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
Slow-Speed Stir Plate			

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

1. Media Preparation

- 1.1. Bring RoosterNourish-MSX-XF components to room temperature, protected from light, for up to four hours.
 - 1.1.1 RoosterBooster™-MSX-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-XF (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 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 vial 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-XF 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-XF medium.

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RoosterHD™-EV 125 mL Spinner Flask Collection

- 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:

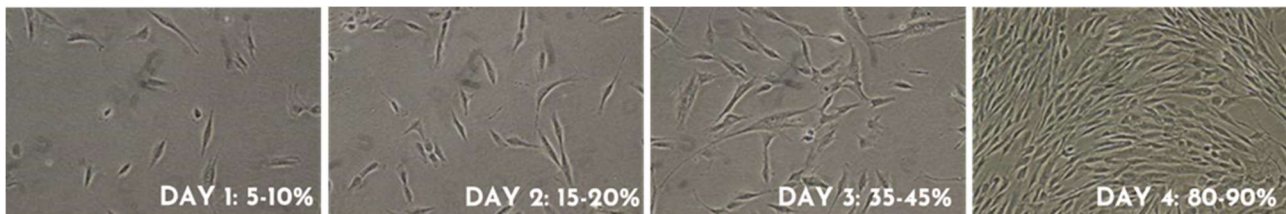
Raw Data		Adjusted Data	
Dilution Factor (=B)	NC-200 Viable Cell Concentration (=C)	Viable Cell Concentration (D)=B*C	Total Viable Cells at Harvest (E)=D*A
	cells/mL	cells/mL	

- 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, 95% RH) 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.

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.

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4. Media Preparation

- 4.1. Allow RoosterBase™-HD warm to room temperature, protected from light, for up to four hours.
- 4.2. Allow RoosterBoost™-HD-EV to thaw at room temperature, protected from light, for no more than 30 minutes.
 - 4.2.1 Note: RoosterBoost-HD-EV should still be cold to touch when added to RoosterBase-HD.
 - 4.2.2 Alternatively, RoosterBoost-HD-EV may be thawed at 2 to 8°C, away from direct light, for up to 36 hours.
- 4.3. Prepare 1 bottle of medium by aseptically adding 1 bottle of RoosterBoost-HD-EV (Part No. S40010) to 1 bottle of RoosterBase-HD (Part No. M43500).
- 4.4. Mix well by capping and gently mixing the bottle.
 - 4.4.1 RoosterHD-EV medium should be used on the day of preparation for best results.

5. Spinner Flask Preparation

Note: For best performance, Steps 5.1-5.2 should be completed the same day as 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.*

- 5.1. In a BSC, weigh 4.2 g of sterile microcarriers into two sterile 50 mL conicals that allow for complete collection of liquid/microcarrier suspension for each bioreactor vessel.
- 5.2. Add 10 mL of complete medium RoosterHD-EV to the microcarriers in each bottle and swirl to wet the microcarriers and equilibrate them for culture.
 - 5.2.1 Proceed immediately to **Step 6.0 Flask Harvest & Bioreactor Inoculation**.

6. Flask Harvest & Bioreactor Inoculation

**The following instruction reflects the steps required for each vessel and can be performed in parallel for all remaining 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 RoosterHD-EV and add to spinner flask for a total of 30ml medium and microcarriers.
- 6.3. Place the spinner in a 37°C, 5%CO₂, 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 RoosterHD-EV 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 360 x g for 6 min on low to medium brake at room temperature.
- 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.

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RoosterHD™-EV 125 mL Spinner Flask Collection

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. Add 2.1×10^6 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.

6.16. Gently swirl.

6.17. Place spinner in a 37°C, 5%CO₂, 95% RH incubator and leave static.

6.18. Incubate for 20 minutes, then swirl the culture to redistribute the cells.

6.19. Repeat previous step 4-5 times.

6.20. After the last 20 minute incubation, remove the spinner from the incubator and place in BSC.

6.21. Add RoosterHD-EV to a final total volume of 100ml.

6.22. Place spinner on stir platform set on 50-60 RPM in a 37°C, 5%CO₂, 95% RH incubator.

6.22.1 The stir speed should be set fast enough to keep microcarriers suspended.

6.22.2 Slightly unscrew cap to vent cultures, if necessary.

7. Bioreactor Feeding (Day 3): Addition of RoosterFeed-HD-EV

7.1. Thaw RoosterFeed-HD-EV (Part No. S45010) to room temperature, protected from light, for up to 30 minutes.

7.1.1 RoosterFeed-HD-EV may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.

7.1.2 Avoid additional freeze and thaw events.

7.2. On day 3 of culture, add 2 mL of RoosterFeed-HD-EV (Part No. S45010), 2% volumetric addition, to the bioreactor as a feed.

7.3. Return to 37°C, 5%CO₂, 95% RH incubator.

7.3.1 As cell cultures become more confluent, adjust stir speed to keep microcarriers suspended.

7.3.2 Slightly unscrew cap to vent cultures, if necessary.

8. Bioreactor Feeding (Day 6): Addition of RoosterFeed-HD-EV

NOTE: If collecting in a 6-day process, proceed with harvest (skip to Step 11).

8.1. Thaw RoosterFeed-HD-EV (Part No. S45010) to room temperature, protected from light, for up to 30 minutes.

8.1.1 RoosterFeed-HD-EV may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.

8.1.2 Avoid additional freeze and thaw events.

8.2. On day 6 of culture, add 2 mL of RoosterFeed-HD-EV (Part No. S45010), 2% volumetric addition, to the bioreactor as a feed.

8.3. Return to 37°C, 5%CO₂, 95% RH incubator.

8.3.1 As cell cultures become more confluent, adjust stir speed to keep microcarriers suspended.

8.3.2 Slightly unscrew cap to vent cultures, if necessary.

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9. Bioreactor Feeding (Day 7): Addition of AgentV-Feed

NOTE: AgentV-Feed is not recommended for use with fibroblast cultures (skip to Step 10).

- 9.1. Thaw AgentV-Feed (Part No. S42001) to room temperature, protected from light, for up to 30 minutes.
 - 9.1.1 AgentV-Feed may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
 - 9.1.2 Avoid additional freeze and thaw events.
- 9.2. On day 7 of culture, add 0.1 mL of AgentV-Feed (Part No. S42001), 1mL per L, to the bioreactor as a feed.
- 9.3. Return to 37°C, 5%CO₂, 95% RH RH incubator.
 - 9.3.1 As cell cultures become more confluent, adjust stir speed to keep microcarriers suspended (~60 RPM).
 - 9.3.2 Slightly unscrew cap to vent cultures, if necessary.

10. Bioreactor Feeding (Day 9): Addition of RoosterFeed-HD-EV

- 10.1. Thaw RoosterFeed-HD-EV (Part No. S45010) to room temperature, protected from light, for up to 30 minutes.
 - 10.1.1 RoosterFeed-HD-EV may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
 - 10.1.2 Avoid additional freeze and thaw events.
- 10.2. On day 9 of culture, add 2 mL of RoosterFeed-HD-EV (Part No. S45010), 2% volumetric addition, to the bioreactor as a feed.
- 10.3. Return to 37°C, 5%CO₂, 95% RH incubator.
 - 10.3.1 As cell cultures become more confluent, adjust stir speed to keep microcarriers suspended.
 - 10.3.2 Slightly unscrew cap to vent cultures, if necessary.

11. Harvest (Day 12): EV Collection

- 11.1. Transfer cell culture vessels, room temperature RoosterHD-EV, and other necessary materials to biosafety cabinet.
- 11.2. Allow cells/microcarriers to settle to the bottom of the bioreactor.
- 11.3. Collect conditioned medium and strain through 100 µm cell strainers into 50 mL centrifuge tubes.
- 11.4. Proceed with further downstream processing and/or analysis.

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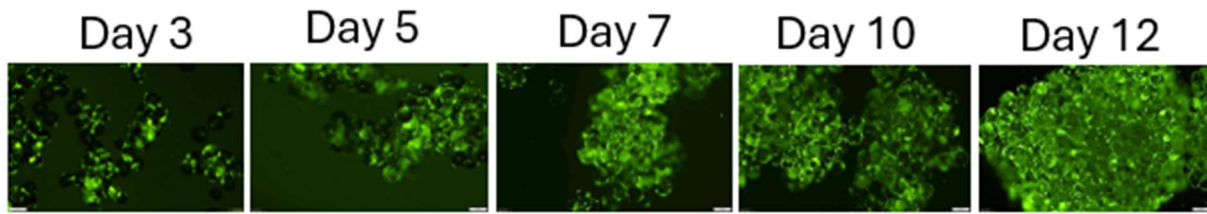
APPENDIX: Process Monitoring (Select Suitable Day(s))

1. Bioreactor Sampling

- 1.1. Borrow the vessels and transfer into a biosafety cabinet.
- 1.2. 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.
 - 1.2.1 Additionally, 1 mL of cell/microcarrier suspension can be sampled to monitor cell attachment, growth, and aggregation via microscopy.
 - 1.2.2 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.
- 1.3. Return the bioreactor vessels to the stir plate and continue agitation.
- 1.4. For the 2 mL cell count sample, allow cells and microcarriers to settle into bottom of tube by gravity, typically 5-10 minutes.
- 1.5. Once the sample of cells and microcarriers settle, carefully collect as much spent medium/supernatant from the tube, without disturbing the cell/microcarrier suspension.
 - 1.5.1 Analyze conditioned media for particle concentration (**Step 12.8**)
- 1.6. 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 15 minutes.
 - 1.6.1 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.
 - 1.6.2 For >Day 5, traditional cell counting may be inaccurate due to increased confluency. Please adjust methods or contact your RoosterBio representative for additional recommendations.
- 1.7. 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

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RoosterHD™-EV 125 mL Spinner Flask Collection


**Representative confluency image of hBM-MSCs stained with Calcein AM cell viability dye.*

1.8. Monitor particle concentration.

Collection Day	Yield Concentration (particles/mL)

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

2. Glucose Monitoring

2.1. Monitor glucose concentration daily.

Collection Day	Concentration (g/L)

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

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3. Glutamine Monitoring

3.1. Monitor glutamine concentration daily.

Collection Day	Concentration (g/L)

3.1.1 If concentration falls below 1.0 mM, adjust to 4 mM with L-glutamine solution (Thermo Fisher Scientific 25030081).

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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