

Recommended Protocol for Fed-Batch hMSC Expansion and Extracellular Vesicle Production 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, and collect conditioned medium you will need the following reagents, materials, and equipment:

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

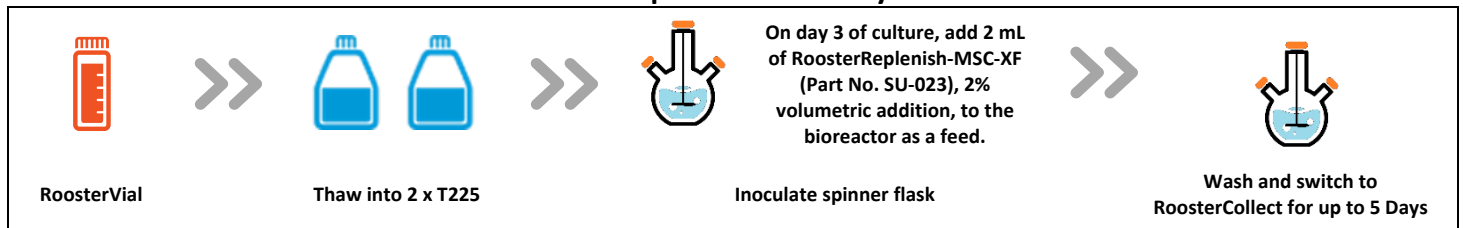
Materials & Equipment

| 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 |
| RoosterCollect™-EV | 2 Bottles | RoosterBio | M2001 |
| T225 CellBIND flasks | 2 Flasks | Corning | 3293 |
| Low Concentration Synthemax™ II Microcarriers | 16.8 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 |
| 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 |
| Slow-Speed Stir Plate | | | |
| Biosafety Cabinet | | | |
| Centrifuge | | | |
| Incubator | | | |
| Water Bath (or ThawSTAR®) | | | |

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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

- Bring RoosterNourish-MSX-XF components to room temperature, protected from light, for up to four hours.
- 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).
- Mix well by capping and gently mixing the bottle.

3. Cell Thawing & Seeding

- Aseptically transfer 10 mL of prepared medium into a 15 mL centrifuge tube
- 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).
- Aseptically transfer vial into a Biosafety Cabinet (BSC).
- Transfer vial contents into the 15 mL centrifuge tube containing prepared medium and mix cell suspension well.
- Centrifuge at 300 x g for 6 min.
- Aspirate the supernatant and resuspend cells in 10 mL of RoosterNourish-MSX-XF medium.
- 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 |

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

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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 4.2 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%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

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K81005 125 mL Spinner Flask Expansion

- 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 |
| | | | |

- 6.15. Add 2.3 x 10⁶ 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. Place spinner in a 37°C, 5%CO₂, 95% RH incubator and leave static.
- 6.17. Incubate for 20 minutes, then swirl the culture to redistribute the cells.
- 6.18. Repeat previous step 4-5 times.
- 6.19. After the last 20min incubation remove the spinner from the incubator and place in BSC.
- 6.20. Add RoosterNourish™-MSC-XF to a final volume of 100ml.
- 6.21. Place spinner on stir platform set on 50-60 RPM in a 37°C, 5%CO₂, 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%CO₂, 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 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.

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- 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. Media Preparation

- 9.1. When cultures reach desired cell density (cells/mL), proceed with the following steps.
- 9.2. 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 spinner flask cap and aspirate as much spent medium from the culture as possible (~70%), without removing the cells/microcarriers.

Total Volume Aspirated

- 10.3.2. Add half the working volume removed (in step 10.3.1) of RoosterCollect-EV, or equivalent wash solution, to the bioreactor and swirl to wash 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.
- 10.4. Add the volume recorded in **10.3.1** of RoosterCollect-EV into the spinner flask.
- 10.5. Return bioreactor to incubation (37°C, 5% CO₂) and return agitation to 60-80 rpm for up to 120 hours.
 - 10.5.1. Agitation may be slightly increased if aggregation is observed (+5 rpm every 24 hours).

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10.6. Monitor glucose concentration daily.

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

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

10.7. 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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