

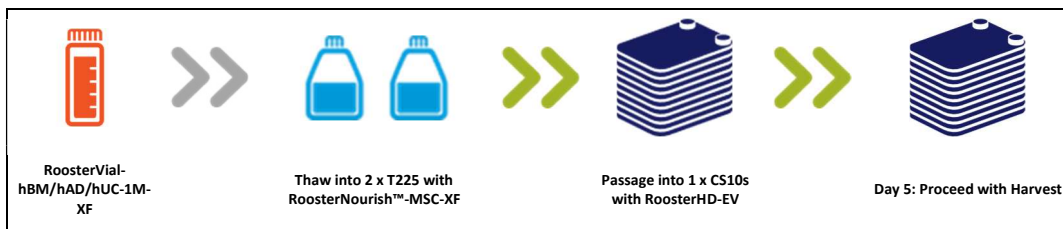
Recommended Collection Protocol for RoosterVial™-1M-XF

Protocol Description

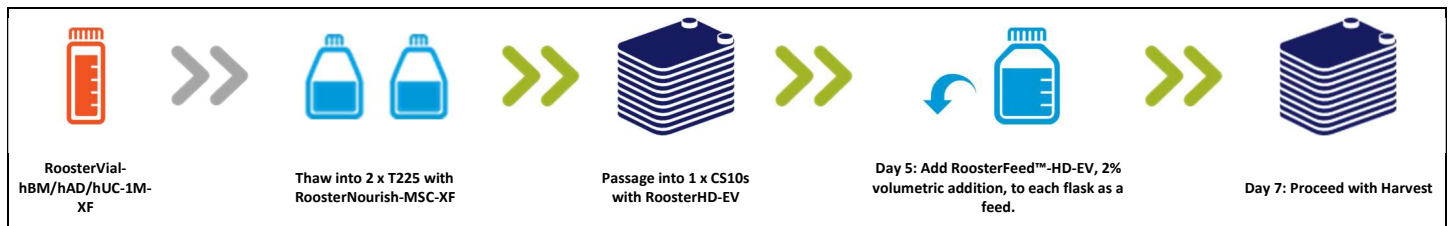
To expand one vial of xeno-free (XF) human bone marrow-, adipose-, or umbilical cord-derived Mesenchymal Stem/Stromal Cells, (RoosterVial-hBM/hAD/hUC-1M-XF), using 2D vessels, you will need the following reagents, cell culture materials, and equipment.

Process Overview

Process Summary: Day 5 Harvest



Process Summary: Day 7 Harvest



**RoosterBio recommends the use of CellBIND surfaces to maximize expansion yields of the Xeno-Free product line.
**Please refer to protocol for full process instructions.*

- Thaw and seed cells at recommended: 3,000 cells/cm² (min. >2,000 cells/cm²).
- Expand cell cultures 3-6 days to >80% confluency at 37°C, 5% CO₂ incubation.
- **NO MEDIA EXCHANGES REQUIRED.** RoosterNourish-MSC-XF does not need to be exchanged, or fed, within 6 days of flask-based culture.

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

Materials & Equipment

Item	Quantity		Vendor	Part Number
	1 st Passage	2 nd Passage		
RoosterVial-hBM/hAD/hUC-1M-XF	1 Vial	-	RoosterBio	MSC-31/C46001AD/C43001UC
RoosterNourish-MSC-XF	1 Bottles	-	RoosterBio	K82016
RoosterHD-EV	-	4 Bottles	RoosterBio	K87206
RoosterFeed-HD-EV (Day 7 Harvest Only)	-	3 Bottles	RoosterBio	S45010
T225 CellBIND Flasks	2	-	Corning	3293
10-layer CellBIND CellStack (CS10)	-	1	Corning	3320
500mL Centrifuge Tube	-	3	Corning	431123
TrypLE Select	1	-	Thermo	12563029

*Note: This is not an exhaustive material list. Common laboratory equipment, reagents, and consumables may be required. For best expansion and functional performance, it is recommended to qualify lots of Corning CellBIND multilayer vessels prior to full-scale production. Alternatively, to bypass these qualification efforts and reduce variability, please refer to RoosterBio's **Alternative Vendor Recommendation** protocol.*

1. Media Preparation

- 1.1. Bring RoosterNourish-MSC-XF 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-MSC-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: 1st Passage

- 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 1mL of RoosterNourish-MSC-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 pelleted cells in 10 mL of RoosterNourish-MSC-XF medium.
 - 2.7.1. Remove supernatant ensuring not to disrupt pellet.
 - 2.7.2. Gently tap conical to dislodge pellet and create cells suspended in remaining solution.
 - 2.7.3. Add RoosterNourish-MSC-XF 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.

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

RoosterHD™-EV 2D Collection

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 four T75 vessels.

2.12. Add additional volume to each flask to achieve a total of 45mLs per T225.

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

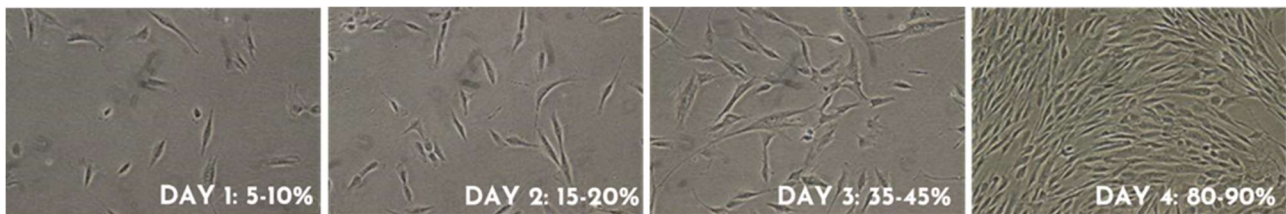
2.14. Transfer unused RoosterNourish-MSX to 2-8°C, away from direct light for up to two weeks.

3. Cell Expansion: 1st Passage

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% confluency. If the cultures reach over confluency, this may result in increased aggregation, decreased cell viability, growth inhibition and loss of differentiation potential.

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

4. Cell Harvest and Passage

*Note: RoosterBio protocol describes methods for maximizing cell number and scale. These recommendations may be modified to best fit your facility and goals. Based upon **Step 4.1**, excess cells may be cryopreserved at an intermediate passage (**Refer to cryopreservation recommendations**).*

If further modification and support is needed, please contact your Application Scientist.

4.1. Determine scale of second passage based upon target yield:

Vessel	Amount of RoosterHD-EV Required (mL)	Amount of RoosterFeed-HD-EV Required (mL; Day 7 Harvest Only)	Total Surface Area (cm ²)
1x CS1	150	3	636
1x CS2	300	6	1272
1x CS5	750	15	3180
1x CS10	1500	30	6360

- 4.2. Allow RoosterBase™-HD warm to room temperature, protected from light, for up to four hours.
- 4.3. Allow RoosterBoost™-HD-EV to thaw at room temperature, protected from light, for no more than 30 minutes.
 - 4.3.1. Note: RoosterBoost-HD-EV should still be cold to touch when added to RoosterBase-HD.
 - 4.3.2. Alternatively, RoosterBoost-HD-EV may be thawed at 2 to 8°C, away from direct light, for up to 36 hours.
- 4.4. Prepare 4 bottles of medium by aseptically adding 4 bottles of RoosterBoost-HD-EV (Part No. S40010) to 4 bottles of RoosterBase-HD (Part No. M43500).
- 4.5. Mix well by capping and gently mixing the bottle.
 - 4.5.1. RoosterHD-EV medium should be used on the day of preparation for best results.
- 4.6. For harvest, transfer vessel into biosafety cabinet and remove spent media.
- 4.7. Add 10 mL TrypLE to the CS5 vessel.
- 4.8. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO₂) incubator. Check culture initially at 10 min, and every 5 min, until cells are >90% detached from surface. Gently tap to dislodge remaining cells from surface.

Total Time Required for Cell Detachment

- 4.9. Add 10mL of RoosterHD-EV to each vessel to stop the TrypLE activity.
- 4.10. Transfer the cell suspension into a 50 mL centrifuge tube
- 4.11. Centrifuge at 500 x g for 10 min on low to medium brake at room temperature.
- 4.12. Remove supernatant ensuring not to disrupt pellet.
- 4.13. Gently tap conical to dislodge pellet and create cells suspended in remaining solution.
- 4.14. Add 10 mL RoosterHD-EV to resuspend pellet.
- 4.15. Add an additional 5 mL of RoosterHD-EV.
- 4.16. Mix thoroughly.
- 4.17. Measure total volume:

Total Volume of Cell Suspension (=A)

4.18. Transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.

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

RoosterHD™-EV 2D Collection

4.19. 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	

4.20. Mix well and aliquot 3mL of cell suspension to each of the 3 prepared RoosterHD-EV bottles.

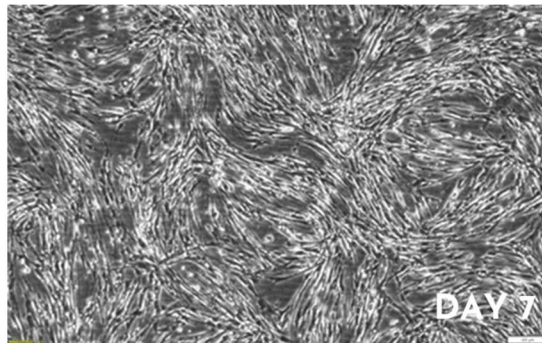
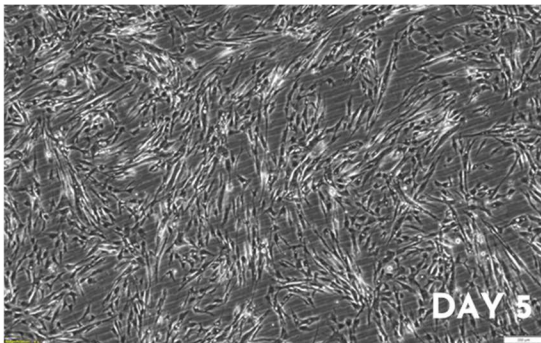
4.21. Mix well and distribute 3x 500mL bottles of cell containing RoosterHD-EV to the 1x CS10s.

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

5. Cell Expansion: 2nd Passage

5.1. Microscopically monitor cell confluency starting on day 3 of culture.

Day	3	4	5	6	7
Cell Confluency					



*Representative confluency image of hBM-MSCs.

6. Harvest (Day 5): 2nd Passage EV Collection

6.1. Weigh empty 500 mL centrifuge conicals:

Weight of Empty Conditioned Media Collection Conicals (grams)

6.2. Transfer vessel into biosafety cabinet and collect spent media into sterile 500 mL centrifuge conicals .

6.3. Weigh 500 mL centrifuge conicals:

Weight of Conditioned Media Collection Conicals (grams)

6.3.1. Proceed with further downstream processing and/or analysis.

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

7. Vessel Feeding (Day 5): Addition of RoosterFeed-HD-EV for Day 7 Harvest

- 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 5 of culture, add RoosterFeed-HD-EV (Part No. S45010), 2% volumetric addition, to each vessel as a feed.
- 7.3. Return to 37°C, 5%CO₂, 95% RH incubator.

8. Harvest (Day 7): 2nd Passage EV Collection

- 8.1. Weigh empty 500 mL centrifuge conicals:

Weight of Empty Conditioned Media Collection Conicals (grams)

- 8.2. Transfer vessel into biosafety cabinet and collect spent media into sterile 500 mL centrifuge conicals .

- 8.3. Weigh 500 mL centrifuge conicals:

Weight of Conditioned Media Collection Conicals (grams)

- 8.3.1. Proceed with further downstream processing and/or analysis.

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.