Recommended Expansion Protocol for RoosterVial-hBM-10M-Dev+

Protocol Summary

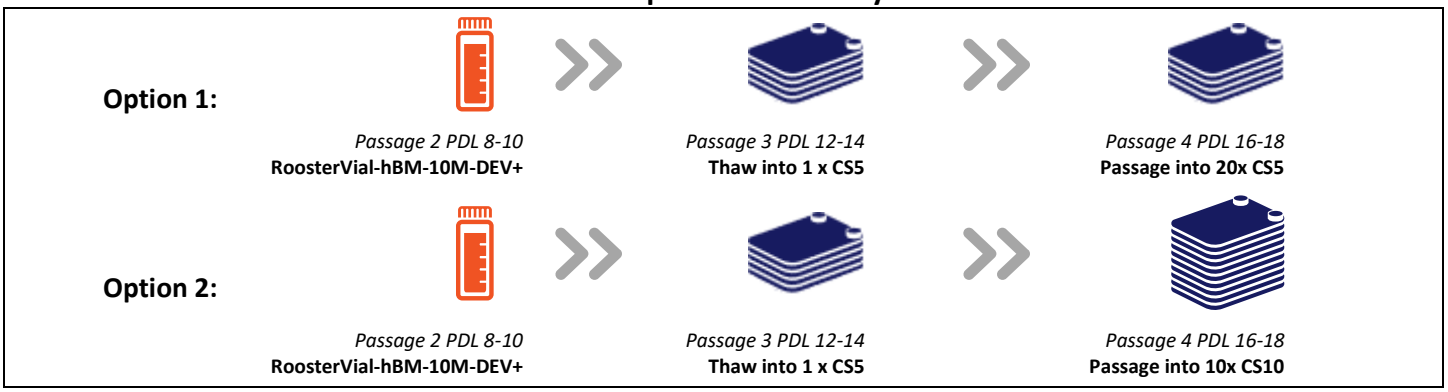
To expand one vial of xeno-free, human bone marrow-derived Mesenchymal Stem/Stromal Cells (RoosterVial-hBM-10M-Dev+) to at least 100 million cells within one week you will need the following reagents, cell culture materials, and equipment.

Materials & Equipment

Item	Quantity	Vendor	Part Number*
RoosterVial-hBM-10M-Dev+	1 Vial	RoosterBio	C44010BM
RoosterNourish™-MSC-XF	32 Bottles	RoosterBio	KT-016
Either of the following: **5-layer CellBIND CellStack (CS5)** **10-layer CellBIND CellStack (CS10)**	*Option 1:* 21 *Option 2:* 1 *Option 1:* 0 *Option 2:* 10	Corning	3311 3312/3320
TrypLE Select Enzyme	6 bottles	Life Technologies	12563029
Biosafety Cabinet			
Centrifuge			
Incubator			
Water Bath (or ThawSTAR®)			

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.

- Thaw and seed cells at recommended: 3,000 cells/cm^2 (min. >2,000 cells/cm^2).
- Expand cell cultures 3-6 days to >80% confluency at 37°C, 5% CO_2 incubation.
- **NO MEDIA EXCHANGES REQUIRED.** RoosterNourish-MSC-XF does not need to be exchanged, or fed, within 6 days of flask-based culture.

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Recommended Protocol

1. Expansion Option

Vessel	Surface Area (cm^2)	Number Vessels Needed	Total Surface Area (cm^2)	Seeding Density (cells/cm^2)	Approx. Yield at Harvest	Days of Culture
CS5	3,180	1	3,180	3,100	≥ 100M	3 to 6

2. Media Preparation

2.1. Bring RoosterNourish-MSC-XF components to room temperature, protected from light, for up to four hours.
2.2. Prepare 2 bottles of medium by aseptically adding 2 bottles of RoosterBooster™-MSC-XF (Part No. SU-016) to 2 bottles of RoosterBasal™-MSC (Part No. SU-005/SU-022).
2.3. Mix well by capping and gently mixing the bottle.

3. Cell Thawing & Seeding

3.1. Aseptically transfer 10 mL of prepared medium into a 50 mL centrifuge tube.
3.2. Thaw RoosterVial-hBM-10M-Dev+ 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).
3.3. Aseptically transfer vial into a Biosafety Cabinet (BSC).
3.4. Transfer vial contents into the 50 mL centrifuge tube containing prepared medium and mix cell suspension well.
3.5. Centrifuge at 280 x g for 10 min.
3.6. Aspirate the supernatant and resuspend cells in 10 mL of RoosterNourish-MSC-XF medium. Transfer cells into 750 mL of RoosterNourish-MSC-XF.
3.7. Mix well and seed cells equally into one CS5 vessels:

Type of Culture Vessel	Final Volume per Vessel
CS5 x 1	750 mL

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

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.

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5. Cell Harvest

5.1. For harvest, transfer vessels into biosafety cabinet and remove spent media.
5.2. Add 125 mL TrypLE to each CS5 vessel.
5.3. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO_2) incubator. Check culture every 5 min until cells are detached from surface. Gently tap to dislodge remaining cells from surface.

Total Time Required for Cell Detachment

5.4. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
5.5. Transfer the cell suspension into a 500 mL centrifuge tube.
5.6. Centrifuge at 500 x g for 10 min.
5.7. Aspirate the supernatant.
5.8. Resuspend cells in medium to achieve desired cell concentration. Measure the total volume of cell suspension:

Total Volume of Cell Suspension (=A)

5.9. Transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.
5.10. 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)	Viable Cell Concentration (=C)	Cell Concentration (D)=B*C	Total Cells at Harvest (E)=D*A

5.11. Cells are ready to be used in your application.

6. Expansion Options —2nd Passage

Note: One passage expansion is described above. If additional cells are required, second passage expansion options are listed below (select option based on required cell number at harvest). It is recommended that cells be used within 8-10 PDLs of the working cell bank.

Vessel	Surface Area (cm^2)	Number Vessels Needed	Total Surface Area (cm^2)	Seeding Density (cells/cm^2)	Approx. Yield at Harvest	Days of Culture
CS5	3,180	20	63,600	2,000-4,000	≥ 1.5B	3 to 6
CS10	6,360	10	63,600	2,000-4,000	≥ 1.5B	3 to 6

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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