Recommended Expansion Protocol for RoosterVial-hUC-1M-XF

Protocol Summary

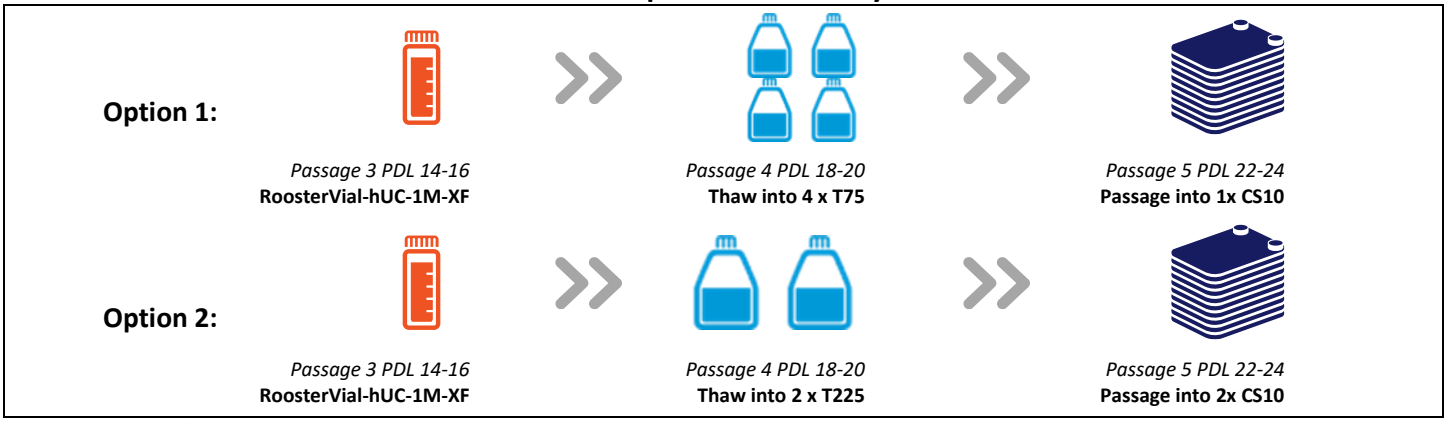
To expand one vial of xeno-free, human umbilical cord-derived Mesenchymal Stem/Stromal Cells (RoosterVial-hUC-1M-XF) to at least 20 million cells within one week you will need the following reagents, cell culture materials, and equipment.

Materials & Equipment

Item	Quantity	Vendor	Part Number*
RoosterVial-hUC-1M-XF	1 Vial	RoosterBio	C43001UC
RoosterNourish™-MSC-XF	1 Bottles	RoosterBio	KT-016
Either of the following: **T75 CellBIND flasks** **T225 CellBIND flasks**	 4 2	Corning	 3290 3293
TrypLE Select Enzyme	20 mL	Life Technologies	12563029
Vitronectin Recombinant Human Protein (500 µg/ml)	ThermoFisher	1 mL: A14700 10 mL: A31804	
DPBS without Calcium, Magnesium (-/-)	ThermoFisher	14190144	
Biosafety Cabinet			
Centrifuge			
Incubator			
Water Bath (or ThawSTAR®)			

Process Overview

Cell Expansion Summary



**RoosterBio strongly recommends the use of CellBIND with Vitronectin or Fibronectin surfaces for expansion of the Xeno-Free hUC 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.

Recommended Protocol

1. Expansion Options

Please refer to the following Expansion Options table to determine the cell culture vessel best suited to your research needs. Note: If larger cell numbers are required, a decrease in seeding density to 2,200 cells/cm^2 (and increase in total vessel seeding surface area to 450 cm^2 and total seeding media to 90 mL) will maximize total cell output at harvest with an increase in total Population Doubling Level (PDL).

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
T75	75	4	300	3,300	≥ 20M	3 to 6
T225	225	2	450	2,200	≥ 30M	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 1 bottle of medium by aseptically adding 1 bottle of RoosterBooster™-MSC-XF (Part No. SU-016) to 1 bottle of RoosterBasal™-MSC (Part No. SU-005/SU-022).
2.3. Mix well by capping and gently mixing the bottle.

3. Flask Preparation

3.1. Upon receipt, thaw the vial of vitronectin and transfer DPBS -/- at room temperature.
3.2. Calculate the working concentration of vitronectin (target 0.15 µg /cm2):

	Culture Surface Area (=A)							
	24-Well	12-Well	6-Well	T25	T75	T225	CellStack 5	CellStack 10
Surface Area (cm^2)	2	4	10	25	75	225	3180	6360

Raw Data		Adjusted Data	
Surface Area (=A)	Target Concentration (=B)	Total µg VN Required (C)=A*B	# mL of VN Stock Solution (D)=C/500 µg/ml
	0.15 µg/cm^2		

Surface Area (=A)	Volume Conversion (=E)	Total Volume of DPBS Required (F)=A*E
	0.1 mL/cm^2	

3.3. Prepare working aliquot of Vitronectin in DPBS -/-.
3.3.1. Aliquot required volume of Vitronectin **(D)**.
3.3.2. Dilute in appropriate volume of DPBS -/- **(F)**.
3.3.3. Gently resuspend by pipetting the vitronectin dilution up and down.

3.3.4. Aliquot remaining Vitronectin and return to -80°C promptly.

3.4. Add total volume **(F)** to each vessel.

3.5. Incubate flasks at room temperature for at least 1 hour (away from direct light).

Total Incubation time

3.6. Aspirate volume within culture flasks (do not allow vessels to completely dry).

4. Cell Thawing & Seeding

4.1. Aseptically transfer 10 mL of prepared medium into a 50 mL centrifuge tube.

4.2. Thaw RoosterVial-hUC-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).

4.3. Aseptically transfer vial into a Biosafety Cabinet (BSC).

4.4. Transfer vial contents into the 50 mL centrifuge tube containing prepared medium and mix cell suspension well.

4.5. Centrifuge at 350 x g for 6 min.

4.6. Aspirate the supernatant and resuspend cells in 20 mL of RoosterNourish-MSC-XF medium.

4.7. Mix well and seed cells equally into four T75 vessels or 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
T75 x 4	5 mL	15 mL
T225 x 2	10 mL	45 mL

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

5. Cell Expansion

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

5.2. 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 difficulty when harvesting, increased aggregation, decreased cell viability, growth inhibition and loss of differentiation potential.

6. Cell Harvest

6.1. For harvest, transfer vessels into biosafety cabinet and remove spent media.

6.2. Add 3 mL TrypLE to each T75 flask or 10 mL TrypLE to each T225 flask.

6.3. 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

6.4. Add equivalent volume of RoosterNourish™-MSC-XF to each vessel to stop the TrypLE activity.
6.5. Transfer the cell suspension into a 50 mL centrifuge tube.
6.6. Centrifuge at 350 x g for 6 min.
6.7. Aspirate the supernatant.
6.8. Resuspend cells in medium to achieve desired cell concentration. Measure the total volume of cell suspension:

Total Volume of Cell Suspension (=A)

6.9. Transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.
6.10. 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

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

7. 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 10-13 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
CS10	6360	1	6,360	3,000-6,000	≥ 500M	3 to 6
CS10	6360	2	12,720	2,200-4,200	≥ 700M	3 to 6