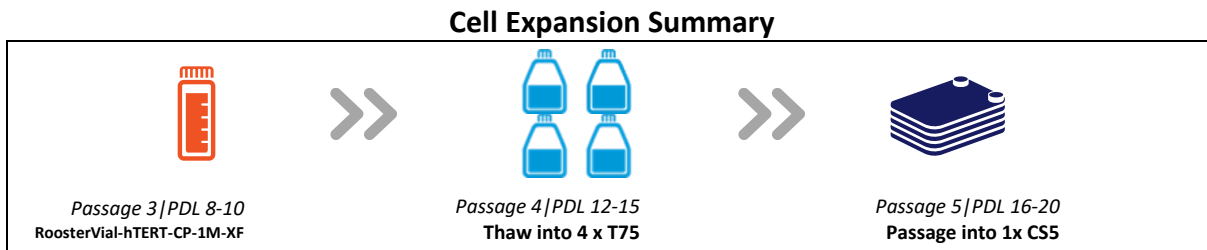


Research Expansion Protocol for RoosterVial-hTERT-CP/BM-1M-XF

Protocol Description

To expand one vial of xeno-free (XF), hTERT-modified human chorionic plate-derived MSCs (RoosterVial-hTERT-CP-1M-XF) or one vial of XF, hTERT modified human bone marrow-derived MSCs (RoosterVial-hTERT-BM-1M-XF) to at least 100 million cells you will need the following reagents, cell culture materials, and equipment. Subsequent passaging to >20 PDLs can be used following similar instructions. For applications such as vessel specific, 2D or 3D bioreactor extracellular vesicle or exosome production refer to appropriate RoosterBio protocols after RoosterVial-hTERT expansion.

Process Summary



**RoosterVial-hTERT is expandable to > 20 PDLs from starting vial.
**See full protocol instructions for subsequent passaging recommendations.*

- Thaw and seed cells at recommended: 3,000 cells/cm² (min. >3,000 cells/cm²).
- Supplement RoosterNourish-MSC-XF with 100ug/ml of Geneticin Selective Antibiotic (G418) for continued expansion
- Expand cell cultures 3-5 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 5 days of flask-based culture.

Materials & Equipment

Item	Quantity		Vendor	Part Number
	Passage 4	Passage 5		
RoosterVial-hTERT-CP-1M-XF OR RoosterVial-hTERT-BM-1M-XF	1 Vial	-	RoosterBio	C51001CP OR C52001BM
RoosterNourish™-MSC-XF	1 Bottles	2 Bottles	RoosterBio	K82016
Geneticin™ Selective Antibiotic (G418 Sulfate)* - 50 mg/ml	1 bottle		ThermoFisher	10131-035
Tissue Culture Treated T75 Flasks	4	-	Nunc, Corning, or equivalent	
5-layer CellStack (CS5)	-	1	Nunc, Corning, or equivalent	
500mL Centrifuge Tube	-	1	Corning	431123
2mL CryoVial	-	1-5	Corning	8671
TrypLE Select	1	1	Thermo	12563029
DPBS	1 Bottles	2 Bottles	Life Technologies	14190144

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

**G418 Addition is necessary during passaging but should be removed when moving to final passage prior to use within application (i.e. EV production).*

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C51001CP RoosterVial™-hTERT-CP-1M-XF / C52001BM RoosterVial-hTERT-BM-1M-XF
1. Media Preparation

- 1.1. Bring RoosterNourish-MSX 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-MSX (Part No. SU-016) to 1 bottle of RoosterBasal™2.0-CC (Part No. M22520).
 - 1.2.1. Aseptically transfer 1ml of 50mg/ml Geneticin (Part No. 10131-035) to 500 ml bottle to reach a concentration of 100ug/ml).
- 1.3. Mix well by capping and gently mixing the bottle.

2. Cell Thawing & Seeding: Passage 4

- 2.1. Aseptically transfer 10 mL of prepared medium into a 15 mL centrifuge tube.
- 2.2. Thaw RoosterVial-hTERT-CP-1M-XF (part no. C51001CP) or RoosterVial-hTERT-BM-1M-XF (part no. C52001BM) 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 1 mL of RoosterNourish-MSX 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-MSX medium.
 - 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	

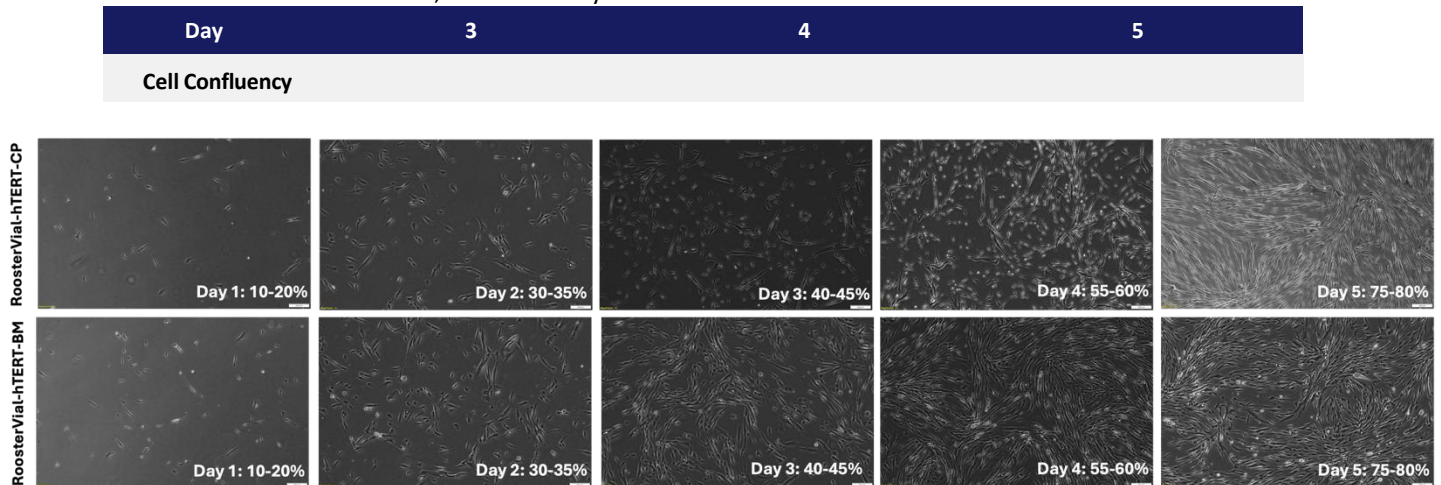
- 2.11. Mix well and seed cells equally into four T75 vessels.
- 2.12. Add additional volume to each flask to achieve a total of 15 mLs per T75.
- 2.13. Transfer vessels into an incubator (37°C, 5% CO₂) and ensure surfaces are covered evenly and leveled with media.
- 2.14. Transfer unused, G418 supplemented RoosterNourish-MSX to 2-8°C, away from direct light for up to two weeks.

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C51001CP RoosterVial™-hTERT-CP-1M-XF / C52001BM RoosterVial-hTERT-BM-1M-XF

3. Cell Expansion: Passage 4

- 3.1. Microscopically monitor cell confluency starting, at least, on day 3 of culture.
- 3.2. When culture is ~75% confluent, cells are ready to harvest.



Note: For best expansion and functional performance, it is recommended to passage the cultures before reaching 75% confluency. If the cultures reach over confluency, this may result in difficulty when harvesting, increased aggregation, decreased cell viability and growth inhibition. Cells cultured >75% confluency may require DPBS rinse prior to TrypLE Select incubation.

4. Recommended Flask Preparation: Passage 5 and Subsequent Passages

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 subsequent passage(s) based upon target yield and number of vessels used:

Subsequent Passage: Expansion Options Per Vessel Seeded at 3,000 cells/cm ²					
Vessel	Amount of Supplemented RoosterNourish Required (mL)	Amount of TrypLE Required (mL)	Amount of Quench (RoosterNourish) Required (mL)	Total Surface Area (cm ²)	Expected Yield
Per T75	15	3	3	75	>3M
Per T225	45	10	10	225	>9M
1x CS1	150	20	20	636	>20M
1x CS2	300	40	40	1272	>40M
1x CS5	750	100	100	3180	>100M

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C51001CP RoosterVial™-hTERT-CP-1M-XF / C52001BM RoosterVial-hTERT-BM-1M-XF
5. Cell Harvest and Passage

Note: Please modify using 4.1 if using different vessel than CS5.

- 5.1. Bring RoosterNourish-MSC-XF components and unused RoosterNourish-MSC-XF to room temperature, protected from light, for up to four hours.
 - 5.1.1. RoosterBooster-MSC-XF may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
- 5.2. Prepare 2 bottles of medium (or necessary volume per 4.1) by aseptically adding 2 bottles of RoosterBooster-MSC-XF (Part No. SU-016) to 2 bottles of RoosterBasal2.0-CC (Part No. M22520).
 - 5.2.1. Aseptically transfer 1ml of 50mg/ml Geneticin (Part No. 10131-035) per 500 ml bottle to reach a concentration of 100ug/ml).
- 5.3. Mix well by capping and gently mixing the bottle.
- 5.4. For harvest, transfer vessel into biosafety cabinet and remove spent media.
 - 5.4.1. If cells are >75% confluent, an DPBS rinse is recommended prior to TrypLE Select application.
- 5.5. Add 3 mL TrypLE to each T75 vessel.
- 5.6. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO₂) incubator. Check culture initially at 5 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

- 5.7. Add 3 mL of RoosterNourish-MSC-XF to each vessel to stop the TrypLE activity.
- 5.8. Transfer the cell suspension into a 50 mL centrifuge tube
- 5.9. Centrifuge at 500 x g for 10 min on low to medium brake at room temperature.
- 5.10. Remove supernatant ensuring not to disrupt pellet.
- 5.11. Gently tap conical to dislodge pellet and create cells suspended in remaining solution.
- 5.12. Add 10 mL RoosterNourish-MSC-XF to resuspend pellet.
- 5.13. Mix thoroughly.
- 5.14. Measure total volume:

Total Volume of Cell Suspension (=A)

- 5.15. Transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.
- 5.16. 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	

- 5.17. Aliquot 750 mL of fresh RoosterNourish-MSC-XF to a sterile container.
- 5.18. Mix well and transfer cell suspension to the 750 mL container with RoosterNourish-MSC-XF.
- 5.19. Mix well and transfer the 750 mL cell suspension to the 1x CS5s.
- 5.20. Transfer vessels into an incubator (37°C, 5% CO₂) and ensure surfaces are covered evenly and leveled with media.
- 5.21. Transfer unused RoosterNourish-MSC-XF to 2-8°C, away from direct light for up to two weeks.

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C51001CP RoosterVial™-hTERT-CP-1M-XF / C52001BM RoosterVial-hTERT-BM-1M-XF
6. Cell Expansion: Passage 5 and Subsequent

- 6.1. Microscopically monitor cell confluency starting on, at least, day 3 of culture.
- 6.2. When culture is ~75% confluent, cells are ready to harvest.

Day	3	4	5
Cell Confluency			

Note: For best expansion and functional performance, it is recommended to passage the cultures before reaching 75% confluence. If the cultures reach over confluence, this may result in difficulty when harvesting, increased aggregation, decreased cell viability and growth inhibition. Cells cultured >75% confluence may require DPBS rinse prior to TrypLE Select incubation.

7. Cell Harvest: Passage 5/Subsequent

Note: Please modify using 4.1 if using different vessel than CS5. G418 Addition is necessary during passaging but should be removed when moving to final passage prior to use within application (i.e. EV production).

- 7.1. Bring unused RoosterNourish-MSX-XF components to room temperature, protected from light, for up to four hours.
- 7.2. Transfer vessels into biosafety cabinet and remove spent media.
 - 7.2.1. If cells are >75% confluent, an DPBS rinse is recommended prior to TrypLE Select application.
- 7.3. Add 100 mL TrypLE to the CS5 vessel.
- 7.4. Distribute TrypLE evenly to cover all the cells and place vessels in 37°C (5% CO₂) incubator. Check culture initially at 5 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

- 7.5. Add 100mL of RoosterNourish-MSX-XF to each vessel to stop the TrypLE activity.
- 7.6. Collect pooled cell suspension into a 500 mL centrifuge tube.
- 7.7. Measure total volume:

Total Volume of Cell Suspension (=A)

- 7.8. 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	

- 7.9. Centrifuge at 500 x g for 10 min on low to medium brake.
- 7.10. Aspirate supernatant without disturbing pellet.
- 7.11. Cells are ready to be passaged further or used in your application.

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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