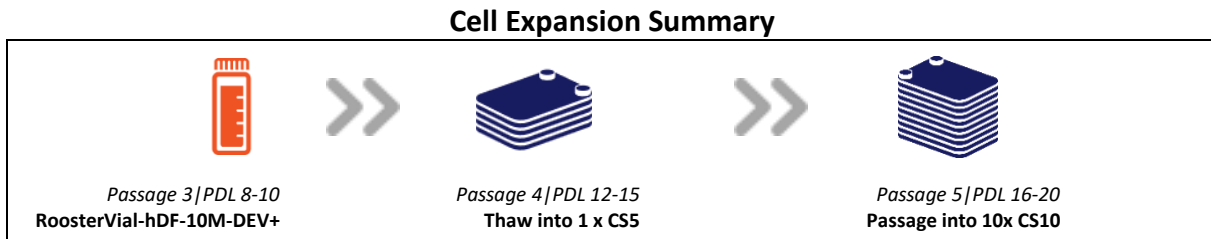


Research Expansion Protocol for RoosterVial-hDF-10M-DEV+

Protocol Description

To expand one vial of xeno-free, human neonatal foreskin-derived Fibroblasts (RoosterVial-hDF-10M-DEV+) to at least 2 billion cells you will need the following reagents, cell culture materials, and equipment.

Process Summary



**RoosterVial-hDF-10M-DEV+ is expandable to > 15 PDLs from starting vial.
**See full protocol instructions for subsequent passaging recommendations.*

- Thaw and seed cells at recommended: 3,000 cells/cm² (min. >2,000 cells/cm²).
- 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 6 days of flask-based culture.

Materials & Equipment

Item	Quantity		Vendor	Part Number
	Passage 4	Passage 5		
RoosterVial-hDF-10M-DEV+	1 Vial	-	RoosterBio	C53010DF
RoosterNourish™-MSC-XF	2 Bottles	36 Bottles	RoosterBio	K82016
5-layer CellStack (CS5)	1	-	Nunc, Corning, or equivalent	
10-layer CellBIND CellStack (CS10)	-	10	Nunc, Corning, or equivalent	
500mL Centrifuge Tube	1	10	Corning	431123
Fill Drain Cap for CellStacks	1	1	Corning	3333
Flexsafe 2D Bag 10L	-	1	Sartorius	FLS130040
Flexsafe 2D Bag 50L	-	1	Sartorius	FLS130146
2mL CryoVial	-	3-10	Corning	8671
TrypLE Select	1	4	Thermo	12563029

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

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

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 2 bottles of medium by aseptically adding 2 bottles of RoosterBooster-MSX (Part No. SU-016) to 2 bottles of RoosterBasal™2.0-CC (Part No. M22520).
- 1.3. Mix well by capping and gently mixing the bottle.

2. Cell Thawing & Seeding: Passage 4

- 2.1. Aseptically transfer 20 mL of prepared medium into a 50 mL centrifuge tube.
- 2.2. Thaw RoosterVial-hDF-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).
- 2.3. Aseptically transfer vials into a Biosafety Cabinet (BSC).
- 2.4. Transfer vial contents into the 50 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 500 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. 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-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	

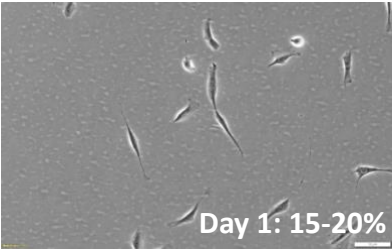
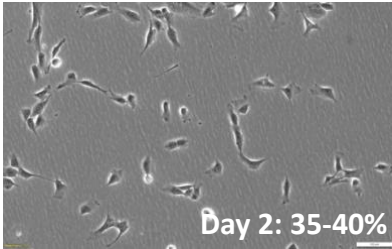
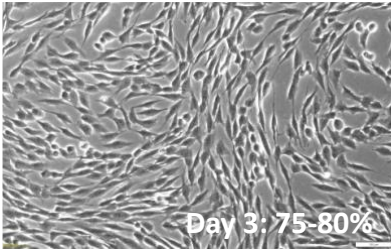
- 1.1. Aliquot 740mL of RoosterNourish-MSX to a sterile 1L bottle.
- 1.2. Transfer cell suspension to 740 mL of RoosterNourish-MSX (~750 mL total).
- 1.3. Mix well and seed cells into one CS5 vessel.
- 1.4. Transfer vessels into an incubator (37°C, 5% CO₂) and ensure surfaces are covered evenly and leveled with media.
- 1.5. Transfer unused RoosterNourish-MSX to 2-8°C, away from direct light for up to two weeks.

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

3. Cell Expansion: Passage 4

- 3.1. Microscopically monitor cell confluency starting on day 2 of culture.
- 3.2. When culture is ~75% confluent, cells are ready to harvest.

Note: Cells cultured >80% confluence may require DPBS rinse prior to TrypLE Select incubation.

Day	2	3	4	5	6
Cell Confluency					
					

Note: For best expansion and functional performance, it is recommended to passage the cultures before reaching 80% confluence. If the cultures reach over confluence, this may result in difficulty when harvesting, increased aggregation, decreased cell viability and growth inhibition.

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 second passage based upon target yield:

Passage 5: Expansion Options					
Amount of CS10s	Amount of RoosterNourish Required (mL)	Amount of TrypLE Required (mL)	Amount of Quench (RoosterNourish) Required (mL)	Total Surface Area (cm ²)	Expected Yield
1	1500	200	200	6360	>200M
2	3000	400	400	12720	>400M
3	4500	600	600	19080	>600M
4	6000	800	800	25440	>800M
5	7500	1000	1000	31800	>1.0B
6	9000	1200	1200	38160	>1.2B
7	10500	1400	1400	44520	>1.4B
8	12000	1600	1600	50880	>1.6B
9	13500	1800	1800	57240	>1.8B
10	15000	2000	2000	63600	>2.0 B

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

5. Cell Harvest and Passage

Note: Please modify according to 4.1 if using different number of vessels.

- 5.1. Bring RoosterNourish-MSX components and unused RoosterNourish-MSX to room temperature, protected from light, for up to four hours.
 - 5.1.1. RoosterBooster-MSX may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
- 5.2. Prepare 30 bottles of medium by aseptically adding 30 bottles of RoosterBooster-MSX (Part No. SU-016) to 30 bottles of RoosterBasal2.0-CC (Part No. M22520).
- 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 >80% confluent, an (optional) DPBS rinse is recommended prior to TrypLE Select application.
- 5.5. Add 100 mL TrypLE to the CS5 vessel.
- 5.6. 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

- 5.7. Add 100mL of RoosterNourish-MSX to each vessel to stop the TrypLE activity.
- 5.8. Transfer the cell suspension into a 500 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-MSX to resuspend pellet.
- 5.13. Add an additional 52 mL of RoosterNourish-MSX.
- 5.14. Mix thoroughly.
- 5.15. Measure total volume:

Total Volume of Cell Suspension (=A)

- 5.16. Transfer 0.5 mL of cells into microcentrifuge tubes for cell counts.
- 5.17. 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	

- 5.18. Mix well and aliquot 2mL of cell suspension (or equal volume) to each of the 30 prepared RoosterNourish-MSX bottles.
- 5.19. Mix well and distribute 3x 500mL bottles of cell containing RoosterNourish-MSX to each of the 10x CS10s.
- 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-MSX to 2-8°C, away from direct light for up to two weeks.

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

6. Cell Expansion: Passage 5 and Subsequent

- 6.1. Microscopically monitor cell confluency starting on day 2 of culture.
- 6.2. When culture is ~75% confluent, cells are ready to harvest.

Day	2	3	4	5	6
Cell Confluency					

Note: For best expansion and functional performance, it is recommended to passage the cultures before reaching 80% confluence. If the cultures reach over confluence, this may result in difficulty when harvesting, increased aggregation, decreased cell viability, and growth inhibition.

7. Cell Harvest: Passage 5/Subsequent

Note: Please modify according to 4.1 if using different number of vessels. Additional materials required for further passaging.

- 7.1. Bring unused RoosterNourish-MSX components to room temperature, protected from light, for up to four hours.
 - 7.1.1. RoosterBooster-MSX may also be thawed at 2-8°C between 12-36 hours before acclimating to room temperature.
- 7.2. Prepare 6 bottles of medium by aseptically adding 6 bottles of RoosterBooster-MSX (Part No. SU-016) to 6 bottles of RoosterBasal2.0-CC (Part No. M22520).
- 7.3. Mix well by capping and gently mixing the bottle.
- 7.4. Weigh empty 10-L cell collection bag (FLS130040):

Total Weight of Empty Collection Bag [grams (=F)]

- 7.5. Transfer vessels into biosafety cabinet and remove spent media into waste bag, sequentially.
 - 7.5.1. Attach one end of the Corning aseptic transfer cap (Corning 3333) to a 50-L waste bag (Sartorius FLS130146) using the MPC connector.
 - 7.5.2. Move each CS10 into the BSC (one at a time) and connect the other end of the aseptic transfer cap to the CS10.
 - 7.5.3. Gravity drain the spent media into the 50-L waste bag from each CS10.
 - 7.5.4. Remove aseptic transfer cap for reuse.
- 7.6. Add 200 mL TrypLE to each CS10 vessel.
- 7.7. 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

- 7.8. Add 200mL of RoosterNourish-MSX to each vessel to stop the TrypLE activity.
- 7.9. Collect pooled cell suspension into a collection bag.
 - 7.9.1. Attach one end of the Corning aseptic transfer cap to a 10-L cell suspension bag (FLS130040) using the MPC connector.
 - 7.9.2. Attach the other end of the Corning aseptic transfer cap to the quenched CS10.
 - 7.9.3. Collect pooled cell suspension by gravity draining into a collection bag.
 - 7.9.4. Remove aseptic transfer cap for reuse.
- 7.10. Weigh 10-L collection bag containing cell suspension:

Total Weight of Collection Bag [grams (=G)]

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

C53010DF RoosterVial™-hDF-10M-DEV+

7.11. Sample the cell collection bag with a sterile syringe and count 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*(G-F)
	cells/mL	cells/mL	

7.12. Distribute cell suspension evenly into (approximately 10x) 500mL centrifuge tubes.

7.13. Centrifuge at 500 x g for 10 min on low to medium brake.

7.14. Resuspend and pool pellets with RoosterNourish-MSX to two 500mL centrifuge tube.

7.14.1. Remove supernatant ensuring not to disrupt pellet.

7.14.2. Gently tap conical to dislodge pellet and create cells in remaining solution.

7.14.3. Add 10 mL of RoosterNourish to resuspend pellet.

7.14.4. Rinse each cell suspension tube with 50mL of RoosterNourish-MSX to collect any remaining cell suspension.

7.15. Measure total volume:

Total Volume of Cell Suspension (=A)

7.16. 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	

7.17. Centrifuge at 500 x g for 10 min on low to medium brake.

7.18. Aspirate supernatant without disturbing pellet.

7.19. 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.

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.