Normal Human Skeletal Muscle Myotubes

A Cells and Media Information

Lonza Cat No	Name	Contain
CC-2580	HSMM Cryopreserved	> 500,000 cells / Amp
CC-3245	SkGM-2 Bullet Kit	SkBM-2 Basal Medium SkGM-2 SingleQuots
CC-3246	SkBM-2 Basal Medium	500 ml
CC-3244	SkGM-2 SingleQuots	rhEGF, 0.5 ml Dexamethasone, 0.5 ml L-Glutamine, 10 ml FBS, 50 ml GA-1000, 0.5 ml
12-719F	DMEM:F12	500 ml
14-403 E	Horse Serum	100 ml
CC-5034	ReagentPack	Trypsin/EDTA Solution, 100 ml HEPES buffered Saline Solution,100 ml Trypsin Neutralizing Solution (TNS), 100 ml

This is the procedure for initial culture of HSM myoblasts and their differentiation to myotubes

B Preparation of Media

1. Decontaminate the external sufraces of all supplement vials and the medium bottle with 70% ethanol .
2. Aseptically open each supplement vial and add the entire amount to the basal medium with a pipette.
3. Rinse each cryovial with the medium.
4. Record the expiration date (one month from the preparation data) on the medium bottle.

C Thawing of Cells/Initiation of Culture Process

1. Recommended seeding density for HSMM is 3500 cells / cm2
2. Determine the total number of flasks by following equation.

   Total # of flasks = Total Cell Count x Percent Viability x Seeding Efficiency / (Growth Area x Recommended Seeding Density)
3. Add 15 ml medium to T75 (1 ml / 5 cm2) to equilibrate at 37C, CO_2, 5% for 30 min.
4. Quickly thaw the cryovial in a 37C water bath. (Do not submerge it, Do not keep longer after ice melt)
5. Resuspend cells in cryovial using a micropipette and transfer to the T75 flasks set up earlier.
6. Gently rock T75 then place it back into incubator.

Note: Centrifugation should not be performed, because centrifugation is more damaging than residual DMSO in the culture.

D Subculturing and Maintenance

1. Subculture when cells are 50% ~ 70% confluent and contain many mitotic figures throughout the flask after 4 to 6 days growth.
2. Aliquot some volume medium and reagents as listed below then warm to room temperature.

Cell Growth vessels	T 75	T175	150 mm
Trypsin/EDTA	2 ml	4 ml	3 ml
D-PBS	15 ml	20 ml	15 ml
Trypsin Neutralizing Solution	4 ml	8 ml	6 ml
Aliquoted medium	15 ml	35 ml	25 ml

The following instructions are for a 75 cm2 falsk. Adjust all volumes accordingly for other size flasks.

3. Aspirate medium from the culture vessel.
4. Rinse the cells with 10 ml of room temperature D-PBS
5. Aspirate the D-PBS from the flask.
6. Cover cells with 2 ml of Trypsin/EDTA solution
7. Keep the T75 flask in incubator for 2 to 6 minutes.
8. Examine the cell layer microscopically.
9. When ~ 90% cells rounded up, rap the flask against palm of hand to release the majority of cells from the culture surface.
10. After cells are released, neutralize the trypsin with 4 ml trypsin neutralizing solution.
11. Re-suspend cells up and down several times with 10 ml pippet.

12 Quickly transfer cells to centrifuge tube (15 ml or 50 ml).

13 Rinse flask with 5ml of D-PBS, combine all cells.

14 Microscope examine the harvested flask to make sure the cells left behind are less than 5%.

15 Centrifuge at 220 xg for 5 min at RT to pellet the cells.

16 Aspirate most supernatant, except of 100 - 200 ul and flick the tube with finger to loosen pellet.

17 Resuspend cells with 5 ml to 10 ml medium and mix with 5 ml or 10 ml pipet to ensure a uniform suspension.

18 Determine cell number and viability (if necessary more dilute cells with D-PBS to count)

19 Determine the total number of flasks to innoculate by using the following equation.

Total # of flasks to innoculate = Total # of viable cells / (Growth area x Rec. seeding Density)

20 Transfer the appropriate amount of growth medium (1 ml / 5 cm2) to the new vessels and warm in incubator for 30 min.

21 Resupend cells with 5 ml or 10 ml pipet about 10 times to make sure cells are seperated from each other very well.

22 Dispense the calculated volume into the prepared subculture flasks.

23 Place the new culture vessels back into a 37C humidified incubator with 5% CO_2 .

24 Change medium the day after seeding, and every other day.
Increase media volume as confluency increase as listed bellow .

Cell confluence	medium volume / area	T75	T175	150 mm
< 25%	1 ml/ 5cm2	15 ml	35 ml	25 ml
25 ~ 45%	1.5 ml/ 5cm2	25 ml	50 ml	40 ml
> 45%	2 ml/ 5cm2	30 ml	70 ml	50 ml

E Myoblast Differentiation to Myotube

1 When HSMM achieved ~ 90% confluence, change SkGM-2 medium to differentiation medium: DMEM/F12 with 2% Horse serum (30 ml/150 mm dish).

2 Replacing the differentiation medium every other day.

3 Differentiation for ~ 5 days till myotube very well formed.

F Large Scale Harvest (> 2E+08 cells)

1 Thaw one Cryovial HSMM cells [> 5 E+05 cells / Amp] into one T75 flask.

2 Change fresh medium next day.

3 Check cell confluence every day, when cells are ~ 70% confluent (need 4 to 6 days growth),
subculture cells (as described above under subculturing) into new vessels.
Each T75 flask can yield ~ 2.9 E+06 cells.

4 Count total cells with hemocytometer and seed as recommended seeding density (3500 cells / cm2) into need number T175 flasks
Total number of flasks depends upon cell yield and seeding density.

5 Subculture cells 1 or 2 more times until the desired cell number (> 5 E+07 cells) is achieved for final harvesting (> 2 E+08 cells).

6 Subculture when these flasks have reached 70% confluence. Each T175 flask can yield ~ 7.5 E+06 cells.
Seed cells as recommended seeding density (3500 cells / cm2) into needed # of 150 mm dishes.

7 When cells ~ 90% confluent (generally need 4 to 6 days) change to differentiation medium 30 ml for 150 mm dish.

8 Change differentiation medium every other day.

9 Differentiation for 5 days and myotubes are very well formed.

10 Aspirate medium from the 150 mm dish..

11 Rinse the dish with 10 ml of room temperature D-PBS.

12 Add 5 ml of Trypsin/EDTA solution into each 150 mm dish.

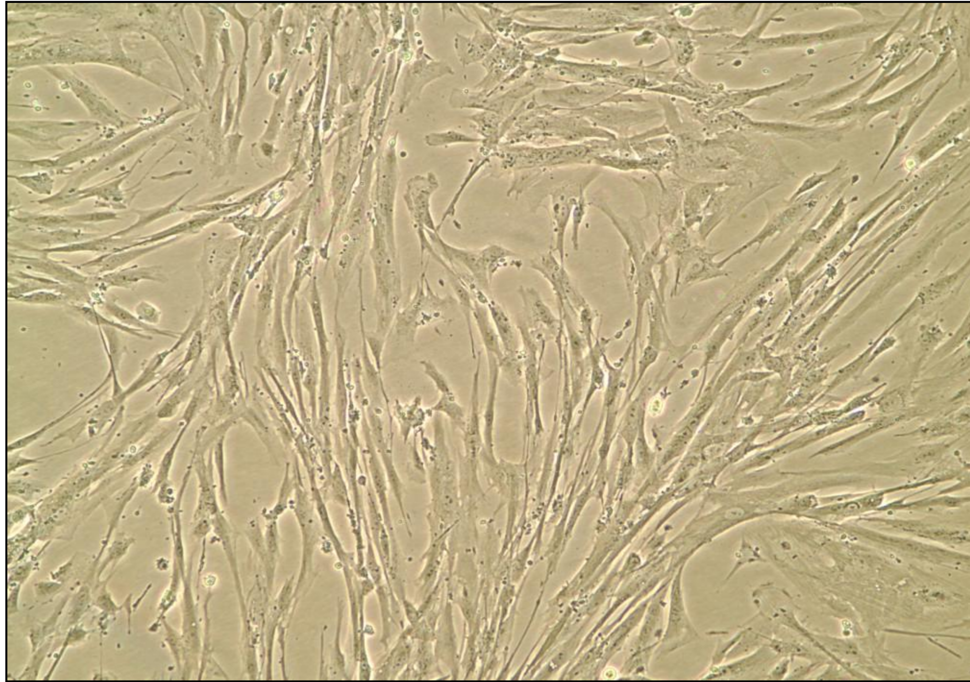
13 Confirm that the myotubes detach microscopically after trypsin added.

14 When only myotubes but not un-differentiated cells detach, quicly collect the trypsined myotubes and transfer to the 50 ml centrifuge tube --
(contains 25 mls TNS). Generally it needs only 1 to 2 min for myotubes detach.
The 50 ml centrifuge tube contains 25 ml TNS and it can hold 5 dishes trypsined cells.

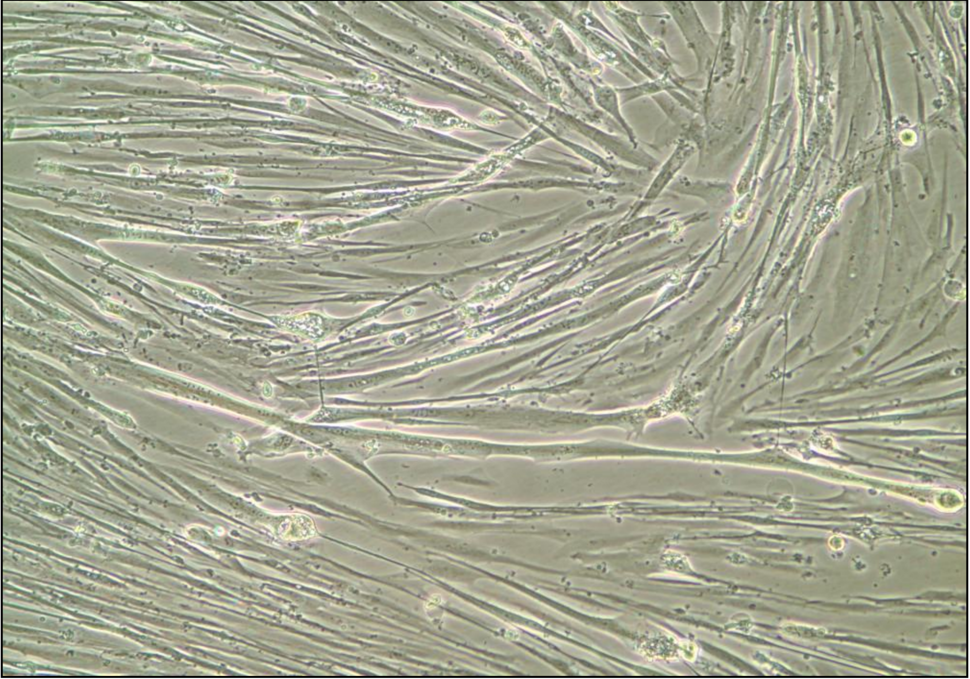
15 Spin at 220 x g for 5 min to pellet myotubes for cross-link.

G Cells and Myotubes Images

Cells before differentiation [10 x]



Myotubes for differentiation 5 days [10 x]



Myotubes for differentiation 5 days [20 x]