

***Primary Aortic Smooth Muscle Cells (AoSMC)***

From: Duke/UNC/UT/EBI ENCODE group

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Prepared by: Chris Mack (UNC)

Source: Lonza: catalog # CC-2571, Lot 7F4356

Cell growth: All conditions were followed as described (see attached) with the following clarifications.

Cell growth conditions:

- **Normal** - cells grown in SmBM basal media (CC-3181) + SmGM-2 SingleQuot serum/growth factors (CC-4149) + 0.5% Pen/Strep. Cells harvested at 80-90% confluence.
- **Serum free** - Cells grown to ~ 80-90% confluence in normal media (above) and then placed in SmBM basal media (CC-3181) + 0.5% Pen/Strep for 36h.
- **TGF-b treated** - Cells serum-starved as above (SmBM basal media + 0.5% Pen/Strep) for 12h, and then treated with TGF-b (1 ng/ml; Calbiochem) for 24h.

Thawing and passaging cells: A 1 ml aliquot of frozen cells (in 20% FBS/5%DMSO at 1 million cells/ml) was thawed in a 100 mm uncoated containing 20 ml of normal basal media + growth factors and antibiotics (see above). Cells were split at ~80-90% confluence using standard trypsinization and re-plated at ~ 20% confluence in uncoated 150mm culture dishes. Media was not changed between splits except for the first day after thawing.

Total number of cell passages: Cells were thawed at passage #7 and harvested on passage #12

## Clonetics<sup>®</sup> Normal Human Smooth Muscle Cell Systems

### Instructions for Use

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#### Safety Statements

**THESE PRODUCTS ARE FOR RESEARCH USE ONLY.** Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or in vitro procedures.

**WARNING: CLONETICS<sup>®</sup> AND POIETICS<sup>®</sup> PRODUCTS CONTAIN HUMAN SOURCE MATERIAL, TREAT AS POTENTIALLY INFECTIOUS.** Each donor is tested and found non-reactive by an FDA approved method for the presence of HIV-1, Hepatitis B Virus and Hepatitis C Virus. Where donor testing is not possible, cell products are tested for the presence of viral nucleic acid from HIV, Hepatitis B Virus, and Hepatitis C Virus. Testing can not offer complete assurance that HIV-1, Hepatitis B Virus, and Hepatitis C Virus are absent. All human sourced products should be handled at the Biological Safety Level 2 to minimize exposure of potentially infectious products, as recommended in the CDC-NIH Manual, [Biosafety in Microbiological and Biomedical Laboratories](#), 1999. If you require further information, please contact your site Safety Officer or Technical Services.

#### Unpacking and Storage Instructions

1. Check all containers for leakage or breakage.
2. For cryopreserved cells – If there is dry ice left in the package, place cryovials immediately into liquid nitrogen. Alternatively, thaw and use the cells immediately. If no dry ice remains, please contact Customer Service.
3. For proliferating cells – Swab down the flask of proliferating cells with 70% ethanol or isopropanol, then place the flask in 37°C, 5% CO<sub>2</sub>, humidified incubator and allow to equilibrate for three to four hours. After cells have equilibrated, remove shipping medium from the flask and replace with fresh medium.
4. BulletKit<sup>®</sup> Instructions: Upon arrival, store Basal Medium at 4-8°C and SingleQuots<sup>®</sup> at –20°C in a freezer that is not self-defrosting. If thawed upon arrival, growth factors can be stored at 4°C and added to basal medium within 72 hours of receipt. After SingleQuots<sup>®</sup> are added to basal medium, use within one month. Do not re-freeze.
5. ReagentPack<sup>™</sup> Subculture Reagents are sterile-filtered and then stored at –20°C until shipment. Subculture reagents may thaw during transport. They may be refrozen once. If you

plan to use within 3 days, store at 4°C.

Trypsin/EDTA Solution has a limited shelf life or activation at 4°C. If, upon arrival, Trypsin/EDTA is thawed, immediately aliquot and refreeze at –20°C. We recommend that the HEPES-BSS and the Trypsin Neutralizing Solution be stored at 4°C for no more than one month.

**NOTE:** To keep Trypsin/EDTA fresh and active after thawing, you may aliquot it into sterile centrifuge tubes and re-freeze at -20°C.

Using media or reagents other than what's recommended will void the cell warranty. Please contact Technical Services if you need help selecting media and/or reagents.

#### Preparation of Media

**For a BulletKit<sup>®</sup>, perform the following steps:**

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
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. It may not be possible to recover the entire volume listed for each cryovial. Small losses, even up to 10%, should not affect the cell growth characteristics of the supplemented medium.
4. Transfer the label provided with each kit to the basal medium bottle being supplemented. Use it to record the date and amount of each supplement added. We recommend that you place the completed label over the basal medium label (Avoid covering the basal medium lot # and expiration date) to avoid confusion or possible double supplementation.
5. Record the new expiration date on the label based on the shelf life.

**Note:** If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be refiltered with a 0.2 µm filter to assure sterility. Routine refiltration is not recommended.

## Thawing of Cells / Initiation of Culture Process

1. The recommended seeding density for AoSMC, BSMC, CASMC, PASM, PrSMC, UASMC, BdSMC and UtSMC is 3,500 cells/cm<sup>2</sup>.
2. To set up cultures calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used. Do not seed cells into a well plate directly out of cryopreservation. Add the appropriate amount of medium to the vessels (1 ml/5 cm<sup>2</sup>) and allow the vessels to equilibrate in a 37°C, 5% CO<sub>2</sub>, humidified incubator for at least 30 minutes.
3. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then retighten. Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Thawing the cells for longer than 2 minutes results in less than optimal results.
4. Resuspend the cells in the cryovial and using a micropipette, dispense cells into the culture vessels set up earlier. Gently rock the culture vessel to evenly distribute the cells and return to the incubator.
5. Centrifugation should not be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMSO residue in the culture.

## Subculturing

The following instructions are for a 25 cm<sup>2</sup> flask. Adjust all volumes accordingly for other size flasks.

### Preparation for subculturing the first flask:

1. Subculture the cells when they are 70 to 80% confluent and contain many mitotic figures throughout the flask.
2. For each 25 cm<sup>2</sup> of cells to be subcultured:
  - a. Thaw 2 ml of Trypsin/EDTA and allow to come to room temperature.
  - b. Allow 7-10 ml of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
  - c. Allow 4 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
  - d. Remove growth medium from 4°C storage and allow to start warming to room temperature.
  - e. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol based on calculated cell count, cell viability, and seeding density.

### In a sterile field:

1. Aspirate the medium from one culture vessel.
2. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the HEPES-BSS from the flask.
4. Cover the cells with 2 ml of Trypsin/EDTA solution.
5. Examine the cell layer microscopically.
6. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 2 to 6 minutes, depending on cell type.
7. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.
8. After cells are released, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. If the majority of cells do not detach within seven minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessel as described above, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available.
9. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
10. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
11. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
12. Centrifuge the harvested cells at 220 x g for 5 minutes to pellet the cells.
  - a. Aspirate most of the supernatant, except for 100 to 200 µl.
  - b. Flick the cryovial with your finger to loosen the pellet.

13. Dilute the cells in 2-3 ml of growth medium and note the total volume of the diluted cell suspension.
14. Determine cell count and viability using a hemacytometer and Trypan Blue. Make a note of your cell yield for later use.
15. If necessary, dilute the suspension with the HEPES Buffered Saline Solution (HEPES-BSS) to achieve the desired "cells/ml" and re-count the cells.
16. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

17. Determine the total number of flasks to inoculate by using the following equation. The number of flasks needed depends upon cell yield and seeding density. If seeding into well plates at this time, the recommended density is 10,000 cells/cm<sup>2</sup>.

$$\text{Total \# of Flasks to inoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Density}}$$

18. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}$$

## Ordering Information

### Cryopreserved Cells – Single Donor

AoSMC	BSMC	CASMC	PASMC	UASMC	UtSMC	PrSMC	BdSMC
CC-2571	CC-2576	CC-2583	CC-2581	CC-2579	CC-2562	CC-2587	CC-2533

### Proliferating Cells – Flasks

	AoSMC	BSMC	CASMC	PASMC	UASMC	UtSMC	PrSMC	BdSMC
T-25	CC-2671	CC-2676	CC-2683	CC-2681	CC-2679	CC-2662	CC-2587T25	CC-2533T25
T-75	CC-0234	CC-0240	CC-0258	CC-0237	CC-0243	CC-0313	CC-2587T75	CC-2533T75
T-150	CC2571T150	CC2576T150	CC2583T150	CC2581T150	CC2579T150	CC2562T150	CC2587T150	CC2533T150
T-225	CC2571T225	CC2576T225	CC2583T225	CC2581T225	CC2579T225	CC2562T225	CC2587T225	CC2533T225

### Proliferating Cells – Multiwell Plates

	AoSMC	BSMC	CASMC	PASMC	UASMC	UtSMC	PrSMC	BdSMC
6-well	CC-2571W6	CC-2576W6	CC-2583W6	CC-2581W6	CC-2579W6	CC-2562W6	CC-2587W6	CC-2533W6
12-well	CC-2571W12	CC-2576W12	CC-2583W12	CC-2581W12	CC-2579W12	CC-2562W12	CC-2587W12	CC-2533W12
24-well	CC-2571W24	CC-2576W24	CC-2583W24	CC-2581W24	CC-2579W24	CC-2562W24	CC-2587W24	CC-2533W24
48-well	CC-2571W48	CC-2576W48	CC-2583W48	CC-2581W48	CC-2579W48	CC-2562W48	CC-2587W48	CC-2533W48
96-well	CC-0148	CC-0180	CC-0096	CC-0152	CC-0192	CC-0089	CC-2587W96	CC-2533W96

- Prepare flasks by labeling each flask with the passage number, strain number, cell type and date.
19. Carefully transfer growth medium to new culture vessels by adding 1ml growth medium for every 5 cm<sup>2</sup> surface area of the flask (1 ml/5 cm<sup>2</sup>).
  20. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks.
  21. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 37°C humidified incubator with 5% CO<sub>2</sub>.

## Maintenance

1. Change the growth medium the day after seeding and every other day thereafter. As the cells become more confluent, increase the volume of media as follows: under 25% confluence then feed cells 1 ml per 5 cm<sup>2</sup>, 25-45% confluence then feed cells 1.5 ml per cm<sup>2</sup>, over 45% confluence then feed cells 2 ml per 5 cm<sup>2</sup>.
2. Warm an appropriate amount of medium to 37°C in a sterile container. Remove the medium and replace it with the warmed, fresh medium and return the flask to the incubator.
3. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container.

## Related Products

### Smooth Muscle Growth Media (Must be purchased separately):

CC-3182	SmGM <sup>®</sup> -2 BulletKit <sup>®</sup>	Kit which contains a 500 ml bottle of SmBM <sup>®</sup> , (CC-3181) and SmGM <sup>®</sup> -2 SingleQuots <sup>®</sup> (CC-4149).
CC-3181	SmBM <sup>®</sup>	Smooth Muscle Basal Medium (no growth factors) (500 ml)
CC-4149	SmGM <sup>®</sup> -2 SingleQuots <sup>®</sup>	Supplements and Growth Factors (hEGF, insulin, hFGF-B, FBS and gentamicin/amphotericin-B)

## Product Warranty

CULTURES HAVE A FINITE LIFESPAN IN VITRO. Lonza warrants its cells in the following manner only if Clonetics<sup>®</sup> Media and Reagents are used.

1. AoSMC, BSMC, CASMC, PASM, UASM and UtSMC cryopreserved cultures are assured for experimental use for fifteen population doublings. PrSMC and BdSMC cryopreserved cultures are assured for experimental use for ten population doublings.
2. Clonetics<sup>®</sup> Smooth Muscle Proliferating Cultures are assured for experimental use for ten population doublings.
3. Additional population doublings and subcultures are possible, but growth rate, biological responsiveness and function deteriorate with subsequent passage.
4. Smooth muscle cells can become irreversibly contact-inhibited if allowed to reach confluence. To avoid the loss of your cells and forfeiture of your warranty, subculture cells before they reach 80% confluence.

## Quality Control

All cell strains test negative by PCR for HIV-1, Hepatitis B and Hepatitis C. Clonetics<sup>®</sup> Smooth Muscle Cells test positive for Alpha Smooth Muscle Actin and negative for von Willebrand Factor VIII. For detailed information concerning QC testing, please refer to the Certificate of Analysis.