Materials and Equipment

Materials

- Cardiac Progenitor Cells (AC015-F)
- Cardiac Cellutions Medium (C-MGRO)
- Cardiomyocyte Cellutions Differentiation Medium (C-MDIFF)
- FGF-2 (20ng/ml, concentration in C-MGRO, to be added – not supplied)
- 5-azacytidine (5-AZA – not supplied; to add when specified: 10 µM in C-MDIFF)
- TGF-β-1 (not supplied; to add only when specified: 1 ng/ml in C-MDIFF)
- Collagen Coating Solution (150 µg/ml)
- 1× PBS (Ca2+/Mg2+-free)
- Fetal Bovine Serum (FBS)
- TrypLE Express or equivalent enzyme solution
- 15.0 ml and 50.0 ml conical centrifuge tubes
- Cell culture vessels
- Dimethyl Sulfoxide (DMSO)

Equipment

- Biosafety Cabinet
- Water Bath, 37°C
- Centrifuge
- Incubator, 37°C, 5% CO_2
- Pipet-Aid
- Pipettes

Procedure

1.	Immediately upon delivery
	• Remove vial from shipping container and check that it is still frozen—if thawed, contact DV Biologics.
	• Transfer frozen vial immediately to liquid nitrogen until you are ready to thaw and begin cell culture.
	• Prior to thawing cells, make sure to have all required reagents and materials ready to use.
	• Coat cell culture vessels with 150 µg/ml collagen coating solution (0.2 ml/cm^2).
	  ○ Incubate at room temperature for 2 hrs.
	  ○ Aspirate collagen coating solution.
	  ○ Wash the culture vessel with PBS.

2.	Thawing Cells
	• Perform and maintain all cell culture using aseptic techniques.
	• Aliquot and warm only media required. To the C-MGRO for use that day, add fresh FGF-2 (20ng/ml).
	• Thaw cells rapidly and with agitation (See How to Thaw Cells).
	  ○ Slowly dilute thawed cells with 3- to 10-fold excess cold FBS (or complete C-MGRO medium).
	  ○ Centrifuge at 400 g for 5 minutes.
	• Remove supernatant. Disperse cell pellet in 1-5 ml fresh 37°C warm C-MGRO medium with FGF-2.
	• Count Cells (See How to Count Cells).

3.	Plating and Maintaining Cells
	• Carefully add cell suspension to collagen-coated culture vessel. Seed at density of 1.0x10^4 cells/cm^2.
	• Leave cells undisturbed for 2 days. Feed cells on the third day by completely replacing the medium. NOTE: There may be many unattached cells. These cells could be progenitors or stem cells that have not yet attached. You may collect these, centrifuge and reseed.
	• Feed cells every 2-3 days with fresh C-MGRO medium (with FGF-2 added) by either:
	  ○ Performing a complete media replacement and discarding the spent media, or
	  ○ Removing the spent media but not discarding until after collecting any unattached cells by centrifugation as noted above. After centrifuging, aspirate supernatant and disperse pellet in C-MGRO with FGF-2 to reseed.
	• Once cells become 60-70% confluent, feed more regularly (every other day).
	• When cells are nearing confluency (approximately 80%), prepare to passage cells either for propagation/expansion of the progenitor cells or for their terminal differentiation. NOTE: Do not let cells become overly confluent.

4.	Passaging of Cells (for Details see How to Passage Cells) • Aspirate and discard medium; wash cells with PBS without Ca^{2+} and Mg^{2+}, and aspirate. • Add TrypLE Express or equivalent enzyme solution at a volume that just covers the cell monolayer. Incubate at 37°C; microscopically observe detachment of cells every 5 minutes; avoid toxic effects of enzymatic over-digestion. • Quench the enzyme digestion by adding 2- to 5-fold volume of C-MGRO medium. • Transfer the entire resulting suspension of detached cells from the culture vessel to a centrifuge tube. • Centrifuge the cell suspension at 400 g at 4°C for 5 min. • Aspirate supernatant and disperse cell pellet in warm C-MGRO medium for counting. Count cells (see How to Count Fresh Cells). • To propagate cardiac progenitor cells, seed cells at 1.0 x 10^4 cells/cm^2 following the differential adhesion/pre-plate method described in the next section. • If more cells are obtained than needed for re-plating, additional cells may be frozen. (See How to Freeze Cells).
5.	Differential Adhesion/Pre-Plate Method • Coat flask with collagen and set aside until time for second seeding of cells. • For the first seeding of cells, plate cells on **NON-coated** flasks at seeding density of 1.0 x 10^4 cells/cm^2 in C-MGRO medium. • Incubate for 30 minutes in 37°, 5% CO^2 incubator. • Transfer medium and non-adhered cells to a tube. • Rinse flask with fresh C-MGRO medium and pool rinse with non-adhered cells. • Centrifuge at 400 g for 5 minutes at 4°C. • Aspirate supernatant and disperse cell pellet in C-MGRO medium for counting. Count cells (see How to Count Fresh Cells). • Seed cells into **collagen-coated** flask at a density of 1×10^3 to 5×10^3 cells/cm^2. • The non-coated flask, which has a mix of cells including some fibroblasts and cardiac muscle cells, can be discarded. • Repeat these steps for each passage until ready to differentiate. • For terminal differentiation use p3 to p5 cells.
6.	Terminal Differentiation • Seed cells in flask/wells using complete growth medium: **C-MGRO (with FGF-2 added),** prepared as described previously**.** • Once confluent, feed cells with complete differentiation medium: **C-MDIFF,** consisting of basal combined with supplement – without any other added component at this step. NOTE: It is important that cells be precisely 100% confluent. Exceeding or not fully reaching 100% confluency could affect success of differentiation. • Let cells acclimate to new C-MDIFF medium for 1 day. (Do not use FGF-2 or TGF-β-1 for this step). This will be Day 0. • On Day 1, feed cells with a complete medium replacement using **C-MDIFF with 5-AZA.** Prepare this by warming fresh aliquot of C-MDIFF and adding 5-AZA (10 µM, final concentration). • Repeat the above step for Day 2 and Day 3, feeding cells by replacing with fresh C-MDIFF with 5-AZA (10 µM) added fresh each time. • On the fourth day, prepare fresh **C-MDIFF with TGF-β-1** WITHOUT 5-AZA. Prepare this by warming fresh aliquot of C-MDIFF and adding TGF-β-1 (1ng/ml, final concentration). • Maintain cells in C-MDIFF with TGF-β-1 from Day 4 onward for 2-3 weeks, feeding cells every 2-3 days with fresh C-MDIFF with TGF-β-1. NOTE: Observe cells regularly for lifting and toxicity.