Scope

The cell culture protocols described in this manual include the in vitro culture of bone marrow mononuclear cells and stromal cells using DV Biologics' complete media solution and optimized reagents. Note that this guide is used for the production and expansion of stromal cells. Mononuclear cells contain various cell types that may not adhere and should be cultured in a different manner. Any changes in the experimental conditions may have negative effects on cell survival and may yield abnormal cell growth.

Materials and Equipment

Materials

- Cells AC006-F, AH002-F, or AH005-F)
- Stromal Cellutions Medium (H-GRO)
- Fetal Bovine Serum (FBS)
- OPTIONAL (See Step 2):
  EDTA (1mM) or DNase (10-100 µg/ml)
- 1× PBS (Ca^{2+}/Mg^{2+}-free)
- TrypLE Express or equivalent enzyme solution
- Tissue culture vessels
- 15.0 ml, 50.0 ml conical centrifuge tubes
- Dimethyl Sulfoxide (DMSO), if freezing cells
- Mr. Frosty
- Ice

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.

2.	Thawing Cells
	• Perform and maintain all cell culture using aseptic techniques.
	• Prepare complete H-GRO media, which may be stored at 4 °C for a maximum of 30 days.
	• Aliquot and warm only media required for use that day at 37°C.
	• Thaw cells rapidly and with agitation, and dilute thawed cells with 3- to 10-fold excess cold FBS (or complete H-GRO medium); add the FBS or H-GRO slowly and drop-wise (See How to Thaw Cells).
	  ○ Please note that cellular aggregation is known to occur with these cell types.
	  ○ Optional – in case of cell aggregation: To facilitate dispersing the pellet of cells, EDTA (1mM) or DNase (10-100 µg/ml) may be added to medium before the centrifugation of thawed cells OR as an additional step followed by re-centrifugation.
	• Centrifuge at 400 g for 5 minutes. Remove supernatant and disperse the cell pellet in 1-5 ml fresh 37°C warm H-GRO (complete medium, NOT with EDTA or DNase).
	• Count cells (see How to Count Fresh Cells).

3.	Cultivating Cells
	• Determine volumes of cell suspension and H-GRO medium required to prepare an aliquot of cells to plate at a seeding density of:
	  ○ 1.0 - 5.0 x 10^4 cells/cm^2 for mononuclear cells
	  ○ 1.0 x 10^3 cells/cm^2 for stromal cells.
	• Gently mix warm media with cells for plating.
	• Carefully add cell suspension prepared to culture vessels (flasks/plates) at correct seeding density.
	• Place culture flasks/plates in a CO_2 incubator.

	• Allow cells to attach to the cell culture vessel before beginning to feed these cultures. ○ For mononuclear cells (AH002-F), let cells sit undisturbed for no less than 3-4 days, and replace medium with fresh H-GRO on the 5th day. ○ For Stromal Cells (AC006-F, AH005-F), let cells sit undisturbed for 2 days, and replace medium with fresh H-GRO on the 3rd day . NOTE 1: Many non-adherent mononuclear cells will be lost at this point when removing the spent medium to feed the cells for first time. See NOTE 2 below if interested in keeping the non-adherent cells in culture – if research/work focus involves culturing the non-adherent (non-stromal) cell types. • Starting with the first replacement of media – at the time indicated above – and every 2-3 days after that, feed the cells each time by completely replacing media as follows: ○ Aspirate spent medium from culture vessel ○ Wash with warm PBS by adding and then aspirating the PBS ○ Add fresh H-GRO media NOTE 2: Collect the supernatant if you are interested in investigating cell types other than stromal. As noted above, mononuclear cells will not adhere; the vast majority of these cells will be floating. • Observe cells every 2-3 days. • Once cells become 70% confluent, prepare cells for passage, collection, or freezing (see How to Freeze Cells). NOTE: Mononuclear cells will take 3-4 days to attach and may take up to 3 weeks to reach 70% confluency. NOTE: Do not let mononuclear cells grow to more than 70% confluency or remain in same culture for more than 14 days.
4.	Passaging Cells (for details see How to Passage Cells) for Sub-cultivation or Cryostorage • Aspirate and discard medium; wash cells with PBS without Ca^{2+} and Mg^{2+}, and aspirate. • Add TrypLE or equivalent enzyme solution at a volume that just covers the cell monolayer. • Incubate at 37°C; microscopically observe detachment of cells every 5 min; avoid toxic effects of enzymatic over-digestion • Quench the enzyme digestion by adding 2- to 5-fold volume of H-GRO medium. Transfer cell suspension to centrifuge tube. • Wash flasks/plate with equal volume PBS to collect any remaining cells. • Centrifuge the collected cell mixture in the conical tube at 400 g for 5 min, and aspirate supernatant. • If plating cells to another cell culture vessel: ○ Disperse cell pellet in pre-warmed media. ○ Count cells (see How to Count Fresh Cells). ○ Plate cells at 1.0 x 10^3 cells/cm^2. ○ Refer to product information sheet for specifics on characterization of cells. • If freezing cells for storage: ○ Disperse cell pellet in cold FBS. ○ Remove aliquot and perform cell count. ○ Place cells on ice. ○ Determine how many vials to freeze. The recommended cell density range for freezing is from 1.0 x 10^6 cells/ml to no more than 5.0 x 10^6 cells per ml; 1ml per cryovial. ○ Slowly and dropwise add to the volume of cell suspension an equal volume of cold FBS containing 20% (v/v) dimethyl sulfoxide (DMSO). This results in 10% (v/v) dimethyl sulfoxide (DMSO) in the final volume. ○ Immediately transfer vials into a control rate freezer or into Mr. Frosty and place at -80°C. After freezing, transfer into long-term Cryostorage freezer.