Primary Cell Culture Protocol

- All cell culture procedures must be conducted in the bio-safety cabinet.
- All cell culture media, supplements, and reagents must be sterilized by filtration through a 0.2 µm filter.
- Use aseptic techniques to prevent microbial contamination.
- Upon receiving the package, cryopreserved cells should be immediately transferred from the dry ice shipping container to a liquid nitrogen storage tank.

Medium

- Review the information provided on the Cell Biologics website for appropriate complete culture media (e.g. serum and other supplements).
- Only pre-warm (37°C) the amount of cell culture media you will be using right away. 5 ML for a T25 or 8-10 ML for a T75 flask is needed when recovering cryo-preserved cells, changing media, or splitting cells.
- Repeated warming of complete culture media is not recommended.

Coating of Flasks or Dishes

Coat sterile culture dishes or flasks with Gelatin-Based Coating Solution (Cell Biologics, Catalog No. 6950) for 2 min and then aspirate the excess solution before seeding cells.

Handling of Live Cells upon arrival

- Upon receiving the live cells in a T25 or a T75 flask, remove the sticker from the filter cap, place the flask with 6-20 ml of the existing medium into 37°C, 5% CO_2 incubator for 1 hour, then replace the medium with desired Cell Biologics' pre-warm (37°C) cell culture medium.
- These cells at 90% confluence can be split at 1:2 ratio for subculturing or frozen down for future usage when 95-100% confluent.

Cell Recovery from Cryovial

1. Quickly thaw cells by placing the vial in a 37°C water bath for <1 min or until there is just a small bit of ice left in the vial.
2. Promptly remove the vial and wipe it down with 70% ethanol.
3. Transfer cells from the vial to a 15 ml sterile conical centrifuge tube with 5 ml of pre-warmed Cell Biologics Cell Culture Medium.
4. Flush the vial with an additional 0.5-1 ml of medium to collect additional cells left in the vial.
5. Centrifuge the cells at 200 x g for 5 minutes. Note: Centrifugation of cells after thawing to remove residual DMSO is recommended.
6. Carefully aspirate the supernatant and resuspend the cell pellet with 5 ml of Cell Biologics' Cell Culture Growth Medium. **Add resuspended cells into a T25 flask** pre-coated with Gelatin-Based Coating Solution (Cell Biologics, Catalog No. 6950).
7. **Place the seeded T25 flask in a humidified, 5% CO_2 incubator at 37°C.** Leave the culture undisturbed for 12-16 hours.
8. Change culture media the following day and then every 24-48 hours afterwards to remove non-adherent cells and replenish nutrients. Please note that the medium should be changed every day when cells are >70-80% confluent.
9. Cells should be checked daily under a microscope for cell confluence and appropriate cell morphology.

Expansion of Cultured Primary Cells

1. Remove culture medium and wash the cells at room temperature 1X phosphate buffered saline solution (without calcium and magnesium).
2. Incubate the cells with pre-warmed (37°C) 0.25% Trypsin-EDTA solution (Cell Biologics, Catalog No. 6914) for 3-5 minutes. Use 3.0 ml of Trypsin-EDTA solution for a T75 flask and 2 ml for a T25 flask, respectively. As soon as the cells have detached (a few firm gentle taps on side of flask may help detachment), add 5-10 ml of Cell Biologics' Cell Culture Medium to the flask (the FBS will neutralize the trypsin) and gently pipette up and down a few times.
3. Seed the cells in fresh flasks/plates precoated with Gelatin, and then return flasks/plates into a humidified, 37°C, 5% CO_2 incubator.

4. Change culture medium the following day and then every 24-48 hours afterwards to remove non-adherent cells and replenish nutrients. Please note that the medium should be changed or add more medium every day when cells are >70-80% confluent.
5. Cells should be checked daily under a microscope for cell confluence and appropriate cell morphology.
6. Cells can be pre-washed with 1X PBS whenever replacing the medium (optional).

We recommend splitting primary cells at the following ratio

- The recommended split ratio for primary cells is 1:2.

Procedure for Freezing Cells

Materials:

1. 1X Phosphate Buffered Saline (PBS-1X)
2. 0.25% Trypsin-EDTA (1X) solution (Cell Biologics, Catalog No. 6914)
3. Tissue Culture Media
4. Cold Freezing Media (10% DMSO, 50% FBS and 40% culture medium, Cell Biologics, Catalog No. 6916).
5. Labeled Cryovials
6. Confluent Cells

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1. Remove and discard the cell culture media from the flask.
2. Flush the adherent layer 2 times with 7 ml sterile PBS (1X, without calcium and magnesium) to remove residual medium.
3. Incubate the cells with pre-warmed (37°C) 0.25% Trypsin-EDTA solution (Cell Biologics, Catalog No. 6914) for 3-5 minutes. Use 3.0 ml of Trypsin-EDTA solution for a T75 flask and 2 ml for a T25 flask, respectively. As soon as the cells have detached (a few firm gentle taps on side of flask may help detachment), add 5 ml of Cell Biologics' Cell Culture Medium to the flask (the FBS will neutralize the trypsin) and gently pipette up and down 1-2 times.
4. Transfer the cell suspension to a centrifuge tube.
5. Centrifuge cells at 200 x g for 5 minutes.
6. Carefully remove supernatant with sterile Pasteur pipette.
7. Quickly resuspend the cell pellet by adding 1 ml freezing media per vial to be frozen.
8. Place vials in Nalgene "Mr. Frosty" freezing container containing100% isopropyl alcohol at -80 °C for 6-12h.
9. Transfer vials to liquid nitrogen tank for indefinite storage.

We recommend freezing primary cells at the following ratio

- A confluent primary endothelial cell grown in a T75 flask may be frozen in 2-3 cryovials.
- A confluent primary endothelial cell grown in a T25 flask may be frozen in 1 cryovial.
- Please send us light microscope cell images (Cell confluence >70-80%) if you have any questions with cultured cells.