## Passaging Protocol:

*Based on Lonza documentation*

**Supplies**:

- Trypsin-EDTA (2mL for each 25 $cm^2$), room temperature
- Trypsin neutralizing solution (4 mL for each 25 $cm^2$), room temperature
- Lonza HEPES-BSS (7 mL for each 25 $cm^2$), room temperature. Can use PBS if out of HEPES-BSS.
- Growth medium (5 mL per 25 $cm^2$), room temperature
- New flasks
- Centrifuge tube (15 or 50 mL depending on number of flasks)
- To count cells (if desired, see separate protocol for instructions): hemocytometer, trypan blue, glass slides, small Eppendorf tube

**Steps:**

1. Passage after reaching 70-80% confluence
2. For each 25 cm^2 of cells to be subcultured:
    a. Thaw 2 mL of trypsin/ETDA to allow to come to room temperature
    b. Allow 7-10 mL HEPES buffered saline solution to come to room temperature
    c. Allow 4 mL Trypsin neutralizing solution to come to room temperature
    d. Remove growth medium from fridge and start warming to room temperature.
3. For each flask, aspirate spent media without disturbing monolayer
4. Rinse cell layer with 5 mL HEPES-BSS to remove residual traces of serum. Then remove and discard HEPES-BSS.
5. Add room temperature Trypsin-EDTA solution (2 mL for every 25 cm^2) to each flask
6. Gently rock the flask to ensure complete coverage over the cells.
7. Observe under the microscope. When cells pull away from each other and round up (usually 2-5 min), remove flask from under the microscope and gently tap it from several side to promote detachment of the cells from the flask surface.
    a. 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.
    b. 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. 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.
8. When majority of cells are detached, quickly add trypsin neutralizing solution to each flask. Gently swirl.
9. Transfer dissociated cells to centrifuge tube (15 mL or 50 mL tube depending on number of flasks you're passaging). Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.

10. 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%. (Can skip if confident that most cells lifted off based on visualization in step 8).
11. Centrifuge cells at 220 xg for 5 min.
    a. Aspirate most of the supernatant, except for 100-200 µl to avoid disturbing pellet.
12. Re-suspend in 1 mL medium, and count cells (if desired). See "Cell Counting using Hemocytometer" protocol for counting instructions.
    a. 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 (Recommended seeding density for fibroblasts is 2500-5000 cells/$cm^2$).

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

13. Prepare subculture flasks (5 mL media for every 25 cm^2)
14. Seed new flasks. Use the following equation to calculate the volume of cell suspension to seed into your flasks:

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

15. Place freshly seeded flasks in 37 degC, 5% CO2 incubator for 24-48 hours before processing cells further.