Cell Passage Protocol
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This protocol describes the process to for cell passage. This protocol should be repeated approximately every 2-3 days when cells have reached ~75-90% confluency in a T-75 flask.

Key point: Sterilization! Sterilization! Sterilization!
Throughout this protocol, the user should constantly sterilize anything of importance with Ethanol:

- Gloves – wipe hands thoroughly and get the Ethanol into all creases.
- Sterilize the flask, by holding by flask neck and spraying all over.
- Sterilize the hood and all objects placed from the outside into the hood.
- Do not remove items from the hood. If you have – resterilize.
- Work close to the hood.
- Ensure a non-compact work area.

Repeat process, every-time something is placed into the hood, or taken out of the incubator.

1. Thaw and Warm all reagents:
    a. Thaw 1-2 mL of Trypsin solution in water bath. Trypsin stocks (1mL aliquots) are stored in freezer. Aliquots are taken directly from a Trypsin stock bottle.
    b. Warm cell media in water bath. (10 mL for centrifuging + 2 mL for resuspension and counting + media volume necessary for the flasks)
    c. Warm 20-30 mL of PBS.

2. Remove flask from incubator. Flask should be tilted and held from the bottom so cells don't touch possibly "infected" neck. Inspect cells on tissue culture scope.

3. STERLIZE flask. Insert it into hood.

4. Tilt the flask so that media pools in the bottom corner against the top-surface of the flask and aspirate the media using the vacuum suction glass pipette.
    a. Tip: When inserting glass pipette, avoid touching all but the pipette head.

5. Using Pipet-aid, add 10 ml of warm PBS buffer to the flask:
    a. Take care to inject buffer into bottom corner of flask (same corner where aspiration took place).
    b. Avoid pipette touching flask rim. If contact occurs - **Replace pipette**.
    c. To avoid subjecting the adherent cells, which are on the bottom surface, to high amounts of shear, pipette onto the top surface of the flask.
    d. Once buffer is injected, then lay the flask flat on the bench to spread the PBS buffer.

6. Pool the PBS in the bottom corner of the flask and aspirate the PBS.

7. Repeat rinse process for a total of 2x PBS buffer rinses.

8. Pipette 1-2 ml of Trypsin solution directly onto the cells on the bottom surface of the flask. Tilt flask to evenly distribute Trypsin solution across the entire bottom area of the flask.

9. Incubate flask for 5-10 minutes.

10. After 5 minutes, visually check to see if the cells have lifted-off of the substrate. If most cells are still adhered to the substrate, continue incubating and check ever 2-3 minutes. Try not to leave cells on Trypsin for more than 10 minutes total. Gently tap sides of flask to promote cell-substrate release until cells have lifted off.

11. Add 10ml of warm media to flask to dilute trypsin. Using the pipet-aid, repeatedly wash flask bottom, pooling the cell-rich media in the bottom corner of the flask each time. Repeat this ~5-10x to ensure all the cells are removed from the flask-substrate and suspended in the media. This step also breaks cell clumps through shear flow.

12. Collect all of the cell-rich media into a 15 ml conical centrifuge tube.

13. Centrifuge the cells at 1000 rpm for 5 minutes.

14. Carefully aspirate pellet – be careful not to aspirate cells.

15. Re-suspend pellet with 1 ml of warm media. Thoroughly pipette mix (~20-30x) to re-suspend cells and break up cell aggregates.

Cell Counting (Hemocytometer)

1. Prepare the Hemocytometer by cleaning all surfaces with ethanol.
    a. Let it fully air dry before flowing cell suspension.

2. Prepare a 1:10 or 1:100 dilution of cells. Make sure cells are well mixed.
    a. Example 1:10 (80 µl media + 10 µl cell stock + 10 µl of trypan blue).
    b. Example 1:100 (980 µl media + 10 µl cell stock + 10 µl of trypan blue).

3. Pipette 10 µl of cell dilution under each side of the Hemocytometer coverslip.

4. Count cells in the 4x4 16 grid corners. Count additional squares for better average, but aim for on order 50 cells in each square.

5. Repeat for both sides of the hemocytometer and ensure that number of cells in each square is not wildly different.

6. Add up the total number of cells from all squares counted and use the conversion factor given below.

$$\frac{Total\ \#\ of\ Cells\ (from\ all\ squares)}{\#\ of\ Squares\ Counted} \times \frac{1\ Square}{100\ nL} \times \frac{1{,}000{,}000\ nL}{1\ mL} \times Dilution$$

Or simplified:

$$\frac{Total\ \#\ of\ Cells\ (from\ all\ squares)}{\#\ of\ Squares\ Counted} \times 10{,}000\ Conversion\ \times Dilution\ Factor = \frac{\#\ Cells}{mL}$$

Seed the new flask with the desired number of cells.

1. Label flasks with
    a. Name
    b. Date
    c. Cell type & Genotype (if applicable)
    d. Passage #

2. In a new T-75 flask, pipette 13 ml of fresh media and tilt to spread the media across the entire bottom surface of the flask.
3. Add calculated volume of dense cell suspension directly onto the bottom surface of the flask. Note that this amount varies based on requirements. It can be more and it can be less. Do not touch the pipette tip to the inner side of the flask.
4. Gently tilt and roll the flask to disperse the concentrated cell mixture.
5. Visually count cells under the tissue culture scope
6. Sterilize and place in incubator.