Materials and Equipment

Materials

- FBS
- DMSO
- Isopropanol
- Cryovials
- Liquid Nitrogen
- Trypsin or similiar
- Mr. Frosty

Equipment

- Centrifuge
- Cryopreservation container (Mr. Frosty)
- Liquid Nitrogen Dewar

Procedure

1. Change media to cells 2-24 hours prior to freezing.

2. Remove flask with cells and flask from incubator.

3. Observe cells under microscope to make sure that they are healthy and have no signs of contamination. In most cases (unless specified) cells should be in "log phase" of growth.

4. Aspirate media.

5. Add warm PBS, gently rock flask/plate to wash, aspirate PBS.

6. Slowly add trypsin to the cells using the volumes indicated in the table below:

Working Volumes for Trypsinization

Flask Type	Flask Size	Volume of Trypsin
Round Dishes	35 mm	0.2-0.5 ml
	60mm	0.5-0.9 ml
	100 mm	2.0 ml
	150 mm	3.0 ml
Multi-well Plates	6-well	0.2-0.3 ml
	12-well	0.1-0.2 ml
	24-well	0.08-0.1 ml
	48-well	0.05 – 0.08 ml
	96-well	0.01 – 0.02 ml
Flasks	T-25	1.0 ml
	T-75	5.0 ml
	T-175	8.0 ml
	T-300	15 ml

7. Neutralize Trypsin by addition of 10% of FBS or enzyme inhibitor. Remove cells in trypsin to conical tube.

8. Wash flask with equal volume (to trypsin used) PBS. Remove cells in PBS to conical vial.

9. Centrifuge cells at 400g for 5 min at 4°C.

10. Aspirate supernatant and gently resuspend cells in cold FBS. Remove aliquot for counting and place cells on ice.

11. Count Cells using a hemocytometer (See How to Count Fresh Cells) or other reliable method. Count a minimum of 200 cells in the hemocytometer (50 cells/square).

12.	Calculate the number of cryovials and volume of freezing solution necessary to freeze collected cells. Freeze 1 x 10^6 to 5 x 10^6 cells per vial in 1 ml of freezing solution. If low viability (less than 60%) increase the concentration of cells so that there are more cells available upon thawing. FOR EXAMPLE: Freezing of 10 x 10^6 cells at a concentration 1 x 10^6 cells/mL = 10 vials of 1mL of freezing solution each.
13.	Prepare the following freezing solutions in a 50 ml conical tube: • Solution I: 50 ml of FBS (50mL) and place on ice • Solution II: 40 ml of FBS and 10 ml of DMSO, mix well and place on ice
14.	Label cryovials with date, name of cell line, and passage number.
15.	Carefully add half the final volume of freezing Solution I to resuspend cells very gently by pipetting 3-4 times up and down. Slowly add the remaining (1/2 of total volume of freezing Solution II—adding Solution II quickly may shock the cells. FOR EXAMPLE: For 10 x 10^6 cells the final volume of freezing medium should be 10 ml. Add 5 ml of Solution I to resuspend cells. Then slowly add cold Solution II to have a final volume of 10 ml. NOTE: DMSO is toxic to cells, therefore, work quickly after adding Solution II.
16.	Place vials in cryopreservation container (“Mr. Frosty”) containing fresh 100% isopropanol. NOTE: Place container in a -80°C freezer for 24 hours.
17.	Transfer cryovials after 24 hours into a liquid nitrogen dewar for extended storage.