SOP: SP072

Freezing Aliquots of THP1 Monocytes cells

Materials and Reagents:

1. Liquid Nitrogen Tank
2. THP1 monocytes cultured in cRPMI (at T75 or T125) (Note 1)
3. Table top centrifuge
4. cRPMI (SOP M023)
5. Dry ice (Note 2)
6. Freezing media
    a. RPMI 1640 75% (ATCC-formulated RPMI-1640 medium, Catalog # 30-2001)
    b. Fetal Bovine Serum (FBS) 20%, not heat inactivated **with-exosomes**, stored at -20 °C. (Note 3)
    c. Dimethylsulfoxide (DMSO) 5% (stored at 4 °C).
7. Material to count cell using hemocytometer (See SOP SP067)
    a. Pipetman 10 µl.
    b. Tips 10 µl.
    c. Hemocytometer
    d. Light microscope
    e. Manual cell counter
    f. Ethanol 70%
    g. Gloves
    h. Trypan blue (0.4%, SIGMA, Cat T8154)
    i. Delicate task wipers (Kim Wipes)
8. Falcon tubes 15 mL and 50 mL
9. T25 flask
10. 0.22 µm syringe filter
11. Sterile syringe 20 ml.
12. Cryogenic vials (1.5 mL)
13. Pipetman 1000 µl.
14. Sterile tips 1000 µl.
15. Serological pipettes 5, 10, 25 ml. and pipette-aid

Protocol:

1. ____Prepare the tissue culture Biosafety cabinet with all materials (points from 7 to 13 and 6a, 6b) and turn on UV light at least 30 minutes. At the same time, Thaw the FBS.
2. ____Clean the flask with the THP1 cell culture using ethanol 70% and put it inside the cabinet vertically. Using a sterile pipette transfer the cells suspension to a 50 mL falcon tube.
3. ____Centrifuge the cells at **125 x g** for 5 minutes (Note 4).
4. ____Retrieve the tube from centrifuge, decontaminate it spraying with 70% ethanol. Inside the cabinet, decant and discard the supernatant.
5. ____Re-suspend the cells in 2 mL of RPMI1640.
6. ____Count the cells (Note 5).

7. ____Based on the total **live-cell** count prepare the precise freezing media volume, in order to obtain a suspension around 2.5 to 3 x 10^6 Cell/mL. (Note 6).
8. ____Filter the freezing media using 0.22 μm syringe filter. After that mix it with the cell suspension.
9. ____Dispense 1 mL of the cell suspension in each cryogenic vial. Save at least the last 500 μl. cell suspension for quality control of contamination (Note 7).
10. ____Label each aliquot properly (THP1 cells, cell concentration, date, name initials).
11. ____Dispense the last 500 μl. of the cell suspension in a T25 flask with 10 mL of RPMI non-supplemented (See point 5a). Incubate it at 37ºC /72 hours, checking every 24 hours. The media must keep orange-red and clear. If you observe a change in color (from orange-red to yellow) and/or cloudiness, then then aliquots are contaminated and should be discarded.
12. ____Freeze the aliquots overnight at -80 ºC and then transfer them to liquid nitrogen tank (Note 2).

Notes:

1. The aliquots to be frozen must be between 2.5 to 3.0 x 10^6 cells/mL. Therefore, the starting cell culture needs to have a very high cell load (from T75 or T125, around 0.8 to 1.0 x 10^6 cells/mL, measured by hemocytometer, see SOP SP067).

2. Transferring the aliquots from -80 to liquid Nitrogen must be very quick (less than 1 minute), never allow the aliquots thaw. If you have more than 10 aliquots, you must keep them on dry ice before they are placed in the liquid Nitrogen tank.

3. The FBS to culture THP1 cells normally is exosomes-free, you must save an aliquot (20 mL) **with exosomes** to use in this procedure. The FBS with exosomes has a positive effect in the THP1 cells when they undergo the freeze-thaw process.

4. To obtain **125 x g,** set the *Allegra Centrifuge* located at C 210 Lab to approximately 750 RPM

5. Transfer 10 μL of cells suspension to a 0.65 μL tube. To avoid contamination do not introduce the pipette body inside the tube, instead of this, place the 15 ml Falcon (containing the cell suspension) almost in horizontal position and take the cells just introducing the sterile tip. Follow the SOP SP067 to Count cells with Hemocytometer.

6. For example, if you have a total live-cell count of 2 x 10^7 Cells (in the 2 mL suspension). Then, you would prepare around 7 mL at 2.85 x 10^6 Cells/mL. To prepare the freezing media, take into account that your cells are already in two milliliters of RPMI. Then, you must always subtract this 2 mL from the 75% of RPMI needed (in this example: for 7 mL of freezing media→ RPMI 75% = 5.25 mL; FBS 20%= 1.4 mL and DMSO 5% + 0.35 mL. In this case you will only need 3.25 mL of RPMI).

7. At this point you must extreme the care to avoid contamination:
    a. Change your gloves.
    b. To take out 1 mL cell suspension use a pipetman and sterile tips (1000 μl.). Never introduce the body of the pipetman in the falcon tube. Instead of this, tilt the falcon and only introduce the sterile tip. Put the lid on the falcon tube.
    c. Open and fill one cryogenic vial at time.