Protocol – Thawing Cryopreserved PBMCs

Materials

10, 25mL Serological pipettes
50 mL conical tube(s)
Sterile Transfer pipettes
Pipette aid
Swinging-bucket centrifuge
Laminar Flow hood
37°C water bath
Microscope

Reagents

Complete growth medium
EDTA Solution, sterile (optional)
DNase I Solution (optional)
Trypan blue (optional)

Storage Recommendation:

Cryopreserved cells stored for <2 weeks can be kept at -80° C. For long-term storage (>2 weeks), it is recommended that vials be stored in the "vapor phase" of a liquid nitrogen tank.

Thawing Procedure:

1. In a 37°C water bath, warm appropriate growth medium (e.g 10% FBS/90%RPMI).
2. Remove the vial from long term storage and quickly clean the frozen vial with 70% alcohol before thawing.
3. In a laminar flow hood, twist the cap a quarter-turn to relieve pressure, and then retighten the cap.
4. In a 37°C water bath, quickly thaw the vial. **Be careful not to submerge the entire vial in the water bath.** Thaw time for a 1 mL sample in a cryovial is approximately 1-2 minutes.
5. Once the tube has thawed to the point that there is only a piece of ice about the size of a grain of rice, remove the tube from the bath.
6. Clean the outside of the vial with 70% alcohol to prevent contamination.
7. Using a sterile transfer pipet, slowly (dropwise) add 1 mL pre-warmed media to the cells while gently swirling the tube (≈ 1 minute).
8. Transfer the cell suspension to a 50 mL conical tube.
9. Rinse the cryovial with 1 mL of growth medium. Add the rinse to the conical tube in a dropwise fashion.
10. Slowly bring up the volume in the conical tube by adding medium dropwise so that there is about 20 times or more than the volume of the original vial.
11. Centrifuge the cell suspension at 200 x *g* at room temperature for 15 minutes.
12. Using a pipette, carefully remove most of the supernatant (save the supernatant in a second tube). Leave a small amount of supernatant behind so the cell pellet is not disturbed (no more than 1 mL). ***Note:*** *DMSO is heavier than medium. Try to aspirate and discard the supernatant soon after centrifugation.*
13. While gently shaking the tube, slowly add an additional 15 to 20 mL of fresh medium to the tube and resuspend the pellet to a uniform suspension.
14. Centrifuge the cell suspension at 200 x *g* at room temperature for 15 minutes.
15. Using a pipette, carefully remove all but 2 mL of supernatant. Gently resuspend the cell pellet in the remaining 2mL of medium and count.
16. Count the cells and determine viability using the Trypan Blue Method (see instructions below) or other preferred methods for cell concentration and viability assessment.
17. If the cell count is lower than expected, centrifuge the supernatant saved in Step 12 at a slightly higher speed, count and combine if necessary.
18. Your cells are now ready for downstream applications.

References

1. Ramachandran *et al.* Optimal Thawing of Cryopreserved Peripheral Blood Mononuclear Cells for Use in High-Throughput Human Immune Monitoring Studies *Cells* 2012, 1, 313-324
2. Hemocytometer Counting Tool: http://www.currentprotocols.com/WileyCDA/CurPro3Tool/toolId-10.htmL

Caution: Treat all products containing human and monkey-derived materials a potentially infectious, as no known test methods can offer assurance that products derived from human or monkey tissues will not transmit infectious agents.

All products are for research use only. Do not use in animals or humans. These products have not been approved for any diagnostic or clinical procedures.

Frequently Asked Questions

My PBMCs cells are clumping, what can I do?
To prevent cell-cell interactions, the initial wash solution may be modified to consist of HBSS (without calcium and magnesium), 10% FBS and 2mM EDTA. If there appear to be stringy fragments in the solution, it may be due to dying cells which release DNA into the media. DNase I, added at final concentration of 0.1 mg/mL (or 200 Kunitz units/mL) and incubated at room temperature for 15 minutes, will reduce the tendency for cells to stick together.

My cells are too dilute, what should I do?
Some cells prefer to be in close contact with each other in culture. The appropriate plate or flask size will vary depending on the number of cells frozen in the cryovial. It is recommended that thawed cells be plated at a high density to optimize recovery. If necessary, try transferring the culture to a smaller flask until the cell density increases.

The viability after thawing is low, why?
The freezing and thawing process is stressful to most cells. Be sure to handle the cells very gently. Do not vortex, bang the vial to dislodge the cells, or centrifuge the cells at high speeds. In addition, decrease the time required to thaw the cells as prolonged exposure to cryopreservative can be toxic to the cells.

FAQs can also be found at http://www.bioreclamationivt.com/faq

Trypan Blue Cell Count Worksheet:

Remove a cell suspension aliquot and perform the following:

- Dilute cells for a Trypan Blue Exclusion cell count.

Example for a 10X dilution:

700 µL Medium or Buffer + 200 µL Trypan Blue* (0.4%) + 100 µL diluted cells

- Mix and incubate for 1 minute
- Apply 10µL aliquot to one side of hemacytometer
- Count cells under 10X magnification
- Calculate total viable cells and percent viability

Cell Count:

Dilution Factor: _____X

Total Viable Cells:_____

Number of squares counted:_____

Total Nonviable Cells:_____

Total Cell Count:_____

% Viability = Total Viable Cells/Total Cell Count x 100 = _____

Dilution of Cell Suspension

$$\text{Cell Concentration (\# Viable Cells/mL)} = \frac{\text{Total Viable Cells}}{\text{\# squares counted}} \times 10{,}000 \times \text{Dilution Factor} \_\_\_\_ = \_\_\_\_ \text{cells/mL}$$

Cell Concentration x _____ mL Total Cell Suspension Volume = _____ Total Yield (cells)

$$\text{Total Resuspension Volume} = \frac{\text{Total Yield (cells)}}{\text{Target Cell Concentration (cells/mL)}} = \_\_\_\_\_ \text{ mL}$$

Resuspension Volume to be added =Total Resuspension Volume – Original Suspension Volume =_____mL

Note: *Over time, trypan blue will form dye aggregates and crystals, making analysis difficult. It is therefore recommended that the solution be filtered using a 0.2 micron filter prior to use.*