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# THAWING & COUNTING PROTOCOL
## Human Primary Cells

CGT Global cells are carefully frozen in a medium containing the cryo-protective agent DMSO (10%) using a controlled rate freezer to ensure maximum viability. Outlined below is our in-house procedure for thawing cells that maintains a high viability. When using this procedure or any thawing procedure, it is important to work quickly as cryopreserved cells are extremely fragile and require the utmost care. Although DMSO protects the cells while frozen, it is toxic to the cells at room temperature for even a short period of time.

Work fast and be gentle to your cells!

## MATERIALS REQUIRED

- Frozen cryovial containing cells
- Thawing Media - RPMI + 10% heat inactivated fetal bovine serum (FBS) or desired medium warmed to 37°C
- Buffer - phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mm EDTA
- Sterile 50 mL centrifuge tube
  70% ethanol
- Trypan blue
- Hemocytometer
- Cover slips
- Propidium iodine
- Detection antibodies
- Flow cytometry staining buffer or an equivalent solution containing BSA and sodium azide
- FACS tubes

## EQUIPMENT

- Water bath warmed to 37°C
- Microscope
- Flow cytometer
- Centrifuge
- 5 mL pipette
- Pipette and pipette tips

## PROTOCOL

1. Warm thawing medium in 37°C water bath.

2. Place the cryovial in 37°C water bath for approximately 1.5 minutes swirling constantly. Be careful not to submerge the entire cryovial.

   **NOTE:** Perform this step immediately after removing the cryovial from the dry ice in the shipment or after removing the cryovial from your liquid nitrogen storage.

3. Remove the cryovial when small ice crystals remain.

   **NOTE:** Prolonged exposure to heat will damage the cells and increase cell death.

4. Remove the cryovial from the water bath to a biosafety hood. Clean the outside of the cryovial with 70% ethanol.

5. Transfer contents of the cryovial to a 50 mL conical tube using a 5 mL pipette.

6. Rinse the cryovial with 1 mL warm thawing medium and transfer to the 50 mL conical.

   **NOTE:** Rinsing the cryovial will help recover remaining cells.

7. Take a small aliquot of the cell suspension for cell counting and viability analysis. Do not let the cells sit long in DMSO as cell viability will decline. Quickly analyze the cells to verify cell count and viability.

8. To remove the DMSO that the cells were transported in, slowly add 15 mL warm thawing medium to the cell suspension and centrifuge at 804 x rcf for 6 min (accel 9, brake 9) at room temperature.

9. Remove the supernatant and gently re-suspend the cells in 2 mL warm buffer.

   **NOTE:** Do not use a vortex mixer to resuspend cells. This will damage the cells and decrease viability.

10. Note the cell suspension volume of the 50 mL conical tube.

11. Clean the hemocytometer and coverslip with 70% ethanol to remove any dust particles with lens paper.

12. Gently swirl the cell suspension to make sure cells are equally distributed in the solution. Failure to mix well will result in inaccurate cell counts.

13. Perform a trypan blue count using the hemocytometer. Make two dilutions and count both. Use a 1:2 dilution for 1 million cells by adding 20 mL well mixed cell suspension to 20 mL 50% trypan blue. Mix gently.

   **NOTE:** Non-viable cells will be blue, viable cells will be unstained.

14. Count four corner squares on each side of the hemocytometer. Count separately the stained and unstained cells to estimate viability.

   **NOTE:** If more than 40 cells per square are counted adjust dilution to 1:5 by adding 20 mL well mixed cell suspension to 80 mL 50% trypan blue and recount. If the counts for the two dilutions are not within approximately 10 cells, make new dilutions and recount.

15. Once the cell count has been taken, the cell concentration can be calculated from the following formula:

$$\textbf{Total cells/mL} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of squares}} \times 10{,}000 \text{ cells/mL}$$

16. Use the total volume from Step 9 to calculate total cell count:

   **Total cells** in 50 mL conical tube = Total cells / mL x Total cell suspension volume

17. Calculate the estimated cell viability:

$$\textbf{Percent Viability} = \frac{\text{Live cell count}}{\text{Live + Dead cell count}}$$

   **NOTE:** Approximately 10-15% cell loss can be expected per wash.

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18. Purity can be determined by staining with appropriate antibodies for the cell phenotype being analyzed. It is recommended to follow the manufacturer's protocol for the antibody being used.

19. (Optional) Viability can be determined using propidium iodine (PI) staining. Stain up to 2x103 cells in approximately 1000 mL of flow cytometry staining buffer. Approximately 1 mL of PI can be added. Mix gently. No incubation is necessary as PI reacts quite fast. Do not wash cells after PI staining.

> **NOTE:** Viability staining with PI can be performed on the same cell suspension used to check for purity in Step 17. Add 1 mL of PI to the sample and begin analysis.

20. Place your cells into an appropriate medium to allow them to recover from the thawing process. Depending on the sensitivity of the cell type, allow them to recover in the medium for a few hours or overnight.

> **NOTE:** Do not centrifuge the cells! They are extremely fragile at this point and may rupture due to the force of the centrifuge. Allow a sufficient amount of time for the cells to recover.

**If you have any questions please contact us at 888-415-4215 or 530-303-3828.**