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Thawing and Measuring Viability of Frozen BM-MSCs

Introduction

This cell count and viability assessment protocol is intended to confirm the quality of cryopreserved cells and should be performed immediately when thawing the cells. After determining the number of viable cells in the culture, thawed bone marrow-derived mesenchymal stem/stromal cells (BM-MSCs) should be then plated in appropriate MSC culture conditions for further maintenance and expansion. Human BM-MSCs show exceptional proliferation and expansion in NutriStem® MSC Medium supplemented with 5% human platelet lysate, but cells may also be cultured in other supportive MSC media.

Required Materials

Reagent	Source	Cat. No.
StemExpress® Human BM-MSCs, Frozen	BI-USA	BMMSC001C BMMSC002C
Trypan Blue	BI-USA	03-102-1B
NutriStem® MSC Medium	BI-USA	05-200-1A-KT
PLTGold® Human Platelet Lysate	BI-USA	PLTGOLD100GMP PLTGOLD100R
Hemocytometer & Cover Slips		

Important Notes

- Cryopreserved cells may be shipped on dry ice. Immediately upon arrival, cells must either be thawed for culture or stored at -135°C or colder (liquid nitrogen) for continued preservation.
- Thawing must be performed as quickly as possible to improve cell viability and recovery from cryopreservation. Be sure to have all equipment and media prepared and accessible prior to beginning the protocol.
- To assess viability, cells must be counted immediately upon thaw.
- Count cells prior to washing. Cell loss during wash steps is expected and may result in 10 to 15% decrease in cell number per wash.
- Purity of cells can also be assessed by staining the cells with appropriate antibodies via flow cytometry.
    - For Human BM-MSCs, appropriate positive antibody markers are CD73, CD90, and CD105.
    - For Human BM-MSCs, appropriate negative antibody markers are CD14, CD34, and CD45.
- Human BM-MSCs, like all products of human origin, must be handled following universal precautions at Biosafety Level 2 or higher.

Protocol for Use

The following protocol outlines the steps to measure viability of cryopreserved cell cultures upon a thaw. For best results, cells should be thawed, plated, and placed in an incubator for culture as quickly as possible. Human BM-MSCs have been validated to efficiently thaw directly into NutriStem® MSC Medium supplemented with 5% PLTGold® Human Platelet Lysate. Follow aseptic techniques and perform cell culture work in a Class II biological safety cabinet.

Thaw BM-MSCs

The BM-MSCs are frozen in a medium containing the cryoprotective agent DMSO. Although DMSO protects the cells while cryopreserved, it is toxic to cells at room temperature, even when exposed for a very short period of time. Work quickly but gently when thawing cells to maximize viability and survival post-thaw.

1. Warm an aliquot of MSC culture medium at 37°C prior to thawing cells. Warm enough medium to thaw, count, and plate cells for culture.
2. Remove the cryovial from liquid nitrogen storage and place on dry ice until ready to thaw.
3. To thaw, immerse the cryovial in a 37°C water bath and swirl the vial gently for approximately 90 seconds, or until only a small ice crystal remains.

   Note: Avoid submerging the cap of the cryovial in the water bath to prevent potential contamination.
4. Sterilize the cryovial with 70% ethanol and transport to a biosafety cabinet.
5. Carefully transfer the cell suspension in the cryovial to a 15 mL conical tube.
6. Add 1 mL of warm MSC medium to the cryovial to rinse, and transfer the rinse to the conical tube containing the cell suspension.
7. Carefully add an additional 5 mL of MSC medium to the conical tube containing the cell suspension to further dilute the DMSO.

   Note: Add medium slowly to prevent osmotic damage to the cells.
8. Centrifuge the cell suspension for 6 minutes at 300 x g at room temperature. If possible, set centrifuge settings to a gradual start and slow brake.
9. Remove the supernatant and gently resuspend the cell pellet in 2 mL of warm MSC medium.

   Note: Resuspend the cells using gentle pipetting – do not vortex.
10. Note the total cell suspension volume in the 15 mL conical tube (Starting Volume).

Count Cells and Assess Viability

In a solution with Trypan Blue, viable cells will appear clear, round, and bright when viewed on a hemocytometer under a microscope. Non-viable cells will appear blue, dull, and may be irregularly shaped.

One square of a hemocytometer = 100 nL

11. Clean a hemocytometer and glass coverslip with 70% ethanol to remove any dust particles, and wipe with lens paper.
12. Gently pipet the cell suspension prepared in step 8 to evenly distribute the cells, and transfer 20 µL of the well-mixed suspension to a small Eppendorf tube.
13. Add 20 µL of Trypan Blue to the same Eppendorf tube containing the cell suspension and mix well to create a 1-in-2 dilution. (Dilution Factor = 2)
14. Load the Trypan Blue/cell solution on each side of a prepared hemocytometer, being careful not to over- or under-fill the chamber.
15. Count viable and non-viable cells in all 4 corner squares on each side of the hemocytometer.

(continued)

Note: If cell count is higher than 100 cells per square, repeat steps 11 through 14 with a more dilute cell suspension. For example, combine 20 µL of well-mixed cell suspension with 80 µL of Trypan Blue. (Dilution Factor = 5)

16. Determine the number of total viable cells in the cell suspension.

% Viability = $\frac{\text{Number of Viable Cells}}{\text{All Cells}}$

Cell Concentration (cells/mL) = (Mean Viable Cells per Square) x (Dilution Factor) x (10^4)

Total Cell Count = (Cell Concentration) x (Starting Volume)

Total Viable Cell Count = (Total Cell Count) x (% Viability)

Plate MSCs for Culture

For best results, plate MSCs initially at a density of 5 x 10^3 cells/cm^2 upon a thaw following the guidelines below. Do not centrifuge cells to concentrate the suspension prior to plating.

17. Plate the cells at a seeding density of 5 x 10^3 viable cells/cm^2 according to Figure 1 below.
18. Immediately transfer the cells to a 37°C humidified incubator and gently rock the plate side to side to evenly distribute the cells across the surface of the culture dish.
19. Incubate the cells overnight at 37°C and 5% CO_2.
20. Observe cells daily to monitor cell health, proliferation, and confluence. Perform a complete medium change every other day or as needed between passages.

Refer to the protocol Passaging MSCs using NutriStem® MSC Medium and Human Platelet Lysate for more information on MSC culture.

Figure 1. Appropriate plating densities for BM-MSC post-thaw. Cell numbers are based on a plating cell density of 5 x 10^3 cells/cm^2 and calculated using the surface area of the culture vessel.

Culture Vessel	Surface Area	Recommended MSC Plating Density at 5 x 10^3 cells/cm^2
6-well Plate	9.6 cm^2 per well	4.8 x 10^4 cells per well
12-well Plate	3.8 cm^2 per well	1.9 x 10^4 cells per well
24-well Plate	2 cm^2 per well	1.0 x 10^4 cells per well
35 mm Dish	9.6 cm^2	4.8 x 10^4 cells
T25 Flask	25 cm^2	1.25 x 10^5 cells

For additional product or technical information, please visit the Biological Industries USA web site at www.bioindusa.com, email techsupport@bioindusa.com, or call customer service at 1-860-269-0596.