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Cryopreservation of Human MSCs

Introduction

Human mesenchymal stem/stromal cells (MSCs) are promising tools for a range of applications in both research and translational studies. Established, well-characterized, and patient-specific cell lines are a valuable resource, and cryopreservation of these MSCs provides an important reserve of cells for future use and experimental repetition, while minimizing changes in cellular genetics over time. Surplus cells can be cryopreserved when harvesting during a routine passage.

The purpose of cryopreservation is to store the cells indefinitely by suspending the cells' metabolism in ultra-low temperatures. The freeze/thaw process is stressful to all cells, and therefore proper techniques and optimized media are essential for efficient recovery and expansion of the cells in culture post-thaw. To reduce damage to cells during the freezing process, the cryoprotectant dimethyl sulfoxide (DMSO) is often used to prevent the formation of ice crystals. Slow freezing at a gradual rate of -1°C per minute using a thermally insulated freezing container or controlled-rate freezer is also essential to minimize cellular damage caused by ice crystal formation.

Serum, typically fetal bovine serum (FBS), has historically been added to freezing medium to protect the cells during cryopreservation. However, serum is not necessary when freezing MSCs, and the highly variable and poorly characterized nature of serum can impact recovery rates and introduce regulatory hurdles in clinical applications. When culturing cells in a defined, serum-free, and xeno-free system required for therapeutic applications, maintaining a serum-free environment during cryopreservation is essential. The chemically defined CryoStem™ MSC Freezing Medium has been specifically optimized to maintain high viability and maximize recovery of MSCs after long-term cryopreservation in a serum-free, animal component-free environment – ideal for research, cell banking, and cell therapy applications.

Advantages

- cGMP-manufactured
- Chemically defined
- Serum-free
- Protein-free
- Animal component-free
- Complete, 1X solution
- Pre-formulated with DMSO
- QC-tested for sterility and endotoxins
- Performance-validated with human MSCs

Required Materials

Reagent	Source	Cat. No.
CryoStem™ MSC Freezing Medium	BI-USA	05-712-1
NutriStem® MSC Medium	BI-USA	05-200-1A-KT
Recombinant Trypsin Solution Or Recombinant Trypsin-EDTA Solution	BI-USA	03-078-1 03-079-1
Dulbecco's Phosphate Buffered Saline (DPBS) no calcium, no magnesium	BI-USA	02-023-1
Soybean Trypsin Inhibitor (50X) (optional)	BI-USA	03-048-1
Trypan Blue Solution (5 mg/mL)	BI-USA	03-102-1B

Important Notes

- CryoStem™ MSC Freezing Medium has been optimized and validated with cells cultured with NutriStem® MSC Medium. However, CryoStem™ MSC Freezing Medium is suitable for cryopreservation of MSCs previously cultured in either serum-containing or other supportive serum-free media.
- For best results, healthy MSCs should be frozen when the cell confluency reaches 60% to 70% and the cells are still actively proliferating. Do not allow MSCs to overgrow in culture prior to harvesting for cryopreservation.
- After cells are cooled to -80°C at a rate of -1°C per minute, vials must be transferred to liquid nitrogen storage (-135°C or lower) for long-term storage.
- Human MSCs, like all products of human origin, must be handled following universal precautions at Biosafety Level 2 or higher.
- Use appropriate personal protective equipment at all times when working with liquid nitrogen and solutions containing DMSO.

Reagent Storage and Notes

- CryoStem™ MSC Freezing Medium is a complete, ready-to-use, 1X freezing solution containing 10% DMSO. There is no need to supplement or dilute this solution with cell culture medium prior to use.
- Store CryoStem™ MSC Freezing Medium at 4°C and keep on ice during use.
- DMSO can absorb water from the air. Minimize exposure of CryoStem™ MSC Freezing Medium to air as much as possible, replacing cap immediately when not in use.

Protocol for Use

The following protocol outlines the steps to cryopreserve human MSCs using CryoStem™ MSC Freezing Medium. For best results, freeze healthy, actively proliferating, low passage cells to ensure highly viable cells upon recovery and continued culture after thawing. Using an optimized cryopreservation medium and freezing at a controlled-rate of -1°C per minute prior to storage in liquid nitrogen are essential steps for efficient cell recovery. Follow aseptic techniques and perform cell culture work in a Class II biological safety cabinet.

Harvest MSCs

1. Using a vacuum aspirator and a sterile aspirator pipette, remove the supernatant from the culture vessel or well(s) to be harvested for cryopreservation.
2. Add a sufficient volume of DPBS (without Ca^{2+} or Mg^{2+}) to wash the culture surface. Use approximately 2 mL of DPBS per 10 cm^2 of culture surface area.
3. Gently rock the culture vessel to wash the cells. Aspirate the DPBS wash.
4. To detach the cells, add a sufficient volume of Recombinant Trypsin Solution to cover the entire cell culture surface, and incubate the cells at room temperature or 37°C for 3 to 5 minutes.

   Note: Recombinant Trypsin-EDTA Solution can be used if cells are over-confluent or difficult to detach after a short incubation with Recombinant Trypsin Solution.

(continued)

5. Observe the cells under a microscope. If less than 90% of the cells are detached from the culture surface, continue incubating and observe again at 1-minute intervals to check for complete detachment.

   Note: Incubation times will vary between cell lines and confluency. Begin checking the cultures after 3 minutes. Do not over-incubate the culture, as MSCs can be sensitive to enzymatic stress. Tap the vessel periodically to expedite cell detachment and monitor the progress of the enzyme solution.

6. Once the cells are detached from the surface, quench the action of the trypsin enzyme by adding a volume of pre-warmed complete medium that is 2 to 4 times the volume of the trypsin solution used.

   Note: Alternatively, 1X Soybean Trypsin Inhibitor (SBTI) solution diluted in DPBS can be used to quench the trypsin.

7. Collect the cell suspension and transfer to a 15 mL centrifuge tube. If needed, rinse the culture vessel with additional media to collect any remaining cells, and transfer to the same tube.

   Note: If desired, steps 8 and 9 may be omitted, providing that the action of the trypsin has been sufficiently quenched and the volume of the cell suspension in step 7 is appropriate for counting. If skipping this first centrifugation, proceed to step 10.

8. Centrifuge at 300 x g for 5 minutes at room temperature.

9. Remove the supernatant and re-suspend the pellet in 3 to 5 mL of complete MSC culture medium.

Count Cells and Assess Viability

Trypan Blue is used at a working concentration of 5 mg/mL. In solution with Trypan Blue, viable cells will appear clear, round, and bright when viewed on a hemocytometer under a microscope. Non-viable cells will take up the dye and appear blue, dull, and may be irregularly shaped. For accurate viability assessment, do not allow the cells to sit for an extended period of time after staining with Trypan Blue, as eventually viable as well as non-viable cells may begin to absorb the dye.

One square of a hemocytometer = 100 nL

10. Clean a hemocytometer and glass coverslip with 70% ethanol to remove any dust particles, and wipe with lens paper.

11. Gently pipet the cell suspension to evenly distribute the cells. Note the total volume of the cell solution. (Starting Volume). Transfer 20 µL of the well-mixed suspension to a 1.5 mL microfuge tube.

12. Add 20 µL of Trypan Blue to the same microfuge tube containing the cell suspension and mix well to create a 1:1 dilution. (Dilution Factor = 2)

13. Load the Trypan Blue/cell solution on each side of a prepared hemocytometer by capillary action, being careful not to over-or under-fill the chamber.

14. Count viable cells and non-viable cells in all 4 corner squares on each side of the hemocytometer under a microscope at 10X magnification.

    Note: If cell count is higher than 100 cells per square, repeat steps 10 through 14 with a more dilute cell solution. For example, combine 20 µL of well-mixed cell suspension with 60 µL of Trypan Blue. (Dilution Factor = 4)

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

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

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

Cryopreserve MSCs

The recommended freezing density for MSCs is between 5 x 10^5 cells/mL and 1 x 10^6 cells/mL. For best results, cells must be kept cold and begin the freezing process immediately upon addition of the DMSO-containing cryopreservation medium. When freezing many vials at once, work in small batches and keep cells on ice at all times to prevent cryovials from warming at room temperature.

Using this method, it is imperative to freeze cells at a slow rate of -1°C per minute. If a specialized controlled-rate freezing apparatus is not available, freeze cells in a thermally controlled freezing container, such as an isopropanol freezing container ("Mr. Frosty"), to -80°C as described in the protocol below before transferring the cryovials to liquid nitrogen for long-term storage.

16. Prepare the workspace by having all materials and equipment on hand and ready. Transfer CryoStem™ MSC Freezing Medium from 4°C to a prepared ice bucket.

17. Centrifuge the cell suspension at 200 x g for 5 minutes at room temperature.

18. While the cells are in the centrifuge, calculate the volume of 1X cryopreservation medium required to bring the cell pellet to the desired freezing density (between 5 x 10^5 cells/mL and 1 x 10^6 cells/mL).

$$\textbf{Freezing Volume} = \frac{\text{Total Viable Cells}}{\text{Freezing Density}}$$

19. Prepare the appropriate number of cryovials to be used based on the freezing volume (1 mL of cell suspension per cryovial). Label vials with the cell line identifier, passage number, and freezing date.

20. Remove the supernatant from the centrifuge tube and quickly but gently re-suspend the pellet in cold 1X CryosStem™ MSC Freezing Medium according to the freezing volume determined in step 18.

21. Dispense 1 mL of the re-suspended cells per cryovial and immediately transfer the cryovials to ice.

Note: If freezing multiple cryovials, keep the of cells on ice at all times. Gently mix the resuspended cell solution frequently to ensure even distribution throughout the vials. Immediately transfer filled cryovials to ice before aliquotting the remaining cell solution.

22. Place cryovials in a controlled-rate freezing container and transfer to -80°C. Allow cells to cool overnight at a rate of -1°C per minute.

23. The following day, transfer frozen vials from -80°C to liquid nitrogen (vapor phase) to maintain cells at -135°C or below for long-term storage.

Note: Long-term storage of MSCs at -80°C is not recommended.

When freezing cell banks, it is recommended to determine the efficiency of cryopreservation by thawing one vial after 24 hours of storage in liquid nitrogen. Evaluate cell cultures for viability and recovery from a thaw according to the protocol **Thawing MSCs using NutriStem® MSC Medium and Human Platelet Lysate.**

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

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 1-860-316-2702.