Protocol: Cryopreservation and thawing of human cells using NutriFreez™ D10 Cryopreservation Medium

Overview

This protocol describes for cryopreservation and thawing of pluripotent stem cells and general mammalian cells using NutriFreez™ D10 Cryopreservation Medium.

Caution

This protocol uses cells that have been stored in liquid nitrogen. Liquid nitrogen is a freezing hazard, and evaporation of liquid nitrogen can generate significant pressures that can rupture closed vessels. Please take appropriate precautions when working with these cells.

Required Materials

PRODUCT DESCRIPTION	CAT. NO.
Dulbecco's PBS w/o Ca^{2+}, Mg^{2+} (DPBS)	Supplied by end user
NutriFreez D10 Cryopreservation Medium	01-0020-50 (BI Cat. No. 05-713-1E)
NutriStem® hPSC XF Medium or other culture medium of your choice	01-0005
iMatrix-511 or other suitable matrix for coating culture plates	NP892-02

Store all required materials according to the manufacturer's recommendations.

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1. Protocol for Cryopreservation of Human Pluripotent Stem Cells (hPSCs)

Note: Human Pluripotent Stem Cells (hPSCs) may be frozen a clumps or as single cells. Either method yields high viability and minimal differentiation.

Note: Keep NutriFreez D10 Cryopreservation Medium on ice at all times during use.

Note: This procedure describes the cryopreservation of cells cultured in a 6-well plate.

Cryopreservation Procedure

1. Remove the culture medium from the wells to be cryopreserved.
2. Rinse the wells with DPBS (about 2 mL per well of 6-well plate), and remove the DPBS.
3. Add the desired dissociation solution. After the cells begin to loosen from the plate, add 2-4 volumes of culture medium to neutralize dissociation solution and suspend the cells.
4. If necessary, determine a cell count on cell suspension.
5. Transfer the cell suspension to a centrifuge tube. Centrifuge at 200 x g for 5 min at room temperature. Aseptically decant the supernatant without disturbing the cell pellet.
6. Resuspend the cell pellet in a sufficient volume of ice-cold NutriFreez D10 Cryopreservation Medium to give a cell density of $1x10^6$ cells per mL of NutrifFreez. Two or three gentle aspirations is usually sufficient to suspend the cells. Try to avoid breaking up aggregates more than necessary.
7. Dispense aliquots of resuspended cells into cryovials.

Note: If freezing multiple cryovials of cells, keep the cells on ice at all times. Prechill the cryovials in ice. Gently mix the resuspended cells to ensure even distribution across the vials. Immediately store filled vials on ice until transfer to freezing apparatus.

8. Use a controlled rate freezing system to cool the cells at 1 °C to 2 °C per minute. Store the cells in the liquid nitrogen vapor phase.

   Alternatively, place the cells in an appropriate freezing container (*e.g*. Mr Frosty™) and store at -80 °C over night. The following day transfer the cells to liquid nitrogen vapor phase storage.

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

Thawing of cryopreserved hPSCs

1. Briefly warm NutriStem® hPSC XF Medium, or other growth culture media of choice, in a 37 °C water bath.
2. Add 9 mL of warmed NutriStem® hPSC XF Medium, or other growth culture media, into a centrifuge tube.
3. Rapidly thaw the cryovial of cells in a 37 °C water bath by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.
4. Disinfect the vial by wiping it down with a cloth moistened with 70% Ethanol or Isopropanol.
5. In a sterile biological safety cabinet, transfer the contents of the cryovial drop by drop into the 9 mL of culture medium in the previously prepared centrifuge tube. Gently rock to continually mix the cells as the new cell droplets are added to the tube.

6. Centrifuge the cells at 200 x g for 5 minutes. Remove and discard supernatant.
7. Gently resuspend the cell pellet in NutriStem® hPSC XF Medium or other growth culture media, and plate on a culture vessel coated with iMatrix-511 or other suitable matrix. Incubate at 37 C.
8. Refresh culture medium 48 hrs after plating.

2. Protocol for Human Mesenchymal Stem Cells (hMSCs)

Note: Keep NutriFreez D10 Cryopreservation Medium on ice at all times during use.

Cryopreservation Procedure

1. Remove the culture medium from the wells to be cryopreserved.
2. Rinse the wells with DPBS (about 2 mL per well of 6-well plate), and remove the DPBS.
3. Add the desired dissociation solution. After the cells loosen from the plate, add 2-4 volumes of culture medium to neutralize dissociation solution and suspend the cells.
4. Transfer the cell suspension to a centrifuge tube. Centrifuge at 300 x g for 5 min at room temperature. Aseptically decant the supernatant without disturbing the cell pellet.
5. Resuspend the cell pellet in 3-5 mL growth medium, and perform a cell count to determine viable cell number.
6. Centrifuge at 300 x g for 5 min at room temperature. Aseptically decant the supernatant without disturbing the cell pellet.
7. While the cells are centrifuging (Step 6), calculate the volume of NutriFreez D10 Cryopreservation Medium needed for a cell density of $1x10^6$ cells per mL of NutriFreez.
8. Quickly and gently resuspend the cell pellet in ice-cold NutriFreez D10 Cryopreservation Medium according to the volume determined in Step 7.
9. Dispense aliquots of resuspended cells into cryovials.

Note: If freezing multiple cryovials of cells, keep the cells on ice at all times. Prechill the cryovials in ice. Gently mix the resuspended cells to ensure even distribution across the vials. Immediately store filled vials on ice until transfer to freezing apparatus.

9. Use a controlled rate freezing system to cool the cells at 1 °C to 2 °C per minute. Store the cells in the liquid nitrogen vapor phase.

   Alternatively, place the cells in an appropriate freezing container (*e.g.* Mr Frosty™) and store at -80 °C overnight. The following day transfer the cells to liquid nitrogen vapor phase storage.

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

Thawing of cryopreserved hMSCs

1. Briefly warm hMSC growth medium in a 37 °C water bath. Add 5-10 mL of warmed hMSC growth medium to a centrifuge tube.
2. Rapidly thaw the cryovial of cells in a 37 °C water bath by gently shaking the vial and remove the vial when only a small frozen cell pellet remains. Do not vortex cells.
3. Disinfect the vial by wiping it down with a cloth moistened with 70% Ethanol or Isopropanol.
4. In a sterile biological safety cabinet, transfer the contents of the cryovial drop by drop into the 9 mL of culture medium in the previously prepared centrifuge tube. Gently rock to continually mix the cells as the new cell droplets are added to the tube.

Note: It is possible to skip the centrifugation step after thawing by simply transferring the thawed cells directly onto a culture vessel with medium at a ratio of at least 1:10 (for the dilution of the DMSO).

5. Centrifuge the cells at 300 x g for 5 minutes. Remove and discard supernatant.
6. Gently resuspend the cell pellet in 0.5-1 mL hMSC culture media.
7. Perform a viable cell count. Resuspend cells to desired density in hMSC growth medium and culture as desired. Incubate in a humidified CO_2 incubator at 37 C.
8. Refresh culture medium 48 hrs after plating.

3. General Instructions for Cryopreservation of Mammalian Cells.

Note: Keep NutriFreez D10 Cryopreservation Medium on ice at all times during use.

Cryopreservation Procedure

1. Detach cells using a dissociation method appropriate for the cell type.
2. To maintain aseptic work conditions, wipe the outer packaging with a cloth moistened in 70% Ethanol/70% Isopropanol before opening the NutriFreez™ D10 Cryopreservation Medium.
3. Centrifuge cells to obtain a cell pellet, 300-400xg for 4-5 minutes, then aseptically decant supernatant without affecting the cell pellet.
4. Suspend the pellet in cold (2 – 8 °C) NutriFreez™ D10 Cryopreservation Medium, mix thoroughly, and transfer the suspension to a cryovial.

Note: If freezing multiple cryovials, keep the 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 aliquoting the remaining cell solution.

5. Freeze the cells gradually (1-2 °C per minute) by using a controlled rate freezing system and store the vials in liquid nitrogen (vapor phase).

   Alternatively, place the vials in appropriate freezing container (e.g. Mr. Frosty) and transfer to -80°C for overnight. The following day transfer cryovials into liquid nitrogen (vapor phase recommended).

Note: long-term storage at -80 °C is not recommended**.**

6. We recommend that the efficiency of cryopreservation be determined by thawing one vial after 24 hours of storage in liquid nitrogen, following the thawing procedure outlined below.

Thawing of cryopreserved cells

1. Briefly warm growth medium in a 37 °C water bath
2. Rapidly thaw the cryovial of cells in a 37 °C water bath by gently shaking the vial. Remove the vial when only a small frozen cell pellet remains. Do not vortex.
3. Disinfect the vial by wiping it down with a cloth moistened with 70% ethanol or isopropanol.
4. Suspend the cell s in warmed growth culture medium at a ratio of at least 1:10 cell suspension to medium.
5. Centrifuge the cells at 300-400 x g for 4-5 minutes. Remove and discard supernatant.
6. Gently resuspend the cell pellet in growth medium as desired and culture the cells at the recommended seeding density according to the usual protocol.

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