Thawing, Passaging, Culturing, and Cryopreservation of Human MSCs

Overview

This protocol describes procedures for thawing one vial (1 million cells) of iMSCs or primary MSCs (details in **Required Materials**).

Caution

This protocol uses cells that have been stored in liquid nitrogen. Liquid nitrogen is a freezing hazard, and the 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.
Cryopreserved Human MSCS: • Repro MSC3 human iPSC-derived MSCs • Repro MSC4 human iPSC-derived MSCs, Phenol red-free • Repro MSC10 human iPSC-derived MSCs, Phenol red-free Cellcolabs BM hMSC primary human MSCs	 RCRP025 RCRP026 RCRP038 CC-BM-hMSC
MSC NutriStem® XF Basal Medium or MSC NutriStem® XF Basal Phenol Red Free Medium	01-0006 01-0007
MSC NutriStem® XF Supplement Mix	05-0061
Growth plate or flask	Supplied by end user
NutriCoat™ Attachment Solution	05-0063
Accutase™	Supplied by end user
DPBS, Ca^{2+} Mg^{2+} free	Supplied by end user

1. Thawing Protocol for MSCs

1. Prepare the working reagents:

    i. Prepare **Complete MSC NutriStem XF Medium** by mixing 500 mL MSC NutriStem XF Basal Medium (with or without Phenol red) with 3 mL MSC NutriStem XF Supplement Mix. Store the unused **Complete MSC NutriStem XF Medium** at 4 °C for up to 30 days.

    ii. Prepare matrix coated appropriate vessel by adding a 1:500 dilution of NutriCoat™ Attachment Solution in DPBS at 37 °C for 1 hour. Coated vessels can be stored wrapped in parafilm for up to 3 days at 4 °C.

Table 1: Incubation times will vary between cell lines and colony sizes. Begin checking the culture after 3 minutes. Do not over-incubate the culture, as MSCs are sensitive to enzymatic stress.

Media and Reagents	6-well	T-25	T-75	T-225
Number of Cells per Vessel	2.9×10^4	7.5×10^4	2.25×10^5	6.75×10^5
Volume of Medium for Thawing Process	13 mL	13 mL	13 mL	13 mL
Volume of Medium for Seeding	2 mL *per well*	10 mL	20 mL	45 mL
Volume of Coating Solution	1 mL *per well*	5 mL	10 mL	25 mL

2. Warm an appropriate amount of **Complete MSC NutriStem XF Medium** (hereinafter referred to as **Culture Medium**) to room temperature. See table above for approximate volumes of Culture Medium needed.

3. Add 7 mL of prewarmed **Culture Medium** to a centrifuge tube.

4. Quickly thaw the cryovial of cells in a 37 °C (antibacterial treated) water bath for approximately 2 min by gently swirling the vial until 2/3 of the frozen cell solution has melted. Do not vortex cells.

5. Disinfect the vial of cells by wiping it down with a cloth moistened with 70% ethanol or isopropanol.

6. Working in a biological safety cabinet (BSC), use a 1 mL pipette to transfer the contents of the cryovial drop by drop into the 7 mL of Culture Medium in the centrifuge tube from step 3. Gently rock to continually mix the cells as the new cell droplets are added to the tube. Rinse the cryovial with 1 mL of Culture Medium and transfer the contents to the centrifuge tube.

7. Centrifuge the cells at 300 × g for 8 minutes. Remove and discard the supernatant.

8. Using a 5 mL pipette, gently resuspend the cell pellet in 5 mL of **Culture Medium**. Count the cells using a hemacytometer or any appropriate cell counting device. Dilute the suspension with Culture Medium to give a suitable concentration for plating, if necessary.

9. Aspirate the NutriCoat™ Attachment Solution from the coated vessel prepared in step1 ii and plate the suspended MSCs at a plating density of 3,000 cells/cm^2. Incubate the plate at 37 °C.

10. Change the culture medium 48 hr after plating.

2. Passaging Protocol

Table 2: Material components for passaging

Media and Reagents	6-well	T-25	T-75	T-225
Number of Cells per Vessel	3,000 cells/cm^2	3,000 cells/cm^2	3,000 cells/cm^2	3,000 cells/cm^2
Volume of Medium for Seeding	2 mL *per well*	10 mL	20 mL	45 mL
Volume of Coating Solution	1 mL *per well*	5 mL	10 mL	25 mL
Volume of Accutase for Passaging	1 mL *per well*	2 mL	7.5 mL	20 mL
Volume of DPBS for Washing	2 mL *per well*	5 mL	15 mL	35 mL

1. Have on hand **Culture Medium** and a coated culture vessel as outlined in in step 1 and Table 2.

2. Warm an appropriate amount of **Culture Medium** at room temperature.

3. Aspirate the media from the cells and rinse with DPBS. Aspirate the DPBS and add Accutase™ (see volume details in Table 1). Incubate the cells at 37 °C for ~5 min.

4. Check the flask periodically to determine when the cells are lifting off the flask.

5. Using a 5 mL pipette, gently add the dissociated cells to 5 mL of warmed **Culture Medium** and transfer to a 15 mL or 50 mL centrifuge tube. Wash the flask with warmed **Culture Medium** using twice the volume as the volume of Accutase added. Pipette up and down the entire surface to wash off the cells and add the wash to the centrifuge tube.

6. Centrifuge the cells at 300 × g for 8 minutes. Remove and discard the supernatant.

7. Using a 5 mL pipette, gently resuspend the cell pellet in an appropriate volume of warmed **Culture Medium**. Count the cells using a hemacytometer or any appropriate cell counting device. Dilute the suspension with **Culture Medium** to give a suitable concentration for plating, if necessary.

8. Aspirate the NutriCoat™ Attachment Solution from the new flask and plate the MSCs on the coated vessel at a density of 3,000 cells/cm^2. Incubate the plate at 37 °C.

9. Change the culture medium 48 hr after plating.

3. Routine Culture

Human MSC cultures should be monitored regularly and media should be refreshed every 48 hours. Human MSCs are generally passaged every 7 days in culture, but the actual passaging schedule and split ratio for each passage will vary depending on each cell line's quality and growth rate. It is important to passage the cells when they reach 70% - 80% confluency; the cells should not be allowed to become overgrown.

For maintenance and expansion, the MSCs should be cultured in Complete MSC NutriStem XF Medium or Complete MSC NutriStem XF Phenol Red Free Medium on NutriCoat™ Attachment Solution or adapted to other proven human MSC culture conditions. It is essential that the MSCs are plated on a coated cell culture surface (i.e. with NutriCoat™ Attachment Solution) when serum-free media (i.e. MSC NutriStem media) is used to culture the MSCs. This coating is not necessary when serum is added to MSC media used to culture the cells.

For the first passage after recovery from cryopreservation and for subsequent passages, the cells should be passaged using Accutase.

4. Cryopreservation of MSCs

Note: This procedure describes the cryopreservation of **cells cultured in a T-75 flask** prior to cryopreservation; scale up or down as appropriate (see **Table 1**).

1. Have on hand an appropriate amount of **Culture Medium** as described in step 1 and **Table 1** on page 2.

Note: Always keep NutriFreez D10 Cryopreservation Medium or NutriFreez D10 Phenol Red Free Cryopreservation Medium on ice during use.

2. Remove the culture medium from the flasks.
3. Rinse the cells with DPBS (about 15 mL for a T-75 flask) and remove the DPBS.
4. Add 7.5 mL of Accutase and incubate the cells at 37 °C for 3-5 minutes. After the cells begin to loosen from the flask, resuspend the cells gently and transfer to a 50 mL centrifuge tube.
5. Wash the flask with Culture Medium twice the volume of the Accutase. Pipette up and down the entire surface to 'wash' off the cells and add the wash to the centrifuge tube.
6. Centrifuge the cells at 300 × g for 8 min at room temperature. Aseptically decant the supernatant without disturbing the cell pellet. Resuspend the cell pellet in an appropriate volume of Culture Medium.
7. Determine a cell count of the cell suspension.

8. Centrifuge the cells at 300 × g for 8 min and resuspend the cell pellet in a sufficient volume of ice-cold NutriFreez D10 Cryopreservation Medium or NutriFreez D10 Phenol Red Free Cryopreservation Medium to give a cell density of 1×10^6 cells per mL of NutriFreez. Two or three gentle aspirations is usually sufficient to suspend the cells.

9. Prechill the cryovials on ice. Dispense aliquots of resuspended cells into cryovials. Gently mix the resuspended cells to ensure even distribution across the vials. Immediately store filled vials on ice until transfer to freezing apparatus.

Note: If freezing multiple cryovials of cells, always keep the cells on ice.

10. 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.culture medium from the well.