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703.3 URMC HTC_Cryopreservation_of_Isolated_Cells_061220

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1 Works for me dx.doi.org/10.17504/protocols.io.biz6kf9e

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ABSTRACT

Lung MAP HTC - BioRepository for Investigation of Neonatal Diseases of the Lung (BRINDL)

Standard Operating Procedures 703.2_HTC102314rev061019

Cryopreservation of Isolated Cells

Purpose and Scope of the Procedure or Laboratory Assay

1. Stable frozen storage of isolated cells from lung, lymphoid tissues and blood while inducing least artifact possible

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KEYWORDS

Cell cryopreservation, Thaw cryoprotected cells

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GUIDELINES

Scientific Principles

Cells may be frozen at a slow controlled rate of minus one degree Celsius per minute in cryoprotectant medium containing 10% dimethylsulfoxide (DMSO). The DMSO acts to prevent ice crystal formation during the slow freezing process, maintaining cell viability. Some cell types such as PBMC may be stored short term (less than one week) at -80^{0}C or long term in a liquid nitrogen freezer. For maximum recovery and viability upon thawing,

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cells should be healthy prior to freezing. Successful cell recovery from the frozen state requires **rapid** but gentle **thawing** of the cells followed by immediate removal of cryoprotectant medium from the cells. Note the protocol suggesting thawing only 1-2 vials at a time and initial 1-2 drop addition of buffer to avoid shocking the cells.

MATERIALS TEXT

1. Beckman Allegra X-14 with SX4750A swinging bucket rotor and inserts with biosafety covers
2. Hemacytometer
3. 0.04% Trypan blue exclusion dye in HBSS
4. Biological Safety Cabinet, Class A2
5. Household bleach (5% sodium hypochlorite) for preparation of a 10% bleach water solution
6. Absorbent towels
7. Sterile serological pipets 10, 5, 2,&1 ml size
8. Pipet aide for serological pipets
9. Sterile pipet tips
10. Single channel air displacement pipets 5-50 uL
11. Waste pan
12. Sterile 15 ml conical polypropylene tubes
13. Ice bath for tubes
14. Racks for cryovials and for 15 ml tubes
15. Freezing medium (10% DMSO and 90% FBS)
16. Dimethylsulfoxide (reagent grade) Fisher BP231-1 or equivalent
17. Fetal Bovine Serum (FBS)(screened for low cell cytotoxicity and of quality to support cell growth with low endotoxin)
18. RPMI 1640 medium or equivalent with 8% FBS
19. Nalgene/Nunc cryogenic vials, 1.5 ml capacity Catalog # 5000-1020
20. Mr. Frosty, Nalgene Cryo 10 C Freezing container Cat# 5100-0001
21. Isopropyl alcohol
22. 0.2 µM sterile filter with sterile plastic bottle (150 to 500 ml)
23. Refrigerated centrifuge with appropriate rotors (Beckman allegra or equivalent) same as 1st item
24. -80^{0}freezer and liquid nitrogen freezer for long term storage

Reagent Preparation:
Cryprotectant/ Freezing medium (90% FBS/10% DMSO): Sterile filter FBS using a 0.2 micron filter with vacuum into a sterile container to remove any particulate matter. Add 10 ml DMSO per 90 ml FBS using a sterile disposable pipet. Be careful not to introduce the pipet beyond the tip to ensure ink on the serological pipet does not enter the DMSO. Do not re-filter after addition of the DMSO. Store the medium at 4^{0}C. Always use sterile technique when entering the solution container. Place the cryoprotectant medium container on ice while using to freeze cells.

For Thawing:
*** FACS media = 1% BSA in DPBS w/v (GB); if cells going into cell culture using Enzyme neutralization buffer = 10% FBS in DPBS (RM)
Freezing container: A Mr Frosty with isopropyl alcohol to the fill line is used to control the rate of cell freezing in the -80^{0}C freezer. Mr Frosty containers are cleaned and new alcohol added after every 5 uses. Mr Frosty is stored at 4^{0}C prior to freezing cells to ensure that cells do not warm as they are placed into the -80^{0}C freezer.

SAFETY WARNINGS

Safety Considerations
1. All work is performed using BSL 2 procedures and following universal precautions for handling human blood and body fluids.
2. Wear thermal gloves to prevent freezer burn while handling frozen cells inside minus 80 freezer and liquid nitrogen tanks.
3. Proper balancing of centrifuge while spinning the cells is requred.

Procedure for Freezing

1 After Centrifugation at 800Xg for 10min at 4^{0}C pour off supernatant and resuspend cell pellet by gently tapping the tube by hand (with fingers) before adding the appropriate amount of freezing medium to tube to yield a cell concentration/vial as indicated in the following table. Immediately pipet 1 ml of cells in cryoprotectant freezing medium into labeled cryovials, close caps tightly and place into a Mr.Frosty slow-cooling freezing container taht was kept at

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room temperature.

2 Place the freezing container with the vials in a -80^{0}C freezer and let freeze for at least 24 hours before transferring to liquid nitrogen freezer unit for long term storage.

3 **Follow the table to determine the number of cells to be frozen per vial**

Total Number of Cells Recovered (106)	# vials to freeze	PBMC Concentration of Cells per Vial (106)	Dissociated Organ Cells
<8	1	All Available	All Available
8-10	2	Split cells between 2 vials	Split cells between 2 vials
>10-50	2-10	** 5 x106 cells per vial	** 10 x106 cells per vial
>50	10	Divide cells evenly between 10 vials	** 10 x106 cells per vial

** Round cells to the nearest vial.(ex. 21**(10^6) PBMC cells equals 4 vials at 5.2(10^6)/vial)**

Procedure for Thawing

4 Move tubes quickly to dry ice from liquid nitrogen freezer for transporting to laboratory. Use protective freezer gloves and a face shield when removing tubes from the liquid nitrogen freezer.

5 **Thaw only 1-2 tubes at a time** in 37^o C water bath while shaking continuously just until the last ice crystal remains. This should take **65-70 seconds** for vials containing 1 ml of freezingmedium.

6 Using a one ml pipet, transfer the entire contents from the thawed tube to a15ml conical polypropylene tube at ambient room temperature. **Slowly add 5ml of cold** FACS*** media 1-2 drops at a time**, while mixing carefully and thoroughly by swirling tube for the first 5 ml. Repeat for the second vial (if applicable). Add an **additional 5 ml of warm FACS media to each tube** before **proceeding directly to next step.**

6.1 After transfering the cells in freezing medium from freezing vials with 1 ml pipette, wash the empty freezing vial with 1 ml thawing medium, mix slowly to collect any leftover cells, add this to the thawed cells.

6.2 ** 6/10/19 this is a change in the SOP from “warm = 25 to 37^{0}C”; however we have been using the cold buffer for some time already

6.3 *** "FACS media" = 1% BSA in DPBS w/v (GB); or if cells going into cell culture suggest using "enzyme neutralization buffer" = 10% FBS in DPBS (RM)

7 Centrifuge tubes at **1000 X g for 10 min at 4^{0}C** remove supernatant, gently tap tube to resuspend cell pellet and add 10 ml warm FACS media. Centrifuge the tube again as above. Discard supernatant.

8 Resuspend to desired volume in medium.

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8.1 While counting the cells under microscope, store the cells on ice.

8.2 Dilute an aliquot of cell suspension in trypan blue exclusion dye for counting in a hemocytometer to determine recovery and viability.

Analysis of Results

9 Determine viability and revised cell number of thawed cell aliquots by trypan blue or live/dead flow cytometry assay

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