UNIVERSITY OF ROCHESTER MEDICAL CENTER

Human Immunology Center Core Laboratory
David H. Smith Center for Vaccine Biology and Immunology
Aab Institute of Biomedical Sciences

STANDARD OPERATING PROCEDURE: NUMBER HIC-1-0007.2

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Title: Cryopreservation of cells: freezing and thawing

Purpose and Scope:

This procedure describes how to freeze and thaw mammalian cells in the Human Immunology Core Laboratory.

Principle:

Cells may be frozen at a slow controlled rate of minus one degree Celsius per minute in cryoprotectant medium containing 10% dimethylsulfoxide (DMSO). Cells are allowed to absorb DMSO into cell membranes for 15 minutes. 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, cells should be healthy with greater than 90% viable prior to freezing. Successful cell recovery from the frozen state requires rapid thawing of the cells followed by immediate removal of cryoprotectant medium from the cells.

References:

Recommendations for Prevention of HIV Transmission in Health-Care Settings: Universal Blood and Body Fluid Precautions Guideline CDC 1987
CDC Biosafety in Microbiological and Biomedical Laboratories 4th Ed U.S.H&HS, Public Heath Service

Safety:

Personnel will adhere to safe work processes outlined in U.S. Public Health Universal Precautions Guidelines for use of human blood and body fluids and follow biosafety level 2 practices.

Reagents and Material:

BD Allegra X-12 with SX4750 swinging bucket rotor and inserts with seals
Hemacytometer
0.4% Trypan blue exclusion dye in saline

Biological Safety Cabinet, Class II
Household bleach (5% sodium hypochlorite) for preparation of a 10% bleach water solution
Absorbent towels
sterile serological pipets 10, 5, 1 mL size
pipet aide for serological pipets
sterile pipet tips
digital variable single channel air displacement pipets 5-50 uL
Waste pan
sterile 15 mL conical polypropylene tubes
ice bath for tubes
test tube racks for cryovials and for 15 mL tubes
Freezing medium (10% DMSO and 90% FBS)
Dimethylsulfoxide (reagent grade) Sigma Cat # 15,493-8
Fetal Bovine Serum (FBS)(screened for low cell cytotoxicity and of quality to support cell growth with low endotoxin)
RPMI 1640 medium or equivalent with 8% FBS (R8)
Nalgene/Nunc cryogenic vials, 2.0 mL capacity Catalog # 5000-1020
Mr. Frosty, Nalgene Cryo 1^0 C Freezing container Cat# 5100-0001
Isopropyl alcohol
0.2 μ sterile filter with sterile plastic bottle (100 to 250 mL)
Refrigerated centrifuge with appropriate rotors (BD allegra or equivalent) same as 1^{st} item
-80^0 mechanical freezer and liquid nitrogen freezer for long term storage

Reagent Preparation:

Cryprotectant 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 insure 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.

Freezing container: A Mr Frosty with isopropyl alcohol to the fill line is used to control the rate of cell freezing in a -80^0 mechanical 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 insure that cells do not warm as they are placed into the -80^0 C freezer.

Procedure for Freezing:

Note: All work is performed using BSL 2 procedures and following universal precautions for handling human blood and body fluids.

1. Count cells in suspension using trypan blue and a hemacytometer. Wash cells with HBSS and spin down at 300 X g for 10 minutes at 4^0 C.
2. 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 1 X 10^6 to 2 X 10^7

cells per mL. Immediately pipet 1 mL of cells in cryprotectant medium into labeled cryovials, close caps tightly and place the vials into ice.

3. Let vials stand in ice bath for 15 minutes before moving to a chilled Mr. Frosty controlled rate freezing container*. Do not let vials stand in ice for longer than 30 minutes as cell viability will be compromised.
4. Place the freezing container with the vials in a -80^0 C freezer and let freeze for 24 hours before transferring to liquid nitrogen freezer unit for long term storage.

* Two Styrofoam 15 mL conical tube racks, placed opening to opening to form a chamber may be used as an alternative to the Mr Frosty container if necessary when freezing PBMC. Tape sides of racks together before placing in freezer.

Procedure for Thawing:

1. 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.
2. 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 freezing medium.
3. Using a one mL pipet, transfer the entire contents from the tube to a15ml conical polypropylene tube at ambient room temperature. Slowly add 5ml of warm** complete medium (RPMI 1640 +8% FCS (R8) at 25 to 37^o C) 1-2 drops at a time, while mixing carefully and thoroughly by swirling tube. Repeat for the second vial (if applicable). Add an additional 8ml of warm R8 to each tube before proceeding directly to next step.
4. Centrifuge tubes at 300 X g for 10 min at 20^0 C, remove supernatant, gently tap tube to resuspend cell pellet and add 10 ml warm R8. Centrifuge the tube again as above. Discard supernatant.
5. Resuspend to desired volume in medium. An aliquot of cell suspension is diluted in trypan blue exclusion dye for counting in a hemacytometer to determine recovery and viability.

** warm = 25 to 37^o C

Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-1007	New/draft only			23-Sept-2005
HIC-1-1007.1	Finalized/word changes only			21-Oct-2006
HIC-1-1007.2	Changed to 25 to 37^0 C temperature for wash medium for thawed cells	Will result in increased viability of frozen human PBMC for antigen specific lymphocyte	JIM 308 (2006) 13-18 Disis, M.L.et al. Maximizing the retention of antigen specific lymphocyte function after cryopreservation	27-Dec-2006

		function	and Quataert previous experience with cell thawing for vaccine trials.	