Freezing and Thawing of Peripheral Blood Mononuclear Cells (PBMCs)

Cryopreserved PBMCs are a common specimen source for future phenotypic and functional analyses. It is important to have an optimized protocol for freezing and thawing PBMCs to retain viability and function for future analyses by flow cytometry.

This protocol describes the freezing of PBMCs (with either 90% FBS/10% DMSO or 12.5% HSA in RPMI/10% DMSO) and the general thawing procedure with complete RMPI medium.

Reagents, Materials, and Equipment

Dimethyl sulfoxide (DMSO), Sigma D2650, or equivalent

Fetal bovine serum (FBS), Sigma F2442, or equivalent

Human Albumin Fraction V, low endotoxin, Serologicals Corp. 82-347-1, or equivalent (for example, human serum albumin [HSA], Gemini Bio-Products)

RPMI-1640, sterile, L-glutamine and HEPES supplemented, Sigma R 7388, or equivalent

Antibiotic/Antimycotic Solution, Sigma A9909, or equivalent

BD Vacutainer® CPT cell preparation tube (Cat. No. 362753), or equivalent

Trypan Blue, 0.4% solution, Sigma T8154, and hemacytometer (or equivalent method for cell number and viability determination)

70% alcohol (isopropanol)

1.8-mL or 3.6-mL cryovials, Nunc or equivalent

37°C water bath

Freezing containers, Nalgene® Mr. Frosty® or equivalent

Refrigerated tabletop centrifuge (for example, Sorvall RT6000 centrifuge)

Biological safety cabinet Class II (NuAire, Baker, or equivalent)

Sterile polypropylene conical tubes, 50 mL, Corning Life Sciences 353077, or equivalent

200-µL micropipettor (PIPETMAN® or equivalent) with sterile tips

Serological pipettor (Pipet-Aid® or equivalent)

2–8°C refrigerator

-80°C freezer (or liquid nitrogen tank for long-term storage)

BD

Procedural Notes

- Before you begin, review the guidelines for preparing freezing medium. Other media options can be considered, for example, X-VIVO™ (pH indicator free) media and/or less DMSO condition (7.5%) or specialized freezing solutions and media (higher grade DMSO). Some preparations include deoxyribonuclease I (Sigma #D5025). A 1% HEPES (of 100 mM) solution is often included to better control the pH. This protocol is intended for non-fixed cells.
- We describe the freezing options for fixed cells in related BD product TDSs or manuals. See the following examples.
    - Intracellular cytokine staining protocol, which includes the freezing option in 10% DMSO + 90% FCS on fixed cells as an alternative protocol (Cat. Nos. 554714/554715/555028) http://www.bdbiosciences.com/ds/ab/others/554714_554715_555028%20Book%20_%20Website.pdf
    - The BrdU Flow Kit staining protocol describes the freezing option in 10% DMSO + 90% FBS on fixed cells (Cat. No. 559619/557891, 552598/557892) http://www.bdbiosciences.com/ds/pm/others/23-12721.pdf
    - The Transcription Factor Phospho Buffer Set protocol describes the freezing option on fixed/permed cells in FBS with 7.5% DMSO and a slow cold thawing. This protocol also describes the use of plates (Cat. No. 563239) http://www.bdbiosciences.com/ds/pm/tds/563239.pdf
- We recommend preparing the reagents for cryopreservation about 24 hours in advance. If they are not prepared in advance, make sure the cell pellet is disrupted, and keep the cells on ice until all reagents are prepared.
- Follow the instructions for thawing cells carefully. It is important for cell viability that the cells are thawed and processed quickly. If PBMCs are not thawed properly, the cell viability and recovery could be compromised, and the cells might not perform optimally in functional assays.
- Once thawed, cells can be used directly or rested overnight at 37°C before performing the functional assay. Following resting, cells can be washed in culture media and re-counted prior to use (for example, for stimulation).
- Each lab may need to determine and optimize the conditions for critical factors that would affect subsequent specific functional studies.

Preparation of Working Reagents

Prepare either reagent A or B for cryopreservation in advance.

A. 2X freezing medium of 90% FBS/10% DMSO

1. Inactivate the FBS in a 56°C water bath for 30 minutes. (The heat-inactivated FBS will be used for resuspension of PBMCs and for preparation of the final freezing medium.)
2. To prepare the final freezing medium, add 5 mL of DMSO to 20 mL of heat-inactivated FBS (as an example).
3. Store at 4°C (in a 2–8°C refrigerator).

B. 2X freezing medium of 12.5% HSA/10% DMSO and PBMC resuspension medium (12.5% HSA in RPMI)

1. Prepare a stock solution of 25% HSA, for example, by dissolving 25 g of human albumin fraction V into 100 mL of RPMI (not complete RPMI).
2. Allow 24 hours to fully dissolve. Store at 4°C (in a 2–8°C refrigerator).
3. Prepare a 12.5% HSA in RPMI solution. For example, combine 10 mL of stock 25% HSA and 10 mL of sterile RPMI-1640 (not complete RPMI).
4. Store at 4°C (in a 2–8°C refrigerator).
5. Prepare the final freezing medium preparation by combining 20 mL of 12.5% HSA in RPMI-1640 medium (prepared in step 3) and 5 mL of DMSO.
6. Store at 4°C (in a 2–8°C refrigerator).

C. Complete RPMI for PBMC resuspension for thawing procedure

1. Supplement sterile RPMI-1640 medium with 10% sterile heat-inactivated FBS and 1% sterile antibiotic/antimycotic solution.
2. Store at 4°C (in a 2–8°C refrigerator).

PBMC Preparation

1. Perform the PBMC isolation using either a Ficoll density gradient method or CPT tube method according to the manufacturer's instructions (for example, BD Vacutainer CPT tube).
2. Transfer the cell suspension collected from either CPT tube(s) or Ficoll tube(s) to a 50-mL conical polypropylene tube, pooling the cells from each tube if there are multiple tubes per donor.
3. Add complete RMPI medium to a total of 40 mL.
4. Remove a 10-μL aliquot of cell suspension (or appropriate volume) for cell counting and viability determination (for example, by the Trypan Blue method or by another cell counter method as available).
5. Centrifuge 50-mL tubes at 250*g* (approximately 1,200 rpm on a Sorvall RT6000 centrifuge) for seven minutes at room temperature.
6. Count cells and determine the viability with a hemacytometer (or other equivalent method) while tubes are centrifuging.
7. When centrifugation is complete, aspirate the supernatant and gently flick the tube with a finger to break up the pellet.

Freezing of PBMCs

Follow step A or B depending on the choice of freezing medium, and then continue with step C.

A. Freezing medium 12.5% HSA /10% DMSO

1. Resuspend PBMCs at 5–10 million viable cells/mL in 4ºC 12.5% HSA in RPMI medium, in a 50-mL conical polypropylene tube.
2. While gently swirling the tube, add enough 4ºC 2X freezing medium (12.5% HSA/10% DMSO), drop-by-drop, to double the volume of the cell suspension.
3. Immediately place the tube on ice.
4. Avoid any further mixing or agitation of the cells.

B. Freezing medium 90% FBS/10% DMSO

1. Resuspend PBMCs at 5–10 million viable cells in 4ºC heat-inactivated FBS.
2. While gently swirling the tube, add enough 4ºC 2X freezing medium (90% FBS/10% DMSO), drop-by-drop, to double the volume of the cell suspension.
3. Immediately place the tube on ice
4. Avoid any further mixing or agitation of the cells.

C. Freezing using cryovials

1. Keep the cryovial(s) on ice before dispensing the cell suspension.
2. Slowly remove the cell suspension into a pipet and dispense 1 mL per cryovial on ice.
3. Place the cryovial(s) in a pre-cooled Mr. Frosty-style freezing container that has been filled with 70% isopropanol according to the manufacturer's instructions.
4. Place the cryovial(s) in the freezing container at -80ºC.
5. [OPTIONAL] Once the cryovial(s) are frozen, transfer them into liquid nitrogen for long-term storage (if available).

Thawing of PBMCs

1. Warm complete RPMI medium to 37ºC in a 37ºC water bath before beginning the thawing procedure.
2. Transfer the cryovial(s) from the liquid nitrogen to a 37ºC water bath. If liquid nitrogen has seeped into the cryovial, loosen the cap slightly to allow the nitrogen to escape during thawing.
3. Hold the cryovial in the surface of the water bath and occasionally flick it gently during thawing. Do not leave the cryovial unattended during the thawing process. Thawing takes only a minute or two.
4. When a small bit of ice chip remains in the cryovial, transfer the cryovial to the biosafety hood.
5. Dry off the outside of the cryovial and wipe with disinfectant (for example, 70% alcohol) before opening to prevent contamination.
6. Add warm complete RPMI medium drop-by-drop into the cryovial containing the cell suspension, slowly over a 40-second period. The final volume should be twice the volume of the cell suspension (for example, add 1 mL of complete RPMI medium to a cryovial containing 1 mL of cell suspension). Be careful not to exceed the capacity of the cryovial.

7. Transfer the diluted cell suspension to a 50-mL polypropylene centrifuge tube containing 8 mL of warm complete RPMI medium for every vial of cells added (multiple cryovials from the same donor can be combined into one 50-mL tube, if desired).
8. Centrifuge the cells at 250*g* (approximately 1,200 rpm on a Sorvall RT6000 centrifuge) for seven minutes.
9. Decant the supernatant and gently flick the tube with a finger to break up the pellet.
10. Resuspend the pellet in desired volume of warm complete RPMI medium.
11. Determine the cell number and viability with a hemacytometer using the Trypan Blue method (or other equivalent method).
12. If cells require concentrating, centrifuge at 250*g* (approximately 1,200 rpm on a Sorvall RT6000 centrifuge) for seven minutes.
13. Decant the supernatant and gently flick the tube with a finger to break up the pellet.
14. Dilute to a final working concentration (for example, 5 x 10^6 PBMCs/mL for flow cytometry) in room temperature complete RPMI medium.
15. Check for clumps and remove them with a pipettor tip.

References

1. Disis ML, dela Rosa C, Goodell V, et al. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. *J Immunol Methods.* 2006;308:13. [PubMed: 16337957]
2. Maecker HT, Moon J, Bhatia S, et al. Impact of cryopreservation on tetramer, cytokine flow cytometry, and ELISPOT. *BMC Immunol.* 2005;6:17. [PubMed: 16026627]
3. Weinberg A, Zhang L, Brown D, et al. Viability and functional activity of cryopreserved mononuclear cells. *Clin Diagn Lab Immunol.* 2000;7:714-716. [PubMed: 10882680]
4. Weinberg A, Song LY, Wilkening C, et al: Pediatric ACTG Cryopreservation Working Group. Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin Vaccine Immunol.* 2009;16:1176-1186. doi: 10.1128/CVI.00342-08. Epub 2009 Jun 10. PubMed PMID: 19515870; PubMed Central PMCID: PMC2725535.
5. Tollerud DJ, Brown LM, Clark JW, et al. Cryopreservation and long-term liquid nitrogen storage of peripheral blood mononuclear cells for flow cytometry analysis: effects on cell subset proportions and fluorescence intensity. *J Clin Lab Anal.* 1991;5:255-261. [PubMed: 1890539]
6. Romeu MA, Mestre M, Gonzalez L, et al. Lymphocyte immunophenotyping by flow cytometry in normal adults. Comparison of fresh whole blood lysis technique, Ficoll-Paque separation and cryopreservation. *J Immunol Methods.* 1992;154:7-10. [PubMed: 1401946]
7. Kreher CR, Dittrich MT, Guerkov R, Boehm BO, Tary-Lehmann M. CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. *J Immunol Methods.* 2003; 278:79-93. [PubMed: 12957398]
8. Costantini A, Mancini S, Giuliodoro S, et al. Effects of cryopreservation on lymphocyte immunophenotype and function. *J Immunol Methods.* 2003;278:145-155. [PubMed: 12957403]
9. Ghanekar SA, Bhatia S, Ruitenberg JJ, et al. Phenotype and in vitro function of mature MDDC generated from cryopreserved PBMC of cancer patients are equivalent to those from healthy donors. *J Immune Based Ther Vaccines.* 2007;5:7. PubMed PMID: 17477875; [PubMed Central PMCID: PMC1868730].
10. Owen RE, Sinclair E, Emu B, et al. Loss of T cell responses following long-term cryopreservation. *J Immunol Methods.* 2007; 326:93-115.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.
Mr. Frosty and Nalgene are registered trademarks of Thermo Fisher Scientific and its subsidiaries.
PIPETMAN is a registered trademark of Gilson, Inc.
Pipet-Aid is a registered trademark of Drummond Scientific Company.
X-VIVO is a trademark of Lonza Group Ltd.
BD, BD Logo and all other trademarks are property of Becton, Dickinson and Company. © 2015 BD
23-18041-00

BD Biosciences
bdbiosciences.com