Department of Health & Human Services
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CHI Standard Operating Procedures for Isolation, Cryopreservation and Storage of Human Peripheral Blood Mononuclear Cells (PBMCs) and RNA

Summary

Human PBMCs isolated using a ficoll gradient are cryopreserved with a freezing medium containing the intracellular cryoprotectant dimethylsulfoxide (DMSO) 10% plus 90% heat inactivated Fetal Bovine Serum (HI-FBS). Cells suspended in the freezing medium are cryopreserved initially in a controlled rate freezer to a temperature of -120°C to minimize cell damage, and are then transferred into a liquid nitrogen (LN2) tank in the vapor phase of the tank at -156°C (+/- 20°C) until thawed for use.

Equipment and Materials

Description	Catalog number	Manufacturer	Specifications
Dimethylsulfoxide DMSO	D128500 or equivalent	Fisher Scientific or equivalent	
Ficoll-Paque Plus (plus ensures low level of endotoxin)	17-1440-03 or equivalent	Amersham Pharmacia Biotech or equivalent	
Falcon Blue Max 50 ml Polypropylene conical tube	352098 or equivalent	Becton Dickinson Labware or equivalent	30 X 115 mm style, Sterile
Legend™ RT Refrigerated Tabletop Centrifuge	75004377 or equivalent	Kendro Laboratory Products or equivalent	Max speed: 15000rpm Max capacity: 3L
HI_FBS	100-106H	Gemini	
Various size pipette(s), pipette tips	NA	Any suitable lab supplier	

Cryogenic vial	5000-0020	Nalgene	
Ice bucket	NA	Any suitable lab supplier	
Cryo-tube rack	NA	Any suitable lab supplier	
Planer 750Plus Controlled Rate Freezer	Kryo 750 - 30	Planer PLC	
QIAzol Lysis Reagent (200 ml)	79306	Qiagen	
Countess cell counter	C10310	Invitrogen	
PBS	10010-072	Invitrogen	
LeucosepTM tube, 50 ml, sterile	227 290	Greiner Bio-One	

Procedure

1. *If cryopreservation is planned for the isolated PBMCs, prepare the freezing medium (90% HI-FBS + 10% DMSO) at this time. Sterilize the freezing medium by 0.22-micron filtration. Freezing medium must be prepared daily (discard any excess from the previous day) and must be chilled to 4℃ in a 4℃ refrigerator prior to use.*

2. *Ensure Ficoll is at room temperature before proceeding.*

3. *Add 15ml of Ficoll-Paque Plus to each Leucosep 50ml tube and centrifuge at 1000xg at RT for 30 seconds so that the Ficoll-Paque Plus passes through the barrier.*

4. *Label the Leucosep 50 ml centrifuge tubes with the subject's number (specific for each subject and each protocol) and the medical record number (MRN). The number of tubes needed for each subject is = total volume of heparinized blood/15-20ml.*

5. *Add 15ml of 1X PBS to each tube*

6. *If blood sample is received in a syringe, draw some air into the syringe before inverting it at least five times.*

7. *Deliver 15-20ml of blood directly from the syringe to the Leucosep tube with Ficoll and PBS present (both 15ml and 20ml blood have been tested and have the same isolation efficiency).*

8. *Invert the tube twice to mix PBS with the blood before centrifugation (Ficoll is locked below the barrier therefore it is safe to invert the tube).*

9. *Set the acceleration and brake speed of the centrifuge to 9 and 1 respectively.*

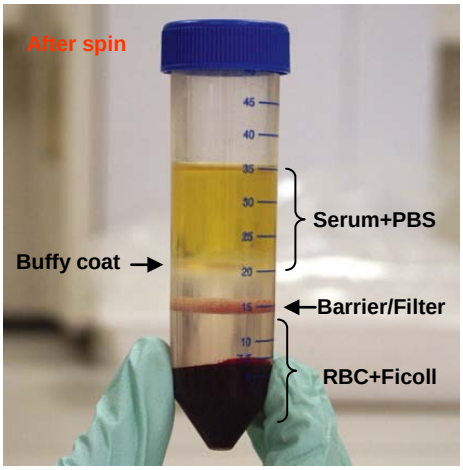
10. *Spin balanced tubes at 863xg for 20 minutes at 22℃ .*

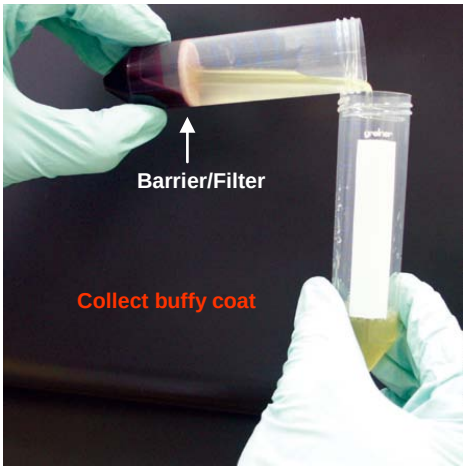
11. *While the tubes are spinning, label 1.8 ml cryovials with a barcode linked to the following information:*

- *Protocol ID*
- *Subject and protocol specific number*
- *Medical record number*
- *Time and date of sample collection*
- *Cell number/ml*
- *Cell type*

We estimate the number of vials needed based on an estimated yield of 1e6 cells/ml blood. We freeze cells based on actual cell count (see below) with 4 vials containing 1 ml of 5e6 cells/ml and the remainder containing 1 ml of 1e7 cells/ml. Place the labeled cryovials in an ice tray and store in a 4℃ refrigerator until needed.

12. *When tubes finish spinning, carefully remove them, and without disturbing the layer place them in a rack.*





13. *Aspirate the top layer (consisting of serum and PBS, see Figure above left) from each tube and leave about 5-7ml of serum without disturbing the buffy layer.*

14. *Pour top layer of buffy coat to a new labeled conical 50ml tube (see Figure above right).*

15. *For the washing step, two buffy coats from the same subjects can be combined into one tube. Bring the total volume up to 50ml with 1XPBS. Invert the tube at least 5 times to facilitate mixing.*

16. *Wash the samples by centrifuging at 300xg for 10 minutes at 22℃ with both acceleration and brake set to 9.*

17. *After centrifugation, using a 2 ml aspirating pipette connected to a vacuumtainer, aspirate the supernatant into the vacuum waste container except the last 2.5ml.*

18. *Resuspend the cell pellet with 10ml 1X PBS by pipetting up and down. Pool multiple tubes from the same subject together and rinse the tube(s) with 1 X PBS and bring the final volume to 50ml if the starting blood volume was 50 ml or greater. If the starting blood volume was less then suspend in the same volume as the starting blood volume.*

19. *Determine the cell recovery and viability by mixing 10μl of the above cell suspension with 10μl of trypan blue, adding to a hemocytometer, and counting on a Countess cell counter. Note: If the original blood volume was greater than 50 ml then the 10μl of the above suspension needs to be diluted with PBS using a dilution factor of calculated as: blood volume ml/50 ml in order to reach a cell concentration of approximately 1e6.*

20. **Turn on the controlled rate freezer and set to wait at 4oC. Move the freezing medium from the refrigerator to an ice bath adjacent to the biologic safety cabinet where the cells are being processed.**

21. *For RNA isolation, aliquot a volume of the cell suspension equal to 1e7 cell to a 15ml tube. Spin this tube and the 50 ml tube containing the rest of the cell suspension at 300xg for 10 min with both the acceleration and brake speed set to 9. Be sure to balance the tubes.*

22. *After centrifugation,*

    a. *Tube for RNA isolation: aspirate the supernatant completely and lyse the cell pellet in 700 ul of Qiazol by pipetting up and down. Transfer the cell lysate to a barcode labeled 1.7ml eppendorf tube and store at -80℃ immediately.*

    b. *Tube with the rest of the PBMC: aspirate the supernatant and resuspend the cell pellet in 1ml of cold freezing medium, mix well by pipetting up and down, then quickly add an additional volume of freezing medium to attain a concentration of 1e7cells/ml and mix well by pipetting up and down.*

23. *Aliquoting for cryopreservation:*

    a. *For aliquots of 1e7 cells: aliquot 1ml samples in freezing medium into 1.8ml barcode labeled cryovials with blue caps*

    b. *For 4 aliquots of 5e6 cells: take 2 ml of the cell suspension and add an additional 2 ml of freezing medium and mix well. Then add 1 ml into a 1.8 ml barcode labeled cryovials with green caps.*

As cryovials are filled place them in a controlled freezer aluminum rack on ice.

24. *Transfer cyrovials in the aluminum rack to CRF when the chamber temperature reaches 4 oC (the green light starts blinking and the CRF starts beeping). Start freezing process by pushing the green light button.*

25. *Cryopreserve according to the following program:*

    (1) 4 oC to -6 oC with rate -1 oC /min (in about 10 min)
    (2) -6 oC to -45 oC with rate -25 oC /min (in about 2min)
    (3) -45 oC to -20 oC with rate +10 oC /min (in about 2.5 min)
    (4) -20 oC to -45 oC with rate -1 oC/min (in about 25 min)
    (5) -45 oC to -120 oC with rate -10 oC/min (in about 8min)
    (6) Transfer sample on dry ice to LN2

26. Record and file freezing chart.

27. Record sample positions in LN