Immune Tolerance Network

ITN091AI VIBRANT: On-site PBMC isolation using SepMate tubes Standard Operating Procedure

Instructions: Study personnel must be trained and authorized by the site Principal Investigator to perform the on-site PBMC isolation using SepMate tubes procedure. Personnel are required to read and understand the following ITN091AI **On-site PBMC isolation using SepMate tubes Standard Operating Procedure**. The following procedure MUST be followed for all specimens.

1. PURPOSE

To describe the procedure for isolating and freezing PBMCs for assessment of longitudinal changes in immune cell populations, numbers and phenotypes.

2. REQUIRED SUPPLIES

Items Supplied by ITN:

- CryoStor CS10 (BioLife Solutions, Cat#210102)
- LymphoPrep (Stem Cell Technologies #085460)
- SepMate tubes (50mL, Stem Cell Technologies #85460)
- 1.8mL cryovials
- Sterile Conical Tubes (50 mL, two labeled “whole blood” and four labeled “PBMC”)
- Mr. Frosty Container (to be filled with isopropanol and pre-chilled at 4°C for at least 4 hours before use)
- Na Heparin blood collection tubes (10mL)

Items Supplied by Clinical Sites:

- Calcium and Magnesium Free DPBS
- 70% Ethanol
- Sterile disposable transfer pipettes
- Sterile Serological Pipettes (1 mL, 10 mL, and 25 mL)
- Ice bucket with wet ice
- Isopropanol

Equipment Supplied by Clinical Sites:

The following laboratory equipment is required for on-site PBMC isolation with SepMate tubes.

- Serological Pipettors
- Benchtop Centrifuge (swinging bucket with aerosol containment buckets, for 50mL conical tubes)
- BSL 1-2 Safety Cabinet
- -70°C to -80°C Mechanical Freezer (preferably on back-up power and with temperature monitoring)
- 4°C Refrigerator
- Flammable Safety Cabinet for Isopropanol Storage (preferred)

4. PROCEDURES

General Precautions

- Informed consent must be obtained from all participants before any research procedures, including specimen collection, are performed.
- Always follow appropriate precautions for handling of human specimens, including appropriate use of personal protective equipment.
- Perform all processing in a BSL 1-2 Safety Cabinet.
- Always use sterile technique.
- Always prepare materials and workspace by wiping with 70% ethanol.
- Before working with isopropanol, individuals should be trained in its proper handling and storage. For reference: http://www.labmanager.com/lab-health-and-safety/2008/12/working-with-isopropyl-alcohol?fw1pk=2#.VsuyffkrLIU.

Storage and Preparation of Reagents

- Store all reagents at 4°C, unless otherwise noted. DPBS and LymphoPrep should be stored at 4°C but brought to room temperature before use.
- Sterile DPBS, LymphoPrep and CryoStor CS10 are cGMP-grade solutions. Use sterile technique when handling these reagents at all times. This includes minimizing exposure of solution to environmental contaminants.
- Be sure the Mr. Frosty container is filled with isopropanol and pre-chilled at 4°C for at least four hours before use. Note that the isopropanol in the Mr. Frosty should be replaced after every five uses, per manufacturer's recommendation.
- Isopropanol: The compound should be stored in a tightly closed container in a cool, dry, well-ventilated area away from incompatible substances. It should be kept away from heat, sparks, flames

and other sources of ignition, as well as strong oxidizers, acetaldehyde, chlorine, ethylene oxide, acids, and isocyanates. Isopropyl alcohol is highly flammable and can easily ignite. Vapors may form explosive mixtures with air, traveling to a source of ignition and flash back. Use of water spray to fight fires may be inefficient. A flammable safety cabinet is the best storage option.

On-site PBMC isolation using SepMate tubes procedure

1. Ensure that blood sample, DPBS, LymphoPrep, and centrifuge are all at room temperature, and Mr. Frosty filled with isopropanol is pre-chilled at 4°C.

2. Using an alcohol-proof black pen, add Participant ID to labels on all tubes. For 40mL of blood this includes eight 1.8 mL cryovials, six 50 mL conical tubes (two labeled "whole blood" and four labeled "PBMC"), and four SepMate tubes. For blood volumes < 40 mL, see Table 1 below to determine how to adjust the procedure and determine appropriate number of tubes and volumes.

Part 1: Prepare blood and SepMate tubes

3. Using a sterile 10 mL serological pipet, transfer the contents of four 10 mL Heparin tubes into a single 50 mL sterile conical tube labeled "whole blood" to combine them.

4. Using a sterile 25 mL serological pipet, transfer half of the pooled blood (~20 mL) into a second 50 mL sterile conical tube labeled "whole blood".

5. Using a sterile 25 mL serological pipet, add an equal volume of DPBS to each 50 mL conical tube of blood (ie, 20 mL DPBS to 20 mL blood) and mix well by slowly pipetting up and down five times.

6. Using a sterile 25 mL serological pipette, carefully add 15 mL of LymphoPrep into the central hole of each of four SepMate tubes. The top of the LymphoPrep will be above the insert in the SepMate tube.

Part 2: Use SepMate tubes to separate PBMCs

7. Using a sterile 25 mL serological pipet, carefully transfer 20 mL of diluted blood to each SepMate tube by pipetting it gently down the side of the tube, being sure to keep the SepMate tube upright. The LymphoPrep will be on the bottom of the tube while the diluted blood will be on top. The diluted blood will mix slightly with the LymphoPrep above the insert.

8. Place the four SepMate tubes in appropriate centrifuge buckets and balance them. Centrifuge the SepMate tubes at 1200g for 10 minutes at room temperature with the brake on. The diluted plasma in the top of the SepMate tubes contains the PBMC that will be collected.

9. Decant the PBMC and diluted plasma from each SepMate tube into a new 50 mL conical tube labeled "PBMC" by inverting quickly and in a single motion. Invert each SepMate tube no longer than 2 seconds to avoid red blood cell contamination. The red blood cells remaining in the bottom of the SepMate tubes can be discarded. Repeat with each SepMate tube being decanted into a separate new 50 mL conical labeled "PBMC".

Part 3: Wash and combine PBMCs

10. Using a sterile 25mL serological pipet, add ~30 mL of DPBS to each conical tube of PBMCs to bring the volume up to 50mL.

11. Place the four 50 mL conical tubes containing the PBMCs in appropriate centrifuge buckets and balance them. Centrifuge at 250g for 10 minutes at room temperature with the brake on.

12. Using a disposable transfer pipet, carefully remove and discard the supernatant from each tube. To prevent cell loss, avoid disturbing the cell pellet at the bottom of the tube.

13. Using sterile 10 mL serological pipettes, add 10 mL DPBS to the cell pellet in each 50 mL conical tube, and re-suspend by gently pipetting up and down 5-10 times. Using a 10mL serological pipet, combine the cells from all four 50mL conical tubes into one of the 50 mL conical tubes. Do not discard the three empty tubes yet.

14. Use 10 mL of DPBS to rinse the three empty conical tubes to recover any remaining cells and add it to the pooled cells (use the same 10mL for all three tubes). The result should be one 50 mL conical tube containing all the cells in 50 mL DPBS, plus three empty conical tubes that can now be discarded.

15. Place the conical tube containing cells in an appropriate centrifuge bucket and balance. Centrifuge at 250g for 10 minutes at room temperature with the brake on.

16. Using a disposable transfer pipet, carefully remove and discard the supernatant. To prevent cell loss, avoid disturbing the cell pellet at the bottom of the tube.

Part 4: Combine, aliquot, and freeze PBMCs

17. Wipe down the outside of the CryoStor CS10 cryopreservation media container with 70% ethanol or isopropanol before opening.

18. Using a sterile 10 mL serological pipet, resuspend the cell pellet in 4 mL of CryoStor CS10 by gently pipetting up and down 5-10 times. (Refer to Table 1 for volume of CryoStor CS10 to be used to resuspend the cell pellet for initial blood volumes < 40 mL.)

19. Using a sterile 1 mL serological pipet, aliquot 0.5 mL of the cells into eight 1.8 ml pre-labeled cryovials, and ensure caps are sealed. (For blood volumes < 40 mL, adjust the number of cryovials so that each cryovial contains 0.5 mL of cells.) If there is any liquid left-over in the 50 mL conical tube containing the resuspended cells, add it to the last cryovial (up to 0.5mL).

20. Place cryovials in a Mr. Frosty container (filled with isopropanol and pre-chilled at 4°C).

21. Keep the Mr. Frosty on ice for 10 minutes, then transfer to a -70 to -80°C freezer.

22. After 15 minutes, slap the Mr. Frosty container to facilitate nucleation, and return the Mr. Frosty to the -70 to -80°C freezer for a minimum of 24 hours and maximum of 7 days.

23. Transfer the cryovials from the Mr. Frosty to a storage box and store at -70 to -80°C until shipment.

Adjustments to SepMate PBMC processing according to available blood volume

If the total blood volume available for SepMate PBMC processing is < 40 mL, adjustments will need to be made to the procedure, according to Table 1, below. Be sure to record the total blood volume processed, as well as the number of SepMate tubes used and enter in LabVantage.

Table 1: Processing adjustments according to blood volume				
Total Blood Volume (undiluted)	10 mL	20 mL	30 mL	40 mL
Total Blood Volume (diluted 1:1)	20 mL	40 mL	60 mL	80 mL
Number of SepMate tubes (Step 6-8)	1	2	3	4
CryoStor CS10 volume (Step 18)	1 mL	2 mL	3 mL	4 mL
Number of cryovials (0.5 mL/vial)	2	4	6	8

For intermediate blood volumes

- For intermediate blood volumes round up to the next highest volume listed in Table 1 to find the appropriate number of SepMate tubes, volume of CryoStor CS10, and number of cryovials.
    - For example, for 24 mL undiluted blood, round up to 30 mL to determine that you will need 3 SepMate tubes, 3 mL of CryoStor CS10, and 6 cryovials.
- Step 5: Dilute blood with an equal volume of DPBS, as in the standard procedure.
    - For example, dilute 24 mL blood with 24 mL DPBS for a total of 48 mL diluted blood.
- Step 7: Divide diluted blood equally between the appropriate number of SepMate tubes. Each SepMate tube should contain a maximum of 20 mL and a minimum of 8 mL of diluted blood.
    - For example, divide 48 mL diluted blood equally between 3 SepMate tubes (16 mL/tube).
- Step 13: If you have 3 conical tubes of cells rather than 4, cells from the third tube can be pooled with either of the other two tubes.
- Step 20: Be sure to use 2, 4, 6 or 8 cryovials, each with 0.5 mL of CryoStor CS10.
    - For example, for 24 mL of blood, purified with 3 SepMate tubes, resuspend the cell pellets in a total volume of 3 mL, and place 0.5 mL in each of 6 cryovials.

Visual Summary of on-site PBMC isolation using SepMate tubes procedure