clsa élcv Canadian Longitudinal Study on Aging Étude longitudinale canadienne sur le vieillissement	**Title:**	**Processing of the Leucosep Tube**		
	Version Date:	2017-APR-25	**Document Number:**	SOP_BCP_0004
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Data Collection Site (DCS)	**Version:**	5.2	**Number of Pages:**	11

1.0 Purpose:

The purpose of this document is to describe the procedure for processing the Leucosep tube as well as the isolation of mononuclear cells from human peripheral blood and cryopreservation for long term storage.

2.0 Scope:

This document is to be used by all DCS laboratory staff when processing blood samples collected in the Leucosep tube.

3.0 Responsibilities:

It is the responsibility of the CLSA laboratory staff to perform procedures as described in the current and approved version of the standard operating procedure.

4.0 Related Documents:

- **CHA_BCP_0003_2** – Matrix Storage Box – Mapping of Biospecimen Aliquots
- **SOP_BCP_0003** – Biospecimen Post Collection Processing
- **SOP_BCP_0030** – VisonMate 2D Barcode Reader – Operation, Maintenance & Calibration
- **SOP_BCP_0031** – Code Reader 3500 - Operation, Maintenance & Calibration
- **SOP_BCP_0032** – VisionMate 1 Wireless Single Tube Scanner – Operation & Maintenance
- **SOP_BCP_0033** - 8 Channel Decapper, Thermo Fischer Scientific
- **SOP_BCP_0034** – Eppendorf adjustable 100-1000ul Pipette– Operation, Maintenance & Calibration
- **SOP_BCP_0037** – Eppendorf 5702R Centrifuge – Operation, Maintenance & Calibration
- **SOP_BCP_0040** – BioCision CoolCell - Operation and Maintenance
- **SOP_BCP_0080** – Packing and Shipping Vapor Shipper from Site to Biorepository and Bioanalysis Centre (BBC)
- **SOP_BCP_0406** – Site Specific - Waste Disposal SOP
- **MAN_BCP_0217_2** – LabWare Guide

5.0 Definitions:

- **Peripheral Blood Mononuclear Cells (PBMC):** Any blood cell having a round nucleus. For example, a lymphocyte, monocyte, or macrophage.

- **Leucosep Cell Preparation Tube:** an evacuated tube separation medium and porous barrier used for the processing of whole blood and separation of mononuclear cells.
- **Hemolysis:** the rupture or destruction of red blood cells.
- **Lipemia:** the presence in the blood of an abnormally high concentration of emulsified fat.

6.0 Equipment:

- Brady Code Reader 3500 – Barcode Scanner;
- Centrifuge, Eppendorf 5702R with rotor A-4-38;
- Freezer, Isotemp Ultra Low Temperature Undercounter;
- Pipette, Eppendorf Model Research plus adjustable 100-1000 µL;
- Pipetting Station, Argos FlexiRack;
- Refrigerator, AGA Marvel Model 6CADM;
- 2D barcode box scanner, Thermo Fischer Scientific Visionmate;
- Individual tube scanner, Thermo Fischer Scientific Visionmate One;
- 8 Channel Decapper, Thermo Fischer Scientific; and,
- BioCision CoolCell, 12 chambers.

7.0 Supplies:

- 10 mL Conical tubes, sterile;
- Non-latex gloves, disposable;
- Cryogenic gloves;
- Liquid waste container;
- 2D coded Matrix Screw top storage tubes (0.5 mL, V-bottom);
- Disposable, sterile transfer pipettes;
- 1x Phosphate Buffered Saline (PBS);
- RPMI 1640 with 12.5% human serum albumin and 12.5% DMSO (StemCell Technologies custom order);
- 70% Isopropyl Alcohol;
- Sharps disposal container;
- Small absorbent gauze pad;

- Leucosep tube, filled;
- 50 mL centrifuge tube;
- 16 mm plastic SAFETY-T-FLEX CAPS;
- Bench protector sheets; and,
- Bleach.

8.0 Procedure Steps:

Overview

This protocol involves separating blood into different layers and extracting the PBMC layer to be washed and stored. Be cognizant when separating layers, where one ends and the other begins so as not to disturb the cell pellets or layers when aspirating supernatant. The preparation of PBMCs requires the use of sterile technique to ensure that cells remain viable, and free of any bacterial/fungal contamination that could negatively affect downstream applications. Leucosep tubes must be processed and frozen within 6 hours.

Sterile techniques involve:

- New gloves when starting the procedure and changing gloves when soiled/contaminated.
- Wearing gloves at all times when handling equipment, supplies or samples.
- Spraying exterior surfaces with 70% isopropyl to clean the bench top, pipettor, pipette tip boxes, PBS bottles, blood tubes and gloves before processing each batch.
- New pipette tip for each aspiration/dispense cycle, sample and tube.
- New transfer pipette for each sample at each step.
- Change tips or pipettes if they have accidentally contacted another surface.
- Closing lids on reagents and samples when not in use.
- Pour off working quantities from large stock bottles rather than insert pipettes into stock bottles.
- Replace soiled bench protector sheets.
- Discarding reagents **if appearance is altered,** e.g. cloudy or colour change.

Keep in mind cells are fragile after the isolation and washing steps. Gradual exposure to freezing temperatures and media will help maximize cell viability. Thus, it is important to "*Dropwise"* add the 4°C freezing media so as not to shock the cells.

It is also important to maximize the yield from each sample with careful pipetting and gentle handling of cells.

8.1 Preparation

8.1.1 Keep the Leucosep and filled 6ml EDTA tubes at room temperature at all times throughout the procedure. The media within the Leucosep tube (Ficoll) only works to separate PBMC at room temperature.

8.1.2 Check that there is an in-date container (within 1 week of thaw date) of RPMI 1640 with 12.5% human serum albumin and 12.5% DMSO (PBMC freezing media) thawed in the Refrigerator.

8.1.3 Discard any empty or out-of-date containers of PBMC freezing media.

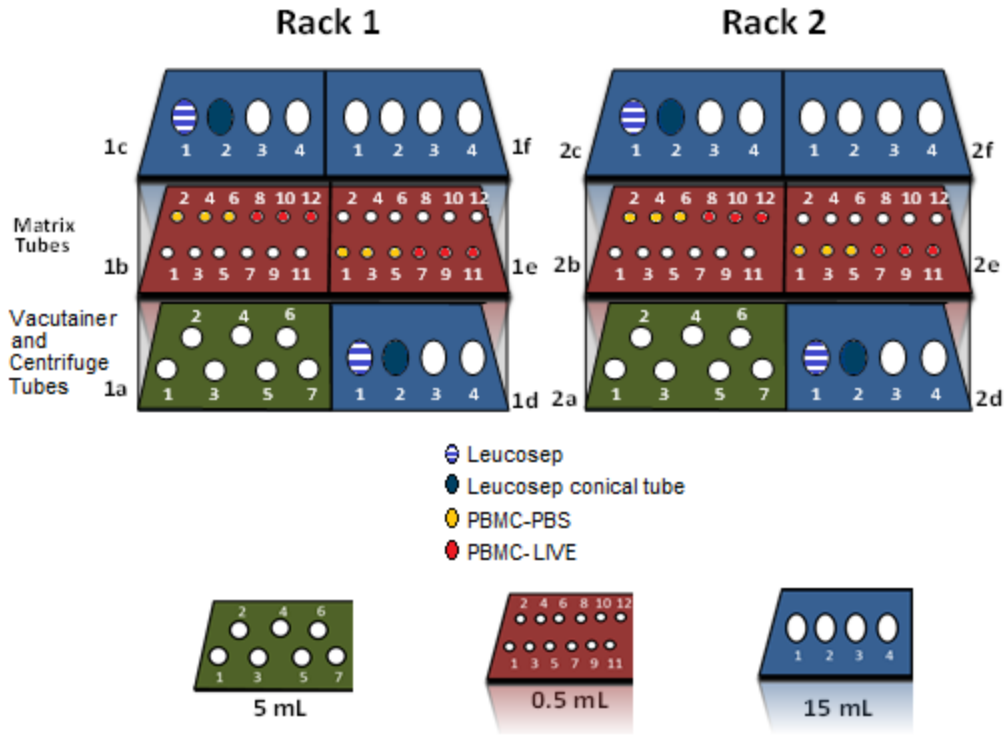
8.1.4 If required, remove a new container of the PBMC freezing media from the Isotemp Ultra Low Temperature Undercounter Freezer and thaw at 2-8°C.

8.1.5 Record thaw date and expiry date (1 week from thaw date) on any container of PBMC freezing media when it is moved from the freezer to the fridge.

8.1.6 Set-up FlexiRack Pipetting Station. Use the appropriate inserts and empty matrix tubes. Refer to **Figure 1.**

8.1.7 Assemble the BioCision CoolCell.

Figure 1. Set-up of the FlexiRack Pipetting Station for Processing of the Leucosep Tube.



8.2 First Centrifugation

8.2.1 Refer to *SOP_BCP_0037 - Eppendorf 5702R Centrifuge – Operation, Maintenance & Calibration* for instructions on using the centrifuge.

8.2.2 Mix filled EDTA tube gently by inversion (8-10 times).

8.2.3 In LabWare, in **Sample Processing**, press the **Pouring** button.

8.2.4 Remove the stopper from the Leucosep tube and filled EDTA tube. Pour contents of filled EDTA tube into the Leucosep tube and recap both.

NOTE: If batching, repeat steps 8.2.2 to 8.2.4 for each participant.

NOTE: Prior to centrifugation, the whole blood sample rests above the porous Leucosep barrier.

8.2.5 In LabWare, in **Sample Processing**, under **Centrifuge Single Sample**, press the **Start** button.

8.2.6 Scan the linear barcode label on the Leucosep tube(s). Ensure the correct date and time are entered in the pop-up window.

8.2.7 Balance the Leucosep tube(s) in the A-4-38 rotor.

8.2.8 Close the centrifuge lid.

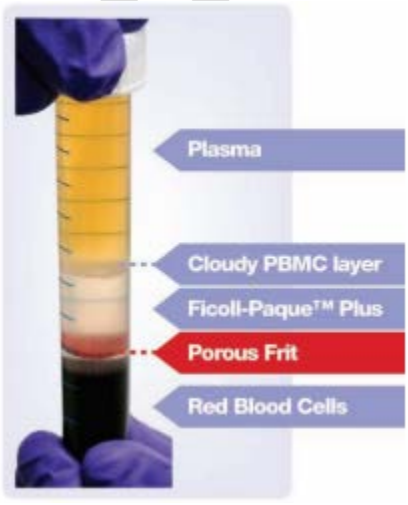
8.2.9 Centrifuge at 1.0 rcf (1000 g) for 10 minutes at 22°C with the SOFT brake turned **ON**.

8.2.10 Remove the Leucosep tube(s).

8.2.11 Confirm that the layers in the Leucosep tube(s) look as they should. Refer to **Figure 2**.

8.2.12 In LabWare, in **Sample Processing**, under **Centrifuge Single Sample**, press the **Stop** button and scan the linear barcode label on the Leucosep tube(s). Ensure the correct date and time are entered in the pop-up window.

Figure 2. Leucosep Tube Post First Centrifugation



8.2.13 Place in the FlexiRack Pipetting Station. Refer to **Figure 1.**

8.3 First Cell Wash

REMINDER

Label one sterile 10 mL conical tube for each Leucosep tube.

NOTE: It is important to label the Leucosep tube and the 10 mL conical tube with sequential numbers from one column of labels for a single participant (e.g., 70029 and 70030). Refer to *SOP_BCP_0003 – Biospecimen Collecting & Processing*.

8.3.1 Remove the cap from the Leucosep tube and place upside down on benchtop.

8.3.2 Use a new transfer pipette and slowly aspirate the plasma layer to within 1-2 mm of the PBMC layer. Starting at the top of the plasma, aspirate this layer at a constant rate, slowly following the liquid down the side of the tube. This will minimize movement and help to avoid disturbing the PBMC cell layer which may at this point resemble a 'cloud' rather than a distinct layer.

8.3.3 Discard the plasma in the liquid waste container.

8.3.4 Make certain that none of the plasma layer falls back into the tube as this will disrupt the PBMC layer and may lead to inadvertent removal of some of the PBMC layer. If you notice any disruption of the PBMC layer, stop collecting plasma and pour off the remaining sample above the porous barrier.

NOTE: Please note that the cell layer you removed must be less than half the volume of the PBS you are adding in order to maximize cell isolation.

8.3.5 Pour off the enriched cell layer into the corresponding sterile 10 mL conical tube. The porous barrier should prevent any mixing of the erythrocytes and granulocytes.

NOTE: If you notice any erythrocytes above the porous barrier please aliquot manually. This should happen very rarely.

8.3.6 Repeat procedure steps 8.3.1 to 8.3.5 for each Leucosep tube.

8.3.7 Pour PBS into each labeled sterile 10 mL conical tube up to the 10 mL mark.

NOTE: Don't pour PBS directly from the stock bottle. Instead, pour PBS into a sterile 50 mL centrifuge tube, correctly labeled, that you will dispose of at the end of the day.

8.3.8 Cap each conical tube and gently invert once by hand to mix. This inversion is to mix the PBS as the cells do not need any agitation for washing.

8.4 Second Centrifugation

8.4.1 In LabWare, in **Sample Processing**, under **Centrifuge Single Sample** press **Start** and scan the linear barcode label on the 10 mL conical tube. Ensure the correct date and time are entered in the pop-up window. Repeat for each 10 mL conical tube.

8.4.2 Centrifuge at 0.3 rcf (300 g) for 10 minutes at 22ºC with the SOFT brake turned **OFF**.

8.4.3 Open the centrifuge lid once the centrifuge has stopped. Remove the conical tubes. An opaque white cell pellet should be visible on the bottom of the conical tube.

8.4.4 In LabWare, in **Sample Processing**, under **Centrifuge Single Sample**, press **Stop** and scan the linear barcode label on the 10 mL conical tube. Ensure the correct date and time are entered in the pop-up window. Repeat for each 10 mL conical tube.

8.4.5 Place the conical tube in the FlexiRack Pipetting Station. Refer to **Figure 1**.

8.5 Second Cell Wash

8.5.1 Uncap each conical tube.

8.5.2 Use a new transfer pipette and aspirate the supernatant of a conical tube down to within 1-2 mL of the pellet (the cone portion of the conical tube), slowly following the fluid volume down the side of the tube at a constant rate.

8.5.3 Discard supernatant into the liquid waste container.

8.5.4 Gently re-suspend the cells in the remaining supernatant by slowly aspirating and dispensing the remaining liquid at least 5 times to lift and break up the pellet.

8.5.5 Repeat procedure steps 8.5.2 to 8.5.4 for each conical tube.

8.5.6 Pour PBS from the 50 mL centrifuge tube labeled as PBS into each labeled 10 mL conical tube up to the 10 mL mark.

8.5.7 Cap each conical tube and gently **invert once or twice** by hand to mix.

8.5.8 In LabWare, in **Sample Processing**, under **Centrifuge Second Spin**, press **Start** and scan the linear barcode label on the 10 mL conical tube. Ensure the correct date and time are entered in the pop-up window. Repeat for each 10 mL conical tube.

8.5.9 Place in the A-4-38 rotor in the centrifuge and balance.

8.5.10 Close the centrifuge lid.

8.5.11 Centrifuge at 0.3 rcf (300g) for 10 minutes at 22ºC with the soft brake turned **OFF**.

8.5.12 Open the centrifuge lid once the centrifuge has stopped.

8.5.13 Remove the conical tubes. An opaque white cell pellet should be visible on the bottom of the conical tube.

8.5.14 Observe the pellet. In LabWare, in **Sample Aliquotting**, click on **Sample Characteristics** and scan the linear barcode on the 10 mL conical tube. Fill in the fields required as described in *MAN_DCS_0217 – LabWare Guide*. Repeat for each 10 mL conical tube.

8.5.15 In LabWare, in **Sample Processing**, under **Centrifuge Second Spin**, press **Stop** and scan the linear barcode on the 10 mL conical tube. Ensure the correct date and time are entered in the pop-up window. Repeat for each 10 mL conical tube.

8.6 Aliquotting and Freezing PBMC

8.6.1 Uncap each 10 mL conical tube.

8.6.2 Use a new transfer pipette to aspirate the supernatant of each 10 mL conical tube down to within 1 mL of the pellet (the cone portion of the conical tube).

8.6.3 Discard supernatant into the liquid waste container.

8.6.4 Using the 1 mL pipette carefully remove the remaining supernatant from one conical tube. All supernatant has to be removed before adding 610 µL of PBS in order to get maximum cell concentration.

8.6.5 With a new pipette tip, add 610 µL of PBS and gently re-suspend. This is the PBMC cell suspension.

> **NOTE:** Pipetting up and down is necessary to break up the pellet but if the cells are re-suspended with too much force then cell damage will occur.

8.6.6 Repeat procedure steps 8.6.4 to 8.6.5 for each 10 mL conical tube.

8.6.7 Uncap the matrix tubes and place the screw tops upside down on a clean surface to avoid contamination.

8.6.8 Using a new pipette tip, add 400 µL of PBS to 3 of the 6 coded Matrix Screw Top storage tubes for each participant.

8.6.9 Using a new pipette tip, transfer 100 µL of the PBMC cell suspension to 6 Matrix Screw Top tubes for each conical tube.

8.6.10 In LabWare, in the **Aliquotting** stage, click on **0.5 mL Matrix Tube** and scan the 10 mL conical tube barcode label. Then, scan the 3 Leucosep PBS matrix tube aliquots with the individual tube scanner. Repeat for each conical tube.

8.6.11 Using a new pipette tip, add 400 µL of PBMC freezing media slowly by dropwise addition down the side of each of the 3 remaining Matrix Screw Top tubes in each set. This should take about 10 seconds per tube. Gently resuspend. Use a new pipette tip for each set of Matrix Screw Top tubes.

> **NOTE:** The PBMC freezing media has a pink colour so the 2 sets of PBMC will be visually differentiable.

8.6.12 In LabWare, in the **Aliquotting** stage, click on **Aliquot Live Cell** and scan the 10 mL conical tube barcode label. Then, scan the 3 matrix tube aliquots with media with the individual tube scanner. Repeat for each conical tube.

8.6.13 In LabWare, in the **Aliquotting** stage, under **1°C/min Freezing** section, press **Start.**

8.6.14 Place the matrix tubes with PBMC freezing media in an assembled BioCision CoolCell.

8.6.15 Cap the BioCision CoolCell and place in the Isotemp Ultra Low Temperature Undercounter freezer for 4 hours.

8.6.16 Place the matrix tubes with PBS in the corresponding Matrix storage box position for each participant. Refer to chart *CHA_BCP_003_2 - Matrix Storage Box – Mapping of Biospecimen Aliquots.*

8.6.17 Return any unused matrix tubes to bulk storage and record the aliquot as insufficient quantity in LabWare. *Refer to MAN_BCP_0217_2 – LabWare Guide*.

8.6.18 Cover the storage box with the lid and lock the matrix box.

8.6.19 In LabWare, in **Sample Aliquotting**, click on **Freeze Samples.** Scan the linear barcode label of the 10 mL conical tube(s)**.** Select the LS-PBS aliquots and check that the number of aliquots is correct.

8.6.20 Once you have selected all the samples you are about to freeze, press **OK**.

8.6.21 If there is any discrepancy between the numbers of aliquots you are going to store and number of aliquots listed in the dialog box or if you mistakenly mark samples with the wrong time, then please contact *lims@clsa-elcv.ca* and create a WebIssue immediately.

8.6.22 In LabWare, in **Sample Aliquotting**, click on **Scan Matrix Box** and scan the Matrix Box linear barcode. Copy the contents of the Matrix Box(es) data and paste the data into the pop-up window.

8.6.23 Place the Matrix Box(es) in the freezer.

> **Very Important:** Before finishing the participant, open the **Sample Folder** and ensure that beakers are full for all processed samples. In the event that the beaker is not full, complete the required steps outlined in the *MAN_BCP_0217_2 – LabWare Guide*. If the issue cannot be resolved, file a WebIssue at https://clsacloud.clsa-elcv.ca/webissues/client.

8.6.24 Verify that deviations in aliquot volumes, hemolysis, lipemia or other specimen comments have been logged in LabWare. *Refer to MAN_BCP_0217_2 – LabWare Guide.*

8.7 Retrieving Matrix Screw Top Tubes from the CoolCell for Long-term Storage

8.7.1 Place hands in Tempshield Water-Resistant Midarm Length Cryo-gloves.

8.7.2 Remove the BioCision CoolCell from the Isotemp Ultra Low Temperature Undercounter freezer.

8.7.3 Carefully remove the tubes from the BioCision CoolCell.

8.7.4 In LabWare, in the **Aliquotting** stage, under **1°C/min Freezing** section, press **Stop.** Scan the linear barcode(s) of the matrix tube(s).

8.7.5 Place the matrix tubes with PBMC freezing media in the corresponding Matrix storage box for each participant. Refer to chart *CHA_BCP_003_2 - Matrix Storage Box – Mapping of Biospecimen Aliquots.*

8.7.6 In LabWare, in **Sample Aliquotting**, click on **Freeze Samples.** Scan the barcode for any one aliquot. Select the LS-Live cells aliquots and check that the number of aliquots is correct. Repeat for each participant.

8.7.7 Once you have selected all the samples you are about to freeze, press **OK**.

8.7.8 Scan the Matrix storage box and place it in the freezer.

8.7.9 Recycle the BioCision CoolCell. The BioCision CoolCell should return to room temperature in 15 minutes if the core black ring is removed.

9.0 Documentation and Forms:

- **CRF_BCP_0001** – Blood Collection Case Report Form

10.0 References:

- Leucosep Instruction Manual
- Eppendorf Centrifuge 5702/ Centrifuge 5702R/Centrifuge 5702RH Manual. 2005

F1 Revision History:

New Version #	Revision Date	Revision Author	Content Approval
5.2	2017-APR-25	Chetna Naik	Cynthia Balion
Summary of Revisions			
Some formatting and other corrections made to clarify steps.			
Under overview added 'Replace soiled bench protector sheets' to list of sterile techniques.			
Removed Step 8.1.6 and 8.4.2 referring to replacing the rotor.			
Under 8.2.4, added **NOTE:** If batching, repeat steps 8.2.2 to 8.2.4 for each participant.			
Removed Figure 3, as it was the same as Figure 1. Changed references to Figure 3 to Figure 1.			
In processing steps, added reference to repeating the step for each tube, in reference to batch processing.			
Moved Step 8.6.13 up to put starting the freezing stage to just before placing the CoolCell in the freezer.			
Made 8.7.3 more general, as each site will remove the matrix tubes differently.			
Moved Step 8.7.9 to the end.			
Updated section 4.0 and 9.0			
Spelling and wording clarification through document.			
New Version #	**Revision Date**	**Revision Author**	**Content Approval**
5.1	2016-AUG-25	Chetna Naik	Cynthia Balion
Summary of Revisions			
Some formatting and other corrections made to clarify steps.			
Added Steps 8.6.15 and 8.7.4.			
Added Section **8.7 Retrieving Matrix Screw Top Tubes from the CoolCell for Long-**			

term Storage			
New Version #	**Revision Date**	**Revision Author**	**Content Approval**
5.0	2015-DEC-01	Chetna Naik	Cynthia Balion
Summary of Revisions			
Correction made to entire protocol to follow new Leucosep tube procedure and collection of live cell. Major changes made to sections: **Procedure Steps: Overview; 8.1 Preparation; 8.2 First Centrifugation; 8.3 First Cell Wash; 8.6 Aliquotting and Freezing PBMC.**			
In all sections, please note minor changes: using **10** mL conical tubes; use the **A-38 rotor**			
Added Section **8.7 Retrieving Matrix Screw Top Tubes from the CoolCell for Long-term Storage**			