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Effective Date: 24Nov20 Uncontrolled Print Copy For Reference Use Only

1. PURPOSE

1.1. This GUIDANCE DOCUMENT is designed to explain the process of isolating Peripheral Blood Mononuclear Cells (PBMCs) and freezing PBMCs for storage at -80°C or colder.

1.2. This GUIDANCE DOCUMENT is intended to convey the process parameters and practices to be followed by each institute associated with the National Cancer Institute (NCI) Serological Sciences Network (SeroNet).

2. SCOPE

2.1. This document applies to all institutes associated with SeroNet through collaborations, grant funding, subcontracts, etc. that perform PBMC isolation and cryopreservation.

2.2. This procedure does not describe the biospecimen collecting process. The biospecimen collecting process is dictated by the institute's protocol.

3. REFERENCES

3.1. VIC_GL_002: Shipping SARS-CoV-2 Associated Specimens to the FNL Central Repository (NCI SeroNet Guidance Document)

3.2. VIC_GL_003: Key Entity Identifier Assignment (NCI SeroNet Guidance Document)

4. RESPONSIBILITIES

4.1. It is the responsibility of the institute performing the PBMC isolation and cryopreservation to:

4.1.1. Perform PBMC isolation and cryopreservation using the indicated reagents, materials, equipment and process parameters in this guidance document.

4.1.2. Ship the PBMCs to the FNL Central Repository following "VIC_GL_002: Shipping SARS-CoV-2 Associated Specimens to the FNL Central Repository (NCI SeroNet Guidance Document)."

4.2. It is the responsibility of the Vaccine, Immunity and Cancer Program (VIC) to:

4.2.1. Generate, review and approve the PBMC isolation and cryopreservation process guidance document.

4.2.2. Distribute the most current version of this guidance document to each institute associated with SeroNet.

5. DEFINITIONS

5.1. Acid Citrate Dextrose (ACD)

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5.2. Biospecimen – a sample of biological material, such as urine, whole blood, blood components, tissue, cells, DNA, RNA, and protein.

5.3. Peripheral Blood Mononuclear Cell (PBMC) – any peripheral cell having a round nucleus; consists of lymphocytes (T cells, B cells, NK cells) and monocytes.

5.4. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

6. REAGENTS, MATERIALS AND EQUIPMENT

6.1. Reagents

6.1.1. Dulbecco's Phosphate-Buffered Saline (DPBS), Ca^{2}+ and Mg^{2}+ free (Life Technologies, Cat # 14190-136 or equivalent)

6.1.2. Ficoll-Hypaque, density of 1.077 g/mL (Amersham Pharmacia Biotech, Cat # 17-1440-02)

6.1.3. RPMI-1640, No L-glutamine (Gibco, Cat # 21870076)

6.1.4. 200 mM L-glutamine (Gibco, Cat # 25030081)

6.1.5. 1M Hepes (Gibco, Cat # 15630-080)

6.1.6. Penicillin/Streptomycin (Sigma, Cat # P-0781)

6.1.7. Dimethyl Sulfoxide (DMSO), Cell Culture Grade (Sigma, Cat # D-2650)

1.1.1. Fetal Bovine Serum (FBS), Heat-Inactivated (Hyclone, Cat # SH30070.03HI)

6.1.8. Vital Stain Dye (e.g., Trypan Blue)

6.2. Consumables

Note: Consumables requiring approval for use as "equivalent" by the NCI SeroNet are indicated with an Asterisk (*).

6.2.1. 50 mL Polypropylene Centrifuge Tubes (Falcon, Cat # 352098 or equivalent)

6.2.2. 2 mL Cryovials (Fisher Scientific, Cat # 12-565-163N or equivalent*)

6.2.3. 15 mL Conical Tube (Falcon, Cat # 352097 or equivalent)

6.2.4. Serological Pipets, various sizes

6.2.5. Pipette Tips, various sizes

6.2.6. Wet Ice

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6.2.7. Media Storage Bottle, various sizes

6.2.8. Labels that can withstand temperatures ≤ -80°C

6.2.8.1. Example: Brady Label (Anthony-Lee Associates, Cat # THT-133-461-SLIT)

6.2.9. BD vacutainer ACD tubes (Thomas Scientific, Cat # 9670A08 or equivalent*)

6.2.10. 2-inch box and 81 slot-grid

6.3. Equipment

6.3.1. Class II Biosafety Cabinet (BSC)

6.3.2. Benchtop Centrifuge

6.3.3. Hemocytometer

6.3.4. Inverted Microscope

6.3.5. Micropipettor

6.3.6. Automated Serological Pipet

6.3.7. Controlled-Rate Freezer

6.3.8. Liquid Nitrogen (LN_2)

6.3.9. Liquid Nitrogen (LN_2) Storage Freezer

6.3.10. 2-8°C Refrigerator

7. HEALTH AND SAFETY CONSIDERATIONS

Note: Each institute's Environment, Health, and Safety department will provide definitive measures for safety when processing human biospecimens as these considerations are provided only as a guideline.

7.1. Proper safety precautions should be taken while working in a laboratory setting. This includes, but is not limited to, proper protective equipment such as lab coats, safety glasses, closed-toe shoes, and non-latex gloves.

7.2. If SARS-CoV-2 positive samples are being processed, additional protective equipment is worn such as double layer of non-latex gloves and disposable arm sleeves.

7.3. A face mask is part of the standard personal protective equipment for the laboratory during the SARS-CoV-2 pandemic.

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7.4. Follow the institute governed Biosafety Level 2 (BSL-2) requirements for handling and processing human biospecimens.

7.5. All human biospecimen processing work is performed inside of a Class II BSC.

7.6. Refer to the respective Safety Data Sheet (SDS) when working with any chemicals.

7.7. Refer to the institute's processes for disposing of biohazardous and chemical waste.

8. PROCEDURE PRINCIPLES

8.1. Refer to "VIC_GL_003: Key Entity Identifier Assignment (NCI SeroNet Guidance Document)" for process of assigning IDs to biospecimens and biospecimen aliquots.

8.2. Image of form "VIC_LAB_001.01, PBMC Isolation and Cryopreservation Form" is attached for institute's reference. The minimum information requiring documentation during the performance of this process is included in this form. See Attachment 1.

8.3. Image of form "VIC_LAB_001.02, PBMC Biospecimen Collection Form" is attached for institute's reference. The minimum information requiring documentation during the performance of the blood biospecimen collection for PBMC isolation and cryopreservation is included in this form. See Attachment 2.

8.4. Phlebotomist should collect blood in ACD tubes.

8.5. It is preferred that all equipment used in this process is maintained, at minimum, per the equipment manufacturer's recommendations.

8.6. It is preferred that all Micropipettors, Laboratory Freezers and Refrigerators, Benchtop Centrifuges, and Automated Cell Counters used in this process be calibrated by a vendor or other qualified party.

8.7. It is preferred that all Laboratory Freezers and Refrigerators used in this process be monitored for temperature by a temperature monitoring system.

8.8. All reagent preparation and human biospecimen handling are performed in a Class II Biosafety Cabinet (BSC) except for centrifugation, freezing cycle and storage.

9. REAGENT PREPARATION

9.1. RPMI-1640 Complete Media + 40% FBS

9.1.1. Combine reagents into appropriately sized media storage bottle. See Table 1 for preparation of 1000 mL; preparation can be scaled up or down as needed.

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Table 1: RPMI-1640 Complete Media + 40% FBS Preparation (1000 mL)

Reagent	Volume (mL)
RPMI-1640, No L-Glutamine	570
Fetal Bovine Serum, Heat-Inactivated	400
200 mM L-Glutamine	10
1M Hepes	10
Penicillin/Streptomycin	10
Total	**1000**

9.1.2. Mix well by inversion.

9.1.3. Label reagent with Reagent Name, Lot Number/Tracking Number, preparation date, expiration date, storage condition and initials.

9.1.4. RPMI-1640 Complete Media + 40% FBS may be stored at 2-8°C for up to two weeks.

9.2. RPMI-1640 Complete Media + 15% DMSO

9.2.1. Prepare reagent day of use.

9.2.2. Combine reagents into appropriately sized media storage bottle. See Table 2 for preparation of 100 mL; preparation can be scaled up or down as needed.

Table 2: RPMI-1640 Complete Media + 15% DMSO Preparation (100 mL)

Reagent	Volume (mL)
RPMI-1640, No L-Glutamine	82
DMSO, Cell Culture Grade	15
200 mM L-Glutamine	1.0
1M Hepes	1.0
Penicillin/Streptomycin	1.0
Total	**100**

9.2.3. Mix well by inversion.

9.2.4. Label reagent with Reagent Name, Lot Number/Tracking Number, preparation date and initials.

9.2.5. Store reagent at 2-8°C or on wet ice until used.

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9.2.6. Do not retain remaining reagent after processing, discard according to the organization's chemical disposal process.

10. PBMC ISOLATION

Note: Maximum allowable time from blood collection (processing of PBMC) to LN_2 storage is 8 hours.

10.1. Upon receipt of blood biospecimen, observe and record the total volume of blood biospecimen collected on form VIC_LAB_001.01.

10.2. Using a 50 mL polypropylene tube or appropriately sized sterile storage bottle/flask dilute the blood biospecimen with an equal volume of DPBS.

10.3. Label 50 mL or 15 mL conical tubes with sample identification number (ID).

10.4. Dispense 15 mL of Ficoll-Hypaque into labeled 50 mL conical tubes, or if using 15 mL conical tubes, dispense 4 mL of Ficoll-Hypaque into labeled tubes.

10.5. Carefully overlay diluted blood from step 10.2 onto the Ficoll-Hypaque from step 10.4.

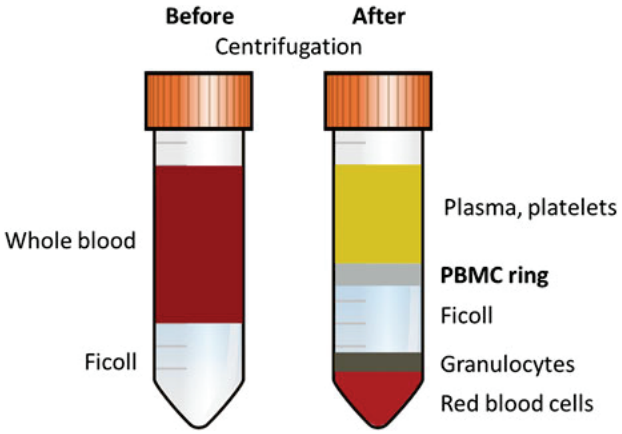
10.5.1. When using 50 mL conical tube, the maximum volume is not to exceed 45 mL.

10.5.2. When using 15 mL conical tube, the maximum volume is not to exceed 13.5 mL. See Figure 1.

10.6. Centrifuge the samples for 20 minutes at 1000 x g at 20°C with the centrifuge brake turned off.

10.7. Using a transfer pipette or serological pipette, remove the PBMC layer and transfer to a single clean 50 mL centrifuge tube labeled with sample ID. See Figure 1.

Figure 1: Image of Blood Overlay and Layers Post Centrifugation



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10.8. Wash the PBMCs by quantum satis (q.s.) to 45 mL with DPBS, then centrifuge for 10 minutes at 470 x g at 20°C with the brake on.

10.9. Decant the supernatant.

10.10. Wash the PBMC pellet one additional time with 45 mL DPBS. Centrifuge for 10 minutes at 300 x g at 20°C with brake on.

10.11. Decant the supernatant.

10.12. Resuspend cells in cold RPMI-1640 Complete Media + 40% FBS (1 mL).

10.13. Perform a cell count using hemocytometer. See Attachment 3 for cell counting using a hemocytometer.

Note: If the institute has an Automated Cell Counter, the institute can perform a second count on the cell counter as For Information Only (FIO).

10.14. Record the hemocytometer cell count and calculate viability (live cells ÷ total cells x 100%). Only proceed with cryopreservation if viability is greater than 80%.

11. CRYOPRESERVATION

Note: It is very important at this point that cells, media, and tubes are kept cold on wet ice.

11.1. Label 2 mL cryovials using Attachment 4. Refer to VIC_GL_003 for biospecimen aliquot ID assignment process. **Use Deidentified Biospecimen Aliquot ID Only**.

11.2. Adjust cell concentration to be 20 x 10^6 cells/mL using RPMI-1640 Complete + 40% FBS.

11.3. Add dropwise an equal volume of cold RPMI-1640 Complete + 15% DMSO giving a final freezing solution of RPMI-1640 Complete containing 20% FBS and 7.5% DMSO. Resuspend cells gently.

11.4. Transfer 1.0 mL of the cell suspension (well suspended) using a pipette with a 1000 µL tip into each of the pre-chilled 2 mL cryovials.

Note: Gently mix cells by inversion after 2-3 minutes has passed for cells to settle, before transferring the cells.

11.5. Maintain the cells on wet ice until all samples are ready for transfer to the controlled-rate freezer. Processing should be performed quickly due to the recognized toxicity of DMSO.

11.6. Controlled-Rate Freezer

11.6.1. See Attachment 5 for the controlled-rate freezer program.

11.6.2. Prechill the controlled-rate freezer to a starting temperature of 4°C.

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11.6.3. Prepare one control vial to regulate the controlled- rate freezer. Use the same volume and concentrations as the final freezing solution (i.e., 0.5 mL of RPMI-1640 Complete + 40% FBS plus 0.5 mL RPMI-1640 Complete + 15% DMSO).

11.6.4. Transfer cryovials immediately to the controlled-rate freezer.

11.6.5. Place the PBMC biospecimen aliquot vials and the control vial into the freezing chamber.

11.6.6. Place the freezer thermocouple into the control vial. Allow the control vial temperature and the chamber temperature to equilibrate to 4°C.

11.6.7. Begin the programmed, controlled-rate freeze.

11.6.8. At the conclusion of the freeze cycle, the cryovials will have reached -90°C and are transferred directly to freeze boxes for liquid nitrogen storage.

11.6.9. Check the freezing report to assure appropriate controlled-rate freezing. Make note on record (form VIC_LAB_001.01) if the parameters were not met. Retain controlled-rate freezer report print out with record.

11.6.10. Record the number of vials frozen. Attached is an example vial label to the record (VIC_LAB_001.01).

11.6.11. If there were problems encountered during PBMC biospecimen processing, note these on the record (form VIC_LAB_001.01). Record any problems with freezing procedure.

11.7. Ship PBMCs in LN_2 shipper to the FNL Central Repository following VIC_GL_002.

12. ATTACHMENTS

12.1. Attachment 1: VIC_LAB_001.01, PBMC Isolation and Cryopreservation Form

12.2. Attachment 2: VIC_LAB_001.02, PBMC Biospecimen Collection Form

12.3. Attachment 3: Counting Cells with a Hemocytometer

12.4. Attachment 4: Vial Label and Box / Rack Label

12.5. Attachment 5: Controlled-Rate Freezer Program Parameters

13. REVISION HISTORY

Version	Change	Reason
1.0	New guidance document for isolation and cryopreservation of PBMC by SeroNet organizations.	Currently no procedure; new initiative requiring communication of expectations.

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2.0	1. Replaced “sample” and “specimen” with “biospecimen” throughout the document. 2. Minor formatting and grammatical changes throughout the document. 3. Added VIC_GL_003 to References section. 4. Added Biospecimen and SARS-CoV-2 to Definitions section. 5. Added Asterix to consumables requiring approval by SeroNet for use as equivalent. 6. Removed Automated cell counter, -80C, -20C and cell freeze device from equipment section. 7. Added SARS-CoV-2 pandemic health and safety guidelines to Health and Safety Considerations section. 8. Added reference to VIC_GL_003 and new form VIC_LAB_001.02 to Procedure Principles section. 9. Reworded equipment requirements to be “preferred” in the Procedure Principles section. 10. Added option to do cell count using Automated Cell Counter; hemocytometer cell count is required. 11. Removed use of cell device as option for cell freeze. 12. New form VIC_LAB_001.02 for collection of PBMC biospecimen. 13. Revised form VIC_LAB_001.01 to have biospecimen receipt, removed automated cell counter and -80C/-20C freezers, removed N/A boxes for required equipment.	1. Consistency between documents and database verbiage. 2. Clarification, ease of use. 3. Referred in the body of the procedure. 4. Clarification. 5. Reflect current practice. 6. Reflect current practice. 7. Clarification. 8. Clarification. 9. Clarification. 10. Clarification; reflect current practice. 11. Reflect current practice. 12. Ease of use. 13. Ease of use, reflect current practice.

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Attachment 1: VIC_LAB_001.01, PBMC Isolation and Cryopreservation Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Program Standard Operating Procedure Form	
Form Title: PBMC Isolation and Cryopreservation Form			
Document ID: VIC_LAB_001.01		Version:	2.0
Associated SOP: VIC_LAB_001		Effective Date:	
Supersedes:	1.0	**Page 1 of 5**	

Biospecimen Receipt

PBMC Biospecimen Processing Laboratory Name:					
Biospecimen Number	Deidentified Biospecimen ID	Volume (mL	Date Received	Time Received (24H)	Initials
1					
2					
3					
4					
5					

Equipment

Equipment Name	Equipment ID	Calibration Due Date
BSC		
Centrifuge		
Pipette		
☐ N/A Pipette		
☐ N/A Pipette		
☐ N/A 2-8°C Refrigerator		
Microscope		
Hemocytometer		
☐ N/A Automated Cell Counter		
Controlled-Rate Freezer		
LN_2 Storage Freezer		

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Reagents

Reagent Name	Catalog Number	Lot Number	Expiration Date
DPBS			
Ficoll-Hypaque			
RPMI-1640, no L-Glutamine			
Fetal Bovine Serum			
200 mM L-Glutamine			
1M Hepes			
Penicillin/Streptomycin			
DMSO, Cell Culture Grade			
☐ N/A Vital Stain Dye (e.g. Trypan Blue)			

Consumables

Consumable Name	Catalog Number	Lot Number	Expiration Date
50 mL Polypropylene Tube			
☐ N/A 15 mL Conical Tube			
☐ N/A 2 mL Cryovial			
☐ N/A Cryovial Label		☐ N/A	☐ N/A
☐ N/A			

PBMC Isolation

Processing Steps

(√)	Process Step
	Dilute blood biospecimen with equal volume of DPBS.
	Dispense Ficoll-Hypaque into conical tubes.
	Overlay diluted blood biospecimen onto Ficoll-Hypaque.
	Centrifuge for 20 min, 1000 x g, 20°C, brake OFF.
	Remove PBMC layer and transfer to 50 mL conical tube. QS to 45 mL with DPBS.
	Centrifuge for 10 min, 470 x g, 20°C, brake ON.
	Decant supernatant. Add 45 mL DPBS.
	Centrifuge for 10 min, 300 x g, 20°C, brake ON.
	Decant supernatant. Resuspend in COLD RPMI-1640 Complete + 40% FBS

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Reagent Preparation

RPMI-1640 Complete + 40% FBS	
Reagent	Volume (mL)
RPMI-1640, no L-Glutamine	
Fetal Bovine Serum	
200 mM L-Glutamine	
1M Hepes	
Penicillin/Streptomycin	

Cell Count – Hemocytometer (Required)

Biospecimen Number	Live Cells	Dead Cells	Total Cells	% Viability
1				
2				
3				
4				
5				

Cell Count – Automated Cell Counter (For Information Only)

Biospecimen Number	Live Cells	Dead Cells	Total Cells	% Viability
1				
2				
3				
4				
5				

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PBMC Cryopreservation

Reagent Preparation

RPMI-1640 Complete + 15% DMSO (Freeze Media)	
Reagent	Volume (mL)
RPMI-1640, no L-Glutamine	
DMSO, Cell Culture Grade	
200 mM L-Glutamine	
1M Hepes	
Penicillin/Streptomycin	

Processing Steps

(√)	Process Step
	Label 2 mL cryovials. Chill at 2-8°C.
	Adjust cell concentration to be 20 x 10^6 cells/mL using RPMI-1640 Complete + 40% FBS.
	Add dropwise an equal volume of cold RPMI-1640 Complete + 15% DMSO. Gently resuspend cells.
	Transfer 1.0 mL of the cell suspension into each labeled 2 mL cryovial.
	Freeze PBMCs using Controlled-Rate Freezer.
	Store PBMCs in LN_2 Freezer.

Controlled-Rate Freezer, see attached printout

Biospecimen Number	Date / Time (24H) Blood Biospecimen Collected	Date / Time (24H) PBMC Biospecimen Stored in LN_2 Freezer	Initials
1			
2			
3			
4			
5			

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Biospecimen Number	Number of Cryovials Frozen	Example Label
1		
2		
3		
4		
5		

Comments: ☐ N/A

Performed by/date:	
Reviewed by/date:	

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Attachment 2: VIC_LAB_001.02, PBMC Biospecimen Collection Form

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Program Standard Operating Procedure Form	
Form Title: PBMC Biospecimen Collection Form			
Document ID: VIC_LAB_001.02		Version:	2.0
Associated SOP: VIC_LAB_001		Effective Date:	
Supersedes:	1.0	**Page 1 of 1**	

Deidentified Biospecimen ID:					
Biospecimen Group:	☐ Positive ☐ Negative ☐ Serosurveillance				
Section I. Vacutainer Collection Tube					
Type:	☐ ACD ☐ Other				
Catalog No.:					
Lot No.:	☐ N/A				
Exp. Date:					
Section II. Blood Biospecimen Collection					
Name of Clinic/Company:					
Date:		Time: (24 Hr)		Initials:	

Reviewed by/date: _____

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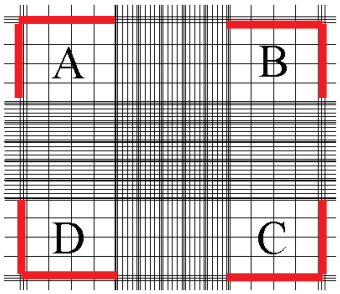
Attachment 3: Counting Cells with a Hemocytometer

- Count cells with a hemocytometer using vital stain dye (Trypan blue).
    - Note: May start with a 1:2 dilution (equal volumes of vital stain dye and cells). However, the dilution may need to change, so the total cell count of quadrants A, B, C, and D is ~80-200 cells.
- Add 10 µL of vital stain dye/cell mixture to the hemocytometer.
- Count cells in quadrants A, B, C, and D (refer to diagram below). Only count cells that fall on two of the four outer edges of each of the four quadrants, as defined by the red lines in the diagram below.



- Record the number of live cells (blue negative), dead cells (blue positive) and total cells (live cells + dead cells).
- To calculate cell concentration, use the following formula:

  (Total cells counted ÷ Number of quadrants counted) x Dilution Factor x 10,000

  For example, a sample that was diluted 1:2 had 100 live cells counted in four quadrants.
  (100 ÷ 4) x 2 x 10,000 = 500,000 cells/mL
- To calculate cell viability, use the following formula:
  (Live cells ÷ Total cells) x 100%

  For example, a sample has 75 live cells and 50 dead cells.
  Total cells = 75 live + 50 dead = 125
  Viability = (75 ÷ 125) x 100% = 60%

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Attachment 4: Vial Label and Box / Rack Label

Vial Label

Biospecimen Aliquot ID
PBMC
Volume
Cell Concentration

Barcode:	Barcode linked to Biospecimen Aliquot ID
Line 1:	Deidentified Biospecimen Aliquot ID
Line 2:	PBMC
Line 3:	Volume (mL)
Line 4:	Final Cell Concentration (x 10^6 cells/mL)

Example Label:

A1_123456_123_1
PBMC
1.0 mL
10 x 10^6 cells/mL

Box / Rack Label

Study: ?????? / ??????
Biospecimen Type: ?????
Date: DDMMMYY
Shipping ID: XXXXXXX
Box ? **of** ?

Line 1:	SeroNet
Line 2:	PBMC
Line 3:	Date in DDMMMYY format
Line 4:	Shipping ID
Line 5:	Box Number

Example Label:

Study: SeroNet
Biospecimen Type: PBMC
Date: 01Jan20
Shipping ID: XXXXXXX
Box 1 **of** 10

Box Label Placement

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Attachment 5: Controlled-Rate Freezer Program Parameters

Step No.	Rate (°C/min)	End Temp (°C)	Hold (m s)	Trigger
1			5m 0s	Chamber
2	-1.00	-4.00		Chamber
3	-25.00	-50.00		Chamber
4	10.00	-20.00		Chamber
5	-1.00	-40.00		Chamber
6	-5.00	-90.00		Chamber
7			0m 0s	Chamber

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