Title:	PBMC Specimen Collection, Preparation, and Freezing for Protein Extraction				Page 1 of 19
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Laboratory of Human Toxicology & Pharmacology

Applied/Developmental Research Directorate, Leidos Biomedical Research, Inc.

Frederick National Laboratory for Cancer Research

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DCTD OD Approval:	Joseph E. Tomaszewski	Date:	03/23/2015

Please check for revision status of the SOP at

http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

and be sure to use the current version.

Change History

Revision	Approval Date	Description	Originator	Approval
H	2/11/2015	Updated contact shipping address and process for advance notification of shipments.	KFG	REP
G	1/8/2013	Guidance for resuspending the initial PBMC isolate added. PBMC pellet preparation updated to allow a maximum concentration of 3 x 10^6 cells/pellet; any pellet with fewer cells will be placed in a single tube and labeled with the total cell number.	YAE, MM	JJ
F	12/29/2010	Update sample snap freeze to dry ice/ethanol bath or liquid nitrogen and add directions for hemocytometer use.	YAE	JJ
E	7/24/2009	Separated PBMC Preparation (SOP34503) and Protein Extraction (SOP34506) SOPs, clarified PBMC processing and handling site issues, added shipping instructions and Appendices 2 and 3, and prepared for publication to the DCTD Biomarkers Web site	YAE	JJ
D	12/01/2008	Updated SOP Web site, SOP title, and moved reagent preparation to Batch Record for technician sign-off	YZ	JJ
C	10/14/2008	Merge PBMC Preparation (SOP34503) with Extraction Method (SOP34506)	KG	JJ
B	9/19/2007	PBMC vial changes	KL	JJ
A	10/13/2006	Format change and revision	YZ	JJ
--	7/13/2006	New document adopted from LHTP	RP	JJ

TABLE OF CONTENTS

1.0 PURPOSE

To standardize the method for collection and handling of peripheral blood mononuclear cells (PBMCs) to enable protein extraction for measurement of pharmacodynamic (PD) markers following treatment with anticancer agents.

2.0 SCOPE

This procedure applies to all personnel responsible for the collection and handling of blood for isolation of PBMCs from patients participating in clinical trials of chemotherapeutic agents. The goal of this SOP and associated training is to ensure consistency of PBMC specimen collection and handling between clinical sites.

3.0 ABBREVIATIONS

CPT	=	Cell Preparation Tube
DCTD	=	Division of Cancer Treatment and Diagnosis
FNLCR	=	Frederick National Laboratory for Cancer Research
ID	=	Identification
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PBMC	=	Peripheral Blood Mononuclear Cells
PD	=	Pharmacodynamic
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

Specimen handling, shipping, and storage procedures (pre-analytical variables) can have a significant impact on the reliability of biomarker measurements in the laboratory. Following detailed steps for sample collection and handling procedures and recording any deviations from this procedure allows retrospective identification of artifactual changes in biomarker readout and increases the reliability of the data and validity of the analytical results.

5.0 ROLES AND RESPONSIBILITIES

Laboratory Director/Supervisor — The Laboratory Director/Supervisor, directs laboratory operations, supervises technical personnel and reporting of findings, and is responsible for the proper performance of all laboratory procedures. The Laboratory Director/Supervisor oversees the personnel who follow the SOPs in the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to handle clinical samples.

Certified Assay Operator and/or PK/PD Support Lab Personnel — A Certified Assay Operator and/or PK/PD Support Lab personnel may be a Laboratory Technician/ Technologist, Research Associate, or Laboratory Scientist who has been certified through DCTD training on this SOP. The Certified Assay Operator and/or PK/PD Support Lab personnel work under the guidance of the Laboratory Director/Supervisor. This person performs laboratory procedures and examinations in accordance with the current SOP(s), as well as any other procedures conducted by a laboratory, including maintaining equipment and records and performing quality assurance activities related to performance.

5.1 It is the responsibility of the Laboratory Director/Supervisor to ensure that all personnel have documented training and qualification on this SOP prior to the actual handling and processing of samples from clinical trial patients. The Laboratory Director/Supervisor is responsible for ensuring the Certified Assay Operator running the SOP has sufficient experience to handle and analyze clinical samples.

5.2 It is the responsibility of the Certified Assay Operator and/or PK/PD Support Lab personnel to confirm scheduled sample collection time points, pre-print all labels and data collection sheets in advance, check documentation for accuracy, and verify that the required collection tubes, supplies, and equipment are available for successful collection and handling of PBMCs.

5.3 It is the responsibility of the Certified Assay Operator to ensure timely transport and processing of the samples, enter and review all of the required collection and processing data, and archive all data sheets in the appropriate files.

5.4 The Certified Assay Operator and/or PK/PD Support Lab personnel responsible for conducting the assay is to follow this SOP and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed in ***real-time*** for each experimental run, with each page ***dated and initialed,*** and placed with the clinical sample information.

5.5 A PD Specimen Collection schedule should be generated for each clinical protocol requiring PBMC collection for PD studies (Appendix 2, Section 1). A Shipping Manifest (Appendix 4) should be prepared for each batch of patient samples prior to shipping to NCTVL for PD biomarker analysis.

5.6 The responsible personnel are to check the DCTD Biomarkers Web site (http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm) to verify that the latest SOP version is being followed.

6.0 MATERIALS AND EQUIPMENT REQUIRED

6.1 Pipettors (100-1000 µL, 50-200 µL, 2-20 µL) and tips
6.2 Electronic pipettor
6.3 1.5-mL Sarstedt o-ring screw cap tubes (Fisher Scientific, Cat#: 72.692.005)
6.4 3-mL Falcon transfer pipette (Fisher Scientific, Cat# 13-711-6)
6.5 15-mL polypropylene tubes (e.g., Fisher Scientific, Cat#: 14-959-49B)
6.6 1 and 5 mL pipettes, sterile, individually wrapped (Fisher Scientific, Cat#: 13-675-15C and 13-675-22)
6.7 Hemocytometer and coverslips
6.8 Vacutainer Cell Preparation Tubes (CPT), 8-mL draw capacity, blue/black conventional closure (Becton Dickinson, Cat#: 362761)
6.9 81-place freezer boxes (Fisher Scientific, Cat#: 12-565-182)
6.10 Thermoflask cooler
6.11 Ice bucket

6.12 Plasma-Lyte A pH 7.4, USP (Baxter, Cat#: 2B2544X)
6.13 100% ethanol
6.14 Trypan blue, 0.4%, sterile (StemCell Technologies, Cat#: 07050)

6.15 Sorvall Legend RT centrifuge with a Swing Bucket Rotor (e.g., Fisher Scientific, Cat#: 75-006-434)
6.16 Sorvall Fresco microcentrifuge, refrigerated
6.17 -80°C freezer
6.18 Dry ice/100% ethanol bath or liquid nitrogen

*If instruments and/or reagents differ from those specified above, the Laboratory processing the clinical specimens must prove their comparability or equivalence to those recommended using the manufacturer's specifications and experimental validation data.

7.0 OPERATING PROCEDURES

Note: One Batch Record (Appendix 1) should be filled out for **each venous blood draw** as indicated in the PD Specimen Collection Schedule (Appendix 2).

7.1 Record the name and certification number of the Certified Assay Operator and/or PK/PD Support Lab personnel performing the SOP, the facility/clinic collecting the specimens, the Patient/Sample ID, and the clinical protocol number in the Batch Record (Appendix 1).

7.2 Though this SOP is for processing a **single** venous blood draw, in practice a PD Specimen Collection Schedule should be generated for each patient as defined in the Pharmacodynamic/Correlative Study Section of the clinical protocol to document both scheduled blood collection times and actual collection times.

7.2.1 Each patient will have a **single** PD Specimen Collection Schedule outlining all samples that are collected (example in Appendix 2, Section 1).

7.2.2 This will ensure any deviations from the clinical protocol collection protocol are recorded for the PD laboratory.

7.3 Four identical, pre-printed sample labels are to be prepared for each time point as defined in the Pharmacodynamic/Correlative Study Section of the clinical protocol (sample pre-printed label in Appendix 2, Section 2).

NCI blood draws for PBMC PD sampling are series 300 with consecutive numbers identifying the collection time point as defined in the Clinical Protocol.

7.4 Blood Collection

Important: PBMCs should be processed through SOP Step 7.7.3 (freezing of the PBMC pellets) within 3 h of blood collection.

7.4.1 Ensure that the phlebotomist is using the recommended 8-mL Becton Dickinson Vacutainer CPTs to draw the blood samples. If necessary, supply the phlebotomist with the correct CPTs.

7.4.2 The research nurse is to notify the laboratory of scheduled PD sample collections, preferably giving at least 24-h notice.

7.4.2.1 The Certified Assay Operator is to arrive at the blood collection site at least 5 min ahead of the scheduled time point(s) to ensure rapid transport to the laboratory after collection.

7.4.2.2 Record the actual time of blood collection should be noted in the PD Specimen Collection Schedule (Appendix 2, Section 1).

7.4.3 Of the 4 pre-printed labels prepared for each sample, one label is placed onto the freshly collected sample CPT, and a second label is given to the research nurse to place into the patient record sheet.

7.4.4 The sample is transported at room temperature (18°C to 25°C) in a double container from the clinical collection site to the sample processing laboratory. A Batch Record is to be started for each venous blood draw (Appendix 1).

Important: Do not place sample(s) on ice. If the clinical staff placed the CPT tube on ice, make a notation in the deviation section of the Batch Record (Appendix 1, Section 5) and bring the tube to 18°C to 25°C before proceeding with sample processing.

7.5 PBMC Isolation

7.5.1 Once in the laboratory, record the Patient/Specimen ID, blood volume, blood draw clinical time point, and time of venous blood draw in the Batch Record (Appendix 1).

7.5.2 The blood sample is mixed by inverting the tube gently 5 to 8 times and then centrifuging in a swing bucket rotor at 1,500 x g for 30 min at 18°C to 25°C, without the brake.

7.5.3 Place the third pre-printed label onto the Batch Record, and place the fourth label onto a sterile 15-mL polypropylene tube. Record the time blood processing begins in the Batch Record (Appendix 1, Section 2).

7.5.4 After centrifugation, using a 3-mL Falcon transfer pipette, carefully remove two-thirds of the upper plasma layer and discard in biological waste. Use care not to disrupt the underlying material. Change pipette tips and transfer the whitish layer that contains the PBMCs into the labeled 15-mL polypropylene tube. Discard the remaining liquid and Vacutainer CPT in the appropriate biohazardous waste container(s).

7.5.5 Using a pipette, slowly add Plasma-Lyte A USP to the PBMCs in the 15-mL polypropylene tube to bring the total volume to 14 mL; cap, then mix by gentle inversion 5 to 8 times.

7.5.6 Centrifuge the sample in a swing bucket rotor at 430 x g for 10 min at 18°C to 25°C, without the brake.

7.5.7 Using a sterile pipette, aspirate as much supernatant as possible without disturbing the cell pellet. Discard the supernatant into biohazardous liquid waste.

7.5.8 Add 1-6 mL of Plasma-Lyte A USP to the 15-mL tube. Resuspension volume is based on PBMC pellet size. Record the total volume of Plasma-Lyte A USP added in the Batch Record (Appendix 1, Section 2).

Guidance:

- 8-mL CPT tubes typically have a solid white PBMC pellet, approximately 0.5 mm thick, with 8-10 x 10^6 cells; pellets of this size should be resuspended in 3 mL Plasma-Lyte A USP.
- 4-mL CPT tubes generally result in a small PBMC pellet (faint to the eye) with 2-4 x 10^6 cells; pellets of this size should be resuspended in 1 mL Plasma-Lyte A USP. **If 4-mL tube is received,** note as a deviation in the Batch Record (Appendix 1, Section 5).

7.5.9 Once the Plasma-Lyte A USP has been added, gently flick the bottom of the tube with the index finger to resuspend the pellet, and then gently pipette up and down 5 times using a 5-mL pipette.

7.6 PBMC Counting and Aliquoting

7.6.1 Immediately after resuspending the cell pellet, prepare a 1:5 dilution of the sample for a hemocytometer count by transferring 20 µL of sample into a microtube containing 60 µL Plasma-Lyte A USP and 20 µL of 0.4% trypan blue. Gently mix by pipetting and set aside for a cell count.

7.6.1.1 Vi-CELL or Moxi Z automated cell counters can be used to perform cell counts. Indicate if an automated cell counter was used in the Batch Record (Appendix 1, Section 2).

7.6.2 Centrifuge the remaining cell suspension in a swing bucket rotor at 430 x g for 10 min at 18°C to 25°C, without the brake.

7.6.3 While centrifuging, determine the total and viable cell count in the trypan blue sample using a hemocytometer (see Appendix 3 for information on hemocytometer use). Optimal cell counts should be 30 to 150 cells in each hemocytometer corner area; additional 1:5 dilutions can be done for higher density samples. Record the following information in the Batch Record (Appendix 1, Section 2):

- Total cell count in each hemocytometer corner that was counted
- Total viable cell count in each hemocytometer corner that was counted
- Calculated viable cells/mL
- Calculated total viable cells in the remaining volume of cell suspension

7.6.3.1 If an automated cell counter was used, record the total viable cells/mL reported by the counter and calculate the total viable cells in the remaining volume of cell suspension in the Batch Record (Appendix 1, Section 2).

7.6.4 Using the total viable cell count calculated in the remaining cell suspension, do one of the following calculations to determine the final volume to resuspend the pelleted PBMCs in SOP Step 7.5.6:

7.6.4.1 If there are $\geq 3 \times 10^6$ total viable cells in the remaining cell suspension, calculate the volume required to make the final PBMC concentration equal to **3×10^6 cells/mL**. Record the total resuspension volume in the Sample Information Table of the Batch Record (Appendix 1, Section 3).

7.6.4.2 If there are $< 3 \times 10^6$ total viable cells in the remaining cell suspension, resuspend the cells in 1 mL total volume. Record "1 mL" for the resuspension volume and then calculate and record the cell concentration in the 1-mL tube in the Sample Information Table of the Batch Record (Appendix 1, Section 3).

7.6.5 After centrifugation of the remaining cell suspension in SOP Step 7.6.2, use a sterile pipette and, without disturbing the cell pellet, remove the supernatant. Discard the supernatant into biohazardous liquid waste.

7.6.6 Add the volume of Plasma-Lyte A USP calculated in SOP Step 7.6.4 to the cell pellet.

7.6.6.1 Resuspend the cell pellet by gently flicking the bottom of the tube with the index finger and then gently pipetting up and down 5 times using a 2-mL pipette for volumes of 2.5 mL or less, or a 5-mL pipette for volumes greater than 2.5 mL.

7.6.7 Aliquot the PBMC cell suspension as follows:

7.6.7.1 For a 3 x 10^6 cells/mL suspension (starting concentration ≥ 3 x 10^6 cells):

- Place 1-mL aliquots into 1.5-mL Sarstedt tubes until the remaining volume of cell suspension is less than 1 mL.
- Record the total number of 1-mL aliquots that have been prepared in the Batch Record (Appendix 1, Section 3).
- Pipette the residual volume of cell suspension into a Sarstedt tube and write "partial" on the top of the tube with a black Sharpie. Note the actual volume in the Sample Information Table (Appendix 1, Section 3). Calculate the cell number present in the partial tube by multiplying 3 x 10^6 by the partial volume in mL. Record the cell concentration in the partial tube in the Sample Information Table (Appendix 1, Section 3)

7.6.7.2 For all other tubes (starting concentration < 3 x 10^6 cells):

- Place the full 1-mL aliquot into a 1.5-mL Sarstedt tube, recording "1" as the total number of 1-mL tubes prepared and "N/A" as the number of partial tubes prepared in the Sample Information Table (Appendix 1, Section 3) Cell concentration should have been recorded in SOP Step 7.6.4.2.
- Depending on the DCTD immunoassay the samples are used for, some PBMC aliquots < 3 x 10^6 cells/mL may be considered unanalyzable.

7.6.8 Place appropriate labels onto each tube, indicating clinical protocol number, sample/patient ID, time point for blood draw (e.g., Pre-dose D1 or D1 4 hr), and final PBMC count in each tube as recorded in the Sample Information Table (Appendix 1, Section 3). The label format should match that used for labeling the CPT to ensure within-laboratory consistency.

7.7 Pellet and Freeze PBMC Aliquots

7.7.1 Centrifuge the Sarstedt tubes in a Sorvall Fresco microcentrifuge swing bucket rotor at 3,000 x g for 10 min at 2°C to 8°C. Be sure to do appropriate RCF/RPM speed conversions. A Sorvall tabletop RCF conversion chart can be found at the following URL: http://www.thermo.com/eThermo/CMA/PDFs/Various/File_661.pdf.

7.7.2 Remove and discard as much supernatant as possible without disturbing the cell pellet. There should be no more than 20 µL residual volume remaining on the pellet. Supernatant should be discarded into the appropriate biohazardous waste container.

7.7.3 Snap-freeze the PBMC cell pellets using liquid nitrogen or a dry ice/ethanol bath.

7.7.4 Store the frozen PBMC samples in 81-place freezer boxes, batched by patient, at -80°C until shipment. Note the date, time, and location of storage in the Batch Record (Appendix 1, Section 3).

7.8 Review and finalize the Batch Record and document **ANY** and **ALL** deviations from this SOP in the Batch Record (Appendix 1, Section 4).

7.9 The Laboratory Director/Supervisor should review the Batch Record and sign to affirm the data contained within are correct (Appendix 1, Section 5).

8.0 SHIP TO FNLCR FOR ANALYSIS (OPTIONAL)

8.1 FedEx return shipment labels will be provided to each approved site sending frozen shipments to FNLCR PD Specimen Central Receiving.

8.1.1 To request return shipment labels send an e-mail to NCI_PD_Support@mail.nih.gov and state "*Protocol Name* Shipment Labels Requested" in the subject line. Specify the address to which the shipment labels should be provided and the number of shipment labels requested. Shipment labels will be provided within 6 business days.

8.2 If possible, ship one patient's PBMCs collected from all cycle time points in one batch. Samples from each patient should be consolidated in a single container (such as an 81-place freezer box). Do not mix samples from different patients in the same freezer box.

8.3 Send an e-mail to FNLCR PD Specimen Central Receiving (NCI_PD_Support@mail.nih.gov) prior to shipping to advise recipient of scheduled shipping time. State "*Protocol Name* PD Specimens Ready for Shipment" in the subject line. Request a confirmation e-mail that personnel will be available on the expected delivery date and time. Personnel are generally available to receive frozen shipments Tuesday through Friday, excluding government holidays. If needed, FNLCR PD Central Receiving can be contacted directly at 240-344-5697.

8.4 Use the PD Sample Shipping Manifest template in Appendix_4 to generate a shipping list containing pertinent sample information and FNLCR PD Specimen Central Receiving shipping address.

Attention: Dan Danner
NCI-F/FNLCR
1073 Beasley Street, Building 1073
Fort Detrick
Frederick, MD 21701
Phone: 301-846-5748

8.5 Make a copy of the Shipping Manifest and specimen Batch Records so one copy can be sent to FNLCR with the PBMC samples and one can be maintained at the collection site for internal records.

8.6 Day of Shipment

8.6.1 Just prior to shipment, place the 81-place freezer box(es) in a shipping container with sufficient dry ice to maintain the samples at -20°C for at least 72 h. All weekly processing samples are recommended to ship out via FedEx on the following Monday afternoon for delivery by 10 AM Tuesday (FedEx First Overnight).

8.6.2 **Verify** the contents of the package match the Shipping Manifest and sign and date the bottom of both copies of the Shipping Manifest. Place one copy of the Shipping Manifest inside the shipping box along with copies of the completed Batch Records for all specimens.

8.6.3 Seal the box and print and attach the shipping address onto the outside of the shipping container; be sure the container is labeled as containing biohazardous specimens.

8.6.4 Record the shipping date, time, tracking number, and shipping information in the Batch Record (Appendix 1, Section 3).

8.6.5 E-mail FNLCR PD Specimen Central Receiving (NCI_PD_Support@mail.nih.gov) a shipment notification. State "*Protocol Name* PD Specimen Shipment" in the subject line and reference the tracking number in the e-mail.

8.6.6 Once specimens arrive at the processing laboratory, they should be immediately placed at -80ºC (or lower) pending delivery to the processing laboratory for protein extraction.

APPENDIX 1: BATCH RECORD

Place PD Specimen Label Here

A **separate** Batch Record should be started for **each patient sample**.

Note*:* A pre-dose and post-dose sample from the same patient would have the same Patient ID, but different Specimen ID numbers.

Note: Record times using **military time** (24-h designation); for example, specify 16:15 to indicate 4:15 PM.

Certified Assay Operator: _____

Certification Number: _____

☐ Check here if PK/PD Support Lab Personnel

Facility/Clinic Collecting Specimens: _____

Clinical Protocol Number: _____

Patient ID: _____

Patient/Specimen ID: _____

Blood Volume: _____

Blood Draw Time Point: _____ Time of Venous Blood Draw: _____

1. Equipment and Reagents

A. Equipment:

Sorvall Legend RT Centrifuge: Make/Model : _____

Serial # : _____

Swing Bucket Rotor: Make/Model : _____

Serial # : _____

B. Consumables:

Vacutainer 8-mL CPT: Product : _____

Lot # : _____

Plasma-Lyte A USP: Lot # : _____

Expiration Date : _____

2. PBMC Isolation and Counting

Time lab processing begins: _____ :_____

Volume of Plasma-Lyte A USP added: _____ mL

Time of cell counts in hemocytometer: _____ :_____

Automated Cell Counter used instead of hemocytometer: _____

Hemocytometer Count (SOP Step 7.6.4 and 7.6.5)				
Hemocytometer area (Appendix 4):	A	B	C	D
Total cells per hemocytometer square counted:				
Viable cells per hemocytometer square counted:				
Hemocytometer dilution factor:	1: ___			

$$\text{Viable CELLS/mL} = \left(\frac{\text{Total Viable Cells A+B+C+D}}{4} * 10{,}000\right) * \text{dilution factor}$$

Calculated total viable CELLS/mL:	CELLS/mL
Total viable cells in remaining cell suspension:	cells

3. PBMC Sample Preparation Table

	Patient Sample	*Example*
Sample/Patient ID		*1234-1023-300*
Collection Time Point		*Pre-dose, D1*
Total Cell No.	cells	*7.2 x10^6 cells*
Resuspension Vol. (mL)	mL	*2.4 mL*
Cell Concentration in 1-mL Tubes	cells	*3 x10^6 cells*
No. of 1-mL Tubes		*2*
Vol. Partial Tube	µL	*400 µL*
Cell Concentration in Partial Tube	cells	*1.2 x10^6 cells*

4. PBMC Pellet Storage

Date/time Sarstedt tubes placed into -80°C storage: _____

Storage location: _____

Date/time samples shipped to FNLCR (optional): _____/_____/_____

BATCH RECORD: INITIALS _____ DATE: _____

5. Notes, including any deviations from the SOP:

6. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: ____/____/_____

BATCH RECORD: INITIALS _____ DATE: _____

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APPENDIX 2: BLOOD SAMPLE COLLECTION AND LABELING

1. Sample PD collection schedule as defined in the Pharmacodynamic/Correlative Study Section of the clinical protocol to ensure all specimens are collected at the designated time points.

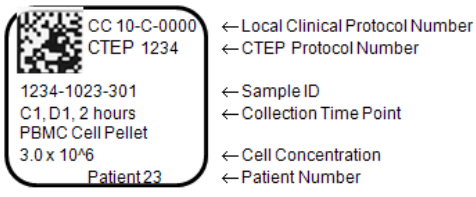
Patient #: **Protocol:** **Clinical Site:** **Cycle:**

All collections in 8-mL draw capacity Vacutainer CPTs

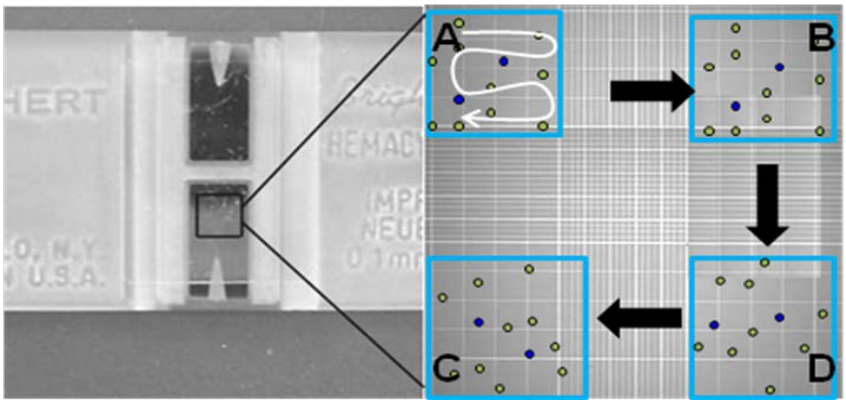
Date Mo/Da/Yr	Planned Time H:Min	Collection	Time Point	Actual Time H:Min	Initials	Comments
_____	:	1	D1 Pre-dose	:		
(Day 1)	:	2	D1 2 h	:		
	:	3	D1 4 h	:		
	:	4	D1 7 h	:		
	:	5	D1 24 h	:		
_____	:	6	D2 2 h	:		
(Day 2)	:	7	D2 4 h	:		
	:	8	D2 7 h	:		
	:	9	D2 24 h	:		

2. **Sample pre-printed label to be brought to clinic for each PD blood collection**

3. **Sample label for tubes for storage and shipping of pelleted PBMC specimens**



APPENDIX 3: HEMOCYTOMETER CELL COUNT



1. Prepare hemocytometer chamber and cover glass for use by cleaning with 70% ethanol and wiping dry with Kimwipes.

2. Place cover glass squarely on top of hemocytometer and transfer 10 to 20 µL of a 1:5 trypan blue stained dilution of cells (20 µL sample + 60 µL 1X PBS + 20 µL 0.4% trypan blue) under cover glass on one side of hemocytometer and allow cells to settle.

3. Using the 20X objective, locate the upper left square of one grid (A).

4. When counting cells follow these general guidelines:

a. The middle of the triple lines separating each corner square (A-D) is the boundary line. Cells that touch the upper or left boundaries are included, but cells that touch the lower or right boundaries are excluded.

b. Optimal cell counts should be 30-150 cells/corner area, do additional 1:5 dilutions for high density specimens

c. If greater than 10% of particles are clusters of cells, try to disperse cells in original trypan blue suspension and repeat the count.

5. Count **total cells** (white and blue cells) in each corner area (A - D) of the hemocytometer using a snake-like motion as indicated in corner B. Record all 4 counts (A, B, C, and D) in the Batch Record.

6. Count **viable cells** (white cells only) in the same manner. Record all 4 counts (A, B, C, and D) in the Batch Record.

7. Total cells and total viable cells/mL can be determined using the following equation:

$$\text{Cells/mL} = \left(\frac{\text{Total Cells A+B+C+D}}{4} * 10{,}000 \right) * \text{dilution factor}$$

APPENDIX 4: PD SAMPLE SHIPPING MANIFEST

From: Phone: E-mail:	**PD Sample Shipping Manifest**	

In Package	Item No	Patient/Specimen ID	Clinical Protocol	Time Point	Collection Date	Collection Time	Description
	Example	*1234-1023-300*	*12-C-0000*	*Pre-dose D1*	*06/12/12*	*08:50*	
	Example	*1234-1023-301*	*12-C-0000*	*C1, D1, 2hr*	*06/12/12*	*16:05*	
☐	1				/ /		
☐	*2*				/ /		
☐	*3*				/ /		
☐	*4*				/ /		
☐	*5*				/ /		
☐	*6*				/ /		
☐	*7*				/ /		
☐	*8*				/ /		
☐	*9*				/ /		
☐	*10*				/ /		

Verification of Contents	**Signature**	**Date**
Contents Verified Collection Laboratory	_____	/ /
Contents Verified FNLCR PD Central Receiving	_____	/ /