

NCTVL Standard Operating Procedure (SOP)

Title:	PBMC Specimen Processing for PAR Immunoassay			Page 1 of 12
Doc. #:	SOP340521	Revision:	RETIRED	Effective Date: 7/24/2009

National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate

SAIC-Frederick, Inc.

NCI-Frederick Cancer Research Facility

Change History

Revision	Approval Date	Description	Originator	Approval
--	7/13/2006	New document adopted from LHTP	RP	JJ
A	10/13/2006	Format change and revision	YZ	JJ
B	9/19/2007	PBMC vial changes	KL	JJ
C	10/14/2008	Merge PBMC Preparation (SOP34503) with Extraction Method (SOP34506)	KG	JJ
D	12/01/2008	Updated SOP Web site, SOP title, and moved reagent preparation to Batch Record for technician sign-off	YZ	JJ
	7/24/2009	RETIRED		

NOTICE:

SOP340521 has been retired.

- SOP340521: PBMC Specimen Processing for PAR Immunoassay, has been separated into 2 SOPs;
 - **SOP340503** outlines PBMC collection and handling, and
 - **SOP340506** outlines protein extraction from the prepared PBMCs.

Processing Samples from Patients Currently on PAR Inhibitor Clinical Trials

- If some of a patient's specimens have been collected following SOP340521, collect and process the remaining samples for that patient following the same SOP to ensure consistency in sample handling.
- For any new patients, use the updated PBMC Specimen Handling SOP, SOP340503, to collect and process samples for shipment to NCTVL.

Please check the DCTD Biomarkers Web site

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

for the current status of SOP340503 and SOP340506



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1.0 PURPOSE

To standardize the method for preparing lysates of peripheral blood mononuclear cells (PBMC) to enable quantification of poly (ADP-ribose) (PAR) levels with an Enzyme-Linked ImmunoSorbent Assay (ELISA) in pharmacodynamic (PD) studies of PAR polymerase (PARP) inhibitors.

2.0 SCOPE

This procedure applies to all personnel responsible for the processing of blood from patients participating in clinical trials of PARP inhibitors to isolate PBMCs and then prepare extracts for the analysis of PAR levels by the PAR Immunoassay (SOP340505).

3.0 ABBREVIATIONS

CEB	=	Cell Extraction Buffer
CPT	=	Cell Preparation Tube
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
ID	=	Identification
NCI	=	National Cancer Institute
PI	=	Protease Inhibitor
PBS	=	Phosphate Buffered Saline
PAR	=	Poly (ADP-Ribose)
PARP	=	Poly (ADP-Ribose) Polymerase
PBMC	=	Peripheral Blood Mononuclear Cells
PD	=	Pharmacodynamic
PMSF	=	Phenylmethanesulfonyl fluoride
SOP	=	Standard Operating Procedure
RT	=	Room Temperature

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4.0 INTRODUCTION

The PAR Immunoassay (SOP340505) has been developed to measure the impact of PARP inhibitors on PAR levels in a variety of biospecimen types. This SOP outlines the recommended procedure for the preparation of PBMCs to ensure consistency in sample extraction of PAR-containing macromolecules.

5.0 RESPONSIBILITIES

- 5.1 It is the responsibility of the Laboratory Supervisor/Manager to ensure that all personnel have documented training and certification on this SOP prior to the actual handling and processing of specimens from clinical trial patients.
- 5.2 It is the responsibility of the Laboratory Supervisor to confirm scheduled specimen collection time points, 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 isolation and extraction of PBMCs.
- 5.3 It is the responsibility of the Laboratory personnel 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 Laboratory Technician responsible for the preparation and processing of the PBMCs is to follow this SOP and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed for each experimental run, with each page ***dated and initialed***, and placed with the clinical specimen information.
- 5.5 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 Sorvall Legend RT centrifuge (Fisher Scientific)
- 6.2 Sorvall Fresco centrifuge (Fisher Scientific)
- 6.3 Ultrasonic Processor (Cole-Parmer Instruments, Model#: CP 130PB)
- 6.4 Hemacytometer
- 6.5 Pipettors (1000 μ L, 200 μ L, 20 μ L) and tips
- 6.6 Electronic pipette

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- 6.7 1.5-mL Sarstedt o-ring screw cap tubes (Fisher, Cat#: 72.694.005)
- 6.8 2.0-mL Sarstedt o-ring screw cap tubes (Fisher, Cat#: 72.694.006)
- 6.9 3-mL Falcon transfer pipette (Fisher, Cat# 13-711-6)
- 6.10 15-mL polypropylene tubes (Fisher, Cat#: 14-959-49B or Becton Dickinson, Cat#: 352097 or 352095)
- 6.11 Vacutainer Cell Preparation Tubes (CPT) (blue/black conventional closure; Becton Dickinson, Cat#: 362760)
- 6.12 Plasma-Lyte A pH 7.4, USP (Baxter, NDC 0338-631703 or 0338-6317-04)
- 6.13 Trypan Blue, 0.4%, sterile (StemCell Technologies, Cat#: 07050)
- 6.14 Phenylmethanesulfonyl fluoride (PMSF) (Sigma, Cat#: 93482-50ML-F)
- 6.15 Protease Inhibitor (PI) Cocktail (Sigma, Cat#: P-2714 or Roche, Cat#: 11 697 498 001)
- 6.16 Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)
- 6.17 100% ethanol
- 6.18 Dry ice
- 6.19 81-cell chipboard storage boxes (Fisher, Cat#: 12-565-182)
- 6.20 Thermoflask cooler
- 6.21 Ice bucket
- 6.22 -80°C freezer

*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

7.1 All reagents for an individual assay are to be prepared for use in one experimental run, and only in the amounts required for the specific assay. All excess reagents are to be discarded following appropriate safety procedures. The buffers to prepare are listed in the Batch Record (Appendix 1, Section 1).

7.2 Blood Collection and PBMC Preparation

7.2.1 Ensure that the phlebotomist is using the recommended 4-mL Becton Dickinson Vacutainer CPTs to draw the blood samples. If necessary, supply the phlebotomist with the correct CPTs. Four identical specimen labels are to be prepared for each time point as defined in the Pharmacodynamic/Correlative Study section of the clinical protocol.

7.2.2 The research nurse is to notify the laboratory of scheduled PD sample collections, preferably giving at least 24-h notice. A laboratory technician is to arrive at the blood collection site at least 5 min ahead of the

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scheduled time point(s) to ensure rapid transport to the laboratory after collection.

- 7.2.3** Of the 4 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.
- 7.2.4** The sample is transported at room temperature (RT) in a double container from the clinical collection site to the sample processing laboratory. A Batch Record is to be started for each specimen (Appendix 1).
- 7.2.5** The blood sample is mixed by inverting the tube gently 5 to 8 times and then centrifuging at 1,500 x g for 30 min at 18°C to 25°C, without the brake.
- 7.2.6** Place the third identical label onto the laboratory tracking sheet, and place the fourth label onto a sterile 15-mL conical tube.
- 7.2.7** 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 conical tube. Discard the remaining liquid and Vacutainer CPT in the appropriate biohazardous waste container(s).
- 7.2.8** Using a pipette, slowly add Plasma-Lyte A USP to the PBMCs in the 15-mL tube to bring the total volume to 14 mL; cap, then mix by gentle inversion 5 to 8 times.
- 7.2.9** Centrifuge the sample at 430 x g for 10 min at 18°C to 25°C, without the brake.
- 7.2.10** Using a sterile pipette, aspirate as much supernatant as possible without disturbing the cell pellet. Discard the supernatant into biohazardous liquid waste.
- 7.2.11** Add 6 mL of Plasma-Lyte A USP to the tube, and 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 5-mL pipette.
- 7.2.12** Immediately after resuspending the cell pellet, transfer 20 µL of sample into a microtube containing 60 µL Plasma-Lyte A USP and 20 µL of 0.4% Trypan Blue, and set aside for a cell count.

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7.2.13 Centrifuge the remaining 5.98-mL cell suspension at 430 x g for 10 min at 18°C to 25°C, without the brake. While centrifuging, after the Trypan Blue sample has incubated for 2 to 5 min, determine the total and viable cell count using a hemacytometer.

7.2.14 Record the following information in Section 2 of the Batch Record (Appendix 1):

- Total cell count in each hemacytometer square that was counted.
- Total viable cell count in each hemacytometer square that was counted.
- Calculated viable cell concentration in the 6.0-mL cell suspension.
- Viable cell yield in the remaining 5.98-mL cell suspension.

7.2.14.1 Based on the total viable cell yield, proceed as follows:

- a. If cell yield is $\geq 6.5 \times 10^6$, then calculate the volume to add in order to make the cell concentration of PBMC suspension equal to 3×10^6 cells/mL.
- b. If cell yield is $< 6.5 \times 10^6$, then calculate the volume required to make the suspension equal to 1.5×10^6 cells/mL.

7.2.15 Without disturbing the cell pellet, remove supernatant and discard in an appropriate biohazardous waste container.

7.2.16 Add to the cell pellet the volume of Plasma-Lyte A USP calculated in SOP Step 7.2.14 to yield a cell suspension containing 3×10^6 , or if required 1.5×10^6 , viable PBMCs per mL. 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.

7.2.17 Based on the PBMC cell suspension concentration, proceed as follows:

- a. For the 3×10^6 cells/mL suspension, aliquot the PBMC suspension into 2.0-mL Sarstedt tubes using 1.0-mL aliquots until the remaining volume of cell suspension is less than 1.0 mL. Record the number of 2.0-mL Sarstedt tubes that have been prepared in the Batch Record (Appendix 1). Discard the remaining cells as biohazardous waste.
- b. For the 1.5×10^6 cells/mL cell suspension, aliquot the PBMC suspension into 1.5-mL Sarstedt tubes using 1.0 mL aliquots until the remaining volume of cell suspension is less than 1.0 mL. Record the number of 1.5-mL Sarstedt tubes that have been prepared in the

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Batch Record (Appendix 1). Pipette the residual volume of cell suspension remaining in the tube into a 1.5-mL Sarstedt tube, noting the actually volume. Write “partial” on the top of the tube with a black Sharpie. Record the preparation of the single residual-volume vial in Section 3 of the Batch Record (Appendix 1).

- **Note:** Based on the initial cell yield determined in Section 7.2.14, either a 2.0-mL (3.0×10^6 cells/mL) or 1.5-mL (1.5×10^6 cells/mL) Sarstedt tube is used to differentiate the final concentration of the cell suspension.

7.2.18 Centrifuge the Sarstedt tubes in Sorvall Fresco centrifuge at 12,000 rpm (12,000 x g) for 10 min at 4°C to 10°C.

7.2.19 Remove and discard as much supernatant as possible without disturbing the cell pellet. Place supernatant into biohazardous waste.

7.2.20 Place appropriate labels onto each Sarstedt tube, indicating specimen type, ID, and date.

7.2.21 Snap-freeze the Sarstedt tube containing the PBMC cell pellets using liquid nitrogen or a dry ice/ethanol bath.

7.2.22 Store the frozen PBMC samples at -80°C until analysis.

7.2.23 Complete Batch Record (Appendix 1), review, obtain required signatures, and file.

7.2.24 All activities with these specimens should be tracked and noted in the specimen file (i.e., extraction and analysis, shipment to another site, etc).

7.3 PBMC Cell Pellet Extraction for PAR Immunoassay

7.3.1 Add 300 µL of Cell Extraction Buffer (CEB) containing 1X protease inhibitor (PI) cocktail and 1 mM PMSF to fresh or frozen cell pellet in a 2.0-mL Sarstedt tube containing 3.0×10^6 cells. The appropriate ratio of buffer to cell number is 100 µL of CEB per 1.0×10^6 cells.

7.3.2 Vortex tube for 3 to 5 sec at medium speed (setting 5-6 on Vortex Genie 2). Ensure the cell pellet is dislodged and mixing gently in the CEB.

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- 7.3.3** Place tube on ice and incubate the cells in the CEB for 30 minutes with 3 to 5 sec of vortexing at 10-min intervals.
- 7.3.4** Move samples to room temperature and add 20% SDS to a final concentration of 1% (e.g., 15 to 300 μ L lysate).
- 7.3.5** Vortex tube for 3 to 5 sec at medium speed to distribute the SDS in the buffer.
- 7.3.6** Boil the cell extract for 5 min in a 100°C heat block or boiling water bath.
- 7.3.7** Clarify the extract by centrifugation of the tube at 13,000 rpm for 5 to 10 min at 4°C. Transfer supernatant into appropriately labeled tubes and hold on ice.
- 7.3.8** If not used immediately for protein or PAR assay, freeze and store at -80°C.
- 7.3.9** Record extraction information in Section 4 of the Batch Record (Appendix 1).

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APPENDIX 1: BATCH RECORD

PAGE 1

Each experimental run must be accompanied by a completed Batch Record with each page *dated and initialed*.

Lab Technician (Certification number): _____ (_____)

Date: _____

Reviewer/Supervisor: _____ Date: _____

Lot numbers:

Vacutainer CPT Product # and Lot #: _____

Plasma-Lyte A USP Lot # and expiration date: _____

15-mL polypropylene tubes (circle one):

Fischer 14-959-49B

Becton Dickinson 352097 or 352095

Other: _____

Sarstedt tube Lot #: _____

Trypan Blue Lot # _____ Dilution Vials _____

Serial numbers of equipment:

P-100 Pipetman: _____

P-1000 Pipetman: _____

Processing Records:

1. Preparation of Reagents

Name	Stock	Working Solution
Cell Extraction Buffer (CEB)	Pre-made 1X buffer	Remove sufficient volume for number of samples to be processed at 500 µL/20 mg tissue.
Protease Inhibitor (PI) Cocktail	25X: 1 tablet in 2 mL ddH ₂ O	1X: 40 µL 25X Stock in 1 mL CEB
PMSF	100 mM Manufacturer's Stock Solution	1 mM: 10 µL of 100 mM Stock in 1 mL CEB (add PMSF immediately before use)

INITIALS _____ DATE: _____



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BATCH RECORD

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NOTE: *Record times using military time (24-h designation). Create a new Batch Record for each patient and each patient sample.*

2. Isolation of PBMCs

Sample ID: _____

Study Project ID: _____

Blood Volume _____ Time of Venous Blood Draw: _____

Time Lab Processing Begins: _____

Time of PBMC Transfer into Plasma-Lyte A USP: _____

Time of Cell Counts in Hemacytometer: _____

Record the following data:

Total cell counts in each hemacytometer square counted: _____

Viable cell counts in each hemacytometer square counted: _____

Hemacytometer dilution factor: _____

Calculated viable cell concentration in suspension: _____

Viable cell yield remaining in the 5.98-mL cell suspension: _____

Volume used to resuspend cell pellet to 3×10^6 viable PBMCs per mL: _____

INITIALS _____ DATE: _____
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BATCH RECORD

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4. Extraction of PBMC Cell Pellet for PAR Immunoassay

Number of PBMC vials: _____

Prepare CEB (_____ PBMC vials + 2) x 300 μ L = _____ μ L

Add 25X PI stock _____ μ L Add 100X (100 mM) PMSF _____ μ L

Add 300 μ L or _____ μ L of CEB into each sample vial.

Lyse cell pellet on ice for 30 min: Start time: _____ Stop time: _____

Add 15 μ L or ____ μ L of 20% SDS into each sample before boiling cell lysate for 5 min at 100°C.

Date of Freezing of Extract: _____ Date of Thawing of Extract: _____

Number	Sample Name/ID	Number of Cells/vial (x10 ⁶)	Add CEB (μ L)	Add 20% SDS (μ L)	PBMC Concentration (10 ⁶ /mL)
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					

INITIALS _____ DATE: _____

