

SOP340506: PBMC Protein Extraction for PAR Immunoassay

Effective Date: 1/8/2013

Please check for revision status of the SOP at

<http://dctd.cancer.gov/drug-discovery-development/assays/validated-biomarker-assays>

and be sure to use the current version.

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VERSION INFORMATION

1. Approvals

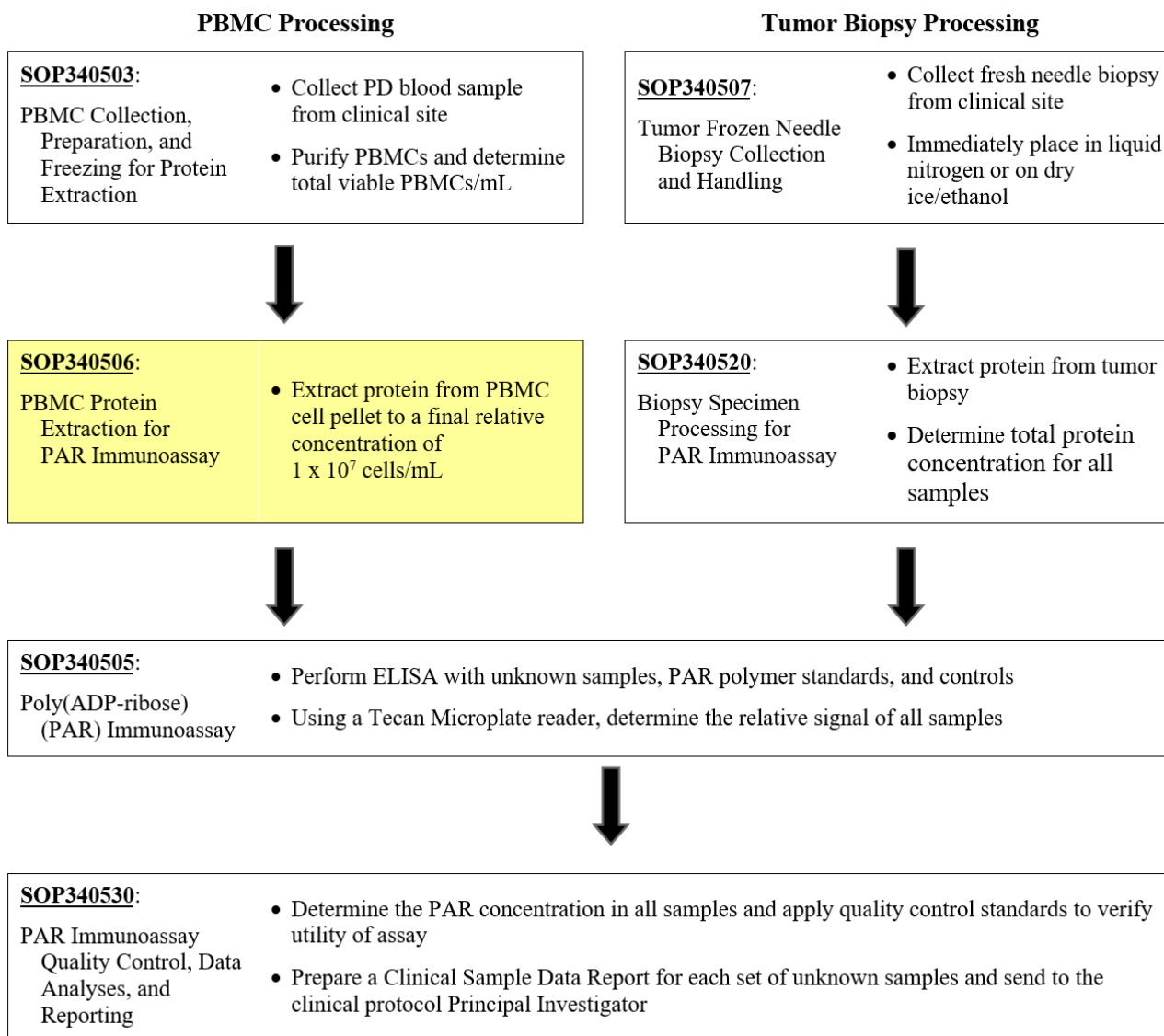
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2. Change History

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
E	8/10/2009	Separate PBMC Preparation (SOP34503) and Protein Extraction (SOP34506) SOPs, and prepare for publication to the DCTD Biomarkers Web site	YAE	JJ
F	4/8/2011	Clarified SOP and Batch Record references and updated Section 5.0 and 6.0	YAE	JJ
G	1/8/2013	Preparation volume of CEB (with PIs) defined to reduce calculation errors, references to 1.5×10^6 cell preparation from SOP340503 removed, minimum PBMC cell number defined, and new quality control and data analysis SOP referenced.	YAE	KFG

OVERVIEW OF PAR IMMUNOASSAY SPECIMEN PROCESSING



1.0 PURPOSE

Standardize the method for preparing lysates from 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 and/or chemotherapeutic agents.

2.0 SCOPE

This procedure applies to all personnel involved in the use of PAR as a PD marker during clinical trials and in the preparation of samples for the analysis of PAR levels by the PAR Immunoassay (SOP340505). The goal of the SOP and associated training is to ensure consistency in PAR measurement across samples and clinical sites.

3.0 ABBREVIATIONS

CEB	=	Cell Extraction Buffer
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HRP	=	Horse Radish Peroxidase
ID	=	Identification
IQC	=	Internal Quality Control
LHTP	=	Laboratory of Human Toxicology and Pharmacology
NCTVL	=	National Clinical Target Validation Laboratory
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PAR	=	Poly(ADP-Ribose)
PARP	=	Poly(ADP-Ribose) Polymerase
PBMC	=	Peripheral Blood Mononuclear Cells
PBS	=	Phosphate Buffered Saline
PD	=	Pharmacodynamic
PI	=	Protease Inhibitor
PMSF	=	Phenylmethanesulfonyl Fluoride
RT	=	Room Temperature
SOP	=	Standard Operating Procedure

4.0 INTRODUCTION

The PAR Immunoassay (SOP340505) has been developed to measure the effect of PARP inhibitors and/or chemotherapeutic agents on PAR levels in a variety of biospecimen types, including PBMCs and tissue/tumor biopsies. An ELISA is used to first capture PAR from total cell extracts on plates coated with a PAR capture monoclonal antibody. The captured protein is then detected using a PAR polyclonal detection antibody followed by addition of an HRP-conjugate to allow chemiluminescent readout and quantitation of PAR levels.

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 also oversees the personnel running SOPs within the laboratory and is responsible for ensuring that only certified and experienced personnel handle clinical samples.

Certified Assay Operator A Certified Assay Operator may be a Laboratory Technician/Technologist, Research Associate, or Laboratory Scientist who has been certified through training on this SOP. The Certified Assay Operator works 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 The Certified Assay Operator 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.
- 5.3 Digital versions of the sample table in the Batch Record (Appendix 1, Section 2) can be created for logging sample information as long as all column information exactly matches the table in the Batch Record. A copy of the completed, digital sample table must be printed and attached to the Batch Record in order to maintain a complete audit trail.
- 5.4 The responsible personnel are to check the DCTD Biomarkers Web site (<http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm>) to verify that the most recent version of the SOP for the assay is being used.

6.0 MATERIALS AND EQUIPMENT REQUIRED

- 6.1** Pipettors (200-1000 μ L, 50-200 μ L, 2-20 μ L) and tips
- 6.2** 1.5-mL Sarstedt o-ring screw cap tubes (e.g., Fisher Scientific, Cat#: 72.692.005)
- 6.3** 2.0-mL Sarstedt o-ring screw cap, skirted tubes (e.g., Fisher Scientific, Cat#: 72.694.006)
- 6.4** Printable microcentrifuge tube labels
- 6.5** 81-place freezer storage boxes (e.g., Fisher Scientific, Cat#: 12-565-182)
- 6.6** Ice bucket
- 6.7** Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)
- 6.8** Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)
- 6.9** Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)
- 6.10** 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)
- 6.11** Liquid nitrogen or dry ice/ethanol bath
- 6.12** Sorvall Fresco microcentrifuge, refrigerated (Fisher Scientific)
- 6.13** Vortex-Genie 2 (Daigger, Cat#: EF3030A)
- 6.14** 100°C heat block or boiling water bath
- 6.15** -20°C and -80°C freezer
- 6.16** 2°C to 8°C refrigerator
- 6.17** PBMC specimens processed following SOP340503 (PBMC Specimen Collection, Preparation, and Freezing for Protein Extraction)

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

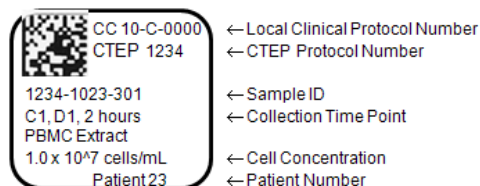
7.0 OPERATING PROCEDURES

- 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. Process a single patient's samples, **batched**, to ensure consistent sample handling. A separate Batch Record should be started for each patient's **batched** samples.
- 7.2 Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record ([Appendix 1](#)). Include reference clinical protocol number(s), if applicable.
- 7.3 Prepare the reagents outlined (Appendix 1, Section 1A). **Note:** Do not add protease inhibitors or PMSF (referred to as PIs) to Cell Extraction Buffer (CEB) until noted in the SOP.
- 7.4 **Cell Lysis**
 - 7.4.1 Fill in the Sample Information Table in the Batch Record (Appendix 1, Section 2) with the Sample/Patient ID and starting PBMC cell number in each PBMC vial.
 - 7.4.1.1 The Sample/Patient ID should include the CTEP protocol number followed by a unique patient identifier and a sequential specimen ID (NCI blood collections for PD sampling are series 300).
 - 7.4.2 Record the total volume of CEB (**with** PIs) needed for each sample in the Sample Information Table in the Batch Record (Appendix 1, Section 3A); 100 μL of CEB (**with** PIs) is added per 1×10^6 PBMCs.
 - 7.4.2.1 **Do not use** samples with $< 1 \times 10^6$ PBMCs for the PAR Immunoassay. A PBMC pellet containing 1×10^6 PBMCs could be resuspended in 100 μL to create a 1×10^7 PBMCs/mL solution; this sample would only be sufficient to run the PAR Immunoassay once.
 - 7.4.2.2 **Do not** prepare samples at $< 1 \times 10^7$ PBMCs/mL for the PAR Immunoassay.
 - 7.4.3 Prepare fresh CEB (**with** PIs) as outlined in the Batch Record (recipe in Appendix 1, Section 1A). Keep on ice.
 - 7.4.4 Place the fresh or frozen PBMC cell pellets on ice and add 100 μL CEB (**with** PIs) per 1×10^6 PBMCs and record the volume CEB (**with** PIs) used for each sample in the Sample Information Table (Appendix 1, Section 2). This should yield a relative cell concentration of 1×10^7 PBMCs/mL.
 - 7.4.5 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 (**with** PIs).
 - 7.4.6 Place tubes on ice and incubate the cells in CEB (**with** PIs) for 30 min; vortex every 10 min for 3 to 5 sec at medium speed. Record the start and stop times for the incubation in the Batch Record (Appendix 1, Section 3).
 - 7.4.7 Move samples to RT and add 20% SDS to a final concentration of 1% SDS (e.g., 15 μL 20% SDS to 300 μL lysate). Record the total volume SDS added to each sample in the Sample Information Table (Appendix 1, Section 2).
 - 7.4.8 Vortex tube for 3 to 5 sec at medium speed to distribute the SDS in the buffer.
 - 7.4.9 Boil the cell extract for 5 min in a 100°C heat block or boiling water bath, and record the start and stop times in the Batch Record (Appendix 1, Section 3).
 - 7.4.10 Snap-cool specimen tube on ice after boiling then vortex tube at maximum speed for 10 sec.

7.5 PBMC Lysate Preparation

7.5.1 Clarify the extract by centrifugation at 12,000 x g for 5 to 10 min at 2°C to 8°C. Transfer the cleared lysate into a labeled Sarstedt tube and hold on ice. Discard the original tube with any precipitated material in the appropriate waste container.

Sample label:



7.6 If the **stock lysates** will be used within 8 h, store on ice or at 2°C to 8°C.

7.7 **Stock lysates** not used immediately for the PAR Immunoassay can be snap-frozen in liquid nitrogen or a dry ice/ethanol bath and then stored in an 81-place freezer box, batched by patient, at -80°C until analysis. Record the date and time lysates are frozen in the Batch Record (Appendix 1, Section 4).

7.8 Review and finalize the Batch Record (Appendix 1). Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 5).

7.9 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within the reports are correct (Appendix 1, Section 6).

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APPENDIX 1: BATCH RECORD

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

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Clinical Protocol Number: _____

Patient ID: _____

A separate Batch Record should be started for each patient's **batched** samples.

1. Equipment and Preparation of Reagents

A. Reagents

Buffers should be prepared based on volumes needed to complete all the steps preparing at least 10% excess volume of buffer to ensure adequate volume to complete the study.

- a. Protease Inhibitor Cocktail Tablets: Dissolve one PI cocktail tablet in 2 mL ddH₂O (25X stock). The 25X stock solution is stable for 1 wk at 2°C to 8°C or 12 wk at -15°C to -25°C. If stored frozen, the material must be prepared as single-use aliquots to prevent repeat freeze-thaw.

Lot#: _____ Expiration Date: _____

- b. PMSF: Manufacturer's stock solution supplied at 100 mM. Label vial with date of receipt from manufacturer; the expiration date should be considered 6 mo after receipt.

Lot#: _____ Expiration Date: _____

- c. Cell Extraction Buffer (CEB [**without** PIs]): Manufacturer's supplied 1X solution.

Lot#: _____ Expiration Date: _____

- d. CEB (**with** PIs): 5.0 mL is sufficient to prepare 15 unknown samples (maximum of 300 µL/sample). Keep on ice.

Reagent	Stock Concentration	Amount Needed	Final Concentration
CEB	stock	4.75 mL	N/A
PI Cocktail	25X	200 µL	1X PI Cocktail
PMSF	100 mM	50 µL	1 mM PMSF