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National Clinical Target Validation Laboratory

Applied Developmental Directorate, Leidos Biomedical Research, Inc.

Frederick National Laboratory for Cancer Research

Technical Reviewer:	Yiping Zhang	Date:	1-22-14
NCTVL Approval:	Jiuping Ji	Date:	1-22-14
IQC Approval:	Katherine V. Ferry-Galow	Date:	1-23-14
LHTP Approval:	Ralph E. Parchment	Date:	Ralph E. Parchment -S (Affiliate) Digitally signed by Ralph E. Parchment -S (Affiliate) DN: c=US, o=U.S. Government, ou=NIH, ou=People, 0.9.2342.19200300.100.1.1=0012115833, cn=Ralph E. Parchment -S (Affiliate) Date: 2014.01.26 18:56:56 -05'00'
DCTD OD Approval:	Joseph E. Tomaszewski	Date:	Joseph E. Tomaszewski -S Digitally signed by Joseph E. Tomaszewski -S DN: c=US, o=U.S. Government, ou=HHS, ou=NIH, ou=People, 0.9.2342.19200300.100.1.1=0010071723, cn=Joseph E. Tomaszewski -S Date: 2014.01.27 08:48:51 -05'00'

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
J	1/22/2014	Thermoconductive plate holder used for all 37°C and 25°C incubation steps; all incubations in fixed-temperature incubators. Minimum protein concentration requirement increased and all samples standardized to same concentration before running assay. Additional 5-sec soak time added to plate washing method to decrease assay variability. Appendix 3 (critical reagent qualification) moved to separate document (QAP-002).	YAE, KFG	KFG
I	1/8/2013	New source for PAR polymer standards; quality control samples now generated from xenograft lysates; unknown sample preparation updated to remove 1 µg/µL starting lysate requirement; all unknown and control samples now have an equivalent volume of matrix/well; new Plate Map set-up for immunoassay; data analysis and quality control sections moved to new SOP340530; web-based macro data analysis tool removed. Critical Reagents supply information generalized in SOP; the shipping manifest will function as the primary source of information for critical reagents. Removed Appendices 3-5 (old quality control and critical reagent sample references and web-based macro directions) and created new Critical Reagent Qualification Appendix.	KFG, YAE	KFG
H	11/30/2011	Changed coating buffer source; provided as a critical assay reagent. Restructure SOP Sections 9.0 and 10.0 for clarity. Removed Appendix 4, Section 2. Laboratory Director/Supervisor signature moved to end of Batch Record.	YZ, KFG	JJ
G	4/8/2011	Added order information for critical reagents, Appendix 3 for tumor control lysate preparation, and Appendix 5 for site recommendations to qualify reagents. QC recommendations for alternative reagents are provided. Batch Record calculations for antibody preparation are now based on dilution factor.	YAE	JJ
F	3/31/2010	Remove Checklist; Update Batch Record; New Lot# PAR mAb	YZ	JJ
E	8/10/2009	Added Data Analysis Macro installation instructions to Appendix 5 and PAR Immunoassay processing flow chart. General edits for consistency.	MF	JJ

Change History (cont.)

Revision	Approval Date	Description	Originator	Approval
D	12/01/2008	Revision of SOP based on first PAR Immunoassay Training Course to include: pictorial flowchart, condensed Batch Record, reorganized appendices, expanded data analysis section, and Program approval	KG	JJ
C	10/15/2008	Revision for SOP Web page – checklists, expanded data analysis, and examples of ranges	KG	JJ
B	1/4/2008	Revision with new standards	YZ	JJ
A	9/20/2007	Revision with new reagents	YZ	JJ
--	10/20/2006	New document	YZ	JJ

TABLE OF CONTENTS

OVERVIEW OF PAR IMMUNOASSAY SAMPLE PROCESSING

PBMC Processing

SOP340503:

PBMC Collection, Preparation, and Freezing for Protein Extraction

- Collect PD blood sample from clinical site
- Purify PBMCs and determine total viable PBMCs/mL

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SOP340506:

PBMC Protein Extraction for PAR Immunoassay

- Extract protein from PBMC cell pellet to a final relative concentration of 1×10^7 cells/mL

Tumor Biopsy Processing

SOP340507:

Tumor Frozen Needle Biopsy Collection and Handling

- Collect fresh needle biopsy from clinical site
- Immediately place in liquid nitrogen or on dry ice/ethanol

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SOP340520:

Biopsy Specimen Processing for PAR Immunoassay

- Extract protein from tumor biopsy
- Determine total protein concentration for all samples

↓

SOP340505:

Poly(ADP-ribose) (PAR) Immunoassay

- Perform ELISA with unknown samples, PAR polymer standards, and controls
- Using a Tecan Microplate reader, determine the relative signal of all samples

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SOP340530:

PAR Immunoassay Quality Control, Data Analyses, and Reporting

- Determine the PAR concentration in all samples and apply quality control standards to verify utility of assay
- Prepare a Clinical Sample Data Report for each set of unknown samples and send to the clinical protocol Principal Investigator

1.0 PURPOSE

Standardize an enzyme-linked immunosorbent assay (ELISA) method for quantifying poly(ADP-ribose) (PAR) levels as a pharmacodynamic (PD) measure of PAR polymerase (PARP) inhibitors and/or chemotherapeutic agents.

2.0 SCOPE

This procedure applies to all personnel involved in the analysis of PAR levels by the PAR Immunoassay during clinical trials. The goal of the SOP and associated training is to ensure consistency in PAR measurement across samples and clinical sites.

3.0 ABBREVIATIONS

BSA	=	Bovine Serum Albumin
C	=	Control
CEB	=	Cell Extraction Buffer
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
HRP	=	Horse Radish Peroxidase
IA	=	Immunoassay
IQC	=	Internal Quality Control
LHTP	=	Laboratory of Human Toxicology and Pharmacology
mAb	=	Monoclonal Antibody
NCTVL	=	National Clinical Target Validation Laboratory
pAb	=	Polyclonal Antibody
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PAR	=	Poly(ADP-ribose)
PARP	=	Poly(ADP-ribose) Polymerase
PBMC	=	Peripheral Blood Mononuclear Cell
PBS	=	Phosphate Buffered Saline
PD	=	Pharmacodynamic
RLU	=	Relative Light Units
SDS	=	Sodium Dodecyl Sulfate
SOP	=	Standard Operating Procedure
Temp	=	Temperature

4.0 INTRODUCTION

The PAR Immunoassay has been developed to measure the effect of PARP inhibitors and/or chemotherapeutic agents on PAR levels in a variety of biospecimen types, including peripheral blood mononuclear cells (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 DCTD 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 DCTD 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 Plate Map Design (Appendix 1) and Batch Record (Appendix 2) 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 2, Section 5 and 6) 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 tables 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 latest SOP version is being followed.

6.0 CRITICAL REAGENTS, MATERIALS, AND EQUIPMENT REQUIRED

6.1 Critical Reagents:

- **6.1.1** PAR polymer standard (Prepared from Trevigen, Cat# 4336-100-01)
- **6.1.2** Xenograft lysate controls, (custom preparation prepared to target low, mid, and high PAR ranges)
- **6.1.3** PDA II Antibody Coating Buffer (custom order; Trevigen, Cat#: 4520-960-13)
- **6.1.4** PAR mouse monoclonal antibody affinity purified, Clone 10HA (PAR mAb; Trevigen, Cat#: 4335-AMC-050)
- **6.1.5** PAR rabbit polyclonal antibody affinity purified (PAR rabbit pAb; Trevigen, Cat#: 4336-APC-050)
- **6.1.6** Goat anti-rabbit HRP-conjugated pAb, 1 mg/mL (KPL, Cat#: 074-15-061)
- **6.1.7** SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Cat#: 37070). Alternative: LumiGLO Chemiluminescent Substrate (KPL, Cat#: 54-61-00). *The KPL substrate has been verified to provide comparable results to the Pierce substrate on the Infinite 200 Microplate Reader, and may be a good alternative for this assay if using an alternate plate reader.*

6.2 Pipettors (200-1000 µL, 50-200 µL, 2-20 µL) and tips
6.3 Multichannel pipettors (50-300 µL, 5-50 µL) and tips
6.4 Reagent reservoirs (e.g., Fisher Scientific, Cat#: 21-381-27C)
6.5 1.5-mL Sarstedt o-ring screw cap tubes (e.g., Sarstedt, Cat#: 72.692.005)
6.6 15-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352097)
6.7 50-mL polypropylene tubes (e.g., Becton Dickinson, Cat#: 352098)
6.8 Aluminum foil
6.9 Ice bucket
6.10 Acetate plate sealers (Thermo Scientific Pierce, Cat#: 3501)
6.11 Reacti-Bind White Opaque 96-well Plate (Thermo Scientific Pierce, Cat#: 15042)

6.12 UltraPure DNase/RNase-free distilled water (e.g., Invitrogen, Cat#: 10977-015) or Milli-Q water
6.13 Tween 20 non-ionic, aqueous solution, 10% w/v (Roche Applied Science, Cat#: 11332465001)
6.14 Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)
6.15 Phenylmethanesulfonyl fluoride solution, 0.1 M (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)
6.16 Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)
6.17 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)
6.18 10X Phosphate Buffered Saline, pH 7.2 (PBS; e.g., Invitrogen, Cat#: 70013-073)
6.19 SuperBlock (TBS) Blocking Buffer (Thermo Scientific Pierce, Cat#: 37535)
6.20 Albumin, bovine serum (BSA; Sigma-Aldrich, Cat#: A 7030)
6.21 Mouse serum (Sigma-Aldrich, Cat#: M 5905)

6.22 Vortex Genie 2 (Daigger, Cat#:EF 3030A)
6.23 Infinite® 200 or Infinite 200Pro Microplate Reader (Tecan US)
6.24 BioTek ELx405 or BioTek ELx405 Select Microplate Washer (BioTek Instruments)
6.25 BioCision CoolSink 96F thermoconductive plate for flat bottom plates (VWR, Cat#: 95045-476); minimum of two are needed per assay (one at 25°C and the other at 37°C)
6.26 37ºC incubator
6.27 Non-humidified, fixed-temperature incubator able to maintain 25°C (± 3°C)
6.28 -80°C freezer
6.29 2°C to 8°C refrigerator

6.30 PBMC samples processed following SOP340506 or tumor biopsy samples following SOP340520; related Batch Records for samples to be assayed are needed

6.31 QAP-002 Critical Reagent Qualification document

7.0 OPERATING PROCEDURES

7.1 Prior to beginning the assay, refer to the Plate Map Design and Batch Record to review all actions required for successful assay setup (Appendices 1 and 2).

7.2 Record the name and certification number of the Certified Assay Operator, facility running the SOP, and associated clinical protocol number in the Batch Record (Appendix 2).

7.3 Critical Reagents

7.3.1 All Critical Reagents are to be labeled with date of receipt and stored under the specified conditions for no longer than the recommended duration.

- Storage conditions and expiration dates for all Critical Reagents are provided on the package insert.
- Do not exchange reagents from one set of qualified Critical Reagents with a set of reagents qualified separately.
- If the Critical Reagents below are purchased directly from the manufacturer, Certified Assay Sites must qualify the reagents using the recommendations provided in the Critical Reagent Qualification document (QAP-002).

7.3.2 Record the date of receipt, lot numbers, stock/supplied reagent concentration, recommended working dilution/concentration, and expiration dates for the Critical Reagents in the Batch Record (Appendix 2, Section 1).

7.3.2.1 **PAR Polymer Standard**: Supplied as a stock solution in SuperBlock (concentration supplied by lot number).

7.3.2.2 **Xenograft Quality Control Lysates**: Lysates prepared from human-origin xenograft tumors grown in athymic nude mice. Control lysates from different xenograft tumors are pooled such that PAR levels meet pre-determined criteria for High, Mid, and Low analyte levels.

7.3.2.3 **PDA II Antibody Coating Buffer**: Stock solution qualified from the manufacturer.

7.3.2.4 **PAR Capture mAb**: Stock solution qualified from the manufacturer. Lots are qualified as a matched set with the PAR Detection pAb. The recommended dilution for the SOP is provided with reagent.

7.3.2.5 **PAR Detection pAb**: Stock solution qualified from the manufacturer. Lots are qualified as a matched set with the PAR Capture mAb. The recommended dilution for the SOP is provided with reagent.

7.3.2.6 **HRP-Conjugated pAb**: Supplied as a 1 mg/mL stock solution in HRP Stabilizer (KPL, Cat#: 54-15-01).

7.3.2.7 **Chemiluminescent Substrate Solutions**: Stock solutions (Peroxide and Pico Luminol/Enhancer Solutions) qualified from the manufacturer. Protect from light.

7.4 Plate Map and Buffer Preparation

7.4.1 Based on the number of patient samples to be analyzed, generate a Plate Map (Appendix 1) to define the location and replicates of unknown samples, tumor controls, and PAR standards. A single patient's samples, **batched**, should be contained on one 96-well plate, not split over two, to ensure consistent sample handling.

Important: The data analysis template (SOP340530) is based on the 96-well sample designations in the Plate Map (Appendix 1). To prevent user errors, always load the plate according to the plate map well designations.

7.4.2 Once the number of wells is known, determine the amount of reagents required for the assay using the Batch Record in Appendix 2. Once these calculations are complete, check that sufficient reagents and supplies are on hand to complete the assay.

7.4.3 Record serial numbers of equipment in the Batch Record (Appendix 2, Section 2A). Prepare the Coating Buffer, Wash Buffer and PBS-BSA Diluent as outlined in the Batch Record (Appendix 2, Section 2B). Do not prepare CEB (**Complete**) until stated in SOP.

7.4.4 Place a sufficient volume of PDA II Antibody Coating Buffer and SuperBlock Blocking Buffer on the bench top to warm 2 h prior to the initiation of the assay.

7.4.5 Both 37°C and 25°C incubation steps for the PAR IA will be carried out in fixed-temperature incubators. Each 96-well plate will be placed on a CoolSink thermoconductive plate during these incubation steps.

7.4.5.1 Place a sufficient number of CoolSink thermoconductive plates inside each incubator **at least 1 h prior** to the initiation of each incubation step. For each assay plate, one thermoconductive plate will be placed in a 25°C incubator and one in a 37°C incubator.

7.4.5.2 These plates should be placed horizontally inside the incubator in direct contact with the incubator bottom or shelf and should not be stacked. The assay plate will be placed and carefully centered onto a prewarmed thermoconductive plate inside the incubator for each incubation step.

IMPORTANT: Do not let plate dry out during wash and aspiration steps.

7.5 Plate Preparation

7.5.1 Use the calculations in the Batch Record (Appendix 2, Section 3A) to prepare 11 mL PAR mAb Coating Solution for the assay. This is sufficient for one 96-well plate (preparing enough for 110 wells). Thaw antibody immediately prior to dilution; do not allow to sit for extended periods upon thawing.

7.5.1.1 If more than one 96-well plate is to be coated, pool antibody aliquots, if necessary, and then dilute appropriately. This will ensure that all plates are exposed to identical coating antibody. Discard excess diluted antibody.

7.5.2 Add 100 µL of the PAR mAb Coating Solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate at 37°C for 2 h on the prewarmed CoolSink thermoconductive plate. Record the coating antibody incubation conditions in the Batch Record (Appendix 2, Section 3B).

7.5.3 Following incubation with the PAR mAb Coating Solution, aspirate the plate using a plate washer (for the BioTek Plate Washer, use the *Aspirate* program). After aspiration, tap the plate on paper towels to remove any residual liquid.

7.5.4 Add 250 µL of SuperBlock to each well for a blocking step. Cover the plate with an acetate sheet and incubate at 37°C for 1-1.5 h on the prewarmed CoolSink thermoconductive plate. Record the incubation conditions in the Batch Record (Appendix 2, Section 4).

7.5.4.1 After blocking, move plate to a fixed-temperature 25°C incubator on the prewarmed CoolSink thermoconductive plate until the washing step (SOP Step 7.9.1).

7.6 Prepare Working Dilutions of Unknown Biopsy Lysates

7.6.1 Samples with total protein concentration of < **0.25 µg/µL** should not be used in the PAR Immunoassay and will be reported as unanalyzable in the Clinical Sample Data report (SOP340530).

7.6.2 Place all stock biopsy lysates to be assayed on ice. Based on the protein measurements for the **stock tumor lysate** (µg/µL), prepare one of the following **Working Lysates** in CEB (**Complete**) on ice for use in the PAR Immunoassay. Do not pipette less than 2 µL. If the calculations below yield volumes of stock lysate less than 2 µL, prepare sufficient volume of a 1:5 pre-dilution of the lysate before proceeding.

Important: Pre- and post-treatment biopsies from a single patient should be prepared with matched protein concentrations (matched to sample with lowest concentration).

7.6.2.1 For unknown stock lysates with stock protein concentrations **≥ 0.5 µg/µL**:

Prepare 70 µL of a **0.5 µg/µL Working Lysate** as follows:

$$\frac{\text{0.5 µg/µL } \textbf{Working Lysate} \;*\; \text{70 µL}}{\text{XXX µg/µL Conc. Stock Lysate}} = \text{XX µL Vol. Stock Lysate to use}$$

- In labeled 1.5-mL tube, add sufficient CEB (**Complete**) to the calculated volume of stock lysate needed to bring the total volume to 70 µL.
- Record the volumes stock lysate and CEB (**Complete**) and final concentration of **Working Lysate** in the Batch Record (Appendix 2, Section 5).

7.6.2.2 For unknown stock lysates with stock protein concentrations **between 0.25 and 0.5 µg/µL**:

Prepare 130 µL of a **0.25 µg/µL Working Lysate** as follows:

$$\frac{\text{0.25 µg/µL } \textbf{Working Lysate} \;*\; \text{130 µL}}{\text{XXX µg/µL Conc. Stock Lysate}} = \underline{\text{XX}}\ \text{µL Vol. Stock Lysate to use}$$

- In a labeled 1.5-mL tube, add sufficient CEB (**Complete**) to the calculated volume of stock lysate needed to bring the total volume to 130 µL.
- Record the volumes stock lysate and CEB (**Complete**) and final concentration of **Working Lysate** in the Batch Record (Appendix 2, Section 5).

7.6.2.3 Keep working lysates on ice. Flash freeze remaining stock lysates and return them to -80°C storage.

7.7 Preparation of Unknown Tumor Biopsy or PBMC Lysate Samples

7.7.1 **Important**: If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculations in SOP340530, load tumor biopsies first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S16. Stock lysates for PBMCs (1×10^7 cells/mL) are prepared according to SOP340506 and tumor lysates are prepared according to SOP340520.

7.7.2 Place all unknown samples to be assayed on ice. Record the sample/patient IDs for all lysates in the Batch Record (Appendix 2, Section 6A). Each unknown biopsy lysate will take up 3 sample wells (e.g., S1, S2, and S3). For tumor biopsy lysates record the stock tumor lysate concentration and for PBMC lysates record the total cells/mL in the lysate (Appendix 2, Section 6A; center and right portions of table, respectively).

- If needed, use the recipe in Appendix 2, Section 2B, to prepare CEB (**Complete**) for preparation of the tumor lysate samples.

7.7.3 Tumor biopsy lysate samples

7.7.3.1 Biopsy samples are prepared according to the total protein concentration of the **Working Lysate** prepared (0.25 or 0.5 µg/µL) as described below.

7.7.3.2 While each well will have 25 µL total loading volume, S1 triplicate wells will hold 4 µg, S2 2 µg, and S3 1 µg total protein from the stock lysate.

- Samples with total protein concentration of < **0.25 µg/µL** should not be used in the PAR Immunoassay and will be reported as unanalyzable in the Clinical Sample Data Report.

7.7.3.3 For unknown **Working Lysates** with protein concentrations of **0.5 µg/µL**:

- Perform the following calculation to prepare 3 different lysate dilutions (4, 2, and 1 µg/well) in 100 µL total volume with SuperBlock. This is sufficient volume to run each dilution in triplicate (+1 well extra). Clearly label each tube with the sample number (e.g., S1, S2).
- Record volume stock lysate and SuperBlock used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 6A).

$$\frac{\text{(4, 2, or 1) µg/well Diluted Lysate}}{\text{0.5 µg/µL Conc. Stock Lysate}} * \text{4 wells} = \text{(32, 16, or 8) µL Vol. Stock Lysate}$$

7.7.3.4 For unknown **Working Lysates** with protein concentrations of **0.25 µg/µL**:

- Perform the following calculation to prepare 3 different lysate dilutions (4, 2, and 1 µg/well) in 100 µL total volume with SuperBlock. This is sufficient volume to run each dilution in triplicate (+1 well extra). Clearly label each tube with the sample number (e.g., S1, S2).
- Record volume stock lysate and SuperBlock used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 6A).

$$\frac{\text{(4, 2, or 1) µg/well Diluted Lysate}}{\text{0.25 µg/µL Conc. Stock Lysate}} * \text{4 wells} = \text{(64, 32 or 16) µL Vol. Stock Lysate}$$

7.7.3.5 Discard remaining **Working Lysates**.

7.7.4 PBMC lysate samples

7.7.4.1 Stock lysates for PBMCs (1×10^7 cells/mL) are prepared according to SOP340506. In the immunoassay, each well will have 25 µL loading volume yielding 2.5×10^5 cells/well.

7.7.4.2 Place 100 µL of the stock lysate into a 1.5-mL tube labeled with the sample number (e.g., S1, S2). No other sample preparation is necessary; this is enough for triplicate well preparation (+1 well extra).

7.7.4.3 Flash freeze remaining stock lysate in liquid nitrogen or dry ice/ethanol bath and return to -80°C freezer.

7.7.4.4 Record the volume set aside for each sample in the Batch Record as well as the stock cell number/mL (Appendix 2, Section 6A).

7.7.5 Keep samples on ice until use. All lysates will be diluted an additional 3-fold with SuperBlock once loaded on the 96-well plate.

7.8 Preparation of PAR Standards and Xenograft Lysate Controls

7.8.1 Preparation of PAR polymer standards; run in duplicate

7.8.1.1 For one 96-well plate, retrieve one PAR standard stock tube from the -80°C freezer and thaw on ice. Vortex and mix by inverting 5-8 times before use. Label eight 1.5-mL tubes, numbered 1 through 8, for the PAR standards.

7.8.1.2 Use the calculations in the Batch Record (Appendix 2, Section 6B) to prepare a 3 ng/mL (3000 pg/mL) PAR standard stock solution in SuperBlock.

7.8.1.3 Prepare the PAR polymer standards by serial dilution as outlined in the Batch Record (Appendix 2, Section 6B) with final concentrations ranging from 3000 to 23.4 pg/mL in SuperBlock.

7.8.1.4 Keep samples on ice until use. Only make enough standards for the assay and discard any excess. Standards will be diluted 3-fold when added to the 96-well plate to generate a reference curve ranging from 1000 to 7.8 pg/mL.

7.8.2 Preparation of xenograft lysate controls; run twice on plate in duplicate

7.8.2.1 For one 96-well plate, retrieve one each High-, Mid-, and Low-C xenograft quality control stock vials from the -80°C freezer and thaw on ice. Controls are provided at a concentration ready for use in the assay and no further dilution is required. Vortex and mix by inverting 5-8 times before use. If more than one 96-well plate is being run, pool the tumor lysate controls from the same lot prior to dilution.

7.8.2.2 Keep samples on ice until use. Controls will be diluted 3-fold with SuperBlock once loaded into the 96-well plate.

7.9 PAR Protein Capture

7.9.1 Following incubation with SuperBlock (SOP Step 7.5.4), aspirate and wash the plates once with 350 µL of Wash Buffer using a plate washer.

For the BioTek Microplate Washer, the settings are:

METHOD	
Number of Cycles:	**1**
Soak/Shake:	**Yes**
Soak Time:	**5 sec**
Shake before soak:	**No**
Prime after soak:	**No**
DISPENSE	
Dispense Volume:	**350 µL/well**
Dispense Flow Rate:	**06**
Dispense Height:	**120 (15.240 mm)**
Horizontal DISP POS:	**00 (0.000 mm)**
Bottom Wash First:	**No**
Prime Before Start:	**No**
ASPIRATE	
Aspirate Height:	**031 (3.937 mm)***
Horizontal ASPR POS:	**-20 (-0.914 mm)***
Aspiration Rate:	**05 (6.4 mm/sec)**
Aspirate Delay:	**1000 MSec**
Crosswise ASPIR:	**No**
Final Aspiration:	**Yes**
Final Aspirate Delay:	**1000 MSec**

*Recommended initial setting, optimize Aspirate Height and Horizontal ASPR POS to optimize complete aspiration for an individual unit following manufacturer's recommendations.

7.9.2 After the wash, tap the plate on paper towels to remove residual buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.9.3 Immediately, add 50 µL of SuperBlock to each well using a multichannel pipettor. Each well will hold a final volume of 75 µL after sample addition.

7.9.4 Use the Plate Map Design (Appendix 1) and the Unknown Sample Calculation Table (Appendix 2, Section 6A) as a guide to set up the 96-well plate for incubation with unknown samples (SOP Step 7.7), PAR polymer standards (SOP Step 7.8.1), and xenograft lysate controls (SOP Step 7.8.2). Pipette reagents in the following order; **do not deviate** from order:

Order	Sample/Reagent and Volume
1	25 µL of specified concentrations of PAR polymer standards into designated duplicate wells. Load the lowest concentration first.
2	25 µL of each unknown sample, tumor biopsy or PBMC, into designated triplicate wells
3	25 µL each of xenograft lysate control (Low-C, Mid-C, and High-C) into both sets of designated duplicate wells
4	25 µL of additional SuperBlock into each of the Background wells

7.9.5 Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 18 ± 2 h. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 7).

7.10 PAR Detection (next day)

7.10.1 Prepare a sufficient amount of the PAR detection pAb 1 h before washing the plate (next step) that has been incubating with samples.

7.10.1.1 Using the calculations in Appendix 2, Sections 8A, prepare the PAR detection pAb working solution in PBS-BSA Diluent; record the lot number of mouse serum used.

7.10.1.2 Incubate the PAR detection pAb working solution in a fixed-temperature incubator for 1 h at 25°C and record the incubation conditions in the Batch Record (Appendix 2, Section 8Ac).

7.10.2 After the 16-h incubation is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (same wash program as SOP Step 7.9.1, except run for 4 cycles). Record the date and stop time of sample incubation in the Batch Record (Appendix 2, Section 7).

7.10.3 After the wash, tap the plate on paper towels to remove residual Wash Buffer. Proceed immediately to the next step; do not allow the plate to dry out.

7.10.4 Add 100 µL of the PAR detection pAb working solution per well using a multichannel pipettor, cover the plate with an acetate sheet, and incubate for 2-2.5 h on the prewarmed CoolSink thermoconductive plate in a fixed-temperature 25°C incubator. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 8B).

7.10.5 One hour before the incubation with PAR detection pAb is complete, prepare a sufficient amount of HRP conjugate for the assay.

7.10.5.1 Using the calculations in Appendix 2, Sections 9A, prepare the HRP conjugate working solution; record the lot number of mouse serum used. Wrap the tube in aluminum foil to keep solution in the dark.

7.10.5.2 Allow the prepared HRP conjugate to incubate in a fixed-temperature 25°C incubator for 1 h and record the incubation conditions (Appendix 2, Section 9Ac).

7.10.6 After the 2 to 2.5 h incubation with the PAR detection pAb is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (same wash program as SOP Step 7.9.1, except run for 4 cycles). Tap plate on paper towels to remove residual liquid and proceed immediately to the next step.

7.10.7 Add 100 µL of the HRP conjugate working solution per well using a multichannel pipettor. Cover the plate with an acetate sheet and then in aluminum foil and incubate for 1-1.5 h on the prewarmed CoolSink thermoconductive plate in a fixed-temperature 25°C incubator. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 9B).

7.11 Signal Detection

7.11.1 Turn on the Tecan Infinite Plate Reader at least 30 min before use.

7.11.1.1 Under "Instrument," select "Heating" and set a Target Temperature of 25°C.

7.11.1.2 For chemiluminescent readings, the plate reader should be set to the following reading parameters:

Shaking duration:	**5 sec**
Mode:	**Linear**
Amplitude:	**1 mm**
Attenuation:	**OD1**
Integration Time:	**100 ms**

7.11.2 Immediately prior to the final wash step (next step), prepare the Chemiluminescent Substrate Solution as outlined in Appendix 2, Section 10A, being sure to note the time of preparation. Cover with aluminum foil.

7.11.3 After the 1 to 1.5 h HRP conjugate incubation is complete, aspirate and wash the wells 4 times with 350 µL of Wash Buffer (same wash program as SOP Step 7.9.1, except run for 4 cycles). Tap plate on a paper towel to remove excess buffer and proceed immediately to the next step.

7.11.4 Add 100 µL of the freshly made Chemiluminescent Substrate Solution per well with a multichannel pipettor, noting the time of addition to wells (Appendix 2, Section 10B).

7.11.5 Immediately place the plate into the Tecan plate reader.

7.11.5.1 Perform the first chemiluminescent reading at approximately 1 min after substrate addition. Record the time of the initial relative light unit (RLU) reading in the Batch Record (Appendix 2, Section 10B).

7.11.5.2 Take a second reading at 5 min after substrate addition (4 min after first reading) using the same instrument settings.

7.11.5.3 Use readings from the readout with the highest RLU values for analysis.

7.11.5.4 In some cases the signal may be too high (no read-out, invalid read-out) from the initial reading, wait an additional 5-10 min and read the plate again at the same instrument setting. Maximum Chemiluminescent Substrate incubation on the plate is 30 min.

7.11.5.5 Record time final RLU reading is taken in Appendix 2, Section 10B.

7.11.6 Save the resulting readings in an Excel file to a secure computer; recommended to label with a unique file name (e.g., SOP number + "Tecan" + run date + unique plate ID). Print a paper copy of the raw Tecan data for inclusion with the Batch Record.

7.12 Proceed to SOP340530 for Quality Control, Data Analyses, and preparation of the Clinical Sample Data Report to send to the clinical protocol Principal Investigator.

7.13 Review and finalize the Batch Records (Appendix 2). Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 11).

7.14 The Laboratory Director/Supervisor should review the Batch Record and sample reports and sign the Batch Record affirming the data contained within are correct (Appendix 2, Section 12).

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APPENDIX 1: PLATE MAP DESIGN

	1	2	3	4	5	6	7	8	9	10	11	12
A	SuperBlock Only*					7.8 pg/mL		SuperBlock Only				
B	High-C	S1	S3	S5	S7	15.6 pg/mL		S9	S11	S13	S15	Low-C
C						31.2 pg/mL						
D	Mid-C					62.5 pg/mL						Mid-C
E		S2	S4	S6	S8	125 pg/mL		S10	S12	S14	S16	
F	Low-C					250 pg/mL						High-C
G						500 pg/mL						
H	SuperBlock Only					1000 pg/mL		SuperBlock Only				
	Control Samples	Unknown Samples, Triplicate				PAR Standards, Duplicate		Unknown Samples, Triplicate				Control Samples

*RLU readings from the 4 corner wells and wells adjacent to the highest standard will not be used to determine background variability.

- S1 through S16 are unknown sample (S) wells in triplicate. If fewer samples are run, fill the empty sample wells with SuperBlock and ignore for data analysis.

  Important: If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculations in SOP340530, load tumor biopsies first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S16

- Background control wells are loaded with SuperBlock only (no sample).
- Document the sample/patient IDs and other pertinent information in the Batch Record (Appendix 2, Section 5A).

  Important: This Plate Map design and well designation is assumed for the format of the Tecan output file that will be used in SOP340530: PAR Immunoassay Quality Control, Data Analysis, and Reporting. Manual adjustment of the output well data is outlined in the SOP if a different Plate Map is used.

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APPENDIX 2: 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: _____

CTEP#/Clinical Protocol#: _____

Plate ID (optional): _____

1. Critical Reagents

Be sure the lot numbers and recommended working solution concentrations on each of the reagents match those determined for the Critical Reagent. Reagents from one set of qualified Critical Reagents **should not** be exchanged with a set of reagents qualified separately.

Reagent Name	Date Received	Lot Number	Provided Reagent	Recommended Dilution/Conc. for Working Solution	Expiration Date
PAR Polymer Standard	/ /		ng/mL	N/A	/ /
Xenograft Lysate Controls (High-, Mid- and Low-C)	/ /		N/A	N/A	/ /
PDA II Antibody Coating Buffer	/ /		N/A	N/A	/ /
PAR Capture mAb	/ /		µg/mL	1 :	/ /
PAR Detection pAb	/ /		µg/mL	1 :	/ /
Goat Anti-Rabbit HRP Conjugate	/ /		1 mg/mL	1:	/ /
SuperSignal Chemiluminescent Substrate Solutions	/ /		N/A	N/A	/ /

2. Equipment and Preparation of Reagents

A. Equipment

BioTek Plate Washer: Make/Model: _____

Serial #: _____

Microplate Reader Make/Model: _____

Serial #: _____

BATCH RECORD: INITIALS _____ DATE: _____

B. Reagents

Buffers should be prepared based on volumes needed to complete all the steps with the number of 96-well plates in the experimental run. Always prepare at least 10% excess volume of buffer to ensure adequate volume to complete the study (scale-up appropriately for additional plates).

a. Coating Buffer: For one 96-well plate (preparing enough for 110 wells), pipette 11 mL PDA II Antibody Coating Buffer into a 15-mL tube. Place on bench top to warm 2 h prior to the initiation of the assay. Discard unused buffer at end of assay run.

b. SuperBlock: For one 96-well plate (preparing for 110 wells), pipette 40 mL SuperBlock into a 50-mL tube. Place on bench top to warm 2 h prior to initiation of the assay. Discard unused buffer at end of assay run.

SuperBlock Lot#: _____

c. Wash Buffer: To prepare 1 L of buffer, pipette 100 mL 10X PBS (1X final) and 10 mL 10% Tween 20 (w/v; 0.1% final) into 890 mL ultrapure DNase/RNase-free water. Keep at 25 for up to 1 wk.

d. PBS-BSA Diluent: To prepare 1 L of buffer, add 20 g BSA (2% final) and 100 mL 10X PBS (1X final) to 900 mL ultrapure DNase/RNase-free water. Keep at 2°C to 8°C for up to 2 wks.

e. Protease Inhibitor Cocktail Tablets: Dissolve one PI cocktail tablet in 2 mL ddH_20 (25X stock). The 25X stock solution is stable for 1 wk at 2°C to 8°C or 12 wk at -20°C ± 5°C. If stored frozen, the material must be prepared as single-use aliquots to prevent repeat freeze-thaw.

Lot#: _____Expiration Date: _____

f. 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: _____

g. Cell Extraction Buffer (CEB): Manufacturer's supplied 1X solution. Store in aliquots at -20°C.

Lot#: _____Expiration Date: _____

h. CEB (Complete): 2 mL CEB (Complete) is sufficient to prepare all unknown sample dilutions. **Note**: If CEB (**with** PIs) is already prepared in the laboratory, simply add SDS to final concentration of 1.0%.

Reagent	Stock Concentration	Amount Needed	Final Concentration
CEB	stock	1800 µL	N/A
PI Cocktail	25X	80 µL	1X PI Cocktail
PMSF	100 mM	20 µL	1 mM PMSF
SDS	20%	100 µL	1.0% SDS

3. Capture Antibody: PAR mAb

A. Preparation of **PAR mAb Coating Solution**

Remove antibody from -20˚C freezer and thaw on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **PAR mAb Coating Solution** using the following calculations:

a. Recommended dilution of PAR mAb **STOCK** = 1: _____

e.g., PAR mAb **STOCK** recommended dilution for Lot# 18733F9 is 1:250 and Lot# M23677 is 1:500.

$$\frac{11\text{ mL}}{\text{Recommended dilution of PAR mAb STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L PAR mAb STOCK}$$

$$\frac{11\text{ mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L PAR mAb STOCK}$$

b. Place the following in a 15-mL polypropylene tube and mix by inversion 5 to 8 times.

11 mL	Coating Buffer
____ µL	PAR mAb Coating **STOCK**

B. Incubation Conditions for Coating Plate

Add 100 µL **PAR mAb Coating Solution** to each well, and incubate at 37°C for 2 h on a prewarmed CoolSink thermoconductive plate.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

4. Block Step

Following the aspiration step after plate coating, add 250 µL SuperBlock to each well and incubate at 37°C for 1 to 1.5 h on a prewarmed CoolSink thermoconductive plate (move to 25°C if blocking longer).

Incubation conditions for blocking plate:

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

5. Preparation of Working Dilutions of Unknown Biopsy Lysates

Normalize unknown biopsy lysates to either a 0.25 or 0.5 µg/µL working dilution prior to preparation of samples for the immunoassay. **Important**: Pre- and post-dose samples from a single patient should be prepared with matched protein concentrations.

Sample No.	Sample/Patient ID	Stock Lysate Conc. xx µg/µL	Working Lysate Conc. 0.25 or 0.5 µg/µL	Vol. Stock Lysate (µL)	Vol. CEB (Complete) 70 (or 130) µL - Vol. Stock Lysate used)
S1		µg/µL	µg/µL	µL	µL
S2		µg/µL	µg/µL	µL	µL
S3		µg/µL	µg/µL	µL	µL
S4		µg/µL	µg/µL	µL	µL
S5		µg/µL	µg/µL	µL	µL
S6		µg/µL	µg/µL	µL	µL
S7		µg/µL	µg/µL	µL	µL
S8		µg/µL	µg/µL	µL	µL
S9		µg/µL	µg/µL	µL	µL
S10		µg/µL	µg/µL	µL	µL
S11		µg/µL	µg/µL	µL	µL
S12		µg/µL	µg/µL	µL	µL
S13		µg/µL	µg/µL	µL	µL
S14		µg/µL	µg/µL	µL	µL
S15		µg/µL	µg/µL	µL	µL
S16		µg/µL	µg/µL	µL	µL

BATCH RECORD: INITIALS _____ DATE: _____

6. Preparation of Unknown Samples (A) and PAR Polymer Standards (B)

A. Unknown Sample Calculation Table: Unknown samples are run in triplicate, 25 µL sample/well (preparing 1 well extra). Sample numbers correspond to those on the Plate Map Design in Appendix 1.

Important: If both tumor biopsy and PBMC samples are being run on the same plate, due to the format of the Data Analysis calculators in SOP340530, load tumor biopsy samples first and then load PBMC samples. For example, load pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S16.

All Samples		**Tumor Biopsy Samples**				**PBMC Samples**	
Sample No.	**Sample/Patient ID**	**Protein Conc. Working Lysate (0.25 or 0.5 µg/µL)**	**Diluted Lysate:** 4, 2, or 1 µg/well			**Stock Cell Number**	**Stock Lysate Vol. Used (µL)**
			Vol. Working Lysate (µL)	**Vol. SuperBlock** (100 µL - Vol. Lysate)	**Final conc. of diluted lysate (µg/well)**	1 x 10^7 cells/mL	100 µL
S1		µg/µL			µg/well	cells/mL	
S2		µg/µL			µg/well	cells/mL	
S3		µg/µL			µg/well	cells/mL	
S4		µg/µL			µg/well	cells/mL	
S5		µg/µL			µg/well	cells/mL	
S6		µg/µL			µg/well	cells/mL	

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All Samples		**Tumor Biopsy Samples**				**PBMC Samples**	
Sample No.	**Sample/Patient ID**	**Protein Conc. Working Lysate (0.25 or 0.5 µg/µL)**	**Diluted Lysate:** 4, 2, or 1 µg/well			**Stock Cell Number**	**Stock Lysate Vol. Used (µL)**
			Vol. Working Lysate (µL)	**Vol. SuperBlock** (100 µL - Vol. Lysate)	**Final conc. of diluted lysate (µg/well)**	1 x 10^7 cells/mL	100 µL
S7		µg/µL			µg/well	cells/mL	
S8		µg/µL			µg/well	cells/mL	
S9		µg/µL			µg/well	cells/mL	
S10		µg/µL			µg/well	cells/mL	
S11		µg/µL			µg/well	cells/mL	
S12		µg/µL			µg/well	cells/mL	
S13		µg/µL			µg/well	cells/mL	
S14		µg/µL			µg/well	cells/mL	
S15		µg/µL			µg/well	cells/mL	
S16		µg/µL			µg/well	cells/mL	

BATCH RECORD: INITIALS _____ DATE: _____

B. PAR Polymer Standards

Calculations for preparation of the 3000 pg/mL (3 ng/mL) PAR standard in tube #1.

Supplied PAR standard = _____ng/mL

e.g., PAR standard **STOCK** Lot# 041612KF is supplied at 10 ng/mL.

$$\left(\frac{3\ \text{ng/mL}}{\text{Conc. of PAR standard STOCK (ng/mL)}}\right) * 200\ \mu\text{L} = \underline{\text{XX}}\ \mu\text{L PAR polymer STOCK solution in 200 µL final}$$

$$\left(\frac{3\ \text{ng/mL}}{\underline{\quad\quad}\ \text{ng/mL}}\right) * 200\ \mu\text{L} = \underline{\quad\quad}\ \mu\text{L PAR polymer STOCK solution in } \underline{\textbf{200 µL}} \text{ final}$$

Serial dilutions of the PAR standards are used to prepare the remaining tubes with final concentrations ranging from 1500 to 23.4 pg/mL in SuperBlock. 25 µL of each diluted standard will be added to the 96-well plate (3-fold dilution), giving a reference curve ranging from 1000 to 7.8 pg/mL PAR standard. Label tubes with final concentration of standard.

Tube # (Plate Row)	Vol. and Source of Concentrated Standard	Vol. SuperBlock	Resulting Conc. of Diluted Standard per Well
1 (H)	_____ µL PAR polymer **STOCK**	_____ µL (bring to 200 µL)	3000 pg/mL
2 (G)	100 µL of tube #1	100 µL	1500 pg/mL
3 (F)	100 µL of tube #2	100 µL	750 pg/mL
4 (E)	100 µL of tube #3	100 µL	375 pg/mL
5 (D)	100 µL of tube #4	100 µL	187.5 pg/mL
6 (C)	100 µL of tube #5	100 µL	93.8 pg/mL
7 (B)	100 µL of tube #6	100 µL	46.9 pg/mL
8 (A)	100 µL of tube #7	100 µL	23.4 pg/mL

7. Plate Incubation

Add 25 µL unknown samples, xenograft lysate controls, and PAR polymer standards to the 96-well plate (wells contain 50 µL SuperBlock), cover plate, and incubate at 2°C to 8°C for 18 ± 2 h.

Date: ___/___/___ Start Time: ____:____ Incubation Temp: _____°C

Date: ___/___/___ Stop Time: ____:____

8. Detection Antibody: PAR pAb

A. Preparation of PAR pAb Working Solution (100 µL/well)

Remove antibody from -20°C freezer and thaw on ice.

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **PAR pAb Working Solution** using the following calculations:

a. Recommended dilution of PAR pAb **STOCK** = 1: _____

e.g., PAR pAb **STOCK** recommended dilution for Lot# 14133L7 is 1:2000.

$$\frac{11\text{ mL}}{\text{Recommended dilution of PAR Detection pAb STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L PAR pAb STOCK}$$

$$\frac{11\text{ mL}}{\underline{\quad\quad}\ \text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \underline{\quad\quad}\ \mu\text{L PAR pAb STOCK}$$

b. Place the following in a 15-mL polypropylene tube:

11 mL	PBS-BSA Diluent
11 µL	Mouse serum (1:1000) Lot #: _____
____ µL	PAR Detection pAb **STOCK**

c. Mix by inversion 5 to 8 times, and then incubate in a fixed-temperature incubator at 25°C for 1 h before use.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

B. Addition of PAR pAb Working Solution

Add 100 µL of the **PAR pAb Working Solution** to each well and incubate for 2 to 2.5 h in a fixed-temperature incubator at 25°C on a prewarmed CoolSink thermoconductive plate.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

9. Reporter: HRP Conjugate

A. Preparation of HRP Conjugate Working Solution (100 µL/well)

For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL. Prepare **HRP Conjugate Working Solution** using the following calculations:

a. Recommended dilution of Goat Anti-Rabbit HRP Conjugate **STOCK** = 1: _____

e.g., HRP Conjugate **STOCK** recommended dilution for Lot# 110373 is 1:1000.

$$\frac{11\text{ mL}}{\text{Recommended dilution of HRP Conjugate STOCK}} * 1000\ \mu\text{L/mL} = \underline{XX}\ \mu\text{L HRP Conjugate STOCK}$$

$$\frac{11\text{ mL}}{\text{(dilution factor)}} * 1000\ \mu\text{L/mL} = \_\_\_\_\_\ \mu\text{L HRP Conjugate STOCK}$$

b. Place the following in a 15-mL polypropylene tube:

11 mL	PBS-BSA Diluent
11 µL	Mouse serum (1:1000) Lot #: _____
___ µL	HRP Conjugate **STOCK**

c. Mix by inversion 5 to 8 times, and incubate in the dark in a fixed-temperature incubator at 25°C for 1 h before use.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

B. Addition of HRP Conjugate Working Solution

Add 100 µL of the **HRP Conjugate Working Solution** to each of the washed wells, cover with aluminum foil, and incubate in the dark at 25°C for 1 to 1.5 h on a prewarmed CoolSink thermoconductive plate.

Start Time: ____:____ Stop Time: ____:____ Incubation Temp: _____°C

10. Chemiluminescent Substrate

A. Preparation of Substrate Solution (100 µL/well)

Calculate volume of substrate required for the experimental run. For one 96-well plate, prepare 110 wells: (100 µL/well*110)/(1000 µL/mL) = 11 mL.

Prepare the following in a 15-mL polypropylene tube wrapped with aluminum foil. Mix by vortexing.

5.5 mL	Pico Stable Peroxide	(50 µL/well*110)/(1000 µL/mL)
5.5 mL	Pico Luminol/Enhancer	(50 µL/well*110)/(1000 µL/mL)

Time of Substrate Preparation: _____ : _____

B. Substrate Solution Incubation and RLU Reading Times

Time of Substrate Addition to Wells: _____ : _____

Time Initial RLU Reading is Captured: _____ : _____

Time Final RLU Reading is Captured: _____ : _____

11. Notes, including any deviations from the SOP:

12. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: _____ (PRINT)

_____ (SIGN)

Date: ____/____/____

BATCH RECORD: INITIALS _____ DATE: _____