National Clinical Target Validation Laboratory (NCTVL)

Applied Developmental Directorate

SAIC-Frederick, Inc.

Frederick National Laboratory for Cancer Research

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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.

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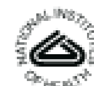

Change History

Revision	Approval Date	Description	Originator	Approval
--	10/20/2006	New document	YZ	JJ
A	9/20/2007	Revision with new reagents	YZ	JJ
B	1/4/2008	Revision with new standards	YZ	JJ
C	10/15/2008	Revision for SOP Web page – checklists, expanded data analysis, and examples of ranges	KG	JJ
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
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
F	3/31/2010	Remove Checklist; Update Batch Record; New Lot# PAR mAb	YZ	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
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
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

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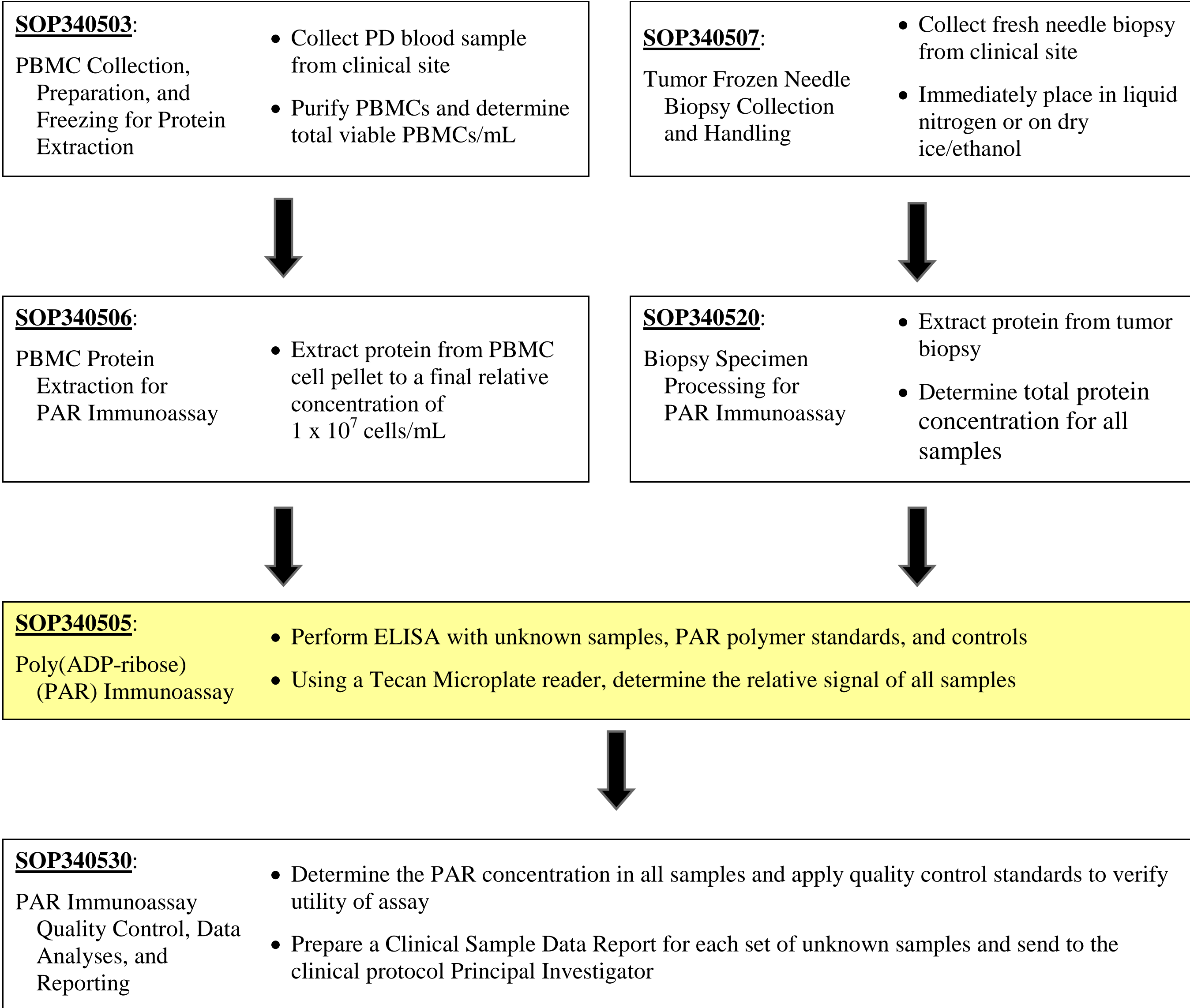
OVERVIEW OF PAR IMMUNOASSAY SAMPLE PROCESSING



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
RT	=	Room Temperature
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.

*Depending on the laboratory, one person may have multiple roles.

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) 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, 1 µg/µL (custom preparations of Low, Mid, and High PAR level controls)
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 polyclonal antibody, 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 Ice bucket
6.9 Acetate plate sealers (Thermo Scientific Pierce, Cat#: 3501)
6.10 Reacti-Bind White Opaque 96-well Plate (Thermo Scientific Pierce, Cat#: 15042)

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

6.21 Vortex Genie 2 (Daigger, Cat#:EF 3030A)
6.22 Infinite® 200 or Infinite 200Pro Microplate Reader (Tecan US)
6.23 BioTek ELx405 Select Microplate Washer (BioTek Instruments)
6.24 -80°C freezer
6.25 2°C to 8°C refrigerator
6.26 37ºC incubator

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

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 Appendix 3.

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. Each stock solution is 1 µg/µL.

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.

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. 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 to 1.5 h. Record the incubation conditions in the Batch Record (Appendix 2, Section 4).

7.5.4.1 After blocking, move plate to RT until washing step (SOP Step 7.8.1).

7.5.4.2 Once coated plates have been blocked they can be stored at 2°C to 8°C for up to one week; do not let wells dry out.

7.6 Preparation of Unknown Tumor Biopsy or PBMC Lysate Samples

7.6.1 **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, it is recommended to 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 x 10^7 cells/mL) are prepared according to SOP340506 and tumor lysates are prepared according to SOP340520.

7.6.2 Place all unknown samples to be assayed on ice. Record the sample/patient IDs for all lysates in the Batch Record (Appendix 2, Section 5A). Each unknown biopsy lysate will take up 3 sample spots (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 5A; 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.

7.6.3 Tumor biopsy lysate samples

7.6.3.1 Biopsy samples are prepared according to the total protein concentration of the sample. 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 < **0.16 µg/µL** should not be used in the PAR Immunoassay and will be reported as unanalyzable in the Clinical Sample Data Report.

7.6.3.2 For unknown **stock lysates with protein concentrations of 0.16 - 2 µg/µL**,

- Perform the following calculation to prepare 3 different lysate dilutions (4, 2, and 1 µg/well) in 100 µL total volume with CEB (Complete). 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 CEB (Complete) used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 5A).

$$\frac{(4, 2, \text{ or } 1)\ \mu g/\text{well}\ \textbf{Diluted Lysate}}{\text{XXX}\ \mu g/\mu L\ \text{Conc. Stock Lysate}} * 4\ \text{wells} = \underline{\text{XX}}\ \mu L\ \text{Vol. Stock Lysate}$$

7.6.3.3 For unknown **stock lysates with protein concentrations > 2 µg/µL**,

- First prepare 45 µL of a 1:3 dilution of the stock lysate (e.g., 15 µL stock lysate into 30 µL CEB [Complete]). In the Batch Record, cross out the original stock lysate concentration and write in the 1:3 diluted concentration and put "1:3" in parenthesis after it.
- Then, perform the following calculation and record volume of the 1:3 diluted stock lysate and CEB (Complete) used to prepare each **Diluted Lysate** in the Batch Record (Appendix 2, Section 5A). These calculations prevent pipetting of volumes < 2 µL while still obtaining a final protein load of 4, 2, and 1 µg/well.

$$\frac{\text{(4, 2, or 1) µg/well Diluted Lysate}}{\text{XXX µg/µL Conc. 1:3 Diluted Stock Lysate}} * \text{4 wells} = \text{XX µL Vol. 1:3 Diluted Stock Lysate}$$

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

7.6.4 PBMC lysate samples

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

7.6.4.2 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).

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

7.6.4.4 Record the volume set aside for each sample in the Batch Record as well as the stock cell number/mL (Appendix 2, Section 5A).

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

7.7 Preparation of PAR Standards and Xenograft Lysate Controls

7.7.1 Preparation of PAR polymer standards; run in duplicate

7.7.1.1 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.

7.7.1.2 Use the calculations in the Batch Record (Appendix 2, Section 5B) to prepare a 3 ng/mL (3000 pg/mL) PAR standard stock solution in SuperBlock.

7.7.1.3 Prepare the PAR polymer standards by serial dilution as outlined in the Batch Record (Appendix 2, Section 5B) with final concentrations ranging from 3000 to 23.4 pg/mL in SuperBlock.

7.7.1.4 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.

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

7.7.2.1 For one 96-well plate, retrieve one each High-, Mid-, and Low-C xenograft quality control stock vials (1 µg/µL) from the -80°C freezer and thaw on ice. 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.

7.7.2.2 Use the dilution scheme in the Batch Record (Appendix 2, Section 5C) to prepare working dilutions of each xenograft lysate control. Label the tubes accordingly.

7.7.2.3 Keep samples on ice until use. Only make enough diluted controls for the assay and discard any excess. Controls will be diluted 3-fold with CEB (Complete) once loaded into the 96-well plate.

7.8 PAR Protein Capture

7.8.1 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:	**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.

7.8.2 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.

7.8.3 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.

7.8.4 Use the Plate Map Design (Appendix 1) and the Sample Calculation Table (Appendix 2, Section 5A) as a guide to set up the 96-well plate for incubation with unknown samples (SOP Step 7.6), PAR polymer standards (SOP Step 7.7.1), and xenograft lysate controls (SOP Step 7.7.2). Pipette reagents in the following order; **do not deviate** from order of addition:

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

7.8.5 Cover the plate with an acetate sheet and incubate at 2°C to 8°C for 16 ± 1 h. Record the date, start time, and incubation temperature in the Batch Record (Appendix 2, Section 6).

7.9 PAR Detection (next day)

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

7.9.1.1 Using the calculations in Appendix 2, Sections 7A, prepare the PAR detection pAb working solution in PBS-BSA Diluent; record the lot number of mouse serum used.

7.9.1.2 Incubate the PAR detection pAb working solution for 1 h at RT and record the incubation conditions in the Batch Record (Appendix 2, Section 7Ac).

7.9.2 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 7.8.1, except run for 4 cycles). Record the date and stop time of sample incubation in the Batch Record (Appendix 2, Section 6).

7.9.3 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.

7.9.4 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 to 2.5 h at RT. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 7B).

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

7.9.5.1 Using the calculations in Appendix 2, Sections 8A, prepare the HRP conjugate working solution; record the lot number of mouse serum used.

7.9.5.2 Allow the prepared HRP conjugate to incubate in the dark for 1 h at RT and record the incubation conditions (Appendix 2, Section 8Ac).

7.9.6 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 7.8.1, except run for 4 cycles). Tap plate on paper towels to remove residual liquid and proceed immediately to the next step.

7.9.7 Add 100 µL of the HRP conjugate working solution per well using a multichannel pipettor. Cover the plate with an acetate sheet and incubate in the dark for 1 to 1.5 h at RT. Discard residual working solution and record the incubation conditions in the Batch Record (Appendix 2, Section 8B).

7.10 Signal Detection

7.10.1 Turn on the Tecan Infinite Plate Reader at least 30 min before use (if using LumiGlo Substrate, move solutions to RT at this point). For chemiluminescence 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**

7.10.2 Just before the HRP conjugate incubation is finished, prepare the Chemiluminescent Substrate Solution as outlined in Appendix 2, Section 9A, being sure to note the time of preparation. This must be made up immediately before use, kept in the dark, and at a sufficient volume for the assay.

7.10.3 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 7.8.1, except run for 4 cycles). Tap plate on a paper towel to remove excess buffer and proceed immediately to the next step.

7.10.4 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 9B).

7.10.5 The first chemiluminescence reading should be within 2 min of substrate addition. Record the time of the initial relative light unit (RLU) reading in the Batch Record (Appendix 2, Section 9B).

7.10.5.1 If the signal is too high from the initial reading, wait 5 min and read the plate again at the same instrument setting, repeat until the RLU signal is on scale. Record time the final RLU reading is taken in Appendix 2, Section 9B.

7.10.6 Save the resulting readings in an Excel file to a secure computer; recommended to label the file with the date and a unique assay identifier (Plate ID). Print a paper copy of the raw Tecan data for inclusion with the Batch Record.

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

7.12 Review and finalize the Batch Records (Appendix 2). Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 2, Section 10).

7.13 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 2, Section 11).

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, it is recommended to 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: ____

Clinical Protocol Number: ____

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)	/ /		1 µg/µL, each	5 µg/well, each	/ /
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 #: _____________________________________

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. Keep at 2ºC to 8°C. 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. Keep at 2ºC to 8°C. 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 RT 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 -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: _____________

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. Keep at 2°C to 8°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	1830 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.

11 mL / Recommended dilution of PAR mAb **STOCK**	*	1000 µL/mL	=	XX µL PAR mAb **STOCK**

11 mL / __________ (dilution factor) * 1000 µL/mL = ______ µL 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.

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

4. Block Step

Following the washing step after plate coating, add 250 µL SuperBlock to each well and incubate at 37°C for 1 to 1.5 h (move to RT if blocking longer).

Incubation conditions for blocking plate:

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

5. Preparation of Unknown Samples (A), PAR Polymer Standards (B), and Xenograft Lysate Controls (C)

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. Stock Lysate (µg/µL)**	**Diluted Lysate:** 4, 2, or 1 µg/well			**Stock Cell Number**	**Stock Lysate Vol. Used (µL)**
			Vol. Stock Lysate (µL)	**Vol. CEB (Complete)** (100 µL - Vol. Lysate)	**Final conc. of diluted lysate (µg/well)**	1 x 10^7 cells/mL	100 µL
	Biopsy Example	*0.85 µg/µL*	*4.7*	*95.3*	*1 µg/well*	*cells/mL*	
	Biopsy Example	*1.43 (1:3)* *~~4.29~~ µg/µL*	*2.8*	*97.2*	*1 µg/well*	*cells/mL*	
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	

Continued on next page.

BATCH RECORD: INITIALS __________________ DATE: ______________

Continued from previous page.

All Samples		Tumor Biopsy Samples				PBMC Samples	
Sample No.	Sample/Patient ID	Protein Conc. Stock Lysate (µg/µL)	Diluted Lysate: 4, 2, or 1 µg/well			Stock Cell Number	Stock Lysate Vol. Used (µL)
			Vol. Stock Lysate (µL)	Vol. CEB (Complete) (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	

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.

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

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

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

C. Xenograft Lysate Controls

The High-, Mid- and Low-C xenograft lysate controls are dilutions prepared from 1 µg/µL stock lysates into CEB (Complete). From the 110 µL preparation volume, 25 µL of each diluted control will be diluted an additional 3-fold in the 96-well plate.

Control Lysate	Vol. and Source of Control Lysate	Vol. CEB (Complete)
High-C	25 µL of High-C	100 µL
Mid-C	25 µL of Mid-C	100 µL
Low-C	25 µL of Low-C	100 µL

6. 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 16 ± 1 h.

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

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

7. 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.

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

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

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 let stand at RT 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 at RT.

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

8. 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.

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

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

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 let stand at RT 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 and incubate in the dark at RT for 1 to 1.5 h.

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

9. 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 inversion 5 to 8 times and store at RT in the dark until use.

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 (opt): ___________:___________

10. Notes, including any deviations from the SOP:

11. Laboratory Director/Supervisor Review of Batch Record

Laboratory Director/Supervisor: ____ (PRINT)

____ (SIGN)

Date: _____/______/______

APPENDIX 3: CRITICAL REAGENT QUALIFICATION

I. Overview

A summary of the specifications and major testing procedures recommended by the PADIS/IQC lab, Frederick National Laboratory for Cancer Research (Frederick, MD), to control the quality of the critical reagents used in the PAR Immunoassay.

II. PAR Polymer Standard

1. Reagent Description

Reagent Name	Poly(ADP-Ribose) PAR Polymer
Manufacturer	Trevigen
Cat#	4336-100-01
Physical State	Provided in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA in 100 µL aliquots at 10 µM
Storage and Stability	A previous lot of PAR polymer concentrated stock material purchased from Biomol was stored and handled as recommended by the manufacturer and gave consistent assay performance for over 3 y. We recommend storing the concentrated stock material at -80°C (or lower) for up to 3 y.

2. Reagent QC

   i. General Recommendations

      a. A sufficient quantity of the PAR polymer should be purchased to ensure assay consistency and limit the frequency of qualification.

      b. A test vial of material should be requested for quality assessment prior to receipt of the large quantity of material.

      c. Once a lot is accepted, store the concentrated stock vials at -80°C (or lower) and prepare working stock solutions for individual assays in SuperBlock as needed at a target concentration of 10 ng/mL. A single 100 µL vial of concentrated stock (10 µM [5589 ng/mL]) is sufficient to prepare several hundred vials of stock solution.

      d. There is no quantitative reference method established for this assay calibrator. A large master lot of working stock solution (10 ng/mL) should be prepared and stored at or below -80°C for assignment of values to subsequent lots to limit drift in assigned PAR values over time.

   ii. Identity

      a. Reagent name and catalog number should be verified against all test and final product vial labels and accompanying Batch Record and Certificate of Analyses.

b. A characteristic elution pattern is expected for the PAR polymer standard analyzed via anion exchange HPLC and can be used to confirm product identity. This method has been developed based on the published method referenced in Kiehlbauch et al (1993. Anal. Biochem 208:26-34). The column used was DiEthylAminoEthane, 3.5 cm x 4.6 mm with Tris-HCl pH 9.0 and a gradient of 0-1 M NaCl and a flow rate of 1 mL/min.

- This method was originally adapted with a concentrated lot of material from Biomol. The predominant polymer species identified was C2 (~88%) which corresponds to an average polymer size of 180 with an average of 5 branches, as described in the Kiehlbauch manuscript. The only other predominant band was C1 (~10%) which corresponds to an average polymer size of 55 with 1 branch. The remaining polymer appeared to be smaller oligomers estimated to range in size from approximately 3 – 50 ADP-ribose residues. The elution time for the C1 and C2 peaks were 27.14 and 29.44 min, respectively.
- Although the Trevigen material is significantly more dilute and supplied in TE buffer, analysis via this method can be performed as a qualitative analysis of identity. In our hands, the Trevigen PAR polymer produces a very similar pattern of elution as the Biomol material. The predominant peak was C2. C1 and the oligomers were evident, but were below the limits of integration.

iii. Functional Testing

a. To qualify a new lot of PAR polymer or a new working stock solution of an existing lot, run the newly prepared solution (target concentration of 10 ng/mL) at a minimum of 3 concentrations in triplicate that span the range of the assay. On the same assay plate, include a qualified master lot of calibrator and use qualified antibodies and controls.

b. Assign an adjusted value to the new stock of PAR polymer based on the value from this qualification run.

c. Confirm comparability of the old and new lots using a proficiency panel of at least 10 specimens. The proficiency panel should include both tumor biopsy and PBMC extracts. The values assigned from the old and new calibrator lots for all specimens should agree within 75%-125%.

III. PAR Mouse Capture Monoclonal Antibody (mAb)

1. Reagent Description

Reagent Name	Anti-PAR Monoclonal Affinity Purified
Manufacturer	Trevigen
Cat#	4335-AMC-050 (50 µL vial size); 4335-AMC-100 (100 µL vial size)
Physical State	Affinity purified IgG from mouse ascites in 1X PBS, containing 50% glycerol
Immunogen	Purified ADP-ribose polymers between 2 and 50 units long
Ig Class	IgG3κ
Specificity	Specific for PAR polymers 2 to 50 units long, but does not recognize structurally related RNA, DNA, ADP-ribose monomers, NAD or other nucleic acid monomers
Storage and Stability	Manufacturer's recommended storage is at -20°C (manual defrost freezer). This material is received frozen on dry ice. We recommend storing the frozen material at -80°C (or lower) until first thaw. After first usage, transfer vial to -20°C and use within 90 d of first thaw. Aliquots stored at -80°C (or lower) have been observed to be stable up to 2 y.

2. Reagent QC

   i. General Recommendations

      a. The coating PAR mAb and detection PAR rabbit pAb should be qualified together and used as a matched set. Lot-to-lot dilution differences for these reagents are expected due to general reagent quality differences in manufacturer-supplied reagents.

      b. A sufficient quantity of the PAR mAb should be purchased to ensure assay consistency and limit the frequency of antibody qualification. The PADIS/IQC lab routinely purchases a minimum of 10 mg of the mAb for each new lot.

      c. A test vial containing 200 µg of material should be requested for quality assessment prior to receipt of the larger quantity of material.

   ii. Identity

      c. Reagent name and catalog number should be verified against all test and final product vial labels and accompanying Batch Record and Certificate of Analyses.

      d. The buffer composition is expected to be 1X PBS containing 50% glycerol. This should be verified on the lot-specific Certificate of Analysis.

iii. Concentration

a. The protein concentration stated on the lot-specific Certificate of Analysis should be ≥ 1.0 mg/mL.

b. Protein concentration must be verified as part of the quality control process when receiving new test vials of material.

c. Verify antibody concentration using the BCA (bicinchoninic acid) Protein Assay Kit from Pierce Thermo Scientific (Cat#: 23227). The standard curve is generated with bovine gamma globulin (BGG) protein concentration reference standards (Pierce Thermo Scientific, Cat#: 23213). BGG is an accepted reference protein for total protein quantitation of purified antibodies.

iv. Functional Testing

a. Evaluation in the PAR Immunoassay and comparison of the new PAR mAb lot to a previously qualified mAb lot must be performed for all new lots of mAb.

- The titration should span above and below the dilution used for the current qualified lot of mAb.
- The initial assay should include a full standard curve of PAR polymer for all conditions, or alternatively points that cover the entire range (e.g., lowest calibrator: 7.8 pg/mL, mid: 62.5 pg/mL, highest calibrator: 1000 pg/mL).
- The background must be measured for all conditions.
- The titration should also include the current qualified lot of PAR mAb at its current recommended dilution.

b. Confirm comparability of the old and new lots using a proficiency panel of at least 10 specimens. The proficiency panel should include both tumor biopsy and PBMC extracts. The values assigned from the old and new calibrator lots for all specimens should agree within 75%-125%. This analysis should include a pAb lot that will be considered “matched” to the mAb (see Section IV for process to qualify pAb).

IV. PAR Rabbit Detection Polyclonal Antibody (pAb)

1. Reagent Description

Reagent Name	Anti-PAR Polyclonal Affinity Purified
Manufacturer	Trevigen
Cat#	4336-APC-050 (50 µL vial size); 4336-APC-100 (100 µL vial size)
Physical State	Affinity purified IgG fraction in 1X PBS, containing 50% glycerol
Immunogen	Poly(ADP-ribose) polymer
Specificity	Detects free PAR and poly-ribosylated proteins
Storage and Stability	Manufacturer's recommended storage is at -20°C (manual defrost freezer). This material is received frozen on dry ice. We recommend storing the frozen material at -80°C (or lower) until first thaw. After first usage, transfer vial to -20°C and use within 90 d of first thaw. Aliquots stored at -80°C (or lower) have been observed to be stable up to 2 y.

2. Reagent QC

   i. General Recommendations

      a. The coating PAR mAb and detection PAR pAb should be qualified together and used as a matched set. Lot-to-lot dilution differences for these reagents are expected due to general reagent quality differences in manufacturer-supplied reagents.

      b. A sufficient quantity of the PAR pAb should be purchased to ensure assay consistency and limit the frequency of antibody qualification. The PADIS/IQC lab routinely purchases a minimum of 5 mg of the pAb for each new lot order.

      c. A test vial containing 200 µg of material should be requested for quality assessment prior to receipt of the large quantity of material

   ii. Identity

      a. Reagent name and catalog number should be verified against all test and final product vial labels and accompanying Batch Record and Certificate of Analyses.

      b. The buffer composition is expected to be 1X PBS containing 50% glycerol. This should be verified on the lot-specific Certificate of Analysis.

   iii. Concentration

      a. The protein concentration stated on the lot-specific Certificate of Analysis should be ≥ 1.0 mg/mL.

      b. Protein concentration must be verified as part of the quality control process when receiving new test vials of material.

      c. Verify the antibody concentration using the BCA (bicinchoninic acid) Protein Assay Kit from Pierce Thermo Scientific (Cat#: 23227). The standard curve is generated with bovine gamma globulin (BGG) protein concentration reference standards (Pierce Thermo Scientific, Cat#: 23213). BGG is an accepted reference protein for total protein quantitation of purified antibodies.

iv. Functional Testing

a. Evaluation in the PAR Immunoassay and comparison of the new PAR pAb to a previously qualified pAb lot must be performed for all new lots of pAb.

- The titration should span above and below the dilution used for the current qualified lot of pAb.
- The initial assay should include a full standard curve of PAR polymer for all conditions, or alternatively points which cover the entire range (e.g., lowest calibrator: 7.8 pg/mL, mid: 62.5 pg/mL, highest calibrator: 1000 pg/mL).
- The background must be measured for all conditions.
- The titration should also include the current qualified lot of pAb at its current recommended dilution. The optimal dilution for the last three lots of PAR pAb has been determined to be 1:2000 (as of June 2012).

b. Confirm comparability of the old and new lots using a proficiency panel of at least 10 specimens. The proficiency panel should include both tumor biopsy and PBMC extracts. The values assigned from the old and new calibrator lots for all specimens should agree within 75%-125%. This analysis should include a mAb lot that will be considered “matched” to the pAb (see Section III for process to qualify pAb).

V. Goat Anti-Rabbit HRP-Conjugated Polyclonal Antibody

1. Reagent Description

Reagent Name	Peroxidase-Labeled Affinity Purified Antibody to Rabbit IgG (H+L) (Xtra Serum Adsorbed) Produced in Goat (Referred to as HRP conjugate)
Manufacturer	KPL
Cat#	074-15-061
Physical State	Lyophilized, 1 mg vials
Description	Affinity-purified antibody is isolated from a pool of serum from goats immunized with purified rabbit IgG and labeled with HRP using a modified periodate method. The molar enzyme to antibody protein ratio is 4:1. Goat serum is added as a protein stabilizer along with a proprietary antibacterial agent. Sodium azide is not compatible with HRP-labeled antibodies and should not be used. Cross-reactivity with bovine, chicken, goat, guinea pig, horse, human, mouse, rat, and sheep serum have been minimized with affinity procedures.
Storage and Stability	Lyophilized HRP conjugate is highly stable. The manufacturer recommends storage of lyophilized vials at 2°C-8°C and states that rehydrated HRP conjugate is stable for up to 1 y at 2°C-8°C; however, the PADIS/IQC lab has noted a measurable decrease in RLU readings in the PAR Immunoassay with storage of rehydrated HRP conjugate for this length of time. The PADIS/IQC lab recommends storage of lyophilized vials at -80°C for up to 2 y and storage of the rehydrated HRP conjugate at 2°C-8°C in the dark (or use amber vials) for no longer than 6 mo.

2. Reagent QC

   i. General Recommendations

      a. Ordering a minimum of 10 x 1 mg vials, or an estimated 2 y supply, of a single lot for each new qualification process.

      b. The 1.0 mg lyophilized vials should be rehydrated in 1.0 mL of HRP Conjugate Stabilizer (KPL, Cat# 54-15-01). Rotate the vial until the lyophilized pellet is totally dissolved and aliquot the vial in 10 x 100 µL vials. Aliquoting the material minimizes the number of times a vial is handled and exposed to temperatures above 2°C-8°C.

   ii. Identity

      a. Reagent name and catalog number should be verified against all product vial labels and the lot-specific Certificate of Analyses. Confirm that the vials are received lyophilized in 1 mg vials.

iii. Functional Testing

a. The lot-to-lot quality of the HRP conjugate from KPL has been very good; however, evaluation in the PAR Immunoassay and comparison of the antibody to a previously qualified pAb lot should be performed for new lots of HRP conjugate. Ideally, the new lot of the HRP conjugate should be compared with a newly hydrated vial of the previously qualified lot of material. The assay concentration used for all lots of HRP conjugate to date have been 0.1 µg/mL (1:1000 dilution) in the recommended PBS-BSA Diluent (containing 2% BSA and mouse serum), as detailed in SOP340505.

b. The assay should pass all acceptance criteria for both the newly identified and the previously qualified lot of HRP conjugate. Carefully compare the new lot and the old lot in terms of the RLU and the signal to background ratio (S/B) for the calibrators. If the RLU of the calibrators using the new lot of material is >15% below that read with the old lot, even if it passes all assay acceptance criteria, the lot should not be accepted. In the event that this reduced signal is observed, the result should be discussed with KPL and an alternative lot should be evaluated.

VI. Chemiluminescent Substrate

1. Reagent Description

i. Thermo Scientific Pierce Substrate

Reagent Name	SuperSignal® ELISA Pico Chemiluminescent Substrate
Manufacturer	Thermo Scientific
Cat#	37070 (50 mL each Reagent A and B); 37069 (125 mL each Reagent A and B)
Description	Substrate is sold as a kit containing Reagent A (Luminol/Enhancer Solution) and Reagent B (Stable Peroxide Solution). These solutions are mixed in a 1:1 ratio immediately before use
Storage and Stability	Store at RT in the dark and discard opened bottles after 6 mo and all remaining material 12 mo after receipt or at manufacturer's assigned expiration date, whichever is first

ii. KPL Substrate

Reagent Name	LumiGLO® Chemiluminescent Substrate
Manufacturer	KPL
Cat#	54-61-02 (30 mL each Substrate A and B); 54-61-00 (120 mL each Substrate A and B)
Description	Substrate is sold as a kit containing Substrate A and Substrate B. These solutions are mixed in a 1:1 ratio immediately before use.
Storage and Stability	Store at 2°C-8°C in the dark and discard opened bottles after 6 mo and all remaining material 12 mo after receipt or at manufacturer's assigned expiration date, whichever comes first. Warm to RT before use.

2. General Information

i. Both of the chemiluminescent substrates listed in the Reagent Description section have been verified for use in the PAR Immunoassay. The assay was developed and validated using the Thermo Scientific Pierce substrate and the Infinite 200 Microplate Reader from Tecan. Due to the high RLU values typical of this assay the KPL substrate was evaluated as an alternative and has been shown to provide comparable results. The KPL substrate is recommended for use on alternate plate readers with lower dynamic range luminometers.

ii. Chemiluminescent substrates are a significant contributor to RLU readout variability in chemiluminescent ELISAs. During the shelf life of a given lot of substrate, a decrease in the signal of known standards to background (S/B) over the range of the assay may be observed; however, the background signal should decline roughly in parallel with the known standards in an optimized assay so that the impact of the S/B is minimal if the substrates are handled as recommended and discarded at the recommended expiration date.

3. Reagent QC

   i. Identity

      a. Reagent name and catalog number should be verified against all product vial labels and the lot-specific Certificate of Analyses.

   ii. Functional Testing

      a. It is advisable to verify the performance of a new lot of chemiluminescent substrate before use in a PAR Immunoassay on unknown specimens. The evaluation assay should pass all acceptance criteria for both the calibrators and quality control samples.

      b. Evaluate the new lot in terms of the RLU and the S/B for the calibrators. If the RLU of the calibrators for the new lot of material is >15% below that read with the old lot (or in a side-by-side comparison), even if it passes all assay acceptance criteria the lot should not be accepted. In the event that this reduced signal is observed, the result should be discussed with the vendor and an alternative lot should be evaluated.

VII. Antibody Coating Buffer

1. Reagent Description

Reagent Name	PDA II Antibody Coating Buffer
Manufacturer	Trevigen
Cat#	4520-960-13 (Custom Order)
Description	PDA II Antibody Coating Buffer is supplied ready to use in 100 mL bottles. This material is used to dilute the mAb for coating the microtiter plate. This material is not a catalog item, but the vendor has agreed to distribute the material as needed for the PAR Immunoassay on clinical specimens.
Storage and Stability	Store at 2°C-8°C. Discard opened bottles after 6 mo and all remaining material 18 mo of receipt or at manufacturer's assigned expiration date (whichever is first).

2. Reagent QC

   i. Identity

      a. Reagent name and catalog number should be verified against all product vial labels and the lot specific Certificate of Analyses.

   ii. Functional Testing

      a. No functional testing is necessary for this material. Assay performance was shown to be reproducible across 3 independently prepared lots of material and no significant difference in lot-to-lot performance of this material is expected.

VIII. Supporting Reagents and Materials

1. General Information

   i. For the supporting reagents and materials listed below, no lot-to-lot differences in performance of the materials are anticipated. The materials specified below from the sources given are recommended. Identity of the materials received should be verified upon receipt and the materials should be stored and used within the recommended shelf life provided by the vendor.

2. Reagent and Material Information

   i. Reacti-Bind White Opaque 96-well Plate (Thermo Scientific Pierce, Cat#: 15042)

   ii. Acetate plate sealers (Thermo Scientific Pierce, Cat#: 3501)

   iii. Tween 20 non-ionic, aqueous solution, 10% w/v (Roche Applied Science, Cat#: 11332465001)

   iv. Protease Inhibitor Cocktail (Sigma-Aldrich, Cat#: P-2714 or Roche, Cat#: 11697498001)

   v. Phenylmethanesulfonyl fluoride solution (PMSF; Sigma-Aldrich, Cat#: 93482-50ML-F)

   vi. Cell Extraction Buffer (CEB; Invitrogen, Cat#: FNN0011)

   vii. 20% sodium dodecyl sulfate (SDS; e.g., Sigma-Aldrich, Cat#: 05030-500ML-F)

   viii. Albumin, bovine serum (BSA; Sigma-Aldrich, Cat#: A 7030)

   ix. Mouse serum (Sigma-Aldrich, Cat#: M 5905)

IX. PAR Immunoassay Xenograft Quality Control Samples

1. General Information

   i. Historically the control samples for the PAR Immunoassay were derived from a tumor cell lysate prepared from MCF7 cells diluted to predefined high, mid and low levels of PAR signal in the ELISA for each assay plate. We are moving to the use of pooled xenograft tumor lysates derived from cell lines that provide a range of PAR signal in the assay at a fixed protein load. These quality control samples more closely match the matrix of the human biopsy lysates than the serially diluted cell lysate controls, providing a better assay quality control sample.

2. Xenograft Quality Control Samples

   i. Preparation and Analysis

      a. Subcutaneous bilateral implanted cells from 3 human cancer cell lines (COLO 829 melanoma, A375 melanoma and A549 ASC-1 lung) are used to produce mouse xenograft tumors in athymic nude mice (nu/nu NCr) with a range of PAR levels.

      b. Xenograft tumors are collected when they reach 250-300 mg in size, cut into quarters and flash frozen in prechilled Sarstedt tubes.

      c. Tumor quarters are then processed and extracted following SOP340520 (Biopsy Specimen Processing for PAR Immunoassay).

      d. Xenograft lysates are adjusted to 1 µg/µL and run in the PAR Immunoassay at a 5 µg total protein per well.

      e. Using the PAR readout for the individual xenograft lysates, lysates from different xenograft quarters are pooled to hit target PAR ranges corresponding to the High-, Mid- and Low-Control PAR pg/mL readout values in the PAR Immunoassay. These pooled lysates are aliquoted, flash frozen, and stored at -80°C (or lower). The target ranges for the low, mid and high quality control lysates are 36-54 pg/mL, 150-200 pg/mL and 400-600 pg/mL, respectively (assay load volume is 75 µL).

      f. Pooled lysates prepared to hit the target High-, Mid- and Low-Control PAR pg/mL readout values are then run on a minimum of 5 individual PAR Immunoassay runs and assigned an acceptable PAR readout range based on the mean value ± 2 SD for the 5 runs. Values assigned to QC specimens are linked to a critical reagent set. Upon qualification of subsequent critical reagents (mAb, pAb or calibrator) new QC specimens should be prepared or new values to an existing lot should be determined.

      g. For distribution, pooled xenograft control lysate aliquots are labeled with the lot number, High/Mid/Low designation, and acceptable PAR readout range for that qualified lot.

   ii. Handling, Storage and Stability

      a. Xenograft control samples should be stored at -80°C (or lower) and should be stable for up to 3 y.

      b. Aliquots of xenograft control samples should be handled as single-use vials and not subjected to repeat freeze/thaw cycles.

      c. Xenograft quality control samples are also used to establish acceptable assay performance as detailed in SOP340530 (PAR Immunoassay Quality Control, Data Analysis, and Reporting SOP). Use of the Westgard Multirule System and a control grid are recommended to identify changes in performance of the xenograft quality control samples across assay runs within a given laboratory.