NIH National Cancer Institute

SOP340530: PAR Immunoassay Quality Control, Data Analyses, and Reporting

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Please check for revision status of the SOP at

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

and be sure to use the current version.

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VERSION INFORMATION

1. Approvals

Technical Reviewer: Yvonne A. Evrard   Date: Jan 22, 2014

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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=HHS, ou=NIH, ou=People, 0.9.2342.19200300.100.1.1=0012310003, cn=Ralph E. Parchment -S (Affiliate) Date: 2014.01.26 19:04:41 -05'00'*

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2. Change History

Revision	Approval Date	Description	Originator	Approval
--	1/25/2013	New Document. Previous data analyses were macro-based and included in SOP340505. The macro has been retired and replaced with Excel and GraphPad Prism to allow for a more user-friendly and adaptable analysis package.	KFG, YAE	KFG
A	1/22/2014	Minimum allowable protein concentration for reporting increased. Definitions of clinical reporting abbreviations added. Percent CV criteria for background wells removed.	KFG, YAE	KFG

OVERVIEW OF IMMUNOASSAY SAMPLE PROCESSING

| **PBMC Processing** | **Tumor Biopsy 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

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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SOP340506:

PBMC Protein Extraction for PAR Immunoassay

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

SOP340520:

Biopsy Specimen Processing for PAR Immunoassay

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

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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 analysis of PAR measurements across samples and clinical sites.

3.0   ABBREVIATONS

C	=	Control
CV	=	Coefficient of Variation
DCTD	=	Division of Cancer Treatment and Diagnosis
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
IA	=	Immunoassay
ID	=	Identification / Identifier
IQC	=	Internal Quality Control
IP	=	Insufficient Protein or PBMCs
LHTP	=	Laboratory of Human Toxicology and Pharmacology
LLQ-RLU	=	Lower Limit of Quantitation for assay in RLU units
LLQ	=	Lower Limit of Quantitation for assay in pg/mL units
NCTVL	=	National Clinical Target Validation Laboratory
NR	=	Not Reportable
PADIS	=	Pharmacodynamic Assay Development and Implementation Section
PAR	=	Poly(ADP-ribose)
PARP	=	Poly(ADP-ribose) Polymerase
PBMC	=	Peripheral Blood Mononuclear Cell
PD	=	Pharmacodynamic
QC	=	Quality Control
RLU	=	Relative Light Unit
SD, STDEV	=	Standard Deviation
SOP	=	Standard Operating Procedure
ULQ	=	Upper Limit of Quantitation

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. Assay quality control criteria are applied to the background, calibrator, and control samples to validate the assay run. Quality control criteria are then applied to the unknown samples and data reporting guidelines are defined.

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 Director/Supervisor oversees the personnel running SOPs within the laboratory and is responsible for ensuring the personnel are certified and have sufficient experience to 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 data analysis and quality control metrics is to follow this SOP and complete the required tasks and associated documentation. The Batch Record (Appendix 1) must be completed in ***real-time***, with each page ***dated and initialed***, and placed with the clinical sample information.

5.3   Neither the Certified Assay Operator nor the Laboratory Director/Supervisor will interpret the assay readout data in terms of drug effect in the Clinical Sample Data Report (Appendix 4). This report should simply state the PAR readout value or define the sample as unquantifiable or unanalyzable.

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   MATERIALS AND EQUIPMENT REQUIRED

6.1   PC

6.2   Microsoft Excel 2003, 2007, or 2010

6.3   GraphPad Prism, Version 5.0 (GraphPad Software, Inc.)

6.4   Data needed from immunoassay results to be analyzed:

6.4.1   Plate Map well assignments, Sample Calculation Table, and Tecan plate readout from SOP340505 (PAR Immunoassay)

6.4.2   Lot-specific ranges for High-, Mid-, and Low-C quality control samples

6.5   Template files supplied during training and available upon request:

6.5.1   PAR immunoassay analysis Excel workbook template Version 001: SOP340530_PAR_template.xslt

6.5.2   PAR immunoassay analysis GraphPad Prism template Version 001: SOP340530_PAR_template.pzf

* If software differs from those recommended above, the Certified Assay Laboratory must prove their comparability or equivalence to those recommended.

7.0   OPERATING PROCEDURES

7.1   A copy of the Tecan Microplate reader output and the Clinical Sample Calculation Table from SOP340505 will be needed. In addition, the lot-specific ranges for the High-, Mid-, and Low-C samples used in this assay run will be needed for quality control analysis. **Important**: The PAR Immunoassay analysis Excel workbook template used in this SOP is based on the 96-well sample designations in the Plate Map from SOP340505.

7.2   Record the name and certification number of the Certified Assay Operator and the facility running the SOP in the Batch Record (Appendix 1). In addition, indicate the date of the original PAR immunoassay run and Plate ID (if available) in the Batch Record.

7.3   Record the version of GraphPad Prism and name of original Tecan data file to be analyzed (Appendix 1, Section 1).

Important: If at any time the **Assay Fails QC**, do not continue with the analysis. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure and notify the Laboratory Director/Supervisor of the assay failure. Rerun the assay with fresh reagents. Batch Records for any assay that fails QC should be maintained per laboratory procedures to track assay and laboratory performance.

7.4   Plate Map Layout QC (Excel, "Tecan Raw Data" and "Plate Layout QC")

7.4.1   Open the Excel Template (SOP340530_PAR_template.xslt) and save as an Excel document with a unique file name (e.g., SOP number + run date + unique plate identifier). Record the file name in the Batch Record (Appendix 1, Section 2A).

7.4.2   Paste the exported Tecan data into the worksheet titled "Tecan Raw Data." This is considered the raw, source data for data analysis; do not edit or modify the data on this worksheet.

7.4.3   If the recommended PAR Plate Map from SOP340505 and illustrated on top of the worksheet titled "Plate Layout QC" was used, copy and paste just the RLU readings from the "Tecan Raw Data" worksheet into the blank plate cells provided at the bottom of the "Plate Layout QC" worksheet (cells C21 - N28). To maintain the background colors, select "Match Destination Formatting" after pasting (not required).

7.4.3.1   If the 96-well plate was set-up in a different orientation than that outlined in SOP340505 and illustrated on top of the worksheet titled "Plate Layout QC" (not recommended), cell-by-cell copy and paste the data from the "Tecan Raw Data" worksheet into the appropriate wells on the "Plate Layout QC" worksheet such that they line up with the PAR color-coded Plate Map layout.

7.4.3.2   Once data has been pasted into the PAR Plate Map QC section of the "Plate Layout QC" worksheet, **do not drag and drop data** from one cell to another. Cells are linked throughout the Excel worksheets and dragging and dropping will break these links.

7.4.4   If any sample wells were blank (i.e., no sample loaded), delete RLU readings from those cells on the Plate Map QC section of the "Plate Layout QC" worksheet.

7.4.5   Indicate in the Batch Record if the recommended PAR Plate Map was used or if cells had to be copy and pasted individually to the "Plate Layout QC" worksheet (Appendix 1, Section 2B). Specify the reason for the deviation from the plate layout map in the Batch Record.

7.5   Background Well QC (Excel, "Assay QC")

7.5.1   On the worksheet titled "Assay QC," all values from the "Plate Layout QC" are auto-filled at the top of the worksheet and formulas within the worksheet will automatically perform QC determination for the background wells. A total of 14 wells are used for background determination; the 4 corner wells and 2 adjacent to the high standard are not used for background level calculation.

7.5.2   A ± 2 SD cut-off is applied to the initial 14-well dataset to identify outliers; if a background well RLU value is ≥ 2 SD from the mean, the cell will be highlighted in red.

7.5.3   To cleanly remove outliers from the background dataset without error, do the following:

7.5.3.1   <u>**First**</u>, copy and paste the RLU <u>values</u> for all wells that are in red (≥ 2 SD from mean) into cells B24 - F25 to document those that will be eliminated as outliers.

7.5.3.2   <u>**Second**</u>, delete those values that were copied and pasted into cells B24 - F25 from the background dataset cells (B18 - E21).

Important:
- The mean and SD values will update as outliers are deleted and this may result in loss of the red coloring; use the copy and pasted values as the primary reference for which cells to delete.

- Upon deletion of outliers from the initial background dataset, some wells may be identified as ≥ 2 SD from the new mean. <u>**Do not**</u> delete any further background wells, the ± 2 SD criteria should only be applied once to the dataset.

7.6   Standard Curve QC (Excel, "Assay QC")

7.6.1   Formulas within the "Assay QC" worksheet will automatically perform QC determination for the low and high standards with the criteria listed below. If any QC section states "**Assay Fails QC**," do not continue with the analysis; notify the Laboratory Director/Supervisor of the assay failure. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.

7.6.2   Low Standard QC and LLQ Assignment:

- In order to use the 7.8 to 15.6 pg/mL range of the standard curve, the mean RLU readout of the 7.8 pg/mL standard must be ≥ 3 SD above the mean RLU readout of the background; this value is referred to as the LLQ-RLU.

- If the 7.8 pg/mL standard fails, then the mean RLU readout of the 15.6 pg/mL standard must be ≥ 3 SD above the mean RLU readout of the background.

- If the 15.6 pg/mL standard also fails, the **Assay Fails QC** (cell H29).

- The lowest passing standard is assigned as the LLQ (pg/mL) for the assay (cell J24).

7.6.3   Signal-to-background (S/B) ratio QC and ULQ Assignment:

- The ratio for the lowest passing standard (7.8 or 15.6 pg/mL) RLU readout to the mean RLU readout of the background must be ≥ 1.1. If not, the **Assay Fails QC** (cells H33).

- The ratio of the highest standard RLU readout (1000 pg/mL) to the mean RLU of the background must be ≥ 15. If not, the **Assay Fails QC** (cells H35).

- If the high standard passes QC, it is assigned as the ULQ (pg/mL) for the assay (cell J25).

7.7   Non-Linear Regression and Data Transformation (GraphPad and Excel, "GraphPad Prism")

7.7.1   Open the GraphPad Prism template file (SOP340530_PAR_template.pzf) and save it with a unique file name mirroring the matched Excel analysis file. Record the file name in the Batch Record (Appendix 1, Section 2A).

- The GraphPad template file has already been set-up with all required criteria for data analyses by non-linear regression and transformation. Numerical values of "1" have been assigned as place holders in the data table. See GraphPad set-up in Appendix 2.

7.7.2   On the Excel worksheet titled "GraphPad Prism" copy cells D8 - G37 from the section "To GraphPad Prism" and paste it into the GraphPad Data Table titled "From Excel" (Data Tables > From Excel). Delete any remaining "1" values in the GraphPad Prism file from unused sample rows.

- All standard, control, and unknown sample RLU values (Replicate Data columns "A:Yn") have the mean background level subtracted.

- The values for the expected standard ranges (column "X") are expressed as a Log_{10} value for non-linear regression analysis.

7.7.3   Go to the "Table of results" sheet (Results > Nonlin fit of From Excel > Table of results) and verify that the "R square (weighted)" value in row 17 is ≥ 0.96.

- If the R square is < 0.96, the **Assay Fails QC**. State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.

7.7.4   Before proceeding, print the following four tables and graphs from GraphPad:

- Data Tables > From Excel

- Results > Table of results

- Results > Transform of Nonlin fit of From Excel

- Graph > From Excel

7.7.5   Go to the results sheet titled "Transform of Nonlin fit of Data" (Results > Transform of Nonlin fit of From Excel). These values have been transformed to represent PAR levels (background subtracted) in pg/mL rather than RLU.

7.7.6   Copy the values in the columns A:Y1, A:Y2, and A:Y3 and paste into the Excel worksheet titled "GraphPad Prism" under the section titled "From GraphPad" (light yellow cells). To maintain the background colors, simply select "Match Destination Formatting" after pasting (not required).

7.7.7   Save and close the GraphPad Prism file.

7.8 **Xenograft Lysate Control QC (Excel, "Control QC")**

7.8.1 On the Excel worksheet titled "Control QC," for each control sample (Low, Mid, and High) enter the pg/mL PAR ranges (top and bottom end) supplied with the **specific lot** of control reagents used for the assay (cells C16 – D21).

7.8.2 Formulas within the worksheet will report the QC determination for the control samples with the following criteria:

- At least one control at each level (Low-, Mid-, and High-C) must have a CV of < 20% for the replicate wells (cells G6 - G11).

- At least one control at each level and at least 4 of 6 controls overall must fall within the defined PAR pg/mL range provided for the **specific lot** of critical reagent (cells E16 - E21).

7.8.3 If any of these criteria are not met, the **Assay Fails QC** (cell F16). State in the Batch Record (Appendix 1, Section 3) the reason for assay failure, and go to SOP Step 7.11. Rerun the assay with fresh reagents.

7.8.4 For any control lysate out of specification, bring the data to the Laboratory Director/Supervisor to review along with recent assay runs to be sure there is not a broader problem with critical reagents or assay equipment.

7.9 **Unknown Sample Replicate QC and LLQ/ULQ QC (Excel, "Unknown QC")**

7.9.1 Replicate values on the "Unknown QC" worksheet are auto-filled from the "GraphPad Prism" worksheet.

7.9.1.1 If during GraphPad Prism analysis all replicate values for a sample are removed (blank cells), review the RLU readings for this sample compared to the lowest and highest calibrator and state in the "Notes" column if the sample was < LLQ or > ULQ, respectively, based on this comparison.

7.9.1.2 In the "Notes" column, specify "no sample" for those rows where blank cells simply indicate no sample was loaded.

7.9.2 Formulas in the "Unknown QC" worksheet will automatically perform %CV QC determination for the unknown sample replicate wells with the criteria listed below:

- Triplicate repeats for each sample must have a CV < 20%. If all samples Pass %CV QC, proceed to SOP Step 7.9.4.

- If an unknown sample has ≥ 20% CV (column H) and is listed as "Fail" in column I, go to SOP Step 7.9.3 and perform a Dixon's test for outliers.

7.9.3 Dixon's Test Outliers on Triplicate Replicates of Unknown Samples (see Appendix 3)

7.9.3.1 If a sample has a CV ≥ 20% (column H) and is listed as "Fail" in column I, copy and paste the triplicate repeat values[*] (columns C - E) into the Dixon's calculator input cells (C26 – E26). [*]**Note**: The values in columns C - E are auto-filled using an Excel formula; when copy and pasting to the Dixon's calculator, "Paste Special" as "Values Only."

7.9.3.2 If either the lowest (X1) or highest (X3) value from the triplicate repeats can be eliminated, the Dixon's calculator will state "Yes, Xn is an Outlier" (cells E33 – E34). Delete that value from the replicate data (columns C - E) and state in the "Notes" column "Outlier removed."

7.9.3.3   If the 2 remaining repeat values (when one has been removed) have a %CV ≥ 20%, **OR** none of the replicates are rejected as an outlier when %CV is ≥ 20%:

- First assess if the mean (column F) is < LLQ or > ULQ (SOP Step 7.9.4); if it is, delete the mean for that dilution (column F), and state "Sample < LLQ or > ULQ."

- If sample is within the dynamic range of the assay, delete the mean for that dilution (column F), and state "Sample Fails %CV QC."

7.9.4   Review the average PAR levels (column F) and identify any values that are < LLQ or > ULQ (highlighted in blue). The assay LLQ and ULQ values have been carried over in cells F26 - F27 from the "Assay QC" worksheet.

7.9.4.1   If the average value for an unknown sample is < LLQ or > ULQ, delete the mean value for that dilution (column F) and in the "Notes" column state "Sample < LLQ" or "Sample > ULQ."

7.9.4.2   If a sample is > ULQ and there is sufficient sample volume, it can be re-run with fresh reagents at a 2-fold lower protein load/well. If a sample is < LLQ and there is sufficient sample volume, it can be re-run at a 2-fold higher protein load/well.

7.10   **Dilution Linearity QC for Unknown Samples (Excel, "PAR Determination")**

Note: The calculations set up in the lower portion of the "PAR Determination" worksheet for dilution linearity QC assume the Plate Map recommendations from SOP340505 were followed.

<u>If both tumor biopsy and PBMC samples are being run on the same plate</u>, the tumor biopsies should have been loaded first followed by the PBMC samples. For example, pre-dose biopsy dilutions in S1 - S3; post-dose in S4 - S6; and PBMC samples in S7 - S16. Every three tumor biopsy samples (e.g., S1, S2, S3) would represent the 1, 2, and 4 µg loads from a single patient's tumor biopsy sample.

7.10.1   On the Excel worksheet titled "PAR Determination" using the Clinical Sample Calculation Table from the SOP340505 Batch Record, enter the following information:

7.10.1.1   Sample/patient ID (cells C6 - C21). Enter "no sample" if the wells were empty.

7.10.1.2   For tumor biopsy samples, enter the total µg protein loaded (e.g., 1, 2, or 4 µg/well) for each sample (cells D6 - D21); for PBMC samples, enter the relative cell number loaded (e.g., 250,000) in each well (cells F6 - F21).

- The total volume loaded per well, 75 µL, is calculated from SOP340505: 25 µL unknown sample plus 50 µL loading buffer per well.

- Since mean PAR values for samples < LLQ or > ULQ were deleted in SOP Step 7.9.4, they are not included in dilution linearity assessment or PAR level determination. If all three dilutions from a single sample are < LLQ or > ULQ, the sample is reported as unquantifiable (see SOP Steps 7.11.3.2/.4.2).

7.10.2   On the bottom half of the "PAR Determination" worksheet, formulas will automatically back-calculate the total PAR protein in pg/µg total protein (cells D27 - D42) for tumor biopsy samples or total PAR in pg/1 x 10^7 PBMCs (cells K27 - K42). Sample/Patient ID information is auto-filled dependent on sample data entered at the top of the worksheet.

If only PBMC samples were run on the plate, no further analysis is required; proceed to SOP Step 7.11.

7.10.3 Dilution linearity for the tumor biopsy dilutions (1, 2, and 4 µg loads) is determined by calculating the percent PAR in each dilution relative to the average of the three. Values between 85% and 115% pass dilution linearity QC; any samples falling outside of this range will appear red in the worksheet (cells F28 - F43). Apply dilution linearity pass/fail criteria as follows:

- If **1 of 3 fails** dilution linearity, delete the PAR value for that sample (cells D27 - D42) and verify that the remaining 2 samples fall within the acceptable dilution linearity criteria. In the "Notes" column state "*Dilution non-linear*."

- If **2 of 3 fail** dilution linearity, delete the PAR value (cells D27 - D42) that is farthest from 100% and verify that the remaining 2 samples fall within the acceptable dilution linearity. In the "Notes" column state "*Dilution non-linear*" for the sample that was deleted.

- If **3 of 3 fail** dilution linearity OR **the 2 remaining** fail dilution linearity (when one has been removed), in the "Notes" column for all three samples state "*All samples failed dilution linearity*." If there is sufficient lysate, re-run the unknown in a new assay; if there is insufficient lysate, report results as described in SOP Step 7.11.3.3.

7.11 **Clinical Sample Data Report**

Important: Decisions regarding levels of pre-dose PAR levels needed to measure a drug effect post-dose will be made by the Clinic or pharmacodynamic Laboratory Director/Supervisor and should not be reported in the Clinical Sample Data Report.

7.11.1 For **each patient**, compile the final PAR assay results using the Clinical Sample Data Report Template (Appendix 4).

7.11.2 Enter the patient and clinical protocol information.

7.11.3 The following abbreviations should be used for consistency. See details in next sections for when to use these abbreviations.

- < LLQ (< X.XX pg/µg) or > ULQ (> X.XX pg/µg), below or above cut-off for reportable assay results, respectively.
- NA, no biopsy provided;
- NR: not reportable – following descriptors should be added when appropriate
  - QC Fail, not reportable due to assay QC failure;
  - IP, not reportable due to insufficient protein

7.11.4 Report biopsy results to the Clinic using the information on the "PAR Determination" worksheet as follows:

7.11.4.1 Unknown samples should be reported as NR, with no value reported, if any of the following are true:

- The assay or sample failed QC and there is insufficient lysate to re-run the unknown: NR, QC Fail.

- A lysate sample had insufficient protein (IP, **≤ 0.25 µg/µL**) by BCA analysis (SOP340520); this sample will not have been analyzed by the PAR Immunoassay: NR, IP.

7.11.4.2   Unknown samples should be reported as < LLQ or > ULQ, if the average PAR levels for **all three** dilutions were < LLQ or > ULQ, and there is insufficient lysate to re-run the unknown sample.

- The GraphPad-transformed PAR readout value for samples < LLQ or > ULQ should not be reported as these are outside the dynamic range of the assay. Instead state if the sample was < LLQ or > ULQ in the "PAR Levels" column of the Clinical Sample Data Report (see below).

- For samples where **all three** dilutions were < LLQ or > ULQ a maximal or minimal possible concentration will be reported to the Clinic in the "QC Note" section of the Clinical Sample Data Report as follows:

  o If a sample was < LLQ, use the calculations at the bottom of the "PAR Determination" worksheet (cells E46 – E48) to report the maximum PAR concentration in pg/µg that *could* have been present in the sample.

    The calculation for the highest protein load for the unknown sample should be used as the LLQ assignment. The worksheet calculation is ([LLQ value * 0.075 mL well volume]/protein load).

Example	PAR Levels pg/µg protein	QC Note
Baseline	16.25	
Post-Dose	< LLQ	0.15 pg/µg; max. possible PAR level

  o If a sample was > ULQ, use the calculations at the bottom of the "PAR Determination" worksheet (cells G46 – G48) to report the minimum PAR concentration in pg/µg that *could* have been present in the sample

    The calculation for the lowest protein load for the unknown sample should be used for ULQ assignment. The worksheet calculation is ([ULQ value * 0.075 mL well volume]/protein load).

Example	PAR Levels pg/µg protein	QC Note
Baseline	35.63	
Post-Dose	> ULQ	75 pg/µg; min. possible PAR level

7.11.4.3 For all other samples, report the PAR value as follows:

- For samples where **≥ 2 dilutions passed** the dilution linearity and other QC criteria; report the calculated average PAR levels in pg/µg protein (cells E27 – E42).

- If PAR levels for **only 1 dilution** was within the dynamic range of the assay (> LLQ or < ULQ), and there is insufficient lysate to re-run the unknown sample; report the PAR readout value for that dilution and state "*xx µg protein load reported; other dilutions < LLQ or > ULQ.*"

- If **3 of 3** OR **the 2 remaining** samples (when one has been removed) fail dilution linearity or other QC criteria, report the PAR levels in pg/µg protein for the dilution (i.e., S1, S2, or S3) with the <u>lowest protein load</u> that falls within the range of the assay. In addition, state "*Lowest protein load reported; sample dilutions non-linear*" in the "QC Note" section.

7.11.5 <u>Report PBMC results</u> to the Clinic using the information on the "PAR Determination" worksheet as follows:

7.11.5.1 Unknown samples should be reported as NR, with no value reported, if any of the following are true:

- The assay or sample failed QC and there is insufficient lysate to re-run the unknown: NR, QC Fail.

- A lysate sample had insufficient PBMCs (IP, **≤ 1 x 10⁶ PBMCs** total; (SOP340506); this sample will not have been analyzed by the PAR Immunoassay: NR, IP.

7.11.5.2 Unknown PBMC samples should be reported as < LLQ or > ULQ, if the average PAR levels for that PBMC sample was < LLQ or > ULQ, and there is insufficient lysate to re-run the unknown sample.

- The Graph-Pad transformed PAR readout value for samples < LLQ or > ULQ should not be reported as these are outside the dynamic range of the assay. Instead state if the sample was < LLQ or > ULQ in the "PAR Levels" column of the Clinical Sample Data Report (see below).

- For samples < LLQ or > ULQ a minimal or maximal possible concentration will be reported to the Clinic in the "QC Note" section of the Clinical Sample Data Report as follows:
  - If a sample was <u>< LLQ (or >ULQ)</u>, use the calculations at the bottom of the "PAR Determination" worksheet (cells K46-L47) to report the maximum (or minimum) PAR concentration in pg/1 x 10⁷ cells that *could* have been present in the sample.

    The fold dilution of cells loaded per well from 1 x 10⁷ cells is used for this calculation. The worksheet calculation is ([LLQ or ULQ value * 0.1 mL well volume]/ [1 x 10⁷ cells / relative cell number in well]).

Example	PAR Levels pg/1E7 PBMCs	QC Note
C1, D1 4h	< LLQ	23 pg/1E7 PBMCs; max. possible PAR level
C1, D18, 6h	> ULQ	3,000 pg/1E7 PBMCs; min. possible PAR level

7.11.5.3 For all other PBMC samples, report PAR in pg/1 x 10⁷ cells (K27 – K42).

7.12 Review and finalize the Batch Records (Appendix 1) and obtain required signatures. Document ANY and ALL deviations from this SOP in the Batch Record (Appendix 1, Section 3).

7.13 The Laboratory Director/Supervisor should review the Batch Record and Clinical Sample Data Report for each patient and date and sign both affirming the data contained within the reports are correct (Appendix 1, Section 4).

7.14 The **signed** Clinical Sample Data Reports for each patient should be sent to the clinical protocol Principal Investigator.

APPENDIX 1: BATCH RECORD

Certified Assay Operator: _____

Certification Number: _____

Facility/Laboratory Running SOP: _____

Data Immunoassay Run: _____ / _____ / _____

Plate ID (optional): _____

1. **Software**

    GraphPad Prism   Version: _____

    Name of original Tecan data file: _____

2. **Plate Map QC**

    A.   <u>Name of saved PAR Excel data analysis workbook</u>

    _____

    <u>Name of saved PAR GraphPad Prism file</u>

    _____

    B.   <u>Plate Map Set Up QC</u>

    ☐   Recommended PAR Plate Map used from SOP340505

    ☐   Alternative plate map used; cells copy and pasted individually to the Plate Layout QC worksheet

    Reason: _____

3. **Notes, including any deviations from the SOP:**

    If assay fails QC, state the specific reason for assay failure and notify the Laboratory Director/Supervisor.

4. **Laboratory Director/Supervisor Review of Batch Record**

    Laboratory Director/Supervisor: _____ (PRINT)

    _____ (SIGN)

    Date: _____

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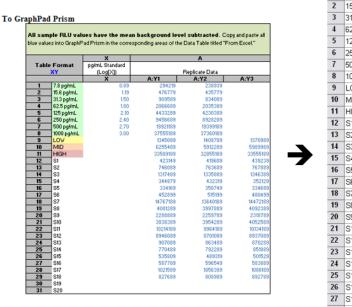U.S. Department of Health & Human Services | National Institutes of Health

APPENDIX 2: GRAPHPAD PRISM SET-UP

While a GraphPad Prism Template document is supplied, below are the steps used to set-up the document from a blank Project File. The PAR immunoassay analysis GraphPad Prism template (SOP340530_PAR_template.pzf) supplied during training has all data tables and graphs set-up with numerical values of "1" assigned as place holders in the data table.

1) GraphPad Prism Non-Linear Regression and Unknown Sample Transformation

   a. Open GraphPad Prism and create an XY graph type with the Y axis showing 3 replicate values in side-by-side sub-columns; plot the Mean only.

   b. Retitle the X and Y headers as shown in the GraphPad image below. Copy and paste the values in blue on the 'GraphPad Prism' Excel worksheet onto the Data Table (see image below for set-up). **Note:** In the supplied GraphPad Prism template, the data table is titled "From Excel," the default name is "Data 1."
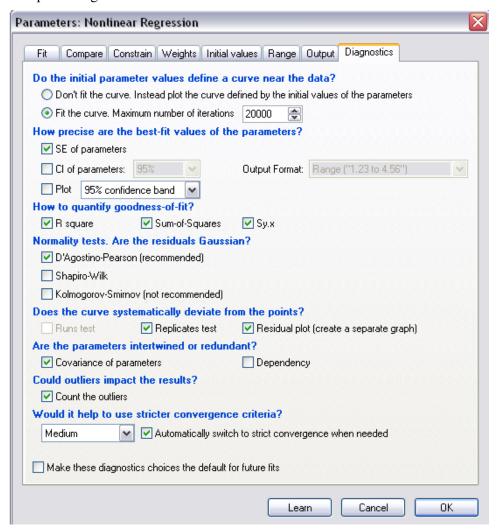
"GraphPad Prism'" Excel worksheet

GraphPad Prism Data Table

Table format: XY	X pg/mL Standard (Log[X])	A Replicate Data		
	X	A:Y1	A:Y2	A:Y3
1 7.8 pg/mL	0.89	294219	238939	
2 15.6 pg/mL	1.19	476779	435779	
3 31.3 pg/mL	1.50	909589	834089	
4 62.5 pg/mL	1.80	2066689	2035389	
5 125 pg/mL	2.10	4433289	4230389	
6 250 pg/mL	2.40	9456689	8928289	
7 500 pg/mL	2.70	2e+007	2e+007	
8 1000 pg/mL	3.00	4e+007	4e+007	
9 LOW		1345089	1408789	1370989
10 MID		6255489	5912289	5989989
11 HIGH		3e+007	3e+007	3e+007
12 S1		1e+007	1e+007	1e+007
13 S2		4001289	3997889	4092389
14 S3		2288889	2259789	2318789
15 S4		3838389	3954289	4052589
16 S5		1e+007	9964189	1e+007
17 S6		8946089	8701089	8937089
18 S7		907089	863489	870289
19 S8		770489	792289	851889
20 S9		535809	489319	501529
21 S10		587789	596549	583889
22 S11		1021589	1056389	1080189
23 S12		827689	808989	892789
24 S13		423149	419609	439239
25 S14		746089	763689	767889
26 S15		1317489	1335889	1346389
27 S16		344879	432319	352129
28 S17		334169	350749	334689
29 S18		452899	515199	480499
30 S19		1945589	1983789	1914589
31 S20		553309	659679	733689

2) In GraphPad Prism, on the menu ribbon in the Analysis box, select Analyze. Under the XY Analysis section, select "Nonlinear regression (Curve fit)" and the A-Replicate Data set should be selected in the right-hand window. Click OK.

3) The non-linear regression (NLR) parameters window will appear. Tab-by-tab, make the following updates:

   a. On the Fit tab, under 'Dose response-Stimulation,' select "log(agonist) vs. response -- Variable slope (four parameters)." At the bottom of the tab, under the 'Fitting Method' select "Least squares (ordinary) fit" and under 'Interpolate' check "Interpolate unknowns" with CI set at None.

   b. On the Weights tab select "Weight by $1/Y^2$ (minimize the relative distances squared)" as the Weighting method and under 'Replicates' select "Consider each replicate Y value as an individual point."

   c. On the Output tab under 'Location of interpolated X values,' select "Y column, maintaining the side-by-side arrangement of replicates." Change Number of digits in output to 2 significant digits.

   d. Set up the Diagnostics tab as follows:

   e. Click OK.

4) A set of Nonlin fit Results sheets will be added to the GraphPad Prism navigator (image on right).

5) The "Table of results" sheet will open by default.

6) Open the "Interpolated X replicates" sheet and on the menu ribbon in the Analysis box, select Analyze. Under the 'Transform, Normalize...' section, select "Transform" and the A-Replicate Data set in the right-hand window should be selected. Click OK.

7) Set up the Transform Parameters with the following items selected: "Standard functions," "Transform Y values using 'Y=10~Y'," and "Transform individual Y values." Under the 'New graph' section, "Create new graph" should be selected.

8) Click OK.

9) A new data sheet will be created called "...Transform of Nonlin fit of Data." This sheet now contains readout values for the plate repeats in pg.

APPENDIX 3: DIXON'S TEST FOR OUTLIERS

1. If the %CV for a triplicate well repeat is $\geq 20\%$ a Dixon's test for outliers can be used to determine if one cell can be eliminated as an outlier

2. The Dixon's test uses the following criteria:

    A. The Dixon's test for outliers is outlined in United States Pharmacopeia (USP) General Chapter <111> "Design and Analysis of Biological Assays" under the "Rejection of Outlying or Aberrant Observations" section. The Dixon's test is used to determine if one (and only one) value from a small set of values can be legitimately rejected from a normally distributed set of data. The Dixon's test can be applied by first arranging the values from the smallest to the largest: triplicate repetitions $X_1 < X_2 < X_3$.

$$X_1 \text{ is an outlier if } \quad \frac{(X_2 - X_1)}{(X_3 - X_1)} > 0.941$$

$$X_3 \text{ is an outlier if } \quad \frac{(X_3 - X_2)}{(X_3 - X_1)} > 0.941$$

    B. The cutoff in USP <111> for rejection of outliers from three values is $P > 0.976$, which gives a 98% confidence level for a one-sided test and a 96% confidence level for a two-sided test. Because an outlier can be either high or low in the triplicate samples, a two-sided approach is suggested. Either the 0.976 or 0.941 confidence limit can be selected as the decision criterion as long as the confidence level is indicated and applied objectively to the data set.

    C. The PAR Immunoassay uses the 0.941 confidence limit criterion.

3. A calculator is provided in the Excel Data Analysis worksheets so that the triplicate repeat numbers only need to be entered into the input cells (light yellow below) and the Dixon's calculations are performed automatically. Example of the highest value being eliminated using the Dixon's calculator:

Dixon's Calculator

INPUT VALUES:	1.10	25.10	1.20

X1 (low)	1.10
X2 (mid)	1.20
X3 (high)	25.10

	Dixon's Calculation	Min. Criteria	Outlier?
Test for Low Outlier (X1)	0.0042	0.941	No
Test for High Outlier (X3)	0.9958	0.941	Yes, X3 is an outlier

This calculation has a confidence level of 96%

4. If either the lowest (X1) or highest (X3) value from the triplicate repeats can be eliminated, the Dixon's calculator will state "Yes, Xn is an Outlier." Delete that value from the replicate data and state in the "Notes" column "Outlier removed."

5. If one replicate is rejected as an outlier, the remaining two repeats must still meet the 20% CV rule. If not, the entire sample fails QC. Delete the mean value so that it is not used for further analysis and state "Sample Fails %CV QC" in the "Notes" column.

6. If the %CV is $\geq 20\%$, but none of the replicates are rejected as an outlier, the entire sample fails QC. Delete the mean value so that it is not used for further analysis and state "Sample Fails %CV QC" in the "Notes" column.

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APPENDIX 4: CLINICAL SAMPLE DATA REPORT

PAR Immunoassay PD Analysis	*Page 1 of 2*

Certified Assay Operator (Print): _____

Certification #: _____   Today's Date: _____

Trial and Patient Information

Patient ID: Trial Site: _____

Clinical Center #: _____   CTEP Protocol #: _____

Assay Readout

Tumor Biopsy Samples

	Patient/Sample ID	Scheduled Collection Cycle, Day	Actual Collection Date	Time	PAR Levels pg/µg protein	QC Note
Baseline						
Post-Dose						

PBMC Samples

	Patient/Sample ID	Scheduled Collection Cycle, Day	Actual Collection Date	Time	PAR Levels pg/1E7 PBMCs	QC Note
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

Designations: NA, no biopsy provided; < LLQ, below minimum cut-off for reportable assay results; > ULQ, above maximum cut-off for reportable assay results; NR, not reportable; QC Fail, not reportable due to assay or sample QC failure; IP, not reportable due to insufficient protein or PBMCs

Additional Information

Raw data available upon request.

To be completed by Laboratory Director/Supervisor

Director/Supervisor Signature*: _____

Today's Date: _____

*Signature indicates assay results have been reviewed and verified.

Biopsy and Treatment Information (for use by Clinical site)

Site of Biopsy: _____ Primary Tumor: _____

Dose Level: _____

Agent Name(s): _____ + _____

Dose and Units: _____ + _____