

Title: Immunoassay for PAR in clinical and pre-clinical specimens

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## 1.0 PURPOSE

- 1.1 Standardize the procedure for assaying specimens from animal models or from human patients for the presence and concentration of poly-adenosylribose (PAR).
- 1.2 Establish the procedures for specimen preparation.
- 1.3 Establish the procedures for data analysis.
- 1.4 Establish the procedures for assay quality control.
- 1.5 Establish the procedures for reporting out data generated by the assay

## 2.0 SCOPE

- 2.1 This assay methodology is to be employed whenever analysis of human xenografts, human biopsy material or human peripheral blood mononuclear cells (PBMCs) are subjected to the determination of PAR levels by chemiluminescent immunoassay.

## 3. DEFINITIONS

## 4. RESPONSIBILITIES

## 5. EQUIPMENT AND SUPPLIES REQUIRED

Wash Buffer: PBS with 0.1% vol/vol Tween 20  
Specimen Diluent: Superblock (Pierce)  
Carbonate Buffer, pH 9.6  
Blocking Agents: Bovine Serum Albumin, 2% W/V in pH 7.2 PBS  
Plate Washer: BioTek ELx450  
Chemiluminometer: Tecan Genios Pro  
Dry Incubator set to 24C  
Dry Incubator set to 37C  
Lysis Buffer

## 6. REFERENCE DOCUMENTS

SOP for protein determination by BCA assay  
SOP003.4.9 PBMC Preparations  
SOP for specimen pretreatment for PAR Immunoassay

## 7.0 PROCEDURE.

- 7.1 Plate coating. Pierce Reacti-Bind plates (CN 15042) are coated at 100ul per well, for 2h at 37C. Plates may be prepared up to 7 days in advance and stored at 2-8C prior to use. For refrigerated storage, perform the 37C heating step immediately prior to use. Coating antibody: MAb # and source, at 2 ug/ml in pH 9.6 Carbonate buffer. Plates are covered with acetate sheets. Do not use Mylar sheets.
- 7.2 On BioTek Plate Washer (ELx405 Select), Aspirate plate to dryness. Block for at least 1 h at 37C. Blocking buffer is pH7.2 PBS with 2% BSA (Sigma ELISA grade), 250 ul per well.
- 7.3 Aspirate plate to dryness and wash 1 time with PBS/Tween, verifying that wells are dry after the wash. All wash steps are carried out on the BioTek plate washer.
- 7.4 Specimens, Standards and Controls Addition.

Specimen diluent: pH 7.2 PBS, 2% BSA, 0.1% Tween. Specimen diluent is added to all assay wells except to the Standards wells as the first step. Cell or Tumor Extracts are assayed diluted in Lysis Buffer (BioSource) at 1:5 or greater dilution prior to addition to the sample wells. Standard tumor extract volume added is 10 ul. Standard PBMC extract volume is 25 ul. Use a single volume calibrated pipettor.

  - a. For tumor extracts, Add 65 ul superbloc to each well that will receive a specimen or a Control using a calibrated multichannel pipettor.
  - b. For PBMC preparations, add 50 ul superbloc to each well.
  - c. Retrieve pure PAR Standard stock from -80 freezer. Thaw the 10ng/ml stock on ice. Vortex and then mix by inversion several times.
  - d. Prepare a 1:5 dilution of the stock in Superblock (2ng/ml final, =2000pg/ml PAR. Use 800 ul Superblock and 200 ul Standard. Mix by repipetting.
  - e. Prepare a 1:2 Serial dilution of the 2000pg/ml stock to yield standards at 1000, 500, 250, 125, 62.5 and 31 ng/ml. The 0ng/ml standard is superbloc.
  - f. Standards are added directly to the wells, 75 ul of standard per well. Standards include a 0 ng/ml PAR, which must be used in each assay.
  - g. Prepare assay controls: retrieve the Colo 829 cell extract High control and Low control from the -80C freezer.
  - h. Prepare dilutions of the controls as follows:
    - i. High Control dilute 1:5 in Superblock.
    - ii. Low Control dilute 1:10 in Superblock.
    - iii. Use the Low Control undiluted and at the 1:10 dilution in the immunoassay.

- i. Prepare specimens according to the appropriate document (see SOPs for PBMC preparation and tumor specimen preparation).
- j. Specimens, standards and controls are added according to this plate map:

	A	B	C	D	E	F	G	H	I	J	K	L	M
3		1	2	3	4	5	6	7	8	9	10	11	12
4	A	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO
5	B	CONTROL	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
6	C	CONTROL	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
7	D	CONTROL	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
8	E	OVERFLOW	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
9	F	OVERFLOW	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
10	G	OVERFLOW	SPECIMEN DUPS	SPECIMEN DUPS	STDS	STDS	SPECIMEN DUPS	SPECIMEN DUPS	CONTROL				
11	H	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO	ZERO

7.5 Sample incubation. Final Assay volume is set to 75 ul. Cover the plate with an acetate sheet, ensuring that all wells are tightly covered. Label the sheet with the assay date, start time and operator initials using a waterproof laboratory marker.

Incubate overnight at 2-8 C (16+/- 1 hours) in a refrigerator.

7.6 Aspirate plate to dryness and wash 4 times with Wash buffer, verifying that wells are dry at the end of the wash cycle. Aspirate again if wells are not dry. Discard plate cover.

7.7 Addition of anti-PAR Probe antibody: Rabbit Anti-PAR (cat.no., source) used at 1:500 dilution in Superblock, 75 ul per well; for this assay step the specimen diluent is supplemented with mouse serum (Sigma) to a final concentration of 1ul/ml.

7.8 Incubate the plate 2 hrs at ambient with a fresh plate cover.

7.9 Aspirate and Wash plate as in step 5. If the underside of the plate cover is not dry, discard and use a new plate cover.

7.10 Detection:

7.10.1 Prepare the Horse Radish Peroxidase conjugate 1 hour prior to use and keep at room temperature, out of direct sunlight.

7.10.2 KPL Goat anti-Rabbit HRP, XSA Grade, is diluted in specimen diluent supplemented with 1:500 mouse serum to 2 ul/mL final concentration.

7.10.3 Conjugate is applied at 75 ul per well, with a calibrated multichannel pipettor and incubated 1 hr at ambient. Keep out of direct sunlight.

7.11 Wash plate as in step 7.6.

7.12 Develop plate with luminescence substrate: Pierce SuperSignal Pico for ELISA, 75 ul per well.

Avoid exposure to sunlight and limit fluorescent light to control blank reads.

7.13 Plates are read at 2 minutes to 10 minutes on Tecan luminometer , 100 ms integration time, after a pre-shaking step in the reader.

7.13.1 Set the preshaking time to 5 seconds and program in a 60 second settling time before the first read. Set the instrument attenuation to NONE.

7.13.2 If after the 2 minute read the signal level is suppressed (too high), reset the instrument to read 25 msec integration time and set instrument attenuation to 1 OD. Set the shaking time to zero and read the plate again.

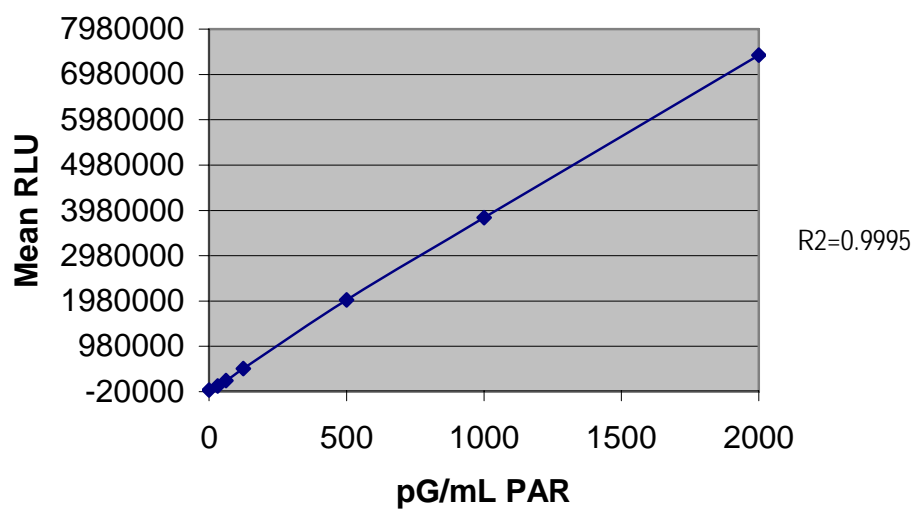
## 8.0 PREPARATION OF ASSAY STANDARDS:

8.1 Currently running at 2000,1000, 500 250,125, 62, 31 and 0 pg/ml.. Assay Standards may be prepared and used for up to 5 days. Assessment of Standards performance is done on the Microsoft Excel program using Linear Regression Analysis.

pG/mL PAR	3/8 curve	3/9 curve	3/10 curve	%CV
2000	7408796	7555822	8290601	6.10
1000	3818188	3749315	3971663	3.00
500	2008138	1832954	1702198	8.31
125	487028	434940	391838	10.88
62	229311	192374	204395	9.02
31	107938	101927	109486	3.75
0	19388	14288	-4142	
0	12589	-16283	-1941	67.28

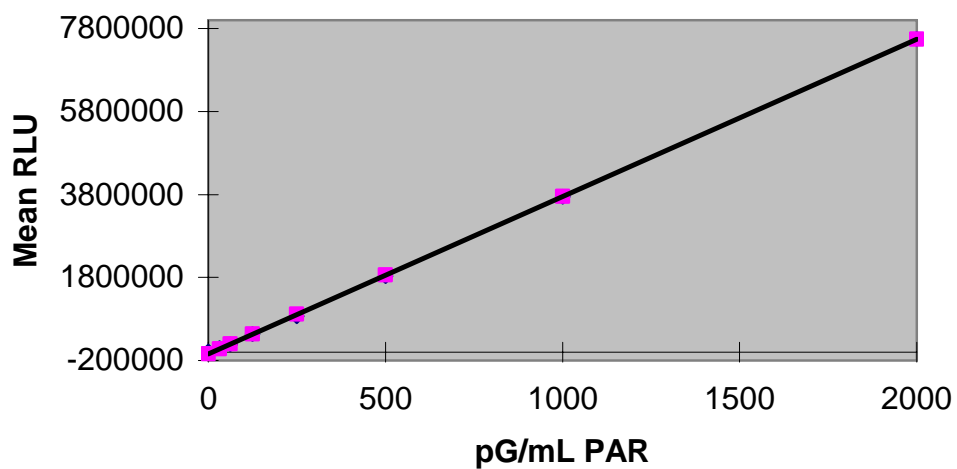
Examples of Standard Curves, used to generate the table above, are inserted below.

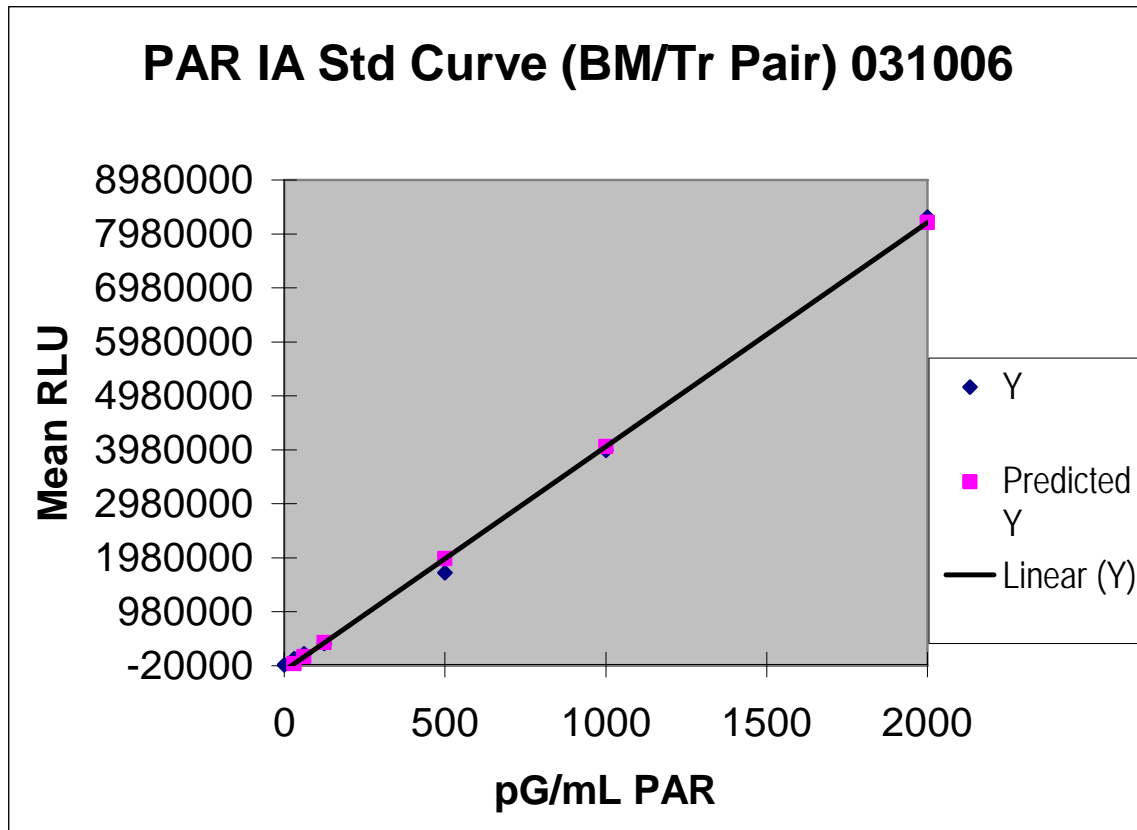
**PAR Standard Curve (BM/Tr) 030806**



**PAR Std Curve (BM/Tr) 030906**

$R^2=0.9999$





#### 9.0 ASSAY QUALITY CONTROL:

- 9.1 Determine the Slope of the Standard Curve, it's intercept and the slope equation.
- 9.2 Use the slope equation to determine the concentration of the assay controls and check this concentration against the range of concentrations expected with the vial of control Colo829 specimens used.
  - 9.2.1 If the  $R^2$  value of the slope is 0.99 or greater, use the linear equation to solve for concentration of the specimens and controls.
  - 9.2.2 If the  $R^2$  value of the slope is less than 0.99, use point-to-point interpolation of the standard curve to determine concentrations of specimens and controls.
- 9.3 If the concentrations of more than one of the 3 controls is out of specifications the assay fails and must be repeated. For one control out of specifications, the range of the standard curve in which the control reads should not be used to

determine concentrations of unknown specimens. These specimens must be re-assayed.

- 9.4 Determine the LLQ of the assay. If the LLQ is greater than 31 pg/ml the assay fails and must be repeated.
  - 9.5 Determine the Signal to Noise Ratio of the 1000 pg/ml standard and the 31 pg/ml standard by dividing the Mean RLU obtained for the standard by the Mean RLU obtained for the Zero.
    - 9.5.1 If the Ratio of the 31 standard to Zero is less than 0.5 do not report values in this range of the standard curve. The specimens should be re-assayed.
    - 9.5.2 If the Ratio of the 1000 standard to zero is less than 10, the assay should be rerun with fresh reagents and performance of the luminometer should be checked.
- 10.0 REPORTING OUT ASSAY DATA:
- 10.1 Determination of Specimen Concentration.
    - 10.1.1 See step 9.2 above for method to determine specimen concentration.
    - 10.1.2 If the %CV of the specimen concentration obtained is greater than 20%, the specimen must be reassayed.
    - 10.1.3 If specimens were run in triplicate and one repetition is discrepant from the other two, apply Dixon's rule to determine if that repetition should be used in reporting the data. The 20% CV rule still applies.