

The Need for a PARP *in vivo* Pharmacodynamic Assay - Update 2010

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Trevigen PARP Pharmacodynamic Assay II. In this article we present information regarding improvements to Trevigen's PARP Pharmacodynamic Assay discussed last year. We provide validation data and accelerated shelf life data regarding key assay components for the new assay format which features: a 96 well assay plate pre-coated with PAR capture antibody, extended dynamic range, and high signal to noise ratios. For readers that missed the discussion of the first assay we have retained much of the same basic information describing the role of PARP and DNA repair.

Poly(ADP-ribose) polymerases are promising therapeutic targets. In response to DNA damage, poly(ADP-ribose) polymerases-1 and 2 (PARP-1, PARP-2) are rapidly activated by DNA strand breaks. Once activated, NAD^+ is consumed for the synthesis of highly negatively charged polymers of ADP-ribose (PAR) on target nuclear proteins that include PARP-1 itself as a major acceptor [1, 2]. These highly branched polymers are in turn rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG). As a consequence of PARP activation, extensive DNA damage can lead to the depletion of NAD^+ in the cell and lead to cell death [3]. An overview of this metabolic response to DNA breakage is shown in Figure 1.

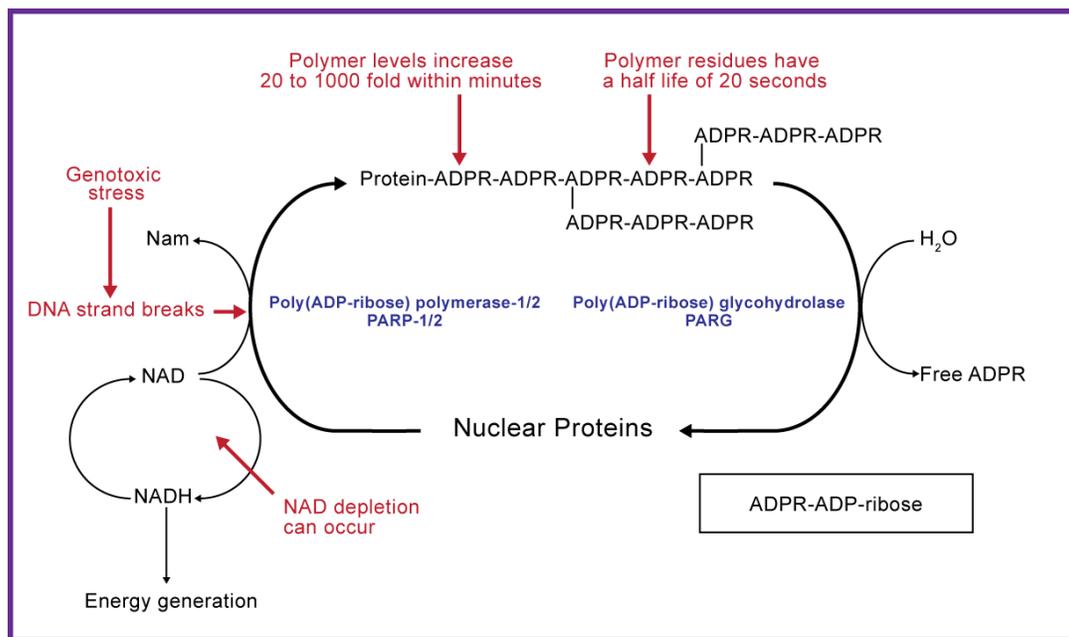


Figure 1:
Relationship between PARP 1 and 2 and PARG in response to DNA damage.
Excess genotoxic stress can lead to the depletion of NAD, resulting in apoptosis or necrosis.

PARP inhibitors are under development by multiple pharmaceutical companies. Figure 2 shows a working model that provides the basis for different approaches for the therapeutic targeting of PARPs that are currently under development by multiple pharmaceutical companies:

1. At relatively low levels of DNA damage, PARP-1, the best understood of the DNA damage responsive PARPs, mediates the repair of single strand breaks in DNA leading to cell recovery (Figure 2, left panel). This has led to the development of PARP inhibitors as chemo- and radio-sensitizing agents for cancer therapy ^[4].
2. Since the discovery that defects in homologous recombination sensitize cancer cells to PARP inhibitors there has been a renewed attention on PARP 1 as a pharmaceutical target for cancer intervention and for other diseases. In cells inhibited for PARP-1 activity (Figure 3 steps 3 and 4) single strand breaks (SSBs) are not repaired. Upon collision with replication forks (Figure 3 step 4), SSBs are converted to double strand breaks (DSBs) and subsequently repaired by homologous recombination (Figure 3, step 5). Exciting new cancer therapies using nontoxic inhibitors of PARP-1 are now under development. In tumors that are deficient in homologous recombination lethal DSBs induced by PARP are not properly repaired (Figure 3 step 6) resulting in cell death ^[5, 6]. This approach offers the exciting possibility of minimizing or eliminating the toxic side effects that frequently limit the effectiveness of the agent, because the oncologist must avoid exceeding the dose that the individual patient can tolerate. Therefore, specifically targeting tumor cells with nontoxic inhibitors, based on metabolic deficiencies, offers tremendous potential benefits for future patient care.

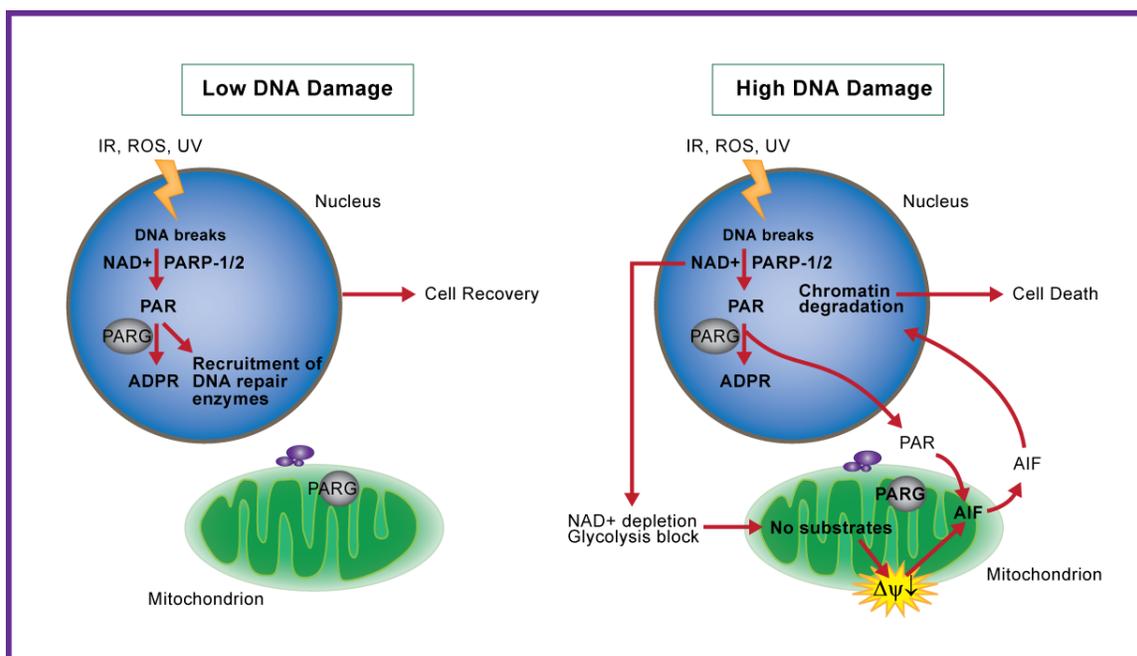


Figure 2:

Illustration of the role of PARP 1 and 2 and PARG in mediating cell death following neurotoxicity or ischemia, reperfusion injury.

The potential for PAR metabolism for targeting neurotoxicity and ischemia-reperfusion injury has been raised by studies demonstrating that PARP-1 knockout animals are extremely resistant to the cell killing effects of these conditions ^[7-9]. This has led to the discovery that PARP-1 activation is required for mitochondrial Apoptosis Releasing Factor (AIF) release ^[10] and that by a number of possible mechanisms including NAD and ATP depletion and release of PAR from the nucleus into the cytoplasm can cause mitochondrial AIF release ^[11] (Figure 2, right panel).

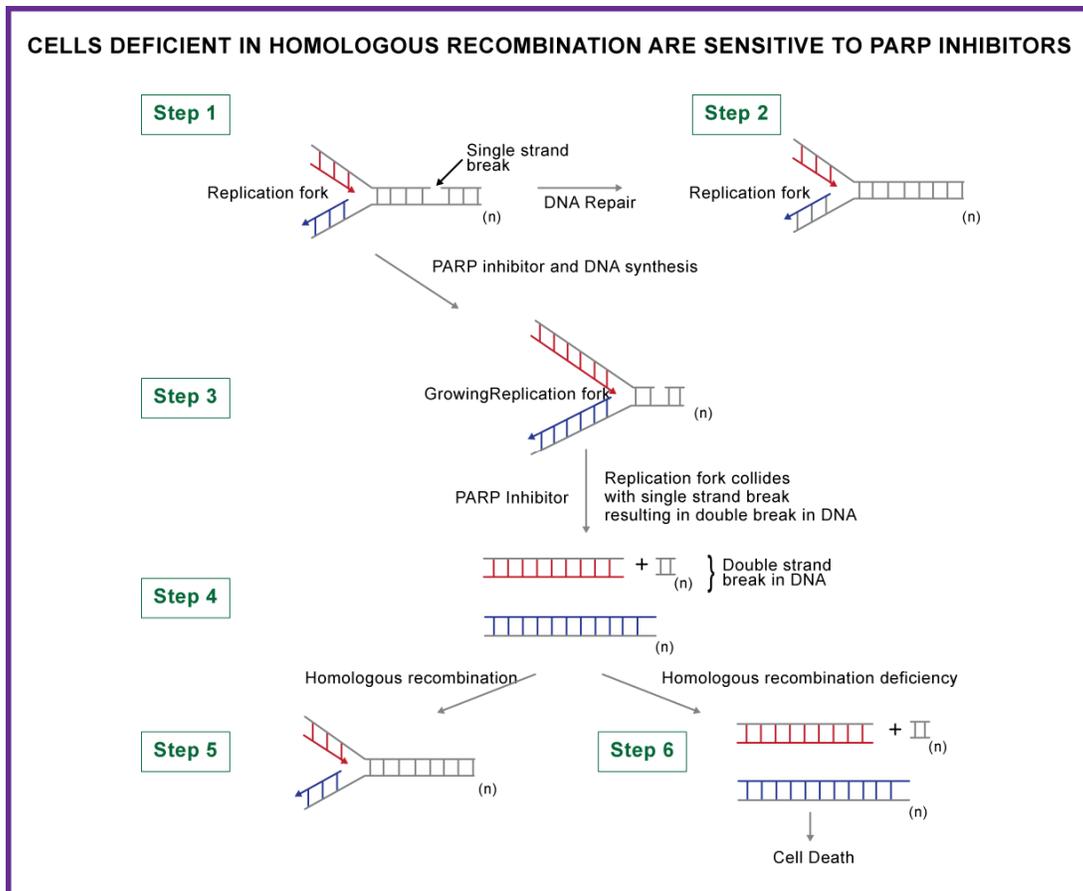


Figure 3:
Illustration of relationship between PARP-1 and homologous recombination.

Poly(ADP-ribose) glycohydrolases are potentially promising therapeutic targets. A number of lines of evidence indicate that PARG should be seriously evaluated as a potential therapeutic target.

1. The activities of PARPs and PARG are closely coordinated as cells respond to DNA damage^[12] (Figure 2).
2. Disruption of this coordinate regulation of PARPs and PARG inhibits DNA repair and sensitizes cells to the cell killing effects of genotoxic stress^[13].
3. Both PARP and PARG inhibitors also show promise for the treatment of shock and ischemia, reperfusion injury^[14, 15] and PARG inhibitors demonstrate protective effects that compare favorably with PARP inhibitors^[16].
4. The activity of PARG is almost certainly required for the release of PAR from the nucleus leading to the release of AIF (Figure 2).

PARP Pharmacodynamic Assay Version 2: Trevigen has introduced a new format in which plates pre-coated with monoclonal capture antibody are provided with the assay kits. The advantages of this assay format are:

1. Few user steps.
2. Reduced inter-assay variability.

3. Extended linear dynamic range of the assay to 1000 pg/ml of PAR from 200 pg/ml observed in the original assay.
4. The concentration of PAR in most samples is in the linear range of the assay meaning fewer dilutions of samples are required to accurately quantify PAR in samples and get accurate base line concentrations.
5. Lower backgrounds with improved signal to noise ratio.

Coated plates are manufactured in an environmentally controlled facility to assure lot to lot consistency. During the development of the second version of the kit Trevigen has developed a proprietary antibody coating buffer which greatly reduces background levels therefore increasing signal to noise ratios. The buffer has been beta tested by members of the scientific community and can now be obtained from Trevigen. Custom manufacture is available to assure single lot availability for critical studies. Shelf life studies for pre-coated plates are shown in figure 8.

Measurement of PAR in clinical samples. To facilitate development of PARP and PARG targeted therapeutics, validated assays to measure *in vivo* response to inhibitors in concurrence with preclinical studies are desirable. Currently therapeutic development is directed to PARP, and therefore, assays should measure response of PARP at the molecular level to treatment with candidate therapeutics. A validated pharmacodynamic assay used in conjunction with clinical trials to measure PARP activity has been described [17-19]

While numerous companies are pursuing the development of PARP targeted therapeutics, until recently there has not been a commercially available validated assay to access the effect of PARP inhibitors *in vivo*. Previously approaches relied on radiometric assays to determine PARP activity in cell extracts. At best these approaches are indirect and depend upon retention of a PARP inhibitor by the enzyme through multiple *in vitro* isolation steps. To circumvent this problem Trevigen has developed an improved PARP Pharmacodynamic Assay II that measures the *in vivo* intracellular concentration of PAR. Now investigators can obtain evidence of drug action on PARP in both *in vivo* and *in vitro* settings.

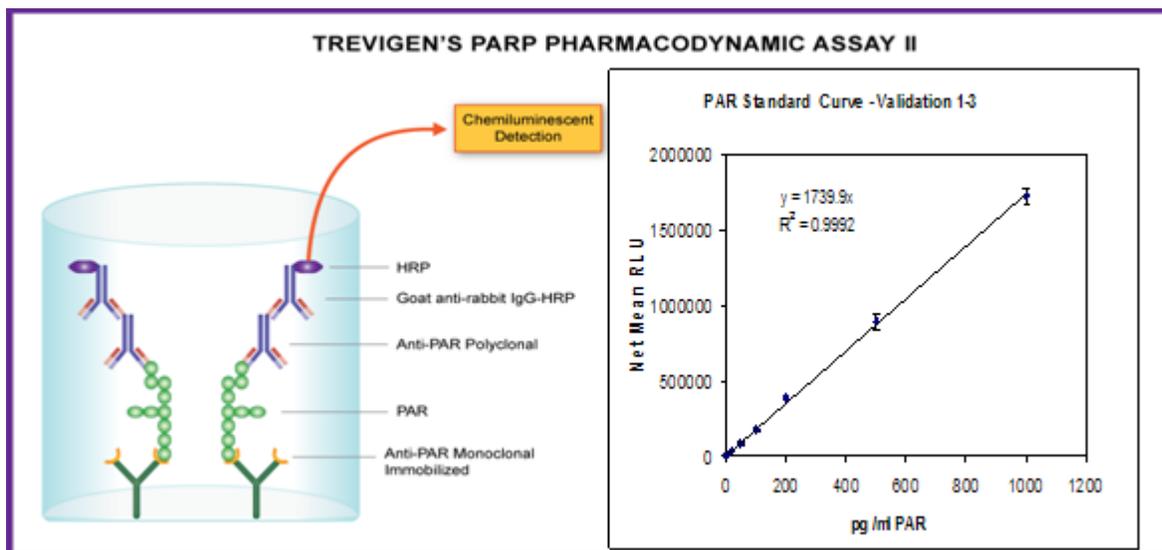


Figure 4:
Illustration of PARP Pharmacodynamic Assay II

Using a capture ELISA format, levels of poly-ADP-ribose (PAR) present in cells are easily determined. In this assay (Figure 4, left panel), free PAR and PAR associated proteins are captured by a monoclonal antibody and subsequently quantified using a PAR directed rabbit polyclonal antibody. A typical standard curve using purified PAR polymer supplied with the kit is shown in Figure 4, right panel.

PAR levels obtained from either frozen cell pellets or the corresponding fresh cells were not significantly different, demonstrating that samples can be collected and stored and subsequently assayed at a later time without deleteriously affecting the results.

In summary, Trevigen has developed a robust assay for determining PARP activity *in vivo*. This is the first commercially available assay to that permits one to access modulation of PARP 1 at the molecular level in response. The assay measures PAR levels *in vivo*. Cellular PAR levels are regulated by the dynamic interaction of PARP and PARG. The development of PARG inhibitors has lagged behind those for PARP. This is in part due to the fact that only recently has PARG been recognized as an important therapeutic target. There is a possibility that this assay will also serve as a tool to monitor the effect on inhibitors of PARG.

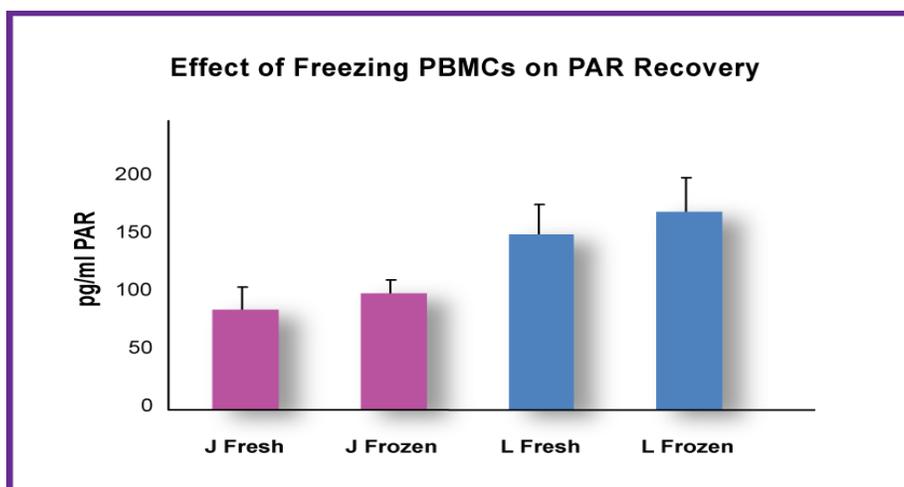


Figure 5:
Comparison of PAR content in extracts prepared from frozen and fresh cells.

Validation of assay performance was determined by obtaining blood from three donors (J, K, L). White cells were isolated, aliquotted, and frozen. Lysates from each donor were prepared on three separate days and were analyzed on three successive days. Variation is minimal indicating the assay is well suited to obtain baseline PAR levels in patients as shown in panel A. Included in the assays are Jurkat cell extracts containing 800, 200 and 10 pg/ml of PAR used as positive controls. The data in Figure 6 demonstrates that the assay provides reproducible results over of this study. The R^2 value for the average of three standard curves was 0.992 (Panel B) and the Jurkat positive control samples were 826 ± 54 , 223 ± 22 and 10 ± 1 . (Panel C).

PARP Pharmacodynamic Assay Validation

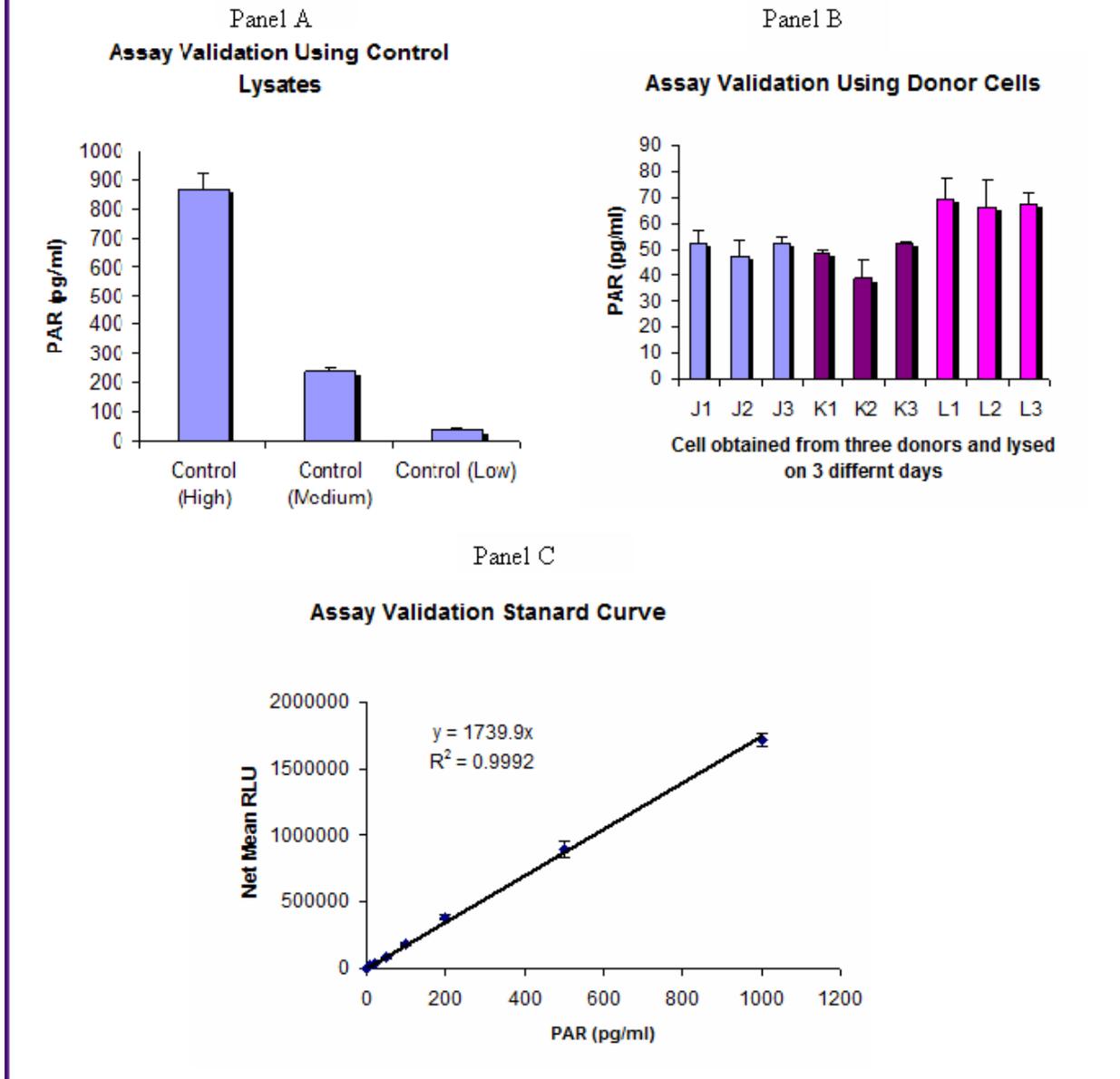


Figure 6
PARP Pharmacodynamic Assay II validation data.

To verify the specificity of the assay Jurkat cells were treated with the PARP inhibitor PJ34 for one hour prior to lysate preparation alternatively lysates were treated with poly-ADP-ribose glycohydrolase (PARG) to degrade PAR. Data in figure 7 reveal a suppression of PAR signal in treated cells compared to untreated Jurkat cell populations.

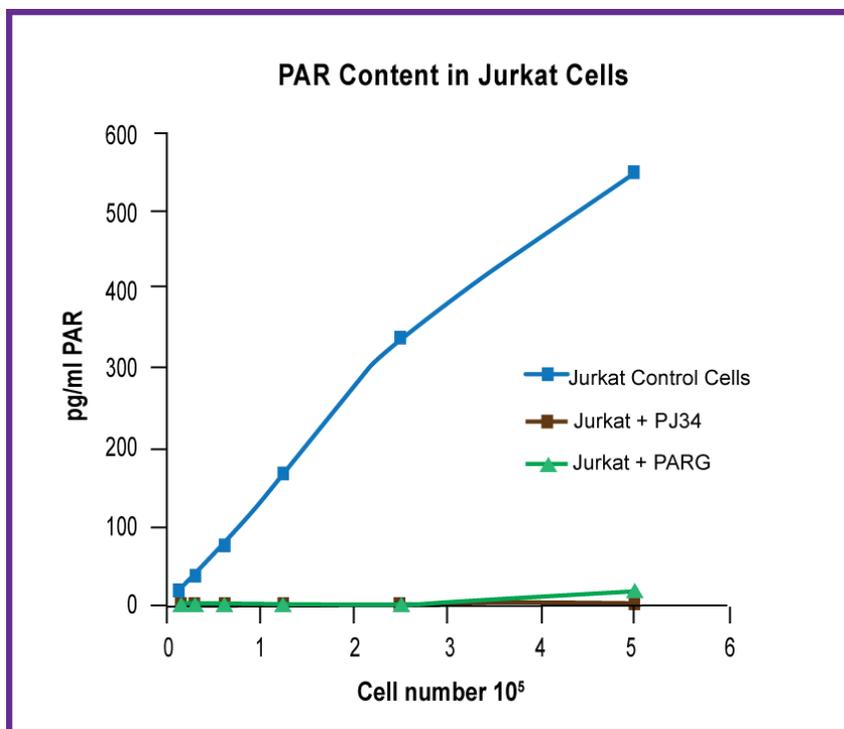


Figure 7:
Illustration of assay specificity for PAR

Accelerated shelf life studies on 96 well assay plates pre-coated with PAR capture antibody were performed according to Scheel^[20]. Plates were incubated for 9 days at 37°C and were used to detect levels of PAR standard and compared to levels of PAR detected on freshly coated plates. The values for each concentration of PAR determined on freshly coated plates was arbitrarily set to be 100% detection as depicted by the blue line in figure 8, Panel A. The pink line represents the percentage of PAR detected on the pre-coated plates compared to the freshly coated plates. In Panel B, PAR levels in Jurkat cell control lysates were determined on freshly antibody coated plates (0 days 37°C), and plates incubated for 5 and 9 days at 37°C. As can be seen in figure 8, the plates are stable after 9 days of storage at 37°C indicating a shelf life of at least one year from the time of manufacture. The other reagents in the kit when stored under the recommended conditions are also stable for minimally one year.

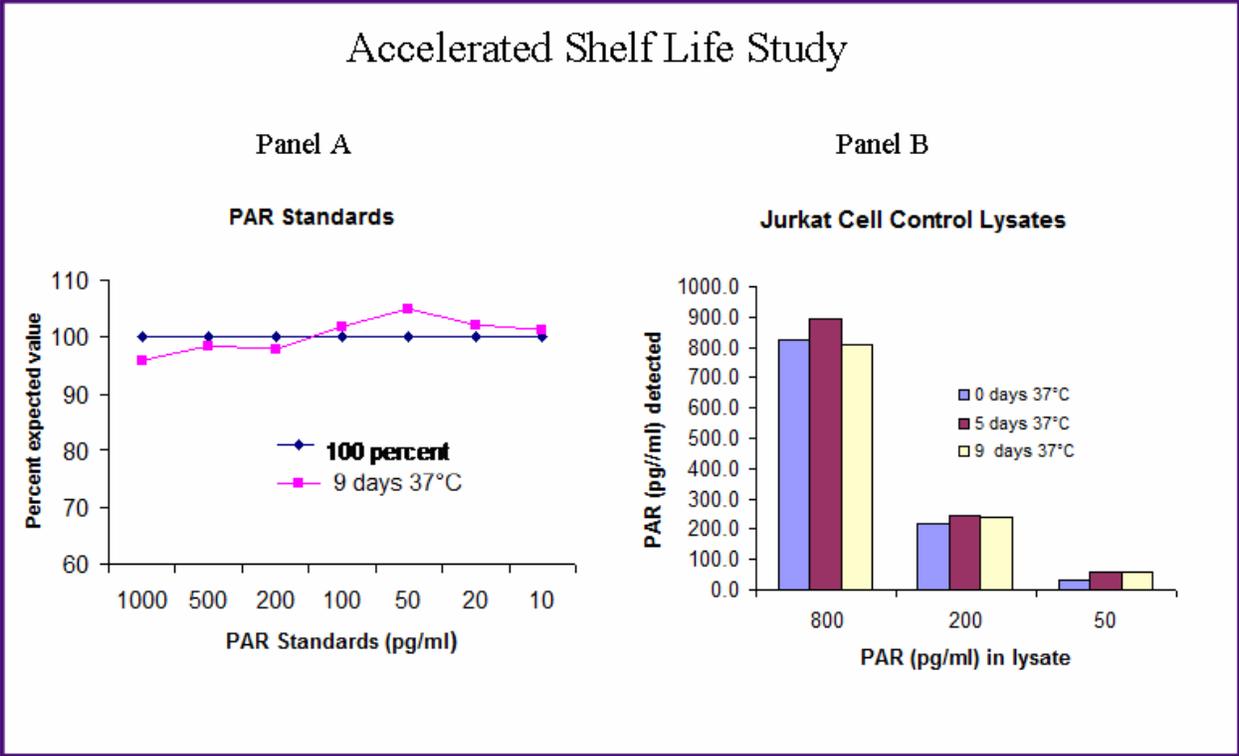


Figure 8:

PARP Pharmacodynamic Assay Version 2 Accelerated Shelf Life Study

In addition to its value as a tool to measure the effect of PARP inhibitors in both *in vivo* and *in vitro* studies the assay is a valuable research tool. In a recent study of head and neck squamous cell cancers, Kato et al ^[21] used the earlier version of this assay to demonstrate that the anticancer agent GMX1777, which targets the NAD⁺ salvage pathway, was highly synergistic when coupled to radiotherapy. Synergy was attributed to interference with DNA damage repair through the NAD⁺/PARP pathway.

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