ELISA

HT PARP *in vivo* Pharmacodynamic ELISA Kit II

Catalog Number 4520-096-K

High throughput chemiluminescent ELISA to quantify poly-ADP-ribose (PAR) in peripheral blood mononuclear cells, tissues, and cultured cells.

Note: The control ranges have changed. Read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

In response to DNA damage, poly-(ADP-ribose) polymerase-1 (PARP-1), which is the main isoform of the PARP family, is rapidly activated by DNA strand breaks that occur during exposure to environmental toxins, cancer therapy, inflammation, ischemia-reperfusion and neurodegeneration (1). Once activated, NAD^+ is consumed for the synthesis of the highly negatively-charged polymer poly-ADP-ribose (PAR), which is found on target nuclear proteins including PARP-1. 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. Therefore, PARP-1 is regarded as a promising target for the development of drugs useful in various regimens of cancer therapy, inflammation, ischemia and neurodegeneration (1-3). For example, the discovery that breast cancers deficient in homologous recombination are sensitive to nontoxic PARP inhibitors, has resulted in efforts by numerous pharmaceutical companies to develop PARP-1 specific drugs.

The HT PARP *in vivo* Pharmacodynamic ELISA Kit II, which measures net PAR levels in cellular extracts, provides the ability to monitor and quantify PARP activity across individuals and within cells. This assay employs a validated sample processing procedure and has been used to document differences in PAR levels among peripheral blood mononuclear cells (PBMCs), tumor lysates, organs, and xenografts (4).

PRINCIPLE OF THE ASSAY

In this assay, immobilized PAR monoclonal antibody in the wells of a 96-well plate captures cellular PAR and PAR attached to proteins. Incubation with a PAR Polyclonal Detecting Antibody, followed by addition of a Goat anti-Rabbit IgG HRP secondary antibody and a chemiluminescent HRP substrate yields relative light units (RLU) that directly correlate with the amount of cellular PAR. This assay is ideal for quantification of PAR in PBMCs, tissues, and cultured cells. Additional uses are for monitoring the efficacy of PARP inhibitors on cellular PAR formation and for verifying enhanced cancer cell cytotoxicity arising from PARP inhibitor/anti-cancer drug combination therapy (3). Important features of the assay include: 1) chemiluminescent, non-radioactive format; 2) high throughput 96 test format with pre-coated capture antibody; 3) broad linear dynamic range to 1000 pg/mL; and, 4) high signal to noise ratio with increased sensitivity at 2 pg/mL of PAR.

The HT PARP *in vivo* Pharmacodynamic ELISA Kit II employs a purified, pre-coated monoclonal PAR antibody as the capture agent, and anti-PAR polyclonal rabbit antibody as the detecting agent. Kit components were subjected to a published validation protocol including procedures facilitating the analysis of human tumor xenografts (4).

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECAUTION

The acute and chronic effects of overexposure to reagents in this kit are unknown. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Use within 3 months from date of receipt.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
PARP Microplate	4520-096-P	96-well polystyrene microplate (12 strips of 8 wells).	Store 2-8 °C.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.
20% SDS	4520-096-12	1 mL	Store at room temperature.	Store at room temperature.
PARP Sample Buffer	4520-096-02	20 mL	Store 2-8 °C.	Store 2-8 °C.
PARP Cell Lysis Reagent	4520-096-05	30 mL		
100X Magnesium Cation	4520-096-07	500 µL		
PARP Antibody Diluent	4520-096-11	15 mL		
PeroxyGlow™ A	4675-096-01	6 mL		
PeroxyGlow™ B	4675-096-02	6 mL		
PAR Polyclonal Detecting Antibody	4520-096-03	30 µL	Store at ≤ -20 °C.	Store at ≤ -20 °C.* Avoid repeated freeze-thaw cycles.
Goat anti-Rabbit IgG HRP	4520-096-04	30 µL		
DNase I, 2 Units/µL	4520-096-06	60 µL		
PAR Standard, 25 pg/µL	4520-096-01	5 vials (20 µL/vial)	Store at ≤ -70 °C.	Discard after use. Use a fresh standard for each assay.
WIL2-NS Cell Lysate Control, Low	4520-096-14	600 µL	Store at ≤ -70 °C.	Store at ≤ -70 °C.
WIL2-NS Cell Lysate Control, Medium	4520-096-15	600 µL		
WIL2-NS Cell Lysate Control, High	4520-096-16	600 µL		
Plate Sealers	N/A	4 Adhesive strips		

OTHER SUPPLIES REQUIRED

Reagents

- 1X PBS
- Tween® 20
- Distilled water
- 200 mM Phenylmethyl Sulfonyl Fluoride (PMSF) in ethanol
- Protease Inhibitor Cocktail (optional)
- Plasma Lyte A (optional)
- Trypsin for detaching adherent cells

Equipment

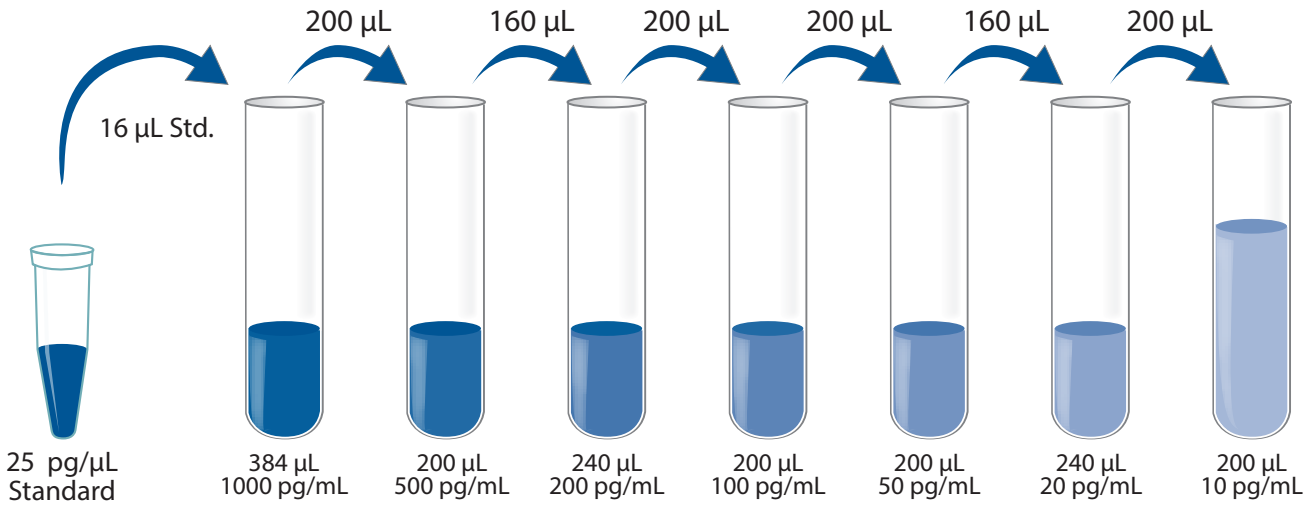
- Pipettes and pipette tips
- Multichannel pipettor 10-100 µL
- Squirt bottle, manifold dispenser, or automated microplate washer
- 96-well chemiluminescent plate reader or luminometer
- Refrigerated centrifuge with swinging bucket rotor
- Microcentrifuge
- 15 mL and 50 mL screw cap centrifuge tubes
- 0.5 mL and 1.5 mL microtubes
- 25 mL solution reservoirs
- Incubator set at 25 °C

REAGENT PREPARATION

PBS + 0.1% Tween 20 Wash Solution (PBST) - Prepare 500 mL of 1X PBST containing 1X PBS and 0.1% Tween 20 in a squirt bottle for washing strip wells.

PAR Standard - The kit contains 5 vials (20 µL/vial) of PAR Standard at a concentration of 25 pg/µL. Thaw each aliquot only once.

Pipette 384 µL of Sample Buffer into the 1000 pg/mL tube. Pipette 200 µL or 240 µL into the remaining tubes indicated below. Use the PAR Standard to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1000 pg/mL standard serves as the high standard. Sample Buffer serves as the zero standard (0 pg/mL).



PAR Polyclonal Detecting Antibody Solution - **One hour before addition**, dilute the PAR Polyclonal Detecting Antibody 250-fold with Antibody Diluent. A total of 50 µL/well of PAR Polyclonal Antibody Solution is required in the assay. For 96 wells, dilute 24 µL of PAR Polyclonal Detecting Antibody into 6 mL of Antibody Diluent.

Goat Anti-Rabbit IgG HRP - **One hour before addition**, dilute the Goat anti-Rabbit IgG-HRP 250-fold with Antibody Diluent. A total of 50 µL/well of diluted Goat anti-Rabbit IgG-HRP conjugate is required in the assay. For a 96-well plate, dilute 24 µL of Goat anti-Rabbit IgG-HRP into 6 mL of Antibody Diluent.

PeroxyGlow™ A and B Chemiluminescent Substrates - Allow PeroxyGlow™ A and B to come to room temperature before use. Immediately before addition, mix equal volumes of PeroxyGlow™ A and B together. A total of 100 µL is required per well. PeroxyGlow™ A and B are Horseradish Peroxidase (HRP) substrates generating light that can be quantified in a suitable chemiluminescent plate reader. For 96 wells, mix 6 mL of PeroxyGlow™ A with 6 mL of PeroxyGlow™ B.

REAGENT PREPARATION *CONTINUED*

Cell Lysis Buffer - Immediately before use, prepare 1 mL of Cell Lysis Buffer by mixing the following and placing on ice:

Part	Volume
Cell Lysis Reagent	985 µL
200 mM PMSF (in ethanol)	5 µL
100X Protease Inhibitor Cocktail*	10 µL

*Optional: Cell Lysis Reagent contains phosphatase inhibitors.

20% SDS - The 20% SDS may precipitate during shipping. To solubilize, warm the tube at 37 °C for 10 minutes and gently vortex periodically.

WIL2-NS Cell Lysate Controls - Three lysate controls, used to monitor assay drift between experiments, contain PAR levels at 533-939 pg/mL (High), 146-219 pg/mL (Medium) and 18-36 pg/mL (Low). These controls are ready for use and have sufficient volume for three experiments. Store each lysate at ≤ -70 °C in working 200 µL aliquots and keep on ice after thawing.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Suspension Cells

1. Grow 2-10 x 10^6 suspension cells in complete medium in a suitable tissue culture plate or flask.
2. Transfer the cells to pre-chilled 15 mL screw cap centrifuge tubes. Count the cells and then centrifuge at 250 x g for 5 minutes at 2-8 °C. Discard the supernatant. Wash the cells one more time with 10 mL of ice-cold 1X PBS.
3. Suspend the cell pellets in 1 mL of ice-cold 1X PBS. Transfer to 1.5 mL microcentrifuge tubes and centrifuge at 10,000 x g or top speed in a microcentrifuge for 10 seconds at 2-8 °C. Discard the supernatant.
4. Resuspend the cell pellet at a cell concentration of 1-5 x 10^7 cells/mL in Cell Lysis Buffer. Incubate the cell suspensions on ice, with periodic vortexing, for 15 minutes.
5. Add 20% SDS to a final concentration of 1%. For example, add 50 µL of 20% SDS to 950 µL of resuspended cells.
6. Incubate cell extracts at 100 °C for 5 minutes. Cool to room temperature.
7. Add 0.01 volume of 100X Magnesium Cation and 2 µL of DNase I (2 Units/µL). Vortex briefly and incubate at 37 °C for 90 minutes. This step degrades cellular DNA and reduces the viscosity of the extract.
8. Centrifuge at 10,000 x g for 10 minutes at room temperature to remove cellular debris. Remove the pellet with a toothpick or pipette tip. Save the supernatant.
9. Measure the protein concentration of the extracts with a BCA protein assay.
10. Assay immediately for PAR or aliquot the extracts and store at ≤ -70 °C.

SAMPLE COLLECTION & STORAGE *CONTINUED*

Adherent Cells

1. Grow 2-10 x 10^6 adherent cells in a suitable tissue culture 60 mm dish or 6 well plate in complete medium until 75% confluent.
2. Remove the medium and gently wash the cells with 5 mL of warm (37 °C) 1X PBS. Carefully pipette out the PBS. Repeat this step one more time.
3. Add 300 µL of cold Cell Lysis Buffer to each well of a 6 well plate, or 500 µL to a 60 mm dish. Place the dish or plate on ice and immediately scrape the cells with a cell scraper to detach the cells. Incubate the cell suspensions on ice, with periodic scraping, for 15 minutes.
4. Transfer the cell suspensions to 1.5 mL tubes. Add 20% SDS to a final SDS concentration of 1%. For example, add 50 µL of 20% SDS to 950 µL of resuspended cells.
5. Incubate cell extract at 100 °C for 5 minutes. Cool to room temperature.
6. Add 0.01 volume of 100X Magnesium Cation and 2 µL of DNase I (2 Units/µL). Vortex briefly and incubate at 37 °C for 90 minutes. This step degrades cellular DNA and reduces the viscosity of the extract.
7. Centrifuge at 10,000 x g for 10 minutes at room temperature. Remove the pellet with a toothpick or pipette tip.
8. Measure the protein concentration of the extracts with a BCA protein assay.
9. Assay immediately for PAR or aliquot the extracts and store at ≤ -70 °C.

Peripheral Blood Mononuclear Cells (PBMC)

Note: *It is important to make the PBMC lysates as quickly as possible after blood drawing to avoid cellular degradation of PAR.*

1. Withdraw 8 mL of blood into one BD Vacutainer CPT tube containing sodium heparin as the anticoagulant. Remix the blood sample by gently inverting the tube 8-10 times and then centrifuge at room temperature (18-25 °C) in a horizontal rotor (swinging bucket) and a proper adaptor for 25 minutes at 1500 x g.
2. After centrifugation, the PBMCs are in a whitish layer under the plasma layer. Carefully transfer the PBMCs into 50 mL centrifuge tube.
3. Add cold Plasma Lyte A to the PBMCs in the 50 mL centrifuge tube to bring the volume up to 45 mL. Cap and mix by inverting 5-8 times.
4. Centrifuge at 330 x g for 10 minutes at 2-8 °C. Discard the supernatant.
5. Resuspend the cell pellet in cold Plasma Lyte A and fill to the 10 mL mark.

SAMPLE COLLECTION & STORAGE *CONTINUED*

6. Count the PBMCs in a hemocytometer. Centrifuge at 330 x g for 10 minutes at 2-8 °C. Discard the supernatant.
7. Resuspend the cell pellet in 1 mL of cold Plasma Lyte A and transfer to a 1.5 mL microcentrifuge tube. Keep it on ice.
8. Centrifuge at 10,000 x g or top speed for 10 seconds at 4 °C. Discard the supernatant.
9. Resuspend the cells at a concentration of 2 x 10^7 cells/mL in cold Cell Lysis Buffer. Incubate the cell suspension on ice, with periodic vortexing, for 15 minutes.
10. Add 20% SDS to a final concentration of 1%. Vortex well. For example, add 50 µL of 20% SDS to 950 µL of resuspended cells.
11. Incubate cell extract at 100 °C for 5 minutes. Cool to room temperature.
12. Add 0.01 volume of 100X Magnesium Cation and 2 µL of DNase I (2 Units/µL). Vortex briefly and incubate at 37 °C for 90 minutes. This step degrades cellular DNA and reduces the viscosity of the extract.
13. Centrifuge at 10,000 x g for 10 minutes at room temperature. Remove the pellet with a toothpick or pipette tip.
14. Assay immediately for PAR or aliquot the extracts and store at ≤ -70 °C.

Biopsies of Tumor Xenografts

1. Anesthetize host and, when unresponsive to toe pinch, disinfect incision area.
2. Make a 2-5 mm incision adjacent to the tumor or cut a flap to expose it and insert a Temno 18 gauge biopsy needle (Cardinal Health, Catalog # T186, or equivalent) into the tumor until fully perforated. Collect biopsy, place in a cryovial and flash freeze in liquid nitrogen (this is important for stabilizing PAR levels).

   Notes: *1) Specimen sizes typically range from 5-20 mm in length, and 3-12 mg in mass. Refer to Kinders J.K. et al. (2008) Clin. Cancer Res.* ***14****:6877.*
3. Add 0.5 mL Cell Lysis Buffer to the frozen tissue and mince completely with fine-point scissors. Vortex to mix and place on ice.
4. Disrupt the extracts by sonication on ice three times for 10 seconds each cycle. Vortex and allow to stand on ice for 15 minutes.
5. Move samples to room temperature and add 20% SDS to a final concentration of 1%. For example, add 25 µL 20% SDS into 475 µL lysate.
6. Vortex again and incubate at 100 °C for 5 minutes. Cool on ice for 1 minute.
7. Centrifuge at 10,000 x g for 2 minutes at 2-8 °C. Collect the xenograft tumor lysate supernatant for each sample.
8. Measure the protein concentration of the extract with a BCA protein assay.
9. Assay immediately for PAR concentration or aliquot the extracts and store at ≤ -70 °C.

ASSAY PROTOCOL

Bring 1X Wash Buffer and PeroxyGlow™ A and B to room temperature before use. It is recommended that all samples, controls, and standards be assayed in triplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL/well of the serially diluted PAR standards, diluted test samples, WIL2-NS Cell Lysate Controls, and Sample Buffer (background control) to appropriate wells in triplicate.

   NOTES:

   i. The extracts must be diluted at least 3- to 5-fold with Sample Buffer to reduce the SDS concentration to below 0.33%.

   ii. Rather than pg PAR/mg protein, it is recommended to report PAR in terms of pg/mL in the PBMC extract or as pg PAR per 10^7 PBMC. The reliability of the protein content may be problematic because of adherence and carryover of plasma proteins to the surface of some PBMC.

   iii. PAR levels in suspension and adherent cell lines may be reported either as pg PAR per 10^7 cells or pg/mg protein extract.

   iv. Xenograft cell extracts are added in the range of 100-2000 ng/well using Sample Buffer. PAR levels in tumor xenograft cell extracts may be reported as pg PAR per 100 µg of protein extract.
4. In order to reach the maximal binding equilibrium, cover the wells with sealing film and incubate the strip wells overnight (16 ± 1 hour) at 2-8 °C.
5. Dilute the PAR Polyclonal Detecting Antibody 1:250 in Antibody Diluent and incubate at 25 °C one hour before use. Bring the incubated stripwells to room temperature.
6. Gently remove film sealer, aspirate each well and wash wells 4 times with PBST (300 µL/well). Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining PBST by aspirating or decanting. Invert the plate and blot it against clean paper towel.
7. Add 50 µL per well of diluted PAR Polyclonal Detecting Antibody. Cover the wells with sealing film and incubate at 25 °C for 2 hours.
8. Dilute the Goat anti-Rabbit IgG HRP 1:250 in Antibody Diluent and incubate at 25 °C one hour before use.
9. Repeat the aspiration and wash as in Step 6.
10. Add 50 µL per well of diluted Goat anti-Rabbit IgG HRP. Cover the wells with sealing film and incubate at 25 °C for 1.0 hour. Place PeroxyGlow™ A and B reagents at 25 °C to pre-warm.
11. Repeat the aspiration/wash as in step 6.
12. Just before use, mix equal volumes of PeroxyGlow™ A and B and add 100 µL per well. Immediately take chemiluminescent readings.

CALCULATION OF RESULTS

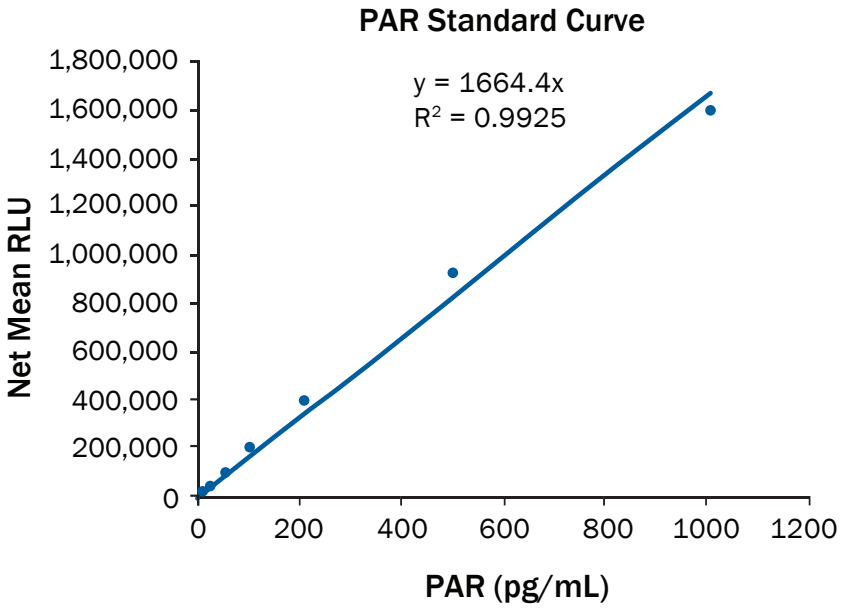
Average the triplicate readings and calculate the net mean RLU (Relative Light Units) values of the PAR standards by subtracting the background (without PAR) from the RLU values. Plot the net mean RLU as a function of PAR values (pg/mL).

Average the triplicate readings and calculate the net RLU values of the WIL2-NS Cell Lysate Controls, PBMC, or Xenograft extracts by subtracting the background from the RLU values. Determine the PAR levels in each sample using a standard curve. Use a linear regression line and set the y-intercept equal to zero.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

STANDARD CURVE

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



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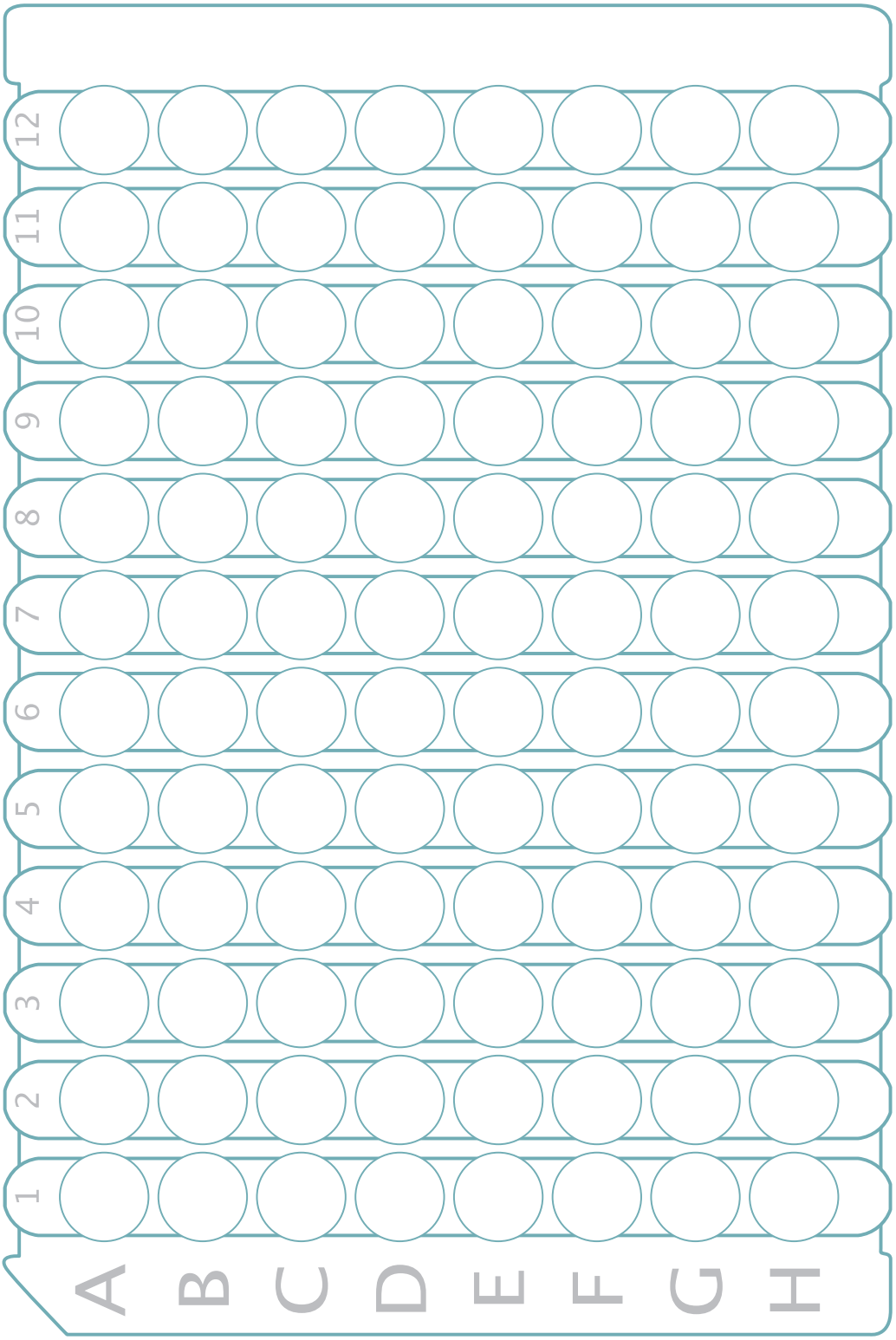
2. Thiemermann, C., J.*et al.* (1997 Proc. Natl. Acad. Sci. USA. **94**:679.
3. Curtin N.J. (2005) Exp. Rev. Mol. Med. **7**:1.
4. Kinders J.K. *et al.* (2008) Clin. Cancer Res. **14**:6877.

TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
No relative light units (RLU) in experimental sample wells but RLU are present in wells with the PAR standard.	PAR levels in the samples are below the sensitivity of the assay.	Increase the number of "cell equivalents" added to each well.
	PARG activity in the cell extract is very high.	Add ADP-HPD (1 µM), a potent PARG inhibitor, to the Cell Lysis Buffer.
		Check that specimen processing included addition of SDS and the boiling step; prepare a new specimen or add SDS and boil existing specimen.
No RLU in wells containing PAR standard.	PAR standards were not added to the wells.	Add serial dilutions of PAR standard to appropriate triplicate wells.
RLU in wells containing cell or tissue extracts too high or above that obtained for the PAR standard curve.	PAR levels in cells and tissues very high.	Extend serial dilutions of extract and check the linearity by back-calculating pg/mL PAR per cell number or per microgram of protein. Expect nonlinearity to appear at the highest dilutions, and choose a value such as the Mean for the linear range; or alternatively, use the first dilution value that plots on the standard curve.
High background in wells with no PAR.	Poor washing.	Increase the number of washes with PBST between steps.
	Failure to preabsorb rabbit anti-PAR with BSA in the Antibody Diluent.	Retest specimens following the preincubation steps in the protocol.
High variability within triplicates.	Uneven distribution of reagents.	Check quality of single and multichannel pipettors.
		Practice repetitive pipetting technique.
		If 2 of 3 replicates agree, the third may be tested for inclusion/exclusion by Dixon's Rule.
	Incomplete solubilization and clarification of the specimen.	Check specimen for viscosity (indication of large quantity of intact DNA) and particulates. Repeat the DNase I treatment and centrifugation steps.
Assay Controls out of range high or low.	Uneven distribution of reagents.	Repeat assay.
Excessive variability in assay controls (>20%).	Poor washing uneven distribution of reagents improper storage and handling failure to preincubate probe.	Repeat assay; if only one control is out of range, do not use specimen values that plot to the same segment of the standard curve; specimens plotting in the standard curve segment of valid controls may be used.
Lower limit of quantitation (LLQ) fails because low standard is within 2 SDEVs of the Mean Zero Read.	Multiple causes: Poor washing; Failure to preincubate Detecting Antibody; Expired standards; or, Expired conjugate.	Check package insert for expiration dates.
		Check performance of plate washer.
		Perform maintenance on plate washer.
		Check handling of PAR Detecting Antibody and conjugate for performance of the preincubation step.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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