

HT Chemiluminescent PARG Assay Kit

Chemiluminescent assay for screening of PARG inhibitors and quantification of PARG activity in cells and tissues. Sufficient reagents for 96 strip wells.

Cat# 4682-096-K

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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Cat# 4682-096-K

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I. Introduction

Poly(ADP-ribose) glycohydrolase (PARG) degrades poly(ADP-ribose) (PAR) polymers synthesized by poly(ADP-ribose) polymerase (PARP1). When activated by DNA strand breaks. PARP1 uses NAD as a substrate to form ADP-ribose polymers on itself and on specific acceptor proteins such as histones, DNA polymerases, DNA ligases, p53, and Fos. These polymers are in turn rapidly degraded by PARG, a ubiquitously expressed exo- and endoglycohydrolase. Excessive activation of PARP1 leads to NAD depletion and cell death during ischemia and other conditions that generate extensive DNA damage. PARG may maintain the active state of PARP1 by continuously removing inhibitory ADP-ribose residues from PARP1. The regulation of PARG activity may therefore, influence PARP-mediated cell death. First, PARG inhibition could slow the turnover of PAR and thereby limit NAD depletion. Second, PARG inhibition could prevent the removal of PAR from PARP1. Because PARP1 is inhibited by extensive poly-(ADP-ribosyl)ation, PARG inhibitors could thereby indirectly inhibit PARP1 activity. 2-10 Prior work has shown that the PARG inhibitor gallotannin can markedly reduce death of astrocytes after oxidative stress.11 Trevigen's HT Chemiluminescent PARG Assay Kit measures the loss of biotinylated PAR from histones attached to strip wells in a 96 well format and is ideal for the screening of PARG inhibitors and for measuring the activity of PARG in cell extracts. Important features of the assay include: (1) Chemiluminescent, non-radioactive format; (2) higher throughput 96 test size; and (3) sensitivity down to 50 pg of PARG per well. Trevigen offers histone-coated 96 white strip well plates (Cat# 4678-096-P) and other components of the PARG assay separately for your convenience.

II. Precautions and Limitations

- 1. For Research Use Only. Not for use in diagnostic procedures.
- 2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the Chemilluminescent PARG Assay Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

III. Wateriais	Oupplica		
Cat#	Component	Amount	Storage
4680-096-01	PARG Enzyme, 1 µg/ml	100 µl	-80°C
4680-096-02	10X PARG Buffer	8 ml	4°C
4680-096-03	DEA, 100 mM	200 µl	-80°C
4668-050-01	PARP-HSA, 10 unif	50 µl	-80°C
4671-096-02	20X PARP Buffer	500 µl	-80°C
4671-096-03	*10X PARP Cockta	300 µl	-80°C
4671-096-06	10X Activated DNA	300 µl	-80°C
4678-096-P	Histone-Coated Str 💟 s	96 wells	4°C
4680-096-04	Strep-HRP80	30 µl	4°C
4675-096-01	PeroxyGlow™ A 🐫	6 ml	4°C
4675-096-02	PeroxyGlow™ B	6 ml	4°C
***************************************	-41 NAD		

*Contains biotinylated NAD.

Note: Components stored at -80°C are defrost freezer.

ble at -20°C for one year in a manual

IV. Materials/Equipment Required But Not Supplied

Reagents:

- 1. Inhibitors or cells/tissue to be tested.
- 2. PBS + 0.1% Triton® X-100
- Distilled water
- 4. Phenylmethyl Sulfonyl Fluoride (PMSF) and other protease inhibitors
- 5. Triton® X-100 or Nonidet P-40 for extract preparation

Disposables:

- 1. 1-200 µl and 100-1000 µl pipette tips
- 2. Eppendorf tubes

Equipment:

- 1. Micropipettes
- 2. Multichannel pipettor 1–50 μl
- 3. Wash bottle or microstrip wells washer (optional)
- 4. 96-well strip wells reader for chemiluminescence

V. Reagent Preparation

1. PBS-Triton® X-100 Wash Solution

Prepare 500 ml of 1X PBS solution containing 0.1% (v/v) Triton® X-100 in a wash bottle for washing the strip wells.

2. Strep-HRP80

Just before use, dilute Strep-HRP80 (Cat# 4680-096-04) 250-fold with 1X PARG buffer. A total of 50 μ l/well of diluted Strep-HRP80 is required in the assay.

3. 1X PARP Buffer

Dilute 300 μ I of the 20X PARP buffer (Cat# 4671-096-02) to 1X (1:20) with 5.7 ml of dH₂O. The 1X PARP buffer is used to dilute the PARP enzyme and to prepare the 1X PARP cocktail.

4. 1X PARP Cocktail

Prepare the 1X PARP cocktail as follows:

10X PARP cocktail (Cat# 4671-096-03) 2.5 μL/well 10X activated DNA (Cat# 4671-096-06) 2.5 μL/well 1X PARP buffer 20 μL/well

5. PARP Enzyme

The kit contains 50 μ l of PARP-HSA enzyme at a concentration of 10 Units/ μ l. The enzyme should be diluted with 1X PARP buffer to 0.008 Units/ μ l just before use. For example, if you intend to ribosylate the whole plate at once, dilute 3.2 μ l of the PARP enzyme with 4 ml of 1X PARP buffer and use 25 μ l per well. **Note:** Diluted enzyme should be used immediately and any remainder discarded.

6. 1X PARG Buffer

Dilute 8 ml of the 10X PARG buffer (Cat# 4680-096-02) to 1X (1:10) with dH_2O . The 1X PARG buffer is used to dilute the enzyme, the Strep-HRP80, the inhibitors to be tested (if required), and to prepare cell extracts.

7. PARG Enzyme

Serially dilute the 1 μ g/ml PARG standard (Cat# 4680-096-01) in microtubes with 1X PARG buffer to make at least 200 μ l each dilution. The recommended final concentrations are 100 ng/ml, 30 ng/ml, 10 ng/ml, 3 ng/ml, 1 ng/ml, 300 pg/ml, and 100 pg/ml. The standard curve requires 50 μ l/well of each PARG dilution and each is performed in triplicate. *Note:* Diluted enzyme should be used immediately and any remainder discarded. The following table describes a serial dilution protocol for PARG:

Final PARG Concentra- tion	100 ng/ml	30 ng/ml	10 ng/ml	3 ng/ml	1 ng/ml	300 pg/ml	100 pg/ml
PARG 1000 ng/ml	60 µl	[™] 200 µl	³ 200 µl	[™] 200 µl	³ 200 µl	³⁴ 200 µl	200 µl
1X PARG Buffer	540 µl	467 µl	400 µl	467 µl	400 µl	467 µl	467 µl

8. PARG Inhibitors

DEA (6,9-diamino-2-ethoxyacridine lactate monohydrate) is provided at 100 mM in DMSO as a control PARG inhibitor. DEA will inhibit the activity of PARG at a wide range of concentrations from 1 μM to 1 mM. In Eppendorf tubes, serially dilute the stock DEA or your PARG inhibitor(s) with 1X PARG buffer to twice their final concentrations. Transfer 100 μI of each 2X concentrated inhibitor to a series of Eppendorf tubes containing 100 μI of 20 ng/ml PARG. Mix well, incubate for 15 min at room temperature, and add to designated wells. The following table describes a serial dilution protocol for DEA:

Final DEA Concentra- tion	2 mM	200 μM	100 μM	40 μM	20 μM	2 μM	200 nM
DEA 100 mM	10 µl	³⁴ 50 µl	[™] 250 µl	[™] 200 µl	[™] 250 µl	[№] 50 µl	³⁴ 50 µl
1X PARG Buffer	490 µl	450 µl	a de de	300 µl	250 µl	450 μl	450 µl

9. Cell Extraction Buffer

Prepare 10 ml of the following c 10X PARG buffer (Cat# 46 5 M NaCl 20% Triton X-100 or 20% N 200 mM PMSF (in ethanol)

action buffer and store at 4°C:

⊢02) 1 ml 800 μl 450 μl 20 μl 7.73 ml

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10. PeroxyGlow™ A and B Chemiluminescent Substrates Just before use mix equal volumes of PeroxyGlow™ A and B together. PeroxyGlow™ A and B are Horseradish Peroxidase (HRP) substrates generating light that can be quantified in a suitable plate reader for luminescence. . A total of 100 µl/well is required in the assay.

VI. PARG Inhibitor Screening Assay Protocol

Principal of the Assay:

Trevigen's 96-well PARG Assay Kit measures the loss of biotinylated PAR from histones attached to strip wells in a 96 well format. The histones attached to the plate wells are first poly(ADP) ribosylated by PARP using a biotinylated NAD substrate. The biotinylated PAR attached to the histones is subsequently hydrolyzed by the action of PARG in the standard or in the experimental samples. Any remaining biotinylated PAR is measured by incubation with Strep-HRP80 (which binds to the biotin) and a chemiluminescent substrate for HRP. The extent of hydrolysis is reflected by the loss in light output compared to that obtained in the absence of PARG. Inhibitors of PARG reduce the hydrolysis of the biotinylated PAR on the histones, thus minimizing the loss in light output compared to PARG alone.

When performing the PARG inhibitor protocol, the assay should be performed in triplicate and include the PARG standard curve. A general plate setup includes a negative PARP control, a negative PARG control, a PARG standard curve, serial dilutions of DEA PARG inhibitor (optional), and serial dilutions of inhibitor to be tested. A suggested plate setup is shown in Figure 3. The wells devoted to the cell extracts would be omitted in this case.

A. Ribosvlation Reaction

Note: Do not premix the PARP-HSA enzyme and the 1X PARP cocktail since PARP will autoribosylate in the presence of NAD.

- 1. During this step PARP-HSA ribosylates the histones on the histone-coated strip wells. Remove the histone-coated strip wells from the wrapper and select the number of strip wells that you need.
- 2. Add 25 µl of diluted PARP enzyme (0.008 Units/µl prepared in Section V.5) to all the wells except that for the PARP negative control (well H12, Fig.3). Substitute 25 µl of 1X PARP Buffer in this well.
- 3. Distribute 25 µl of 1X PARP cocktail (prepared as described in Section V.4) into each well using a multichannel pipettor.
- Volume Order of Addition 4. The final reaction volume is 50 μl: Diluted PARP-HSA enzyme (0.008 Unit/ul) 25 ul 1 2 1X PARP cocktail 25 µl
- 5. Incubate the strip wells at room temperature for 60 min.

6. Wash strip wells 4 times with 1X PBS + 0.1% Triton® X-100 (200 µl/well). Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.

B. Hydrolysis Reaction

- 1. Prepare negative control and standard curve samples:
 - a. PARG Standard Curve: Serially dilute the 1 µg/ml PARG standard in microtubes with 1X PARG buffer as described in Section V.7 above. Add 50 ul of each PARG dilution to triplicate wells (columns 1 to 3, rows B to H, Fig.
 - b. PARG Negative control: Include a negative control without PARG to provide the 100% reference light output (columns 1 to 3, row A, Fig. 3).
 - c. PARP Negative control: This control measures the nonspecific binding of Strepatavidin-HRP80 to the histones in the strip wells (well H12).

2. Prepare inhibitor samples:

- a. In 1.5 ml tubes, prepare twice-concentrated serial dilutions of the control DEA PARG inhibitor and/or your inhibitor in 1X PARG buffer as described in Section V.8 above. Include one tube containing 1X PARG assay buffer without inhibitor. This tube provides the 100% activity reference point.
- b. Transfer 100 ul of each 2X concentrated inhibitor to a series of Eppendorf tubes containing 100 µl of 20 ng/ml PARG. Mix well, incubate for 15 min at room temperature, and add to designated wells. Note: Always premix your inhibitor with PARG before adding to the strip wells.
- c. Each 1.5 ml tube will contain sufficient inhibitor and PARG for triplicate wells at 50 µl per well:

Component	<u>Volume</u>	Order of Addition
20 ng/ml PARG	100 µl	1
Diluted test inhibitor, DEA, or 1X PARG buffer	<u>100 μ</u> l	2
Total volume	200 µl	

Note: The final concentration of PARG will be 10 ng/ml and the inhibitors will be at their final 1X concentrations.

3. Add the PARG and PARP nega and inhibitor/PARG mixtures to

(see Fig. 3).

4. Incubate the strip wells at room

ontrols. PARG standard curve dilutions ate ribosylated strip wells at 50 µl/well

rature for 60 min.

C. Detection

- Wash strip wells 4 times with 1X PBS + 0.1% Triton® X-100 (200 µl/well). Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.
- 2. Add 50 µl per well of diluted Strep-HRP80 (prepared in Section V.2). Incubate at room temperature for 60 min.
- 3. Wash strip wells 4 times with 1X PBS + 0.1% Triton® X-100 (200 µl/well). Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.
- 4. Just before use mix equal volumes of PeroxyGlow™ A and B together and add 100 µl per well. Immediately take chemiluminescent readings.

VII. PARG Activity in Cell and Tissue Extracts

When performing the PARG activity protocol, the assay should be performed in triplicate. A general plate setup includes a negative PARP control, a negative PARG control, a standard curve, and serial dilutions of extract to be tested. A general plate setup is shown in Figure 3 for testing of four extracts. The wells devoted to the PARG inhibitors would be omitted in this case.

A. Processing Cells

Suspension cells: Centrifuge 2×10^6 to 1×10^7 suspension cells at $400 \times g$ for 10 min at 4°C. Discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge at $10,000 \times g$ for 10 sec at 4°C. Discard the supernatant and proceed to step VII.C.

Adherent cells: Wash 2×10^6 to 1×10^7 adherent cells with 1X PBS. Adherent cells may be harvested by scraping in 5 ml of ice-cold 1X PBS or by gentle trypsinization. Transfer to a prechilled 15 ml tube. Centrifuge at 400 x g for 10 min at 4°C and discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge at $10,000 \times g$ for $10 \times g$. Discard the supernatant and proceed to step VII.C.

B. Processing Tissue

- Remove tissue and place in cold PBS in a 50 ml conical tube. Repeatedly wash the tissue with cold PBS to remove blood clots and other debris.
- 2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
- Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 ml of cold 1X PBS.

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- 4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
- 5. Transfer the cell suspension to a 50 ml conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 min to allow large aggregates of tissue to settle out of solution.
- 6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 ml conical centrifuge tube. Determine the number of cells at this point. Centrifuge at 400 x g for 10 min at 4°C. Discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge at 10,000 x g for 12 sec at 4°C. Discard the supernatant and proceed to step VII.C.

C. Preparation of Extracts

- Suspend the cell pellet in 5–10 pellet volumes of cold cell extraction buffer from Step V.9. Incubate the cell suspensions on ice, with periodic vortexing, for 30 min.
- 2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 min at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. Occassionally, the pellet may float and can easily be removed with a pipet tip.
- Determine the protein concentration of the cleared cell lysate. Use at least 10 μg of protein per well in the assay. *Note:* 1X PARG buffer contains 0.1 mg/ml BSA.
- Snap-freeze the cleared cell extract in small aliquots and store at −80°C. Avoid repeated freezing and thawing of the extract.

D. Ribosylation Reaction

Note: Do not premix the cell extract and the 1X PARP cocktail since PARP will autoribosylate in the presence of NAD.

1. Perform the ribosylation reaction as described in Section VI A.

E. Hydrolysis Reaction

1. Each sample will be in 3 wells the designated 3 wells. *Note:* assay buffer may be required to 対対 株式会社

oute 50 µl of your sample into each of dilutions of your sample in 1X PARG ize PARG activity determination.

2. Controls:

- PARG Standard Curve: Serially dilute the 1 µg/ml PARG standard in microtubes with 1X PARG buffer such that the concentration is 100 ng/ml, 30 ng/ml, 10 ng/ml, 3 ng/ml, 1 ng/ml, 300 pg/ml, and 100 pg/ml. Add 50 µl of each standard to triplicate wells (columns 1 to 3, rows B to H, Fig. 3).
- PARG Negative control: Include a negative control without PARG to provide the 100% reference light output (columns 1 to 3, row A, Fig. 3).
- iii. PARP Negative control: This control measures the nonspecific binding of Strepatavidin-HRP80 to the histones in the strip wells (well H12, Fig.
- Incubate the strip wells at room temperature for 60 min.

Detection

Follow Section VI. C in the PARG Inhibitor Screening Assay Protocol.

VIII. Data Interpretation

Typical PARG activity curves and inhibition curves for the PARG inhibitor DEA are graphically represented in Figures 1 and 2, respectively. The activity of PARG is expressed as the percent cleavage of PAR and is calculated as follows:

Let A = Light output in the absence of PARG B = Light output in the presence of PARG

% Cleavage of PAR =
$$(A - B) \times 100$$

Plot the percent cleavage of PAR versus log [PARG, pg/ml]. Determine the PARG activity in your cell extract from the standard curve. Express the results as pg of PARG/mg protein.

Chemiluminescent PARG Activity Curve

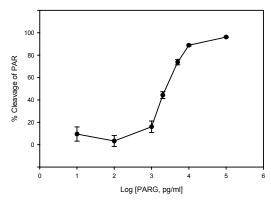


Figure 1. Graphical representation of PARG Activity curve

The inhibition of PARG caused by your inhibitor will be reflected as an increase in the observed light output readings relative to that caused by 10 ng/ml PARG in the absence of inhibitor.

Let C = Light output in the presence of PARG inhibitor

D = Light output in the absence of PARG inhibitor

% Inhibition of PARG =
$$(C - D) \times 100$$

IC50 = the concentration of inhibitor that reduces the activity of PARG by 50%

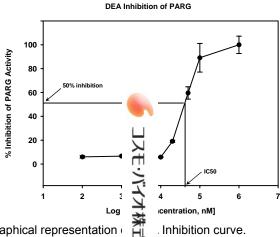


Figure 2. Graphical representation

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4 5 6 7 8 9 10 11 12	1 mM DEA 1 mM DEA 1 mM DEA CellTissue CellTissue <th> 100 µM 100 µM 100 µM CellTissue Ce</th> <th>50 µM DEA 50 µM DEA 50 µM DEA CellTissue CellTissue</th> <th>20 µM DEA 20 µM DEA 20 µM DEA CellTissue CellTissue</th> <th>10 µM DEA 10 µM DEA 10 µM DEA CellTissue CellTissue</th> <th> 1 MA DEA</th> <th>100 pM 100 pM CellTissue CellTissue</th> <th> No DEA No DEA No DEA No DEA Cell/Tissue Cell/Tissue Cell/Tissue Cell/Tissue No PARP + + + + + + + Extract #2 Extract #2 Extract #4 Extract #4 Extract #4 Extract #4 Extract #4 10 ng/ml</th>	100 µM 100 µM 100 µM CellTissue Ce	50 µM DEA 50 µM DEA 50 µM DEA CellTissue CellTissue	20 µM DEA 20 µM DEA 20 µM DEA CellTissue CellTissue	10 µM DEA 10 µM DEA 10 µM DEA CellTissue CellTissue	1 MA DEA	100 pM 100 pM CellTissue CellTissue	No DEA No DEA No DEA No DEA Cell/Tissue Cell/Tissue Cell/Tissue Cell/Tissue No PARP + + + + + + + Extract #2 Extract #2 Extract #4 Extract #4 Extract #4 Extract #4 Extract #4 10 ng/ml
8	No PARG 1n	PARG 1	900 pg/ml 10	PARG 201	PARG 101	PARG 11, 10 ng/ml 10	PARG 1	PARG N 100 ng/ml
2	No PARG	PARG 100 pg/ml	PARG 300 pg/ml	PARG 1 ng/ml	PARG 3 ng/ml	PARG 10 ng/ml	PARG 30 ng/ml	PARG 100 ng/ml
1	No PARG	PARG 100 pg/ml	PARG 300 pg/ml	PARG 1 ng/ml	PARG 3 ng/ml	PARG 10 ng/ml	PARG 30 ng/ml	PARG 100 ng/ml
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Figure 3. Suggested layout for a PARG standard curve, PARG inhibitor serial dilutions, and for assay of PARG in cell extracts. Note that the histones on the plate must first be poly(ADP) ribosylated following Section VI.A.

IX. References

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X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
High light output in wells with PARG Inhibitor at all inhibitor dilutions.	PARG inhibitor is extremely potent.	Increase the serial dilutions of your inhibitor.
High light output in wells with PARG alone in PARG inhibitor studies.	PARG enzyme was not added to the wells.	Add 100 µL of 20 ng/ml PARG to each 1.5 ml tube as described in Section VI.B.2.
Low light output in wells containing cell extracts.	PARG expression in cells too high.	Increase the serial dilutions of your cell extracts in 1X PARG Assay Buffer.
High light output in PARG standard curve in wells with high concentrations of PARG	High background due to poor washing	Increase the number of washing steps.

XI. Related Products Available From Trevigen

Catalog #	Description	Size
4335-MC-100	Anti-poly(ADP-ribose), monoclonal	100 µg
4336-PC-100	Anti-poly(ADP-ribose), polyclonal	100 µl
4338-MC-50	Anti-poly(ADP-ribose) polymerase (PARP), monoclonal	50 μg
4500-050-P	Poly(ADP-ribosylated) protein control for WB	50 µl
4667-50-03	3-Aminobenzamide	60 µl
4667-50-06	Activated DNA for PARP Assay	500 µl
4667-50-09	4-Amino-1,8-Naphthalimide	100 µl
4667-50-10	6(5H)-Phenanthridinone	100 µl
4667-50-11	Benzamide	100 µl
4668-100-01	Recombinant Human PARP Enzyme (HSA)	1,000 U
4668-2K-01	Recombinant Human PARP Enzyme (HSA)	20,000 U
4668-500-01	Recombinant Human PARP Enzyme (HSA)	5,000 U
4670-500-01	Biotinylated-NAD 250 μM	500 µl
4671-096-K	Universal Colorimetric PARP Assay Kit with Histone Reagent	96 samples
4675-096-K	Universal Chemiluminescent PARP Assay Kit with Histone Reagent	96 samples
4676-096-K	Universal Chemiluminescent PARP Assay Kit with Histone Coated Strip Wells	96 samples
4677-096-K	Universal Colorimetric PARP Assay Kit with Histone Coated Strip Wells	96 samples
4678-096-P	White 96 Well Histone-coated plate (strip wells)	Each
4870-500-6	10X PBS, pH 7.4	6 x 500 ml
4683-096-K	HT Colorimetric PARG Assay Kit	96 samples

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