TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Universal Chemiluminescent PARP Assay Kit With Histones and Coating Buffer

96 samples

Cat# 4675-096-K

Chemiluminescent assay kit for screening of PARP inhibitors, and quantitation of PARP activity in cells, and cell and tissue extracts.

HT Universal Chemiluminescent PARP Assay Kit 96 samples

Cat# 4675-096-K: Kit with Histone Reagent

Table of Contents

I. Introduction

Poly ADP-ribosylation of nuclear proteins is a post-translational event that occurs in response to DNA damage. Poly (ADP-ribose) polymerase (PARP) catalyzes the NAD-dependent addition of poly (ADP-ribose) to itself and adjacent nuclear proteins such as histones. PARP contributes to the sequence of events that occurs during DNA base excision repair.[1] Whereas PARP-mediated induction of necrosis can occur by extensive depletion of the intracellular NAD pool,[2] the cleavage of PARP-1 promotes apoptosis by preventing DNA repair-induced survival and by blocking energy depletion-induced necrosis.[3] Experimental models have shown that PARP inhibition prevents tissue damage in animal models of myocardial and neuronal ischemia, diabetes, septic shock, and vascular stroke.[4-11] Moreover, PARP inhibition promotes chemosensitization and radiosensitization of tumors.[12]

Trevigen's Universal 96-well PARP Assay Kit measures the incorporation of bio-tinylated poly(ADP-ribose) onto histone proteins, or protein of interest, in a 96 microtiter well format. This assay is ideal for the screening of PARP inhibitors and for measuring the activity of PARP 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 10 mU of PARP per well. Trevigen offers two formats of the kit: Cat# 4675-096-K (Chemiluminescent Kit with histone reagent) and Cat# 4676-096-K (Chemiluminescent Kit with histone-coated strip wells). Histone-coated 96-well white strip wells (Cat# 4678-096-P) are available 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 HT Universal Chemiluminescent PARP Assay Kits 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

Catalog #	Component	Amount	Storage
4668-050-01	PARP-HSA, 10 U/µl	50 µl	-80 °C
4671-096-02	*20X PARP Buffer	500 µl	-80 °C
4671-096-03	*10X PARP Cocktail**	300 µl	-80 °C
4667-50-03	*200 mM 3-Aminobenzamide	60 µl	-80 °C
4667-50-07	*Histone Solution	700 µl	-80 °C
4671-096-03	10X Strep-Diluent	2 ml	4 °C
4671-096-05	5X Coating Buffer	1.4 ml	4 °C
4800-30-06	Strep-HRP	30 µl	4 °C
4675-096-01	PeroxyGlow™ A	6 ml	4 °C
4675-096-02	PeroxyGlow™ B	6 ml	4 °C
4671-096-06	*10X Activated DNA	300 µl	-20 °C
4675-096-03	White Strip Well Plate	each	Room Temp.

*Components marked with an asterisk can be stored at -20 °C for one year in a manual defrost freezer.

**Contains biotinylated NAD.

IV. Materials/Equipment Required But Not Supplied

Reagents:

1. PARP inhibitors, or cells/tissue to be tested
2. 1X PBS + 0.1% Triton X-100
3. Distilled water
4. 0.2M HCl or 5% Phosphoric acid
5. Phenylmethyl Sulfonyl Fluoride (PMSF) or other protease inhibitors
6. Triton X-100 or Nonidet P-40, and 1M NaCl for extract preparation
7. Purified test protein of interest.

Disposables:

1. 1 - 200 µl and 100-1000 µl pipette tips

Equipment:

1. Micropipettes
2. Multichannel pipettor 10 - 200 µl
3. Wash bottle or microstrip wells washer (optional)
4. 96-well plate luminometer/chemiluminescent plate reader

V. Reagent Preparation

1. Histones

Dilute the Histone solution (Cat# 4667-50-07) for coating your 96-well plate as follows:

Histone Solution (Cat# 4667-50-07)	5 µl/well
5X Coating Buffer (Cat# 4671-096-05)	10 µl/well
dH_2O	35 µl/well

2. 10X Strep-Diluent

This solution is used as a diluent for the Strep-HRP. Dilute **1:10** in **1X** PBS + 0.1% Triton X-100 before use.

3. 20X PARP Buffer

Dilute the 20X PARP Buffer to **1X (1:20)** with dH_2O. The **1X** PARP Buffer is used to dilute the enzyme, PARP Cocktail, the inhibitors to be tested (if required) and to prepare cell extracts.

4. 10X PARP Cocktail

Dilute the 10X 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 µl of PARP-HSA enzyme at a concentration described in the enclosed Product Data Sheet. The enzyme should be diluted appropriately with **1X** PARP Buffer just before use. **Note: Diluted enzyme should be used immediately and any remainder discarded.**

6. PARP Inhibitors
The 3-aminobenzamide (3-AB) is provided at 200 mM in ethanol as a control inhibitor. 3-AB will inhibit the activity of PARP at a wide range of concentrations from 2 µM to 10 mM. Serially dilute the stock 3-AB or your PARP inhibitor(s) with **1X** PARP Buffer and add to designated wells.

7. Strep-HRP
Just before use, dilute Strep-HRP (Cat# 4800-30-06) 500-fold with **1X** Strep-Diluent (Cat# 4671-096-04). A total of 50 µl/well of diluted Strep-HRP is required in the assay.

8. 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 chemiluminescent plate reader.

VI. PARP Inhibitor Assay Protocol

A. Plate Coating

1. If working with the provided histones, aliquot 50 µl of diluted histones per well (prepared in Section **V. 1**) of the white protein-binding plate (4675-096-03). Otherwise, coat positive control wells with the provided histones. Cover plate with lid, adhesive plate cover, or parafilm and incubate overnight at 4ºC.

2. If working with another protein, as a rule of thumb, select a binding buffer with an effective buffering capacity (pH) at or near the isoelectric point of your test protein. Start with a 3 µM solution of your protein for coating and use 50 µl per well. Coat positive control wells with the provided histones. Cover plate with a lid, adhesive plate cover, or parafilm and incubate overnight at 4ºC.

B. Plate Blocking

1. Wash plate 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 plate onto paper towels.

2. Block the wells by adding 100 µl of **1X** Strep-Diluent to every well.

3. Incubate at room temperature for 1 hour or overnight at 4°C, covered.

4. Wash plate 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 plate onto paper towels.

C. Ribosylation Reaction

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

1. Add serial dilutions of inhibitor of interest (prepared in section **V.6**) to appropriate wells.

2. Add diluted PARP enzyme (0.5 Unit/well prepared in Section **V.5**) to the wells containing inhibitor. Incubate for 10 minutes at room temperature.

3. Controls:
i. Activity control: 0.5 unit/well PARP-HSA without inhibitors. These wells provide the 100% activity reference point. The use of histone-coated wells should be included as positive controls if other test proteins are used.

ii. Negative control: A negative control without PARP should be prepared to determine background absorbance.

4. Distribute 25 µl of **1X** PARP Cocktail into each well using a multichannel pipettor.

5. The final reaction volume is 50 µl:

	Volume	Order of Addition
Diluted test inhibitor	X µl	1
Diluted PARP-HSA enzyme (0.5 Unit)	Y µl	2
1X PARP cocktail	25 µl	3
Total volume	50 µl	

Where X + Y = 25 µl

Note: If X = 10 µl, make the concentration of your inhibitor 5-fold that of the final inhibitor concentration in the reaction since the reaction volume is 50 µl. In this example, Y = 15 µl. Therefore, dilute the PARP-HSA enzyme to 0.5 units/15 µl in **1X** PARP Buffer.

6. Incubate the strip wells at room temperature for 30-60 minutes.

D. Detection

1. 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-HRP (prepared in section **V.7**). Incubate at room temperature for 30 minutes.

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. PARP Activity in Cell and Tissue Extracts

A. Processing Cells

1. **Non-adherent cells**: Centrifuge 2 x 10^6 to 1 x 10^7 non-adherent cells at 400 x g for 10 minutes 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 10 sec at 4°C. Discard the supernatant.

Adherent cells: Wash the 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 minutes 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 x g for 10 sec at 4°C. Discard the supernatant.

B. Processing Tissue

1. Remove tissue and place in cold PBS in a 50 ml conical tube. Repeatedly wash the tissue with 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.

3. 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.

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 PBS containing the single 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 minute 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. Centrifuge at 400 x g for 10 minutes 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.

C. Preparation of Extracts

1. Suspend the cell pellet obtained above in 5-10 pellet volumes of cold **1X** PARP Buffer containing 0.4 mM PMSF, other protease inhibitors, 0.4 M NaCl, and 1% Triton X-100 or 1% NP-40 nonionic detergent. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.

2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. Occasionally, the pellet may float and can easily be removed with a pipette tip.

3. Determine the protein concentration of the cleared cell lysate. Use at least 20 µg of protein per well in the assay.

4. Snap-freeze the cleared cell extract in small aliquots and store at -80°C. Avoid repeated freezing and thawing of the extract.

D. Plate Coating and Plate Blocking

1. Follow steps A and B in Section **VI** (PARP Inhibitor Assay Protocol).

E. Ribosylation Reaction

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

1. Each sample will be in 3 wells. Add X µl of **1X** PARP Buffer and Y µl of your sample (containing at least 20 µg of protein) into each of the designated 3 wells such that X + Y = 25 µl.

2. Controls:
   i. PARP Standard Curve: Serially dilute the PARP-HSA standard in cold microtubes with **1X** PARP Buffer such that the total activity is 1 Unit/25 µl, 0.5 Units/25 µl, 0.1 Units/25 µl, 0.05 Units/25 µl, and 0.01 Units/25 µl, 5 mUnits/25 µl, and 0.001 Units/25 µl. Add 25 µl of each standard to triplicate wells.

   ii. Negative control: A negative control without PARP or cell extract should be included to provide the background absorbance that is subtracted from the experimental samples in the analysis of the data.

3. Distribute 25 µl of **1X** PARP Cocktail into each well using a multichannel pipettor.

4. The final reaction volume in each well is 50 µl:

	Volume	Order of Addition
1X PARP Buffer	X µl	1
Cell Extract or PARP Standard	Y µl	2
1X PARP cocktail	25 µl	3
Total volume	50 µl	

   Where X + Y = 25 µl.
   Notes: Y = 25 µl for the PARP Standards and X = 0 µl
   X = 25 µl for the background wells.

5. Incubate the strip wells at room temperature for 60 minutes. The incubation time can be extended if required.

F. Detection

1. Follow **Section VI D** in the PARP Inhibitor Assay Protocol.

VIII. Data Interpretation.

Typical chemiluminescent PARP standard curves and inhibition curves for the PARP inhibitors 3-aminobenzamide (provided in the kit), benzamide and 4-amino-1,8-naphthalimide (available from Trevigen) are graphically represented in Figure 1. Determine the PARP activity in your cell extract from the standard curve. Express the results as Units of PARP/mg protein.

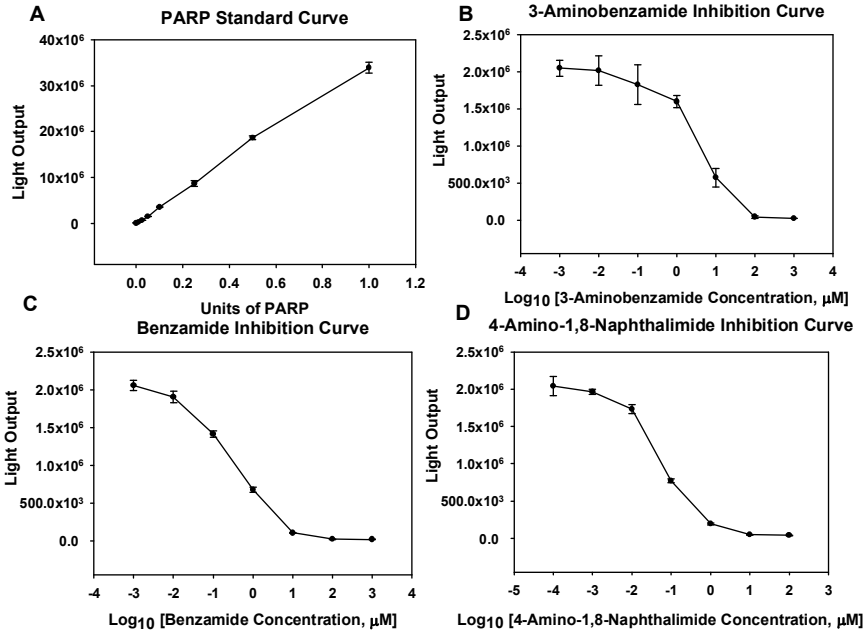

Figure 1. Graphical representation of the chemiluminescent readout of the PARP standard curve (Panel **A**) and inhibition curves for 3-aminobenzamide (Panel **B**), benzamide (Panel **C**), and 4-amino-1,4-naphthalimide (Panel **D**). Each point represents the median value from triplicates.

IX. References

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X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No light output in wells with Inhibitor but present in wells with PARP alone	PARP inhibitor is extremely potent	Increase the serial dilutions of your inhibitor
No light output in wells with PARP alone	PARP enzyme was not added to the wells	Add 0.5 Unit of PARP to each well
	Insufficient coating of histones or test protein	Coat overnight with histones as the positive control or use pre-coated strip wells (Cat#4676-096-P)
No light output in wells containing cell or tissue extracts	PARP expression in cells and tissues very low	Extend development time with Cell extracts to 1.5 hours.
		Add 1M NaCl to a final concentration of 0.4M in the cell extraction buffer
		Increase the volume and/or concentration of cell extract added to each well
No light output in wells containing cell or tissue extracts	PARG activity in the extracts very high	Add ADP-HPD (Calbiochem, Cat.# 118415), a specific inhibitor of PARG, to a final concentration of 100 nM
High background in wells with no PARP	Insufficient blocking with Strep-Diluent	Increase blicking time to overnight at 4 °C.
		Increase volume of 1X Strep-Diluent to 200 µl /well
	Poor washing	Increase the number of washes with **1X** PBS + 0.1% Triton X-100 after the ribosylation reaction and incubation with Strep-HRP.

XI. Related Products Available From Trevigen

Catalog #	Description	Size
4671-096-K	**HT Universal Color PARP Assay Kit/w Histone Reagents**	**96 samples**
4676-096-K	**HT Universal Chemiluminescent PARP Assay Kit/w Histone Coated Strip Wells**	**96 samples**

Catalog #	Description	Size
4677-096-K	Universal Colorimetric PARP Assay Kit/w Histone Coated Strip Wells	96 samples
4684-096-K	HT Colorimetric PARP Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP Apoptosis Assay	96 tests
4667-250-01	Recombinant Human PARP Enzyme	250 µl
4668-100-1	Recombinant Human PARP (High Specific Activity)	1000 Units
4682-096-K	HT Chemiluminescent PARG Assay Kit	96 tests
4683-096-K	HT Colorimetric PARG Assay Kit	96 tests
4667-50-11	Benzamide PARP inhibitor (8 mM)	100 µl
4667-50-10	6(5H)-Phenanthridinone PARP inhibitor (160 µM)	100 µl
4667-50-9	4-Amino-1,8-naphthalimide PARP inhibitor (800 µM)	100 µl

Accessories

Catalog #	Description	Size
4870-500-6	10X PBS, pH = 7.4	6 x 500 ml
4869-500-6	Apoptosis Grade™ H_2O	6 x 500 ml
4670-500-1	Biotinylated-NAD 250 µM	500 µl
4668-100-01	Recombinant Human PARP (HSA)	100 U
4667-50-06	Activated DNA	500 µl
4677-096-P	Histone-coated strip wells	96

XII. Appendix

Reagent composition:

1. **1X PBS (pH 7.4):** 7.5 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 145 mM NaCl.
2. **Histone Solution:** 1 mg/ml Histones.
3. **10X Strep Diluent:** Biotin-reduced proprietary blocking solution.
4. **20X PARP Buffer:** Proprietary buffer solution.
5. **10X PARP Cocktail:** Proprietary solution containing biotinylated NAD.
6. **PARP-HSA Enzyme:** PARP-HSA is provided at a concentration described in the enclosed Product Data Sheet.
7. **3-Aminobenzamide:** 200 mM 3-aminobenzamide in Ethanol.
8. **PeroxyGlow™ A and PeroxyGlow™ B:** Chemiluminescent peroxidase substrates which, when mixed together, emit light in the presence of HRP.
9. **10X Activated DNA:** Activated Herring Sperm DNA in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA.
10. **Strep-HRP:** Provided at 500X Concentration

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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