TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

Universal Chemiluminescent PARP Assay Kit With Histones and Coating Buffer

Chemiluminescent assay kit for screening of PARP inhibitors, and quantitation of PARP activity in cells. Sufficient reagents for one 96-well plate.

Cat# 4675-096-K

Universal Chemiluminescent PARP Assay Kit 96 Tests

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

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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) is activated when it becomes attached to regions of damaged DNA. It then catalyzes the NAD-dependent addition of poly(ADP-ribose) to itself and to adjacent nuclear proteins such as histones. PARP plays an important role in DNA repair[1] but can also lead to cell death by depleting the cellular NAD pool[2]. Experimental models have shown that PARP inhibition prevents tissue damage in animal models of myocardial and neuronal ischemia, diabetes, septic shock, and vascular stroke[3-10]. Recent data implicate a synergistic function of Ku80 and PARP-1 in minimizing chromosome aberrations and cancer development[8]. Trevigen's Universal 96-well PARP Assay Kit measures the incorporation of biotinylated Poly(ADP-ribose) onto histone proteins in a 96-well plate 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 0.001 unit of PARP per well. Trevigen offers two kits: 4675-096-K (Kit with histone reagent) and 4676-096-K (Kit with histone-coated strip wells). Histone-coated 96-well white strip wells (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 Universal Chemiluminescent PARP 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

Catalog Number	Component	Amount Provided	Storage Temperature
4668-50-01	PARP-HSA	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	*3-Aminobenzamide	60 µl	-80˚C
4667-50-07	*Histone Solution	700 µl	-80˚C
4671-096-04	10X Strep-Diluent	2 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
4671-096-05	5X Coating Buffer	1.4 ml	4˚C
4671-096-06	*10X Activated DNA	300 µl	-80˚C

*Components marked with an asterisk can be stored at -20˚C for one year.

**Contains biotinylated NAD.

IV. Materials/Equipment Required But Not Supplied

Reagents:

1. Inhibitors or cells/tissue to be tested.
2. 1X PBS + 0.1% Triton X-100
3. Distilled water
4. 0.2 M HCl or 5% Phosphoric acid
5. Phenylmethyl Sulfonyl Fluoride (PMSF) and other protease inhibitors
6. Triton X-100 or NP-40 and 1M NaCl for cell extract preparation.

Disposables:

1. 1 - 200 µl and 100-1000 µl pipette tips
2. 96 well protein-binding (via hydrophobic interactions) white plate with lid

Equipment:

1. Micropipettes
2. Multichannel pipettor 1 - 50 µl
3. Wash bottle or microplate 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 blocking reagent as well as a diluent for the Strep-HRP. Dilute **1:10** with 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 a ws:

10X PARP Cocktail (Cat# 4 96-03)	2.5 µl/well
10X Activated DNA (Cat# 4 96-06)	2.5 µl/well
1X PARP Buffer	20 µl/well

5. PARP Enzyme

The kit contains 50 µl of PARP- nzyme at a concentration described in the enclosed Product Data t. The enzyme should be diluted appropriately with **1X** PARP Bu st before use. **Note: Diluted enzyme should be used immediately a y remainder discarded.**

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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) 1000-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 Screening Assay Protocol

A. Plate Coating

1. Aliquot 50 µl of diluted histones into each well (prepared in Section **V.1**) of a white plate. 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 these wells containing inhibitor.

3. Controls:

i. Activity control: 0.5 unit/well PARP-HSA without inhibitors. These wells provide the 100% activity reference point.

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. Note: the final reaction volume is 50 µl:

	Volume	Order of Addition
Diluted test inhibitor	X µl	1
Diluted PARP-HSA enzyme (0.1 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 final reaction volume is 50 µl. In this example, Y = 15 µl. Therefore, dilute the PARP-HSA enzyme to 0.5 units/15 µl.

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

D. Detection

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. Add 50 µl per well of diluted Strep-HRP (prepared in section **V.7**). Incubate at room temperature for 30 minutes.

3. 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.

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 an sue Extracts

A. Processing Cells

1. Non-adherent cells: Centrifug 0^6 to 1 x 10^7 non-adherent cells at 400 x g for 10 minutes at 4°C. Disc e supernatant. Suspend the cell pellet in 1 ml of ice-cold **1X** PBS ar sfer to a prechilled 1.5 ml microtube. Centrifuge at 10,000 x g for 10 4°C. Discard the supernatant.

Adherent cells: Wash the adh cells with **1X** PBS. Adherent cells may be harvested by scraping in of ice-cold **1X** PBS or by gentle trypsinization. Transfer to a pr 15 ml tube. Centrifuge at 400 x g for 10 minutes at 4°C and discard pernatant. Suspend the cell pellet in 1

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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 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 isolated 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 pipet 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 Screening 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, 0.005 Units/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 plate at room temperature for 30-60 minutes. The incubation time can be extended if required.

F. Detection

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

VIII. Data Interpretation

Typical chemiluminescent PARP s[illegible]d curves and inhibition curves for the PARP inhibitors 3-aminobenzamid[illegible]vided in the kit), benzamide and 4-amino-1,8-naphthalimide (available [illegible]revigen) are graphically represented in Figure 1. Determine the PARP Ac[illegible]n your cell extract from the standard curve. Express the results as Units [illegible]RP/mg protein.

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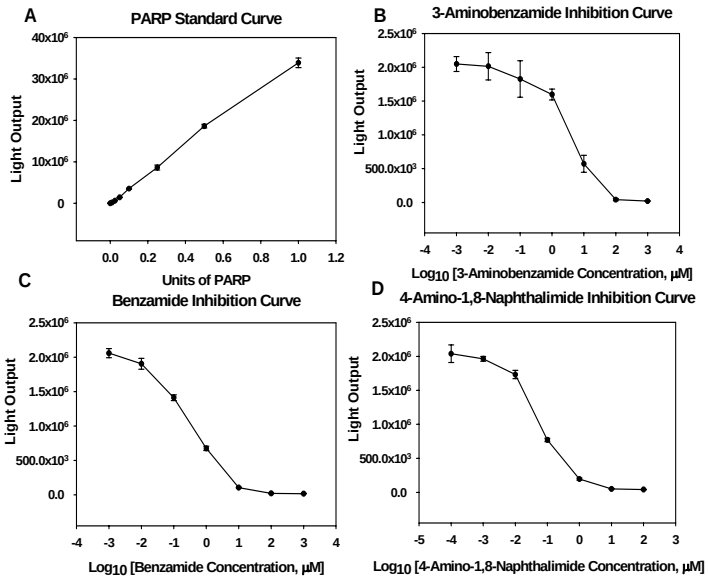

Figure 1. Graphical representation of the chemiluminescent readout of the PARP standard curve (Panel **A**) and inhibition curves for 3-aminobenzamide (Panel **B**), benzamide (Panel **C**), and 4-amino-1,4-naphthalimide (Panel **D**). Each point represents the median value from triplicates.

IX. References

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X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No light output in PARP Inhibition assay	Insufficient coating of histones	Use white chemiluminescent plates treated to bind proteins
		Use Trevigen's precoated strip wells (Cat# 4678-096-P)
	PARP enzyme was not added to the wells.	Add 0.5 Unit of PARP to each well.
No light output in PARP activity assay in cells or tissues.	Insufficient coating of histones	Coat overnight with 50 µl/well histones.
		Use white chemiluminescent plate treated to bind proteins
		Use Trevigen's precoated strip wells (Cat# 4676-096-P)
	PARP expression in cells and tissues very low	Increase incubation time with cell extracts to 1.5 hours.
		Increase the volume and/or concentration of cell extract added to each well.
	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	Insufficient blocking with Strep-Diluent.	Increase blocking 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 coating and blocking.

XI. Related Products Available From Trevigen

Catalog #	Description	Size
4870-500-6	10X PBS, pH 7.4	6 X 500 ml
4670-500-01	Biotinylated-NAD 2	500 µl
4335-MC-100	Anti-poly(ADP-ribo	onoclonal 100 µg
4338-MC-50	Anti-poly(ADP-ribo	ymerase (PARP) 50 µg
4500-050-P	Poly(ADP-ribosylat	otein 50 µl
4668-100-01	Recombinant Hum	RP Enzyme (HSA) 100 U
4668-500-01	Recombinant Hum	RP Enzyme (HSA) 500 U
4668-2K-01	Recombinant Hum	RP Enzyme (HSA) 2000 U
4667-50-06	Activated DNA	500 µl
4678-096-P	Histone-coated 96	hite strip wells Each
4667-50-03	3-Aminobenzamide	100 µl
4667-50-09	4-Amino-1,8-Napht	de 100 µl

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4667-50-10	6(5H)-Phenanthridinone	100 µl
4667-50-11	Benzamide	100 µl

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. **5X Coating Buffer:** Proprietary buffer solution that enhances protein binding.
10. **10X Activated DNA:** Activated Herring Sperm DNA in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA.

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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