TREVIGEN® Instructions

For Research Use Only. Not for Use In Diagnostic Procedures.

Universal Colorimetric PARP Assay Kit With Histones and Coating Buffer

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

Cat# 4671-096-K
Kit with Histone Reagent

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Universal Colorimetric PARP Assay Kit 96 Tests

Cat# 4671-096-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) catalyzes the NAD-dependent addition of poly (ADP-ribose) to adjacent nuclear proteins. PARP may orchestrate the sequence of events that occurs in the DNA repair process[1]. Induction of apoptosis occurs by depletion of 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[3-7]. 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) colorimetric, non-radioactive format; 2) higher throughput 96 test size; and 3) sensitivity down to 0.01 units of PARP per well. Trevigen offers two formats of the kit: Cat# 4671-096-K (Kit with histone reagent) and Cat# 4672-096-K (Kit with histone-coated plate). Histone-coated 96-well plates (Cat# 4671-096-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 Colorimetric 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
4667-50-07	*Histone Solution	700 µl	-80°C
4667-50-03	*3-Aminobenzamide	60 µl	-80°C
4671-096-04	10X Strep-Diluent	2 ml	4°C
4800-30-06	Strep-HRP	30 µl	4°C
4822-96-08	TACS-Sapphire™	10 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. PBS + 0.1% Triton X-100
3. Distilled water
4. 0.2 N HCl or 5% Phosphoric acid
5. Phenylmethyl Sulfonyl Fluoride (PMSF) and other protease inhibitors
6. Triton X-100 or Nonidet P-40, and 1M NaCl for cell extract preparation.

Disposables:

1. 1 - 200 µl and 100-1000 µl pipette tips
2. Plate lid or cover
3. 96 well protein-binding plate (via hydrophobic interactions).

Equipment:

1. Micropipettes
2. Multichannel pipettor 1 - 50 µl
3. Wash bottle or microplate washer (optional)
4. 96-well plate reader with 630 nm or 450 nm filters

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** 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) 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. TACS-Sapphire™

TACS-Sapphire is a Horseradish Peroxidase (HRP) substrate generating a soluble blue color with maximum absorbance at 630 nm. Development of the colorimetric reaction can be stopped by the addition of an equal volume of 0.2 M HCl or 5% phosphoric acid, generating a yellow color stable for up to 60 minutes that can be read at 450 nm. The absorbance at 450 nm will be significantly higher than the absorbance at 630 nm.

VI. PARP Inhibitor Screening Assay Protocol

A. Plate Coating

1. Aliquot 50 µl of diluted histones into each well (prepared in Section **V.1**). 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 (prepared in Section **V.2**) 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 (1.0 Unit/well prepared in Section **V.5**) to the wells containing inhibitor.

3. Controls:
i. Activity control: 1.0 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 (1.0 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 1 Unit/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 20 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. Add 50 µl per well of TACS-Sapphire™ colorimetric substrate and incubate, **in the dark**, for 10-30 minutes. Color development should be monitored for observable blue color in your samples and the plate read at 630 nm. The reaction can be stopped by adding 50 µl per well of 0.2 M HCl or 5% Phosphoric Acid and the absorbance read at 450 nm.

VII. PARP Activity in Cell and Tissue Extracts

A. Processing Cells

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 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 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 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 colorimetric 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.

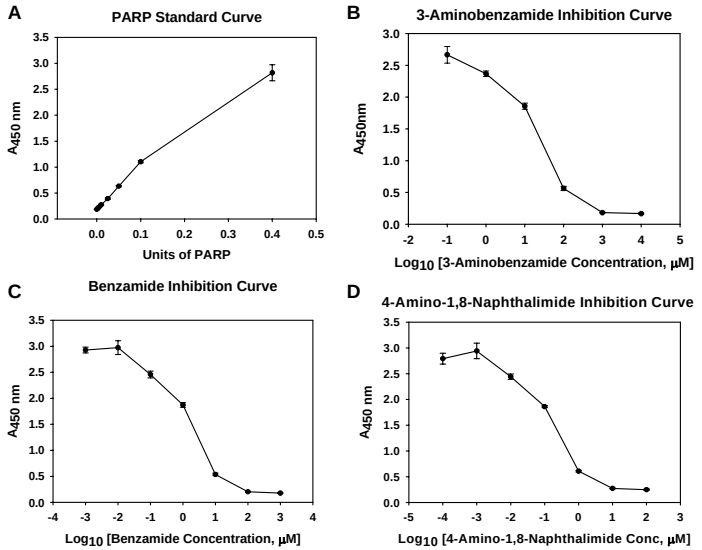

Figure 1. Graphical representation of the chemiluminescent readout of the PARP standard curve (Panel **A**) and inhibition curves for 3-aminobenzamide (Panel **B**), benzamide (Panel **C**), and 4-amino-1,4-naphthalimide (Panel **D**). Each point represents the median value from triplicates.

IX. References

1. Satoh MS, Lindahl T. 1992. Role of poly(ADP-ribose) formation in DNA repair. *Nature*; **356**: 356–358.
2. Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA. 1997. Ischemic brain injury is mediated by the activation of poly(ADPribose) polymerase. *J Cereb Blood Flow Metab*; **17**: 1143–1151.
3. Eliasson, M., K. Sampei, and A.J. Mandir. 1997. Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med*. **3**:1089-1095.
4. Miller, M.S., C. Zobre, and M. Lewis. 1993. In vitro neuroprotective activity of inhibitors of poly-ADP ribose polymerase. *Soc Neurosci Abstr* **19**.1656
5. Piper, A.A., A.Verma, J. Zhang, and S.H. Snyder. 1999. Poly(ADP-ribose) polymerase, nitric oxide and cell death. *Trends in Pharmacological Sciences* **20**:171-181.
6. Thiemermann, C., J. Bowes, F.P. Myint, and J.R. Vane. 1997. Inhibition of the activity of poly(ADP-ribose) synthase reduces ischemia-reperfusion injury in the heart and skeletal muscle. *Proc Natl Acad Sci USA* **94**:679-683.
7. Virag, L., and Szabo, C. 2002. The therapeutic potential of Poly(ADP-Ribose) Polymerase inhibitors. *Pharmacological Reviews* **54**:375-429.
8. Tong WM, *et al*. 2002. Synergistic role of Ku80 and poly(ADP-ribose) polymerase in suppressing chromosomal aberrations and liver cancer formation. *Cancer Res*. **62**:6990-6996.
9. Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL. 2004. NAD-Dependent Modulation of Chromatin Structure and Transcription by Nucleosome Binding Properties of PARP-1. *Cell* **119**:803–814.
10. Curtin NJ. 2005. PARP inhibitors for cancer therapy. *Expert Rev Mol Med*. **7**:1-20.

X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No color in PARP Inhibition assay	Insufficient coating of histones	Use plate treated to bind proteins such as NUNC Polysorp plates
		Use Trevigen's precoated plates (Cat# 4671-96-P)
	PARP enzyme was not added to the wells.	Add 1 Unit of PARP to each well.
No color in PARP activity assay in cells.	Insufficient coating of histones	Coat overnight with 50 µl/well histones.
		Use plate treated to bind proteins such as NUNC Polysorp plates
		Use Trevigen's precoated plates (Cat# 4671-96-P)
	PARP expression in cells and tissues very low	Extend development time with TACS Sapphire to 1 hour.
		Add 1M NaCl to 0.4M final concentration in cell extraction buffer
		Increase the volume and/or concentration of cell extract added to each well.
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 250 µM	500 µl
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)	50 µg
4500-050-P	Poly(ADP-ribosylated) protein	50 µl
4668-100-01	Recombinant Human PARP Enzyme (HSA)	100 U
4668-500-01	Recombinant Human PARP Enzyme (HSA)	500 U
4668-2K-01	Recombinant Human PARP Enzyme (HSA)	2000 U
4667-50-06	Activated DNA	500 µl
4671-096-P	Histone-coated 96 well plates	Each
4667-50-03	3-Aminobenzamide	100 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

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. **TACS-Sapphire™ (Cat# 4822-96-08):** Peroxidase substrate readable at 630 nm (blue) or at 450 nm (yellow) after stopping reaction with 0.2 M HCl or 5% Phosphoric Acid.
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.

Trevigen, Inc.
8405 Helgerman Ct.
Gaithersburg, MD 20877
Tel: 1-800-873-8443 • 301-216-2800
Fax: 301-560-4973
e-mail: info@trevigen.com
www.trevigen.com