TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Chemiluminescent PARP/Apoptosis Assay

96 Tests

Cat# 4685-096-K

ELISA kit for Measuring PARP Activity in Cell Lysates before and during Apoptosis

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Table of Contents

I. Introduction

The control of apoptosis--the most intensely studied form of programmed cell death--has been a long sought after goal for the treatment of cardiovascular, neurological, autoimmune and malignant diseases.[1,2] Poly (ADP-ribose) polymerase (PARP-1) becomes a mediator of cell death by triggering the translocation of apoptosis-inducing factor from the mitochondria to the nucleus.[3] In experimental models, PARP-1 inhibition can prevent unwanted tissue damage following myocardial and neuronal ischemia, diabetes, septic shock, and vascular stroke.[4-8] Apoptosis involves many changes in cell component structure including exposure of phosphatidylserine in the outer plasma membrane, caspase activation, cytochrome C release from the mitochondria, chromatin condensation in the nucleus, and DNA ladder formation.[1] During apoptosis, PARP-1 which catalyzes the NAD-dependent addition of poly (ADP-ribose) (PAR) onto various cytoplasmic and nuclear proteins, is cleaved from about 116 kDa to 85 kDa.[9,10]

Trevigen's **HT PARP/Apoptosis Assay** is ideal for measuring PARP activity in cell extracts prepared before and during apoptosis. The **HT PARP/Apoptosis Assay** is an ELISA which semi-quantitatively detects PAR deposited onto immobilized histone proteins in a 96-well format. An anti-PAR monoclonal antibody, goat anti-mouse IgG-HRP conjugate, and HRP substrate are used to generate a chemiluminescent signal. Thus, light output correlates with PARP activity. Etoposide is a topoisomerase II inhibitor that stabilizes this enzyme after it cleaves DNA.[11] It is included as a control apoptosis inducer. Important features of the assay include: 1) chemiluminescent, non-radioactive format; 2) higher throughput 96 test size, and 3) sensitivity down to 0.1 mUnits of PARP. Trevigen offers two formats of the **HT PARP/Apoptosis Assay**: cat# 4684-096-K (Colorimetric) and cat# 4685-096-K (Chemiluminescent).

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 PARP/Apoptosis Assay 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
4684-096-01	PARP-HSA, 10 mUnits/µl	100 µl	-20 °C
4684-096-07	20X I-PAR Assay Buffer	2.5 ml	-20 °C
4684-096-02	20 mM NAD	300 µl	-20 °C
4685-096-P	Histone-Coated White Strip Well Plate, I-PAR	96 wells	4 °C
4684-096-06	10 mM Etoposide	100 µl	-20 °C
4684-096-03	5X Antibody Diluent	3 ml	4 °C
4684-096-04	Anti-PAR monoclonal antibody	20 µl	-20 °C
4684-096-05	Goat anti-mouse IgG-HRP	20 µl	-20 °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

IV. Materials/Equipment Required But Not Supplied

Reagents:
1. PARP inhibitors, or cells/tissue to be tested.
2. PBS (cat# 4870-500-6)
3. PBS + 0.1% Triton X-100
4. Distilled water
5. Phenylmethyl Sulfonyl Fluoride (PMSF) or other protease inhibitors
6. Triton X-100, and 5M NaCl for extract preparation.

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 chemiluminescent plate reader or luminometer

V. Reagent Preparation

1. PBS Wash Solution
Prepare 500 ml of 1X PBS in a wash bottle for washing strip wells.

2. PBS + 0.1% Triton X-100 Wash Solution
Prepare 500 ml of 1X PBS containing 0.1% Triton X-100 in a wash bottle for washing the strip wells.

3. 1X I-PAR Assay Buffer (contains 0.1 mg/ml BSA)
Dilute the 20X I-PAR Assay Buffer (cat# 4684-096-07) to **1X (1:20)** with dH_2O. The **1X** I-PAR Assay Buffer is used to rehydrate the histone coated wells, dilute the PARP standard, prepare cell extracts, and set up the PARP reactions.

4. PARP Substrate Cocktail
Make a PARP Substrate Cocktail as follows (a total of 25 µl/well is required):

	Volume/well	/plate
20X I-PAR Assay Buffer (cat# 4684-096-07)	1.25 µl	100 µl
10X Activated DNA (cat# 4671-096-06)	2.5 µl	250 µl
20 mM NAD (cat# 4684-96-02)	2.5 µl	250 µl
dH_2O	18.75 µl	1875 µl
total:	25 µl	2500 µl

5. PARP Enzyme
The kit contains 100 µl of 10 mUnits/µl PARP-HSA enzyme (cat# 4684-096-01). Serially dilute the PARP-HSA with **1X** I-PAR Assay Buffer to make at least 100 µl of each dilution just before use. The recommended final concentrations are 10 mUnits/25 µl, 5 mUnits/25 µl, 2.5 mUnits/25 µl, 1 mUnit/25 µl, 0.5 mUnits/25 µl, 0.25 mUnits/25 µl, and 0.1 mUnits/25 µl. The

standard curve requires 25 µl/well of each PARP dilution and each is performed in triplicate (see Figure 3, page 7). **Note: Diluted enzyme should be used immediately and any remainder discarded.** The following table describes a serial dilution protocol for PARP:

mUnits PARP 25 µl/well	10 mUnits well	5 mUnits well	2.5 mUnits well	1 mUnits well	0.5 mUnits well	0.25 mUnits well	0.1 mUnits well
PARP 10 mUnits/µl	8 µl	100 µl	100 µl	80 µl	100 µl	100 µl	80 µl
1X I-PAR Buffer	192 µl	100 µl	100 µl	120µl	100 µl	100 µl	120 µl

6. Etoposide

Etoposide is provided at 10 mM as a control apoptosis inducer. Excellent and reproducible results are obtained when used at a final concentration of 50-100 µM.

7. Antibody Diluent

This solution is used as a diluent for the antibodies. Dilute the 5X Antibody Diluent (cat# 4684-096-03) **1:5** with dH_2O before use.

8. Anti-PAR Monoclonal Antibody

Just before use, dilute the anti-PAR monoclonal antibody (cat# 4684-096-04) 1,000-fold with **1X** Antibody Diluent (cat# 4684-096-03). A total of 50 µl/well of diluted anti-PAR monoclonal antibody is required in the assay.

9. Goat Anti-Mouse-IgG-HRP Conjugate

Just before use, dilute the goat anti-mouse IgG-HRP conjugate (cat# 4684-096-05) 1,000-fold with **1X** Antibody Diluent (cat# 4684-096-03). A total of 50 µl/well of diluted goat anti-mouse IgG-HRP conjugate is required in the assay.

10. PeroxyGlow™ A and B Chemiluminescent Substrates

Prewarm PeroxyGlow A and B to room temperature before use. Just before use, mix equal volumes of PeroxyGlow™ A and B together and add 100 µl per well. PeroxyGlow™ A and B are Horseradish Peroxidase (HRP) substrates generating light that can be quantified in a suitable chemiluminescent plate reader.

11. Cell Extraction Buffer

Prepare 10 ml of the following cell extraction buffer and store at 4°C (a total of 100 µl/well is required):

	Volume/well	/plate
20X I-PAR Assay Buffer (cat# 4684-096-07)	5 µl	500 µl
5 M NaCl	8 µl	800 µl
20% Triton X-100	4.5 µl	450µl
200 mM PMSF	0.2 µl	20 µl
dH_2O	82.3 µl	8230µl
total:	100 µl	10 ml

VI. Assay Protocols

A. Monitoring PARP Activity Before and During Apoptosis

PARP, expressed endogenously in all cells, undergoes transient activation following DNA damage, followed by inactivation due to autoribosylation and cleavage by Caspase 3 during apoptosis. Trevigen's HT PARP/Apoptosis Assay is sufficiently sensitive to capture these events in a small number of cells per test and can, therefore, monitor the extent of apoptosis under a variety of experimental conditions. The following suggested protocol will help you to set up these types of experiments:

1. On Day 0, seed actively-growing cells: 5 x 10^3 to 5 x 10^4 cells/200 µl fresh medium/well in a 96 well flat-bottom plate for adherent cells, or a V-bottom plate for non-adherent cells. Be sure to set aside triplicate wells containing healthy cells for controls.

2. Early on Day 1, add 1 µl of 10 mM Etoposide, and/or other apoptosis-inducing agents to triplicate wells, for 50 µM final concentration, and incubate overnight at 37°C/5% CO_2. These wells will be the 6-8 hour time points.

3. Continue as above (add 1 µl of 10 mM Etoposide, and/or other agents to triplicate wells) to set up the wells for the 4 hr, and 2 hr and remaining time points.

4. Prepare extracts right in the wells:
Non-adherent cells: Centrifuge the V-bottom plate at 1,000 x g for 5 minutes at 4°C, and carefully aspirate off the supernatants. Wash the cell pellets twice with 200 µl/well ice cold 1X PBS. Add 100-200 µl Cell Extraction Buffer (Section V, item 11), and incubate lysates on ice (or in the cold room) with periodic mixing for 30 minutes. Using a multi-channel pipettor, transfer 25 µl of each lysate to corresponding wells of the histone coated plate and proceed with the ribosylation reaction. Otherwise, seal the plate using a product such as the ThermalSeal™ (Excel Scientific, Inc.) and store at -80°C.

Adherent cells: Carefully aspirate the medium from the wells and wash the cells twice with 1X PBS (200 µl/well). Centrifugation may be necessary to avoid loss of apoptotic cells. Add 100 µl/well Cell Extraction Buffer (Section V, item 11). Incubate the cell lysates on ice (or in the cold room) with periodic mixing for 30 minutes. Using a multi-channel pipettor, transfer 25 µl of each lysate to corresponding wells in the histone-coated plate and proceed with the ribosylation reaction. Otherwise, seal the plate using a product such as the ThermalSeal™ (Excel Scientific, Inc.) and store at -80 °C.

Notes:

1. For less than 5 x 10^4 cells, centrifugation of the lysates is usually not required. For 1 x 10^5 or more cells, 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. Alternatively, remove the highly viscous pellet with a pipette tip.

2. Determine the protein concentration of the extracts, and adjust for at least 200 ng protein/25 µl test volume. **Note:** 1X I-PAR Assay Buffer contains 0.1 mg/ml BSA.

3. Assay immediately, or snap-freeze the extracts in plates or small aliquots and store at -80°C. Avoid repeated freezing and thawing of the extracts.

B. Ribosylation Reaction

Note: Do not premix cell extracts with the PARP Cocktail, because PARP will autoribosylate in the presence of NAD.

1. Remove the strip wells from the Ziploc bag and add 50 µl/well of 1X I-PAR Assay Buffer to rehydrate the histones. Incubate at room temperature (25°C) for 30 minutes. In order to eventually obtain data expressed in terms of mU PARP/cell, make serial dilutions of the PARP standard (Section V, item 5). The assay is sufficiently sensitive to measure PARP activity in as little as 500 Jurkat cells. The amount of protein derived from so few cells may not be measurable. In this case, adjust the volume of your extract so that 25 µl are theoretically derived from 1,000-5,000 cells. We recommend that you start with 200 ng protein /25 µl test volume. **Note:** 1X I-PAR Assay Buffer contains 0.1 mg/ml BSA.

Notes: It may be necessary to make serial dilutions of your extract down to 10 ng/25 µl test volume to obtain signals within the standard curve.

2. Remove the 1X I-PAR Assay Buffer from the wells by tapping the strip wells on paper towels. Add 25 µl, in triplicate, of the serial dilutions of PARP standard (Section V, item 5). Add 25 µl/well, in triplicate, the cell lysates directly from the tissue culture plate or from the 1.5 ml microtubes.

3. **Negative Control**: Include wells without PARP or cell extract to provide the background absorbance that will be subtracted from the experimental sample values.

4. Distribute 25 µl of the PARP Substrate Cocktail (prepared in Section V, item 4) into each well using a multichannel pipettor.

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

	Volume	Order of Addition
Cell Extract, PARP Standard, or 1X I-PAR Buffer	25 µl	1
1X PARP substrate cocktail	25 µl	2
Total volume	50 µl	

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

C. Detection

1. Wash strip wells 2 times with **1X** PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. 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 anti-PAR monoclonal antibody (prepared in section V, item 8). Incubate at room temperature (25°C) for 30 minutes.

3. Wash strip wells 2 times with **1X** PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.

4. Add 50 µl per well of diluted goat anti-mouse IgG-HRP conjugate (prepared in section V, item 9). Incubate at room temperature (25°C) for 30 minutes.

5. Wash strip wells 2 times with **1X** PBS + 0.1% Triton X-100 (200 µl/well) followed by 2 washes with 1X PBS. Ensure that all the liquid is removed following each wash by tapping strip wells onto paper towels.

6. Just before use, mix equal volumes of prewarmed PeroxyGlow™ A and B together and add 100 µl per well. Immediately take chemiluminescent readings.

VII. Data Interpretation.

The loss of PARP activity in Jurkat cells correlates with PARP cleavage during apoptosis as shown in Figure 1:

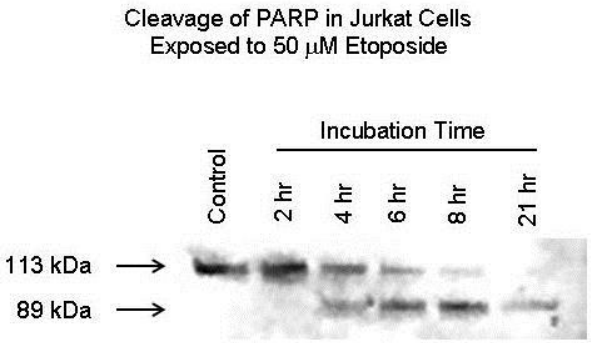

Figure 1. Western blot of a time course of Jurkat T cells treated with 50 µM etoposide for the indicated time periods. The amount of extract theoretically derived from 100,000 cells were resolved, per lane, on an 8%-16% SDS-PAGE gel and analyzed by immunoblotting for PARP-1 using the monoclonal antibody C2-10 (Trevigen cat# 4338-MC-50).

Example results obtained using Jurkat T cells and Trevigen's HT Chemiluminescent PARP/Apoptosis Assay are shown in Figure 2.

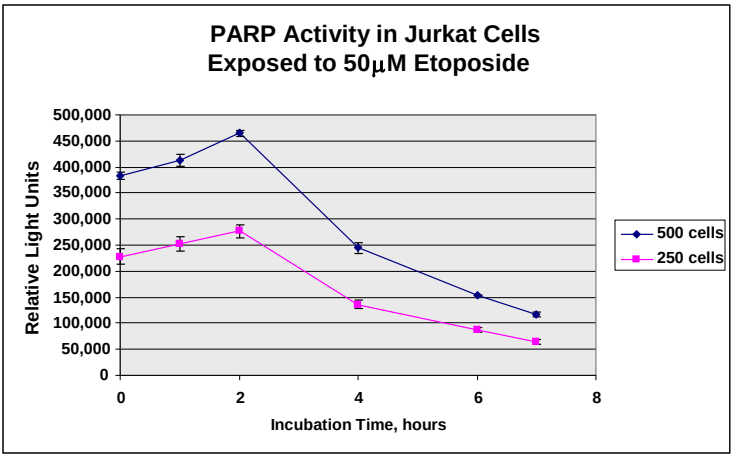

Figure 2. HT PARP/Apoptosis Assay results: PARP activity in Jurkat cells, exposed to 50 µM etoposide, a known and well-characterized apoptosis inducer, decreases as a function of time. Each point represents the mean value from triplicate determinations and each reading represents the equivalent of PARP activity in 250 and 500 cells.

A typical Chemiluminescent PARP standard curve is graphically represented in Figure 3. Determine the PARP Activity in your cell extract from the standard curve. Use of a standard curve allows for expression of the results in mUnits PARP/ng protein, or Units PARP/10^6 cells, or µUnits PARP/cell depending upon your preference.

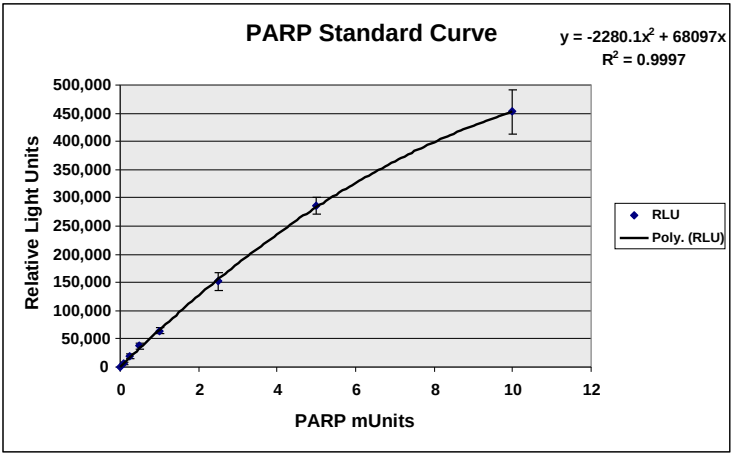

Figure 3. Graphical representation of an example Chemiluminescent readout of a PARP standard curve. Each point represents the mean value from triplicate determinations.

Some investigators may wish to express results as a percent inhibition relative to the untreated control. The inhibition of PARP caused by caspase-mediated cleavage will be reflected as a *decrease* in the observed light output readings relative to that observed in the absence of apoptosis induction. Subtract the

mean background relative light units (mean negative control value) from those of all the experimental wells.

Let C = Net Relative Light Units in the absence of induced apoptosis
D = Net Relative Light Units determined during apoptosis

$$\% \text{ Inhibition of PARP} = \frac{(C - D)}{C} \times 100$$

VIII. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No light output in wells with PARP alone	Active PARP enzyme was not added.	Order fresh PARP-HSA (cat# 4684-096-01) and add 10 mUnits of PARP to each positive control well
Light output in wells containing cell or tissue extracts too high or above that obtained for the PARP standard curve	PARP expression in cells and tissues can be very high	Extend serial dilutions of extract down to 10 ng of protein or equivalent to 500-1000 cells per well
High background in wells with no PARP	Poor washing	Increase the number of washes with **1X** PBS + 0.1% Triton X-100 after the ribosylation reaction and after incubation with antibodies

IX. References

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X. Related Products Available From Trevigen

Catalog #	Description	Size
4684-096-K	HT Colorimetric PARP/Apoptosis Assay	96 tests
4520-096-K	HT PARP In Vivo Pharmacodynamic Assay II	96 tests
4676-096-K	Universal Chemiluminescent PARP Assay Kit/w Histone Coated Strip Wells	96 samples
4701-096-K	Tankyrase 1 Chemiluminescent Activity Assay	96 tests
4677-096-K	Universal Color PARP Assay Kit/w Histone Coated Strip Wells	96 samples
4700-096-K	Tankyrase 1 Colorimetric Activity Assay	96 tests
4817-60-K	FlowTACS™ Apoptosis Detection Kit	60 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 tests
4830-01-K	TACS® Annexin V FITC Kit	100 samples
4835-01-K	TACS® Annexin V Biotin Kit	100 samples
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 tests
6305-100-K	MitoShift™ Mitochondrial Potential Assay Kit	100 tests
4815-30-K	TumorTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4823-30-K	NeuroTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4827-30-K	CardioTACS ™ *In Situ* Apoptosis Detection Kit	30 samples
4829-30-K	DermaTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4826-30-K	VasoTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4828-30-DK	TACS•XL® DAB *In Situ* Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4810-30-K	TACS® 2 TdT DAB *In Situ* Apoptosis Detection Kit	30 samples
4811-30-K	TACS®2 TdT Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4812-30-K	TACS® 2 TdT Fluorescein Apoptosis Detection Kit	30 samples
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	20 samples
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
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests

Accessories

Catalog #	Description	Size
4667-250-EB	Recombinant Human PARP Enzyme	250 µl
4668-100-1	Recombinant Human PARP (High Specific Activity)	1000 Units
4869-500-6	Apoptosis Grade™ H_2O	6 x 500 ml
4870-500-6	10X PBS, pH = 7.4	6 x 500 ml
4670-500-1	Biotinylated-NAD 250 µM	500 µl

Antibodies

Catalog #	Description	Size
2281-MC-100	Anti-Bax Monoclonal Antibody (Clone YTH-6A7)	100 µg
2291-MC-100	Anti-Bcl-2 Monoclonal Antibody (Clone YTH-8C8)	100 µg
4411-PC-100	Anti-Phosphorylated Histone-γ-H2AX polyclonal	100 µl
6361-PC-100	Anti-human/mouse-PBR polyclonal	100 µl
4335-MC-100	Anti-PAR polymer mAb (10HA)	100 µl
4336-BPC-100	Anti- PAR polymer polyclonal	100 µl
4338-MC-50	Anti-human/murine-PARP mAb (clone C2-10)	50 µg

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