ELISA

PARP/Apoptosis Colorimetric Assay Kit

Catalog Number: 4684-096-K

ELISA kit for measuring PARP activity in cell lysates before and during apoptosis.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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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) poly-merase (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).

PRINCIPLE OF THE ASSAY

The PARP/Apoptosis Colorimetric Assay Kit is ideal for measuring PARP activity in cell extracts prepared before and during apoptosis. This ELISA 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 colorimetric signal. Thus, absorbance correlates with PARP activity. Etoposide is a topoisomerase II inhibitor that stabilizes this enzyme after it cleaves DNA (11), is included as a control apoptosis inducer.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed and protective clothing should be worn when handling kit reagents.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Use within 3 months from date of receipt.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Histone-Coated White Strip Well Plate, I-PAR	4684-096-P	1 plate (96-wells)	Store 2-8 °C.
5X Antibody Diluent	4684-096-03	3 mL	
TACS-Sapphire™	4822-96-08	10 mL	
PARP-HSA (10 mUnits/µL)	4684-096-01	100 µL	Store at ≤ -20 °C in a manual defrost freezer.
20X I-PAR Assay Buffer	4684-096-07	2.5 mL	
20 mM NAD	4684-096-02	300 µL	
10 mM Etoposide	4684-096-06	100 µL	
Anti-PAR Monoclonal Antibody	4684-096-04	20 µL	
Goat Anti-Mouse IgG-HRP	4684-096-05	20 µL	
10X Activated DNA	4671-096-06	300 µL	
Plate Sealers	N/A	4 adhesive strips	Room temperature

Equipment:

- Pipettes and pipette tips
- Squirt bottle, manifold dispenser, or automated microplate reader
- 96-well plate reader with 450 nm filter
- Microcentrifuge
- Refrigerated centrifuge (for plate centrifugation)

Reagents:

- PARP inhibitors
- 1X PBS
- PBS + 0.1% Triton™ X-100
- Distilled water
- Phenylmethyl Sulfonyl Fluoride (PMSF) or other protease inhibitors
- Triton™ X-100
- 5M NaCl

REAGENT PREPARATION

1X PBS - Prepare 500 mL of 1X PBS in a wash bottle for washing strip wells.

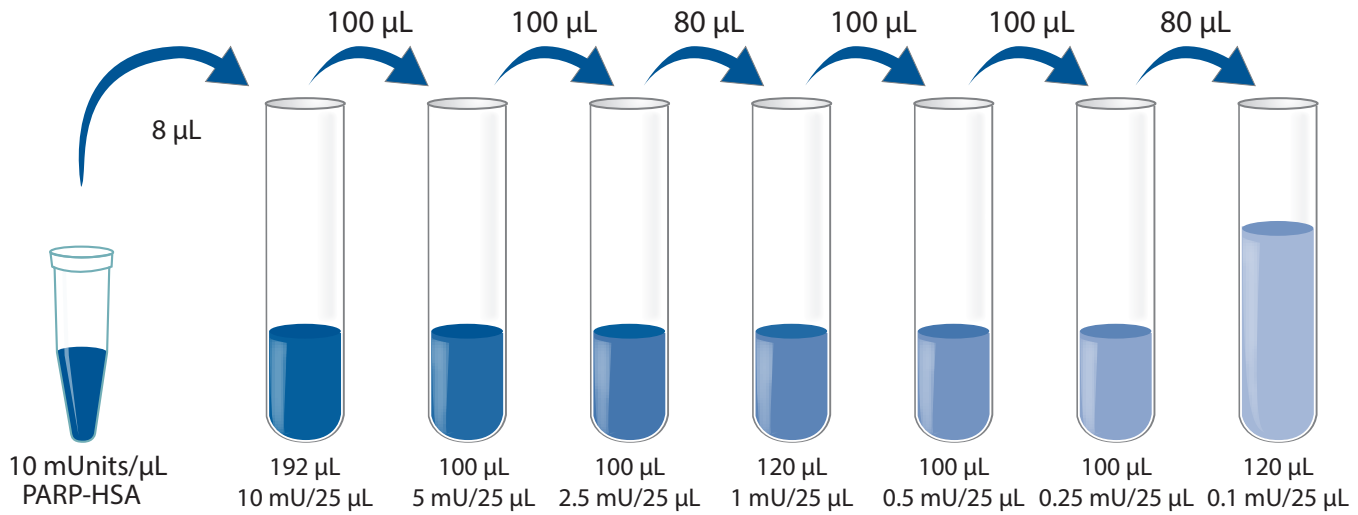
PBS + 0.1% Triton X-100 Wash Solution - Prepare 500 mL of 1X PBS containing 0.1% Triton X-100 in a wash bottle for washing the strip wells.

1X I-PAR Assay Buffer (contains 0.1 mg/mL BSA) - Dilute the 20X I-PAR Assay Buffer 1:20 with distilled water to generate 1X I-PAR Assay Buffer. This buffer is used to rehydrate the histone-coated wells, dilute the PARP standard, prepare cell extracts, and set up the PARP reactions.

PARP Substrate Cocktail - Make a PARP Substrate Cocktail as follows (a total of 25 µL/well is required):

Reaction Component	Volume/well	Volume/plate
20X I-PAR Assay Buffer	1.25 µL	125 µL
10X Activated DNA	2.50 µL	250 µL
20 mM NAD	2.50 µL	250 µL
Distilled water	18.75 µL	1875 µL
Total	25 µL	2500 µL

PARP Standard - The kit contains 100 µL of 10 mUnits/µL PARP-HSA enzyme. Pipette 192 µL of 1X I-PAR Assay Buffer into the 10 mUnits/25 µL tube. Pipette 100 µL or 120 µL into the remaining tubes indicated below. Use the PARP-HSA enzyme to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 mUnits/25µL serves as a high standard. 1X I-PAR Assay Buffer serves as the zero standard (0 mUnits/25µL). Diluted enzyme should be used immediately and any remainder discarded.



REAGENT PREPARATION *CONTINUED*

Etoposide - Etoposide is provided at 10 mM as a control apoptosis inducer. Use at a final concentration of 50-100 µM.

1X Antibody Diluent - Before use, dilute the 5X Antibody Diluent 1:5 using distilled water to generate 1X Antibody Diluent. For 96 wells, a suggested dilution can be achieved by adding 2.4 mL 5X Antibody Diluent to 9.6 mL distilled water.

Anti-PAR Monoclonal Antibody - Just before use, dilute the Anti-PAR Monoclonal Antibody 1000-fold using 1X Antibody Diluent. A total of 50 µL/well of diluted Anti-PAR Monoclonal Antibody is required in the assay. For 96 wells, a suggested 1000-fold dilution can be achieved by adding 10 µL Anti-PAR Monoclonal Antibody to 90 µL 1X Antibody Diluent, followed by adding 50 µL of the diluted antibody to 4950 µL 1X Antibody Diluent.

Goat Anti-Mouse-IgG-HRP Conjugate - Just before use, dilute the Goat Anti-Mouse IgG-HRP 1:1000 using 1X Antibody Diluent. A total of 50 µL/well of diluted Goat Anti-Mouse IgG-HRP Conjugate is required in the assay. For 96 wells, a suggested 1000-fold dilution can be achieved by adding 10 µL Anti-PAR Monoclonal Antibody to 90 µL 1X Antibody Diluent, followed by adding 50 µL of the diluted antibody to 4950 µL 1X Antibody Diluent.

TACS-Sapphire - Pre-warm TACS-Sapphire to room temperature before use. TACS-Sapphire is a colorimetric substrate that turns blue in the presence of Horseradish Peroxidase (HRP). The addition of an equal volume of 0.2 M HCl or 5% Phosphoric Acid stops the reaction to generate a yellow color stable for up to 60 minutes that can be read at 450 nm.

Cell Extraction Buffer - Prepare 10 mL of the following cell extraction buffer and store at 2-8 °C (a total of 100 µL/well is required):

Reaction Component	Volume/well	Volume/plate
20X I-PAR Assay Buffer	5.0 µL	500 µL
5 M NaCl	8.0 µL	800 µL
20% Triton X-100	4.50 µL	450 µL
200 mM PMSF	0.20 µL	20 µL
Distilled water	82.3 µL	8230 µL
Total	100 µL	10 mL

ASSAY PROTOCOL

MONITORING PARP ACTIVITY BEFORE AND DURING APOPTOSIS

It is recommended that all standards, controls, and samples be assayed in triplicate.

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. PARP/Apoptosis Colorimetric Assay Kit 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 10^3-5 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. 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 2-8 °C to remove insoluble material. Recover the supernatant to a fresh tube pre-chilled on ice. Alternatively, remove the highly viscous pellet with a pipette tip.

2. Early on Day 1, add 1.0 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 indicated in Step 2, (add 1.0 µL of 10 mM Etoposide, and/or other agents to triplicate wells) to set up the wells for the 4 hour and 2 hour and remaining time points.

4. Prepare extracts directly in the wells:

   Non-adherent cells: Centrifuge the V-bottom plate at 1,000 x g for 5 minutes at 2-8 °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, and incubate lysates on ice (or in the cold room) with periodic mixing for 30 minutes.

   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. Incubate the cell lysates on ice (or in the cold room) with periodic mixing for 30 minutes.

5. 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.

6. Assay immediately, or snap-freeze the extracts in plates (using plate sealers) or small aliquots and store at ≤ -70 °C. Avoid repeated freezing and thawing of the extracts.

RIBOSYLATION REACTION

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

1. Remove the strip wells from the bag and add 50 µL/well of 1X I-PAR Assay Buffer to rehydrate the histones, cover with an adhesive strip and incubate at room temperature for 30 minutes. Prepare the PARP Standard as directed in the Reagent Preparation section. The assay is sufficiently sensitive to measure PARP activity in as little as 500 Jurkat cells. The amount of protein derived from less 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: *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 PARP Standard and the cell lysates directly from the tissue culture plate.
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 into each well using a multichannel pipettor.
5. The final reaction volume in each well is 50 µL:

Reaction Component	Volume	Order of Addition
Cell Extract, PARP Standard, or 1X I-PAR Buffer	25 µL	1
1X PARP Substate Cocktail	25 µL	2
Total Volume	50 µL	

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

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. Cover with a new adhesive strip and incubate at room temperature for 30 minutes.
3. Repeat the wash as in Step 1.
4. Add 50 µL per well of Goat Anti-Mouse IgG-HRP Conjugate. Cover with a new adhesive strip and incubate at room temperature for 30 minutes.
5. Repeat the wash as in Step 1.
6. Add 50 µL per well of pre-warmed TACS-Sapphire colorimetric substrate and incubate, **in the dark**, for 15 minutes at room temperature. Stop the reactions by adding 50 µL per well of 0.2 M HCl or 5% Phosphoric Acid and read the absorbance at 450 nm.

DATA INTERPRETATION

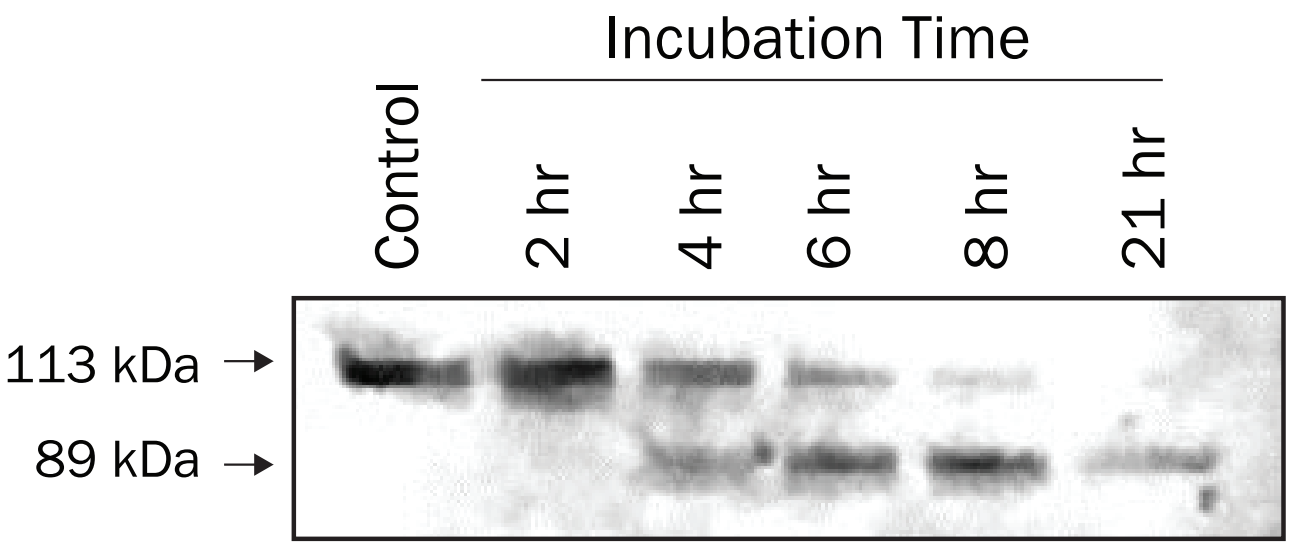


Figure 1. Cleavage of PARP in Jurkat Cells Exposed to Etoposide. Western Blot of a time course of Jurkat T cells treated with 50 µM Etoposide for the indicated time periods. The amount of extract theoretically derived from 100,000 cells were resolved, per lane, on an 8-16% SDS-PAGE gel and analyzed by immunoblotting for PARP-1 using the monoclonal antibody C2-10. The loss of PARP activity in Jurkat cells correlates with PARP cleavage during apoptosis.

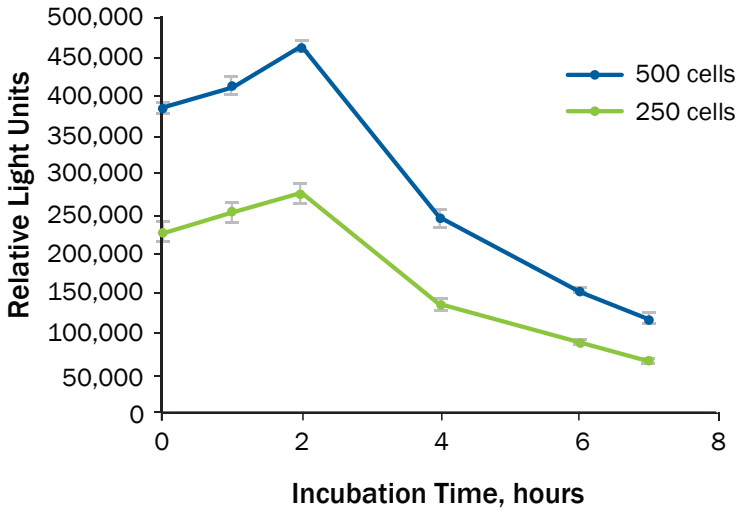


Figure 2. Etoposide reduces PARP Activity in Jurkat Cells. PARP activity in Jurkat cells exposed to 50 µM etoposide decreases as a function of time. Each point represents the mean value from triplicate determinations and each reading represents the equivalent of PARP activity in 250 and 500 cells.

A typical Chemiluminescent PARP standard curve is graphically represented in Figure 3. Determine the PARP Activity in your cell extract from a 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.

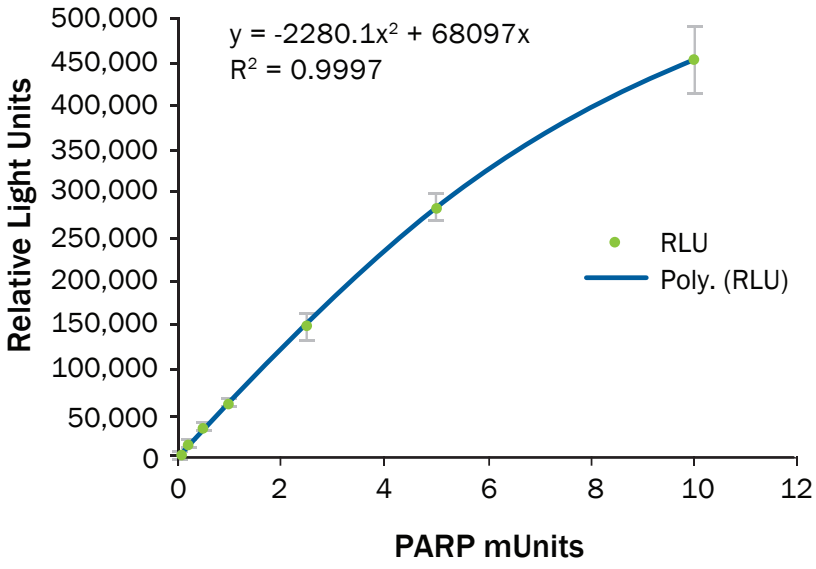


Figure 3. Example Chemiluminescent PARP Assay Standard Curve. Each point represents the mean value from triplicate determinations. Determine the PARP Activity in your cell extract from a standard curve. Use of a standard curve allows for expression of the results in mUnits PARP/ng protein, or Units PARP/10^6 cells, or µUnits PARP/cell depending upon your preference.

DATA INTERPRETATION *CONTINUED*

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 absorbance readings relative to that observed in the absence of apoptosis induction. Subtract the mean background absorbance (mean negative control value) from those of all the experimental wells.

C = Net Relative Light Units in the absence of induced apoptosis

D = Net Relative Light Units determined during apoptosis

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

TROUBLESHOOTING

PROBLEM	CAUSE	SOLUTION
No light output in wells with PARP alone.	Active PARP enzyme was not added.	Order fresh PARP-HSA and add 10 mUnits of PARP-HSA 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/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.

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