

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

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Histone-Coated Natural 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
- 0.2M HCl or Phosphoric acid

REAGENT PREPARATION

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

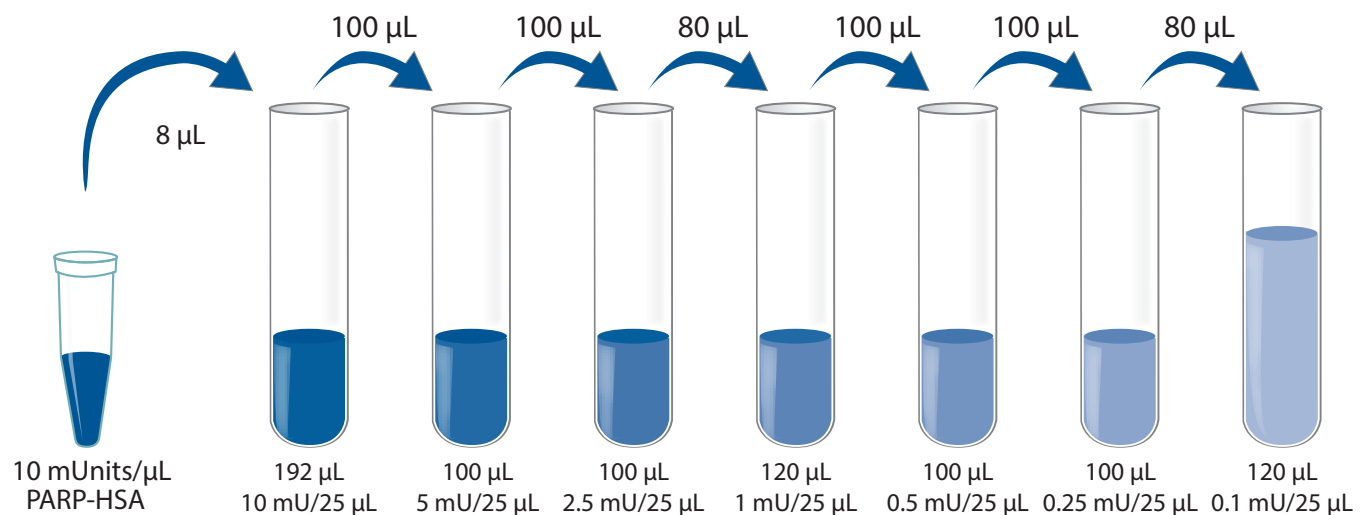
PBS + 0.1% Triton X-100 Wash Solution - Prepare 500 mL of 1X PBS containing 0.1% Triton X-100 in a wash bottle for washing the strip wells.

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 μ L/well is required):

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

PARP Standard - The kit contains 100 μ L of 10 mUnits/ μ L PARP-HSA enzyme. Pipette 192 μ L of 1X I-PAR Assay Buffer into the 10 mUnits/25 μ L tube. Pipette 100 μ L or 120 μ L 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/25 μ L serves as a high standard. 1X I-PAR Assay Buffer serves as the zero standard (0 mUnits/25 μ L). 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 μ M.

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 μ L/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 μ L Anti-PAR Monoclonal Antibody to 90 μ L 1X Antibody Diluent, followed by adding 50 μ L of the diluted antibody to 4950 μ L 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 μ L/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 μ L Anti-PAR Monoclonal Antibody to 90 μ L 1X Antibody Diluent, followed by adding 50 μ L of the diluted antibody to 4950 μ L 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 μ L/well is required):

Reaction Component	Volume/well	Volume/plate
20X I-PAR Assay Buffer	5.0 μ L	500 μ L
5 M NaCl	8.0 μ L	800 μ L
20% Triton X-100	4.50 μ L	450 μ L
200 mM PMSF	0.20 μ L	20 μ L
Distilled water	82.3 μ L	8230 μ L
Total	100 μ L	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: 5×10^3 - 5×10^4 cells/200 μ L 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 5×10^4 cells, centrifugation of the lysates is usually not required. For 1×10^5 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 μ L of 10 mM Etoposide, and/or other apoptosis-inducing agents to triplicate wells, for 50 μ M final concentration, and incubate at 37 °C/5% CO₂. These wells will be the 6-8 hour time points.
3. Continue as indicated in Step 2, (add 1.0 μ L 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 μ L/well ice cold 1X PBS. Add 100-200 μ L 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 μ L/well). Centrifugation may be necessary to avoid loss of apoptotic cells. Add 100 μ L/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 μ L 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.

ASSAY PROTOCOL *CONTINUED*

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 Substrate Cocktail	25 μL	2
Total Volume	50 μL	

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

ASSAY PROTOCOL *CONTINUED*

DETECTION

1. Wash strip wells 2 times with 1X PBS + 0.1% Triton X-100 (200 μ L/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 μ L 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 μ L 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 μ L 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 μ L per well of 0.2 M HCl or 5% Phosphoric Acid and read the absorbance at 450 nm.

DATA INTERPRETATION

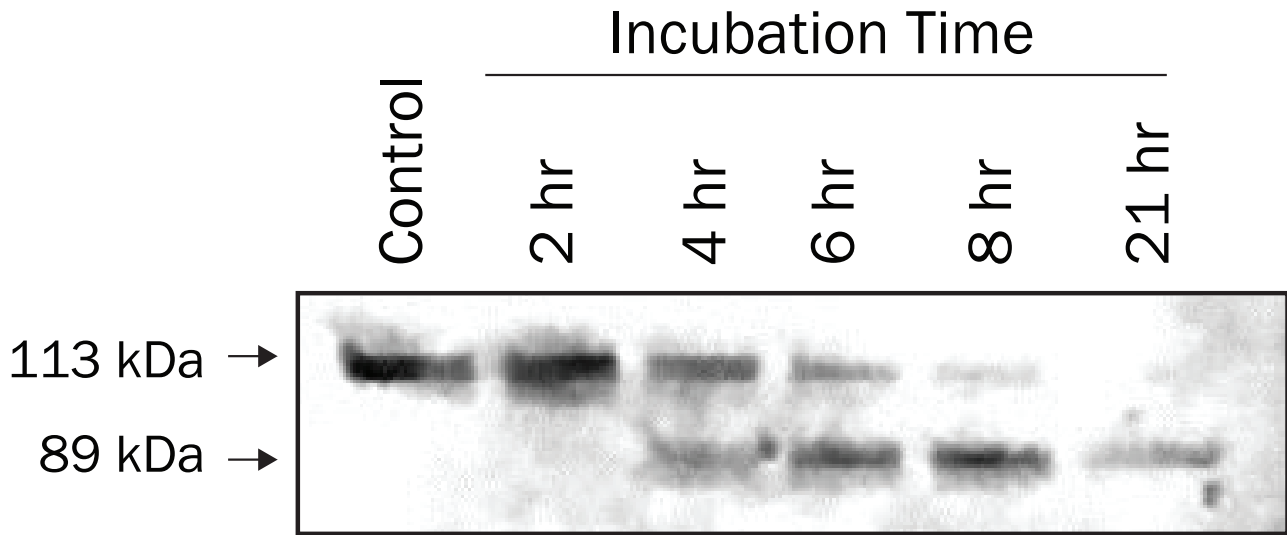


Figure 1. Cleavage of PARP in Jurkat Cells Exposed to Etoposide. Western Blot of a time course of Jurkat T cells treated with 50 μ M Etoposide for the indicated time periods. The amount of extract theoretically derived from 100,000 cells were resolved, per lane, on an 8-16% SDS-PAGE gel and analyzed 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.

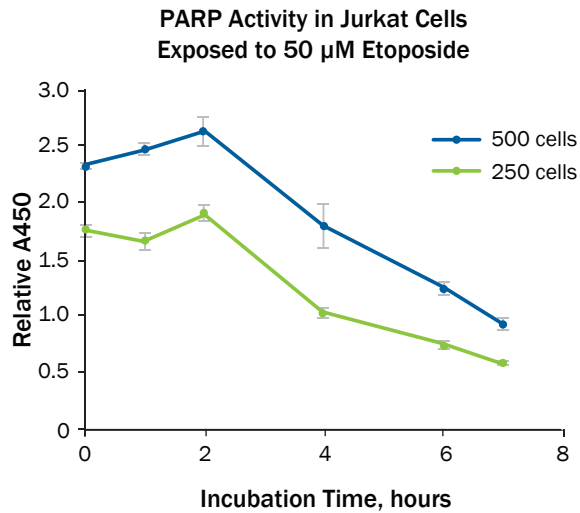


Figure 2. HT PARP/Apoptosis Assay results. PARP activity in Jurkat cells, exposed to 50 μM etoposide, a known and well-characterized apoptosis inducer, decreases as a function of time. Each point represents the mean value from triplicate determinations and each reading represents the equivalent of PARP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

A typical colorimetric 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/10⁶ cells, or μUnits PARP/cell depending upon your preference.

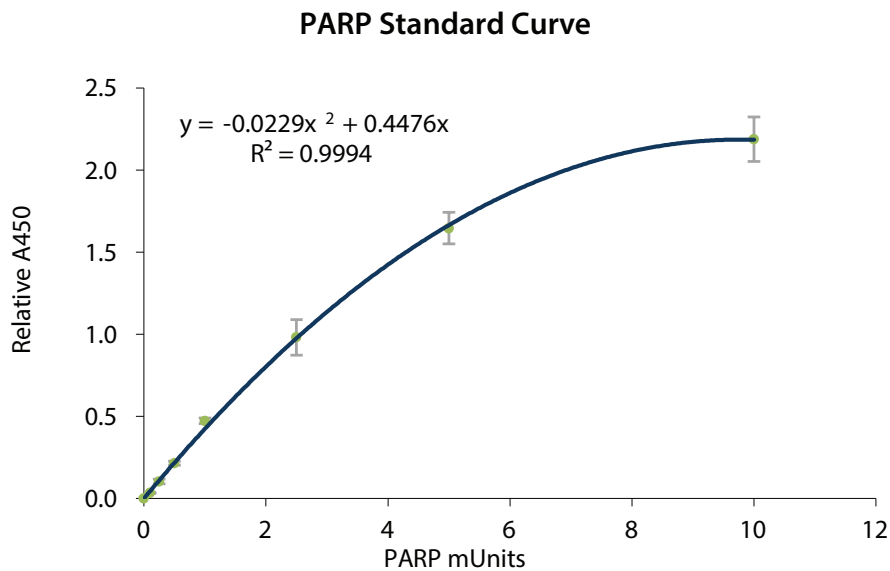


Figure 3. Graphical representation of an example colorimetric readout of a PARP standard curve. Each point represents the mean value from triplicate determinations.

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 absorbance in the absence of induced apoptosis

D = Net absorbance determined during apoptosis

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

TROUBLESHOOTING

PROBLEM	CAUSE	SOLUTION
No color in wells with PARP alone	If no color develops in the wells, then active PARP enzyme was not added.	Add PARP-HSA or Order fresh PARP-HSA (catalog # 4684-096-01) and add serial dilutions of PARP in triplicate.
Color development 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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