Promega

Technical Bulletin

Anti-PARP p85 Fragment pAb

INSTRUCTIONS FOR USE OF PRODUCT G7341.

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Anti-PARP p85 Fragment pAb

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1. Description

Anti-PARP p85 Fragment pAb[a] is a polyclonal antibody directed against the neo N-terminus of the 85kDa caspase-cleaved fragment (p85) of human poly (ADP-ribose) polymerase (PARP; 1). Anti-PARP p85 Fragment pAb specifically detects the 85kDa (apparent molecular weight) fragment of PARP that results from caspase cleavage and does not detect the 116kDa intact PARP molecule. Its specificity for the cleaved form of PARP makes this antibody a novel marker to detect apoptosis. The antibody is designed for use as an immunocytochemical/immunohistochemical marker for apoptosis in cells and tissues and can be used with standard fixatives and immunohistochemical procedures. Anti-PARP p85 Fragment pAb provides a convenient and simple method to monitor apoptosis in situ. This antibody can be used with dual-labeling techniques in conjunction with TUNEL assays for a more complete analysis of the various stages of apoptosis.

Most cells from higher eukaryotes have the ability to self-destruct by activating an intrinsic cellular suicide program referred to as programmed cell death or apoptosis (2,3). Apoptosis is important in development, homeostasis and in several diseases (3–6) and is characterized by certain cellular morphological features, including membrane blebbing, nuclear and cytoplasmic shrinkage, and chromatin condensation. The morphological changes observed in the nucleus of apoptotic cells may, in part, result from the process of generating DNA fragments through the action of endogenous endonucleases (7). Typically, the DNA of apoptotic cells is cleaved to a population of multimers of 180–200bp fragments, readily observed as a ladder on agarose gels.

1. Description (continued)

PARP is a nuclear DNA-binding protein that detects DNA strand breaks and functions in base excision repair (8–11). Once PARP is cleaved, it no longer supports the enzymatic DNA repair function, and there is some evidence that cleaved PARP may inhibit access by other repair enzymes (12). Although PARP is not absolutely required for apoptosis to proceed, the cleavage of PARP may contribute to the irreversibility of apoptosis (8,12).

Activation of a cascade of caspases is essential for apoptosis in many systems. Caspase activation results in cleavage of PARP into two characteristic fragments of molecular mass 85kDa and 25kDa [by sequence data the fragments are now designated 89kDa and 24kDa (13,14)]. Detection of caspase-3 cleavage fragments of PARP has been established as a hallmark of apoptosis (15–17). Anti-PARP p85 Fragment pAb was raised against an N-terminal peptide from the p85 fragment. The resulting antibody is specific for the p85 fragment and does not recognize the 116kDa intact molecule.

Antibody Production and Affinity Purification: The PARP immunogen is a synthetic peptide, gly-val-asp-glu-val-ala-lys (GVDEVAK), representing the N-terminus of the large C-terminal fragment of human PARP that results from caspase-3 cleavage. The peptide was modified to contain a short spacer molecule and a C-terminal cysteine and coupled to keyhole limpet hemocyanin (KLH) carrier protein. The antigen complex was injected into rabbits to produce antiserum using standard methods. An affinity purification column was made by covalently linking the peptide antigen via the C-terminal cysteine to Sulfolink resin (Pierce Chemical, Rockford, IL, USA) at 1 mg peptide/ml resin. The antiserum was then affinity purified on this column (1).

Figure 1 shows that the Anti-PARP p85 Fragment pAb strongly stains Fas monoclonal antibody-treated Jurkat cells (Panel A) with minimal background staining of untreated cells (Panel B). This example of immunocytochemical staining illustrates that Anti-PARP p85 Fragment pAb is useful as a marker to detect apoptosis. Western blots (Figure 2) further demonstrate that Anti-PARP p85 Fragment pAb does not recognize full-length PARP but specifically recognizes the p85-cleaved fragment. In Western blots of cell extracts from model apoptosis systems (Fas monoclonal antibody-treated Jurkat cells or anisomycin-treated HL-60 cells), the Anti-PARP p85 Fragment pAb specifically recognizes an 85kDa band of PARP from extracts of cells induced to undergo apoptosis (Figure 2). Therefore, the Anti-PARP p85 Fragment pAb is ideal for immunocytochemical analysis of apoptosis. The antibody provides an early marker to detect apoptosis, since cleavage of PARP occurs prior to DNA fragmentation, measured using TUNEL assays.

This Technical Bulletin includes a detailed immunocytochemical protocol for detection of apoptosis in Fas mAb-treated Jurkat cells. This model system demonstrates the utility of the Anti-PARP p85 Fragment pAb for staining apoptotic cells.

2. Product Components and Storage Conditions

Product	Size	Cat.#
Anti-PARP p85 Fragment pAb	50µl	G7341

Contains sufficient antibody for 50 immunocytochemical reactions at the suggested working dilution of 1:100.

Storage Conditions: For long-term storage, store the undiluted antibody at –20°C. For daily/weekly use, store in aliquots at –20°C. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes.

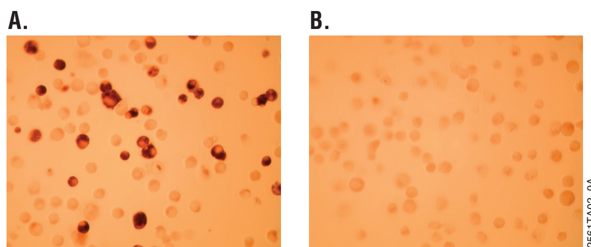

Figure 1. Immunocytochemical staining of Jurkat cells using the Anti-PARP p85 Fragment pAb. Jurkat cells were treated with 0.05µg/ml anti-Fas mAb for 6 hours (**Panel A**) or were untreated (**Panel B**). Cells were mounted on slides and fixed in 10% buffered formalin. The Anti-PARP p85 Fragment pAb was used at a 1:100 dilution, and the secondary Ab was donkey anti-rabbit biotin (Jackson Cat.# 711-065-152) followed by streptavidin-HRP and DAB. There are numerous labeled apoptotic cells in the treated preparation and virtually no labeled cells in the untreated control.

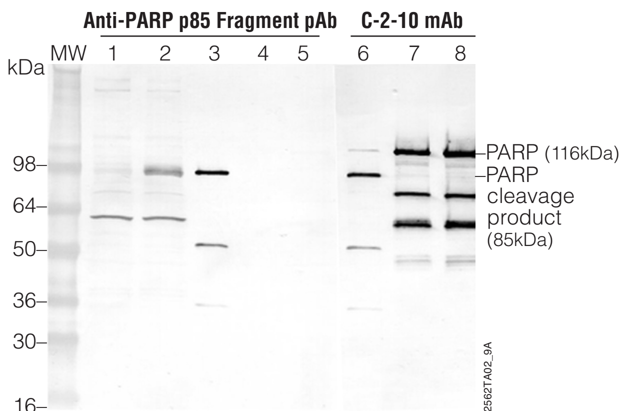

Figure 2. Western analysis of Jurkat cell extracts and bovine PARP immunostained with Anti-PARP p85 Fragment pAb and the anti-PARP monoclonal antibody, C-2-10. Anti-PARP p85 Fragment pAb staining of 10µg of extracts from untreated (lane 1) or anti-Fas antibody-treated (lane 2) Jurkat cells reveals the 85kDa cleaved PARP fragment in treated cells only. Anti-PARP p85 Fragment pAb staining of 50ng of caspase-3 (CPP32)-cleaved bovine PARP (lane 3), 500ng of bovine PARP (lane 4) or 1µg of bovine PARP (lane 5) demonstrates that the Anti-PARP p85 Fragment pAb only recognizes the caspase-3-cleaved PARP. Even with a 20-fold excess of full-length PARP, the antibody shows complete specificity for the p85 fragment of PARP. C-2-10 anti-PARP mAb staining of 50ng of caspase-3 (CPP32)-cleaved bovine PARP (lane 6) and 500ng and 1µg of bovine PARP (lanes 7 and 8, respectively) shows both the 85kDa cleaved PARP and 116kDa full-length PARP. (One unknown band at ~60kDa is present in both treated and untreated extracts but does not affect immunocytochemical staining. Additional bands seen in lane 3 and lane 6 represent cleavage products from PARP fragments that were not full-length, which were present in the bovine PARP preparation [see lanes 7–8]).

Jurkat cells were treated with 0.05µg/ml anti-Fas mAb for 6 hours or were untreated. Cells were harvested, washed with cold PBS and resuspended in lysis buffer (25mM HEPES, 5mM $MgCl_2$, 5mM EDTA, 5mM DTT, 2mM PMSF, 10µg/ml pepstatin A, 10µg/ml leupeptin). After four freeze-thaw cycles, extracts were subjected to centrifugation at top speed in a microcentrifuge for 10 minutes, and supernatants were harvested. To cleave the PARP protein, 2µg of CPP32 enzyme (Pharmingen Cat. #66281T) was added to 20µg of bovine PARP (Biomol Cat.# SE-165) in PIPES cleavage buffer (20mM PIPES, 100mM NaCl, 10mM DTT, 10mM EDTA, 0.1% CHAPS, 10% sucrose, [pH 7.2]), incubated at 37°C for 2 hours, then frozen at –20°C. The samples were run on an SDS-polyacrylamide gel and transferred to nitrocellulose by standard methods. The nitrocellulose blots were blocked in Tris-buffered saline (TBS) + 1% BSA, then incubated with Anti-PARP p85 Fragment pAb (diluted 1:400 in TBS + 0.05% Tween® 20 + 0.1% BSA) or C-2-10 anti-PARP mAb (1.5µg/ml in TBS + 0.05% Tween® 20 + 0.1% BSA). Secondary antibodies were donkey anti-rabbit AP (Jackson Cat.# 711-055-152), 1:5,000, for the Anti-PARP

p85 Fragment pAb and donkey anti-mouse AP (Jackson Cat.# 715-055-150), 1:5,000, for the C-2-10 anti-PARP mAb. The blots then were stained with Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841).

Note: For additional examples using the Anti-PARP p85 Fragment pAb, go to the Apoptosis Assistant at: **www.promega.com/apoasst/**. See also the Antibody Assistant at: **www.promega.com/techserv/tools/abasst/**

3. Example Protocol Overview for Anti-PARP p85 Fragment pAb Immunocytochemistry Using HRP-Conjugated Secondary Antibody

Flow Diagram	Notes
Attach sections or cells to microscope slides ↓ **Fix**	Immerse slide in appropriate fixative (e.g., 4% paraformaldehyde or 10% buffered formalin in PBS).
↓ Wash ↓ **Permeabilize**	Permeabilize tissue sections or cells with 0.2% Triton® X-100 in PBS.
↓ Wash ↓ **Block nonspecific immunostaining**	Block with normal serum from the host species identical (or similar) to that used to generate the secondary antibody (usually 2–10% serum).
↓ **Incubate with Anti-PARP p85 Fragment pAb**	The recommended starting dilution is 1:100 in blocking buffer.
↓ Wash ↓ **Block with hydrogen peroxide for HRP-conjugated secondary antibody**	Incubate in 0.3% hydrogen peroxide to block endogenous peroxidases.
↓ Wash ↓ **Incubate with secondary antibody**	Incubate in anti-rabbit secondary antibody diluted in PBS.
↓ Wash ↓ **Add DAB for HRP-conjugated secondary antibody**	Combine DAB components and add to slides. Develop color for approximately 10 minutes.
↓ Wash ↓ **Add mounting medium and analyze sample**	Observe under a light or fluorescent microscope.

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4. Procedure for Immunocytochemical Detection of Apoptosis in Cultured Cells

This protocol is a standard immunocytochemical assay demonstrating the use of the Anti-PARP p85 Fragment pAb as a marker for apoptosis in a model system. In this example protocol, Jurkat cells are treated with a Fas monoclonal antibody to induce apoptosis. Fas ligand or Fas antibody binds to the Fas receptor (a member of the TNF-α death receptor family) and initiates an apoptotic pathway (18,19). The Anti-PARP p85 Fragment pAb labels Fas mAb-treated Jurkat cells with minimal background staining of untreated Jurkat cells.

Materials to Be Supplied by the User

- PBS
- 10% neutral buffered formalin, 4% paraformaldehyde, or other standard fixative
- mounting medium
- 0.2% Triton® X-100 solution in PBS
- secondary antibody with labeled conjugate
- normal serum (same or similar species that used to generate secondary antibody)
- chromogenic substrate for colorimetric secondary antibodies
- 0.3% hydrogen peroxide for blocking endogenous peroxidases (if using HRP-conjugate)
- microscope slides (Cel-Line® HTC-coated chamber slides or other standard slides)
- coplin jars
- forceps
- 37°C incubator
- micropipettors
- glass coverslips
- microscope (fluorescence microscope if fluorescent secondary antibody is used)

Additional Materials for Cultured Cells

- poly-L-lysine (Sigma Cat.# P8920)
- anti-Fas monoclonal antibody (clone #CH-11, MBL International Cat.# SY-001)

4.A. Preparation of Poly-l-Lysine-Coated Slides

1. Prepare sufficient poly-L-lysine-coated slides for appropriate positive and negative controls as well as all experimental samples.
2. Pipet an aqueous solution of poly-L-lysine (Sigma Cat.# P8920, diluted 1:10 in water) onto the surface of each precleaned glass slide (8-chambered Cel-Line® HTC-coated or other standard slides). Distribute a thin layer of the poly-L-lysine solution throughout the areas to be used for fixing cells. As soon as the slides have dried, rinse in deionized water, then allow the coated slides to air-dry. Poly-L-lysine-coated slides may be stored at 4°C for up to 7 days before use.

4.B. Fas mAb Induction of Apoptosis in Jurkat Cells

Treatment with anti-Fas monoclonal antibody induces apoptosis in the human Jurkat cell line (15). Following the procedure described below for Fas-induced apoptosis of Jurkat cells, approximately 50% of cells should be apoptotic.

1. Grow Jurkat cells in RPMI-1640 medium containing 10% fetal bovine serum in a humidified 5% CO_2 incubator at 37°C.

2. Resuspend cells in fresh medium to 1×10^5 cells/ml. Two to three days later, resuspend in fresh medium to 5×10^5 cells/ml and add anti-Fas mAb to a final concentration of 0.05µg/ml. Incubate for 6 hours in a 37°C incubator. As a negative control, incubate untreated cells (no Fas mAb) under the same conditions.

3. Collect cells by centrifugation at 300–350 × *g* for 5 minutes. Remove all medium ,and resuspend in PBS. Repeat and resuspend the cell pellet in PBS to 1.5×10^6 cells/ml.

4. Add a thin layer of anti-Fas mAb-treated and untreated cells to the poly-L-lysine-coated slides. **Do not** allow cells to dry before fixing. The cells will adhere to the poly-L-lysine rapidly.

5. Fix cells on slides in 10% neutral buffered formalin or other standard fixative (e.g., 4% paraformaldehyde) in coplin jars for 25 minutes at room temperature in a fume hood.

6. Wash slides in 1X PBS for 5 minutes at room temperature. Repeat. Transfer to fresh PBS, and store at 4°C (upright). These prepared slides are good for several weeks.

   For adherent cells, grow in chamber slides. Following control or experimental treatment to induce apoptosis, fix cells directly on the slide as described above after removing serum-containing culture medium.

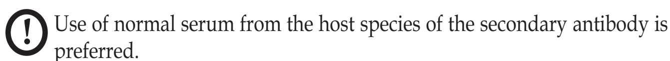
4.C. Immunocytochemistry Using Anti-PARP p85 Fragment pAb

Do not allow slides to dry during the immunocytochemistry procedure.

1. Permeabilize cells fixed on slides in 0.2% Triton® X-100/PBS for 5 minutes at room temperature.

2. Wash in 1X PBS in coplin jars for 5 minutes at room temperature. Repeat twice for a total of 3 washes.

3. Drain slides, and add blocking buffer (PBS/0.1% Tween® 20 + 5% normal serum). Cover cells with blocking buffer (200µl per slide). Lay slides flat in a humidified chamber, and incubate for 2 hours at room temperature.


Use of normal serum from the host species of the secondary antibody is preferred.

4. Rinse once in PBS.

4.C. Immunocytochemistry Using Anti-PARP p85 Fragment pAb (continued)

5. Add 100µl of Anti-PARP p85 Fragment pAb diluted in blocking buffer. We recommend a starting dilution of 1:100. Include a slide with no Anti-PARP p85 Fragment pAb as a negative control. Incubate slides in a humidified chamber overnight at 4°C.
6. The following day, wash the slides twice for 10 minutes in 1X PBS, twice for 10 minutes in PBS/0.1% Tween® 20, and twice for 10 minutes in 1X PBS at room temperature.
7. If the secondary antibody is a horseradish peroxidase (HRP)-conjugate, block endogenous peroxidases by incubating with 0.3% hydrogen peroxide for 4–5 minutes at room temperature. If you are using a different method of detection with a secondary antibody, proceed to Step 9.
8. Wash in 1X PBS in coplin jars for 5 minutes. Repeat twice for a total of three washes.
9. Drain slides, and add 100–200µl of diluted secondary antibody to each slide. We recommend donkey anti-rabbit biotin conjugate (Jackson Cat.# 711-065-152) or donkey anti-rabbit Cy®3 conjugate (Jackson Cat.# 711-165-152) diluted 1:500 in PBS/0.1% Tween® 20. Lay slides flat in a humidified chamber, and incubate for 2 hours at room temperature.
10. Wash several times in 1X PBS.
11. For the biotin conjugate, drain slides, and add 100–200µl of Streptavidin-HRP solution to each slide. (We recommend peroxidase-labeled streptavidin from KPL, Cat. #14-30-00, diluted to 1µg/ml in PBS). Lay slides flat in a humidified chamber, and incubate for 45 minutes at room temperature. For HRP-conjugated secondary antibodies, proceed to Step 13. For other secondary antibodies, proceed to Step 15.
12. Wash in 1X PBS in coplin jars for 5 minutes. Repeat twice for a total of three washes.
13. Add 100–200µl of freshly made diaminobenzidine (DAB) solution to each slide. We recommend the DAB kit from Zymed (Cat.# 00-2014). Lay slides flat, and incubate for ~10 minutes at room temperature.
14. Rinse slides in NANOpure® water. Bleach is frequently used to inactivate the DAB before disposal; however, local requirements for hazardous waste should be followed.
15. Drain the liquid, and mount the slides in a permanent or aqueous mounting medium (slides mounted in 70% glycerol can be stored for several weeks at 4°C or –20°C).

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6. Related Products

Apoptosis Reagents

Product	Size	Cat.#
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml**	G7792
Caspase-Glo® 3/7 Assay*	2.5ml**	G8090
Caspase-Glo® 8 Assay*	2.5ml**	G8200
Caspase-Glo® 9 Assay*	2.5ml**	G8210
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl**	G7461
CaspACE™ Assay System, Colorimetric*	50 assays**	G7351
Caspase Inhibitor Z-VAD-FMK	50µl**	G7231
Caspase Inhibitor Ac-DEVD-CHO	100µl	G5961
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
DeadEnd™ Colorimetric TUNEL System	20 reactions**	G7360
Anti-pS^{473} Akt pAb	40µl	G7441
GSH-Glo™ Glutathione Assay	10ml**	V6911

*For Laboratory Use.
**Additional sizes are available.

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP, luminescent)	10ml	G7570
	10 × 10ml**	G7571
CellTiter-Fluor™ Cell Viability Assay (fluorescent)*	10ml	G6080
	5 × 10ml**	G6081
CellTiter-Blue® Cell Viability Assay (resazurin, fluorometric)	20ml	G8080
	100ml**	G8081
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays**	G7890
CytoTox 96® Non-Radioactive Cytotoxicity Assay*	1,000 assays	G1780
CytoTox-Fluor™ Cytotoxicity Assay*	10ml**	G9260
CytoTox-Glo™ Cytotoxicity Assay*	10ml**	G9290
CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS, colorimetric)*	200 assays**	G3582
CellTiter 96® AQ$_{ueous}$ MTS Reagent Powder*	250mg**	G1112

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Protease Assays

Product	Size	Cat.#
Calpain-Glo™ ProteaseAssay*	10ml**	G8501
DPPIV-Glo™ Protease Assay*	10ml**	G8350
DUB-Glo™ Protease Assay (DUB/SENP/NEDP)**	10ml**	G6260
Proteasome-Glo™ Cell-Based Assay*	10ml**	G8660
Proteasome-Glo™ Chymotrypsin-Like Assay	10ml**	G8621
Proteasome-Glo™ Trypsin-Like Assay	10ml**	G8631
Proteasome-Glo™ Caspase-Like Assay*	10ml**	G8641
Proteasome-Glo™ 3-Substrate System*	10ml**	G8531

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Multiplex Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml**	G6410
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml**	G9200
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml**	G9270

For Laboratory Use.
**Additional sizes are available.

[a]U.S. Pat. Nos. 6,350,452 and 7,037,664.

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