



**ab110215**

# **PARP-1 (cleaved) Human In-Cell ELISA Kit (IR)**

## **Instructions for Use**

For the quantitative measurement of Human PARP-1 (cleaved) concentrations in cultured adherent and suspension cells.

This product is for research use only and is not intended for in vitro diagnostic use.

**[www.abcam.com](http://www.abcam.com)**

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# 1. Introduction

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In-Cell ELISA Assay Kits use quantitative immunocytochemistry to measure protein levels or post-translational modifications in cultured cells. Cells are fixed in a microplate and targets of interest are detected with highly specific, well-characterized monoclonal antibodies, and levels are quantified with IRDye®-labeled Secondary Antibodies. IR imaging and quantitation is performed using a LI-COR® Odyssey® or Aeries® system.

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Ab110215 (MSA43) is a highly specific and high-throughput assay for measuring the larger (89 kDa) fragment of poly (ADP-ribose) polymerase 1 (PARP-1) generated by caspase cleavage between Asp214 and Gly215 of human PARP-1.

PARP-1 is a 113 kDa nuclear DNA-repair enzyme that transfers ADP-ribose units from NAD<sup>+</sup> to variety of nuclear proteins including topoisomerases, histones and PARP-1 itself. Via poly ADP ribosylation, PARP-1 is responsible for regulation of cellular homeostasis including cellular repair, transcription and replication of DNA, cytoskeletal organization and protein degradation. In response to DNA damage, PARP-1 activity is increased upon binding to DNA strand nicks and breaks. Excessive DNA damage leads to

generation of large branched ADP-ribose polymers and activation of a unique cell death program. During apoptosis, PARP-1 is cleaved by activated caspase-3 between Asp214 and Gly215, resulting in the formation of an N-terminal 24 kDa fragment containing most of the DNA binding domain

In-Cell ELISA (ICE) technology is used to perform quantitative immunocytochemistry of cultured cells with a near-infrared fluorescent dye-labeled detector antibody. The technique generates quantitative data with specificity similar to Western blotting, but with much greater quantitative precision and higher throughput due to the greater dynamic range and linearity of direct fluorescence detection and the ability to run 96 samples in parallel. This method rapidly fixes the cells in situ, stabilizing the in vivo levels of proteins, and thus essentially eliminates changes during sample handling, such as preparation of protein extracts. Finally, the cleaved PARP-1 signal can be normalized to cell amount, measured by the provided Janus Green whole cell stain, to further increase the assay precision.

The assay is designed for use with cultured adherent and suspension cells in a 96-well microplate format. The adherent cells undergoing apoptosis readily detach from a culture plate. The cell detachment often leads to their loss and thus underestimating the proportion of apoptotic cells. This assay was developed to eliminate the loss of apoptotic cells. In addition, this assay is also applicable for suspension cells.

## 2. Assay Summary

### Adherent Cell Seeding

- Seed cells into Assay Plate.
- Allow cells to adhere.
- Treat cells as desired in total volume of 100  $\mu$ l.

### Suspension Cell Seeding

- Seed cells into a plate or dish (not provided).
- Treat cells as desired.
- Concentrate & transfer the cells into the Assay Plate in 100  $\mu$ l.

### Cell Fixation (30 minutes)

- Centrifuge the plate at 500x g for 8 minutes at room temperature.
- Immediately add 100  $\mu$ l of 8% Paraformaldehyde Solution to fix and crosslink the cells to the plate.
- Immediately centrifuge the plate at 500x g for 8 minutes at room temperature.
- Incubate for additional 15 minutes.
- Wash wells with PBS (may be stored at 4 °C at this point).

### Permeabilization and Blocking (2.5 hours)

- Dilute the 100X Triton X-100 stock one hundred times in 1X PBS and add the 1X Permeabilization Buffer. Incubate 30 minutes at RT.
- Dilute the 10X Blocking Solution five times in 1X PBS and add the 2X Blocking Solution. Incubate 2 hours at RT.

### Primary Antibody Incubation (Overnight)

- Dilute the primary antibody stock 500X in 1X Incubation Buffer and add the diluted primary antibodies.
- Incubate overnight at 4 °C.
- Wash thoroughly.

### Secondary Antibody Incubation (2 hours)

- Dilute the secondary antibody stock 1000X in 1X Incubation Buffer and add the diluted secondary antibodies.
- Incubate 2 hours at RT.
- Wash thoroughly.

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### Measure Plate

- Image plate with a LI-COR® Odyssey® scanner and analyze data using ICW settings.
- If desired, stain with Janus Green and measure relative cell seeding density in a microplate spectrophotometer or IR imager.
- Perform data analysis.

### 3. Kit Contents

Part Number	Item	Quantity
8209706	10X Phosphate Buffered Saline (PBS)	100 ml
8209707	100X Triton X-100 (10% solution)	0.5 ml
8209708	400X Tween – 20 (20% solution)	2ml
8203023	10X Blocking Solution	15 ml
8209737	500X Primary Antibody	42 µl
8209724	1000X IRDye800-labeled Secondary Antibody	24 µl
8209709	1X Janus Green Stain	11 ml
8200010	Repackaged Sterile 96-well Assay Plate	2 EA
5201089	Plate Seals	2 EA

### 4. Storage and Handling

Store all components at 4 °C. The kit is stable for at least 6 months.

## 5. Additional Materials Required

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- A LI-COR® Odyssey® or Aeries® Infrared imaging system
- Centrifuge equipped with standard microplate holders
- 20% paraformaldehyde
- Deionized water
- Multichannel pipette (recommended)
- 0.5 M HCl (optional for Janus Green cell staining procedure)

## 6. Preparation of Reagents

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***Note: Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.***

*Preparation of sufficient buffers and working solutions to analyze a single microplate.*

1. Prepare 1X PBS by diluting 45 ml of 10X PBS in 405 ml Nanopure water or equivalent. Mix well. Store at room temperature.



2. Prepare 1X Wash Buffer by diluting 0.625 ml of 400X Tween-20 in 250 ml of 1X PBS. Mix well. Store at room temperature.
3. Immediately prior to use prepare 8% Paraformaldehyde Solution by mixing 6.25 ml Nanopure water, 1.25 ml 10X PBS and 5.0 ml 20% paraformaldehyde. *Note – Paraformaldehyde is toxic and should be prepared and used in a fume hood. Dispose of paraformaldehyde according to local regulations.*
4. Immediately prior to use prepare 1X Permeabilization Buffer by diluting 0.25 ml 100X Triton X-100 in 24.75 ml 1X PBS.
5. Immediately prior to use prepare 2X Blocking Solution by diluting 5 ml 10X Blocking Solution in 20 ml 1X PBS.
6. Immediately prior to use prepare 1X incubation Buffer by diluting 2.5 ml 10X Blocking Solution in 22.5 ml 1X PBS.

## 7. Assay Method

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### A. Cell Seeding

#### ***Adherent Cells:***

1. Adherent cells can be seeded directly into the Assay Plate and allowed to attach for several hours to overnight. The optimal cell seeding density is cell type dependent. For suggestions regarding the cell seeding see Appendix. As an example, HeLa cells should be seeded at 50,000 cells per well.
2. The attached cells can be treated as desired with a drug of interest to induce apoptosis. For suggestions regarding the treatment to induce apoptosis, positive and negative controls see Appendix. *Note – When treatment with drug of interest is performed, it is recommended to include wells with untreated cells and cells treated with the vehicle only.*
3. After treatment proceed to Step B1.

***Suspension Cells:***

1. To ensure efficient crosslinking of the suspension cells to Assay Plate, cells must be grown and treated in a different plate or dish of choice (not provided).
2. The treated suspension cells are then transferred to the provided Assay Plate in 100  $\mu$ l of media per well. The cell seeding density of the Assay Plate is cell type-dependent. For suggestions regarding the cell culture and seeding, see Appendix. If necessary, cells can be concentrated by centrifugation and re-suspended in PBS (preferred) or in media to desired concentration. As an example, HL-60 and Jurkat cells should be seeded, respectively, at 300,000 and 200,000 cells per well in 100  $\mu$ l of PBS (preferred) or media. Note – The media should contain no more than 10 % fetal serum otherwise efficiency of the cell crosslinking to the plate may be compromised.
3. After treatment proceed to Step B1.

**B. Cell Fixation**

1. Centrifuge the Assay Plate at 500x g for 8 minutes at room temperature.

2. Immediately add 100 µl of 8 % Paraformaldehyde Solution to the wells of the plate.
3. Immediately centrifuge the plate at 500x g for 8 minutes at room temperature.
4. Incubate for additional 15 minutes.
5. Gently aspirate the Paraformaldehyde Solution from the plate and wash the plate 3 times briefly with 1X PBS. For each wash, rinse each well of the plate with 300 µl of 1X PBS. Finally, add 100 µl of 1X PBS to the wells of the plate. The plate can now be stored at 4°C for several days. Cover the plate with provided seal. For prolonged storage supplement PBS with 0.02% sodium azide. *Note – The plate should not be allowed to dry at any point during or before the assay. Both paraformaldehyde and sodium azide are toxic, handle with care and dispose of according to local regulations.*

### C. Assay Procedure:

*Note – It is recommended to use a plate shaker (~300 rpm) during incubation steps. Any step involving removal of buffer or solution should be followed by blotting the plate gently upside down on a paper towel.*

6. Remove PBS and add 200  $\mu$ l of freshly prepared 1X Permeabilization Buffer to each well of the plate. Incubate 30 minutes.
7. Remove 1X Permeabilization Buffer and add 200  $\mu$ l 2X Blocking Solution to each well of the plate. Incubate 2 hours.
8. Prepare 1X Primary Antibody Solution by diluting 21  $\mu$ l 500X Primary Antibody into 10.5 ml 1X Incubation Buffer.
9. Remove 2X Blocking Solution and add 100  $\mu$ l 1X Primary Antibody Solution to each well of the plate. Incubate overnight at 4 °C.  
*Note – To determine the background signal it is essential to omit primary antibody from at least one well containing cells for each experimental condition.*
10. Remove Primary Antibody Solution and wash the plate 3 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 250  $\mu$ l of 1X Wash Buffer. Do not remove the last wash until step 8.
11. Prepare 1X Secondary Antibody Solution by diluting 12  $\mu$ l 1000X IRDye800-labeled Secondary Antibody into 12 ml 1X Incubation Buffer.

*Note – Protect fluorescently labeled antibodies from light.*

12. Remove the 1X Wash Buffer and add 100 µl 1X Secondary Antibody Solution to each well of the plate. Incubate 2 hours.
13. Remove Secondary Antibody Solution and wash 5 times briefly with 1X Wash Buffer. For each wash, rinse each well of the plate with 250 µl of 1X Wash Buffer. Do not remove the last wash.
14. Wipe the bottom of the plate and the scanner surface with 70% ethanol and scan the plate on the LI-COR® Odyssey® system using 800 channel according to manufacturer's instructions. *Note – The absolute value of the IR signal is dependent on the 800 channel intensity setting. Value 6.0 is recommended for initial scanning.*

#### **D. Whole Cell Staining with Janus Green (Optional)**

*Note – The IR signal can be normalized to the Janus Green staining intensity to account for differences in cell seeding density. It is recommended to use a plate shaker (~300 rpm) during incubation steps.*

1. Remove last 1X Wash and add 50  $\mu$ l of 1X Janus Green Stain per well. Incubate plate for 5 minutes at room temperature.
2. Remove dye, wash plate 5 times in deionized water or until excess dye is removed.
3. Remove last water wash, blot to dry, add 200  $\mu$ l of 0.5 M HCl and incubate for 10 minutes.
4. Measure using a measure OD595 nm using a standard microplate spectrophotometer.

## 8. Data Analysis

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*Note – Analyze data using a suitable data analysis software, such as Microsoft Excel or GraphPad Prism.*

1. Correct the IR800 raw signals (Integrated Intensities) for the background signal by subtracting the mean IR800 of well(s) incubated in the absence of the Primary Antibody from all other IR800 readings.
2. This step is optional. Correct the Janus Green signal for the background signal by subtracting the mean Janus Green

signal of well that do not contain cells from all other Janus Green readings.

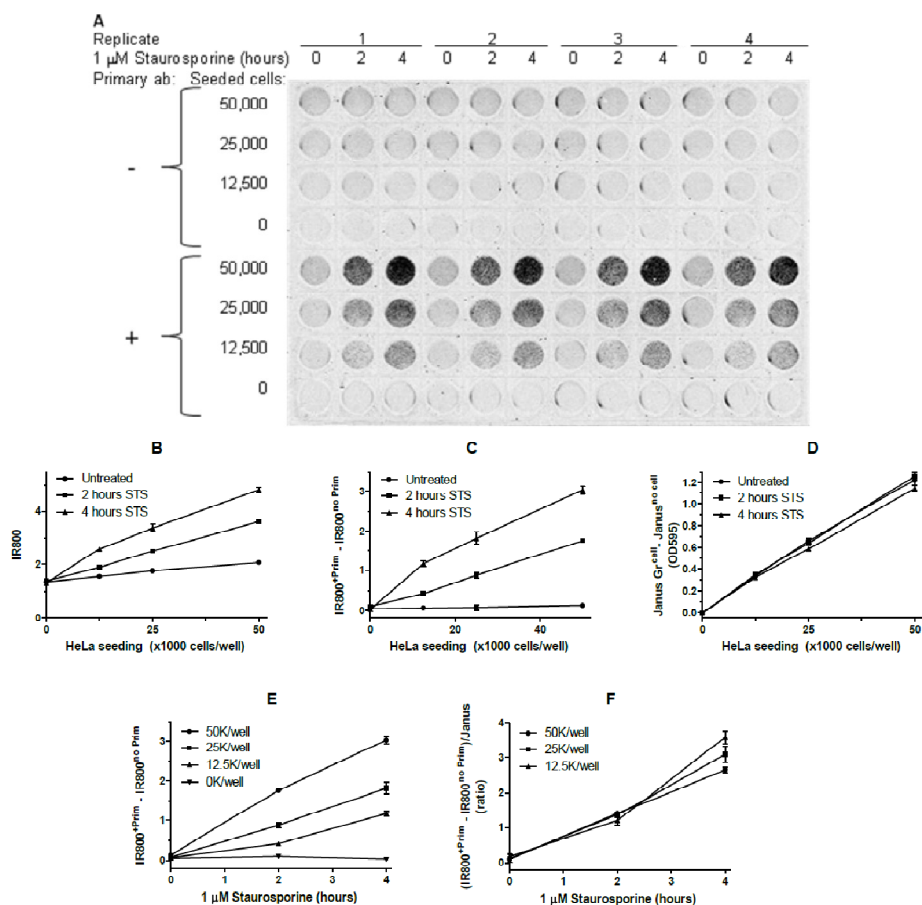
3. Normalize the IR800 signal. Divide the background-corrected IR800 signal by the (background-corrected) Janus Green signal.

#### **A. Utility**

Assay utility can be demonstrated using a drug to induce apoptosis measured as a cleavage of PARP-1. Figure 1 shows the cell number- and time-dependence of PARP-1 (cleaved) ICE analysis of adherent HeLa cells treated with the protein kinase C inhibitor Staurosporine to induce apoptosis. The figure also shows various stages of data analysis. Figure 2 shows determination of Staurosporine IC<sub>50</sub> of PARP-1 cleavage using the ICE in both adherent (HeLa and HepG2) as well as suspension (HL-60) cells treated with Staurosporine. Figure 3 shows the PARP-1 (cleaved) ICE analysis of various adherent (HeLa, HepG2, H196) and suspension (HL-60, Jurkat) cells treated with Staurosporine or Fas antibody. Note the cell type-specific response of cleaved PARP-1 to Staurosporine (Figure 2 and Figure 3).

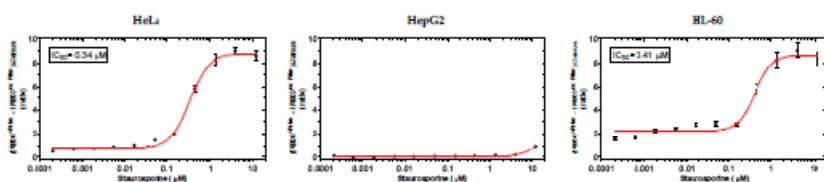


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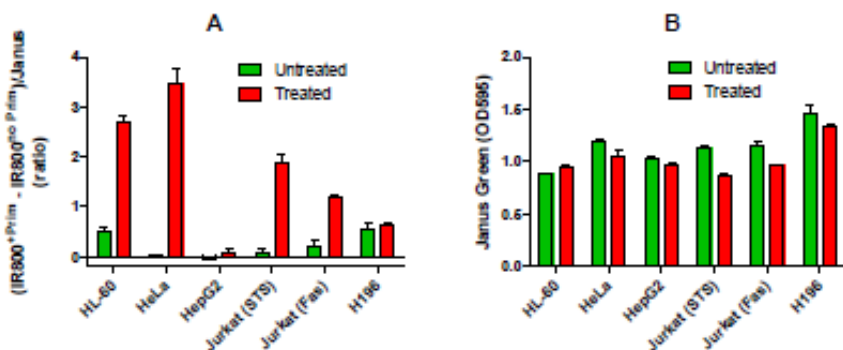


**Figure 1. Cell number- and treatment time-dependence of PARP-1 cleavage in adherent cells induced to undergo apoptosis.** HeLa cells were seeded as indicated in the Assay Plate, allowed to attach overnight, treated with Staurosporine (STS) and fixed. The plate was analyzed by ICE to measure cleaved PARP-1 using ab110215 protocol. (A) Image of the

assay plate. (B-F) Sequential stages of data analysis and various plots. Mean and standard error of the mean ( $n=4$ ) is shown. (B) Raw values of IR800 signal (Integrated Intensities). (C and E) Cleaved PARP-1 IR800 signal after subtraction of background signal (signal in the absence of primary antibody [Prim]). (D) Cell amount measured by Janus Green whole cell stain. Note no or very small differences between Janus Green staining of treated and untreated cells indicating that the treated cells undergoing apoptosis are efficiently crosslinked to the assay plate. (F) Cleaved PARP-1 normalized to cell amount as ratio of IR800 signal (after background signal subtraction) to Janus Green. Note that the normalized cleaved PARP-1 signal is independent of number of cells seeded.



**Figure 2. Determination of Staurosporine  $\text{EC}_{50}$  of the PARP-1 cleavage.** Adherent cell lines (HeLa and HepG2) were seeded at 50,000 per well directly in Assay Plate, allowed to attach overnight and treated for 6 hours with Staurosporine as indicated. Suspension cells (HL-60) were treated for 6 hours with Staurosporine as indicated in a 96 well plate, and about 300,000 of treated cells was directly transferred (in media containing 10% serum) from each well into wells of the Assay Plate. The treated cells were then fixed and the plate was analyzed by ICE to measure the cleaved PARP-1 using MSA43. All steps were performed as described in MSA43 Protocol. Mean and standard error of the mean ( $n=3$ ) of the cleaved PARP-1 normalized to cell amount is shown. The Staurosporine  $\text{EC}_{50}$  of PARP-1 cleavage in HeLa and HL-60 cells are, respectively,  $0.34 \mu\text{M}$  and  $0.41 \mu\text{M}$ . Note HepG2 cells are resistant to undergoing apoptosis when treated for 6 hours with up to  $12 \mu\text{M}$  Staurosporine, consistent with all of our previous observations.



**Figure 3. Cell-line dependent induction of PARP-1 cleavage.** Adherent cell lines were seeded (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) directly in Assay Plate, allowed to attach overnight and treated with 1  $\mu$ M Staurosporine (STS) as indicated. Suspension cells were treated with 1  $\mu$ M STS or 50 ng/mL Fas antibody as indicated and transferred (HL-60 at 300,000 per well, Jurkat at 200,000 per well) in media containing 10% serum to the assay plate. Cells were fixed and the plate was analyzed by ICE to measure the cleaved PARP-1. All steps were performed as described in MSA43 Protocol. Mean and standard error of the mean ( $n=3$ ) is shown. (A) Cleaved PARP-1 normalized to cell amount measured by Janus Green whole cell stain. Note HepG2 and H196 cells are resistant to undergoing Staurosporine-induced apoptosis under these conditions, consistent with all of our previous observations. (B) Cell amount measured by Janus Green. Note no or very small differences between Janus Green staining of treated and untreated cells indicating that the treated cells undergoing apoptosis are efficiently crosslinked to the assay plate.

## B. Reliability

ICE results provide accurate quantitative measurements of antibody binding and hence cellular antigen concentrations.

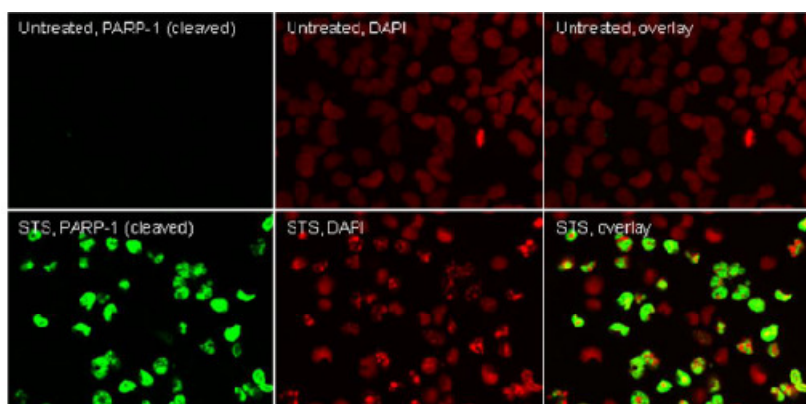
The **coefficient of the intra- and inter-assay of variation** of this assay for HeLa cells seeded between 12,500 and

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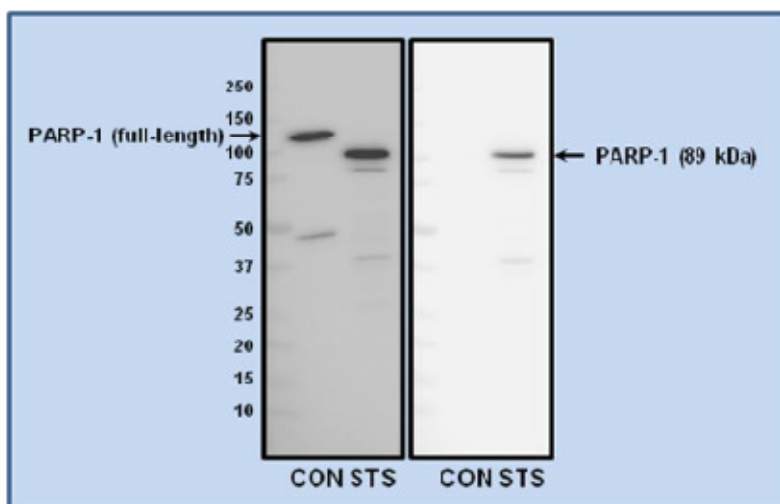
50,000 cells per well and treated for 4 hours with 1  $\mu$ M Staurosporine is less than 15%.

However, ICE does not provide internal confirmation of antibody binding specificity with each experiment, unlike traditional Western blots or immunocytochemistry which allow confirmation by molecular weight or subcellular localization, respectively. Therefore, confidence in antibody specificity is critical to ICE data interpretation. All of Abcam's ICE-validated antibodies have been rigorously tested for specificity by fluorescence immunocytochemistry and by Western blotting under the conditions used for the ICE assay. Examples demonstrating the immunocytochemical and Western blot specificities of the primary antibody used in ab110215 are shown in Figures 4 and 5.

As mentioned in the Introduction section, the adherent cells undergoing apoptosis often detach from the culture plate. The detachment often leads to the cell loss and thus underestimating the proportion of apoptotic cells. Figure 1D and Figure 3B shows that there is no significant (or very minimal) loss of apoptotic cells if cells are fixed according to the protocol provided in ab110215.



**Figure 4. PARP-1 (cleaved) antibody specificity demonstrated by immunocytochemistry.** Immunocytochemical analysis of HeLa cells either untreated or treated with 1  $\mu$ M Staurosporine (STS) for 4 hours to induce apoptosis. The same PARP-1 (cleaved) antibody as in the ab110215 was used in this analysis (in green). Cells well co-stained with DNA stain DAPI (in red). Note that the PARP-1 (cleaved) antibody specifically labels nucleus of only STS-treated cells.



**Figure 5. PARP-1 (cleaved) antibody specificity demonstrated by Western blotting.** Western blot analysis of HeLa cells either untreated (CON) or treated with 1  $\mu$ M Staurosporine (STS) for 4 hours to induce apoptosis. Blots were incubated with an antibody that recognizes both the full-length PARP-1 and its 89 kDa fragment (**left panel**), or with the same PARP-1 (cleaved) antibody as the one used in ab110215 (**right panel**). Appropriate HRP-conjugated secondary antibodies followed by ECL detection were used. Note that the PARP-1 (cleaved) antibody recognizes the apoptosis-specific 89 kDa fragment of PARP-1 but it does not recognize the 113 kDa full-length PARP-1.

## 9. Specificity

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The antibody in this kit reacts with the N-terminal end formed by the cleavage adjacent to Asp214. It recognizes the apoptosis-specific 89 kDa catalytic domain fragment, but it does not recognize full-length PARP-1 or the 24 kDa DNA binding domain fragment.

**Species Reactivity:** Human

## 10. Appendix

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Cell seeding density, culture medium and other growth conditions are important and cell-type specific parameters should be defined by the user.

Adherent cells can be seeded and treated directly in the Assay Plate. The coating of the Assay Plate does not interfere with the cell growth and enhances the cell attachment. Cell attachment can be checked with a microscope. When the cells are fully attached the media can be removed and replaced with media containing drug of interest to induce apoptosis. Culture media containing up to 10% fetal serum does not interfere with the cell fixation and crosslinking to the plate.

Suspension cells should be grown and treated as desired in a separate dish or plate of choice. We recommend simple (not treated)

polystyrene vessels to minimize cell attachment during the cell culture and/or drug treatment. Do not use the provided Assay Plates for cell culturing (including drug treatment) or the efficiency of cell crosslinking to the Assay Plate may be compromised. At the end of the treatment or other experimental condition, the cells are transferred into the Assay Plate in PBS (preferred) or in media containing up to 10% fetal serum. No major difference in the efficiency of crosslinking of HL-60 cells to the Assay Plate was observed whether the cells were in RPMI1640 media containing 10% fetal serum or in 1X PBS. However, media containing more than 10% serum may compromise the cell crosslinking and may lead to significant cell loss.

Since normal healthy cells usually have very low endogenous levels of cleaved PARP-1, we recommend including an appropriate positive control. For HeLa, HL-60 and Jurkat cells we recommend cells treated for 4 hours with 1  $\mu$ M Staurosporine.

The cell seeding density of the Assay Plate is cell type dependent. It depends on the cell size (diameter, in case of the suspension cells; in case of adherent cells on their ability to spread) and the abundance of the target protein. As a general guideline, if the fixed cells form a monolayer, ICE assays using ICE-validated antibodies give robust signals. The cell seeding can be determined experimentally by microscopic observation of cell density of serially diluted cells. For adherent cells, prepare serial dilution of the cells in



a plate (of similar well dimensions) and allow them to attach prior to observation. For suspension cells, prepare a serial dilution of the cells in a plate (of similar well dimensions) and observe the cell density in a microscope after a simple centrifugation step (8 min at 500x g). The provided Assay Plate has flat bottom wells of bottom surface 29.8 mm<sup>2</sup>. Working on the high end of cell densities will generate stronger signals and allow small signal increases to be measured accurately. As an example, HeLa cells should be seeded at 50,000 cells/well, HepG2 cells from 25,000 to 50,000 cells per well, human fibroblasts (HdFN) at 25,000/per well, Jurkat cells at 200,000 cells per well and HL-60 at 300,000 cells per well.

It is essential to omit primary antibody in at least one well (3 wells recommended) to provide a background signal for the experiment which can be subtracted from all measured data. This should be done for each experimental condition.

The assay can be also performed in 384-well plate format. Contact Abcam to inquire about 384 well Assay Plates.

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### **Abcam in the USA**

Abcam Inc  
1 Kendall Square, Ste B2304  
Cambridge,  
MA 02139-1517  
USA

Toll free: 888-77-ABCAM (22226)  
Fax: 866-739-9884

### **Abcam in Europe**

Abcam plc  
330 Cambridge Science Park  
Cambridge  
CB4 0FL  
UK

Tel: +44 (0)1223 696000  
Fax: +44 (0)1223 771600

### **Abcam in Japan**

Abcam KK  
1-16-8 Nihonbashi  
Kakigaracho,  
Chuo-ku, Tokyo  
103-0014  
Japan

Tel: +81-(0)3-6231-094  
Fax: +81-(0)3-6231-0941

### **Abcam in Hong Kong**

Abcam (Hong Kong) Ltd  
Unit 225A & 225B, 2/F  
Core Building 2  
1 Science Park West Avenue  
Hong Kong Science Park  
Hong Kong

Tel: (852) 2603-682  
Fax: (852) 3016-1888