

Fluoro ssDNA Caspase 3 – DNACasp™

A dual antibody assay to detect DNA damage (Single stranded DNA) and active Caspase 3 in Apoptotic Cells

PROTOCOL

Contact Information

Address	Cell Technology Inc 950 Rengstorff Ave Suite D Mountain View CA 94043 USA
Telephone	650-960-2170
Fax	650-960-0367
General Information	info@celltechnology.com
Sales	sales@celltechnology.com
Technical Questions	techsupport@celltechnology.com
Website	www.celltechnology.com



CELL TECHNOLOGY

Introduction

1. Antibody to Single Stranded DNA

A widely used cytochemical technique for evaluation of DNA damage associated with apoptosis is the terminal deoxynucleotidyl transferase-mediated in situ end labeling or TUNEL assay. However the TUNEL assay has its drawbacks in that false positive staining makes the assay unreliable as a marker for apoptosis¹⁻⁵. A more universal and specific marker for detecting apoptosis associated DNA damage is to measure the morphological changes in nuclei that reflect chromatin condensation into compact masses.⁶⁻⁷

Further biochemical and cytochemical studies have demonstrated the increased susceptibility of apoptotic DNA to thermal denaturation. Analysis of nuclei by scanning calorimetry to detect thermal induced DNA denaturation and analysis of DNA fragmentation by electrophoresis have shown that intact apoptotic DNA is susceptible to denaturation at lower temperatures than that of non-apoptotic cells⁸.

2. Antibody to Active Caspase 3

Apoptosis is an evolutionarily conserved form of cell suicide, which follows a specialized cellular process. The central component of this process is a cascade of proteolytic enzymes called caspases. These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in cleavage of protein substrates, causing the disassembly of the cell¹³.

Caspases have been identified in organisms ranging from *C. elegans* to humans. The mammalian caspases play distinct roles in apoptosis and inflammation. In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly (effector caspases), and are involved in upstream regulatory events (initiator caspases). An active caspase consists of two large (~20 kD) and two small (~10 kD) subunits to form two heterodimers which associate in a tetramer¹⁴⁻¹⁶. As is common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity¹⁷.

Caspase 3, also known as CPP-32, Apopain or Yama, is a key effector caspase in the apoptotic pathway¹⁸. It is present in many different cell lineages and is responsible for the cleavage of a variety of molecules such as poly ADP-ribose polymerase (PARP), protein kinase C δ , actin and DNA-dependent protein kinase¹⁹⁻²⁰.

Assay Principle

Cell Technology introduces a dual parameter antibody based assay to detect DNA damage (heat denatured single stranded DNA: ssDNA)⁹⁻¹² and active caspase 3 in apoptotic cells. The assay utilizes a monoclonal antibody generated against ssDNA and a primary rabbit affinity purified polyclonal antibody raised against amino acid 163-175 of murine caspase 3¹². This neo epitope is present on the p18 subunit of cleaved caspase 3²¹.

DNA Damage is detected using the FL1 Channel (Ex: 488nm, Em: 530nm) and Active Caspase 3 using the FL4 Channel (Ex: 633nm, Em: 670nm)

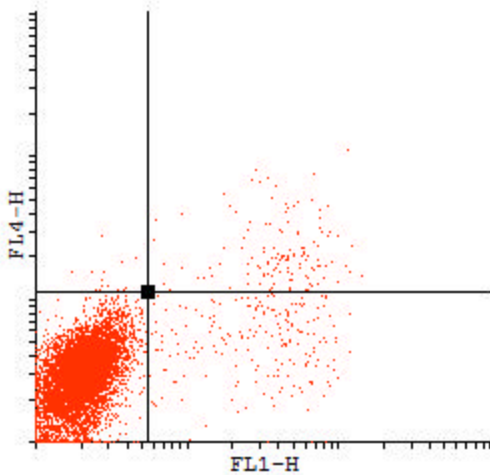


Figure 1 (A)

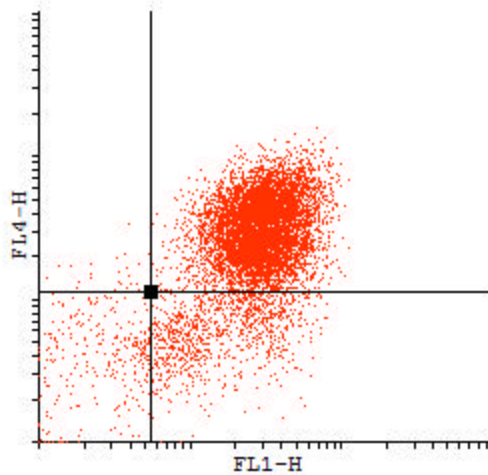


Figure 1 (B)

Fig 1 (A): Negative control: Jurkat cells treated with DMSO for 3 hours and Fig1(B): Positive Control: Jurkat cells treated with 1 μ M staurosporine for 3 hours. The cells were stained with Fluoro ssDNA/caspase 3 kit as described in the protocol. Key: FL 1-H = Anti active ssDNA stain (DNA damage), FL 4-H = anti active caspase 3 stain.

Section A. Warnings and Precautions

1. This kit is only for usage on cell lines or primary cell cultures.
2. For Research Use Only. Not for use in diagnostic procedures.
3. Gloves, protective clothing and eyewear should be worn and safe laboratory practices followed.
4. 1X DNA Denaturing Buffer: **Caution Toxic, irritant use only in vented chemical hood. Read MSDS before using this product.**
Wear protective gloves and clothing when handling.

Section B. Storage and Shelf Life

1. Kit storage conditions: The Apo ss DNA and Active caspase 3 kit has multiple storage conditions, please see individual storage conditions below for long-term storage. If stored and handled properly (see table below) the performance of this product is guaranteed until the expiration date stated on the kit,

Kit Components	Part Number	Long Term Storage Undiluted Material
1. Mouse anti single stranded DNA	1003	-70°C aliquot
2. Rabbit anti active caspase 3	1006	4-8 °C
3. Goat anti Mouse FITC	2006	4-8°C
4. Goat anti Rabbit APC	2007	4-8°C Do not freeze
4. Fixative	3033	-20°C
5. 10 X Wash Buffer	3037	-20°C
6. 1X DNA Denaturing Buffer	3036	Room Temperature

Section C. Additional Materials Required, But Not Supplied

1. Solutions

- a. Casein Block: Pierce Cat# 37532
- b. FACS tubes
- c. Di Water
- d. Phosphate Buffered saline (PBS)

2. Equipment

- a. Flow Cytometer with 488nm laser for excitation and emission in FL1 and FL4 channels.
- b. FACS tubes.
- c. Fluorescent Microscope or plate reader.
- d. Centrifuge.
- e. General laboratory supplies.

Section D. REAGENT PREPARATION

1. Mouse anti ssDNA (Part# 1003) and anti active caspase 3 (Part# 1006)

A. The antibodies are ready to use. Thaw the vials and centrifuge the vial as antibody maybe trapped in the cap. Aliquot the antibody into single use vials. Avoid repeated freeze thaw.

2. Anti secondary labeled antibodies

A. The antibodies are ready to use. Centrifuge the vial as antibody maybe trapped in the cap.

3. Dilution of 10X Wash Buffer.

- A. If necessary warm the 10X Wash Buffer until any salt crystals are completely dissolved.
- B. Make a 1X wash buffer by diluting the 10X 1:10 with Di water (e.g. 1ml 10X Wash Buffer + 9ml Di water).
- C. The diluted buffer can be stored for several weeks at 4-8⁰C.

4. Fixative.

A. Ready to use. Store at -20⁰C until ready to use, the fixative will be added (as mentioned below in section E-3) to the samples at -20⁰C.

6. Denaturation Buffer

A. Ready to use. Store at room temperature.

Section E. Assay

E-1: Suspension cells.

1. Cells should be cultured to a density not to exceed 1x 10⁶ cells/mL.
Note: Each cell line should be evaluated on an individual basis to determine optimal density for cell culture and apoptosis induction.
3. Induce apoptosis according to your specific protocol or add test compounds.

Negative control - (solvent that was used to dissolve your test compounds) should also be set up at this time point).

4. After the required activation time transfer the cells for each test sample to any suitable tube.
5. Proceed to the 'Fixation' section (E3) section below.

E-2: Adherent Cells

1. Culture cells to confluence.
2. Activate cells according to your protocol.
3. After the required activation time according to your experimental protocol, detach the cells according to standard tissue culture techniques (Trypsin EDTA, Accutase: Sigma Cat# A6964). Keep in mind that adherent apoptotic cells may have detached and are floating in the media. To recover these cells collect the supernatant and spin at ~500g for 5-10 minutes for the cells to pellet. Mix with the rest of the harvested cells.
4. Proceed to the 'Fixation' section (E3) below.

E-3: Fixation

1. Wash the cells (~500g for 5-10') with 2mL of PBS twice.
3. After the final wash, decant the supernatant and gently vortex the cell pellet.
4. Add 1mL of the fixative (at -20°C) while vortexing the sample.
- 5 Allow the samples to fix for 24 hours at -20°C . The samples may be stored, in the fixative solution, for up to 72 hours in this fixative at -20°C .
6. Spin the samples and pipette out the fixative solution.
7. Vortex the cell pellet gently and add 250 μL of 1X DNA Denaturing Buffer, gently vortex the samples again.
Note: After the addition of the DNA denaturing buffer the cell will become translucent and it maybe difficult to see a cell pellet at step 9 below.
8. Place the samples in a water bath between $70-80^{\circ}\text{C}$ for 10 minutes with gently agitation every five minutes.
9. After heating, allow the samples to reach room temperature (~15') and spin down the sample.
10. Pipette out the 1X DNA Denaturing Buffer.
11. Add 1mL of the diluted 1% block buffer. Allow the samples to block for 15-30 minutes at room temperature.
12. Wash the samples two times with 2mL of 1X wash buffer (prepared from above).

E-4. Staining

1. After the final wash, decant the wash buffer, vortex the cell pellet and add 10uL of the anti ssDNA and anti active caspase 3 antibodies to the samples. Gently vortex the sample and stain for 30-60 minutes at room temperature.

Note: Each investigator should optimize staining time and concentration for their particular experimental protocol. Titrate out the staining concentration of the antibodies for optimal results.

2. Wash the samples two times with 2mL of 1X Wash Buffer to remove excess antibody.
3. After the final wash, decant the supernatant and vortex the cell pellet. Add 10µL of the secondary labeled antibodies. Vortex and stain for 30-60 minutes at room temperature in the dark.

After staining wash the samples two times with 2mL of 1X Wash Buffer to remove excess antibody.

Note: Each investigator should optimize staining time and concentration for their particular experimental protocol. Titrate out the staining concentration of the secondary antibodies for optimal results.

4. After the final washes decant the supernatant and add 300-500µL of 1X wash buffer.
5. Samples are ready for flow analysis – see section F below

Section F. Sample Analysis

Flow Cytometer

Analyze samples using two color histogram;

DNA Damage Detection - Ex: 488 nm; Em: 530 nm - FL-1

Active caspase 3 Detection - Ex: 633 nm; Em: 670 nm (LP) - FL-4.

Fluorescent Plate Reader

Analyze samples;

DNA Damage Detection - Ex: 488nm; Em: 515-530 nm

Active Caspase 3 Detection- Ex: 633 nm; Em: 660 nm (peak)

Please note that if your plate reader is a bottom reader you will need to use black clear bottom plates.

Fluorescent Microscope.

Analyze samples;

DNA Damage Detection - Ex: 488nm; Em: 515-530 nm

Active Caspase 3 Detection- Ex: 633 nm; Em: 660 nm (peak)

References

1. Charriaut-Marlangue C, Ben-Ari J (1995) A cautionary note on the use of TUNEL to determine apoptosis. *NeuroReport* 7:61–64
2. Grasl-Kraipp B, Ruttkau-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R (1995) *In situ* detection of fragmented DNA (TUNEL) fails to discriminate among apoptosis, necrosis and autolytic cell death: a cautionary note. *Hepatology* 21:1465–146
3. Didenko VV, Hornsby PJ (1996) Presence of double-stranded DNA breaks with single-base 3' overhangs in cells undergoing apoptosis but not necrosis. *J Cell Biol* 135:1369–1376
4. Ohno M, Takemura G, Ohno A, Misao J, Hayakawa Y, Minatoguchi S, Fujiwara T, Fujiwara H (1998) "Apoptotic" myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with *in situ* nick end-labeling. *Circulation* 98:1422–1430.
5. Stadelmann C, Bruck W, Bancher C, Jellinger K, Lassmann H (1998) Alzheimer disease: DNA fragmentation indicates increased neuronal vulnerability, but not apoptosis. *Neuropathol Exp Neurol* 57:456–464
6. Willingham MC (1999) Cytochemical methods for the detection of apoptosis. *J Histochem Cytochem* 47:1101–1109
7. Zamzani N, Kroemer J (1999) Condensed matter in cell death. *Nature* 401:127–128.
8. Allera C, Lazzarini G, Patrone E, Alberti I, Barboro P, Sanna P, Melchiori A, Parodi S, Balbi C (1997) Condensation of chromatin in apoptotic thymocytes shows a specific structural change. *J. Biol Chem* 272:10817–10822.
9. Frankfurt OS (1990) Decreased DNA stability in cells treated with alkylating agents. *Exp Cell Res* 191:181–185.
10. Frankfurt OS, Robb JA, Sugarbaker EV, Villa L (1996) Monoclonal antibody to single-stranded DNA is a specific and sensitive cellular marker of apoptosis. *Exp Cell Res* 226:387–397.
11. Frankfurt OS (1994) Detection of apoptosis in leukemic and breast cancer cells with monoclonal antibody to single-stranded DNA. *Anticancer Res* 14:1861–1870.
12. Oskar S. Frankfurt and Awtar Krishan (2001). Identification of Apoptotic Cells by Formamide-induced DNA Denaturation in Condensed Chromatin. *The Journal of Histochemistry & Cytochemistry* Volume 49(3): 369–378, 2001
13. Slee, E. A., C. Adrain, and S. J. Martin. 1999. Serial Killers: ordering caspase activation events in apoptosis. *Cell Death and Differ.* 6:1067-1074.
14. Walker, N. P., R. V. Talanian, K. D. Brady, L. C. Dang, N. J. Bump, C. R. Ferenz, S. Franklin, T. Ghayur, M. C. Hackett and L. D. Hammill. 1994. Crystal Structure of the Cysteine Protease Interleukin-1 β -Converting Enzyme: A (p20/p10) $_2$ Homodimer. *Cell* 78:343-352.

15. Wilson, K. P., J. F. Black, J. A. Thomson, E. E. Kim, J. P. Griffith, M. A. Navia, M. A. Murcko, S. P. Chambers, R. A. Aldape, S. A. Raybuck, and D. J. Livingston. 1994. Structure and mechanism of interleukin-1 beta converting enzyme. *Nature* 370: 270-275.
16. Rotonda, J., D. W. Nicholson, K. M. Fazil, M. Gallant, Y. Gareau, M. Labelle, E. P. Peterson, D. M. Rasper, R. Ruel, J. P. Vaillancourt, N. A. Thornberry and J. W. Becker. 1996. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nature Struct. Biol.* 3(7): 619-625.
17. Kumar, S. 1999. Mechanisms mediating caspase activation in cell death. *Cell Death and Differ.* 6: 1060-1066.
18. Alnemri, E.S. et al (1996) *Cell* 87:171
19. *Trends Biochem Sci* 22,388 (1997)
20. *Nature* 376, 37 (1995).
21. Srinivasan A, et al *Cell Death and Differentiation* (1998) 5: 1004-1016