CELL TECHNOLOGY

Apo ssDNA

An antibody assay to detect DNA damage (single stranded DNA) in Apoptotic Cells

PROTOCOL

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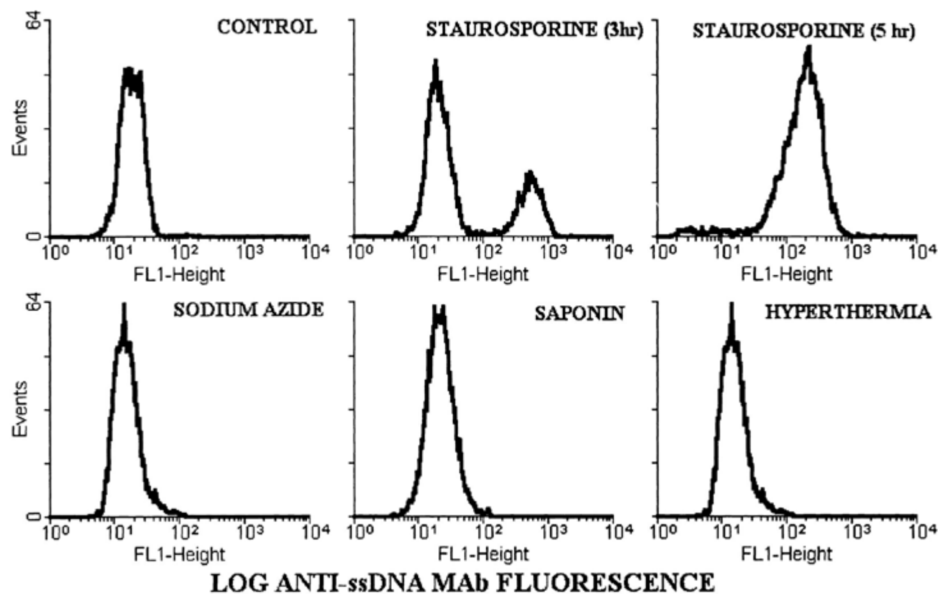
Introduction

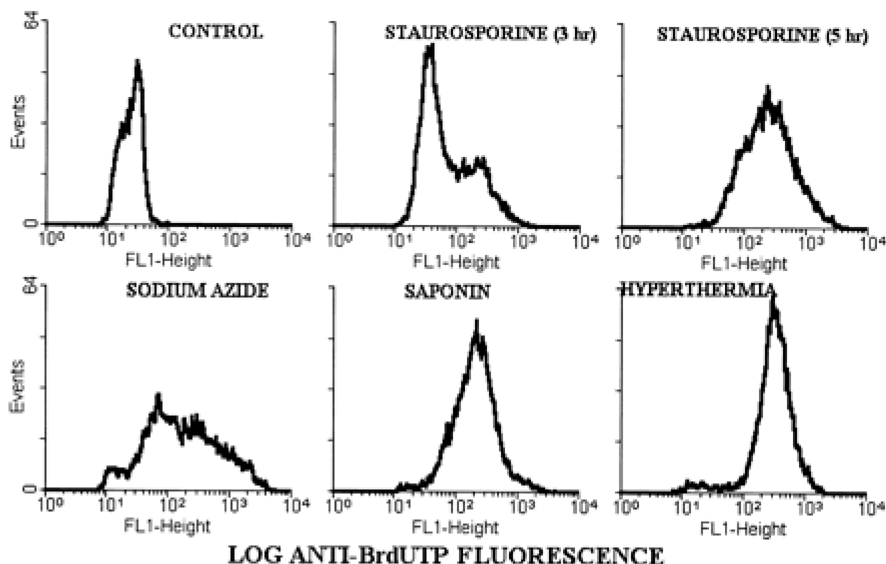
A widely used cytochemical technique for evaluation of DNA damage associated with apoptosis is the terminal deoxynucleotidyl transferase-mediated in situ end labeling or TUNEL assay. However the TUNEL assay has its drawbacks in that false positive staining makes the assay unreliable as a marker for apoptosis (see figure 1) [1-5]. A more universal and specific marker for apoptosis associated DNA damage is 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 then that of non-apoptotic cells [8].

Assay Principle

Cell Technology introduces Apo ssDNA, an antibody based assay to detect DNA damage (single stranded DNA: ssDNA) in apoptotic cells.
The assay utilizes an antibody generated against ssDNA. This antibody recognizes large stretches of heat-denatured ssDNA only in apoptotic cells [9-12] (see Figure 1).



(A) Monoclonal anti ssDNA



(B) TUNEL Assay

Figure 1.
A. MAb to ssDNA staining of apoptotic but not of necrotic cells. Fluorescence distributions of MDA-468 cells heated and stained with MAb F7-26 were generated on a flow cytometer. Apoptosis was induced by staurosporine and necrosis was induced by sodium azide, saponin, or hyperthermia. Note that apoptotic cells are intensely stained with the MAb, whereas the fluorescence profiles of necrotic and control cells are similar. **B.** TUNEL staining of apoptotic and necrotic cells. Fluorescence distributions of MDA-468 cells stained with TUNEL were generated on a flow cytometer. Apoptosis was induced by staurosporine and necrosis was induced by sodium azide, saponin, or hyperthermia. Note that cells at early stage of apoptosis (staurosporine 3 hr) are weakly stained, whereas late apoptotic cells (staurosporine 5 hr) and necrotic cells are intensely stained by TUNEL.

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. Store the kit at -20°C for short term (1 –2 weeks). 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. Anti mouse IgM FITC labeled	2005	-20°C
3. Fixative	3033	-20°C
4. 10 X Wash Buffer	3037	-20°C
5. 1X DNA Denaturing Buffer	3036	-20°C

Cat # ApoDNA-1 – (25 Tests), Cat# ApoDNA-2 (100 Tests).

Section C. Additional Materials Required, But Not Supplied

1. Solutions
    a. Casein Block: Pierce Cat# 37532
    b. PBS (pH 7.0 – 7.4)

2. Equipment
    a. Flow Cytometer with 488nm laser for excitation and emission in FL1 and FL2 channels.
    b. FACS tubes.
    c. Fluorescent Microscope or plate reader.

Section D. REAGENT PREPARATION

1. Mouse anti ssDNA (PRIMARY ANTIBODY)

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

2. Anti mouse IgM FITC labeled (SECONDARY ANTIBODY)

A. Spin down vial as antibody may be trapped in the tube cap. Recommended dilutions**:** Dilute amount just enough, as per your experimental requirement. Dilute the stock antibody 1:10 in with the 1XWash Buffer (see step 3 below). For example to 90 □l of 1 XWash Buffer add 10□l of the anti mouse IgM FITC labeled. Mix by gentle vortexing. Use 10ul of the diluted antibody for every 100ul of cells such that the final dilution of the secondary antibody is 1:100.
Note: The diluted primary and secondary antibody can be stored frozen at –20C for several weeks. Avoid freeze thaw cycles.

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 4-8^{0}C.

4. Fixative.

A. Ready to use. Store at –20^{0}C until ready to use, the fixative will be added (as mentioned below in section E-3) to the samples at –20^{0}C.

6. Denaturation Buffer

A. Ready to use. Store at -20°C.

Section E. Assay

E-1: Suspension cells.

1. Cells should be cultured to a density not to exceed 1x 10^6 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 treated 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). 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^{0}C) while vortexing the sample.

5 Allow the samples to fix for 24 hours at –20^{0}C. The samples may be stored for up to 72 hours in this fixative at –20^{0}C.

6. Spin the samples and pipette out the fixative solution.

7. Vortex the cell pellet gently and add 250 □L 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^{0}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 Casein 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 antibody. Gently vortex the sample and stain for 15-60 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 anti ssDNA antibody 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 10 □L of the 1:10 DILUTED Goat anti Mouse IgM FITC labeled antibody. Vortex and stain for 30 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 Goat anti Mouse IgM FITC antibody for optimal results.

4. After the final washes decant the supernatant and add 300-500□Lof 1Xwash buffer.

5. Samples are ready for flow analysis – see section F below

Section F. Sample Analysis

Flow Cytometer

Analyze samples using single color histogram; Ex: 488nm Em: FL1 (FITC Channel).

Fluorescent Plate Reader

Analyze samples; Ex: 488nm Em: 515-530

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

Fluorescent Microscope.

Analyze samples; Ex: 488nm Em: 515-530nm.

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