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ab66110
In situ BrdU-Red DNA Fragmentation (TUNEL) Assay Kit

For the detection of DNA fragmentation in apoptosis by flow cytometry and fluorescence microscopy in live cells.

View kit datasheet: www.abcam.com/ab66110
(use www.abcam.cn/ab66110 for China, or www.abcam.co.jp/ab66110 for Japan)

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

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1. Overview

In situ BrdU-Red DNA Fragmentation (TUNEL) Assay Kit (ab66110) is a convenient and sensitive method to detect DNA fragmentation by flow cytometry and fluorescence microscopy in live cells. The kit uses Br-dUTP (bromolated deoxyuridine triphosphate nucleotide), which can be more readily incorporated into DNA strand breaks that other dUTP labels such as fluorescein, biotin or dioxigenin. The greater incorporation rate produces a brighter signal when the Br-dUTP sites are detected with an anti-BrdU monoclonal antibody directly labeled with a red fluorochrome.

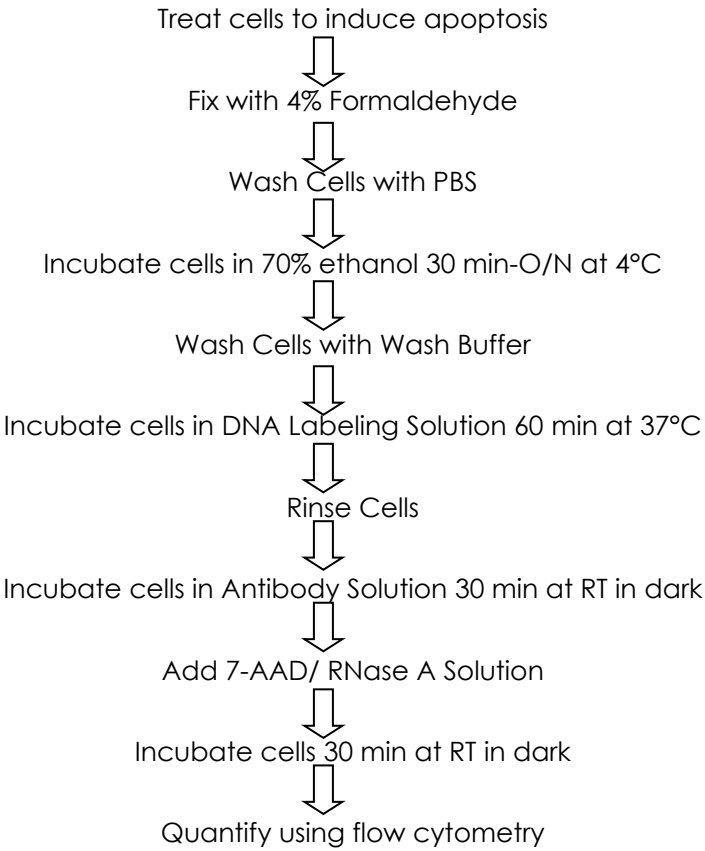
The kit includes cells that can be used as positive or negative control.

This assay is especially suitable for studying DNA fragmentation in GFP-transfected cells.

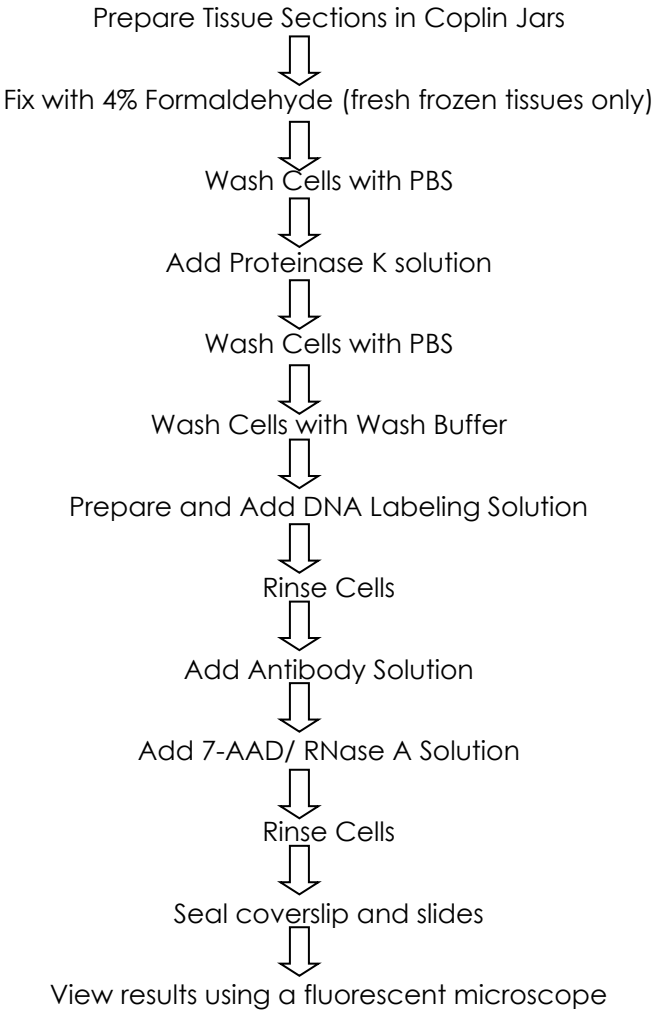
Internucleosomal DNA fragmentation is a morphological hallmark of end stages of apoptosis in mammalian cells.

Traditionally, DNA fragmentation have been studied by examining fragmented genomic DNA on an agarose gel. This method is semi-quantitative and can be tricky to perform. An alternative method for detecting DNA fragmentation involves the identification of nicks (or strand breaks) using TUNEL (TdT dUTP nick end labeling) labeling. TUNEL is based on the ability of terminal deoxynucleotidyl transferase (TdT) to end-label nicks in the DNA caused by the caspase-activated DNase. TdT has the ability to label 3' blunt ends of double stranded DNA with deoxyuridine, independently of the template.

2. Protocol Summary – Flow cytometry



3. Protocol Summary – IHC detection



4. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

5. Storage and Stability

Store components of kit at -20°C or 4°C in the dark immediately upon receipt, according to storage conditions described in Materials Supplied section. Kit has a storage time of 1 year from receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

6. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

7. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Positive Control Cells	5 mL	-20°C	-20°C
Negative Control Cells	5 mL	-20°C	-20°C
Wash Buffer	120 mL	4°C	4°C
Reaction Buffer	0.6 mL	4°C	4°C
TdT Enzyme	45 µL	-20°C	-20°C
Br-dUTP	0.48 mL	-20°C	-20°C
Rinse Buffer	120 mL	4°C	4°C
Anti-BrdU (red) antibody	0.3 mL	4°C	4°C
7-AAD/RNase Staining Buffer	30 mL	4°C	4°C

8. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Flow cytometer capable of detecting fluorescence at Ex/Em =488/576 nm (orange, TRITC channel) and Ex/Em = 488/655 nm (red channel, 7-AAD)
- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Paraformaldehyde solution
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 12 x 75 mm test tubes designed for flow cytometer
- General tissue culture supplies
- 70% Ethanol

For IHC-P protocol:

- Fluorescence microscopy capable of measuring fluorescence at Ex/Em =488/576 nm (orange, TRITC channel) and Ex/Em = 488/655 nm (red channel, 7-AAD)
- Coplin jars
- Glass slides
- Xylene
- Ethanol in the following percentages: 100% - 95% - 85% - 70% - 50%
- Coverslips (plastic and glass)
- 0.85% NaCl solution
- 10 mg/ml Proteinase K
- 100 mM Tris-HCl, pH 8.0 + 50mM EDTA solution
- (Optional) Anti-fading mounting solution - we recommend Fluoroshield Mounting Medium (ab104136)
- (Optional) Nail polish or rubber cement

9. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

10. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

10.1 Positive Control Cells (HL-60 cells treated with camptothecin):

Ready to use as supplied. Thaw cells on ice. Take 1 mL aliquot (1 x 106 cells/mL) to use as positive control in the assay. Aliquot the rest of the cells in 1 mL aliquots and store at -20°C.

10.2 Negative Control Cells (HL-60 cells):

Ready to use as supplied. Thaw cells on ice. Take 1 mL aliquot (1 x 106 cells/mL) to use as positive control in the assay. Aliquot the rest of the cells in 1 mL aliquots and store at -20°C.

10.3 Wash Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10.4 Reaction Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10.5 TdT Enzyme:

Ready to use as supplied. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C.

10.6 Br-dUTP:

Ready to use as supplied. Aliquot Br-dUTP so that you have enough volume to perform the desired number of assays. Store at -20°C.

10.7 Rinse Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10.8 Anti-BrdU antibody:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

10.9 7-AAD/RNase Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C.

11. Assay Procedure – Flow cytometry

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density.
- Control cells included in the kit are already fixed. You can start using them from Step 11.3.2. Thaw cells on ice.

11.1 Grow and treat cells:

11.1.1 Grow cells (adherent or suspension) and induce apoptosis by your desired method.

Δ Note: Concurrently incubate a control culture without induction.

11.1.2 Collect 1-5 x 10^6 cells and resuspend in 0.5 mL PBS.

11.1.2.1 Suspension cells: collect cells by centrifugation.

11.1.2.2 Adherent cells: trypsinize cells in trypsin/EDTA for 1-2 min, stop trypsinization by adding culture medium and pellet by centrifugation.

Δ Note: take cell supernatant from adherent cell culture and centrifuge together with trypsinized cells.

11.2 Cell fixation:

11.2.1 Fix the cells by adding 5 mL of 4% paraformaldehyde (w/v).

11.2.2 Place cells on ice for 15 minutes.

11.2.3 Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.

11.2.4 Wash cells in 5 mL of ice-cold PBS. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.

11.2.5 Repeat washing step one time.

11.2.6 Re-suspend cells in 0.5 mL PBS

11.2.7 Add cells to 5 mL of ice-cold 70% (v/v) ethanol. Let cells stand for 30 minutes – overnight on ice (or at -20°C).

11.3 Label cells:

11.3.1 Resuspend fixed cells by swirling the vials.

11.3.2 Remove 1 mL aliquots of the cell suspension at 10^6 cells/mL and place 12 x 75 mm tubes.

Δ Note: use this procedure for control cells included in the kit.

11.3.3 Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and carefully aspirate ethanol.

11.3.4 Resuspend cells in 1 mL Wash Buffer. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and aspirate supernatant carefully.

11.3.5 Repeat washing step one time.

11.3.6 Resuspend cells in 50 µL of the DNA Labeling Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
TdT Reaction Buffer	10	100
TdT Enzyme	0.75	75
Br-dUTP	8	80
ddH_2O	32.25	322.5
TOTAL VOLUME	51	510

11.3.7 Incubate cells in DNA labeling solution for 60 minutes at 37°C. Shake cells every 15 minutes to resuspend.

11.3.8 Add 1 mL of Rinse Buffer to each tube. Centrifuge for 5 minutes at 300 xg in a cold centrifuge and aspirate supernatant.

11.3.9 Repeat rinsing step one more time.

11.3.10 Resuspend cells in 100 µL of Antibody Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
Anti-BrdU-Red antibody	5	50
Rinse Buffer	95	950

11.3.11 Incubate cells in the Antibody Solution in the dark for 30 minutes at room temperature.

11.4 Detection by flow cytometry:

11.4.1 Add 500 µL of 7-AAD/RNase A solution.

11.4.2 Incubate cells in the dark for 30 minutes at room temperature.

11.4.3 Analyze cells by flow cytometry: Ex/Em = 488/576 nm for BrdU-Red and Ex/Em = 488/655 nm for 7-AAD.

Δ Note: analyze cells within 3 hours of staining.

ALTERNATIVE PROTOCOL FOR GFP-TRANSFECTED CELLS

Ethanol will affect the GFP signal. To detect DNA fragmentation in GFP-expressing cells, we recommend the following alternative protocol:

- Proceed with general protocol until Step 11.1.2
- Fix cell by adding 5 mL of 1% paraformaldehyde/PBS and incubate on ice 15 minutes
- Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Wash cells in 5 mL of ice-cold PBS. Centrifuge cells for 5 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Repeat washing step one time.
- Wash cells in 5 mL of ice-cold PBS.
- Resuspend cells in 2 mL of the following detergent solution:

  PBS solution (pH 7.2-7.4)

  0.1% Triton X-100

  4% FBS
- Incubate cells in detergent solution 3-5 minutes at room temperature.
- Centrifuge cells for 10 minutes at 300 x*g* in a cold centrifuge and discard supernatant.
- Continue with Step 11.3.6 from the general protocol.

Δ Note: we recommend performing an optimization run with control cells (GFP-transfected untreated cells) and look at the GFP signal. You might need to vary incubation times and paraformaldehyde percentages to suit your experimental settings.

12. Assay Procedure – IHC detection

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Each cell line should be evaluated on an individual basis to determine the optimal cell density.

12.1 Deparaffinization and rehydration protocol:

Δ Note: this section describes the preparation of formalin-fixed, paraffin-embedded apoptotic tissue section mounted on glass slides. If using fresh-frozen tissue sections, proceed directly to Step 12.2.1.

12.1.1 Remove paraffin by immersing slides in a Coplin jar containing fresh xylene. Incubate 5 minutes at room temperature.

12.1.2 Repeat previous step in a second Coplin jar containing fresh xylene.

12.1.3 Immerse slides in a Coplin jar containing 100% ethanol and incubate 5 minutes at room temperature.

12.1.4 Re-hydrate slides by sequential 3-minutes/room temperature incubations in Coplin jars containing:

- 100% ethanol
- 95% ethanol
- 85% ethanol
- 70% ethanol
- 50% ethanol

12.1.5 Immerse slides in a Coplin jar containing 0.85% NaCl and incubate 5 minutes at room temperature.

12.1.6 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at room temperature.

12.1.7 Proceed to Step 12.2.3

12.2 Tissue section preparation:

12.2.1 FOR FRESH-FROZEN TISSUE SECTIONS ONLY: fix slides by immersing them in a Coplin jar containing fresh 4% formaldehyde/PBS (w/v). Incubate for 15 minutes at room temperature.

12.2.2 Wash slides by immersing them in a Coplin jar containing PBS and incubate 5 minutes at room temperature.
12.2.3 Repeat washing step in a new Coplin jar containing fresh PBS. Allow liquid to drain thoroughly and place slides on a flat surface.
12.2.4 Prepare 1 mL of 20 µg/mL Proteinase K solution (2 µL Proteinase K 10 mg/mL + 998 µL Tris-HCl pH 8.0 + 50 mM EDTA).
12.2.5 Cover each tissue section with 100 µL and incubate 5 minutes at room temperature.
12.2.6 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at room temperature.
12.2.7 Transfer slides to a Coplin jar containing 4% formaldehyde/PBS and incubate 5 minutes at room temperature.
12.2.8 Immerse slides in a Coplin jar containing PBS and incubate 5 minutes at room temperature.

12.3 Label cells:

12.3.1 Remove slides from PBS and tap gently to remove excess liquid. Cover section with 100 µL of Wash Buffer.
12.3.2 Using forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid and incubate 5 minutes at room temperature. Remove plastic coverslip and gently tap the slides to remove excess liquid.
12.3.3 Repeat Step 12.3.2 one more time. Carefully blot dry around the edges with tissue paper.
12.3.4 Cover slides with 50 µL of the DNA Labeling Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
TdT Reaction Buffer	10	100
TdT Enzyme	0.75	7.5
Br-dUTP	8	80
ddH_2O	32.25	322.5
TOTAL VOLUME	51	510

12.3.5 Using forceps, gently place a piece of plastic coverslip on top pf the cells to evenly spread the liquid.
12.3.6 Place slides in a dark humidified 37°C incubator for 1 hour.

Δ Note: ensure high humidity by placing wet paper towels in the bottom of the dry incubator.

12.3.7 Remove the plastic coverslips with forceps. Rinse slides in a fresh Coplin jar filled with PBS for 5 minutes.

12.3.8 Repeat Step 12.3.7 one more time. Carefully blot dry around the edges with tissue paper.

12.3.9 Cover slides with 100 µL of Antibody Solution, prepared as described in the table below:

Component	1 test (µL)	10 tests (µL)
Anti-BrdU-Red antibody	5	50
Rinse Buffer	95	950

12.3.10 Using forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid. Incubate slides in the Antibody Solution in the dark for 30 minutes at room temperature.

12.4 Detection by fluorescence microscopy:

12.4.1 Wash cells by transferring slides to a fresh Coplin jar filled with ddH_2O and incubate for 5 minutes at room temperature.

12.4.2 (Optional DNA counterstaining) Add 100 µL of 7-AAD/RNase A Staining Buffer.

Δ Note: other DNA stains such PI or DRAQ5™ can be used.

12.4.3 Using forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid. Incubate slides in the Staining Buffer in the dark for 30 minutes at room temperature.

12.4.4 Wash cells by transferring slides to a fresh Coplin jar filled with ddH_2O and incubate for 5 minutes at room temperature.

12.4.5 Repeat previous washing step.

12.4.6 (Optional) Add a drop of anti-fading solution and cover the treated portion of the slide with a glass coverslip.

12.4.7 (Optional) Seal the edges of the coverslip with rubber cement or clear nail polish.

12.4.8 Analyze cells by fluorescence microscopy at Ex/Em = 488/576 nm (BrU-Red) and Ex/Em = .488/655 nm (if using 7-AAD).

Δ Note: View slides as soon as possible. Analyze cells within 3 hours of staining.

13. Data Analysis

FOR FLOW CYTOMETRY

The following are general guidelines. Specific methods of analysis will vary with different flow cytometer analysis programs.

- Establish appropriate FSC vs SSC gates to exclude debris and cell aggregates.
- Collect BrdU-Red and 7-AAD fluorescence in the appropriate channels (typically, FL2 and FL3 respectively).
- Using fluorescence intensity, determine fold change between control and treated cells.

FOR FLUORESCENCE MICROSCOPY

- We recommend acquiring several images per well.
- We recommend data analysis after coding and mixing images to ensure unbiased results.
- For manual analysis, if you do not have a specific software installed in your microscope, you can download ImageJ, an open source image processing designed for scientific multidimensional images by the National Institute of Health (NIH).

14. Typical data

Data provided for **demonstration purposes** only.

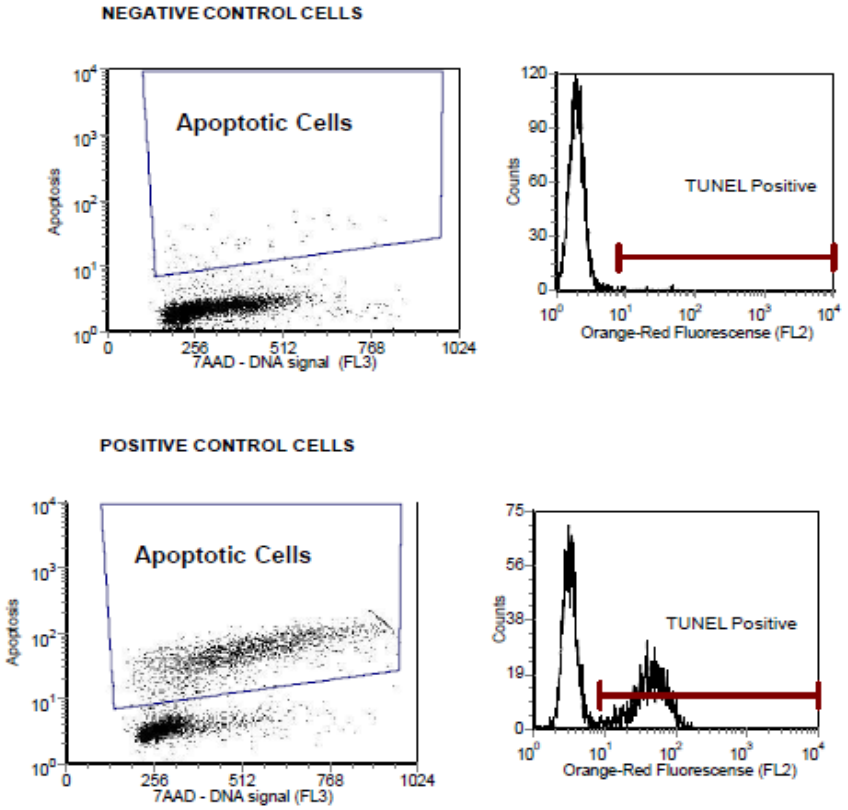


Figure 1. Detection of DNA fragmention (TUNEL staining) using the negative and positive control cells (HL-60 untreated and treated with camptothecin). Cells were stained following the assay protocol. The fluorescence signal was detected and analyzed using BD FACScan System (Becton Dickinson).

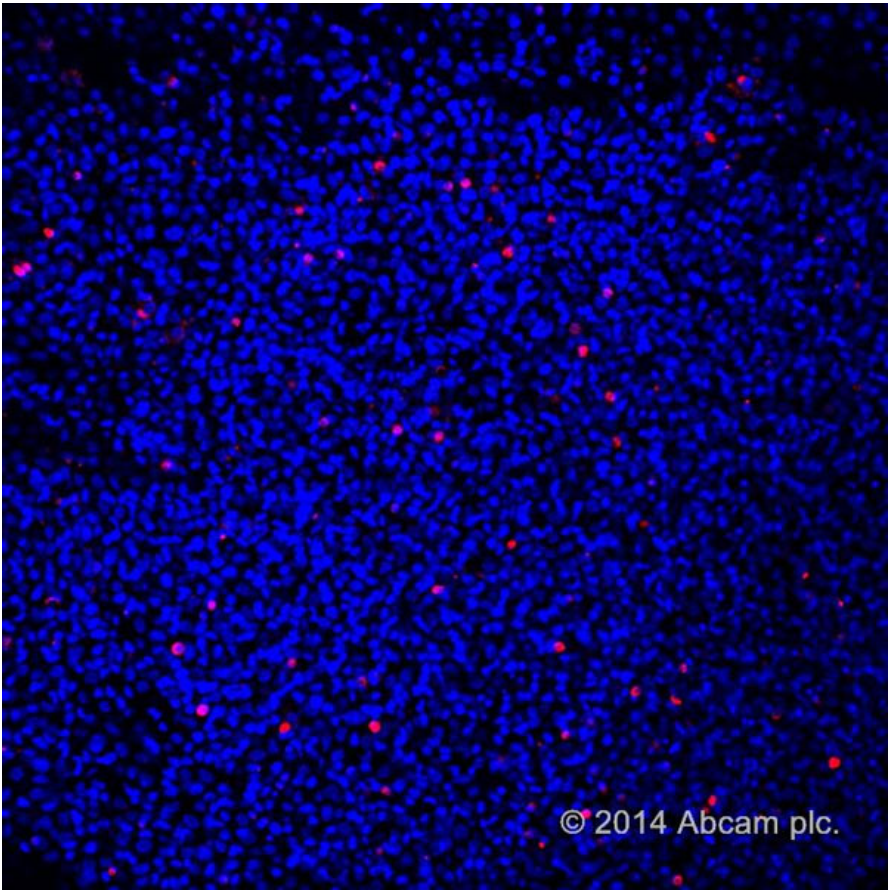


Figure 2. TUNEL staining in whole mount *Hydractinia echinata.* Animals were fixed in 4% paraformaldehyde/PBS and processed as per assay protocol without proteinase K treatment. Instead of proteinase K, animals were permeabilized with 3% Triton/PBS for 15 minute. Red dots represent DNA fragmentation/TUNEL staining. Animals were counter-stained with DAPI (blue). *Image courtesy of James Gahan, submitted as part of Abcam's Abreview program.*

15. FAQs

Q. Can you give me any tips to perform this assay successfully?

A. Here are some tips:

- If using adherent cells, cells remaining in the supernatant have a higher probability of being apoptotic than do the cells adhering to the bottom of the plate or flask. Save cells in the supernatant for the assay prior to the trypsinization step.
- Cell fixation using a DNA crosslinking chemical fixative is an important step in analyzing apoptosis. Unfixed cells may lose smaller fragments of DNA that have not been chemically fixed inside the cell during the washing steps.
- To minimize loss during the assay, restrict the assay to the use of a single 12 x 75 mm test tube. If polystyrene plastic test tubes are used, an electrostatic charge can build up on the sides of the tube. Cells will adhere to the side of the tube and the sequential use of multiple tubes can result in significant cell loss during the assay.
- Occasionally a mirror image population of cells at lower intensity is observed in the flow cytometry dual parameter display. The population appears because during the DNA labeling reaction some cells have become stuck to the side of the test tube and are not fully exposed to the reaction solution. This issue can be overcome by washing all the cells from side of the tube and properly resuspend the cells at the beginning of the labeling reaction.

Q. Can I perform a staining for detecting surface marker by flow at the same time?

A. Staining of proteins such as cellular surface antigens can be accomplished by first incubating the cells with the fluorescent labeled antibody and then using a commercially available fixative and permeabilization solution to rapidly fix and permeabilize the cells in preparation for the assay.

Q. Can I do dual staining for microscopy assay?

A. Yes, it is possible. It is preferable to follow these recommendations:

- For the second target, use an antibody that works best with proteinase K retrieval. We recommend that you run a control test with the same sample and antibody for the other target and see if proteinase K can retrieve the antigen.
- Use a fluorophore-based detection system for the second target as opposed to an HRP-based approach.
- Perform the BrdU-Red staining (TUNEL staining) before adding the second antibody
- Stain samples with individual targets only as controls.

16. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abcan.com | +64-(0)9-909-7829