ApoMark™ FL
Fluorescent Apoptosis Detection Kit Manual

Features

- Easy to use
- Reagents titered for success
- Proven protocol

Ordering Information

Catalog Number
ApoMark™ FL488
- X2835K1 (30 Tests)
- X2835K2 (60 Tests)

ApoMark™ FL549
- X2836K1 (30 Tests)
- X2836K2 (60 Tests)

ApoMark™ FL594
- X2837K1 (30 Tests)
- X2837K2 (60 Tests)

ApoMark™ FL649
- X2838K1 (30 Tests)
- X2838K2 (60 Tests)

Size
30 or 60 Slides

Format
Immunofluorescence Kit

Species Reactivity
Ubiquitous

Company Information
Exalpha Biologicals, Inc.
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fax: 866.924.5100
978.425.1376
info@exalpha.com
www.exalpha.com

Related Kits Available from Exalpha Biologicals

ApoMark™ Colorimetric (DAB) Apoptosis Detection Kit
X2044K1 (30 Tests)
X2044K2 (60 Tests)

ApoMark™ FL Fluorescent Apoptosis Detection Kit

ApoMark™ FL488
- X2835K1 (30 Tests)
- X2835K2 (60 Tests)

ApoMark™ FL549
- X2836K1 (30 Tests)
- X2836K2 (60 Tests)

ApoMark™ FL594
- X2837K1 (30 Tests)
- X2837K2 (60 Tests)

ApoMark™ FL649
- X2838K1 (30 Tests)
- X2838K2 (60 Tests)

BrdU Colorimetric Cell Proliferation ELISA Kit
X1327K1 (200 Tests)
X1327K2 (1000 Tests)
X1327K3 (5000 Tests)

BrdU Chemiluminescent Cell Proliferation ELISA Kit
X1623K1 (200 Tests)
X1623K2 (1000 Tests)
X1623K3 (5000 Tests)

BrdU Immunohistochemistry Kit
X1545K (50 Sections)

BrdU Immunofluorescence Kit
X2839K (FL488, 50 Sections)
X2840K (FL549, 50 Sections)

Anti-Fade Mounting Media
X2841 (7 mls)

BrdU Reagent for In Vivo Injection
X2834 (5 x 5 mg)

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ApoMark™ FL

Apoptosis Detection Kit

Intended Use

Exalpha's ApoMark™ FL Apoptosis Detection Kit is a non-isotopic system for the labeling of DNA breaks in apoptotic cell nuclei in paraffin-embedded tissue sections, tissue cryosections, and in cell preparations fixed on slides.

This assay is for research use only and not for use in diagnostic or therapeutic procedures.

Storage of Kit Components

Exalpha's ApoMark™ FL kit components are shipped on cold pack. Upon receipt, store kit at -20°C in a non-frost-free freezer. Thirty (30) minutes prior to use of each component, thaw component and keep on cold block or on ice. Return to -20°C for long term storage or 4-8ºC for short term storage (up to 2 weeks) imediately after use. Special care should be taken to keep TdT (Component 4), Labeling Reaction Mix (Component 3) and Conjugate (Component 7) cold.

Reagent Stability

All of the reagents included with the ApoMark™ FL Apoptosis Detection Kit have been tested for stability. Reagents should not be used beyond the stated expiration date.

Background

Cell death occurs by two major mechanisms, necrosis and apoptosis. Apoptosis is also known as programmed cell death or ankoikis (a form of apoptosis which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix). Apoptosis leads to the elimination of cells without releasing harmful substances into the surrounding area. Too little or too much apoptosis plays a role in a great many diseases.

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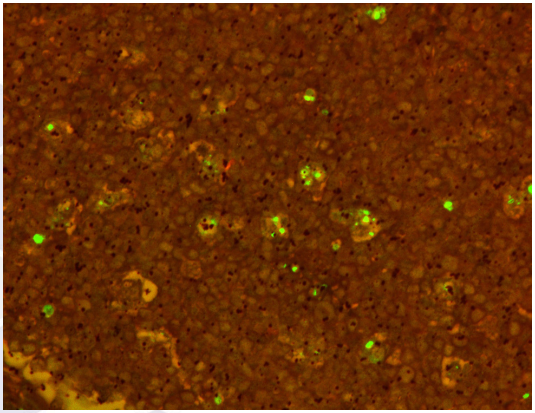
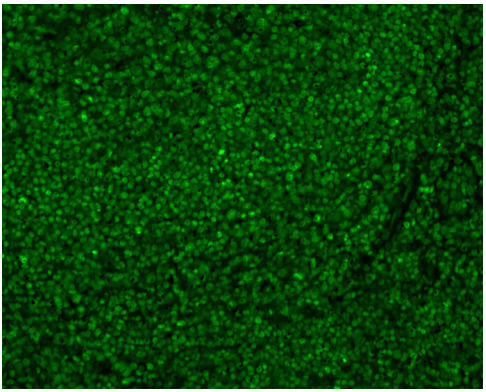
When apoptosis functions inappropriately, cells that should be eliminated survive and potentially become immortal, as in cancer or leukemia. When apoptosis works overly well, too many cells may 'die' and the result may be grave tissue damage. This is the case in stroke and neurodegenerative disorders such as Alzheimer, Huntington and Parkinson diseases. The term 'apoptosis' refers only to the structural changes a cell goes through during the process of programmed cell death and not to the process itself. Classical necrotic cell death occurs due to noxious injury or trauma to the cell while apoptosis is an energy dependent mechanism that takes place during normal cell development. While necrotic cell death results in cell lysis, cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane. Apoptosis is the result of a cascade of molecular and biochemical events involving endogenous endonucleases that cleave DNA into the prototypical 'ladder of DNA fragments' that may be visualized in agarose gels. Observation of oligonucleosomal DNA fragments by DNA laddering has long been the most acceptable and only available assay for the detection of apoptosis.

Exalpha's ApoMark™ FL assay exploits the fact that apoptotic endonucleases not only affect cellular DNA by producing the classical DNA ladder but also



ApoMark™ FL488 kit (X2835K) using paraffin fixed human tonsil tissue, 10 μm sections (20X). (above) Staining clearly illustrate the level of positive staining that can be achieved in apoptotic cells within the germinal centers of tonsil tissue.



ApoMark™ FL488 kit (X2835K), using paraffin fixed human tonsil tissue, 10 μm sections (20X). (above) Section was treated with DNase I in order to generate a positive control slide. Note all nuclei stain positive. The use of DNase I generates free 3'-OH groups on cellular DNA. These free 3'-OH groups are then labeled with biotinylated-nucleotide by the TdT in the ApoMark™ FL488 kit.

generate free 3'-OH groups at the ends of these DNA fragments. These free 3'-OH groups are end-labeled by the ApoMark™ FL Apoptosis Detection Kit allowing for the detection of apoptotic cells using a molecular biology-based, end-labeling technique.

Principles of the Assay

Exalpha's ApopMark™ FL Apoptosis Detection Kit allows for the recognition of apoptotic nuclei in paraffin-embedded tissue sections, frozen tissue sections, or in preparations of single cell suspensions fixed on slides. In this assay Terminal deoxynucleotidyl Transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-fluorochrome conjugate which reacts with biotin.

Materials Provided

The ApoMark™ FL kit supplies sufficient reagents to stain 30 or 60 specimens of approximate size 2.5 cm^2. Components shown below are for 30 specimen kit

Component 1: Proteinase K, pH-stabilized Solution (50 μl)
Component 2: TdT Equilibration Buffer (4 ml)
Component 3: TdT Labeling Reaction Mix - Optimized mix of labeled and unlabeled nucleotides (1.3 ml)
Component 4: TdT Enzyme: Terminal Deoxynucleotidyl Transferase (40 μl)

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Component 5: Stop Buffer (4 ml)
Component 6: Blocking Buffer (12 ml)
Component 7: Ready to use Conjugate: SA-Fluorochrome (3.5 ml)

Materials Required But Not Provided

- Xylene
- Ethanol, 100, 90, 80, 70%
- Methanol
- 30% hydrogen peroxide for quenching endogenous peroxidase activity and autofluorescence from red blood cells (RBCs)
- Tris-buffered saline (1X TBS, 20 mM Tris pH 7.6, 140 mM NaCl)
- DNase I (optional, for use in generating positive control)
- Distilled de-ionized water
- Coplin jars, glass or plastic with slide holders
- Humidified chamber
- Glass or plastic coverslips
- Mounting media suitable for use with fluorescent dyes, such as our Anti-Fade Mounting Media (Cat. No. X2841).
- Microscope equipped with suitable excitation and emission filter sets for fluorochrome.
- 1-20 μl, 20-200 μl, and 200-1000 μl precision pipettors
- Sterile DNase/RNase free disposable pipette tips
- Microcentrifuge tubes
- Absorbent wipes (such as Kimwipes)
- Cold block or ice bath
- Pap pen
- Sudan Black B (optional)

Precautions and Recommendations

READ ALL INSTRUCTIONS COMPLETELY BEFORE PERFORMING ASSAY

- For optimal results read these instructions completely before using this kit.
- The TdT Enzyme contains glycerol and will not freeze solid at -20°C. To preserve the activity of this enzyme, do not remove it from the -20°C freezer storage until immediately before use in preparing the labeling reaction mixture. Pulse-spin the TdT enzyme tube in a microcentrifuge prior to opening. Place the TdT enzyme in a -20°C storage device

(cold block or ice bath) for use. To preserve enzyme activity, return immediately to -20°C for long term storage or 4-8ºC for short term storage (up to 2 weeks) imediately after use. Components containing glycerol should not be stored at -80°C.

- **Ready-to-Use Conjugate must be kept in the dark**
- All other ApoMark™ FL kit components, with the exception of Stop Buffer (Component 5) and Blocking Buffer (Component 6), should be kept on ice or in a cold block during usage, and then promptly returned to -20°C for long term storage or 4-8ºC for short term storage (up to 2 weeks) imediately after use.
- To avoid reagent loss in tube caps, briefly pulse spin all solutions before removing caps and before use.
- Cacodylic acid is a component of the TdT Equilibration Buffer and Labeling Reaction Mix. Cacodylic acid is toxic and carcinogenic. Avoid contact with eyes and skin. Do not ingest.
- Gloves, lab coat, and protective eyewear should be worn. Refer to your institutions health and safety guidelines for appropriate procedures.
- Separate protocols have been provided for the end labeling of paraffin-embedded tissue sections, tissue cryosections, and cell preparations fixed on slides.

See 'Application Notes' at the end of this manual for tips on performing these procedures.

- Incubation time for Proteinase K, DNase I, and labeling may need to be empirically determined for your particular cell type and slide preparation. Use this protocol as a guideline.
- The use of coverslips is recommended during the labeling step to assure even distribution of the reaction mixture and to prevent loss due to evaporation during incubation.
- A humidified chamber should be used for all steps indicated to prevent reagent loss from evaporation

Procedure Outline

Paraffin-Embedded Sections

Activity	Time (Mins.)
Rehydrate samples	29

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Activity	Time (Mins.)
Rinse with TBS	5
Permeabilize samples using Component 1	20
Rinse with TBS	5
Inactivate endogenous peroxidases. Incubate with 3% H_2O_2	5
Rinse with TBS	5
Equilibrate samples with Component 2	30
Add Component 4 to Component 3 and incubate	90
Rinse with TBS	5
Terminate reaction with Component 5	5
Rinse with TBS	5
Block samples with Component 6	10
Add Component 7 to each slide	30
Rinse with TBS	5
Mount using mounting media with antifade and coverslip	-

Detailed Assay Protocol

DO NOT LET THE SPECIMEN DRY OUT DURING OR BETWEEN ANY STEP!

(If necessary, cover or immerse the specimen in 1X TBS to keep hydrated)

1. Immerse slides in xylene for 5 minutes at room temperature. Repeat (total two 5 minute incubations). NOTE: Xylene should be changed frequently.
2. Immerse slides in 100% ethanol for 5 minutes at room temperature. Repeat (total two 5 minute incubations).
3. Immerse slides in 90% ethanol for 3 minutes at room temperature.
4. Immerse slides in 80% ethanol for 3 minutes at room temperature.
5. Immerse slides in 70% ethanol for 3 minutes at room temperature.
6. Rinse slides briefly with 1X TBS for 5 minutes and

carefully dry the glass slide around the specimen.

* To help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen or a hydrophobic slide marker (Pap Pen).

1. PERMEABILIZATION OF SPECIMEN

1.1. Dilute Proteinase K (Component 1) 1:100 in dH_2O (mix 1 μl of Proteinase K plus 99 μl dH_2O per specimen).

1.2. Cover the entire specimen with 100 μl of Proteinase K solution prepared above and incubate at room temperature for 20 minutes.

1.3. Rinse slide with 1X TBS for 5 minutes.

1.4. Gently tap off excess liquid and carefully dry the glass slide around the specimen using a kimwipe or other adsorbent material. Care should be take to not touch the specimen.

2. QUENCHING: INACTIVATION OF ENDOGENOUS PEROXIDASES

2.1. Dilute 30% H_2O_2 1:10 in methanol (mix 10 μl 30% H_2O_2 with 90 μl methanol per specimen).

2.2. Cover the entire specimen with 100 μl of 3% H_2O_2. Incubate at room temperature for 5 minutes.

2.3. Rinse slide with 1X TBS for 5 minutes.

2.4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

3. EQUILIBRATION

3.1. Cover the entire specimen with 100 μl of TdT Equilibration Buffer provided (Component 2). Incubate at room temperature for 30 minutes. During the last five minutes of this incubation prepare the Labeling Reaction Mixture.

4. LABELING REACTION

4.1. Prepare the working TdT Labeling Reaction Mixture as follows:
pulse-spin the TdT Enzyme tube in a microcentrifuge prior to opening. Prepare only enough TdT

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Labeling Reaction Mixture for the number of samples/ slides to be labeled. For each sample to be labeled add 1 µl TdT Enzyme (Component 4) to 39.0 µl TdT Labeling Reaction Mix (Component 3) in a clean microfuge tube. Mix gently and keep on ice or a cold block until use.

4.2. Carefully blot the TdT Equilibration Buffer from the specimen, taking care not to touch the specimen.

4.3. Immediately apply 40 µl of TdT Labeling Reaction Mixture (prepared above) onto each specimen and cover the specimen with a coverslip to assure even distribution of the reaction mixture and prevent loss due to evaporation during incubation.

4.4. Place slides in a humidified chamber and incubate at room temperature (at least 22ºC) for 1.5 hours. NOTE: If room temperature is below 22ºC the use of a 37ºC incubator for 1.5 hours is recommended.

5. TERMINATION OF LABELING REACTION

5.1. Locate the Stop Buffer (Component 5). If a precipitate is present, warm the Stop Buffer to 37ºC for five minutes or until precipitate is no longer evident.

5.2. Remove coverslip* and rinse slide with 1X TBS for 5 minutes.

* HELPFUL HINT: Cover slip is best removed by submerging the slide in TBS solution in a Coplin jar or beaker and allowing cover slip to gently slide off specimen. A glass cover slip is best but plastic cover slip may be used.

5.3. Cover the entire specimen with 100 µl of Stop Solution. Incubate at room temperature for 5 minutes.

5.4. Rinse slide with 1X TBS for 5 minutes.

5.5. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

6. BLOCKING

6.1. Cover the entire specimen with 100 µl of Blocking Buffer (Component 6). Incubate at room temperature for 10 minutes.

7. DETECTION

7.1. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen. Immediately

apply 100 µl of ready to use conjugate (Component 7) to the specimen.

7.2. Place slides in a humidified chamber and incubate at room temperature **in the dark** for 30 minutes.

7.3. Rinse slides with 1X TBS for 5 minutes.

7.4 Background fluorescence elimination: Optional treatment with Sudan Black or other compatible treatment to decrease autofluorescence of sample can be applied at this point.

Sudan Black treatment

1. 0.2% Sudan Black (w/v) in 70% EtOH stirred in the dark for 2 hours; **Sudan Black solution should be prepared fresh each time it is used.**
2. Apply to slide for 15 minutes after the fluorescent label application.
3. Rinse quickly with TBS or PBS 8 times and mount. Addition of 0.02% tween 20 may be required.

8. STORAGE

8.1 Mount a glass coverslip using a mounting media with antifade, such as Exalpha's Anti-Fade Mounting Media (Cat. No. X2841), over the specimen. Allow to dry following manufacturer's recommendations.

8.2 Store in the dark at -20ºC

APPLICATION NOTES:

A. How to make a humidified chamber.

A simple humidified chamber may be constructed using an empty plastic pipette box, Tupperware™ or other plastic container with tight-sealing lid. Place a moist paper towel or Kimwipe™ on the bottom taking care not to over wet. Place slides carefully onto moist surface or construct a support scaffolding using plastic pipettes and place slides onto this support.

B. ApoMark™ FL OF TISSUE CRYOSECTIONS

This protocol is similar to ApoMark™ FL of paraffin-embedded tissue sections EXCEPT that the deparaffinization step is replaced with a short hydration step and permeabilization with proteinase K is performed for only 10 minutes. Fixation of cryopreserved tissue is required prior to performing the assay.

* To avoid loss of tissue from glass slides during washing steps, it is recommended that slides be gently dipped

2-3 times into a beaker of 1X TBS rather than rinsed with a wash bottle.

* DO NOT LET THE TISSUE DRY OUT BETWEEN OR DURING ANY STEP. If additional time is needed between steps (e.g. to prepare reagents etc.), cover or immerse the slides in 1X TBS to keep hydrated until use.

TISSUE FIXATION and HYDRATION

1. Immerse slides in 4% formaldehyde (prepared in 1X PBS) for 15 minutes at room temperature.

2. Gently drain off excess liquid and carefully dry the glass slide around the specimen.

3. Immerse slides in 1X TBS for 15 minutes at room temperature.

4. Carefully dry the glass slide around the specimen.

* To help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen (Pap pen) or a hydrophobic slide marker.

PERMEABILIZATION OF SPECIMEN

1. Dilute Proteinase K (Component 1) 1:100 in dH_2O (mix 1 μl of Proteinase K plus 99 μl, dH_2O per specimen)

2. Cover the entire specimen with 100 μl of Proteinase K solution prepared above and incubate at room temperature for 10 min.

DO NOT OVERINCUBATE

3. Rinse slide with 1X TBS for 5 minutes.

4. Gently tap off excess liquid and carefully dry the glass slide around the specimen using a Kimwipe™ or other absorbent material. Care should be take to not touch the specimen.

All remaining steps are identical to those steps outlined for ApoMark™ FL of paraffin-embedded tissue sections. Proceed from **2. Quenching: Inactivation of Endogenous Peroxidases** through the end of the protocol.

Care should be taken during wash steps to avoid losing tissue sections. Washing by gentle emersion is recom-

mended.

C. ApoMark™ FL OF FIXED CELL PREPARATIONS

Procedure for fixing cell suspensions or cell lines as preparation for performing the ApoMark™ FL procedure.

FIXING CELL PREPARATIONS

Cells grown in suspension (suspension cultures etc.) can be fixed and attached to slides.

Cells are pelleted by gentle centrifugation for 5 minutes at 4°C.

Wash cells 2X with cold (4°C) PBS.

Cells are then resuspended in 4% formaldehyde (in PBS) at a cell density of $1x10^6$/ml and incubated at room temperature for 10 minutes.

Cells are pelleted by gentle centrifugation for 5 minutes at room temperature and resuspended, at the same concentration, in 80% ethanol.

Fixed cells may be stored at 4°C until assayed. Fixed cells (100-300 μl) can be immobilized onto glass slides by directly placing the cell suspension onto the slide and allowing to air dry or by the use of a Cytospin may also be used – follow manufacturers recommendation for slide preparation.

NOTE: Precoating slides with poly-L-lysine may enhance cell adherence. Cytospun slides may be stored for up to 6 months at -20°C until use.

This protocol varies from the standard ApoMark™ FL procedure for paraffin-embedded tissue sections. Replace the deparaffinization step with a rehydration step. Permeabilization with Proteinase K is performed for only 5 minutes (see below).

NOTE: To avoid loss of cells from glass slides during washing steps, it is recommended that slides be dipped 2-3 times into a beaker of 1X TBS rather than rinsed with a wash bottle.

NOTE: DO NOT LET THE CELLS DRY OUT BETWEEN OR DURING ANY STEP. If necessary cover or immerse the slide in TBS to keep hydrated.

REHYDRATION

Immerse slides in 1X TBS for 15 min at room temperature.

Carefully dry the glass slide around the specimen. To

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help contain small reaction volumes around the specimen, it may be helpful at this point to encircle the specimen using a waxed pen or a hydrophobic slide marker (pap pen).

PERMEABILIZATION OF SPECIMEN

Dilute Proteinase K (Component 1) 1:100 in 10 mM Tris pH 8 (mix 1 µl of Proteinase K plus 99 µl 10 mM Tris per specimen).

Cover the entire specimen with 50 -100 µl of prepared Proteinase K solution. Incubate at room temperature for 5 min. DO NOT OVERINCUBATE.

Dip slide 2-3 times into a beaker of 1X TBS.

Gently tap off excess liquid and carefully dry the glass slide around the specimen.

All remaining steps are identical to those steps outlined for ApoMark™ FL of paraffin-embedded tissue sections. Proceed from **2. Quenching: Inactivation of Endogenous Peroxidases** through the end of the protocol.

D. Elimination of background auto fluorescence in sample.

Numerous methods exist for the elimination of background or autofluorescence in samples. Most common methods for dealing with autofluorescence entail avoiding it in sample if at all possible. Filtering it out post-staining is useful but not always practical due to broad emission spectra of many fluorchromes. Chemical removal of autofluorescence is useful but this may also decrease specific signal and hence sensitivity.

Empirical data is always the best. Try different methods on your target tissue/specimen.

A quick and useful method is the use of SUDAN BLACK.

Sudan Black treatment

1. 0.2% Sudan Black (w/v) in 70% EtOH stirred in the dark for 2 hours; **Sudan Black solution should be prepared fresh each time it is used.**
2. Apply to slide for 15 minutes after the fluorescent label application.

3. Rinse quickly with TBS or PBS 8 times and mount. Addition of 0.02% tween 20 may be required.

Excitation and Emmission Spectra of ApoMark™ FL kits.

Fluorochrome	Maximum Excitation (nm)	Maximum Emission (nm)
488	493	518
549	556	571
594	592	617
649	655	670

Evaluation of Results

Since 3'-OH ends of DNA fragments generated during apoptosis are concentrated within the nuclei and apoptotic bodies, morphology as well as fluorescent staining can and should be used to interpret ApoMark™ FL results. Characteristic morphological changes during apoptosis are well characterized and should be used as verification of programmed cell death. Non-apoptotic cells do not incorporate significant amounts of biotin labeled nucleotide since they lack free 3'-OH ends (indicative of apoptosis).

After performing the ApoMark™ FL test, careful evalu-

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ation of the slides should be performed using a fluorescent microscope.

Generation of Control Samples

A. GENERATION OF NEGATIVE CONTROL

An appropriate negative control to employ is the elimination of the TdT enzyme from a duplicate slide. Simply perform the ApoMark™ FL test as outlined and substitute dH_2O for the TdT in the reaction mixture or keep the specimen in reaction buffer (with a cover slip to prevent drying out) during the labeling step. Perform all other steps as described in the manual. This is a suitable control for endogenous peroxidases and non-specific conjugate binding or background in the assay. A nonapoptotic control is also a useful control. A delay in fixation or routine mechanical manipulation may result in the unwanted breakage of DNA that could be read as apoptosis.

B. GENERATION OF POSITIVE CONTROL

A positive control can be generated from your slides (or any commercially available slide preparation of any species or tissue - slides of 10 μm thickness are preferred).

To generate a positive slide/sample, treat a slide(s) with 1 μg/μl DNase I in TBS/1 mM $MgSO_4$ for 20 minutes at room temperature immediately following the Proteinase K treatment step in the ApoMark™ FL manual.

Perform all other steps as described in the ApoMark™ FL manual.

The DNase I treatment will fragment DNA in normal cells, generating free 3'-OH groups identical to those generated during apoptosis.

Notes

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