

Apoptosis Kit

NeuroTACS™ *In Situ* Apoptosis Detection Kit

Catalog Number: 4823-30-K

Reagent kit for *in situ* detection of apoptosis in tissue sections and cells.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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BACKGROUND

For many cell types in culture, identification of apoptosis and quantification of cell death can be readily achieved using a combination of morphological criteria, extraction and analysis of DNA by agarose gel electrophoresis, and *in situ* detection of DNA fragmentation in immobilized cells. Other approaches include measuring the activation of caspases, flow cytometry, binding and detection of annexin to the cell surface, and cleavage of poly-ADP ribose polymerase. For neuronal cell types there can be inherent technical difficulties associated with analysis of apoptosis. Many neuronal cell types are available only in primary culture, limiting the cell number for DNA extraction and analysis, or labeling and FACS analysis. Neuronal cells tend to be more fragile, are less amenable to extensive handling and lose morphology when removed from the substratum. Primary neuronal cell cultures are often maintained in mixed culture, the ability to identify specific cells using criterion other than morphology is important. Similarly, it can be difficult to identify cell types within central nervous system tissue, particularly in the cortex.

The NeuroTACS™ II *In Situ* Apoptosis Detection Kit has been designed for cells or tissue sections from the nervous system, and is compatible with immunocytochemistry to identify cell types and antigens by using cell specific antibodies, (*e.g.*, NCAM, GFAP), or antibodies against other proteins of interest (*i.e.*, Bax and p53).

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between sample additions and reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

NeuroTACS *In Situ* Apoptosis Detection Kit contains reagents that are harmful if swallowed or in contact with skin, and irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Refer to the SDS on our website prior to use.

Dispose of DAB in accordance with all local, state, and federal regulations.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Proteinase K	4800-30-01	50 µL	Store at ≤ -20 °C.
TdT dNTP	4810-30-04	35 µL	
TdT Enzyme	4810-30-05	30 µL	
50X Mn ²⁺	4810-30-14	50 µL	
DAB Solution	4800-30-07	3.75 mL	
DAB Enhancer	4800-30-09	1 mL	
TACS-Nuclease™	4800-30-15	15 µL	Store 2-8 °C.
NeuroPore™	4820-30-01	5 mL	
10X TdT Labeling Buffer	4810-30-02	100 mL	
10X TdT Stop Buffer	4810-30-03	100 mL	
Strep-HRP	4800-30-06	30 µL	
TACS-Nuclease Buffer™	4800-30-16	1.5 mL	Store at room temperature.
Blue Counterstain	4820-30-13	50 mL	

OTHER MATERIALS REQUIRED

Equipment:

- Pipette and pipette tips
- 37 °C incubator
- 50 and 500 mL graduated cylinders
- 2 coplin jars
- ≤ -20 °C and 2-8 °C storage
- Ice bucket
- Fluorescence microscope
- Flow cytometer
- Cryostat or microtome
- Humidity chamber
- 57 °C incubator or slide warmer

Reagents:

- 10X Phosphate Buffered Saline (PBS)
- 37% formaldehyde
- o- or p- xylene
- 30% hydrogen peroxide
- 95% and 100% ethanol
- Mounting Medium or alternative mounting solution
- Distilled water
- 30% ammonium hydroxide

Disposables:

- Treated Glass Microscope Slides (or alternative support)
- 50 mL tubes
- 1-200 µL and 200-1000 µL pipette tips
- Microcentrifuge tubes
- 1.5 and 10 mL serological pipettes
- Hydrophobic coverslips
- Glass coverslips

REAGENT PREPARATION

Reagents marked with an asterisk (*) should be prepared immediately before use.

The volumes given for each reagent are based on processing samples of up to 4 cm² immobilized on glass slides. Different configurations of chamber slides, culture plates, free floating sections, and the use of glass coverslips may require adjustments to the stated volumes.

1X PBS - Approximately 500 mL of 1X PBS is used to process 1-10 slides. Dilute 10X PBS to 1X using distilled water. Store 1X PBS at room temperature.

NeuroPore - 50 µL of NeuroPore is used per sample. NeuroPore is ready for use. Discard if solution is cloudy.

***3.7% Buffered Formaldehyde** - If required, 50 mL of freshly prepared fixative is used to process 1-10 samples. To prepare add:

Reaction Component	Volume
37% Formaldehyde	5 mL
10X PBS	5 mL
Distilled water	40 mL

Wear gloves and exercise caution when handling formaldehyde solutions.

***Proteinase K Solution** - Use 50 µL of Proteinase K Solution per sample. Store on ice. Thaw provided Proteinase K at room temperature, then place on ice. To prepare add:

Reaction Component	2 Samples	10 Samples	n Samples
Distilled water	100 µL	500 µL	n x 50 µL
Proteinase K	2 µL	10 µL	n x 1 µL

Under some circumstances, the Proteinase K may be used at a 1:200 dilution.

***Quenching Solution** - Use 50 mL of Quenching Solution to process 1-10 samples. To prepare add:

Reaction Component	Volume
Methanol	45 mL
30% hydrogen peroxide	5 mL

Always use fresh 30% hydrogen peroxide. It is recommended that 6 mL aliquots of fresh 30% hydrogen peroxide be made and stored at 2-8 °C. For each labeling procedure, use a fresh 30% hydrogen peroxide aliquot then discard the unused portion.

1X TdT Labeling Buffer - Dilute the 10X TdT Labeling Buffer to 1X using distilled water. Leave at room temperature until use. Use 50 mL of 1X Labeling Buffer to process 1-10 samples. Remove an aliquot of 50 µL per sample for preparing the Labeling Reaction Mix and place on ice.

REAGENT PREPARATION *Continued*

***Labeling Reaction Mix** - Thaw TdT dNTP Mix at room temperature, then place on ice. To maintain optimal enzyme activity, remove the TdT Enzyme tube from freezer only long enough to pipette the required volume. Alternatively, place the TdT Enzyme in a $\leq -20\text{ }^{\circ}\text{C}$ freezer block. Prepare the Labeling Reaction Mix just before use and keep the prepared reaction mix on ice. Prepare 50 μL per sample in the sequence given below:

Reaction Component	2 Samples	10 Samples	n Samples
TdT dNTP Mix	2 μL	10 μL	n x 1 μL
TdT Enzyme	2 μL	10 μL	n x 1 μL
50X Mn ²⁺	2 μL	10 μL	n x 1 μL
1X TdT Labeling Buffer	100 μL	500 μL	n x 50 μL

1X TdT Stop Buffer - Dilute the 10X TdT Stop Buffer to 1X using distilled water. Leave at room temperature until use. Use 50 mL of 1X TdT Stop Buffer to process 1-10 samples.

***Strep-HRP Solution** - Use 50 μL of Strep-HRP Solution per sample. Store prepared Strep-HRP Solution on ice until use. To prepare add:

Reaction Component	2 Samples	10 Samples	n Samples
1X PBS	100 μL	500 μL	n x 50 μL
Strep-HRP	2 μL	10 μL	n x 1 μL

***Working DAB Solution** - Thaw DAB Solution at 37 $^{\circ}\text{C}$ for 30 minutes. If not using the full bottle, store the remaining stock solution back at $\leq -20\text{ }^{\circ}\text{C}$. It is not recommended to freeze thaw DAB stock solution more than three times. If required, aliquot in smaller volume and store at $\leq -20\text{ }^{\circ}\text{C}$ protected from light. To prepare add:

Reaction Component	per control
1X PBS	50 mL
DAB Solution	250 μL
DAB Enhancer	0-50 μL
30% hydrogen peroxide	50 μL

Use only fresh 30% hydrogen peroxide. It is recommended that 6 mL aliquots of fresh 30% hydrogen peroxide are made and stored at 2-8 $^{\circ}\text{C}$. For each labeling procedure use a fresh 30% hydrogen peroxide aliquot then discard any remaining solution.

Note: Do not place the DAB Solution on ice after thawing, otherwise the DAB will precipitate. Prepare Working DAB Solution no more than 30 minutes before use. Use 50 mL of DAB Solution to process 1-10 samples. Working DAB Enhancer may be used to intensify and darken DAB staining; the concentration must be optimized experimentally.

REAGENT PREPARATION *Continued*

***TACS Nuclease Solution** - For the preparation of a Nuclease-treated positive control sample, it is recommended that the DNA breaks be generated in a separate step in this case, TACS-Nuclease should be diluted 1:50 in TACS Nuclease Buffer, as below:

Reaction Component	2 Samples	10 Samples	n Samples
TACS-Nuclease Buffer	100 μ L	500 μ L	n x 50 μ L
TACS-Nuclease	2 μ L	10 μ L	n x 1 μ L

It is also possible to obtain acceptable, but lower intensity, positive control staining by incubating the TACS Nuclease with the Labelling Reaction Mix. In this case, per positive control, prepare:

Reaction Component	per positive control
1X TdT Labeling Buffer	50 μ L
TdT dNTP Mix	1 μ L
TdT Enzyme	0-50 μ L
TACS-Nuclease	1 μ L
50X Mn ²⁺ Cation	1 μ L

Blue Counterstain - Blue Counterstain is ready for use. Blue Counterstain may be reused many times. Store in a closed container to prevent evaporation. If a precipitate forms, filter sample through Whatman 3MM paper.

o- or p-Xylene - Mixed xylenes may be used for deparaffinization and for clarification prior to mounting coverslips onto the samples. Xylenes used for deparaffinization may be reused several times. Xylenes used in deparaffinization should not be used for clarification.

100%, 95%, 70% ethanol - Either 100% (200 proof) or denatured alcohol (90% ethanol, 5% methanol, 5% isopropanol) may be used. Dilute with deionized water to prepare 95% and 70% solutions. Ethanol used for deparaffinization may be reused several times. Ethanol used in deparaffinization should not be used for dehydration.

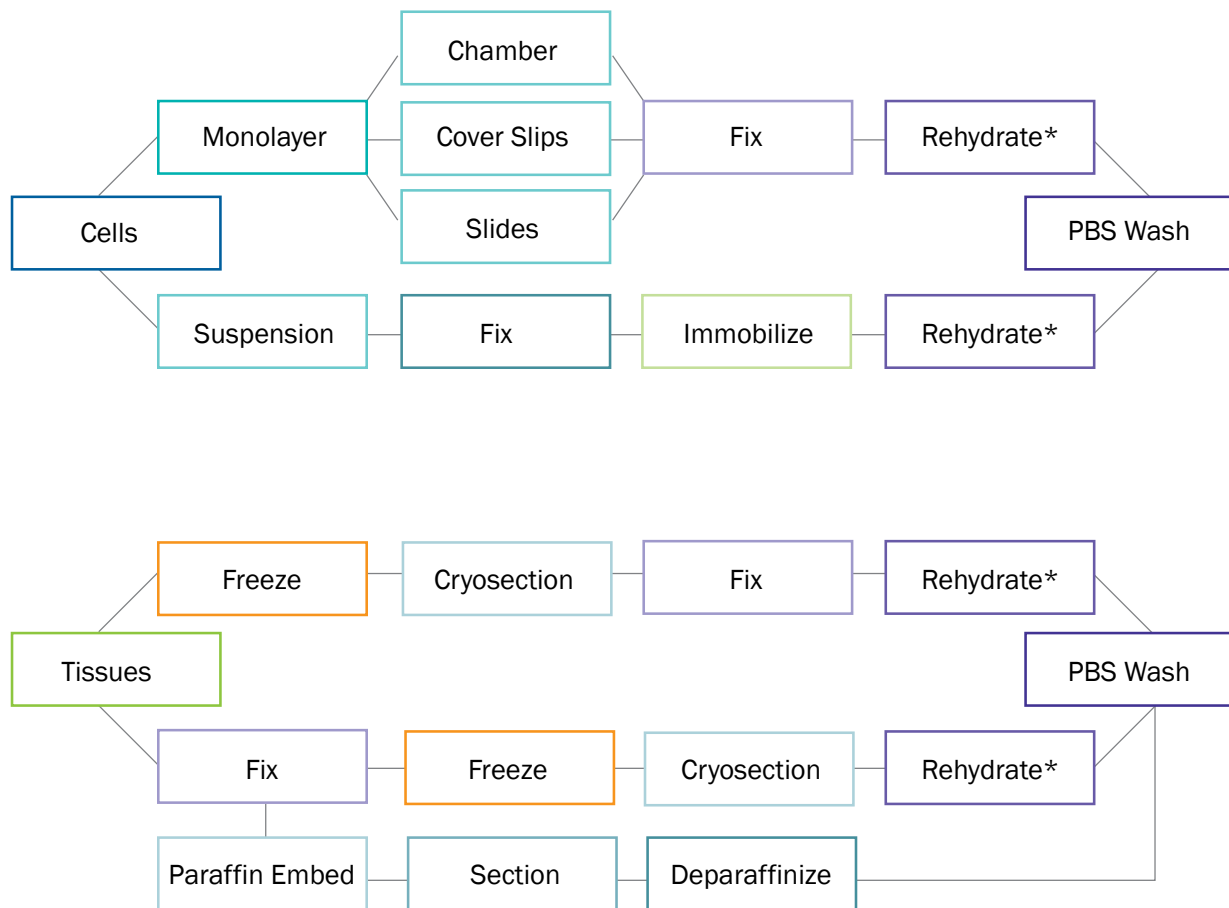
***Ammonium Water** - 500 mL is used to process 1-10 slides. Prepare Ammonium Water using tap water; **do not use deionized water**. To prepare add:

Reaction Component	per positive control
30% ammonium hydroxide	0.25 mL
Tap water	500 mL

The Ammonium Water is used as a bluing agent during counterstaining.

ASSAY PROTOCOL

It is important to read through the instructions before preparing tissue or cell samples for labeling. There are key steps that are very important for successful labeling. This section includes instructions for sample preparation, *in situ* labeling and viewing. The assay protocol for labeling is in tabulated form and details the steps involved in the labeling reaction and in preparing the sample for viewing. Prior to labeling, the samples may need to be rehydrated and washed in PBS. The labeling procedure begins with samples in PBS regardless of the fixation and immobilization method.



*Rehydration may not be required if samples are not dried.

Follow the appropriate flow diagram to determine the correct sequence of steps needed to prepare samples. Details for preparing cells and tissues before labeling. The tissue or cell type, source, storage conditions, facilities and equipment available will help determine which method is most appropriate. In addition, careful consideration of each method's advantages and disadvantages should be made. The Appendix provides alternative preparation methods including analysis by electron microscopy.

SAMPLE PREPARATION AND FIXATION

PREPARATION OF CELLS IN CULTURE

Preparation of Suspension Cells - Cells grown in suspension or prepared from dissociated tissues can be fixed in solution, and then spotted onto pretreated glass microscope slides for processing. This method is quick and easy and requires no special equipment. Cells immobilized onto glass slides can be stored for several months.

1. Harvest cell suspension by centrifugation at 500 x g for 5 minutes at room temperature.
2. Discard media and resuspend at 1×10^6 cells/mL in 3.7% Buffered Formaldehyde. Let stand for 10 minutes at room temperature.
3. Centrifuge at 500 x g for 5 minutes at room temperature and discard fixative.
4. Resuspend at 1×10^7 cells/mL in 80% ethanol. Cells can be stored in 80% ethanol at 2-8 °C for several weeks. Signal intensity in positive cells will reduce with time due to loss of small DNA fragments.
5. Spot 1×10^5 cells onto clean glass microscope slide. Dry for 20 minutes on slide warmer at 45 °C.
Note: *Glass slides pretreated for electrostatic adherence are recommended. Slide treatments (i.e. collagen, gelatin, poly-L-lysine) can cause increased background staining.*
6. Immerse slide in 70% ethanol for 10 minutes, then air dry overnight at room temperature or dry at 45 °C for 2 hours. Samples may be stored at this point. Store samples at 2-8 °C in airtight containers with desiccant for up to several months. After storage, rehydrate through a decreasing alcohol series and wash in 1X PBS prior to the labeling reaction.
7. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
8. Immerse in 1X PBS and proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

PREPARATION OF CELLS IN MONOLAYER

On Sterile Chamber Slides - For optimal outcomes, cells should be grown on a surface that allows for both fixation and direct labeling, such as sterile chamber slides. Remove the chamber walls and gasket after fixation. The chamber walls and gasket may be left in place during the labeling reaction if different treatments (*i.e.* no enzyme and nuclease treatment) are required for adjacent samples on the same slide.

On Sterile Slides - Other cell culture methods include culturing directly on microscope slides. The slides must be sterile and, if necessary, pretreated to ensure cell adhesion. Sterilize microscope slides by autoclaving in a large glass petri dish. If needed, coat slides with sterile poly-L-lysine or collagen, these slide pretreatments may increase background staining. Place sterile microscope slides in culture vessel directly before plating cells.

On Sterile Glass Coverslips - Cells can be cultured directly on sterile coverslips that are placed into a 12- or 24-well tissue culture plate. Sterilize coverslips by autoclaving in a large glass petri dish. Coat coverslips with sterile poly-L-lysine or collagen, these slide pretreatments may increase background staining. Place sterile glass coverslips in wells of tissue culture dishes (12 mm coverslips fit into 24-well tissue culture plates) using fine tipped sterile forceps. Handle only at edges prior to cell plating.

1. Remove media from cells and rinse once with 1X PBS at room temperature.
2. Fix cells for 10 minutes at room temperature in 3.7% Buffered Formaldehyde.
3. Wash cells one time in 1X PBS. Samples can be stored at this point, using one of the following methods:
 - a) Dehydrate the cells by passing through an increasing alcohol series of 70%, 95%, and 100% ethanol for 5 minutes each followed by air drying for 10 minutes. Store at 2-8 °C with desiccant.
 - b) Fixed cells can be stored for up to 1 week in NeuroPore at 2-8 °C. The samples must be covered to prevent contamination and evaporation. If experimental design dictates a time course extending over several days, storage in NeuroPore is recommended.
Note: *Labeling directly after fixation is optimal as the labeling of some samples is less efficient after storage. If possible, a pilot study should be performed to ensure that stored fixed samples can be labeled.*
4. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

PREPARATION OF TISSUES

Use of glass slides pretreated for electrostatic adherence is recommended for all tissues.

Preparation of Fresh Frozen Sections - Frozen samples are easily permeabilized for labeling. Some disadvantages include the difficulty in collecting good quality sections, the need to cut thicker sections, and poor retention of morphology. Frozen sections are less resistant to protease treatments and can lift off if not collected onto the appropriately pretreated slides and dried thoroughly. **Samples must be fixed prior to labeling.**

1. Rapidly freeze tissue or biopsy immediately after removal by immersing in liquid nitrogen or on dry ice. Store frozen tissue below ≤ -70 °C.
2. Samples may be embedded in a cutting matrix. Position the sample within cutting matrix in a suitable container. Immerse embedded tissue in isopentane chilled on dry ice. Frozen samples may be stored for many months at ≤ -70 °C.
3. Using the cutting matrix, attach the sample to cutting block and equilibrate to the temperature of the cryostat before sectioning. Collect sections between 6-15 μm on glass slides pretreated for electrostatic adherence.
4. Individual expertise and tissue type will determine the thickness of the sections. Sections between 10-15 μm provide the best results. Sections between 6-9 μm tend to tear during cutting, resulting in rough edges that can increase the background staining. Up to 3 sections can be placed per slide; each spaced well apart to prevent reagents from mixing between samples.
5. Fixation After Cryosectioning
 - a. It is critical to dry the samples thoroughly after sectioning. Dry overnight at room temperature or for at least 2 hours at 45 °C on a slide warmer. Samples can be stored at this point. Store slides at ≤ -70 °C with desiccant for up to 3 months. After storage equilibrate samples to room temperature and re-dry for 2 hours at room temperature or 2 hours at 45 °C on a slide warmer. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
 - b. Wash once in 1X PBS for 5 minutes.
 - c. Fix samples by immersing in 3.7% Buffered Formaldehyde for 10 minutes at room temperature.
 - d. Wash cells 1 time in 1X PBS.
6. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

Preparation of Fixed Samples Before Sectioning – Immersion or Perfusion

Fixation Samples - Fixation samples are routinely fixed by immersion or perfusion methods. After fixation, samples are cryosectioned or paraffin embedded. Formaldehyde is the recommended fixative based on laboratory testing. Other fixatives that maintain DNA integrity may be used. These include other cross linking agents such as paraformaldehyde and glutaraldehyde. Bouin's reagent should be avoided due to the high acidity. If alternative fixatives are used, it is recommended that a pilot study is performed to ensure that the fixative allows for permeabilization and labeling. Regardless of the fixative used, it is important not to fix cells and tissues for extended periods of time. Post-fixation in acetone, ethanol or methanol is common in preparation of tissues.

Immersion Fixation - The fixation time should ensure good cross-linking but prevent tissue from becoming hard and brittle. Some empirical determination of the optimal fixation time may be required. Immerse relatively small pieces of tissue (1 cm³) in at least 10 volumes of 3.7% Buffered Formaldehyde. After 30 minutes change to fresh 3.7% Buffered Formaldehyde and leave at room temperature up to 24 hours. Tissues with high cellularity may require longer fixation times.

Perfusion Fixation - Standard laboratory procedures should be followed for perfusion fixation. Formaldehyde, paraformaldehyde, or glutaraldehyde may all be used as fixatives. After tissue perfusion, the dissected tissue should be immersed in fresh 3.7% Buffered Formaldehyde for up to 24 hours.

Storage of Fixed Samples - Fixed samples may be stored for long periods. For long term storage, use 70% ethanol or sterile 1X PBS at 2-8 °C to avoid extended exposure to fixative. Archival material that has been stored in fixative for months or years will be more difficult to permeabilize and may not be useful for *in situ* detection of apoptosis due to DNA degradation.

Sectioning of Fixed Tissue

1. Cryosection Fixed Tissues - Immerse fixed tissue in 20% sucrose in water at room temperature until the sample sinks. Embed the cryoprotected sample in cutting matrix and freeze. Collect sections of 6-10 µm onto slides pretreated for electrostatic adherence of samples and dried as described in Preparation of Fresh Frozen Sections.

Note: *When collecting onto slides from buffer use either a low salt buffer or distilled water to ensure that samples adhere to slides.*

2. Storage of Sectioned Tissues and Rehydration - Sections of fixed frozen tissue may be stored at ≤ -70 °C, with desiccant, for up to one month. After storage, the slides should be equilibrated to room temperature and dried for 2 hours at room temperature or at 45 °C on a slide dryer. Rehydrate samples before labeling by immersing for 5 minutes each in 100%, 95%, then 70% ethanol and wash for 10 minutes in 1X PBS.

SAMPLE PREPARATION AND FIXATION *Continued*

Section Paraffin Embedded Tissues - Paraffin embedding is a routine procedure in many laboratories and is commonly performed by automated equipment. The temperature of the molten paraffin must not exceed 65 °C, otherwise additional DNA damage can occur leading to spurious positives and high background. Do not bake slides after sectioning.

Sections between 6-10 µm should be collected onto slides pretreated for electrostatic adherence. Prior to the labeling reaction the samples must be deparaffinized. Optimal labeling is achieved when the samples are processed within days of sectioning.

Deparaffinization of sections prepared from paraffin blocks is required prior to the labeling reaction.

1. Warm slides to 57 °C for 5 minutes.
2. Immerse sections in 2 changes of xylenes, 5 minutes each.
3. Immerse sections in 100%, 95% then 70% ethanol, 5 minutes each.
4. Wash 2 times in 1X PBS, 5 minutes each.
5. Proceed to Labeling Procedure.

Storage

It is preferable to store the uncut paraffin block at room temperature, as opposed to the sections.

Note: *The xylenes and ethanols used for deparaffinization can be reused several times (up to 100 slides may be processed in 200 mL) but they must not be used for rehydration of non-embedded samples or for dehydration after performing the labeling reaction.*

LABELING PROCEDURE

Details on the labeling procedure are provided in the table on the following page.

Labeling Samples on Slides - Wash slides using small Coplin histology jars. Each jar holds up to 50 mL of buffer and up to 10 slides. For procedural steps involving 50 µL per sample, place slides on a flat surface and spot reagent from above using a pipette tip; do not touch the sample with the pipette tip. Small biopsy samples are easily covered with 50 µL. If 50 µL does not cover the sample, hydrophobic coverslips may be used after pipetting the 50 µL volume. Lower the hydrophobic coverslip from one edge and press down gently to expel any air bubbles. Remove hydrophobic coverslips by dipping the slide vertically in distilled water.

Labeling Samples in Chamber Slides - Remove chamber walls and gasket after fixation and process as described for slides. Hydrophobic coverslips may be used for all steps involving 50 µL reaction volumes. If different labeling reactions are performed on samples on the same slide, leave the plastic walls in place until after the labeling reaction, then remove the plastic walls and rubber gasket and proceed as described above.

LABELING PROCEDURE *Continued*

Labeling Samples on Glass Coverslips - Process the 12 mm glass coverslips with the cell-side facing up in the 24-well tissue culture plate. Wash by filling the wells with buffer and removing with a pasteur pipette (use a gentle vacuum if available). Spot the 50 μ L reaction volumes directly onto the coverslip. Alternatively, spot the 50 μ L reaction buffers onto a clean glass slide, then remove 12 mm glass coverslip from the well and flip it over, cell-side down, on top of the reagent. Use fine tipped forceps and handle glass coverslips only at the very edges. For dehydration and clarification, dip the 12 mm glass cover slips individually in ethanol series and xylenes for 20 seconds.

Note: *Xylenes will dissolve plastics, therefore, do not add xylenes to tissue culture plates.*

Step	Instructions	Notes
1	Place samples in 1X PBS for 10 minutes at room temperature after rehydration in ethanols. Carefully dry glass slide around sample.	Do not allow sample to dry at any stage prior to completion of protocol.
2	Cover sample with 50 μ L of NeuroPore and incubate 15-30 minutes at room temperature, or cover sample with 50 μ L of Proteinase K Solution for 15-30 minutes at 37 °C. If necessary, use cover slips.	Samples may be stored in NeuroPore, covered at 2-8 °C, for up to one week. A 1:50 dilution of the Proteinase K is appropriate for some tissues.
3	Wash 2 times with distilled water, 2 minutes each.	
4	Immerse slides in Quenching Solution for 5 minutes at room temperature.	Do not leave longer than 5 minutes since hydrogen peroxide can damage DNA.
5	Wash samples in 1X PBS for 1 minute at room temperature.	Refer to Reagent Preparation section.
6	Immerse slides in 1X TdT Labeling Buffer for 5 minutes.	Refer to Reagent Preparation section.
7	Cover sample with 50 μ L of Labeling Reaction Mix and incubate at 37 °C for 1 hour in a humidity chamber. If necessary, use hydrophobic coverslips.	Use humidity chamber during incubation time.
8	Immerse samples in 1X TdT Stop Buffer for 5 minutes at room temperature to stop labeling reaction.	Refer to Reagent Preparation section.
9	Wash samples 2 times with distilled water for 5 minutes each at room temperature.	This step removes unbound conjugate.
10	Cover sample with 50 μ L of Strep-HRP Solution and incubate for 10 minutes at 37 °C. If necessary, use hydrophobic coverslips.	Refer to Reagent Preparation section.
11	Wash samples 2 times with 1X PBS for 2 minutes each.	
12	Immerse samples in Working DAB Solution for 2-7 minutes.	Use caution when handling Working DAB Solution.
13	Wash samples in several changes of distilled water for 2 minutes each.	
14	Proceed to Counterstaining and Preparation for Viewing.	

COUNTERSTAINING AND PREPARATIONS FOR VIEWING

Cells and tissues may be counterstained using either of the methods described below. Glass coverslips can be held in fine tipped forceps and dipped individually into the stains and ethanols. Spot only 25 μL mounting medium onto a clean glass slide and mount the coverslip, cell side down, onto the slide. If a plastic support was used for cell culture, do not pass through xylenes.

Method A - For most cells and tissues

1. Immerse samples in distilled water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in Blue Counterstain.
3. Wash slides sequentially by dipping ten times each in:
 - a. Distilled water 70% ethanol, 2 changes
 - b. 95% ethanol, 2 changes
 - c. 100% ethanol, 2 changes
 - d. Xylenes, 2 changes
4. Wipe off excess xylenes from the back of the slide and lay slide flat.
5. Place one drop, about 50 μL , of mounting medium from a 100 μL pipette onto sample.
6. Lower glass coverslip onto sample and apply gentle even pressure to expel air bubbles.
7. Leave slide flat overnight to allow mounting medium to harden.
8. Store slides **in the dark**.

Method B - Use if Method A does not counterstain

1. Immerse samples in distilled water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in Blue Counterstain.
3. Wash slides sequentially by dipping ten times each in:
 - a. 1-butanol
 - b. 1-butanol, 1 time to rinse
 - c. Xylene, 2 times 5 to 10 seconds
4. Wipe off excess xylenes from the back of the slide and lay slide flat.
5. Place one drop, about 50 μL , of mounting medium from a 100 μL pipette onto sample.
6. Lower glass coverslip onto sample and apply gentle even pressure to expel air bubbles.
7. Leave slide flat overnight to allow mounting medium to harden.
8. Store slides **in the dark**.

CONTROLS

It is critical to run controls using the provided TACS-Nuclease to assess and optimize cell/tissue permeabilization; the recommended experimental controls are listed below.

TACS-Nuclease-treated Control Sample - Treat one sample with TACS-Nuclease to generate DNA breaks in every cell. Avoid repeated freeze-thaw cycles. The TACS-Nuclease-treated control will confirm that the permeabilization and labeling reaction has worked. The information can help optimize the conditions for the labeling procedure. The majority of cells should exhibit pale brown nuclear staining.

1. After Step 5, wash 2 times in deionized water, 2 minutes each.
2. Prepare TACS-Nuclease Solution.
3. Cover sample with 50 μ L of TACS-Nuclease Solution.
4. Incubate at room temperature for 10-60 minutes in a humidity chamber. If necessary, use Hydrophobic Coverslips.
5. Wash 2 times in 1X PBS for 2 minutes each.
6. Continue from Step 6, Labeling Procedure.

Though the above method is recommended, to save time, TACS-Nuclease can be added directly to the Labeling Reaction Mix of the positive control sample. Add 1 μ L of TACS-Nuclease to 50 μ L of complete labeling mix and incubate for the regular labeling time. The signal intensity obtained, using this method, is usually lower than the recommended method.

Unlabeled Experimental Control Sample - The TdT Enzyme should be omitted from the Labeling Reaction Mix for one sample. This control will indicate the level of background labeling (DAB) associated with non-specific binding of the Strep-HRP. This control should not have any brown staining.

Experimental Negative Control Sample - An appropriate experimental control should be included in each experiment and will depend upon the system under study. Typically the experimental negative control will be untreated sample, or normal cells/tissues. Many normal or untreated cells and tissues will have a small number of apoptotic cells so a few cells may be positive for brown staining.

CONTROLS *Continued*

Counterstaining Controls - Although uncommon, some cells and tissues may take up excessive amounts of the Blue Counterstain, obscuring the brown DAB staining. It is recommended to process one or two samples up to and including the 1X PBS wash step after the Quenching Step of the Labeling Procedure. Process through counterstaining. Staining times of 5 seconds to 5 minutes have been noted. It is recommended to start with a 5 second immersion in Blue Counterstain and alter accordingly.

DATA INTERPRETATION

Apoptosis is often defined by morphological criterion. Morphological data obtained from standard microscopy and histochemistry should always be considered in conjunction with biochemical assays used to confirm apoptosis.

Blue Counterstain allows all cells in the specimen to be visualized. Cells that are condensed (pyknotic, mitotic or apoptotic) will exhibit increased Blue Counterstain uptake. Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit a brown nuclear staining that may be very dark after labeling. This dark brown staining is typically associated with cell condensation. Brown staining in the cytoplasm as well as the nucleus of enlarged or swollen cells may occur in instances of necrosis. In tissue sections where cells have been torn open during sectioning or the edges of the specimen are ragged there may be non-specific brown staining that is not associated with nuclei.

The controls listed are important in data interpretation. These controls allow optimization of in situ detection of apoptosis without expending valuable test samples. Under optimal conditions the Unlabeled Control (*e.g.* enzyme omitted) should show no brown staining, the TACS-Nuclease treated sample should show pale brown staining in almost all cells, and the Experimental Negative Control should have less than 20% brown stained cells. The brown staining of Treated Control Sample cells is paler and usually more diffuse than the staining of truly apoptotic cells. This is due to the difference in chromatin structure between nuclease treated normal cells and the fragmented chromatin of apoptotic cells. The Counterstaining Control should show pale blue staining of all cells with some variability in intensity between cell types and darker staining of any condensed cells within that sample. Refer to the Troubleshooting section for information if the controls do not provide the expected result.

TROUBLESHOOTING

Rule out major problems by checking the labeling in the control samples first.

Problem	Cause	Action
Brown staining of cells when the TdT Enzyme is omitted from the Labeling Reaction Mix.	Endogenous peroxidase activity inadequately quenched.	Use fresh aliquots of 30% hydrogen peroxide.
	Excessive peroxidase activity in sample (rare).	Increase concentration of hydrogen peroxide in Quenching Solution to 5%.
	Non-specific binding of Strep-HRP conjugate.	Increase number of washes after binding. Prepare Strep-HRP Solution in 1X PBS, 1% BSA.
		Decrease concentration of Strep-HRP by diluting stock solution up to 1:200.
Sample dried out during the labeling procedure.	Use hydrophobic coverslips (or lids for plates or chamber slides) and incubate in humidity chamber.	
No staining in TACS-Nuclease-treated sample.	Poor permeabilization and/or excessive fixation with cross-linking fixative (common with archival tissue) preventing enzyme access.	Increase incubation time with Proteinase K (up to 60 minutes) or NeuroPore (up to overnight at 2-8 °C).
	No DNA left in sample due to hydrolysis (poor storage of samples or sections).	Read Sample Preparation and Fixation prior to labeling.
	Excessive (removed all DNA) or inadequate TACS-Nuclease treatment.	Optimize time for TACS-Nuclease treatment (5 minutes up to 2 hours).
	TdT Enzyme is inactive. The enzyme is the most labile component in the kit.	TdT Enzyme must be stored at ≤ -20 °C in a manual defrost freezer. Do not bring enzyme up to ice temperature. Place in ≤ -20 °C block or remove aliquot from tube directly in freezer.
	Color development reaction failed.	Use freshly prepared Quenching Solution made from fresh 30% hydrogen peroxide. To check reaction, spot 1 μ L of Strep-HRP onto 1 cm ² of Whatman 3MM paper and air dry. Immerse the filter paper in DAB Solution to check for brown coloration compared to a sheet of white paper.
Experimental sample shows extensive cytoplasmic staining.	High rate of cell death, late apoptosis or necrosis.	Necrotic samples will exhibit cytoplasmic staining. Apoptosis in cell culture will progress to necrosis. Reduce time of treatment in cell culture.
Methyl Green counterstain is dark blue.	Overstaining.	Reduce time in Methyl Green. Increase the number of washes in 95% ethanol or butanol.
Labeling of majority of cells in the negative experimental control (i.e. normal tissue or untreated cells) when there is no labeling if the enzyme is omitted and satisfactory labeling of the TACS-Nuclease-treated control.	High level of apoptosis (or necrosis) in negative control.	Select a more appropriate negative control or inhibit apoptosis in cell culture (i.e. with protein synthesis inhibitors). Check morphology of cells prior to assay for evidence of excessive apoptosis.
	Prolonged incubation with Working DAB Solution leads to a precipitate over entire sample.	Reduce time of DAB treatment and follow color development under the microscope.
	Excessive Proteinase K treatment.	Reduce incubation time in Proteinase K solution to 5-15 minutes. Or dilute Proteinase K 1:200 in distilled water and incubate sample for 15 minutes.
No labeling in experimental sample.	No apoptosis (or necrosis) occurring in sample.	If all controls gave the expected results and were processed at the same time as the experimental sample there may be no DNA fragmentation in cells within the sample.

APPENDICES

Appendix A. Fixation Methods

There are several fixation methods commonly used that are appropriate for the protocol described in the Instructions for Use. Formaldehyde is the recommended fixative based on laboratory testing. Other fixatives that maintain DNA integrity may be used. These include other cross linking agents such as paraformaldehyde and glutaraldehyde. Bouin's fixative should be avoided due to the picric acid content. Regardless of the fixative used, it is important not to fix cells and tissues for extended periods of time. Your fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments.

To store the immobilized fixed cells, *i.e.* on slides, chamber slides or coverslips, post-fix in 100% methanol after fixation. Wash in 1X PBS, then store in NeuroPore at 2-8 °C for up to one week. After storage, wash in 1X PBS then continue with the labeling reaction starting at Step 4 in the protocol (quenching step).

Note: *If cells are fixed using alcohol, e.g. ethanol, there will be leakage of small DNA fragments from apoptotic cells during storage and labeling intensity of apoptotic cells will be reduced.*

Appendix B. Double Labeling Hints and Tips

The *in situ* labeling protocol described here is useful for double labeling experiments when the occurrence of apoptosis can be correlated with the presence of cellular antigens that can be detected using the appropriate antibodies.

Note: *The antibodies must recognize the fixed form of the antigen of interest.*

The key to double labeling experiments is determining fixation and permeabilization conditions under which antigen and DNA integrity are maintained. Appropriate fixatives for DNA labeling are described above.

Post-treatments used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with NeuroPore may be sufficient for many antibodies and additional treatment may not be needed. Protease treatment is not recommended on most samples because the sample will often disintegrate later during immunocytochemistry or DNA labeling. Strong acid or base treatment should be avoided. Microwaving is an option that has given excellent results in double labeling experiments but requires careful empirical determination for correct wattage, time and cooling cycles for each sample.

Empirically determine optimal conditions for immunohistochemistry and *in situ* detection of apoptosis in separate experiments first. Combine the two methodologies only after optimizing separately on the same samples. Plan carefully and include controls to allow interpretation of double labeled samples. Controls for immunohistochemistry may include omission of primary antibodies to determine binding of the secondary antibody. In addition, blocking the primary antibody binding site with antigens may establish and demonstrate specificity.

The selection of the color reaction products should be considered ahead of time. Red Label provides excellent contrast with DAB and Blue Counterstain.

A standard immunohistochemistry protocol is provided for using phosphatase-conjugated secondary antibody and color development with Red Label Solution. Antibody concentrations, incubation times and temperatures and buffers may have to be optimized empirically for each system under study.

Note: *Phosphatase-conjugated reagents are inhibited by PBS or other phosphate-containing buffers. Tris buffers should be substituted for PBS.*

Method

1. After fixation, permeabilize with NeuroPore at room temperature for 1 hour. Use Hydrophobic Coverslips and a humidity chamber to prevent evaporation.
2. Wash 1 time in 100 mM Tris, pH 7.4.
3. Incubate at 2-8 °C overnight with primary antibody diluted in NeuroPore. Use Hydrophobic Coverslips and a humidity chamber to prevent evaporation.
4. Wash 3 times in 100 mM Tris, pH 7.4.
5. Incubate with phosphatase-conjugated secondary antibody diluted in NeuroPore at room temperature for 1 hour.
6. Wash 3 times in 100 mM Tris pH 7.4.
7. Prepare Red Label Solution:
 - a. To new micro tube add 50 μ L of deionized water and 5 μ L of Red Label Solution 1.
 - b. To new microtube add 1 μ L each of Red Label Solution 2 and Red Label Solution 3, tap microtube to mix. Centrifuge briefly and let stand for 3 minutes at room temperature. Transfer this solution to microtube in Step 7a.
8. Cover sample with Prepared Red Label Solution and incubate for up to 30 minutes in the dark.
9. Wash in deionized water 3 times to stop reaction.
10. Proceed with *in situ* detection beginning with Quenching Solution.

Many options are available for double labeling experiments. If the antigen is nuclear, carefully select the detection label and counterstains. Labeling nuclear antigens means the signal from the DNA labeling and immunocytochemistry will be in the same subcellar compartment and one signal may obscure the other. Similarly, many counterstains are not compatible with some color reaction products, *e.g.* Methyl Green cannot be used with Red Label. Some options are listed below.

- If a peroxidase-linked secondary antibody is preferred, use Quenching Solution prior to incubation with primary antibody and again prior to *in situ* detection of apoptosis. DAB Solution may be used for color reaction if alternative peroxidase based color development is used for detection of apoptosis.
- The Strep-HRP may be replaced with a streptavidin-phosphatase conjugate and developed using a phosphatase-based system such as Red Label.
- Similarly, fluorescent streptavidin conjugates and secondary antibodies may be used for a fluorescent read-out.

Appendix C. Electron Microscopy

The protocol given here can be adapted for electron microscopy. Both pre- and post-embedding labeling can be performed depending upon the system under study. For pre-embedding, fix sample and post-fix in osmium. After embedding and ultrathin sectioning, process sample for DNA labeling up to and including the washes of the labeling procedure prior to incubation with streptavidin. For detection of incorporated biotin use streptavidin conjugated to colloidal gold and incubate overnight at 2-8 °C. Stain with uranyl acetate.

For some samples post-embedding may be more convenient. Use fixed floating sections and process for in situ labeling up to and including the washes in the labeling procedure prior to streptavidin binding. Incubate in streptavidin conjugated to colloidal gold overnight at 2-8 °C. Wash; then proceed with standard embedding procedure and ultra thin sectioning.

Appendix E. Humidity Chamber

To prevent evaporation it is recommended that incubations at 37 °C are carried out in a humidity chamber. A humidity chamber can be made using a plastic box with a tight fitting lid and two glass rods or other support. Place paper towel on the bottom of the box and wet thoroughly with water. Lay the glass rods parallel to each other and less than one slide length apart on the wet tissue. Position the slides on the glass rods and place the plastic box, with lid, in a 37 °C incubator. Ensure that the slides are horizontal.

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