# Apoptosis Kit

## DermaTACS™ *In Situ* Apoptosis Detection Kit

Catalog Number: 4829-30-K

Reagent kit for *in situ* detection of apoptosis in dermal 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

Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Detection of nuclear DNA fragmentation is a widely accepted method to assay for apoptosis and can be performed *in situ* by incorporating labeled nucleotides onto the free 3' OH ends of DNA fragments using a terminal deoxynucleotidyl transferase enzyme (TdT). Terminal dUTP nick-end labeling (TUNEL) allows monitoring of apoptotic events among cells and within tissue sections.

The DermaTACS™ *In Situ* Apoptosis Detection Kit was developed to improve tissue staining and decrease non-specific background that can compromise data interpretation. Incorporation of brominated deoxyuridine (BrdU), which is more efficiently incorporated by TdT than biotinylated nucleotides at DNA fragmentation sites, is detected using a highly specific and sensitive biotinylated anti-BrdU antibody in combination with a streptavidin-peroxidase conjugate. Labeled cells are visualized using TACS Blue Label™ which provides unprecedented signal to noise ratios in competitive testing.

The DermaTACS *In Situ* Apoptosis Detection Kit provides all the reagents for the detection of DNA fragmentation among skin cells and dermal tissue sections. DermaTACS generates an intense blue staining in cells with DNA fragmentation which is easily visualized against a pale red counterstain. This kit also includes, Cytonin™, a non-lipophilic detergent based buffer optimized for the permeabilization of cells prior to labeling and Strep- Diluent, a proprietary blocking reagent that contributes to the low background obtained with DermaTACS.

## 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.

This 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.

## 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. |
| TACS-Nuclease™ | 4800-30-15 | 15 µL | |
| TdT Enzyme | 4810-30-05 | 30 µL | |
| B-dNTP Mix | 4828-30-04 | 30 µL | |
| Cytonin™ | 4876-05-01 | 6 mL | Store 2-8 °C. |
| 10X TdT Labeling Buffer | 4810-30-02 | 100 mL | |
| 10X TdT Stop Buffer | 4810-30-03 | 100 mL | |
| Strep-Diluent | 4828-30-12 | 7.5 mL | |
| Strep-HRP | 4800-30-06 | 30 µL | |
| TACS Blue Label™ | 4800-30-11 | 3 mL | |
| Anti-BrdU Antibody | 4828-30-06 | 30 µL | |
| TACS-Nuclease Buffer™ | 4800-30-16 | 1.5 mL | |
| Red Counterstain C | 4800-30-19 | 50 mL | Store at room temperature. |

## 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
- Slide warmer
- 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
- Methanol
- Mounting medium
- Tween® 20
- Distilled water

**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 $cm^2$ 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.

**PBST** - Approximately 200 mL of PBS-0.05% Tween® 20 is used to process 1-10 slides. To prepare add:

| Reaction Component | Volume |
|---|---|
| 1X PBS | 200 mL |
| Tween 20 | 100 µL |

***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 |

Proteinase K may be used at a 1:200 dilution. For more information read the Troubleshooting section.

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

**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 | Volume |
|---|---|
| Strep-Diluent | 800 µL |
| Strep-HRP | 1 µL |

## REAGENT PREPARATION *Continued*

***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 TdT 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.

***Labeling Reaction Mix -** Thaw B-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 ≤ -20 °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 |
|---|---|---|---|
| B-dNTP Mix | 2 µL | 10 µL | n x 1 µL |
| TdT Enzyme | 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.

**Antibody Solution** - Use 50 µl of diluted antibody per sample. To prepare add:

| Reaction Component | 2 Samples | 10 Samples | n Samples |
|---|---|---|---|
| Anti-BrdU | 2 µL | 10 µL | n x 1 µL |
| Strep-Diluent | 100 µL | 500 µL | n x 50 µL |

## REAGENT PREPARATION *Continued*

**TACS Blue Label** - 50 µL of TACS Blue Label is used per sample. It is provided ready to use. A faint blue coloration may be observed during storage. This does not affect stability or activity.

***TACS-Nuclease** - 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 µL | 500 µL | n x 50 µL |
| TACS-Nuclease | 2 µL | 10 µL | n x 1 µL |

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

| Reaction Component | per positive control |
|---|---|
| 1X TdT Labeling Buffer | 50 µL |
| TdT dNTP Mix | 1 µL |
| TdT Enzyme | 0-50 µL |
| TACS-Nuclease | 1 µL |

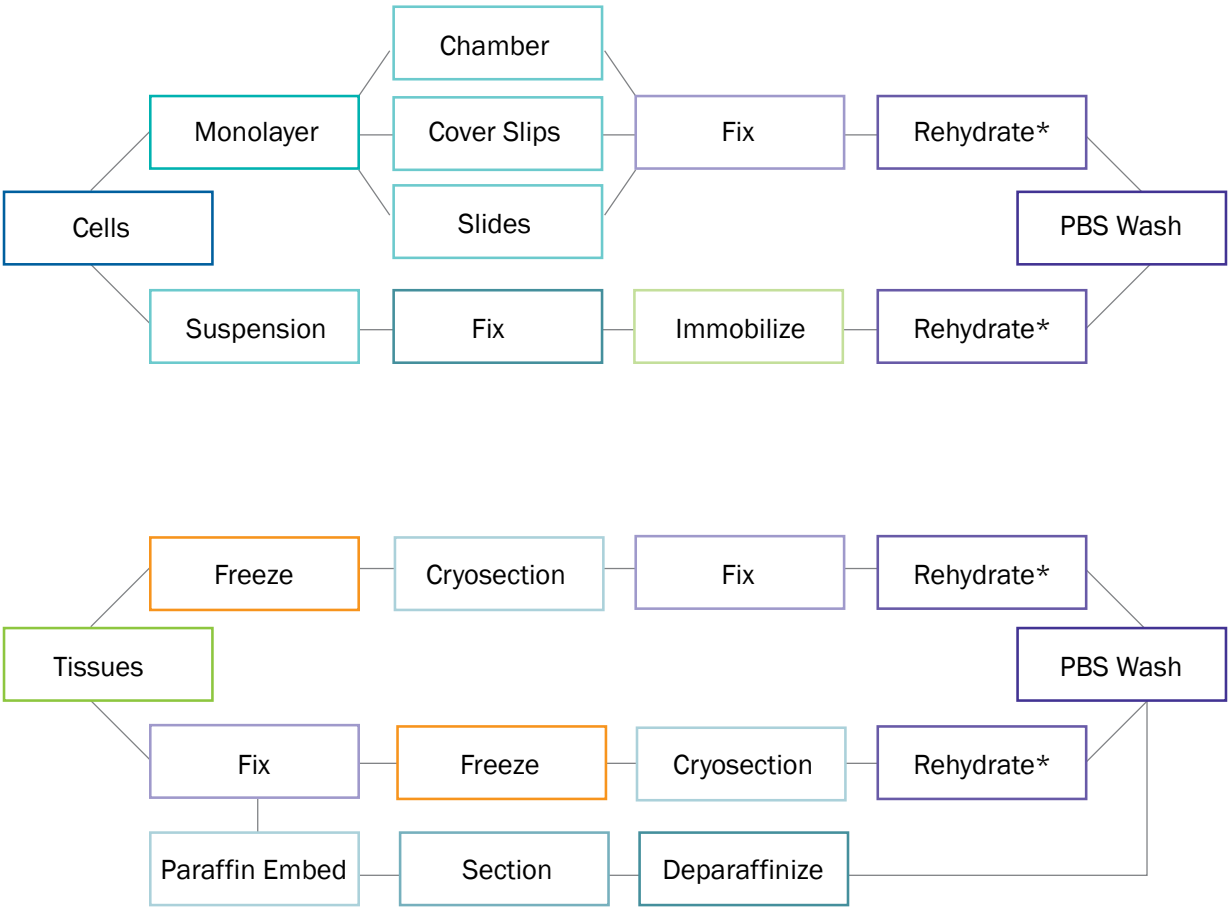
**Red Counterstain** - Red Counterstain C is ready to use. Red Counterstain C can be reused many times. Store in a closed container to prevent evaporation. For long term storage, keep away from light.

**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. Ethanols used for deparaffinization may be reused several times.

## 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** - Adherent cultured cells, such as keratinocytes, may float into the medium, and it may be necessary to recover these cells for analysis. Harvested floating cells, or suspension cells prepared from dissociated tissues can be fixed in solution, 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 x $10^6$ 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 x $10^7$ 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 x $10^5$ 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

For optimal labeling of keratinocytes, the cells should be grown on a surface that allows for both fixation and direct labeling, such as sterile chamber slides, cover slips or directly onto slides.

**On 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, e.g. 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. If needed, 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 Cytonin 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 Cytonin 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** - 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 and is usually compatible with DermaTACS.

**Immersion Fixation** - Skin samples are routinely fixed by immersion. It is useful to shave the skin prior to fixation to prevent air bubble formation on immersion. 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 $cm^3$) in at least 10 volumes of 3.7% buffered formaldehyde. After 30 minutes change to fresh fixative and leave at room temperature up to 24 hours.

**Perfusion Fixation** - Standard laboratory procedures should be followed for perfusion fixation. Formaldehyde, paraformaldehyde or glutaraldehyde may all be used as fixatives. After perfusion, the dissected skin tissue should be immersed in fresh fixative 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 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. Spot the 50 µL reaction volumes directly onto the coverslip. Alternatively, spot the 50 µL 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 µL of Proteinase K Solution and Incubate 15-30 minutes at room temperature, or cover sample with 50 µL of Cytonin and incubate for 15-30 minutes at 37 °C. If necessary, use cover slips. | |
| 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 µL 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 µL of Antibody Solution and incubate for 10 minutes at 37 °C. If necessary, use hydrophobic coverslips. | Refer to Reagent Preparation section. Incubation may be extended to 60 minutes if desired. |
| 11 | Wash samples 3 times with 1X PBS for 2 minutes each. | Refer to Reagent Preparation section. |
| 12 | Cover sample with 50 µL of Strep-HRP Solution for 10 minutes at room temperature. If necessary, use hydrophobic coverslips. | Refer to Reagent Preparation section. |
| 13 | Wash samples 2 times in 1X PBST for 2 minutes each at room temperature. | |
| 14 | Wash sample 1 time in deionized water, 2 minutes | |
| 15 | Cover sample with 50 µl of TACS Blue Label for 2-5 minutes at room temperature. | Monitor color development under a microscope for optimal results. |
| 16 | Wash samples in several changes of deionized water for 2 minutes each. | |
| 17 | Proceed to Counterstaining and Preparation for Viewing. | |

## COUNTERSTAINING AND PREPARATIONS FOR VIEWING

Skin cells and samples may be counterstained with Red Counterstain C. 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**

1. Immerse samples in distilled water for 2 minutes.
2. Immerse samples for 30 seconds to 5 minutes in Red Counterstain C.
3. Wash slides sequentially by dipping ten times in 2 changes of distilled water.
4. Air dry or dehydrate by dipping ten times in 95%, then 100% ethanol.
5. Clarify by dipping ten times, in two changes of o- or p-xylene. Wipe off excess xylene from the back of the slide and lay slide flat.
6. Place one drop, about 25 µL , of mounting media from a 100 µL pipette onto sample.
7. Lower glass cover slip onto sample and apply gentle even pressure to expel air bubbles.
8. Leave slide flat overnight to allow mounting media to harden. 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 µL 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 Reaction Labeling Mix of the positive control sample. Add 1 µL of TACS-Nuclease to 50 µL 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 associated with non-specific binding of the Strep-HRP. This control should not have any blue 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 blue staining.

**Counterstaining Controls** - It is recommended to process one or two samples up to and including the deionized water 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 1 minute immersion in Red Counterstain C 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.

Red Counterstain C allows all cells in the specimen to be visualized. Cells that are condensed (pyknotic, mitotic or apoptotic) will exhibit increased Red Counterstain C uptake. Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit a blue nuclear staining that may be very dark after labeling. This dark blue staining is typically associated with cell condensation. Blue 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 blue staining that is not associated with nuclei.

The controls 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 blue staining, the TACS-Nuclease Treated Control Sample should show pale blue staining in almost all cells, and the Experimental Negative Control should have few, if any blue stained cells. The blue staining of TACS-Nuclease Treated Control Sample 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 red/pink 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 |
|---|---|---|
| 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. |
| 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. |
| Blue 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. |
| | | Decrease concentration by diluting stock solution up to 1 in 800. |
| | 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 Cytonin (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 Storage of Samples, 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. |
| | Mixed xylenes were used during clarification and Blue Label was solubilized. | Use only o- or p-xylene that do not contain contaminating benzenes. |
| Red Counterstain C is too dark. | Overstaining | Reduce time in Red Counterstain C. |
| Labeling of majority of cells in the negative experimental control (e.g. normal skin sample of untreated cells) when there is no labeling if the enzyme is omitted and satisfactory labeling of the Nuclease-Treated Control Sample. | High level of apoptosis (or necrosis) in negative control. | Select a more appropriate negative control or inhibit apoptosis in cell culture (e.g. with protein synthesis inhibitors). Check morphology of cells prior to assay for evidence of excessive apoptosis. |
| | Excessive Proteinase K treatment. | Reduce incubation time in Proteinase K solution to 5-15 minutes. Or dilute Proteinase K 1:200 in water and incubate sample for 15 minutes. |

# 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, cover with Cytonin at 2-8 °C for up to one month. After storage, wash in 1X Labeling buffer then continue with the labeling reaction.
**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. Suggestions for Assay Optimization

When using paraffin-embedded sections, permeabilization is often a limiting step, therefore, start with a 1:50 dilution of Proteinase K for permeabilization at 37 °C for 15 minutes. If background is high, reduce the Proteinase K concentration and incubation time and temperature. If insufficient permeabilization is suspected, incubate the sample with a 1:50 dilution of Proteinase K at 37 °C for 30 minutes. When using fresh frozen sections, nominal permeabilization is required, therefore, start with Cytonin for permeabilization. If there is no labeling, permeabilize with Proteinase K at 1:200 for 15 minutes at 37 °C.

Freshly prepared cells usually do not require Proteinase K treatment, therefore, use Cytonin. If there is no labeling, permeabilize with Proteinase K at 1:200 for 15 minutes at 37 °C.

## 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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