

TREVIGEN[®] Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

TACS•XL[®] DAB *In Situ* Apoptosis Detection Kit

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

Catalog# 4828-30-DK

TACS•XL[®] DAB *In Situ* Apoptosis Detection Kit

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I. Quick Reference Procedure for TACS•XL® DAB (Cat# 4828-30-DK):

Read through the complete Instructions for Use prior to using this kit. Some incubation times and washes must be optimized using the controls to maximize labeling. All steps performed at room temperature unless otherwise noted.

This page is designed to be photocopied and used as a checklist:

- 1. Immerse hydrated, fixed, and immobilized sample in 1X PBS (page 4) for 10 minutes.
- 2. Cover sample with 50 µl of Proteinase K Solution (page 4) for 15-30 minutes, or cover sample with 50 µl of Cytonin™ (page 4) for 30 minutes.
- 3. Wash two times in deionized water, 2 minutes each.
- 4. Immerse sample in Quenching Solution for 5 minutes (page 4)
- 5. Wash sample in 1X PBS for 1 minute.
- 6. Immerse sample in 1X TdT Labeling Buffer for 5 minutes (page 5).
- 7. Cover sample with 50 µl of Labeling Reaction Mix (page 5). Incubate for 60 minutes at 37 °C in a humidity chamber (page 22).
- 8. Immerse sample in 1X TdT Stop Buffer (page 5) for 5 minutes.
- 9. Wash two times in 1X PBS, 2 minutes each.
- 10. Cover sample with 50 µl of Antibody Solution (page 5), and incubate for 30 minutes at 37 °C.
- 11. Wash two times in 1X PBS, 2 minutes each.
- 12. Cover sample with 50 µl of Strep-HRP Solution (page 5), and incubate for 10 minutes at room temperature.
- 13. Wash two times in 1X PBS, 2 minutes each.
- 14. Immerse in DAB Solution (page 5) for 2 to 7 minutes.
- 15. Wash in deionized water 2 times, 2 minutes each.
- 16. Immerse in 1% Methyl Green (page 6) for 30 seconds up to 5 minutes.
- 17. Dip slides ten times each in 2 changes of deionized water, 95%, and then 100% ethanol (page 6).
- 18. Dip 10 times each in 2 changes of xylenes (page 6).
- 19. Mount glass coverslips using mounting medium (page 18).

II. 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). This type of assay, often referred to as the TUNEL assay, allows monitoring of apoptosis in cell samples or in tissue sections, providing histological localization of the apoptotic events.

TACS•XL[®] was developed by Trevigen's scientists to improve on background problems that can compromise data interpretation. The new approach utilizes a brominated nucleotide (BrdU) which is more efficiently incorporated by TdT than biotinylated nucleotides at the sites of DNA fragmentation. Incorporated BrdU is detected using a highly specific and sensitive biotinylated anti-BrdU antibody and visualized thereby providing unprecedented signal to noise ratios in competitive testing.

The TACS•XL[®]-DAB *In Situ* Apoptosis Detection Kit provides all the reagents for the detection of DNA fragmentation in cells and tissue sections. TACS•XL[®]-DAB generates dark brown staining in cells with DNA fragmentation that is easily visualized against a green counterstain. In addition, the TACS•XL[®]-DAB Kit provides 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 TACS•XL[®]. The kit can be supplemented with additional reagents for double labeling using immunocytochemistry for antigen detection and *in situ* detection for apoptosis.

Please see pages 17-19 for information on additional Trevigen products available for the study of apoptosis.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical and toxicological properties of these products may not yet have been fully investigated therefore Trevigen recommends the use of gloves, lab coats and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. The TACS•XL[®]-DAB 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. Material safety data sheets are available on request.
4. Dispose of DAB in accordance with all local, state, and federal regulations.

IV. Materials Supplied

| Component | Quantity | Storage | Catalog# |
|-------------------------|----------|---------|------------|
| Proteinase K Solution | 50 µl | -20 °C | 4800-30-01 |
| Cytonin [™] | 6 ml | 4 °C | 4876-05-01 |
| 10X TdT Labeling Buffer | 100 ml | 4 °C | 4810-30-02 |

| Component | Quantity | Storage | Catalog# |
|-----------------------|-----------------|----------------|-----------------|
| 10X TdT Stop Buffer | 100 ml | 4 °C | 4810-30-03 |
| B-dNTP Mix | 30 µl | -20 °C | 4828-30-04 |
| TdT Enzyme | 30 µl | -20 °C | 4810-30-05 |
| Strep-HRP | 30 µl | 4 °C | 4800-30-06 |
| DAB Solution | 3.75 ml | -20 °C | 4800-30-07 |
| DAB Enhancer | 1 ml | -20 °C | 4800-30-09 |
| anti-BrdU antibody | 30 µl | 4 °C | 4828-30-06 |
| Strep-Diluent | 7.5 ml | 4 °C | 4828-30-12 |
| TACS-Nuclease™ | 15 µl | -20 °C | 4800-30-15 |
| TACS-Nuclease™ Buffer | 1.5 ml | 4 °C | 4800-30-16 |
| 1% Methyl Green | 50 ml | RT | 4800-30-18 |

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
2. 37 °C incubator
3. 50 and 500 ml graduated cylinders
4. 2 coplin jars
5. -20 °C and 4 °C storage
6. ice bucket
7. standard light microscope
8. cryostat or microtome
9. humidity chamber
10. 57 °C incubator or slide warmer
11. pipette helper
12. timer

Reagents

1. •Apoptosis Grade™ Water
2. •10X PBS
3. 37% formaldehyde
4. Tween® 20
5. xylenes
6. 30% hydrogen peroxide
7. 95% and 100% ethanol (or denatured alcohol)
8. methanol
9. butanol
10. •Mounting Medium or alternative mounting solution e.g. Permount

Disposables

1. •Treated Glass Microscope Slides (or alternative support)
2. 50 ml tubes
3. 1 - 200 µl and 200 - 1000 µl pipette tips
4. microcentrifuge tubes
5. 1.5 and 10 ml serological pipets
6. gloves
7. •Hydrophobic Coverslips (optional)
8. glass coverslips

•Available from Trevigen, Inc. (please see pages 18-19)

VI. 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.

1. 1X PBS

Please see page 18 for purchase or preparation of 10X PBS.

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

2. PBST

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

| | |
|-----------|--------|
| 1X PBS | 200 ml |
| Tween® 20 | 100 µl |

3. *3.7% Buffered Formaldehyde

If required, 50 ml of freshly prepared fixative is used to process 1 to 10 samples. To prepare add:

| | |
|--------------------|-------|
| 37% formaldehyde | 5 ml |
| 10X PBS | 5 ml |
| ddH ₂ O | 40 ml |

Wear gloves and exercise caution when handling formaldehyde solutions. Refer to Appendix C (page 20) for alternative fixation methods.

4. *Proteinase K Solution

Use 50 µl of Proteinase K Solution per sample. Store on ice. Thaw provided Proteinase K (Cat# 4800-30-01) at room temperature, then place on ice. To prepare add:

| | <u>2 samples</u> | <u>10 samples</u> | <u>n samples</u> |
|--------------------------------|------------------|-------------------|------------------|
| Deionized water | 100 µl | 500 µl | n x 50 µl |
| Proteinase K (Cat# 4800-30-01) | 2 µl | 10 µl | n x 1 µl |

Under some circumstances, the Proteinase K may be used at a 1:200 dilution. For guidelines please refer to pages 20-21 (Appendices C and D).

5. Cytonin™ (Cat# 4876-05-01)

If required, 50 µl of Cytonin is used per sample. Cytonin is ready for use. Store at 4 °C. Discard if solution is cloudy.

6. *Quenching Solution

Use 50 ml of Quenching Solution to process 1 to 10 samples. To prepare add:

| | |
|-----------------------|-------|
| 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 4 °C. For each labeling procedure, use a fresh 30% hydrogen peroxide aliquot then discard the unused portion.

7. 1X TdT Labeling Buffer

Dilute the 10X TdT Labeling Buffer (Cat# 4810-30-02) to 1X using distilled water. Leave at room temperature until use. Use 50 ml of 1X Labeling Buffer to process 1 to 10 samples. Remove an aliquot of 50 µl per sample for preparing the Labeling Reaction Mix (see below) and place on ice.

8. *Labeling Reaction Mix

Thaw B-dNTP Mix (Cat# 4828-30-04) at room temperature, then place on ice. To maintain optimal enzyme activity, remove the TdT Enzyme (Cat# 4810-30-05) 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:

| | <u>2 samples</u> | <u>10 samples</u> | <u>n sample</u> |
|---------------------------------|------------------|-------------------|-----------------|
| B- dNTP Mix (Cat# 4828-30-04) | 2 µl | 10 µl | n x 1 µl |
| TdT Enzyme (Cat# 4810-30-05) | 2 µl | 10 µl | n x 1 µl |
| 1X TdT Labeling Buffer (Step 7) | 100 µl | 500 µl | n x 50 µl |

9. 1X TdT Stop Buffer

Dilute the 10X TdT Stop Buffer (Cat# 4810-30-03) to 1X using distilled water. Leave at room temperature until use. Use 50 ml of 1X TdT Stop Buffer to process 1 to 10 samples.

10. Antibody solution

Use 50 µl of diluted antibody per sample. To prepare add:

| | <u>2 samples</u> | <u>10 samples</u> | <u>n samples</u> |
|---------------------------------|------------------|-------------------|------------------|
| Anti-BrdU (Cat# 4828-30-06) | 2 µl | 10 µl | n x 1 µl |
| Strep-Diluent (Cat# 4828-30-12) | 100 µl | 500 µl | n x 50 µl |

11. *Strep-HRP Solution

Use 50 µl of Strep-HRP Solution per sample. Store prepared Strep-HRP Solution at room temperature until use. To prepare add:

| | <u>2 samples</u> | <u>10 samples</u> | <u>n samples</u> |
|-----------------------------|------------------|-------------------|------------------|
| 1X PBS | 100 µl | 500 µl | n x 50 µl |
| Strep-HRP (Cat# 4800-30-06) | 2 µl | 10 µl | n x 1 µl |

12. *DAB Solution

Thaw DAB (Cat# 4800-30-07) at 37°C for 30 min. If not using the full bottle, store the remaining stock solution back at -20°C. It is not recommended to freeze thaw DAB stock solution more than three times. If required, aliquot in smaller volume and store at -20°C protected from light.

To prepare add:

| | |
|--------------------------------|------------|
| 1X PBS | 50 ml |
| DAB (Cat# 4800-30-07) | 250 µl |
| DAB Enhancer (Cat# 4800-30-09) | 0 to 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 4 °C. For each labeling procedure use a fresh 30% hydrogen peroxide aliquot then discard any remaining solution.

Note: Do not place the DAB (Cat# 4800-30-07) on ice after thawing, otherwise the DAB will precipitate. Prepare DAB Solution no more than 30 minutes before use. Use 50 ml of DAB Solution to process 1 to 10 samples. DAB Enhancer may be used to intensify and darken DAB staining; the concentration must be optimized experimentally.

13. 1% Methyl Green (Cat# 4800-30-18)

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

14. Xylenes

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.

15. 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.

16. *TACS-Nuclease™ and Buffer

For the preparation of a Nuclease-treated positive control sample, it is recommended that the DNA breaks be generated in a separate step (see Section VIII, pg 13), in this case, TACS-Nuclease™ should be diluted 1:50 in TACS Nuclease buffer, as below:

| | <u>2 samples</u> | <u>10 samples</u> | <u>n samples</u> |
|--|------------------|-------------------|------------------|
| TACS-Nuclease™ Buffer (Cat# 4800-30-16) | 100 µl | 500 µl | n x 50 µl |
| TACS-Nuclease™ (Cat# 4800-30-15) | 2 µl | 10 µl | n x 1 µl |

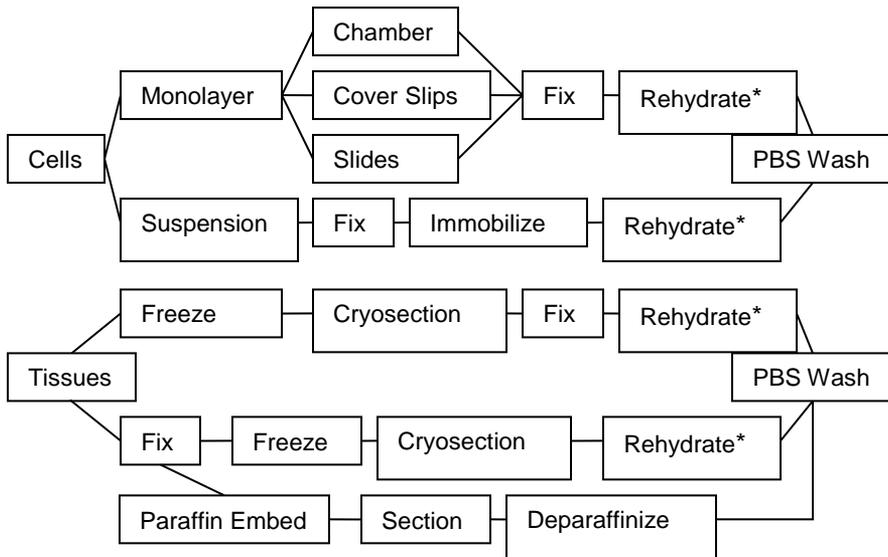
It is also possible to obtain acceptable, but lower intensity, positive control staining by incubating the TACS Nuclease with the Labelling Reaction Mix. In this case, per positive control, prepare:

| | <u>per positive control</u> |
|---------------------------------|-----------------------------|
| 1X TdT Labeling Buffer (Step 6) | 50 µl |
| B- dNTP Mix (Cat# 4828-30-04) | 1 µl |
| TdT Enzyme (Cat# 4810-30-05) | 1 µl |
| TACS-Nuclease™ | 1 µl |

Avoid repeated freeze-thaw of TACS-Nuclease™.

VII. 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 must be rehydrated, if necessary, 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 are provided in Section VI. A. Sample Preparation and Fixation (below). 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. Contact Trevigen's Technical Service for additional information about other methods not described.

A. Sample Preparation and Fixation

A.1 Preparation of Cells in Culture

a. Preparation of Suspension Cells

Cells grown in suspension or 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.

Method

1. Harvest cell suspension by centrifugation at 500 x *g* for 5 minutes at room temperature.
2. Discard media and resuspend at 1 x 10⁶ cells per 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 10⁷ cells per ml in 80% ethanol. Cells can be stored in 80% ethanol at 4 °C for several weeks. However, signal intensity in positive cells will reduce with time due to loss of small DNA fragments.
5. Spot 1 x 10⁵ 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 (e.g. Trevigen's Three Ring Treated Microscope Slides, Cat# 4864-100). Other slide treatments (e.g. 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 4 °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 (step 7 and 8 below).
7. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
8. Immerse in 1X PBS and proceed to Labeling Procedure, page 11.

b. 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, 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, however, 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, however, 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.

Method

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 4 °C with desiccant.
 - b) Fixed cells can be stored for up to 1 week in Cytonin™ at 4 °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 on page 11.

A.2 Preparation of Tissues

Use of glass slides pretreated for electrostatic adherence is recommended for all tissues (e.g. Trevigen's Treated Glass Microscope Slides, Cat# 4861-72).

a. 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.**

i) Freeze Tissue

Rapidly freeze tissue or biopsy immediately after removal by immersing in liquid nitrogen or on dry ice. Store frozen tissue below -70 °C.

ii) Cryosection Frozen Tissue

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 -80 °C.

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.

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.

iii) Fixation after cryosectioning

Method

1. 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 -80 °C with desiccant for up to 3 months. After storage equilibrate samples to room temperature and redry for 2 hours at room temperature or 2 hours on a slide warmer at 45 °C. Proceed with rehydration from step 2 below.
2. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
3. Wash once in 1X PBS for 5 minutes.
4. Fix samples by immersing in 3.7% buffered formaldehyde for 10 minutes at room temperature.
5. Wash cells 1 time in 1X PBS.
6. Proceed to Labeling Procedure on page 11.

b. Preparation of Fixed Samples Before Sectioning – Immersion or Perfusion

i) 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. However, 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 TACS•XL®-DAB.

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 fixative 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 perfusion, the dissected 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 4 °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.

ii) Sectioning of Fixed Tissue

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* on page 8.

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

Storage of Sectioned Tissues and Rehydration after Storage Sections of fixed frozen tissue may be stored at -80 °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 drier. Rehydrate samples before labeling by immersing for 5 minutes each in 100%, 95%, then 70% ethanol and wash for 10 minutes in 1X PBS.

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.

Sections between 6-10 µm should be collected onto slides pretreated for electrostatic adherence (e.g. Trevigen's Treated Glass Microscope Slides, Cat# 4861-72). Prior to the labeling reaction the samples must be deparaffinized. Optimal labeling is achieved when the samples are processed within days of sectioning.

iii) Deparaffinization

Sections prepared from paraffin blocks require removal of the paraffin prior to the labeling reaction.

Method

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 below.

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.

B. *In Situ* Labeling Procedure

Details on the labeling procedure are provided in the table below.

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 (Cat# 4867-100) 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 (use a gentle vacuum if available). 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 melt plastics, therefore, do not add xylenes to tissue culture plates.

Labeling Procedure for Immobilized, Fixed, and Rehydrated Samples in 1X PBS

| 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 to 30 minutes at room temperature, or cover sample with 50 µl of Cytonin. | Some empirical determination may be required. A 1:200 dilution of the Proteinase K (Cat# 4800-30-01) is appropriate for some tissues. |
| 3 | Wash 2 times in 1X PBS, 2 minutes each. | |
| 4 | Immerse slides in Quenching Solution for 5 minutes at room temperatures. | Refer to page 4 for Reagent Preparation. 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 page 4 for Reagent Preparation. |

| | | |
|------|--|--|
| 6 | Immerse slides in 1X TdT Labeling Buffer for 5 minutes. | Refer to page 5 for Reagent Preparation. |
| 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. | Refer to page 5 for Reagent Preparation. Use humidity chamber during incubation time. For details see Appendix F. To generate a positive control using TACS-Nuclease™, refer to page 13. |
| 8 | Immerse samples in 1X TdT Stop Buffer for 5 minutes at room temperature to stop labeling reaction. | |
| Step | Instructions | Notes |
| 9 | Wash samples 2 times in 1X PBS for 2 minutes each at room temperatures. | |
| 10 | Cover sample with 50 µl of Antibody Solution and Incubate for 30 minutes at 37 °C, If necessary, use Hydrophobic Coverslips. | Refer to page 5 for reagent preparation. |
| 11 | Wash samples 2 times in 1X PBST for 2 minutes each at room temperatures. | |
| 12 | Cover sample with 50 µl of Strep-HRP Solution and incubate for 10 minutes at room temperature. If necessary, use Hydrophobic Coverslips. | Refer to page 5 for Reagent Preparation. |
| 13 | Wash samples 2 times in 1X PBS, 2 minutes each. | |
| 14 | Immerse samples in DAB Solution for 2 to 7 minutes. | Use caution when handling DAB Solution. Refer to page 5 for Reagent Preparation. |
| 15 | Wash samples in several changes of deionized water, 2 minutes each. | |
| 16 | Proceed to Counterstaining and Preparation for Viewing. | |

C. Counterstaining and Preparation for Viewing

Cells and tissues may be counterstained with Methyl Green. 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 deionized water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in Methyl Green.
3. Wash slides sequentially by dipping ten times each in:
 - Deionized water; 70% ethanol, 2 changes
 - 95% ethanol, 2 changes
 - 100% ethanol, 2 changes
 - 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 deionized water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in Methyl Green.
3. Wash slides sequentially by dipping ten times each in: 1-butanol, until sample turns from blue to green 1-butanol, 1 time to rinse 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 μ l, of mounting medium from a 100 μ l 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.

VIII. Controls

If you wish to use a pre-prepared control you may use either the Cell Culture Control Slides (Cat# 4800-30-20) or the Tissue Control Slides (Cat# 4800-30-40). These controls allow you to run through the procedure to become familiar with handling the samples, etc. Each set of Control Slides is shipped with a product information sheet that provides information on the recommended permeabilization method, incubation times, and interpretation of data. It is critical to run controls using the provided TACS-NucleaseTM to assess and optimize cell/tissue permeabilization; the recommended experimental controls are listed below.

TACS-NucleaseTM-Treated Control

Treat one sample with TACS-NucleaseTM to generate DNA breaks in every cell. Avoid repeated freeze-thaw cycles. The TACS-NucleaseTM-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.

Method

1. After Step 5 (page 11), wash 2 times in deionized water, 2 minutes each.
2. Prepare TACS-NucleaseTM Solution by diluting TACS Nuclease 1:50 in the TACS Nuclease buffer (See Reagent Preparation, pg 6).
3. Cover sample with 50 μ l of TACS-NucleaseTM Solution.
4. Incubate at RT for 10 to 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, page 11.

To save time, TACS-NucleaseTM can be added directly to the labeling mix of the positive control sample. In this case, add 1 μ l of TACS-NucleaseTM to 50 μ l of complete labeling mix (see Reagent Preparation, page 6), and incubate for the regular labeling time. However, it should be noted that, this method generates lower signal intensity compared to the recommended method above.

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.

Counterstaining Controls

Although uncommon, some cells and tissues may take up excessive amounts of the Methyl Green 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 (after step 4, page 11) through counterstaining. Staining times of 5 seconds to 5 minutes have been noted. It is recommended to start with a 30 second immersion in Methyl Green Counterstain and alter accordingly.

IX. 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. Methyl Green allows all cells in the specimen to be visualized. Cells that are condensed (pyknotic, mitotic or apoptotic) will exhibit increased Methyl Green 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 experimental controls listed above (page 13), are important for data interpretation. These controls allow optimization of *in situ* detection of apoptosis without expending valuable test samples. Under optimal conditions the Unlabeled Control (i.e. 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 typically have less than 20% brown stained cells. The brown staining of TACS-Nuclease™ treated 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 Counterstain Control should show a pale green staining of all cells with some variability in intensity between cell types and darker staining of any condensed cells. Refer to Troubleshooting, for information if the Controls do not provide the expected result.

X. Troubleshooting

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

| Troubleshooting Guide | | |
|--|---|--|
| 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 in 200. |
| | Sample dried out during the labeling procedure. | Use Hydrophobic Coverslips (or lids for plates or chamber slides) and incubate in humidity chamber. |
| Problem | Cause | Action |
| 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 Cytonin™. Samples can be stored for up to 1 week in Cytonin at 4 °C. |
| | No DNA left in sample due to hydrolysis (poor storage of samples or sections). | Read Section VII, A, <i>Sample Preparation and Fixation</i> , page 7, for information on storage. |
| | 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 µl of Strep-HRP onto 1 cm ² of 3MM paper and air dry. Immerse the filter paper in DAB Solution to check for brown coloration compared with a piece of white paper. |
| Labeling of majority of cells in the negative experimental control (e.g. normal tissue or untreated cells) when there is no labeling if the enzyme is omitted and satisfactory | 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. |

| | | |
|---|--|--|
| labeling of the TACS-Nuclease™ treated control. | | |
| | Prolonged incubation with DAB leads to precipitate over entire sample. | Reduce time of DAB treatment. |
| 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. |

| Problem | Cause | Action |
|---|--|--|
| 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. |

XI. References

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XII. Related products available from Trevigen.

Apoptosis kits

| Catalog # | Description | Size |
|------------|--|-------------|
| 4817-60-K | FlowTACS™ Kit | 60 samples |
| 4822-96-K | HT TiterTACS™ Assay Kit | 96 tests |
| 4830-01-K | TACS® Annexin V FITC Kit | 100 samples |
| 4835-01-K | Annexin V Biotin Kit | 100 samples |
| 6300-100-K | DePsipher™ Mitochondrial Potential Assay Kit | 100 tests |
| 6305-100-K | MitoShift™ Mitochondrial Potential Assay Kit | 100 tests |
| 4815-30-K | TumorTACS™ <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4823-30-K | NeuroTACS™ <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4827-30-K | CardioTACS™ <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4829-30-K | DermaTACS™ <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4826-30-K | VasoTACS™ <i>In Situ</i> Apoptosis Detection Kit | 30 samples |

| Catalog # | Description | Size |
|------------|---|------------|
| 4828-30-BK | TACS•XL® Blue Label <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4810-30-K | TACS® 2 TdD DAB <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4811-30-K | TACS® 2 TdT Blue Label <i>In Situ</i> Apoptosis Detection Kit | 30 samples |
| 4812-30-K | TACS® 2 TdT Fluorescein Apoptosis Detection Kit | 30 samples |
| 4850-20-ET | TACS® Apoptotic DNA Laddering Kit EtBr | 20 samples |
| 4855-20-K | TACS® Apoptotic DNA Laddering Kit Chemiluminescent | 20 samples |
| 4857-20-K | TACS® Apoptotic DNA Laddering Kit Colorimetric | 20 samples |
| 4850-20-K | TACS® Apoptotic DNA Laddering Kit Isotopic | 20 samples |
| 4892-010-K | Cultrex® Calcein-AM Cell Viability Kit | 1000 tests |
| 4684-096-K | HT Colorimetric PARP Apoptosis Assay | 96 samples |
| 4685-096-K | HT Chemiluminescent PARP Apoptosis Assay | 96 samples |

Antibodies

| Catalog # | Description | Size |
|--------------|--|--------|
| 2281-MC-100 | Anti-Bax Monoclonal Antibody (Clone YTH-6A7) | 100 µg |
| 2291-MC-100 | Anti-Bcl-2 Monoclonal Antibody (Clone YTH-8C8) | 100 µg |
| 4411-PC-100 | Anti-Phosphorylated Histone-γ-H2AX polyclonal | 100 µl |
| 6361-PC-100 | Anti-human/mouse-PBR polyclonal | 100 µl |
| 6370-MC-100 | Anti-human/murine-Cytochrome C | 100 µg |
| 6380-MC-100 | Anti-human/murine-Holocytochrome C | 100 µg |
| 4335-MC-100 | Anti-PAR polymer mAb (10HA) | 100 µl |
| 4336-BPC-100 | Anti- PAR polymer polyclonal | 100 µl |
| 4338-MC-50 | Anti-human/murine-PARP mAb (clone C2-10) | 50 µg |

Accessories

| Catalog # | Description | Size |
|-------------|--|------------|
| 4800-30-40 | Tissue Control Slides | 2 ea. |
| 4864-100 | Treated Glass Microscope Slides w/3 sample Hydrophobic Barrier | 100 ea. |
| 4800-30-20 | Cell Culture Control Slides | 2 ea. |
| 4867-100 | Hydrophobic Coverslips | 100 ea. |
| 4865-25 | Mounting Medium | 25 ml |
| 4800-30-14 | Strep-Fluorescein | 30 µl |
| 4870-500-6 | 10X PBS | 6 x 500 ml |
| 4830-100-03 | Propidium Iodide | 30 µl |

| | | |
|-------------------|--|-------------------|
| 4869-500-6 | Apoptosis Grade™ H₂O | 6 x 500 ml |
| 4820-30-13 | Blue Counterstain | 50 ml |
| 4825-30-01 | Red Label™ | 5 ml |
| 4862-10 | Coverslips 24 x 60 mm, No. 1.5 | 2 ea. |
| 4878-05-02 | Cytonin™ IHC | 2 x 5 ml |

XIII. Appendices

Appendix A. Reagent and Buffer Composition

10X PBS, pH 7.4 (Cat# 4870-500-6) – Sold Separately:

75 mM disodium hydrogen phosphate (Na₂HPO₄)

25 mM sodium dihydrogen phosphate (NaH₂PO₄)

1.45 M sodium chloride (NaCl)

Apoptosis Grade™ Water (Cat# 4869-500-6) – Sold Separately:

Deionized (18 mΩ) sterile water

Distilled autoclaved water can be used

Cytonin™ (Cat# 4876-05-01):

Proprietary permeabilization and blocking agent

Proteinase K (Cat# 4800-30-01)

1 mg/ml proteinase K

10X TdT Labeling Buffer (Cat# 4810-30-02):

1 M TACS Safe-TdT™ Buffer

0.5 mg/ml BSA (RIA Grade)

0.6 mM 2-mercaptoethanesulfonic acid (MESNA)

10X TdT Stop Buffer (Cat# 4810-30-03):

0.1 M EDTA, pH 8.0

B-dNTP Mix (Cat# 4828-30-04):

Optimized nucleotide mix of brominated dNTPs

Strep-Diluent (Cat# 4828-30-12)

Optimized blocking agent

Anti-BrdU (Cat# 4828-30-06)

Biotinylated mouse monoclonal antibody to bromodeoxyuridine

TACS-Nuclease™ (Cat# 4800-30-15):

Proprietary endonuclease

TACS-Nuclease™ Buffer (Cat# 4800-30-16):

50 mM Tris-Cl, pH 8.0

1 mM magnesium chloride

100 µg/ml BSA

1% Methyl Green (Cat# 4800-30-18):

0.1 M sodium acetate, pH 4.0

1.0 % methyl green

Appendix B. 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. However, 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. Fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments (see Appendix D: *Double Labeling Hints and Tips*, page 20). Post-fixation in acetone, ethanol, or methanol is common in preparation of tissues and is usually compatible with TACS-XL[®]-DAB. To store the immobilized fixed cells, i.e. on slides, chamber slides or coverslips, post-fix in 100% methanol after fixation. Wash in PBS, then store in Cytonin[™] at 4 °C for up to 1 week. After storage, wash in 1X PBS then continue with the labeling reaction starting at Step 4 in the protocol on page 11 (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 C. Suggestions for Assay Optimization

The following table shows examples of conditions that have been used for labeling the tissues listed and acts as a guide only. Actual incubation times and the permeabilization method selected will require empirical determination for optimal results.

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.

| | Cytonin [™] | Pro K (1:50) | Pro K (1:200) |
|-----------------------------|----------------------|--------------|---------------|
| Brain | * | * | * |
| Heart | | * | |
| Lung | * | | * |
| Liver | | * | |
| Kidney | | * | |
| Spleen | | * | |
| Duodenum | | * | |
| Colon | * | * | |
| Small Intestine | * | * | |
| Large Intestine | * | * | |
| Skin | | * | |
| Bone/Cartilage | * | * | |
| Tumor | | * | |
| Epithelium | | * | * |
| Endothelium | | * | * |
| Cultured Cells (stored dry) | * | * | |
| Cultured Cells (fresh) | * | | * |

Appendix D. 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 cellular antigens against which antibodies are available.

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

The key to double labeling experiments is determining fixation and permeabilization conditions under which both antigen and DNA integrity is maintained. Appropriate fixatives for DNA labeling are provided in Appendix B: *Fixation Methods*, page 19. Post-treatments

used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with Cytonin™ IHC (Cat# 4878-05-02) 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. Trevigen offers Red Label™ (Cat# 4825-30-RL) for use with secondary antibodies conjugated to phosphatases. Red Label provides excellent contrast with DAB and Blue Counterstain (Cat# 4820-30-13).

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 Cytonin™ IHC 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 4 °C overnight with primary antibody diluted in Cytonin™ IHC. 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 Cytonin™ IHC at room temperature for 1 hour.
6. Wash 3 times in 100 mM Tris pH 7.4.
7. Prepare Red Label Solution:
 - a. To fresh microtube add 50 µl of deionized water and 5 µl of Red Label Solution 1.
 - b. To new microtube add 1 µl 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 step on page 11.

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 E. Electron Microscopy

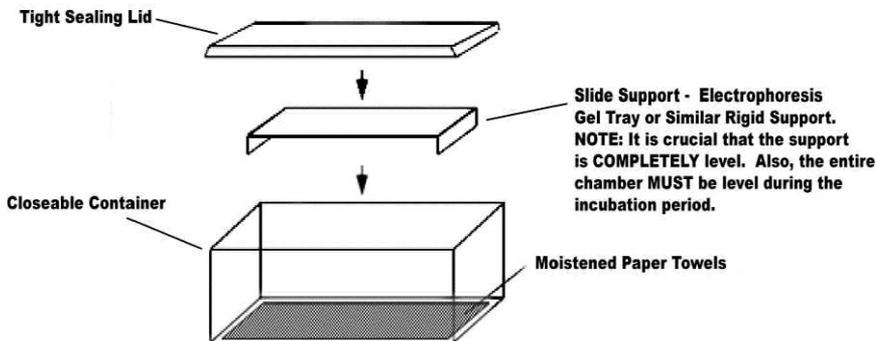
The protocol given here can be adapted for electron microscopy. Both pre- and post-embedding labeling can be performed depending upon the system under study. For pre-embedding, fix sample and post-fix in osmium. After embedding and ultrathin sectioning, process sample for DNA labeling up to and including 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 4 °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 4 °C. Wash, then proceed with standard embedding procedure and ultra thin sectioning. Contact Trevigen® for more details.

Appendix F. 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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