

**TumorTACS™ *In Situ* Apoptosis
Detection Kit**

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Apoptosis Detection Kit**

Catalog# 4815-30-K

Reagent kit for *in situ* detection
of apoptosis in tumor-derived sections and cells.

30 samples

Catalog# 4815-30-K

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I. Quick Reference Procedure for TumorTACS™ (cat# 4815-30-K):

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 are 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 3) for 10 minutes.
 - 2. Cover sample with 50 µl of diluted Proteinase K Solution (page 4) for 5 minutes, **or** cover sample with 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 4).
 - 7. Cover sample with 50 µl of Labeling Reaction Mix (page 4). Prepare controls (page 12). Incubate for 60 minutes at 37 °C in a humidity chamber (Appendix E).
 - 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 Strep-HRP Solution (page 5), and incubate for 10 minutes at room temperature in a Humidity Chamber to avoid evaporation (page 19).
 - 11. Wash two times in 1X PBS, 2 minutes each.
 - 12. Immerse in DAB solution (page 5) for 2 to 7 minutes.
 - 13. Wash 4 times in deionized H₂O, 1-2 minutes each.
 - 14. Immerse in 1% Methyl Green for 30 seconds.
 - 15. Dip slides ten times each in 2 changes of deionized water, 95%, then 100% ethanol (page 5).
- OR** Dip 10 times in two changes of butanol.
- 16. Dip 10 times each in 2 changes of o- or p-xylene (page 5).
 - 17. Mount glass coverslips using mounting medium (cat# 4865-25).

II. Background

A common biochemical marker of apoptosis is DNA fragmentation. During apoptosis the chromosomal DNA is cleaved by endonucleases to generate DNA fragments with free 3'-hydroxyl residues. This DNA cleavage is not sequence specific, however, the short DNA linker regions between nucleosomes are more susceptible to cleavage. Extraction of total DNA from apoptotic cells and analysis by agarose gel electrophoresis reveals the typical DNA ladder pattern of multimers of approximately 180 base pairs: the average length of DNA within each nucleosome. *In situ*, the 3' ends of cleaved DNA fragments provide a substrate for the enzyme terminal deoxynucleotidyl transferase (TdT) which adds nucleotides at the site of DNA breaks. The incorporation of biotinylated nucleotides allows chromosomal DNA fragmentation to be visualized by binding streptavidin-horseradish peroxidase followed by reaction with diaminobenzidine (DAB) to generate a dark brown precipitate. When viewed under a standard light microscope apoptotic cells are clearly distinguished by the dark brown staining.

Trevigen's **TumorTACS™ *in situ* Apoptosis Detection Kit** is designed specifically for the detection of DNA fragmentation in tumor tissues and cancer cells in culture. The kit is derived from Trevigen's TACS® 2 *In Situ* Apoptosis Detection Kit and developed from laboratory testing of tumor samples analyzed through our in-house start up labeling service. The TumorTACS™ kit is supplemented with a cation that specifically enhances the signal in apoptotic tumor cells. In addition, TACS-Nuclease™ allows positive controls to be generated for each experimental system. TumorTACS™ is also ideal for the detection of apoptosis in frozen samples, and paraffin embedded tumor specimens. Please see pages 15-16 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 TumorTACS™ 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

<u>Component</u>	<u>Quantity</u>	<u>Storage</u>	<u>Catalog#</u>
Cytonin™	5 ml	4 °C	4876-05-01
Proteinase K	30 µl	-20 °C	4800-30-01
10X TdT Labeling Buffer	100 ml	4 °C	4810-30-02
10X TdT Stop Buffer	100 ml	4 °C	4810-30-03
TdT dNTP Mix	30 µl	-20 °C	4810-30-04
TdT Enzyme	30 µl	-20 °C	4810-30-05
50X Mn ²⁺	30 µl	-20 °C	4810-30-14
Strep-HRP	30 µl	4 °C	4800-30-06
DAB Solution	3.75 ml	-20 °C	4800-30-07

Component	Quantity	Storage	Catalog#
DAB Enhancer	1 ml	-20 °C	4800-30-09
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 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
- 37°C incubator
- 50 ml and 500 ml graduated cylinders
- 2 coplin jars
- 20 °C and 4 °C storage
- ice bucket
- standard light microscope
- cryostat or microtome
- humidity chamber
- 57°C incubator or slide warmer
- pipette helper
- timer

Reagents

- Apoptosis Grade™ Water
- 10X PBS
- 37% formaldehyde
- o- or p-xylene
- 30% hydrogen peroxide
- 95% and 100% ethanol (or denatured alcohol)
- Butanol (n-butanol, 1-butanol)
- methanol
- Mounting Medium (cat# 4865-25) or alternative e.g. Permount

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 pipets
- gloves
- Hydrophobic Coverslips (optional)
- glass coverslips
 - Available from Trevigen, Inc. (please see pages 15-16)

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 17 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. 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.

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 B (page 18) 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.

5. *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.

6. 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 (next item) and place on ice.

7. *Labeling Reaction Mix

Thaw 50X Mn²⁺ (cat# 4810-30-14) and TdT dNTP Mix (cat# 4810-30-04) 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>
TdT dNTP Mix (cat# 4810-30-04)	2 µl	10 µl	n x 1 µl
50X Mn ²⁺ Cation (cat# 4810-30-14)	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 (item 6)	100 µl	500 µl	n x 50 µl

Also prepare a separate mix for the positive control (see item 11, below).

8. 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.

9. *Strep-HRP Solution

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

	2 samples	10 samples	n samples
1X PBS	100 μ l	500 μ l	n x 50 μ l
Strep-HRP (cat# 4800-30-06)	2 μ l	10 μ l	n x 1 μ l

10. *DAB Solution

Thaw DAB (cat# 4800-30-07) at 37 °C, then leave at room temperature. **Note:** Do not place on ice or the DAB solution will precipitate. Prepare DAB Solution no more than 30 minutes before use. Use 50 ml of DAB Solution to process 1 to 10 samples. To prepare add:

1X PBS	50 ml
DAB (cat# 4800-30-07)	250 μ l
DAB Enhancer (cat# 4800-30-09)	0 to 50 μ l
30% hydrogen peroxide	50 μ l

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.

11. *TACS-Nuclease™ and Buffer

For the preparation of a Nuclease-treated control sample, TACS-Nuclease™ may be used directly in the labeling mix (see below) or used diluted in a separate step (please see Section VIII, page 12). Avoid repeated freeze-thaw of TACS-Nuclease™. Per positive control prepare:

	per positive control
1X TdT Labeling Buffer (item 6)	50 μ l
TdT dNTP Mix (cat# 4810-30-04)	1 μ l
TdT enzyme (cat# 4810-30-05)	1 μ l
TACS-Nuclease™ (cat# 4800-30-15)	1 μ l
50X Mn ²⁺ Cation (cat# 4810-30-14)	1 μ l

12. 1% Methyl Green (cat# 4800-30-18) 1% Methyl Green is ready for use, and may be reused many times. Store in a closed container to prevent evaporation. If a precipitate forms, filter sample through Whatman 3MM paper.

13. o- or p-Xylene

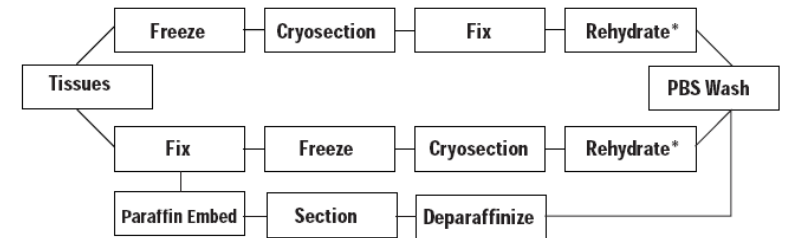
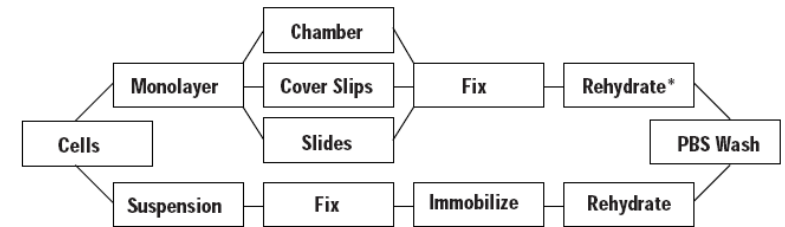
Mixed xylenes may be used for deparaffinization and for clarification prior to mounting coverslips onto the samples. Xylenes used for deparaffinization may be reused several times. Xylenes used in deparaffinization should not be used for clarification. Limolene terpenes offer a nontoxic alternative to xylenes however, longer immersion times may be required.

14. 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.

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 VII. 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

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 1,500 x g for 5 min. at room temp.
2. Discard media and resuspend at 1×10^6 cells per ml in 3.7% buffered formaldehyde. Let stand for 10 minutes at room temperature.
3. Centrifuge at 1,500 x g for 5 minutes at room temperature and discard fixative.
4. Resuspend at 1×10^7 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 1×10^5 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 (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 (next steps 7 and 8).
7. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
8. Immerse in 1X PBS and proceed to *In Situ* Labeling Procedure, page 10.

b. Preparation of Cells in Monolayers

On Sterile Chamber Slides

For optimal outcomes, cells should be grown on a surface that allows for 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 medium 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 10.

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

Fresh tissue requires minimal fixation and 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 a 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 tumor 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 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.
2. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
3. Wash 2X in 1X PBS for 5 minutes each.
4. Fix samples by immersing in 3.7% buffered formaldehyde for 10 minutes at room temperature.
5. Wash cells 2 times in 1X PBS, 5 minutes each.
6. Proceed to Labeling Procedure on page 10.

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.

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 2 hours of 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* (previous page).

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. Do not bake slides.

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 Cytonin™ and incubate 30 minutes at room temperature, or cover sample with 50 µl of Proteinase K Solution (page 4) for 5 to 30 minutes at 37 °C. If necessary, use Cover Slips.	Cytonin™ is recommended for frozen sections and when protease treatment will destroy antigens of interest in double labeling experiments. Time of proteinase K treatment will vary between cell type. Start at 5 minutes and increase if labeling is insufficient.
3	Wash 2 times in dH ₂ O, 2 minutes each.	

Step	Instructions	Notes
4	Immerse slides in Quenching Solution for 5 minutes at room temperature.	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 3 for Reagent Preparation.
6	Immerse slides in 1X TdT Labeling Buffer for 5 minutes.	Refer to page 4 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 (Appendix E). If necessary, use Hydrophobic Coverslips.	Refer to page 4 for Reagent Preparation. To generate a positive control using TACS-Nuclease™, add 1 µl of TACS-Nuclease™ to 50 µl of Labeling Reaction Mix and cover sample. Incubate at 37 °C for 1 hour. Refer to page 12 for Controls.
8	Immerse samples in 1X TdT Stop Buffer for 5 minutes at room temperature to stop labeling reaction.	Refer to page 5 for Reagent Preparation.
9	Wash samples 2 times in 1X PBS for 2 minutes each at room temperature.	This step removes unincorporated biotinylated nucleotides.
10	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.
11	Wash samples 2 times in 1X PBS for 2 minutes each at room temperatures.	This step removes unbound conjugate.
12	Immerse samples in DAB Solution for 2 to 10 minutes.	Use caution when handling DAB Solution. Refer to page 5 for reagent preparation.
13	Wash samples in 4 changes of deionized water, 2 minutes each.	
14	Proceed to <i>Counterstaining and Preparation for Viewing</i> .	

C. Counterstaining and Preparation for Viewing

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

Method A - For most cells and tissues

1. Immerse samples in deionized water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in 1% Methyl Green Counterstain.
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 pipet 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. DAB and Methyl Green stained samples are stable for 1 year in the dark.

Method B - Use for Bouin's fixed tissues and if Method A does not counterstain well

1. Immerse samples in deionized water for 2 minutes.
2. Immerse samples for 5 seconds to 5 minutes in 1% Methyl Green Counterstain.
3. Wash slides sequentially by dipping ten times each in:
 - 1-butanol (removes excess stain)
 - 1-butanol, 1 time to rinse (specimen should turn from blue to green)
 - Xylene, 2 times 5 to 10 seconds
4. Wipe off excess xylenes from the back of the slide and lay slide flat.
5. Place one drop, about 50 µl, of mounting medium from a 100 µl pipette onto sample.
6. Lower glass coverslip onto sample and apply gentle even pressure to expel air bubbles.
7. Leave slide flat overnight to allow mounting medium to harden.
8. Store slides in the dark.

VIII. Controls

If you wish to use a pre-prepared control you may use either Cell Culture Control Slides (cat# 4800-30-20) or 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-Nuclease™ to assess and optimize cell/tissue permeabilization; the recommended experimental controls are listed below.

TACS-Nuclease™-Treated Control

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

Method

For most cells and tissues TACS-Nuclease™ can be added directly to the labeling mix of the positive control sample. Add 1 µl of TACS-Nuclease™ per 50 µl of complete labeling mix and incubate for the regular labeling time. Wash slide separately in PBS to stop nuclease activity.

Alternatively, if the incubation time with the TACS-Nuclease™ needs to be reduced, use the following method:

1. After treatment with Cytonin™ or Proteinase K Solution, wash 2 times in deionized water, 2 minutes each.
2. Prepare TACS-Nuclease™ Solution:

	<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
3. Cover sample with 50 µl of TACS-Nuclease™ Solution.
4. Incubate at 37 °C for 10 to 60 minutes in a humidity chamber (Appendix E). If necessary, use Hydrophobic Coverslips.
5. Wash 2 times in 1X PBS for 2 minutes each.
6. Continue with Step 4, Labeling Procedure, page 11.

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 through counterstaining. Staining times of 5 seconds to 30 minutes have been noted. For most tissues, 1 minute is sufficient.

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

The Controls listed in Section VIII are important in data interpretation. These Controls allow optimization of *in situ* detection of apoptosis without expending valuable test samples. Under optimal conditions the Unlabeled Control (e.g. enzyme omitted) should show no brown staining, the TACS-Nuclease™-treated sample should show pale brown staining in almost all cells, and the Experimental Negative Control should have less than 20% brown stained cells. The brown staining of 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 pale green 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 Guide 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
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	
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 1 X PBS, 1% BSA (Bovine Serum Albumin)
			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 (page 20).	
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 maximum 60 minutes) or Cytonin™ (up to overnight at 4 °C).	
	No DNA left in sample due to hydrolysis (poor storage of samples).	Read <i>Sample Preparation and Storage</i> sections, pages 6-10, prior to labeling.	
	Excessive (removed all DNA) or inadequate TACS-Nuclease™.	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 ul of Strep-HRP onto 1 cm ² of 3M paper and air dry. Immerse the filter paper in DAB solution to check for brown coloration compared to a white sheet of paper.	
	Labeling of majority of cells in the negative experimental control (e.g. normal tissue) when there is no labeling if the enzyme is omitted and satisfactory labeling of the Nuclease-treated control.	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.
Prolonged incubation with DAB leads to a precipitate over entire sample.		Reduce time of DAB treatment and follow color development under the microscope.	
Excessive Proteinase K		Reduce time of Proteinase K treatment.	

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 or intensity of treatment.
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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3. Dakhama, A. R.G. Hegele (1996) A nonradioactive method for rapid and sensitive detection of polymerase chain reaction products by use of bromodeoxyuridine. *Modern Pathology* **9**: 849-853.
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5. Kerr, J.F., G.C. Gobe, C.M. Winterford and B.V. Harmon (1995) Anatomical methods in cell death. *Methods in Cell Biology* **46**:1-27.
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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	TACS® 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
4823-30-K	NeuroTACS™ II <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
4828-30-DK	TACS•XL® DAB <i>In Situ</i> Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label <i>In Situ</i> Apoptosis Detection Kit	30 samples
4810-30-K	TACS® 2 TdT 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

Catalog #	Description	Size
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	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™ H ₂ O	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	10 oz.
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

Proteinase K (cat# 4800-30-01)

1 mg/ml proteinase K

Cytonin™ (cat# 4876-05-01):

Proprietary permeabilization and blocking agent

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

0.1 M EDTA, pH 8.0

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)

TdT dNTP Mix (cat# 4810-30-05):

0.25 mM biotinylated dNTPs

TACS-Nuclease™ (cat# 4800-30-15):

Proprietary endonuclease. Avoid repeated freeze-thaw.

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 Counterstain (cat# 4800-30-18):

0.1 M sodium acetate, pH = 4.0

1% 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. Your fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments (see Appendix C).

To store the immobilized fixed cells, i.e. on slides, chamber slides or coverslips, post-fix in 100% methanol after fixation. Wash in 1X PBS, then store in Cytonin™ at 4 °C for up to one month. 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. Double Labeling Hints and Tips

The *in situ* labeling protocol described here is useful for double labeling experiments when the occurrence of apoptosis can be correlated with the presence of cellular antigens that can be detected using the appropriate antibodies. **Note:** The antibodies must recognize the fixed form of the antigen of interest.

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

Post-treatments used in immunocytochemistry to permeabilize or expose antigenic determinants include treatment with proteases, acid or base, detergent and microwaving. Permeabilization with Cytonin™ 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™ at room temperature for 1 hour. Use Hydrophobic Coverslips and a humidity chamber (Appendix E) 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™. 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™ at room temperature for 1 hour.
6. Wash 3 times in 100 mM Tris pH 7.4.
7. Prepare Red Label Solution:
 - a. To new 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 subcellular 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.

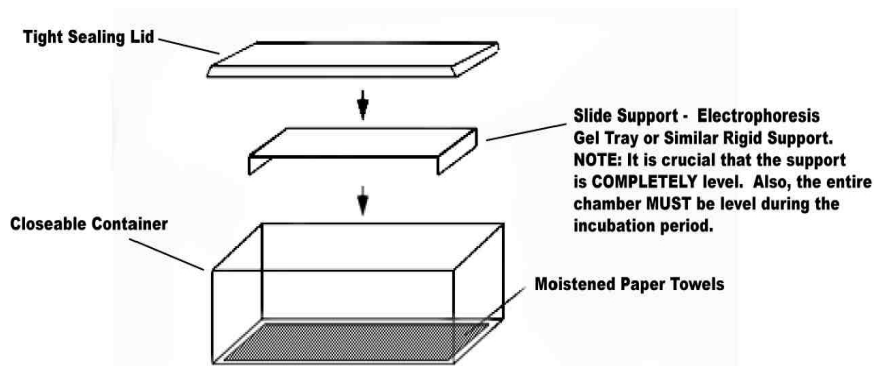
Appendix D. Electron Microscopy

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 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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