Apoptosis Kit

TACS® 2 TdT-Fluor *In Situ* Apoptosis Detection Kit

Catalog Number: 4812-30-K

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

30 Samples

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

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INTRODUCTION

For many cell types in culture, identification of apoptosis and quantification of cell death can be readily achieved using a combination of methods, including morphological criteria, extraction and analysis of DNA *in situ* detection of DNA fragmentation in immobilized cells. Other approaches include measuring the activation of ICE-like proteases, flow cytometry, detection of Annexin V at the cell surface, and cleavage of poly-ADP ribose polymerase. For many cell types, there are inherent technical difficulties associated with analysis of apoptosis. Many cell types are available only in primary culture thereby limiting the cell number for DNA extraction immunostaining, or FACS analysis. Many cells are fragile and less amenable to extensive handling when removed from the substratum for analysis. Many cells are maintained in mixed culture therefore the ability to identify cells using criterion other than morphology is important. Further, it can be difficult to identify cell types in tissue samples.

PRINCIPLE OF THE ASSAY

The TACS 2 TdT-Fluor *In Situ* Apoptosis Detection Kit can be used for cells or tissue sections. This kit enables samples to be double labeled using immunocytochemistry to determine cell type or other protein of interest. In addition, this kit comes complete with Cytonin™, a detergent based buffer optimized for the permeabilization of cells prior to labeling.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

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

PRECAUTIONS

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

The TACS 2 TdT-Fluor *In Situ* Apoptosis Detection Kit contains reagents that are harmful if swallowed or in contact with skin, and irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Material safety data sheets are available on request.

MATERIALS PROVIDED & STORAGE CONDITIONS

Use within 3 months from date of receipt.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Proteinase K Solution	4800-30-01	50 µL	Store ≤ -20 °C.
TdT dNTP Mix	4810-30-04	35 µL	
TdT Enzyme	4810-30-05	30 µL	
Strep-Fluorescein	4800-30-14	30 µL	
50X Co^{2+}	4810-30-09	30 µL	
50X Mg^{2+}	4810-30-10	30 µL	
50X Mn^{2+}	4810-30-14	50 µL	
TACS-Nuclease	4800-30-15	15 µL	
TACS-Nuclease Buffer	4800-30-16	1.5 mL	Store at 2-8 °C
Cytonin	4876-05-01	6 mL	
10X TdT Labeling Buffer	4810-30-02	100 mL	
10X TdT Stop Buffer	4810-30-03	100 mL	

OTHER MATERIALS REQUIRED

Equipment:

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

Reagents:

- 10X Phophate Buffered Saline (PBS)
- 37% formaldehyde
- Xylenes
- 30% hydrogen peroxide
- 70%, 95%, and 100% ethanol (or denatured alcohol)
- Methanol
- Butanol
- Fluorescent/aqueous mounting medium
- Tween® 20

Disposables:

- Treated glass microscope slides (or alternative support)
- 50 mL tubes
- Microcentrifuge tubes
- 1.5 and 10 mL serological pipettes
- Gloves
- Hydrophobic coverslips (optional)
- Glass coverslips

REAGENT PREPARATION

The volumes given for each reagent are based on processing samples of up to 4 cm^2 immobilized on glass slides. Different configurations of chamber slides, culture plates, free floating sections, and the use of glass coverslips may require adjustments to the needed volumes.

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

1X PBS - Approximately 500 mL of 1X PBS is used to process 1 to 10 slides. Dilute 10X PBS 1:10 using distilled water. Store 1X PBS at room temperature. For 1X PBST, add 0.05% Tween® 20 to 1X PBS and mix thoroughly by gentle inversion.

1X PBST - For 1X PBST, add 0.05% Tween® 20 to 1X PBS and mix thoroughly by gentle inversion.

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

Reaction Component	Volume
37% Formaldehyde	5.0 mL
10X PBS	5.0 mL
ddH20	40 mL

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

Reaction Component	2 Samples	10 Samples	n Samples
Distilled water	100 µL	500 µL	n x 50 µL
Proteinase K	2.0 µL	10 µL	n x 1.0 µL

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

Cytonin - If required, 50 µL of Cytonin is used per sample. Cytonin is ready for use. Store at 2-8 °C. Discard if solution is cloudy.

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

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

Reaction Component	2 Samples	10 Samples	n Samples
TdT dNTP Mix	2.0 µL	10 µL	n x 1.0 µL
TdT Enzyme	2.0 µL	10 µL	n x 1.0 µL
50X Cation Stock**	2.0 µL	10 µL	n x 1.0 µL
1X TdT Labeling Buffer	100 µL	500 µL	n x 50 µL

*See appendix to select the proper 50X citation stock.

REAGENT PREPARATION *Continued*

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

***Strep-Fluorescein Solution** - Use 50 µL of Strep-Fluorescein Solution per sample. Store prepared Strep-Fluorescein Solution at room temperature **in the dark** until use. To prepare add:

Reaction Component	4 Samples	10 Samples	n Samples
1X PBST	200 µL	500 µL	n x 50 µL
Strep-Fluorescein	1.0 µL	2.5 µL	n x 0.25 µL

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.

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

***TACS-Nuclease Solution** - TACS-Nuclease should be diluted 1:50 in TACS-Nuclease Buffer, as below:

Reaction Component	2 Samples	10 Samples	n Samples
TACS-Nuclease Buffer	100 µL	500 µL	n x 50 µL
TACS-Nuclease	2.0 µL	10 µL	n x 1.0 µL

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

Reaction Component	Per Positive Control
1X TdT Labeling Buffer	50 µL
TdT dNTP Mix	1.0 µL
50X Cation Stock**	1.0 µL
Tdt Enzyme	1.0 µL
TACS-Nuclease	1.0 µL

**Use the same cation as in the Labeling Reaction Mix.

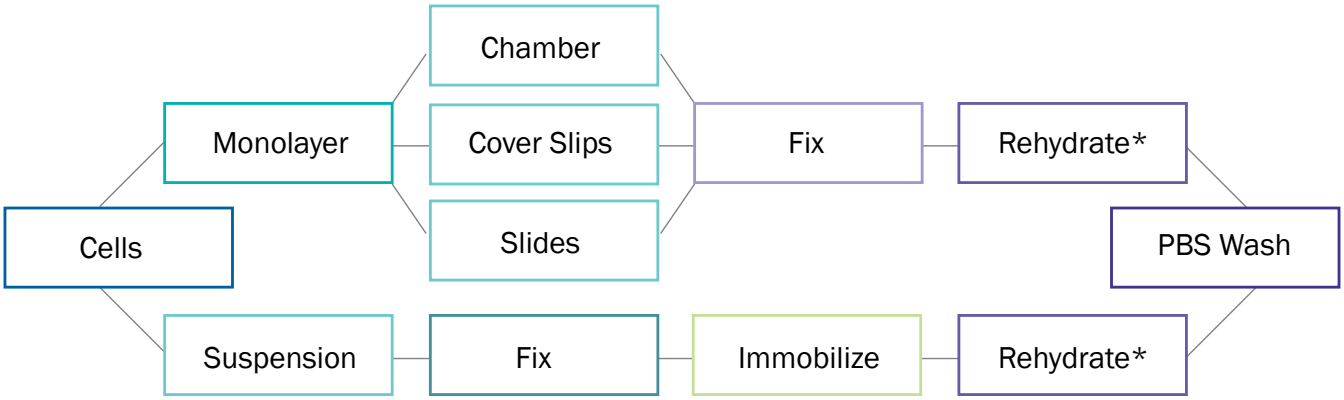
ASSAY PROTOCOL

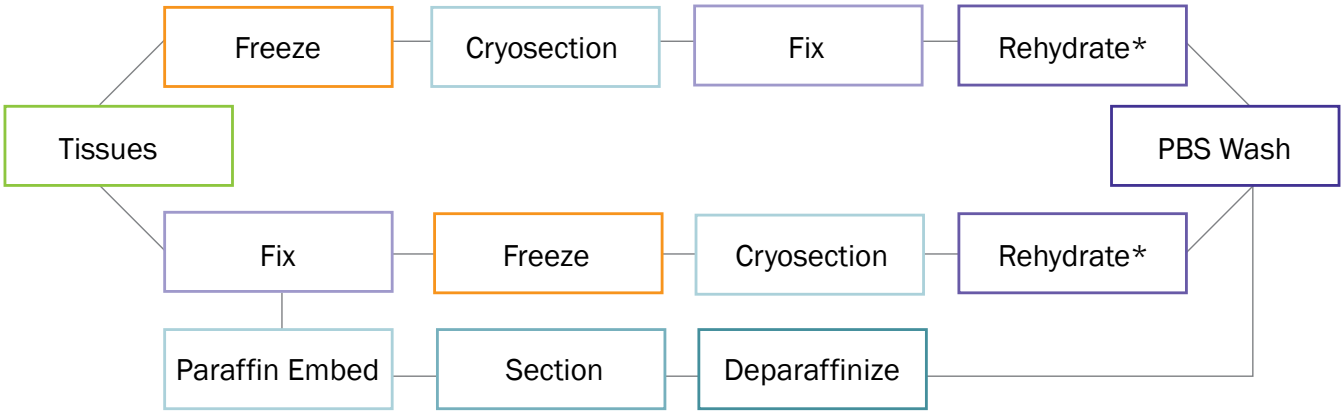
It is important to read through the Assay Protocol before preparing tissue or cell samples.

There are key steps that are very important for successful labeling. This section includes instructions for sample preparation, *l*abeling, 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.

Follow the appropriate flow diagram to determine the correct sequence of steps needed to prepare samples.

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.





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

ASSAY PROTOCOL *Continued*

PREPARATION OF CELLS IN CULTURE

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

1. Harvest cell suspension by centrifugation at 500 x g for 5 minutes at room temperature.
2. Discard media and resuspend at 1 x 10^6 cells/mL in 3.7% Buffered Formaldehyde. Let stand for 10 minutes at room temperature.
3. Centrifuge at 500 x g for 5 minutes at room temperature and discard fixative.
4. Resuspend at 1 x 10^7 cells/mL in 80% ethanol. Cells can be stored in 80% ethanol at 2-8 °C for several weeks. Signal intensity in positive cells will reduce with time due to loss of small DNA fragments.
5. Spot 1 x 10^5 cells onto clean glass microscope slide. Dry for 20-60 minutes on slide warmer at 45 °C.

Note: *Glass slides pretreated for electrostatic adherence are recommended. Slide treatments (i.e. collagen, gelatin, poly-L-lysine) can cause increased background staining.*

6. Immerse slide in 70% ethanol for 10 minutes, then air dry overnight at room temperature or dry at 45 °C for 2 hours. Samples may be stored at this point. Store samples at 2-8 °C in airtight containers with desiccant for up to several months. After storage, rehydrate through a decreasing alcohol series and wash in 1X PBS prior to the labeling reaction.
7. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.
8. Immerse in 1X PBS and proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION

PREPARATION OF CELLS IN MONOLAYER

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

On Sterile Slides - Other cell culture methods include culturing directly on microscope slides. The slides must be sterile and, if necessary, pretreated to ensure cell adhesion. Sterilize microscope slides by autoclaving in a large glass petri dish. If needed, coat slides with sterile poly-L-lysine or collagen, 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.

1. Remove media from cells and rinse once with 1X PBS at room temperature.
2. Fix cells for 10 minutes at room temperature in 3.7% Buffered Formaldehyde.
3. Wash cells one time in 1X PBS. Samples can be stored at this point, using one of the following methods:

    a) Dehydrate the cells by passing through an increasing alcohol series of 70%, 95%, and 100% ethanol for 5 minutes each followed by air drying for 10 minutes. Store at 2-8 °C with desiccant.

    b) Fixed cells can be stored for up to 1 week in Cytonin at 2-8 °C. The samples must be covered to prevent contamination and evaporation. If experimental design dictates a time course extending over several days, storage in Cytonin is recommended.

    Note: *Labeling directly after fixation is optimal as the labeling of some samples is less efficient after storage. If possible, a pilot study should be performed to ensure that stored fixed samples can be labeled.*

4. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

PREPARATION OF TISSUES

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

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

1. **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.

2. **Cryosection Frozen Tissue**

   a. Samples may be embedded in a cutting matrix. Position the sample within cutting matrix in a suitable container. Immerse embedded tissue in isopentane chilled on dry ice. Frozen samples may be stored for many months at ≤ -70 °C.

   b. 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.

   c. 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.

3. **Fixation After Cryrosectioning**

   a. It is critical to dry the samples thoroughly after sectioning. Dry overnight at room temperature or for at least 2 hours at 45 °C on a slide warmer. Samples can be stored at this point. Store slides at ≤ -70 °C with desiccant for up to 3 months. After storage equilibrate samples to room temperature and re-dry for 2 hours at room temperature or 2 hours on a slide warmer.

   b. Rehydrate by immersing for 5 minutes each in 100%, 95%, then 70% ethanol.

   c. Wash once in 1X PBS for 5 minutes.

   d. Fix samples by immersing in 3.7% Buffered Formaldehyde for 10 minutes at room temperature.

   e. Wash cells 1 time in 1X PBS.

   f. Proceed to Labeling Procedure.

SAMPLE PREPARATION AND FIXATION *Continued*

Preparation of Fixed Samples Before Sectioning – Immersion or Perfusion

Samples are routinely fixed by immersion or perfusion methods. After fixation, samples are cryosectioned or paraffin embedded. Formaldehyde is the recommended fixative based on laboratory testing. Other fixatives that maintain DNA integrity may be used. These include other cross-linking agents such as paraformaldehyde and glutaraldehyde. Bouin's reagent should be avoided due to the high acidity. If alternative fixatives are used, it is recommended that a pilot study is performed to ensure that the fixative allows for permeabilization and labeling.

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 cm^3) in at least 10 volumes of 3.7% Buffered Formaldehyde. After 30 minutes change to fresh 3.7% Buffered Formaldehyde and leave at room temperature up to 24 hours. Tissues with high cellularity may require longer fixation times.

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

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

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

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

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

SAMPLE PREPARATION AND FIXATION *Continued*

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

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

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

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

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

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

LABELING PROCEDURE

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

Labeling Samples on Slides - Wash slides in 1X PBS using small coplin histology jars. Each jar holds up to 50 mL of buffer and up to 10 slides. For procedural steps involving 50 µL per sample, place slides on a flat surface and spot reagent from above using a pipette tip; do not touch the sample with the pipette tip. Small biopsy samples are easily covered with 50 µL. If 50 µL does not cover the sample, hydrophobic coverslips may be used after pipetting the 50 µL volume. Lower the hydrophobic coverslip from one edge and press down gently to expel any air bubbles. Remove hydrophobic coverslips by dipping the slide vertically in distilled water.

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

LABELING PROCEDURE *Continued*

Labeling Samples on Glass Coverslips - Process the 12 mm glass coverslips with the cell-side facing up in the 24-well tissue culture plate. Wash by filling the wells with 1X PBS and removing with a 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.

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 and incubate for 30-120 minutes at room temperature or 2-8 °C. If necessary, use hydrophobic coverslips.	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 15 minutes and increase if no labeling occurs.
3	Wash 2 times in distilled water, 2 minutes each.	
4	Immerse slides in 1X TdT Labeling Buffer for 5 minutes.	
5	Cover sample with 50 µL of Labeling Reaction Mix and incubate at 37˚C for 1 hour in a humidity chamber. If necessary, use hydrophobic coverslips.	Use humidity chamber during incubation time. A positive control using TACS-Nuclease may be needed.
6	Immerse samples in 1X TdT Stop Buffer for 5 minutes at room temperature to stop labeling reaction.	
7	Wash samples 2 times in 1X PBS for 5 minutes each at room temperature.	
8	Cover sample with 50 µL of Strep-Fluorescein Solution and incubate for 20 minutes at room temperature **in the dark**. If necessary, use hydrophobic coverslips.	Follow labeling intensity under the microscope to determine the optimal incubation period.
9	Wash samples 2 times in 1X PBS for 2 minutes each.	
10	Proceed to Preparation for Viewing.	

Preparation for Viewing - Cells and tissues may be viewed directly. Use an appropriate mounting medium for fluorescent samples and follow the manufacturers instructions. Glass coverslips can be held in fine tipped forceps for mounting: spot only 25 µL mounting medium onto a clean glass slide and mount the coverslip, cell side down, onto the slide.

CONTROLS

If you wish to use a pre-prepared control you may use the Tissue Control Slides (R&D Systems, Catalog # 4800-30). 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 Sample - Treat one sample with TACS-Nuclease to generate DNA breaks in every cell. Avoid repeated freeze-thaw cycles. The TACS-Nuclease-Treated Control will confirm that the permeabilization and labeling reaction has worked. The information can help optimize the conditions for the Labeling Procedure. The majority of cells should exhibit fluorescent green nuclear staining, using a FITC filter.

1. After Step 2 of the Labeling Procedure, wash 2 times in distilled water, 2 minutes each.
2. Prepare TACS-Nuclease Solution by diluting TACS-Nuclease 1:50 in the TACS-Nuclease Buffer.
3. Cover sample with 50 µL of TACS-Nuclease Solution.
4. Incubate at room temperature for 10-60 minutes in a humidity chamber. If necessary, use hydrophobic coverslips.
5. Wash 2 times in 1X PBS for 2 minutes each.
6. Continue from Step 3, Labeling Procedure.

Though the above method is recommended, to save time, TACS-Nuclease can be added directly to the Labeling Reaction Mix of the positive control sample. Add 1.0 µL of TACS-Nuclease to 50 µL of complete Labeling Reaction Mix and incubate for the regular labeling time. The signal intensity obtained, using this method, is usually lower than the recommended method.

Unlabeled Experimental Control Sample - The TdT Enzyme should be omitted from the Labeling Reaction Mix for one sample. This control will indicate the level of background labeling (fluorescein) associated with non-specific binding of the Strep-HRP Fluorescein.

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 an 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 have fluorescent staining.

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.

Cells containing fragmented nuclear chromatin characteristic of apoptosis will exhibit fluorescent staining after labeling. Fluorescent 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 fluorescent staining that is not associated with nuclei.

Controls are important in data interpretation. These controls allow for optimization of *in situ* detection of apoptosis without expending valuable test samples. Under optimal conditions the Unlabeled Experimental Control Sample should show minimal background fluorescent staining, the TACS-Nuclease-Treated Control Sample should show fluorescent staining of almost all cells, and the Experimental Negative Control should resemble the Unlabeled Control. The fluorescent staining of TACS-Nuclease-treated cells is less intense 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. Refer to the TroubleShooting Guide for information if the controls do not provide the expected result.

TROUBLESHOOTING

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

Problem	Cause	Action
Fluorescent staining of cells when the TdT Enzyme is omitted from the Labeling Reaction Mix.	Non-specific binding of Strep-Fluorscein.	Increase number of washes after binding. Decrease concentration of Strep-Fluorscein by diluting stock solution up to 1:500.
	Sample dried out during the labeling procedure.	Use hydrophobic coverslips (or lids for plates or chamber slides) and incubate in humidity chamber.
No staining in TACS-Nuclease Treated Control sample.	Poor permeabilization and/or excessive fixation with cross-linking fixative (common with archival tissue) preventing enzyme access. No DNA left in sample due to hydrolysis (poor storage of samples or sections). Excessive (removed all DNA) or inadequate TACS-Nuclease treatment. TdT Enzyme is inactive. The enzyme is the most labile component in the kit.	Increase incubation time with Proteinase K (up to 60 minutes) or Cytonin (up to overnight at 2-8 ˚C). Read Sample Preparation and Storage, prior to labeling. Optimize time for TACS-Nuclease treatment (5 minutes up to 2 hours). 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.
Labeling of majority of cells in the negative experimental control (i.e. normal tissue or untreated cells) when there is no labeling if the enzyme is omitted and satisfactory labeling of the TACS-Nuclease-Treated Control.	High level of apoptosis (or necrosis) in negative control.	Select a more appropriate negative control or inhibit apoptosis in cell culture (i.e. using protein synthesis inhibitors). Check morphology of cells prior to assay for evidence of excessive apoptosis.
	Excessive Proteinase K treatment	Reduce incubation time in Proteinase K Solution to 5-15 minutes. Or dilute Proteinase K 1:200 in distilled water and incubate sample for 15 minutes.
No labeling in experimental sample.	No apoptosis (or necrosis) occurring in sample.	If all controls gave the expected results and were processed at the same time as the experimental sample there may be no DNA fragmentation in cells within the sample.
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.

APPENDICES

Appendix A. Fixation Methods

There are several fixation methods commonly used that are appropriate for the protocol described in the Instructions for Use. Formaldehyde is the recommended fixative based on laboratory testing. Other fixatives that maintain DNA integrity may be used. These include other cross linking agents such as paraformaldehyde and glutaraldehyde. Bouin's fixative should be avoided due to the picric acid content. Regardless of the fixative used, it is important not to fix cells and tissues for extended periods of time. Your fixation method will likely be dictated by immunocytochemistry protocols in double labeling experiments. Post fixation in acetone, ethanol, or methanol is common in preparation of tissues and is usually compatible with TACS 2 TdT DAB *In Situ* Apoptosis Detection Kit.

To store the immobilized cells on slides, chamber slides, or coverslips, post-fix in 100% methanol after fixation, wash in 1X PBS and then store in Cytonin at 2-8 °C for up to one week. After storage, wash in 1X Labeling Buffer then continue with the labeling reaction at the quenching step.

Appendix B. 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 and try manganese cation in the labeling reaction. If background is high, reduce the Proteinase K concentration, incubation time, temperature, and use cobalt cation in the labeling reaction. 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 insufficient labeling, permeabilized with Proteinase K at 1:200 for 15 minutes at 37 ˚C.

	Cytonin	Proteinase K (1:50)	Proteinase K (1:200)	Mg^{2+}	Co^{2+}	Mn^{2+}
Brain	x		x			x
Heart		x			x	x
Lung	x		x		x	
Liver		x			x	x
Kidney		x				x
Spleen		x			x	
Duodenum		x			x	
Colon	x	x			x	
Small Intestine	x	x			x	x
Large Intestine	x	x			x	x
Skin		x				x
Bone/Cartilage	x	x				x
Tumor		x		x	x	
Epithelium		x	x			x
Endothelium		x	x			x
Cultured Cells (stored dry)	x	x			x	x
Cultured Cells (fresh)	x		x		x	x

Note: Use only one cation.

APPENDICES *Continued*

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 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 A. Post-treatments used in immunocytochemistry to permeabilized 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. Red Label provides excellent contrast with DAB and Blue Counterstain. A standard immunohistochemistry protocol is provided for using phosphatase-conjugated secondary antibody and color development with Red Label Solution. Antibody concentrations, incubation times and temperatures and buffers may have to be optimized empirically for each system under study.
Note: Phosphatase-conjugated reagents are inhibited by PBS or other phosphate-containing buffers. Tris buffers should be substituted for PBS.

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