ApopTag® Peroxidase *In Situ* Oligo Ligation (ISOL) Apoptosis Detection Kit

S7200

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

EMD Millipore Corporation, 28820 Single Oak Drive, Temecula, CA 92590 USA 1-800-437-7500
Technical Support T 1-800-MILLIPORE (1-800-645-4576) F 1-800-437-7502
www.millipore.com

This page left blank intentionally.

TABLE OF CONTENTS

I. INTRODUCTION

Using this Manual

This manual is intended for use with the ApopTag® Peroxidase *In Situ* Oligo Ligation (ISOL) Kit, Cat #S7200. The ApopTag® ISOL Kit utilizes a proprietary hairpin oligonucleotide labeling process to detect apoptosis in paraffin-embedded tissue, cell suspensions and adherent cells. Positive control slides are provided to serve as a test for the integrity of the reagents and to exemplify the relationship of apoptotic morphology.

These protocols are presented in a streamlined manner. The novice user is advised to read the entire manual, including the bullet points, which provide additional details, prior to using the kit. For additional questions, assistance is available from Chemicon® Technical Service at (800) 437-7500 or at techserv@chemicon.com.

Background

Apoptosis is a mode of cell death that actively eliminates damaged or nonessential cells. Multiple signaling and effector pathways that mediate active responses to death factors, cellular damage, external growth, and survival of the cell, regulate its specific metabolic apparatus. A comprehensive body of review literature is available as an introduction to this field, including many books in print, hundreds of tutorial and academic review articles, and internet sites (refer to Page 37).

Apoptotic cell death is distinguished from necrosis or 'accidental' cell death (such as direct oncosis, or ischemic necrosis, 10, 31) by both morphological and biochemical criteria (as summarized in Table 1). Apoptotic cell death is rapid, and the minute, late stage apoptotic bodies are transient. The percentage of apoptotic cells apparent in a tissue (apoptotic index) may be deceptive because a rate of tissue regression as rapid as 25% per day can be associated with an apoptotic index of only 2%-3% (6). Special staining for apoptotic DNA fragmentation, as in the ISOL method, eliminates the need for fixing tissues.

Caspase-activated endonuclease activity is associated with the large DNA fragments that are derived from higher-order chromatin structures. In many model systems, the fragments of 50 kb and 300 kb can be seen using pulsed field electrophoresis (5, 45, 46). Subsequently, Ca^{2+}- and Mg^{2+}-dependent endonucleases further cleave these fragments at linker DNA sites between

nucleosomes (3, 7). The terminal DNA fragments are multimers of a nucleosomal unit length of about 180-200 bp and comprise a "DNA ladder" when viewed on agarose gels.

Table 1: Differences Between Classic Apoptosis and Necrosis

	Apoptosis	Necrosis
Morphological Criteria		
Incidence	Scattered	Shrunken
Volume	Shrunken	Swollen
Surface	Sealed, blebbed	Leaky
Shape	Fragmented	Intact
Chromatin	Condensed, Marginated	Clumped
Tissue reaction	Phagocytosis	Inflammation
Biochemical Criteria		
DNA Double Strand Breaks	Blunt ends, some short 3'-overhangs	Blunt ends, 3' and 5' overhangs of varying lengths
Onset	Regulated or Programmed	Accidental
Enzyme cascade	Complete	Truncated
Metabolism & Biosynthesis	Active	Inert
Death Signal	Phosphatidylserine	Lytic Discharge
DNA fragment lengths	Non-random (Laddered)	Random (smear)

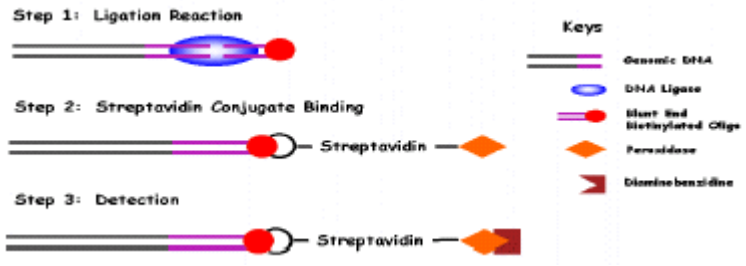
Principles of the Technique

Chemical Mechanism

The ApopTag® *In Situ* Oligo Ligation (ISOL) technique is based upon the biochemical specificity of the enzyme T4 DNA ligase. This enzyme covalently joins exactly complementary ends of a pair of double-stranded DNA molecules. The ends to be joined are those of the genomic DNA in the sample and those of the synthetic, biotinylated oligonucleotide in the ISOL kit. Detection is subsequently performed by binding the streptavidin-peroxidase conjugate. Peroxidase causes the deposition of the substrate diaminobenzadine (DAB) from solution that can be visualized by bright field microscopy. Labeling is found to coincide with apoptotic cell morphology, and subcellular localization becomes apparent within the apoptotic chromatin.

Unique, synthetic oligonucleotide reagents are provided in the ISOL kit. These self-annealing oligos contain two internal complementary base sequences that spontaneously form a linear duplex segment. At one ‘pole’ of the linear segment are both the 3' and 5' strand ends of the molecule. These may form either a blunt or an overhanging type of duplex end, depending on the absence or presence of a one base 3' extension. Both kinds of duplex ends are provided in the ISOL kit to confer alternate specificities for target DNA ends. At the 'pole' of the duplex opposite to the strand ends is a hairpin loop secondary structure. A biotin label is positioned upon a spacer arm extension in the middle of this loop. The oligos provided are not phosphorylated. Since the DNA target to be ligated must have a 3'-hydroxyl group and a 5'-phosphate group for a covalent reaction to occur, a hairpin oligo cannot be dimerized. Use of a hairpin oligonucleotide minimizes nonspecific annealing to a sample because it is 100% duplex, thus reducing the need for differential morphological evaluation (13, 15). This single-chain duplex hairpin structure replaces an earlier version that was prepared using PCR (12).

Figure 1: ApopTag® ISOL Chemistry Using Oligo B (Blunt End Detection)



The ISOL Kit does not label nicks, gaps, single-stranded DNA, 3'-recessed ends or 3'-overhanging ends longer than one dT base. Table 2 summarizes the differences in analytical enzyme specificity between four different *in situ* assays for fixed cells that offer comparable functionality. These are the DNA ligase assay (12-15, or Chemicon®'s ApopTag® ISOL Kit (#S7200)); the terminal transferase (22,23), TUNEL (19) or tailing assay (the Chemicon® ApopTag® *In Situ* Apoptosis Detection Kits (cat.# series S71XX)); the DNA polymerase I or nick translation assay (20, 23, 48); and the Klenow DNA polymerase I or ISEL assay (48).

A schematic showing a blunt end ligation reaction using biotinylated Oligo B (blunt ended oligo) and the subsequent detection reaction is presented in Figure 1. The illustrated reaction detects only blunt ends. Similarly, a reaction sequence using Oligo A (3'-dA overhang) only labels and detects cellular DNA ends having a complementary single base 3'-dT overhang. The DNA target must have a 5'-phosphate group. As Oligo A and B are not phosphorylated, these Oligos cannot self-ligate.

Table 2: Specificity of* In Situ *DNA End Labeling Assays

Type of DNA End	T4 DNA Ligase	Terminal Transferase (TUNEL)[2]	DNA Pol I (ISNT)	Klenow DNA Pol. (ISEL)
Single strand DNA	No	Yes	No	No
Duplex, single strand nick	No	Yes	Yes	No
Duplex, single strand gap	No	Yes	Yes	Yes
Duplex, 3' recess	No	Yes	Yes	Yes
Duplex, 3' overhang*	Yes[1]	Yes	No	No
Duplex, blunt end	Yes[1]	Yes	No	no
Which strand end is reactive	5'-phosph.	3'-OH	3'-OH	3'-OH

**This type of end is believed to be most characteristic of apoptosis.*

[1]The ISOL Kit offers two options: Oligo B labels only blunt ends and Oligo A labels only one base 3'-dT overhang ends.

[2]See reference 12 for the specificity of terminal deoxynucleotidyl transferase (TdT) in cobalt-containing buffer.

The detection reagent is a streptavidin molecule conjugated with a reporter moiety. Upon application, it binds tightly to the biotin affinity label on the ligated oligo. There is one biotin moiety per oligo molecule that binds to a single streptavidin-peroxidase conjugate molecule (in a 1:1 ratio). The specimen is further stained with DAB (diaminobenzidine) substrate to form a brown precipitate. The apoptotic cells are then visualized via bright field microscopy. Very high concentrations of 5'-phosphorylated, blunt, and shallow 3'-overhanging ends are found to be localized in apoptotic nuclei and in apoptotic bodies.

Ligation reactions may occur within a temperature range between 16°C and 23°C. This range is acceptable because the rate of annealing is not an important factor. Although the activity of ligase enzyme is higher with increasing temperature, much of the enzyme activity will be lost due to thermal protein denaturation. An incubation time of 8-16 hours is optimal for paraffin tissue sections.

Cellular Selectivity

In the ISOL method, T4 DNA ligase is utilized to specifically ligate DNase I-type ends to biotin-labeled hairpin oligonucleotides; subsequently, localization of labeled oligos is observed to be restricted to areas of chromatin that are characteristic of apoptotic nuclei. ISOL avoids labeling randomly damaged DNA better than the TUNEL method which can cause false-positive signals and background. The ApopTag® ISOL Kit stains induced apoptotic cells, starting with the onset of chromatin condensation through their division into apoptotic bodies. Normal or proliferative nuclei, lacking significant numbers of doublestrand breaks, do not stain with ISOL.

In situ staining for DNA fragmentation (as in the ISOL method) is both a means of detection for rare cells and an analytical test of those cells' DNA. ApopTag® ISOL Kits facilitate the differentiation of apoptotic cells from necrotic or transiently damaged cells. While conventional *in situ* detection techniques such as ISEL (Klenow DNA polymerase), TUNEL (terminal deoxynucleotidyl transferase, TdT) and ISNT (DNA Polymerase I) are useful in detecting internucleosomal DNA cleavage, they do not differentiate DNase I-type cleavage which results from the activation of apoptotic endonucleases.

In classic apoptosis, as exemplified in glucocorticoid-treated thymic lymphocytes, a very large percentage of the DNA ends are found by *in situ* ligation to be either blunt or very short 3' overhangs (12, 13, 14). This is consistent with biochemical findings (11, 38, 47). By comparing different apoptotic and necrotic systems, Didenko *et al*. have shown that specific ligation of one base overhanging DNA ends (as opposed to blunt ends) could fine-tune oligo ligation to be more selective than TUNEL for apoptosis over 'oncosis' (classic necrosis). This extra selectivity has been confirmed and exploited in other studies of placenta (2), atherosclerotic lesions (28) and cardiac myocytes (9, 17, 18, 25, 41). When ISOL was used for direct comparison with TUNEL in specimens without necrosis, the results have been concordant. In specimens presenting necrosis, the better selectivity of ISOL was proven.

Although Oligo A confers the most selective discrimination between apoptosis and 'oncotic' necrosis, apoptotic DNA contains fewer one base overhanging ends than blunt ends (37 and Chemicon®, unpublished). The researcher can choose to use Oligo A, which has a one base overhanging dA end to detect complementary dT one base overhangs. The researcher can also choose to use Oligo B (blunt end) which is more sensitive, but less selective, on another sample. In specimens not presenting necrosis, blunt end ligation may be preferred for extra sensitivity.

With either oligo, ISOL provides the most selective technique for *in situ* detection of targeted DNA ends (please see Table 2).

Some variant forms of cell death appear difficult to classify as either classic apoptosis, necrosis or oncosis (10, 31) by DNA fragmentation and cell ultrastructure analysis. DNA fragmentation may be absent or incomplete in induced apoptosis (see review 9). 3'-recessed ends, which should not to stain with ISOL, may predominate in some atypical specimens, such as in cerebral ischemia (30). Besides apoptotic nuclei, some non-apoptotic nuclei that show signs of active gene transcription may be labeled by the TUNEL technique (27). ISOL can provide analytic information about the presence or the absence of precisely defined, double-stranded DNA ends (i.e. blunt or 3'-dT).

Detection Options

The ApopTag® ISOL technique (Peroxidase) can be analyzed using bright field microscopy by following one of the protocols selected from the following table:

Table 3: Detection Options for Different Sample Types

	SAMPLE TYPE		
	Adherent paraffin tissue	**Adherent cells or frozen tissue**	**Cell suspensions**
Oligo A	Protocol 1	Protocol 2	Protocol 2
Oligo B	Protocol 1	Protocol 2	Protocol 2

The technique has been adapted for fluorescence detection (see Sec. VI. *Appendix*, *TECH NOTE #3*).

Specificity and Reactivity

The ApopTag® *In Situ* Oligo Ligation Kit has been qualified for use in histochemical and cytochemical staining of the following specimens: formalinfixed, paraffin-embedded tissues, frozen tissue sections, and adherent or suspended cultured cells. Formalin-fixed, paraffin-embedded tissues tested include dexamethasone-treated rat thymus, human thymus, and rat regressing mammary gland (ApopTag® Positive Control Slides, Cat #S7115). Whole mount methods that have been adapted for TUNEL staining (37) have not yet been validated with ISOL.

Apoptotic cells on the ApopTag® Positive Control Slides (formalin-fixed, paraffin-embedded rat mammary gland) stain vividly using either Oligo A or B when detected with peroxidase. Oligo A is adequately sensitive for peroxidase detection on paraffin sections and for other specimen types. However, there may be fewer 3'-dT ends, as opposed to blunt ends, present for ligation and detection.

Data Analysis for Microscopy

To obtain valid results, it is very important to examine stained cells for typical apoptotic morphological changes and to search for minute, stained apoptotic bodies which are present in only certain tissue types. Counterstaining for total DNA is highly recommended (8, 32). Visualization of positive ApopTag® ISOL results will reveal focal *in situ* staining inside apoptotic nuclei, in apoptotic bodies and usually in punctate cytoplasmic organelles that are a result of phagocytosis. Early-stage apoptotic cells also may stain positive before significant nuclear pycnosis or cytoplasmic shrinkage has occurred. Intense staining of even a very few cells indicates a technically successful assay. However, not all pycnotic nuclei may be stained, particularly in paraffin-embedded tissue samples. Diffuse, even staining of cytoplasm or of extracellular matrix should be held as suspect (see Sec. V. *Troubleshooting*.)

An understanding of normal and apoptotic cellular morphology is critical for data interpretation. The nuclear morphology of positive cells should be carefully observed at high magnification (400X-1000X). In some tissues, cytoplasmic shrinkage may be indicated by a clear space surrounding the cell. *In situ* staining by TdT/TUNEL has been visualized using electron microscopy (4, 33, 42), but the ISOL technique has not yet been used in this way. Early staged positive, round nuclei have observable chromatin margination if lightly stained. Condensed nuclei of middle stages and apoptotic bodies are usually stained. Apoptotic bodies may be found either in the extracellular space or inside of

phagocytic cells. It is highly recommended that less experienced observers should refer to illustrations of dying cells for comparison (*e.g.* 10, 26, 31).

When researching a new system, the staging and correlation of apoptotic morphology and DNA fragmentation should be characterized (see below). In addition to ISOL staining, biochemical confirmation of positive results may be obtained by detection of DNA fragmentation on agarose gels. If a sufficient number of apoptotic cells are available, electrophoresis of extracted DNA can be done to detect a DNA ladder. DNA can be extracted from unfixed tissue or from paraffin tissue sections using Chemicon®'s #S4520 or #S4530 kits, respectively. Apoptosis detection via ISOL histochemistry is more sensitive at the single cell level than standard DNA electrophoretic analysis. DNA laddering data of comparable sensitivity may be obtained in several other ways. These include using methods for selectively extracting low molecular weight DNA (21), for preparing radiolabeled DNA (36, 43) and for PCR amplification (38).

II. KIT COMPONENTS

Table 4: ApopTag® Peroxidase ISOL Kit

Component	Part #	Vol/Qty
DNA Ligase Enzyme	90430	0.25 mL
Equilibration Buffer	90431	3.5 mL
Oligo A	90432	2.2. mL
Oligo B	90433	2.2 mL
Streptavidin-Peroxidase Conjugate	90434	2.5 mL
Plastic Coverslips	90421	100 pieces
Positive Control Slides*	90422	2 pieces
DAB Substrate	90423	130 µL
DAB Dilution Buffer	90424	6.5 mL
Proteinase K	90435	25 mg

**The Positive Control Slides (#07) are 5 µm thick sections of paraffin-embedded rat mammary gland tissue obtained at the fourth day after weaning (39) and fixed for 18 hours in 10% neutral-buffered formalin. Sections were mounted on silanized slides.*

Storage and Shelf Life

1. Store the kit at -20°C until the first use.
2. After the first use, all the components other than DNA Ligase Enzyme (90430) can be stored at 4°C.
3. For maximum shelf life, DNA Ligase Enzyme (90430) should be stored continuously at -20°C. Hold DNA Ligase Enzyme on ice as briefly as possible during use. DNA Ligase Enzyme contains glycerol and will not freeze at -20°C. The best way to store DNA Ligase Enzyme is in a cold-block type of tube carrier which will maintain its temperature at -20°C when briefly removed from the freezer.
4. The stability of this product is guaranteed for six months from the date of purchase if stored and handled properly.

Precautions

1. For Research Use Only. Not for use in diagnositc procedures.
2. DAB (3,3' diaminobenzidine) Substrate (90423) has been demonstrated to be a potential carcinogen and skin contact should be avoided. If skin contact does occur, flush with copious amounts of dH_2O.
3. Oligo A, Oligo B and 1X Equilibration Buffer (90432, 90433 & 90431) contain 0.08% sodium azide as a preservative.
4. Do not store DNA Ligase Enzyme (90430) in a self-defrosting (frost-free) freezer.
4. Each ApopTag® ISOL Kit provides sufficient reagents for testing a total of 40 samples. However, to allow the researcher to choose between Oligo A and Oligo B, a sufficient amount of each oligo has been provided so that 40 samples may be tested using either. In this way, it is possible for the researcher to select either Oligo A or Oligo B based on which reagent best serves his/her experimental needs. Therefore, expect to have some oligo left over which can be discarded.

Materials Required But Not Supplied

Select from the following list those materials that are required for particular protocols (See Sec. IV. *Protocols*)

Solvents and Media

1. Deionized water (dH_2O)
2. Xylene or xylene substitute (keep separate stocks to use for deparaffinization and for washing before mounting the stained tissue)
3. Ethanol: absolute, 95%, 70% diluted in dH_2O
4. Ethanol:acetic acid, 2:1 (v/v)
5. Coverslip mounting medium (e.g. Permount)

Solutions

1. PBS (see Sec. VI. *Appendix*)
2. 10% (v/v) neutral buffered formalin (see Sec. III. *Beginning Considerations*)
3. 1% pure (methanol-free) (para) formaldehyde diluted in PBS (See Sec. III. *Beginning Considerations*)

Materials

1. Silanized glass slides (Silanization is highly recommended in order to avoid detachment of tissue sections during processing.)
2. Glass coverslips
3. Adjustable micropipettors
4. Glass or plastic coplin jars
5. 37°C covered water bath or incubator
6. Squeeze bottle containing PBS
7. Tissue paper wipes
8. Humidified incubation chamber – This chamber can consist of a water-tight plastic box with a close-fitting lid containing a damp paper towel to maintain high humidity. Warm and maintain at 37°C either by placing the box in an incubator or by floating it on a covered water bath. The water bath cover prevents dehydration of the specimen as a result of condensation on the inside of the lid of the box.

Equipment

1. Light microscope equipped with bright field optics (20X, 40X and 100X objectives recommended)
2. Film or digital camera (optional)

III. BEGINNING CONSIDERATIONS

Specimen Fixation and Pretreatment

Specimen Fixation

Use of a cross-linking fixative is believed to tether the small chromatin fragments to the tissue, so that they will not be lost during processing steps. The preferred fixative for use before embedding tissue in paraffin is standard 10% (v:v) neutral-buffered formalin (NBF, see Sec. III. *Beginning Considerations, Reagent Preparation and Set-Up*).

Fixation times from 1 hour to 24 hours in NBF at 4°C are preferred for these tissue pieces. Longer fixation times could decrease the assay's sensitivity.

Immediate fixation is recommended, although holding tissue pieces at 4°C for 18 hours in PBS was found to cause no quantitative change in the staining.

After sectioning a paraffin block, seal the exposed tissue with liquid paraffin to maintain the freshness of the block.

Tissue Pretreatment

Paraffin-embedded tissue sections require rehydration and proteinase K treatment or an alternative pretreatment to further expose the DNA by opening up some of the cross-links caused by the formaldehyde. The tissue type, tissue size, specimen location and fixation time can affect the strength of protease pretreament needed. Alternatives to proteinase pretreatment are available, particularly if double staining of a proteinase-sensitive antigen is a goal (see Sec. VI. *Appendix, Tech Note #1, Alternate Tissue Pretreatments* and *Tech Note #2, Double-labeling for Microscopy*).

Changing Small Volumes of Solution on Slides

Do not let the specimen dry out between solutions. Take up the solution to be applied next into a pipette tip. Remove one slide at a time from a wash bath and tap off excess water, wiping water from the back of the slide on a paper towel. Then, carefully blot or aspirate around the section. Tilt the slide, and carefully wick most of the fluid off of the section with a corner of a re-folded paper wipe so as not to disturb the specimen. Promptly apply the next reagent from the pipette and return to the humidified chamber. Many samples can be processed sequentially while maintaining a consistent incubation time. Apply reagent at accurate time intervals (*e.g.* one slide every 30 seconds) and then end the incubations separately and sequentially at the next wash step.

Reagent Preparation and Set-Up

First, choose the Protocol to be performed (see Table 3: Detection Options).

Set-Up

1. Prepare chipped ice in which to hold working strength DNA Ligase Enzyme
2. Pre-warm incubator or covered water bath to 37°C.

Reagent Volumes

Table 5 outlines the recommended reagent volumes sufficient to assure adequate coverage of the tissue surface area.

Table 5: Working Reagent Volumes

Reagent	Vol/cm^2	Vol/5 cm^2
Working Strength DNA Ligase & Oligo	10-11µL	50-55 µL
Equilibration Buffer	10-12 µL	50-60 µL
Working Strength Streptavidin Conjugate	10-12 µL	50-60 µL
Working Strength DAB Substrate	15-20 µL	75-100 µL

Reagent Preparation

10% Neutral-buffered Formalin (For Use In Protocol 1)

Combine 10 mL of commercial formalin solution with 90 mL PBS as a buffer (see Sec. VI. *Appendix*). Use for tissue samples.

Notes:
NBF (netural-buffered formalin) is a 1:10 (v:v) dilution of commercial formalin (which is 37%-40% (w:v) formaldehyde hydrate preserved with 10%-15% methanol) in PBS. Result is 3.7%-4% formaldehyde (w/v).

Upon aging, the NBF solution accumulates impurities that are associated with harsher tissue fixation, as compared to a freshly prepared solution. Discard unused portions frequently.

1% (w/v) Pure Formaldehyde (For Use In Protocol 2)

Combine 1 mL of methanol-free, 16% (w/v) pure formaldehyde solution and 15 mL of PBS (see Sec. VI. *Appendix*). Prepare fresh. Use for frozen tissue sections or adherent cells (prevents clumping).

Note: *Pure formaldehyde can be prepared from solid paraformaldehyde polymer by hydrolysis in water aided by heat and titration with base. A 16% (w/v) pure formaldehyde solution, methanol-free, is available in ampoules sealed under nitrogen to prevent oxidation.*

Working Strength Hydrogen Peroxide (H_2O_2)

Dilute 30% stock H_2O_2 to 3% in PBS (i.e., a 10-fold dilution) for paraffin sections. For example, to prepare enough reagent to use in a coplin jar, combine 36 mL of PBS with 4 mL of the 30% stock. For frozen sections, dilute the H_2O_2 stock to 0.5%.

Proteinase K or Protein Digesting Enzyme (For Use In Protocol 1)

Reconstitute Proteinase K in 5 mL of PBS (making a 5 mg/mL stock). Dilute the concentrated 5 mg/mL Proteinase K stock to 50 µg/mL in PBS just before use, according to the direction described under the preferred method of application.

Using coplin jars for Proteinase K application

If the application of the Protease K to tissue sections is to be performed by immersing the slides in coplin jars, then aliquot 1 mL into each of 5 vials for later use to make the Working Strength Stock Dilution. Store at -20°C. The stock solution may be thawed and re-frozen 3 times.

Prepare 35 mL of Working Strength Proteinase K per coplin jar. Add 350 µL from a 5 mg/mL stock to 35 mL of PBS. Two or three batches of slides may be processed in quick succession. Discard diluted enzyme within one hour after preparation.

The amount of Proteinase K supplied in the kit, when diluted, is sufficient to fill 14 coplin jars. As the entire kit will process 40 specimens, at least three specimens must be processed together per 35 mL of diluted Proteinase K.

Addition of Proteinase K directly to the slide

If application of the Protease K to the slde is your preferred method, then aliquot 1 mL of the 5 mg/mL stock into each of 4 vials. Sub-aliquot the remaining 1 mL as 10 x 0.1 mL in 0.5 mL vials. Store at -20°C. The stock solution may be thawed and refrozen 3 times.

Add 1.5 µL of the 5 mg/mL stock to 150 µL of PBS per section, scaled up for the number of sections. Use 150 µL of diluted enzyme per 5 cm^2 specimen. Discard diluted enzyme within one hour after preparation.

Working Strength DNA Ligase Enzyme (For Use In Protocols 1 & 2)

DNA Ligase Enzyme (90430) is an 8.5X concentrate. It must be diluted by mixing with either Oligo A or Oligo B to prepare Working Strength Ligase.

Notes:
Minimize the length of time the DNA Ligase Enzyme is kept on ice.

The DNA Ligase Enzyme vial (90430) may have to be centrifuged briefly in a refrigerated micro-centrifuge at 4°C to 8°C to retrieve all contents in the tip of the tube.

Vortex the Oligo solutions (90432, 90433) before use to assure homogeneity.

Due to high viscosity of DNA Ligase Enzyme and Oligo solutions, pipette slowly and mix thoroughly but gently.

The Working Strength Ligase/Oligo mixture should be freshly prepared within 30 minutes of use.

1. For one 5 cm^2 specimen, add to a fresh, cold microfuge tube:

   54 µL Oligo A or B (90432 and/or 90433)
   6 µL T4 DNA Ligase Enzyme (90430)
   ---
   60 µL Total

2. Rinse all DNA Ligase Enzyme from inside the tip with mixture.
3. Mix well by brief vortexing at medium speed (3 x 2 sec.). Hold on ice.

   Note: *In order to determine the quantity of reagent to be prepared, multiply this volume by the number of 5 cm^2 specimens to be assayed or prepare a volume proportional to the number and area of specimens if the samples differ in size.*

Working Strength Peroxidase Substrate (For Use In Protocols 1 & 2)

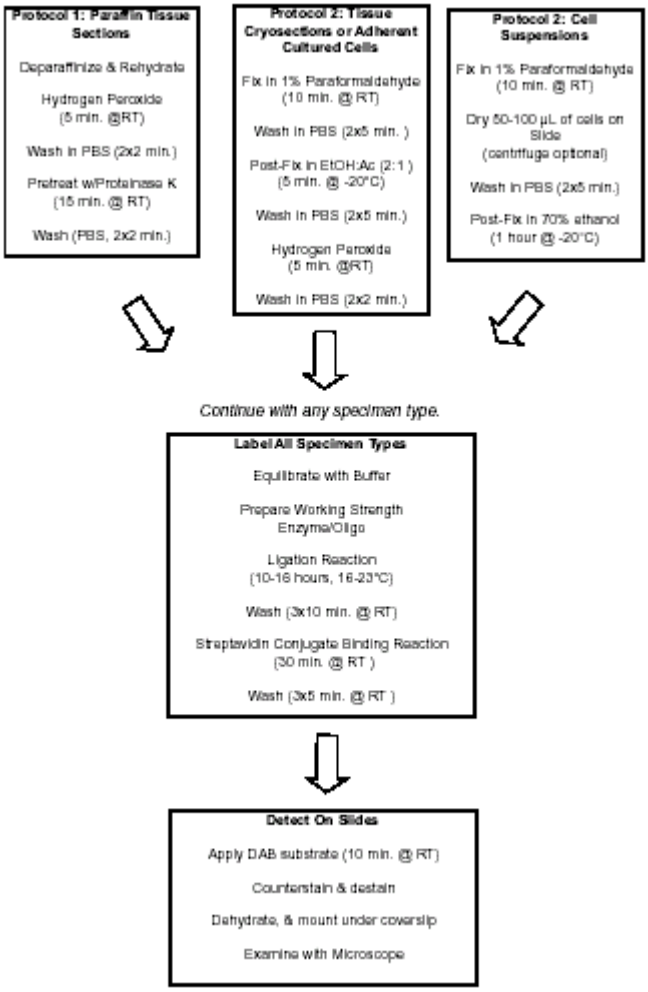
50X DAB and 1X DAB Dilution Buffer are provided in the kit. Prepare Working Strength Peroxidase Substrate by mixing in a clean tube:

147 µL DAB Dilution Buffer (90424)
3 µL DAB Substrate (90423)
150 µL Total

After dilution, warm to room temperature and store in the dark until use. This amount of Working Strength Peroxidase Substrate is sufficient to treat two 5 cm^2 tissue specimens. After mixing, unused solution is stable in the dark for 7 days at room temperature or up to 14 days at 4°C.

IV. PROTOCOLS

Figure 2: Flow Chart for Peroxidase Staining Protocols 1 & 2



Protocol 1: Peroxidase Staining of Paraffin-embedded Tissue

DO NOT ALLOW SAMPLES TO DRY OUT DURING PROCESSING.

Deparaffinize Tissue Section (in a coplin jar)

1. Wash the specimen in 3 changes of Xylene for 5 minutes each wash.
2. Wash the specimen in 2 changes of Absolute Ethanol for 5 minutes each wash.
3. Wash the specimen once in 95% Ethanol and once in 70% Ethanol for 3 minutes each wash.
4. Wash the specimen in one change of PBS for 5 minutes.

Quench Endogenous Peroxidase

1. Apply 3.0% hydrogen peroxide in PBS for 5 minutes at room temp. (either on a slide or in a coplin jar).
2. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen
3. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

Pretreat Tissue

1. Apply freshly diluted Protein Digestion Enzyme or Proteinase K to the specimen for 15 minutes at room temp. in a coplin jar or directly on the slide (~60-150 µl/5 cm^2).
2. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
3. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

Apply Equilibration Buffer

1. Gently remove any excess liquid from the section.
2. Immediately apply 1X Equilibration Buffer (60 µL/5 cm^2, #S7200-2) directly on the specimen. Incubate for at least 10 seconds at room temperature.

   Note: *Slides may be left in Equilibration Buffer or water for up to 24 hours at 4°C in a humidified chamber.*

Apply Working Strength DNA Ligase Enzyme

1. Gently remove any excess liquid from the section (see Sec. III. *Beginning Considerations, Changing Small Volumes of Solutions on Slides*).
2. Immediately pipette Working Strength DNA Ligase Enzyme onto the section (60 µl/5 cm^2; see Sec. III. *Beginning Considerations, Reagent Preparation*).
3. Incubate in a humidified chamber at 16°C to 22°C for 10-16 hours.

   Note: *The DNA Ligase Enzyme is susceptible to thermal denaturation during an extended ligation reaction at temperatures above 22℃. For best results, incubate ligation reactions between 16℃ and 22℃.*

   Note: *After incubating in Working Strength DNA Ligase Enzyme, slides can be held in wash buffer at 4℃ in a coplin jar overnight. After this, they can be held in 70% EtOH in a coplin jar and stored at -20℃ for at least 3 days. After storage in 70% EtOH, samples should be washed with three changes of PBS for 2 minutes per change before continuing the protocol.*

Wash

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
2. Wash the specimen in 3 changes of dH_2O in a coplin jar for 10 minutes each wash.

Apply Streptavidin-Peroxidase Conjugate

1. Immediately pipette Streptavidin-Peroxidase on the section (50-60 µL/5 cm^2 of 90434).
2. Incubate in a humidified chamber for 30 minutes at room temp.

Wash in PBS

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
2. Wash the specimen in 3 changes of PBS in a coplin jar for 5 minutes each wash at room temperature.

Develop Color in Peroxidase Substrate

1. Gently remove excess liquid from the section.
2. Apply Working Strength Peroxidase Substrate (75-100 µl/5 cm^2; see Sec. III. *Beginning Considerations, Reagent Preparation*).
3. Stain for ~10 minutes at room temp. A humidified chamber is optional.

   Note: *Specimen color may be visually monitored during development in order to determin the optimal stopping time. Stop development if the gross specimen color should approach a pale beige; this is the highest acceptable background level. By monitoring color development under a microscope, one can also determine the time needed to stain positive cells to an adequate intensity.*

Wash Specimen

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of dH_2O **above, not on** the section, allowing the dH_2O to gently trickle over the specimen.
2. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

Counterstain Specimen

1. Counterstain in 0.5% (w:v) methyl green in a coplin jar for 10 minutes at room temp.
2. Wash the specimen in 3 changes of dH_2O in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.
3. Wash the specimen in 3 changes of 100% n-butanol in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.

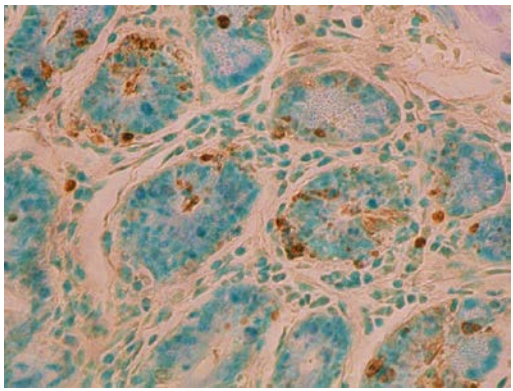
Mount Specimen (for specimens on silanized glass slides)

Note: *Specimens on plastic supports must be viewed in other compatible mounting media.*

1. Dehydrate the specimen by moving the slide through three jars of xylene, incubating for 2 minutes in each jar.
2. Remove the slides one at a time from the coplin jar. Gently tap the edge of the slide to drain, but do not allow the specimen to dry.
3. Immediately mount under a glass coverslip in a mounting medium such as Permount.
4. View under microscope.

   Note: *Slides may also be stored indefinitely at room temperature.*

Figure 3: Detection of apoptotic cells in rat intestine using ApopTag® ISOL Kit, Protocol 1



Protocol 2: Peroxidase Staining of Frozen Tissue Sections, Adherent Cells or Cell Suspensions

DO NOT ALLOW SAMPLES TO DRY OUT DURING PROCESSING.

Note: *In adherent cultures, apoptotic cells typically detach themselves from the substrate, and so supernatents should be tested, if possible, by using cytospin processing.*

Fix Specimen According to Type

Fix Tissue Cryosection or Adherent Cultured Cells

1. Fix in 1% pure (para)Formaldehyde (methanol-free formaldehyde) in PBS, pH 7.4 in a coplin jar(or cell culture vessel) preferably for 10 minutes at room temperature, or for up to 15 hours at 4°C. Drain off excess liquid.
2. Wash in 2 changes of PBS for 5 minutes each wash.
3. Skip to Quench Endogenous Peroxidase Step.

Post-Fix Tissue Cryosections or Adherent Cultured Cells

1. Post-fix in precooled Ethanol:Acetic acid 2:1 (v:v) for 5 minutes at -20°C in a coplin jar. Drain, but do not allow to dry (this solvent permeabilizes cells).
2. Wash in 2 changes of PBS for 5 minutes each wash.
3. Skip to Quench Endogenous Peroxidase Step (for cryosections) or Apply Equilibration Buffer Step (for cultured cells).

Fix Cell Suspensions for Microscopy

1. Fix the cells, in a tube, at a density of approximately 5 X 10^7 cells/mL in freshly diluted 1% pure Formaldehyde (methanol-free formaldehyde) in PBS, pH 7.4 for 10 minutes at room temperature.
2. Dry 50-100 µL of cell suspension on a microscope slide (alternatively, cytospin cells).
3. Optional: Post-fix in precooled Ethanol:Acetic acid 2:1 (v:v) for 5 minutes at -20°C om a coplin jar. Drain, but do not allow to dry (this solvent permeabilizes cells).
4. Wash in 2 changes of PBS for 5 minutes each wash.

5. Skip to Apply Equilibration Buffer Step.

***Note**: Tissue pretreatment with Proteinase K is unnecessary.*

Quench Endogenous Peroxidase (not necessary for most types of cultured cells)

1. Apply 0.5% hydrogen peroxide in PBS for 5 minutes at room temp. (either on a slide or in a coplin jar).

   ***Note:** Hydrogen peroxide concentration is lowered because tissue cryosections, more so than paraffin sections, may tend to detach from the slide.*

2. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
3. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

Apply Equilibration Buffer

1. Gently remove any excess liquid from the section.
2. Immediately apply 1X Equilibration Buffer (60 µL/5 cm^2, 90431) directly on the specimen. Incubate for at least 10 seconds at room temperature.

   ***Note:** Slides may be left in Equilibration Buffer or water for up to 24 hours at 4°C in a humidified chamber.*

Apply Working Strength DNA Ligase Enzyme

1. Gently remove any excess liquid from the section (see Sec. III. *Beginning Considerations, Changing Small Volumes of Solutions on Slides*).
2. Immediately pipette Working Strength DNA Ligase Enzyme onto the section (60 µL/5 cm^2; see Sec. III. *Beginning Considerations, Reagent Preparation*).
3. Incubate in a humidified chamber at 16°C to 22°C for 10-16 hours.

   ***Note:** The DNA Ligase Enzyme is susceptible to thermal denaturation during an extended ligation reaction at temperatures above 22°C. For best results, incubate ligation reactions between 16°C and 22°C.*

Note: *After incubating in Working Strength DNA Ligase Enzyme, slides can be held in wash buffer at 4℃ in a coplin jar overnight. After this, they can be held in 70% EtOH in a coplin jar and stored at -20℃ for at least 3 days. After storage in 70% EtOH, samples should be washed with three changes of PBS for 2 minutes per change before continuing the protocol.*

Wash

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
2. Wash the specimen in 3 changes of dH_2O in a coplin jar for 10 minutes each wash.

Apply Streptavidin-Peroxidase Conjugate

1. Immediately pipette Streptavidin-Peroxidase on the section (50-60 µL/5 cm^2 of 90434).
2. Incubate in a humidified chamber for 30 minutes at room temp.

Wash in PBS

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of PBS **above, not on** the section, allowing the PBS to gently trickle over the specimen.
2. Wash the specimen in 3 changes of PBS in a coplin jar for 5 minutes each wash at room temperature.

Develop Color in Peroxidase Substrate

1. Gently remove excess liquid from the section.
2. Apply Working Strength Peroxidase Substrate (75-100 µL/5 cm^2 ; see Sec. III. *Beginning Considerations, Reagent Preparation*).
3. Stain for ~10 minutes at room temp. A humidified chamber is optional.

Note: *Specimen color may be visually monitored during development in order to determin the optimal stopping time. Stop development if the gross specimen color should approach a pale beige; this is the highest acceptable background level. By monitoring color development under a microscope, one*

can also determine the time needed to stain positive cells to an adequate intensity.

Wash Specimen

1. For the first rinse, hold the slide vertically over a sink or container. Use a squeeze bottle to direct a stream of dH_2O **above, not on** the section, allowing the dH_2O to gently trickle over the specimen.
2. Wash the specimen in 2 changes of dH_2O in a coplin jar for 2 minutes each wash.

Counterstain Specimen

1. Counterstain in 0.5% (w:v) methyl green in a coplin jar for 10 minutes at room temp.
2. Wash the specimen in 3 changes of dH_2O in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.
3. Wash the specimen in 3 changes of 100% n-butanol in a coplin jar, dipping the slide 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash.

Mount Specimen (for specimens on silanized glass slides)

Note: *Specimens on plastic supports must be rehydrated and mounted in aqueous mounting media instead of using the following method.*

1. Dehydrate the specimen by moving the slide through three jars of Xylene, incubating for 2 minutes in each jar.
2. Remove the slides one at a time from the coplin jar. Gently tap the edge of the slide to drain, but do not allow the specimen to dry.
3. Immediately mount under a glass coverslip in a mounting medium such as Permount.
4. View under microscope.

Note: *Slides may also be stored indefinitely at room temperature.*

V. TROUBLESHOOTING

Table 6: Troubleshooting Recommendations

Problem	Suggested Troubleshooting Test	Interpretation
Pale cell staining	Whole protocol	See comment B below
Uneven cell staining	Whole protocol	See comment C below
Nonspecific DAB staining	Sham (negative) protocol without ligase enzyme	See Sec. VI. *Appendix* and continue diagnosis using these controls
No staining	Only Peroxidase Substrate	Tissue peroxidase quenched or absent; skip to 2nd test below
DAB staining seen	Only Peroxidase Substrate	Tissue peroxidase present; see comment A.1 below
DAB staining seen	Only Streptavidin-Peroxidase & Substrate	Consider endogenous biotin present OR Over-developed in Substrate; see comments A.1 and A.3 below
Pale counterstaining	Whole protocol	See comment D below

**First perform the regular pretreatments with proteinase K and hydrogen peroxide.*

Protocol Modifications for Optimizing Specimen Staining

A. Reducing diffuse cytoplasmic or matrix backgrounds

Infrequently, certain tissues may give diffuse backgrounds and false positive staining. Specimens and preparation techniques vary, and not all relevant specimen/process parameters have been fully identified. The suggested development time, ten minutes, is approximate because the rate of color development is affected by differences in the specimen composition and room temperature.

1. If staining of both nuclei and cytoplasm is apparent, the nonspecific component often can be mitigated by reducing the development time in DAB substrate. To obtain the best ratio of specific staining to diffuse background, develop for the minimum time needed for good staining of positive cells. The most positive cells should turn deep tan, then brown, while the diffuse background is still absent or is beginning to turn pale tan.

   Background may be further reduced in some epithelial tissues as follows. The concentraton of oligo (A or B) in Working Strength Ligase Enzyme can be reduced 25% so as to reduce the nonspecific oligo binding. Prepare the suggested total volume of Working Strength Ligase enzyme but substitute Equilibration Buffer for 25% of the Reaction Buffer volume in the mix. In addition, the wash after the Ligase Enzyme step may be done in 10X PBS preheated to 55 °C (instead of dH_20 at room temp.), followed by one change of 1X PBS at room temp. The development time in DAB substrate should not be extended.

2. Tissue peroxidase, if present, should be quenched by the recommended hydrogen peroxide pretreatment. Red blood cells are one of the cell types that may present peroxidase activity.

3. As with any biotin/avidin-based technique, there is a possibility that endogenous biotin may be present which could cause inappropriate staining. This artifact can be diagnosed by preparing controls using no DNA Ligase Enzyme, or heat-inactivated Enzyme, and it is observable in simple unreacted tissue. A standard method to mitigate this problem is to block the endogenous biotin before doing the DNA ligation step, by applying avidin. Avidin is tetravalent, and therefore it is then necessary to block all remaining biotin binding sites on avidin by applying biotin, immediately before the ligation step. This procedure can be performed by applying avidin, 50 µg/mL in PBS, for 20 minutes, then rinsing briefly 3X in PBS. This treatment is followed by the application of d-biotin, 5 µg/mL in PBS, for 10 minutes and

again rinsing briefly 3X in PBS. Commercial Avidin/Biotin Blocking Kits are available (*e.g.* Vector Cat. #SP- 2001).

4. In necrosis small DNA fragments may leak into the cytoplasm after the metabolic demise of the cell. ISOL is relatively insensitive to these fragments, but a pale signal may be seen.

B. Optimizing Pale Staining

Observation of even a few very deeply stained cells in a section is sufficient to indicate that the Kit reagents are functional. If none are seen, or if only lightly stained cells are seen, the reagents may be tested on the Positive Control Slide provided (90422) to assure functionality.

1. In the development step, if diffuse beige background does not appear within ten minutes, the development time in Working Strength DAB Substrate can be increased.
2. Although the proportion of Oligo used to prepare Working Strength DNA Ligase can be decreased (i.e. below 88% v/v), so as to increase the working concentration of DNA Ligase Enzyme, doing this will decrease the total volume of this mixture and the number of samples that can be processed.
3. The temperature of the ligation reaction must remain in the proper range, 16°C to 22°C.
4. Avoid prolonged over-fixation of sample in NBF, or prolonged storage of an exposed tissue surface in an unprotected paraffin block after sectioning.

C. Optimizing Uneven Staining

1. Both Equilibration Buffer and Working Strength DNA Ligase Enzyme are viscous media, so bulk amounts may not mix quickly on the slide. Uneven staining may result from an excessive residue of Equilibration Buffer remaining on the section when Working Strength DNA Ligase Enzyme is applied. This may also cause a dilution effect (see Sec. IV. *Protocols*).
2. An edge artifact of apparent hypersensitivity is an occasional occurrence, as with all variants of immunoperoxidase staining. Extraordinary numbers of positive cells and a higher background around the edges of a specimen should be considered as localized hypersensitivity of the assay.
3. Poor staining and poor morphology could result from inadequate fixation, particularly in the interior of a bulky tissue specimen.
4. Both nucleus and cytoplasm may be lightly stained in necrosis.
5. All pycnotic nuclei should not be expected to stain identically, just as with other comparable in situ methods.

D. Optimizing Counterstaining With Methyl Green

1. Normal nuclei should stain pale to medium green if the correct pH (pH 4) is used (make sure the stain does not contain any crystal violet).
2. Ethanol should not be substituted for butanol in the destaining step as it will destain nuclei.
3. For destaining after methyl green, number the coplin jars of dH_2O, of n-butanol and of xylene, and always use in this sequence. Replace the first water jar after each slide. When the first jar of the n-butanol series appears light blue, replace it with the second, replace the second with the third, and replace the third with new stock. Rotate xylene stocks at the same time, though no color will be seen.
4. Nonspecific peroxidase staining will competitively reduce counterstaining. Use the minimum development time in DAB that gives distinctly positive apoptotic staining.

VI. APPENDIX

Reagent Preparation

1X PBS (100 mM sodium phosphate, 200 mM NaCl, pH 7.4)

1. Prepare two stocks: 1.0 M Na_2HPO_4 and 1.0 M NaH_2PO_4

   1.0 M Na_2HPO_4: Combine 14.2 g Na_2HPO_4 and H_2O to final volume of 100 mL

   1.0 M NaH_2PO_4: Combine 12.0 g NaH_2PO_4 and H_2O to final volume of 100 mL

2. Mix 2X buffered phosphate as follows: 77.4 ml of Na_2HPO_4, 22.6 mL of NaH_2PO_4 and dH_2O to 1.0 L

3. Prepare a stock of 2X NaCl

   0.4 M NaCl: Combine 23.4 g and dH_2O to 1.0 L

4. Mix PBS as 1 volume 2X buffered phosphate and 1 volume of 2X NaCl

0.5% (w/v) Methyl Green

1. Prepare 0.1 M sodium acetate, pH 4.0 as follows

   Dissolve 1.36 g sodium acetate ($C_2H_3O_2Na{:}3H_2O$) in 80 mL of dH_2O. Adjust the pH to 4.0 with acetic acid. Add dH_2O to a final volume to 100 mL.

2. Dissolve 0.5 g methyl green in 100 mL of 0.1 M sodium acetate, pH 4.0.

3. Filter through a 0.45 or 0.2 micron filter prior to use.

Tech Notes

TECH NOTE #1: Alternate Tissue Pretreatments

- Besides the standard protease pretreatment, there are two other pretreatment methods for exposing the DNA in tissue sections. These may be useful if double-staining for a second antigen of interest is the goal and the antigen is proteinase sensitive.

- In the heating method, the slide is placed in 10 mM citrate buffer, pH 6.0, in a coplin jar, and gently boiled for 3-5 cycles of 3 minutes each in a microwave oven (40). Refill with fresh buffer between cycles, and do not let the sample dry out. A pressure cooker or an autoclave can be used instead of a microwave. Let the solution sit on the bench until it reaches a warm, but not hot, temperature before proceeding. In the detergent pretreatment method, 0.5% Triton X-100 can be applied for 10 minutes at room temperature (44).

TECH NOTE #2: Fixatives and fixation

- *In Situ* Oligo Ligation (ISOL) has been used together with TdT/TUNEL (terminal uridine nick end labeling) (13).

- TdT end-labeling (TUNEL), for example, has been used in combination with immunochemical proliferation markers, including halodeoxyuridine labeling of DNA (29; 34) or Ki67/MIB1 (Chemicon®, unpublished data). The antigen should be tested for susceptibility to proteinase K, and if this lowers the immunoreactivity, another pretreatment should be tested. Perform thermal tissue treatment before doing ApopTag® (see Tech. Note 1).

- A method for double-labeling of cultured cells for apoptosis with the ApopTag® *In Situ* TUNEL Peroxidase Apoptosis Detection Kit, (Catalog #'s: S7100 and S7101) for necrosis using trypan blue has been described (35).

TECH NOTE #3: Fluorescent Applications

For high sensitivity fluorescent staining of tissue sections using ISOL, a signal amplification method is useful in conjunction with ISOL. Cell suspensions; however, can be stained for flow cytometry without using signal amplification, by simply using a streptavidin-fluorophore conjugate following the ligation and wash steps. For further information, please call Chemicon® Technical Service at (800) 437-7500 or email techserv@chemicon.com.

Controls

Negative controls

Negative controls are used to validate the specific reactivity of the assay and to detect other potential artifacts. Two methods may be used to prepare a specificity (negative or sham) control reaction. Either substitute Equilibration Buffer (90431) for DNA Ligase Enzyme (90430), or remove an aliquot of complete Working Strength DNA Ligase Enzyme and heat to 65°C for 20 minutes to inactivate the DNA Ligase Enzyme. Equilibaration Buffer (90431) is the same medium in which the Oligos are dissolved.

Positive controls

These controls are used to validate the chemical functionality and sensitivity of the assay reagents at the time of use, based on staining of a known positive sample.

Biological apoptosis

In normal female rodent mammary gland, extensive apoptosis occurs 3-5 days after weaning of rat pups; this is the tissue on the included slides. (Extra positive control slides, #S7115, are sold separately.) Apoptosis can be induced in young adult rat thymic lymphocytes by dexamethasone (3, 12, 19), or in many kinds of in vitro systems by various treatments (32). Another source of apoptotic cell is in normal rodent testis where apoptotic spermatogonia occur in the seminiferous tubules (1).

Biochemically modified tissue

By cutting DNA with DNase I *in situ*, a positive control sample can be prepared from most kinds of tissue sections. A protocol for endonuclease treatment using DNase I (2, 19, 47) is as follows.

1. Pretreat section with DN Buffer (see below).
2. Dissolve DNase I (#7052) in DN Buffer to a concentration of 1.0-10 Kunitz (enzyme activity) Units/mL.
3. Remove DN Buffer and apply DNase solution. Incubate for 10 minutes at room temperature.
4. Rinse with 5 changes of dH_2O, for 3 minutes each change.

Note: *Over-digestion or under-digestion with DNase I will not allow forsensitive detection. As the tissue composition and its prior processing may vary, an optimization procedure using of a range of enzyme concentrations is recommended.*

DN buffer (30 mM Tris Base, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT)

i. Prepare 1 M Tris base, pH 7.2

   Dissolve 12.1 g of Tris base in 80 mL of dH_2O. Adjust the pH to 7.2 with concentrated HCl and add dH_20 to a final volume of 100 mL.

ii. Prepare 1.0 M $MgCl_2$

   Dissolve 20.3 g of $MgCl_2 \cdot 6H_2O$ in sufficient dH_2O for a final volume of 100 mL.

iii. Prepare DN buffer

   Combine 3 mL of 1.0 M Tris, pH 7.2, 400 µL of 1.0 M $MgCl_2$, 1.54 mg of DTT and sufficient dH_2O for a final volume of 100 mL.

Related Products

Table 7: ApopTag® DNA Fragmentation Detection Kits

Cat #	Product	Quantity
S7100	ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit	40 Assays
S7101	ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit	40 Assays
S7110	ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit	40 Assays
S7111	ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit	40 Assays
S7160	ApopTag® Fluorescein Direct *In Situ* Apoptosis Detection Kit	40 Assays
S7165	ApopTag® Red In Situ Apoptosis Detection Kit	40 Assays

Table 8: Apoptosis Reagents

Cat #	Product	Quantity
S7114	Antifade Solution	1 mL
S7106	ApopTag® Equilibration Buffer	15 mL
S7115	ApopTag® Positive Control Slides	5 slides
S7105	ApopTag® Reaction Buffer	1 mL
S7108	ApopTag® Stop/Wash Buffer	20 mL
S7107	ApopTag® TdT Enzyme	300 µL
S7113	DAPI/Antifade Solution	1 mL
S7112	Propidium Iodide/Antifade Solution	1 mL
S7109	Propidium Iodide Solution	1 mL

Positive Control Slides are supplied in the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (S7101), ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (S7111) and by the package (S7115). The slides contain unstained rat mammary glands obtained at the fourth day after weaning (36) that were fixed for 18 hours in 10% neutral buffered formalin. After embedding in paraffin, 5 micron thick sections were cut from the middle of the tissue and mounted on silanized slides.

Table 9: Caspase Assays

Cat #	Product	Quantity
APT400	CaspaTag™ Pan-Caspase *In Situ* Assay Kit	100 tests
APT403	CaspaTag™ Caspase 3 *In Situ* Assay Kit, Fluorescein	100 tests
APT500	CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Sulflrhodamine	100 tests
APT503	CaspaTag™ Caspase 3 *In Situ* Assay Kit, Sulforhodamine	100 tests

Table 10: Mitochondrial Membrane Permeabilization

Cat #	Product	Quantity
APT142	MitoLight® Mitochondrial Apoptosis Detection Kit	25 Assays

VII. REFERENCES

Internet Sites

1. Chemicon® Corporation: www.chemicon.com
2. Images obtained using the ApopTag® Peroxidase ISOL Kit at: www.chemicon.com/
3. APOPTOSIS Online: The Apoptosis Information & Communication Center at www.apopnet.com
4. Purdue Cytometry Mailing List: www.cyto.purdue.edu/hmarchive/Cytometry/index.html
5. PubMed: www.ncbi.nlm.nih.gov/pubmed

References Cited in this Manual

1. Allen, D.J., Roberts, S.A., and Harmon, B.V. (1993) Spontaneous spermatogonial apoptosis shows three distinct morphological phases and no circadian rhythm in the rat. *Cell Proliferation* 25: 241-250.
2. Al-Lamki, R.S., Skepper, J.N., Loke, Y.W., King, A. and Burton, G.J. (1998) Apoptosis in the early human placental bed and its discrimination from necrosis using the in-situ DNA ligation technique. *Human Reproduction* 13:3511-3519.
3. Arends, M.J., Morris, R.G. and Wyllie, A.H. (1990) Apoptosis The role of the endonuclease. *Amer. J. Pathol.* 136: 593-608.
4. Attanasio, A., and Schiffer, D. (1995) Ultrastructural detection of DNA strand breaks by in situ end-labeling techniques. *J. Pathol*. 176:27-35.
5. Brown, D.G., Sun, X.M., and Cohen, G.M. (1993) Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation . *J. Biol. Chem.* 268: 3037-3039.
6. Bursch, W., Kleine, L. and Tenniswood, M. (1990) Determination of the length of the histological stages of apoptosis in normal liver and altered hepatic foci of rats. *Carcinogenesis* (London) 11: 847-853.
7. Bursch, W., Paffe, S., Barthel, G. and Schulte-Harmann, R. (1990) Biochemistry of cell death by apoptosis. *Biochem. Cell Biol.* 68: 1071-1074.

8. Chapman, R., Chresta, C.M., Herberg, A.A., Beere, H.M., Heer, S. and Whetton, J.A. (1995) Further characterization of the in situ terminal deoxynucleotidyl transferase (TdT) assay for the flow cytometric analysis of apoptosis in drug resistant and drug sensitive leukemia cells. *Cytometry* 20:245-256.

9. Chen, Z., Chua, C.C., Ho, Y.S., Hamdy, R.C. and Chua, B.H. (2001) Overexpression of Bcl-2 attenuates apoptosis and protects against myocardial I/R injury in transgenic mice. *Am J. Heart Circ. Physiol.* 280: H2313-20.

10. Darzynkiewicz, Z. Juan, X., Gorzyca, W., Murakami, T. and Traganos, F. (1997) Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 27:1-20.

11. Deng, G. and Wu, R. (1983) Terminal transferase-catalyzed addition of nucleotides to the 3' terminal of DNA. *Methods in Enzymol*. 65:43-62

12. Didenko, V.V., and Hornsby, P.J. (1996) Presence of double-stranded breaks with single-base 3' overhangs in cells undergoing apoptosis but not necrosis. *J. Cell Biol*. 135:1369-1376.

13. Didenko, V.V., Tunstead, J.R., and Hornsby, P.J. (1998) Biotin-labeled hairpin oligonucleotides: probes to detect double-strand breaks. DNA in apoptotic cells. *Am. J. Pathol*. 152:897-902.

14. Didenko, V.V., and Hornsby, P.J. (1996) Assay for detecting apoptotic cells. U.S. Patent Application Ser. No. 08/758,027.

15. Didenko, V.V. and Hornsby, P.J. Didenko, V.V., Boudreax, D.J. and Baskin, D.S (1999) Substantial background reduction in ligase-based apoptosis detection using newly designed hairpin oligoprobes. *Biotechniques* 27: 1130-1132.

16. Didenko, V.V., Wang, X., Yang, L. and Hornsby, P.J. (1999) DNA damage and p21 (WAF/CIP1/SDI1) in experimental injury of the rat adrenal cortex and trauma-associated damage of the adrenal cortex. *J. Pathol.* 189: 119-26.

17. Ding, B., Price, R.L., Goldsmith, E.C., Borg, T.K., Yan, X., Douglas, P.S., Weinberg, E.O., Bartunek, J., Thielen, T., Didenko, V.V., & Lorell, B.H. (2000) Left ventricular hypertrophy in ascending aortic stenosis mice: anoikis and the progression to early failure. *Circulation* 101:2854-62.

18. Frustaci, A.F., Chimenti, C., Setoguchi, M., Guerra, S., Corsello, S., Crea, F., Leri, A., Kajstura, J., Anversa, P. & Maseri, A. (1999) Cell death in acromegalic cardiomyopathy. *Circulation* 99:1426-1434.

19. Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol*. 119: 493-501.

20. Gold, R. (1994) Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab Invest.* 71: 219-225.

21. Gong, J., Traganos, F. and Darzynkiewicz, Z. (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem*. 218:314-319.

22. Gorczyca, W., Bruno, S., Darzynkiewicz, J., Gong, J. and Darzynkiewicz, Z. (1992) DNA strand breaks occurring during apoptosis - Their early in situ detection by terminal deoxy-nucleotidyl transferase and nick translation assays and prevention by serine protease inhibitors. *Int. J. Oncol.* 1: 639-648.

23. Gorczyca, W., Gong, J. and Darzynkiewicz, Z. (1993) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53: 1-7.

24. Halicka, H.D., Seiter, K., Feldman, E.J., Traganos, F., Mittelman, A., Ahmed, T. and Darzynkiewicz, Z. (1997) Cell cycle specificity of apoptosis during treatment of leukemias. *Apoptosis* 2:25-39.

25. Kanoh, M., Takemura, G., Misao, J.M., Hayakawa, Y., Aoyama, T., Nishigaki, K., Noda, T., Fujiwara, T., Fukuda, K., Minatoguchi, S. & Fujiwara, H. (1999) Significance of myocytes with positive in situ nick end-labeling (TUNEL) in hearts with dilated cardiomyopathy. *Circulation* 99:2757-2764.

26. Kerr, J.F.R., Gobe, G.C., Winterford, C.M. and B.V. Harmon (1995) anatomical methods in cell death. In: *Methods in Cell Biology* Vol. 46, Academic Press, chapt. 1, pp. 1-27.

27. Kockx, M.M., Muhring, J., Knaapen, M.W.M., and de Meyer, G.R.Y (1998) RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. *Am J. Pathol*. 152:885-888.

28. Kunishima, A., Takemura, G., Takatsu, H., Hayakawa, Y., Kanoh, M., Qui, X., Fujiwara, T. & Fujiwara, H. (1999) Mode and role of cell death during progression of atherosclerotic lesions in hypercholesterolemic rabbits. *Heart Vessels* 14:295-306.

29. Li, X., Melamed, M.R. and Darzynkiewicz, Z. (1996) Detection of apoptosis and DNA replication by differential labeling of DNA strand breaks with fluorochromes of different color. *Exper. Cell Res*. 222:28-37

30. Macmanus, J.P., Fliss, H., Preston, E., Rasquinha, I., & Tuor, U. (1999) Cerebral ischemia produces laddered DNA fragments distinct from cardiac ischemia and archetypal apoptosis. *J. Cerebral Blood Flow Metab*. 19:502-510.

31. Majno, G. and Joris, I. (1995) Apoptosis, oncosis and necrosis, an overview of cell death. *Am. J. Pathol*. 146:3-15.

32. McGahon, A., Bisonnette, R., Schmitt, M., Cotter, K.M., Green, D.R. and Cotter, T.G. (1994) BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. *Blood* 83: 1179-1187.

33. Migheli, A.M., Attanasio, A. and Schiffer, D. (1995) Ultrastructural detection of DNA strand breaks in apoptotic neural cells by in situ end-labeling techniques. *J. Pathol.* 176:27-35.

34. Mundle, S., Iftikhar, A., Shetty, V., Dameron, S., Wright Quinones, V., Marcus, B., Loew, J., Gregory, S. and Raza, A. (1994) Novel in situ doublelabeling for simultaneous detection of proliferation and apoptosis. *J. Histochem. Cytochem*. 42:1533-1537.

35. Perry, S.W., Epstein, L.G. and Gelbard, H.A. (1997) Simultaneous in situ detection of apoptosis and necrosis in monolayer cultures by TUNEL and trypan blue staining. *BioTechniques* 22:1102-1106.

36. Rosl, F. (1992) A rapid and simple method for detection of apoptosis in human cells. *Nucleic Acids Res.* 20: 5243.

37. Smith, S. & Cartwright, M. (1997) Spatial visualization of apoptosis using a whole-mount in situ DNA end-labeling technique. *Biotechniques* 22:832-834.

38. Staley, K., Blaschke, A.H. and Chun, J. (1997) Apoptotic DNA fragmentation is detected by a semi-quantitative ligation-mediated PCR of blunt DNA ends. *Cell Death Different*. 4:66-75.

39. Strange, R., Friis, R.R., Bemis, L.T. and Geske, F.J. (1995) Programmed cell death during mammary gland involution. In: chapt. 15 of Cell Death, *Methods In Cell Biol*. 46:355-366, pub. by Academic Press.

40. Strater, J, Gunthert, A.R., Bruderlein, S. and Moller, P. (1995) Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for in situ detection of apoptotic cells. *Histochem. Cell Biol.* 103:157-160.

41. Takemura, J., Kato, S., Aoyama, T., Hayakawa, Y., Kanoh, M., Maruyama, R., Arai, M., Nishigaki, K., Minatoguchi S., Fukuda, K., Fujiwara, T and Fujiwara, H. (2001) Characterization of ultrastructure in its relation with DNA fragmentation in Fas-induced apoptosis of cultured cardiac myocytes. *J. Pathol.* 193:546-56.

42. Thiry, M. (1992) Highly sensitive immunodetection of DNA on sections with exogenous termal deoxynucleotidyl transferase and non-isotopic nucleotide analogs. *J. Histochem. Cytochem*. 40: 411-419.

43. Tilly, J.L., Kowalski, K.I., Johnson, A.L. and Hsueh, A.J. (1991) involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129: 2799-2801.

44. Tornusciolo, D., Schmidt, R.E. and Roth, K.A. (1995) Simultaneous detection of TdT-mediated dUTP-biot nick end labeling (TUNEL)-positive cells and multiple immunohistochemical markers in single tissue sections. *BioTechniques* 19:800-805.

45. Walker, P.R., Kokileva, L., LeBlanc, J. and Sikorska, M. (1993) Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques* 15: 1032-1047.

46. Walker, P.R., Weaver, P.M., Lach, B., LeBlanc, J. and Sikorska, M. (1994) Endonuclease activities associated with high molecular weight and internucleosomal DNA fragmentation in apoptosis. *Exp. Cell. Res.* 213: 100-106.

47. Widlak, P.Li, P. and Garrard, W.T. (2000) Cleavage preferences of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates. *J. Biol. Chem.* 275: 8226-32.

48. Wijsman, J.H., Jonker, R.R., Keijzer, R., Van De Velde, C.J.H., Cornelisse, C.J. and Van Dierendonck, J.H. (1993) A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA. *J. Histochem. Cytochem*. 41: 7-12.

Disclaimers

Warranty

EMD Millipore Corporation (“EMD Millipore”) warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **EMD MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of EMD Millipore products appearing in EMD Millipore’s published catalogues and product literature may not be altered except by express written agreement signed by an officer of EMD Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, EMD Millipore Corporation’s sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies EMD Millipore Corporation promptly of any such breach. If after exercising reasonable efforts, EMD Millipore Corporation is unable to repair or replace the product or part, then EDM Millipore shall refund to the Company all monies paid for such applicable Product. **EMD MILLIPORE CORPORATION SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY COMPANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

Unless otherwise stated in our catalog or other company documentation accompanying the product(s), our products are intended for research use only and are not to be used for any other purpose, which includes but is not limited to, unauthorized commercial uses, in vitro diagnostic uses, ex vivo or in vivo therapeutic uses or any type of consumption or application to humans or animals.

Cat. No.: S7200

July 2016
Version 6: 41430