# PRODUCT INFORMATION & MANUAL

## APO-BRDU-IHC (TUNEL) Apoptosis Kit

NBP2-31164

For research use only. Not for diagnostic or therapeutic procedures.

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# APO-BRDU-IHC™

## *A Complete Kit for Measuring Apoptosis by Dual Color ImmunoHistoChemistry*

## Description of Kit

The **APO-BRDU-IHC™** reagent kit is a two color TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay for labeling DNA breaks to detect apoptotic cells by immunohistochemistry (1). The kit contains the instructions and reagents required for measuring apoptosis in cells including; positive/negative control slides for assessing reagent perfor-mance; reaction and blocking buffers for processing individual steps in the assay; proteinase K; terminal deoxynucleotidyl tranferase enzyme (TdT), bromodeoxyuridine triphosphate (Br-dUTP), biotin labeled antiBrdU antibody for labeling DNA breaks, horseradish peroxidase streptavidin conjugate, DAB, $H_2O_2$/Urea tablets for color generation and methyl green solution for counter staining the cells.

## Contents of the APO-BRDU™ IHC Kit

The **APO-BRDU-IHC™** Kit is shipped in one package containing frozen ice packs and the reagent contents should be stored at -20C upon arrival. Phoenix Flow Systems, Inc. has determined this shipping method is adequate to maintain the integrity of the kit components.

Once the kit is defrosted, two components, the 5X-reaction buffer, biotin~PRB-1 monoclonal antibody should not be refrozen but stored in the refrigerator and the methyl green solution should be stored at room temperature.

Reagent bottles have color coded caps to aid in their identification. Sufficient reagents are provided to process 50 slides including 2 positive/negative control slides. The control slides are derived from a human promyelocitic leukemia cell line (HL60) and are incubated with 0.5 μg/ml actinomycin D for 19 hours to induce apoptosis.

# APO-BRDU-IHC™ Kit Components:

| COMPONENT | COLOR CODE | PART NUMBER | VOLUME (ml) | STORAGE CONDITIONS |
|---|---|---|---|---|
| Control Slides-pos/neg | natural box | CC1003 | 2 ea. | -15 to -25C |
| Blocking Buffer | white cap | AHBB23 | 22.000 | -15 to -25C |
| $H_2O_2$/Urea Tablets | amber vial | AHTAB20 | 6 ea. | -15 to -25C |
| Proteinase K | pink cap | AHPK25 | 0.110 | -15 to -25C |
| DAB Tablets | amber vial | AHDAB24 | 6 ea. | -15 to -25C |
| TdT Enzyme | yellow cap | AHTD15 | 0.041 | -15 to -25C |
| Br-dUTP | violet cap | AHBU16 | 0.440 | -15 to -25C |
| 200X Conjugate | natural cap | AHHR22 | 0.035 | -15 to -25C |
| 5X-Reaction Buffer | green cap | AHRXB14 | 1.750 | 2 to 8C |
| Biotin~PRB-1 mAb | orange cap | AHBIO18 | 0.275 | 2 to 8C |
| Methyl Green | natural cap | AHMG21 | 6.000 | room temp. |

## Precautions and Warnings

**1.** For optimal results: **READ THESE INSTRUCTIONS COMPLETELY BEFORE USING THIS KIT.**

2. The components of this kit are for **Research Use Only** and are not intended for diagnostic procedures.

**3.** After initial defrosting of this kit, the following components should be stored at 2 - 8 C: 5X Reaction Buffer (AHRXB14) and Biotin-PRB1 mAb (AHBIO18). Methyl Green Counterstain (AHMG21) should be stored at room temperature. **Do Not Re-freeze any of these components.**

4. Component part numbers **AHRXB14** and **AHBU16** contain cacodylic acid (dimethylarsenic) as a buffer; **AHBI018** contains 0.05% (w/v) sodium azide as a preservative.; **AHDAB24** contains (Diaminobenzidine). These materials are harmful if swallowed and potentially carcinogenic; avoid skin contact, wash immediately with water. See Material Safety Data Sheets for more information.

5. TdT Enzyme (AHTD15) will not freeze at -20C, because it is in a 50% (v/v) glycerol solution. Use carefully-it is the most expensive component of the kit.

6. To avoid reagent loss of thawed solutions, centrifuge vials for 30 seconds to force all the liquid to the bottom of the vial before using.

7. Gloves, lab coat and protective eye wear should be worn while staining.

# Reagents and Materials Required, but not supplied:

1. Xylene-100%
2. Ethanol-100%, 90%, 80%, 70% v/v with $dH_2O$
3. Methanol-100%
4. Hydrogen peroxide-30%
5. Phosphate buffered **or** tris-buffered saline (1X TBS, 20mM Tris pH 7.6, 140 mM NaCl)
6. 10 mM Tris pH 8.0
7. 1 mM $MgSO_4$ in 1X TBS (optional, for use in generating positive control)
8. DNase I (optional, for use in generating positive control)
9. 1-4% Formaldehyde in PBS pH 7.4
10. Distilled water
11. Coplin jars, glass or plastic with slide holders
12. Wash bottle or beaker for rinsing slides
13. Slide humidifing chamber
14. Glass coverslips
15. Mounting media (such as Permount [r])
16. Microscope
17. 2-20 µl, 20-200 µl and 200-1000 µl precision pipetters with disposable tips
18. Microcentrifuge tubes
19. Parafilm [r]
20. Absorbent wipes
21. Ice-crushed

Parafilm (r) is a registered trademark of the American National Can Company
Permount (r) is a registered trademark of Fisher Scientific

# Description of Apoptosis

Apoptosis is the term that describes regulated or programmed cell death and is believed to take place in the majority of animal cells. Apoptosis is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle culminating in the disappearance of the cell. It is common during embryogenesis (3,4), normal tissue and organ involution (5,6), cytotoxic immunological reactions (7,8) and occurs naturally at the end of the life span of differentiated cells (9,10). Apoptosis can also be induced in cells by the application of a number of different agents including physiological activators, heat shock, bacterial toxins, oncogenes, chemotherapeutic drugs or ultraviolet and gamma radiation (11). When apoptosis occurs, the nucleus and cytoplasm of the cell often fragments into membrane-bound apoptotic bodies that are then phagocytized by neighboring cells. The alternative mode of cell death, necrosis, occurs as a result of gross injury to cells resulting in cellular lysing and release of cytoplasmic components into the surrounding environment often inducing an inflammatory response in the surrounding tissue. A landmark of cellular self destruction by apoptosis is the activation of nucleases that degrade the higher order chromatin structure of the DNA into fragments of 50 to 300 kilobases and subsequently into smaller DNA pieces of about 200 base pairs in length (12). Numerous reviews of the events accompanying apoptosis are available and several well-researched model systems have been described (13,14,15).

# Measurable Features of Apoptosis

One of the most easily measured features of apoptotic cells is the break-up of the genomic DNA by cellular nucleases. These DNA fragments can be extracted from apoptotic cells and result in the appearance of "DNA laddering" when the DNA is analyzed by agarose gel electrophoresis (12). The DNA of non-apoptotic cells which remains largely intact does not display this "laddering" on agarose gels during electrophoresis. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3′-hydroxyl ends in the DNA. This property can be used to identify apoptotic cells by labeling the 3′-hydroxyl ends of the DNA with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). The enzyme terminal deoxynucleotidyl transferase

(TdT) catalyzes this template independent addition of deoxyribonucleotide triphosphates to the 3′-hydroxyl ends of double- or single-stranded DNA with either blunt, recessed or overhanging ends (16). A substantial number of these sites are available in apoptotic cells providing the basis for the method utilized in the **APO-BRDU-IHC™** Kit (1,17). Non-apoptotic cells do not incorporate significant amounts of the Br-dUTP owing to the lack of exposed 3′-hydroxyl DNA ends. After labeling the 3′-hydroxyl ends of the DNA with Br-dUTP, an anti-BrdU antibody directly conjugated to biotin is attached to the Br-dUTP's. Horseradish peroxidase conjugated to avidin is attached to the Br-dUTP's. Then a standard contrast microscope stain reaction, DAB-$H_2O_2$-Urea, is used to label the incorporated Br-dUTP's. Recent evidence has demonstrated that Br-dUTP is more readily incorporated into the genome of apoptotic cells than are the

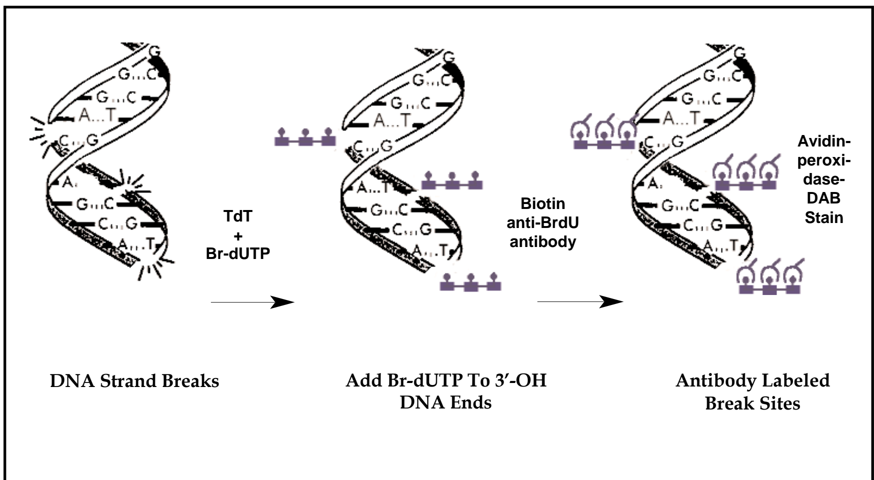


***Figure 1: Diagrammatic representation of the addition of bromodeoxyuridine triphosphate (Br-dUTP) catalyzed by terminal deoxynucleotidyl transferase (TdT) to the 3′-OH sites of DNA strand breaks induced in the genome of apoptotic cells.***

deoxynucleotide triphosphates complexed to larger ligands like fluorescein, biotin or digoxigenin (1). This greater incorporation gives rise to a stronger flow cytometry signal when the Br-dUTP sites are identified by a fluorescein labeled antiBrdU monoclonal antibody and may increase the signal to noise ratio in this immunohistochemical staining method.

# Flow Diagram of APO-BRDU™ IHC Apoptosis Assay

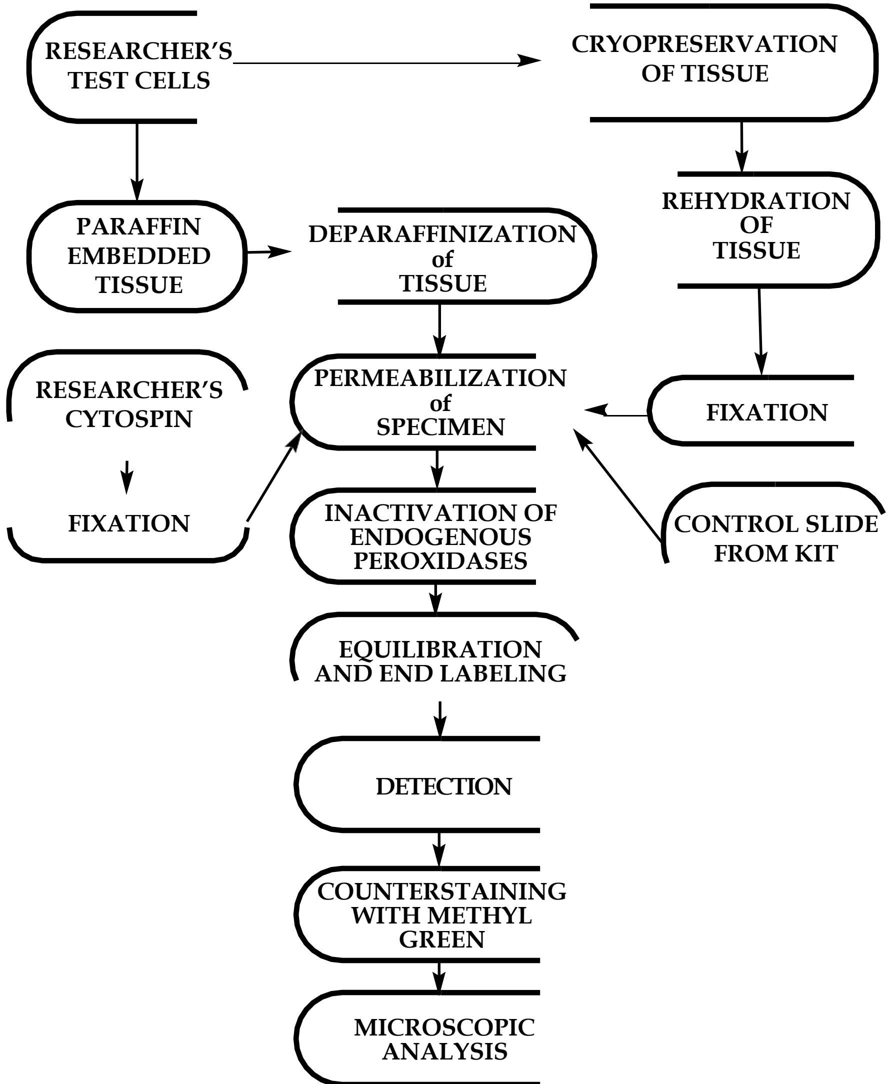


*Figure 2: Flow diagram used in the APO-BRDU-IHC™ Apoptosis Assay. The positive and negative control cells supplied in the kit are from a cytospin preparation and do not need to be deparaffinized.*

# General Procedural Notes

**1.** Three protocols are provided in this manual for apoptosis measurement of: paraffin-embedded sections, cell preparations fixed on slides, and tissue cryosections. **See the section appropriate to your sample.**

2. The control slides provided should be labeled using the Cell Preparations Fixed on slides. (CFS- p.13 section A)

3. The control slides contain a mixture of apoptotic and non-apoptotic cells. This allows the visualization of both positive and negative labeling within the same microscope field.

4. Incubation time for proteinase K, DNase I and the end labeling of the DNA may need to be empirically determined for your particular cell type and slide preparation. Use this protocol as a starting guideline.

5. Since the reaction mixture in the end labeling step of this protocol is such a small volume, the use of a Parafilm coverslip is recommended during this step to ensure even distribution of the reaction mixture on the sample and to prevent the failure of the reaction due to evaporation during incubation. To make a cover slip, cut a piece of Parafilm just slightly larger than the specimen. Fold up one corner of the Parafilm to aid in its application and removal. Lay the Parafilm coverslip on top of the sample during the incubation.

6. To construct a humidified chamber, wet several paper towels with water and place them along the bottom of a glass or plastic container with sides. Place two pipets parallel to each other on top of the towels. Keeping slides level, lay them face up across the pipets. To avoid unwanted drainage of reagents from slides make sure that the towels do not contact the slides in any way. Cover the container with a lid or plastic wrap to provide a humidified environment.

7. Cells grown in suspension or attached to T-flasks can be fixed and attached to slides as follows:

   -Pellet cells at 300xg for 5 minutes at 4C. Remove media. Add enough 1-4% formaldehyde (in PBS pH 7.4) to the pelleted cells to create a cell density of $1x10^6$ cells/ml and incubate 15 minutes at room temperature.
   -Centrifuge at 300xg for 5 minutes at room temperature and resuspend at the same density in 70% ethanol. An aliquot of fixed cells (100-300 µl) can then be adhered to glass slides by directly placing the suspension onto the slide or by using a Cytospin[r]. Slides precoated with poly-L-lysine may enhance cell adherence.

# Immunohistochemical Staining Protocols

## Paraffin Embedded Tissue (PET)

## Cell Preparations Fixed on Slides (CFS)

## Tissue Cryosections (TCS)

# Staining of Paraffin Embedded Tissue (PET)

**PET-A** DEPARAFFINIZATION AND REHYDRATION

1. Immerse slides in xylene (or xylene substitute) for 5 minutes at room temperature. Repeat using fresh xylene for second 5 minute incubation.

2. Immerse slides in 100% ethanol for 5 minutes at room temperature. Repeat using fresh 100% ethanol for second 5 minute incubation.

3. Immerse slides in 90% ethanol for 3 minutes at room temperature.

4. Immerse slides in 80% ethanol for 3 minutes at room temperature.

5. Immerse slides in 70% ethanol for 3 minutes at room temperature.

6. Immerse slides briefly into 1X PBS and carefully dry the glass slide around the specimen.

If processing the kit's control slides simultaneously with unknown samples, please refer to the CFS protocol, section A page 13 at this point.

**Do not let the tissue specimen dry out during or between any step!**
**(If necessary, cover or immerse the specimen in 1X PBS to keep hydrated)**

*** At this point it may be helpful to encircle the specimen using a waxed pen or a hydrophobic marker. ***

**PET-B** PERMEABILIZATION OF SPECIMEN

1. Dilute only enough **Proteinase K (pink cap)** needed 1:100 in 10 mM Tris pH 8.

2. Cover the entire specimen with 100 µl proteinase K. Incubate at room temperature for 20 minutes. DO NOT OVER INCUBATE.

3. Rinse slide with 1X PBS.

4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

**(Optional)** GENERATION OF A POSITIVE CONTROL **(Optional)**

1. Cover the entire specimen with 1 µg/µl DNase I in 1X PBS/1 mM $MgSO_4$. Incubate at room temperature for 20 minutes.

2. Rinse with 1X PBS.

3. Gently tap off excess liquid and carefully dry the glass around the specimen.

## Staining of Paraffin Embedded Tissue (PET)(cont.)

### PET-C INACTIVATION OF ENDOGENOUS PEROXIDASES

1. Dilute 30% $H_2O_2$ 1:10 in methanol.

2. Cover the entire specimen with 100 µl of 3% $H_2O_2$. Incubate at room temperature for 5 minutes. DO NOT OVER INCUBATE.

3. Rinse slide with 1X PBS.

4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

### PET-D EQUILIBRATION AND END LABELING REACTION

1. Dilute only enough **5X Reaction Buffer (green cap)** as needed 1:5 with $dH_2O$. (**NOTE:** This buffer does NOT contain nucleotides or enzyme).

2. Cover the entire specimen with 100 µl of the 1X Reaction Buffer. Incubate at room temperature for 10 to 30 minutes while preparing the labeling reaction mixture below.

3. Prepare the **Complete Labeling Reaction Mixture** as follows:

| DNA LABELING SOLUTION | 1 ASSAY | 5 ASSAYS | 10 ASSAYS |
|---|---|---|---|
| 5X Reaction Buffer (green cap) | 10.00 µl | 50.00 µl | 100.00 µl |
| TdT Enzyme (yellow cap) | 0.75 µl | 3.75 µl | 7.50 µl |
| Br--dUTP (violet cap) | 8.00 µl | 40.00 µl | 80.00 µl |
| Distilled $H_2O$ | 32.25 µl | 161.25 µl | 322.50 µl |
| Total Volume | 51.00 µl | 255.00 µl | 510.00 µl |

The appropriate volume of Staining Solution to prepare for a variable number of assays is based upon multiples of the component volumes combined for 1 Assay. **Mix only enough DNA Labeling Solution to complete the number of assays prepared per session.** The DNA Labeling Solution is active for approximately 24 hours.

4. Carefully blot the 1X Reaction Buffer from the specimen, taking care not to touch the specimen.

5. Immediately apply **50 µl** of Complete Labeling Reaction Mixture (prepared above) onto each specimen except for the control slides which require only 25 **µl each**.

## Staining of Paraffin Embedded Tissue (cont.)

*** The use of a cover slip at this point assures even distribution of the reaction mixture and prevents loss due to evaporation during incubation. ***

6. Cover the specimen with a piece of Parafilm cut slightly larger than the specimen. HINT: Folding up one corner of the Parafilm cover slip will aid in its application and removal.

7. Place slides in a humid chamber and incubate at 37C for 1 to 1.5 hours.

**NOTE:** The DNA End Labeling Reaction can also be carried out at 22-24C overnight for the control slides. For samples other than the control slides provided in the kit, incubation times at 37C may need to be adjusted to longer or shorter periods depending on the characteristics of the tissue supplied by the researcher.

8. Remove Parafilm cover slip and rinse slide with 1X PBS.

9. Gently tap off excess liquid and carefully dry the glass around the specimen.

**PET-E** DETECTION

1. Cover the entire specimen with 100 µl of **Blocking Buffer (white cap)**. Incubate at room temperature for 10 minutes.

2. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen.

3. Immediately cover specimen with 100 µl of **Antibody Solution** (prepared as described below).

| ANTIBODY SOLUTION | 1 ASSAY | 5 ASSAYS | 10 ASSAYS |
|---|---|---|---|
| Biotin~PRB-1 (orange cap)<br>Blocking Buffer (white cap) | 5.00 µl<br>95.00 µl | 25.00 µl<br>475.00 µl | 50.00 µl<br>950.00 µl |
| Total Volume | 100.00 µl | 500.00 µl | 1000.00 µl |

4. Incubate with the **Biotin~PRB-1 Antibody Solution** in the dark for 1-1.5 hours at room temperature. Hint: Cover slides with aluminum foil.

5. Rinse slide in PBS.

6. Gently tap off excess liquid and carefully dry the glass around the specimen.

7. Cover the entire specimen with 100 µl of **Blocking Buffer (white cap)**.

## Staining of Paraffin Embedded Tissue (cont.)

8. Dilute only enough of the **200X Conjugate (natural cap)** as needed 1:200 in Blocking Buffer (white cap). Prepared as described below.

| CONJUGATE SOLUTION | 1 ASSAY | 5 ASSAYS | 10 ASSAYS |
|---|---|---|---|
| 200x Conjugate (natural cap) | .5 µl | 2.5 µl | 5 µl |
| Blocking Buffer (white cap) | 100.0 µl | 500.00 µl | 1000.00 µl |

9. Carefully blot the Blocking Buffer from the specimen, taking care not to touch the specimen. Immediately apply 100 µl of diluted conjugate to the specimen.

10. Incubate at room temperature for 30 minutes.

11. **Five minutes** before concluding incubation prepare DAB solution by dissolving one tablet of **DAB (amber vial)** and one tablet of **$H_2O_2$/Urea** (amber vial) in one ml of **TAP $H_2O$**. This yields enough DAB solution for 10 specimens.

*** Tap $H_2O$ may contain metal ions that enhance the DAB reaction ***

***NOTE:*** *DAB is highly carcinogenic and care should be taken when handling.*

12. Rinse slides with 1X PBS.

13. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

14. Cover the entire specimen with 100 µl of DAB solution. Incubate at room temperature for 15 minutes.

15. Rinse slides with $H_2O$ and blot.

**PET-F** COUNTERSTAIN

1. Immediately cover the entire specimen with 100 µl of **Methyl Green Counterstain (natural cap)** solution.
2. Incubate at room temperature for 3 minutes.
3. Press edge of the slide against an absorbent towel to draw off most of the counterstain and place in a coplin jar slide holder.
4. Dip slides 2 times briefly into 100% ethanol.
5. Blot slides briefly on an absorbent towel.
6. Repeat step 4 using fresh 100% ethanol.

7. Blot slides briefly on an absorbent towel.

8. Dip slides into xylene (or xylene substitute).

9. Wipe excess xylene from back of slide and around specimen.

10. Mount a glass cover slip using a mounting media such as permount (r) over the specim

# Staining of Cell Preparations Fixed on Slides (CFS)

The following protocol describes the method for measuring apoptosis in the positive and negative control slides that are provided in the APO-BRDU-IHC™ kit. The same procedure should be employed for measuring apoptosis in the slide specimens provided by the researcher.

**Important points to remember before starting this assay:**

*The cells must be fixed prior to performing this assay***.** (page7 point7)

*This protocol is similar to staining of paraffin-embedded tissue sections on slides EXCEPT that the deparaffinization step is replaced with a short rehydration step* ***and*** *permeabilization with proteinase K is performed for only 5 minutes.*

*Reagent volumes may be decreased to account for the lower surface area that usually accompanies cells fixed on slides.*

*DO NOT LET THE CELLS DRY OUT BETWEEN OR DURING ANY STEP!!! (if necessary cover or immerse the slide in 1X PBS to keep hydrated).*

*To avoid loss of cells from glass slides during washing steps, it is recommended that slides be dipped into a beaker of 1X PBS rather than rinsed with a wash bottle.*

**CFS-A** REHYDRATION

1. Immerse slides in 1X PBS for 10 minutes at room temperature.

2. Carefully dry the glass around the specimen.

*** At this point it may be helpful to encircle the specimen using a waxed pen or a hydrophobic slide marker. ***

**CFS-B** PERMEABILIZATION OF SPECIMEN

1. Dilute **Proteinase K (pink cap)** 1:100 in Tris pH 8.

2. Cover the entire specimen with 50 - 100 µl of the diluted proteinase K. Incubate at room temperature for **5 minutes**. DO NOT OVERINCUBATE.

3. Dip slide 2-3 times into a beaker of 1X PBS.

4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.
5. Follow these steps to complete the staining.

All the remaining steps of staining cells fixed on slides are identical to those steps outlined in the previous section for staining of paraffin-embedded tissue sections:

**INACTIVATION OF ENDOGENOUS PEROXIDASES**-page 10 section PET-C
**EQUILIBRATION AND END LABELING REACTION**-page 10 section PET-D
**DETECTION**-page 11 section PET-E
**COUNTERSTAIN**-page 12 section PET-F

# STAINING OF TISSUE CRYOSECTIONS (TCS)

**Important points to remember before starting this assay:**

*Fixation of cryopreserved tissue is **required** prior to performing this assay.(page7 pt7)*

*This protocol is similar to staining of paraffin-embedded tissue sections on slides EXCEPT that the deparaffinization step is replaced with a short rehydration step **and** permeabilization with proteinase K is performed for only 10 minutes.*

*DO NOT LET THE TISSUE DRY OUT BETWEEN OR DURING ANY STEP!!! (if necessary cover or immerse the slide in 1X PBS to keep hydrated).*

*To avoid loss of tissue from glass slides during washing steps, it is recommended that slides be dipped 2-3 times into a beaker of 1X PBS rather than rinsed with a wash bottle.*

## Staining of Tissue Cryosections on Slides (cont.)

**TCS-A** TISSUE FIXATION AND HYDRATION

1. Immerse slides in 4% formaldehyde (in PBS, pH 7.4) for 15 minutes at room temperature.

2. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

3. Immerse slides in 1X PBS for 15 minutes at room temperature.

4. Carefully dry the glass slide around the specimen.

*At this point it may be helpful to encircle the specimen using a waxed pen or hydrophobic slide marker.*

**TCS-B** PERMEABILIZATION OF SPECIMEN

1. Dilute **proteinase K (pink cap)** 1:100 in 10 mM Tris, pH 8.

2. Cover the entire specimen with 50 - 100 µl of the diluted proteinase K solution. Incubate at room temperature for **10 minutes**. DO NOT OVER INCUBATE.

3. Dip slide 2 - 3 times into a beaker of 1X PBS.

4. Gently tap off excess liquid and carefully dry the glass slide around the specimen.

All the remaining steps of staining tissue cryosections on slides are identical to those steps outlined for staining of paraffin-embedded tissue sections:

**INACTIVATION OF ENDOGENOUS PEROXIDASES**-page 10 section PET-C
**EQUILIBRATION AND END LABELING REACTION**-page 10 section PET-D
**DETECTION**-page 11 section PET-E
**COUNTERSTAIN**-page 12 section PET-F

5. Follow these steps to complete the staining.

# Technical Tips and Frequently Asked Questions About the APO-BRDU-IHC™ Assay

1. High background on slides....all the cells are brown,
   There are many possible explanations for this:
   -Did the sample dry out at any time during the staining?
   -Did the $H_2O_2$/methanol solution evaporate off the sample in the inactivation of endogenous peroxidases step (PET-C)?
   -Is this a false positive due to improper fixation or overhandling of the tissue?
   -Was there a period of time between removal of the tissue and fixation when apoptosis (or necrosis) could have occurred?
   -Is their endogenous peroxidase activity?
   -Or non-specific binding of strep-avidin HRP? Try staining a control with no TDT.
   -Some people suggest increasing the $H_2O_2$ concentration to 5%, but over incubation with $H_2O_2$ or proteinase K can also damage DNA and create a false brown background.

2. Cell fixation using a DNA crossing linking chemical fixative is an important step in analyzing apoptosis. Unfixed cells may lose smaller fragments of DNA that are not chemically fixed in place inside the cell during washing steps. The researcher may have to explore alternative fixation and permeabilization methods to fully exploit their systems.

3. Wash, don't squirt your slides.

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