



# Product Manual

## **PATHO-GENE<sup>®</sup> AP-NBT/BCIP HPV *in situ* screening assay**

Catalog #: ENZ-32879



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**FOR RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**



Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

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## PROPOSED USE

Enzo Life Sciences' **PATHO-GENE® AP-NBT/BCIP HPV *in situ* screening assay**, Cat. No. ENZ-32879, provides reagents and materials for (a) preparation and pretreatment and (b) hybridization/detection and typing of HPV DNA (using alkaline phosphatase-NBT/BCIP for signal generation) in 20 tissue biopsy specimens. This product is for research use only and is not to be used for diagnostic procedures.

## BACKGROUND<sup>2-7,9-15</sup>

Anogenital papilloma is a common sexually transmitted disease that has been strongly associated with the presence of human papillomavirus (HPV). Particular types of HPV have been found in relatively benign diseases such as genital warts (condylomata accuminata) while other specific types are frequently associated with anogenital malignancies such as cervical carcinoma. More than 60 HPV types have been identified by the use of Southern blot DNA hybridization using radioactive probes. Cytological methods such as Papanicolaou staining or histological criteria alone cannot be used to identify the different viral types, nor can they be used to identify unequivocally specimens that contain HPV.

The **PATHO-GENE® AP-NBT/BCIP HPV *in situ* screening assay** employs a mixture of HPV-specific probes that have been specially formulated to detect most HPV types that have been associated with anogenital papillomas. Among the HPV types detected with this assay system are those commonly associated with benign lesions presenting as condylomas and mild dysplasia, as well as types commonly associated with cervical intraepithelial neoplasia (CIN) and carcinoma *in situ* (CIS). The **PATHO-GENE® AP-NBT/BCIP HPV *in situ* screening assay** can be used to detect HPV DNA in tissue sections under conditions that preserve cellular morphology.

## ASSAY PRINCIPLE<sup>1,3,7,8</sup>

DNA hybridization assays make use of the fundamental chemical and physical properties of DNA molecules to detect and identify specific sequences of DNA and thus, specific organisms. The DNA of most organisms and viruses, HPV included, is double-stranded, i.e., composed of two complementary strands. When double-stranded DNA is heated, the complementary strands separate (denature) to form single strands. Under appropriate conditions, separated, complementary strands of DNA can join together to reform a double-stranded DNA molecule. This process is called hybridization or renaturation. The hybridization of complementary DNA strands is an extremely faithful process. If DNA strands are not complementary, they will not hybridize to each other. Furthermore, the presence of unrelated DNA will not affect the hybridization of DNA strands that are complementary.

Hybridization with a DNA probe may be used to detect and identify specific sequences of DNA. A DNA probe is a segment of DNA that is specific for and complementary to the DNA of the organism or virus that is to be detected and identified. Procedures for hybridization of a DNA probe to a DNA target include three steps: 1) denaturation of the DNA probe and the DNA of the sample; 2) hybridization of the probe with the specimen DNA and 3) measurement of the amount of hybridization between the DNA probe and the DNA in the specimen. The amount of DNA probe hybridized is directly related to the amount of complementary DNA in the specimen. If there is no DNA complementary to the DNA probe, no probe DNA will hybridize.

The **PATHO-GENE® AP-NBT/BCIP HPV *in situ* screening assay** is designed for use in determining the presence of HPV DNA in infected tissue biopsy sections. Formalin-fixed, paraffin-embedded tissue sections are fixed to pretreated slides, deparaffinized, treated with Proteinase K (to make the specimen DNA accessible to the biotinylated DNA probes) and then dehydrated. Following these pretreatment procedures, the biopsy sections are stained for the *in situ* detection and identification of HPV DNA.

The HPV DNA Probe Mix (HPV DNA sequences covalently labeled with biotin) detects but does not distinguish among HPV types that have been shown to be associated with sexually transmitted papillomavirus. The probe mixture is denatured and hybridized to the tissue section fixed and pretreated on the microscope slide. Specific hybridization between the HPV DNA probe and DNA in the specimen is determined by the detection of biotin. Detection of biotin is accomplished in two steps. First, a streptavidin-alkaline phosphatase complex is bound to the biotin of

the hybridized HPV DNA probe. In the second step the entire complex is visualized after conversion of the substrate and chromogen (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) into a localized blue/purple precipitate. Following counterstaining with eosin, the stained cells are observed by light microscopy. The appearance of a blue/purple precipitate within the nuclei of epithelial cells is indicative of the presence of HPV DNA.

## SAFETY WARNINGS & PRECAUTIONS

**FOR RESEARCH USE ONLY. NOT TO BE USED FOR *IN VITRO* DIAGNOSTIC PURPOSES.**



Handle  
with care



Avoid  
freeze /  
thaw cycles

- **Use a protein-free water bath for floating sections** when using the provided pretreated specimen slides.
- Use a safety pipetting device for all pipetting. Never pipet by mouth.
- The NBT Reagent and the BCIP Reagent are prepared using dimethylformamide. These solutions can cause irritation upon exposure to the skin. Therefore, avoid contact with skin. Should the solution come in contact with the skin, wash the area with soap and water.
- The HPV DNA Probe Mix and the Hybridization Wash Reagent contain formamide. Formamide is combustible. Keep away from open flame. Formamide is classified as a teratogen. Pregnant workers should keep exposure to a minimum. Avoid inhalation, ingestion and contact with unprotected skin. Should the solution come in contact with the skin, wash the area thoroughly with soap and water. Dispose of solutions containing formamide in accordance with local regulations.
- The Counterstain Concentrate is prepared in alcohol, which is highly flammable. Keep away from open flame, spark, etc. The counterstain (eosin) readily stains skin. Avoid contact with unprotected skin. Should the solution come in contact with the skin, wash the area thoroughly with soap and water.
- The AP Detection Reagent contains sodium azide as preservative. Avoid direct contact with eyes and skin. Azide can form explosive compounds when in contact with copper pipe. Therefore, upon disposal of this solution, flush sink and drain with a large volume of water. Dispose of solutions containing sodium azide in accordance with local regulations.



Reagents  
require  
separate  
storage  
conditions.

## REAGENTS SUPPLIED

### **HPV DNA Probe Mix**, 1ml (small, *white*)

Mixture of biotin-labeled HPV-specific DNA probes in buffered formamide and hybridization enhancers

### **Hybridization Wash Reagent**, 15ml (large, *white*)

Buffered formamide

### **AP Detection Reagent**, 15ml (*orange*)

Streptavidin-biotinylated alkaline phosphatase in buffered saline with stabilizers and preservative

### **NBT Reagent**, 0.4ml (*amber*)

Nitroblue tetrazolium in solvent

### **BCIP Reagent**, 0.4ml (*amber*)

Bromochloroindolyl phosphate in solvent

### **AP Reaction Buffer**, 4 x 5ml (*gray*)

Buffered saline and alkaline phosphatase enhancers

### **Counterstain Concentrate**, 30ml

Eosin Y (certified) in alcohol

### **Proteinase K**, 5 mg

Lyophilized powder

### **SIGNASURE<sup>®</sup> Wash Buffer Salts**, 4 packets

Each packet yields 1 liter of buffer

### **Pretreated Specimen Slides**, 20 slides

20 single-well slides for mounting formalin-fixed paraffin-embedded tissue specimens

## OTHER MATERIALS NEEDED

- Formalin-fixed biopsy specimens embedded in quality paraffin
- Equipment for making sections
- Micropipets
- One clean 500ml plastic squeeze (wash) bottle equipped with a spout to deliver an even stream of Wash Buffer
- Coplin jars or equivalent
- Timer
- One heating block adjusted to  $95^{\circ}\text{C} \pm 3^{\circ}\text{C}$
- One heating block (slide warmer), set at  $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$
- Surface thermometer
- Heating oven set at  $\geq 60^{\circ}\text{C}$
- Coverslips
- Absorbent wipes
- Light microscope equipped for 10X to 400X magnification
- Xylenes or equivalent
- 50%, 70%, 90% and 100% Alcohol (Reagent Grade)
- Permanent mounting medium
- Deionized or distilled water

## STORAGE AND SHELF-LIFE

1. Upon receipt, store all kit reagents at  $2-8^{\circ}\text{C}$ .
2. After reconstitution of the **SIGNASURE**® Wash Buffer, store at  $28^{\circ}\text{C}$  when not in use.
3. Store the reconstituted 10X Proteinase K Stock Solution at or below  $-20^{\circ}\text{C}$ . This solution is stable for at least one year when kept frozen. Once defrosted, **do not refreeze**.
4. Store dilute Counterstain (1X) at ambient temperatures ( $20-30^{\circ}\text{C}$ ) in a well-sealed container.
5. The kit is stable until the expiration date indicated on the box when used and stored as directed.

### Indications of Instability or Deterioration of Reagents:

Chemical or microbial contamination of kit components and reconstituted **SIGNASURE**® Wash Buffer could produce erroneous results. Care should be exercised when dispensing these reagents. If any sign of contamination (discoloration or cloudiness) appears, the reagent should be discarded. Use control slides to detect major problems.

## PERFORMANCE CONSIDERATIONS

1. Do not use reagents past their expiration date.
2. Do not allow the slides to dry completely during the hybridization and detection procedures, or erroneous results may occur. Avoid drying by ensuring that the entire specimen is covered with sufficient amounts of buffers and reagents as recommended in the procedures. While incubating, the slides may be covered to help prevent drying.
3. Do not substitute reagents from kits from other manufacturers or from other ENZO LIFE SCIENCES, INC. kits.
4. The components of this kit are provided at precise concentrations. Other than where specified, dilution or other adulteration of these reagents could cause erroneous results.
5. Use care when preparing slides from more than one specimen. Keep specimen within the perimeter of the slide wells.
6. Allow all kit components to warm to room temperature (20-30°C) before beginning the test procedure.
7. Proper temperature of both the heating block ( $95^{\circ} \pm 3^{\circ}\text{C}$ ) and slide warmer ( $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) is important. A temperature of at least  $92^{\circ}\text{C}$  is necessary for denaturation of the biotinylated probe and the specimen DNA within a reasonable period of time. Perform all incubations at the temperatures designated. Room temperature in this procedure is defined as 20-30°C.
8. Incubation times and temperatures other than those specified may give erroneous results.
9. Improper specimen preparation may cause false results.
10. Failure to use pretreated slides may result in loss of sections from the slides during the pretreatment and hybridization procedures.

## REAGENT PREPARATION

**SIGNASURE® Wash Buffer:** As needed, dissolve a packet of **SIGNASURE® Wash Buffer** in one liter of deionized or distilled water. The pH of the solution should be  $8.0 \pm 0.2$ . When not in use, reconstituted **SIGNASURE® Wash Buffer** should be stored refrigerated. Warm to room temperature before use. Dispense into Coplin jars and squeeze (wash) bottle as needed.

**10X Proteinase K Stock Solution:** Just before use, add 2.0ml of **SIGNASURE® Wash Buffer** to the vial containing the Proteinase K and mix gently. Pipet 0.5ml of 10X Proteinase K Stock Solution into separate tubes and freeze all tubes that are not required for immediate use at  $\pm 20^{\circ}\text{C}$ . These separate frozen tubes of 10X Proteinase K Stock Solution are to be used for later batches of slides. Once frozen, the enzyme is stable for at least one year. Once defrosted, **do not refreeze**.

**1X Proteinase K Working Solution:** Immediately before use thaw one vial of 10X Proteinase K Stock Solution and dilute 10-fold with **SIGNASURE® Wash Buffer** to make 1X Proteinase K Working Solution. At room temperature 1X Proteinase K Working Solution is stable for **1 hour**; therefore, use immediately after dilution and discard any remaining diluted material.

**NBT/BCIP Reaction Mixture:** Prepare **NBT/BCIP Reaction Mixture** fresh for use for each test run as directed in the **Hybridization/Detection Procedure (STEP 15)**. Each kit contains enough reagent for preparing this mixture twice. Keep **NBT/BCIP Reaction Mixture** well protected from direct bright light. Discard unused **NBT/BCIP Reaction Mixture** according to local regulations. **Do not re-use previously prepared NBT/BCIP Reaction Mixture.**

**NOTE:** *If desired, an alternative NBT/BCIP Reaction Mixture that produces strong signal without causing excessive blue coloration of the specimen can be prepared: first add 10 $\mu\text{l}$  of NBT for each milliliter of AP Reaction Buffer, mix and then add 10 $\mu\text{l}$  of BCIP and mix.*

**Counterstain (1X):** Dilute **Counterstain Concentrate** to a final volume of 200ml with anhydrous alcohol.

**NOTE:** Mix **Counterstain Concentrate** vigorously prior to dilution as a precipitate may have formed on standing. Upon dilution, **Counterstain (1X)** may be stored at ambient temperatures in a **well-sealed container**. **Keep away from open flame, spark, etc.**

## **SPECIMEN COLLECTION AND STORAGE**

**NOTE:** The **ENZO PATHO-GENE<sup>®</sup> AP-NBT/BCIP HPV in situ screening assay** has been optimized for use on formalin-fixed, paraffin-embedded tissue sections. Use on other tissues or with other fixatives has **NOT** been examined nor optimized.

Specimens should be fixed in 10% buffered formalin and **processed within 4-18 hours** for best results. **Do not leave in formalin for longer than 48 hours.** Long term storage in formalin may result in a reduction of the positive signal intensity. If necessary, formalin-fixed tissues may be stored in 70% alcohol at 2-8°C prior to embedding. Embedding procedures using automated tissue processors can be used to prepare samples for this procedure.

## **SPECIMEN SLIDE PREPARATION AND PRETREATMENT**

The **PATHO-GENE<sup>®</sup> AP-NBT/BCIP HPV in situ screening assay** can be performed on formalin-fixed, paraffin-embedded biopsy sections. Tissue sections must contain epithelial cells; sections that are all stromal tissue will not provide useful information. For preparation of biopsy specimen slides, begin at STEP 1 of the following procedure.

As procedure and reagent checks, fixed tissue sections or cells known to be positive with HPV may be run with the biopsy specimen slides. These known positive samples may be used as controls to monitor the performance of the hybridization and detection steps.

## **PREPARATION AND PRETREATMENT OF BIOPSY SLIDES**

**STEP 1:** Apply a section (4-6 microns thick) of each biopsy specimen to the well of a specimen slide. These slides are pretreated to enhance retention of the tissue sections during the Pretreatment and Hybridization/Detection Procedures. Bake tissue-mounted slides vertically for 2-18 hours at 60-80°C to fix the slides. Fixed slides may be stored at room temperature.

**STEP 2:** The biopsy specimen slides must be deparaffinized prior to continuing the test procedure. To deparaffinize, bake the biopsy specimen slides at 60-80°C for 5-15 minutes to melt the paraffin. Soak the slides sequentially in a series of Coplin jars containing the following solutions, for the times indicated:

Soak Number	Reagent	Duration of Soak
1	Xylenes	10 minutes
2	Xylenes	2 minutes
3	100% Alcohol	1 minute
4	100% Alcohol	1 minute
5	90% Alcohol	1 minute
6	70% Alcohol	1 minute
7	50% Alcohol	1 minute
8	Deionized water	1 minute

Use fresh reagents in each jar for each batch of slides and discard in appropriate waste containers when finished.

**Caution:** *Xylenes saturate rapidly with paraffin. Use fresh xylenes for each batch of 5-8 slides.*

**STEP 3:** After the final soak (number 8, deionized water), allow slides to dry completely by incubation for 5-10 minutes at 37°C.

**STEP 4:** To each biopsy specimen and tissue control slide well, add **0.35ml to 0.5ml of freshly prepared 1X Proteinase K Working Solution** (see **REAGENT PREPARATION**). Incubate the slides at **37°C for 15 minutes**. Tap off Proteinase K and soak slides at room temperature for 1 minute in a Coplin jar filled with **SIGNASURE® Wash Buffer**. Gently agitate the Coplin jar while soaking the slides. Tap off wash buffer.

**STEP 5:** Dehydrate all slides by incubating at room temperature in the following series of Coplin jars for the times indicated:

Soak Number	Reagent	Duration of Soak
1	Deionized water	1 minute
2	50% Alcohol	1 minute
3	70% Alcohol	1 minute
4	100% Alcohol	1 minute

**STEP 6:** Dry the slides at 37°C for 5 to 10 minutes. The slides can be stored desiccated at 4°C for up to one week, if necessary.

## HYBRIDIZATION/DETECTION PROCEDURE

**STEP 7:** Remove the slides from the 37°C slide warmer. Apply **1 drop (~40µl) of HPV DNA Probe Mix** (*small, white*) to each specimen well and control slide well. **Cover each well with a coverslip being careful not to trap bubbles under the coverslips.**

**STEP 8:** Place slides on a **95°C ± 3°C heating block and incubate for 8-10 minutes.**

**STEP 9:** Move slides to the **37°C heating block (slide warmer) and incubate for 30-60 minutes.** If stronger signal is desired, hybridize for 2 hours or longer.

**STEP 10:** Removing one slide at a time from the 37°C block in the order in which it was placed on the block, remove the coverslip by tilting the slide and allowing the coverslip to slide off. If a coverslip does not slide off easily, dip the slide in **SIGNASURE® Wash Buffer** until the coverslip comes off. Discard coverslips and place slide in a Coplin jar containing **SIGNASURE® Wash Buffer**. Allow slides to stand in wash buffer at room temperature for **2 to 5 minutes**. Gently agitate the Coplin jar.

**STEP 11:** Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the well, being careful not to disturb the specimen. Place slide on the **37°C heating block and add enough (approximately 6-8 drops, or 0.5ml) Hybridization Wash Reagent** (*large, white*) to each well. **Incubate for 10 minutes.**

- STEP 12:** Removing slides from the 37°C heating block in the order and timing in which they were placed on the block, tap off **Hybridization Wash Reagent** and soak slides at room temperature for **1 minute in each of three** Coplin jars filled with fresh **SIGNASURE® Wash Buffer**. **Gently** agitate the Coplin jars during each wash.
- STEP 13:** Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the well, being careful not to disturb the specimen. Place the slide on the **37°C heating block and add enough (approximately 6-8 drops, or 0.5ml) AP Detection Reagent (orange)** to each well. **Incubate for 30 minutes.**
- STEP 14:** Removing one slide at a time from the 37°C heating block in the order and timing in which it was placed on the block, rinse the well with a **gentle** stream of **SIGNASURE® Wash Buffer** from the plastic squeeze (wash) bottle for **10 seconds**. Place the slide in a Coplin jar filled with fresh room temperature **SIGNASURE® Wash Buffer**. When the last slide has been placed in the Coplin jar, **continue incubating for 1 minute**. Then soak the slides at room temperature for 1 minute in each of two Coplin jars filled with fresh **SIGNASURE® Wash Buffer**. **Gently** agitate the Coplin jars during each wash.
- STEP 15:** During the last (third) soak, **STEP 14**, prepare the **NBT/BCIP Reaction Mixture**. Remove the cap and dropper tip from the vial (*gray*) containing **5ml AP Reaction Buffer** and add **12.5µl of NBT Reagent (amber vial)**. Mix gently. Then add **50µl of BCIP Reagent (amber vial)**. Replace the dropper tip and cap and mix thoroughly. One 5ml vial of this mixture is sufficient for development of up to 10 specimen wells. If more than 10 specimen wells are being tested at one time, prepare additional **NBT/BCIP Reaction Mixture**. **Keep NBT/BCIP Reaction Mixture well-protected from direct bright light. Discard unused NBT/BCIP Reaction Mixture.**

**NOTE:** In order to prepare smaller volumes of NBT/BCIP Reaction Mixture, pipet the desired amount of AP Reaction Buffer (gray) into a glass or polypropylene test tube (**DO NOT USE POLYSTYRENE**). For each milliliter of AP Reaction Buffer, add 2.5µl NBT Reagent, and mix gently. Then add 10µl of BCIP Reagent and mix thoroughly.

- STEP 16:** Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the well, being careful **not to disturb or dry the specimen**. Place the slide on the **37°C heating block and add enough (approximately 6-8 drops, or 0.5ml)** freshly prepared **NBT/BCIP Reaction Mixture** to each well. **Incubate for 20 minutes, well-protected from direct bright light.**
- STEP 17:** Removing slides one at a time from the 37°C heating block in the order and timing in which they were placed on the block, tap off **NBT/BCIP Reaction Mixture** and place slide in a Coplin jar filled with fresh room temperature **SIGNASURE® Wash Buffer**.
- STEP 18:** After the last slide has been placed in the Coplin jar of wash buffer, transfer all slides to a Coplin jar filled with **water**. Slides may be allowed to stand in water for up to two hours before counterstaining and permanent mounting.
- STEP 19:** For counterstaining, soak the slides at room temperature sequentially in a series of Coplin jars containing the following solutions for the times indicated:

Soak Number	Reagent	Duration of Soak
1	Water	10 minutes
2	<b>*Counterstain (1X)</b>	2 minutes
3	95% Alcohol	1 minute
4	100% Alcohol	1 minute
5	100% Alcohol	1 minute
6	Xylenes	1 minute

**\*Prepare Counterstain (1X) as described in Preparation of Reagents**

**NOTE:** Use all fresh solutions for each batch of 5-8 slides.

**STEP 20:** Place slides on a clean, flat surface, add 1-4 drops of a permanent histological mounting medium (e.g., Permount<sup>®</sup>, or equivalent), and cover with a coverslip. Allow mounting medium to harden. Observe results by light microscopy.

## **CONTROLS**

To assure that the staining procedures are performed correctly, a control slide should be run with the first set of specimen slides. It serves as a hybridization/detection control and as an aid in interpretation of the specimen slides. Each laboratory can prepare tissue control slides from known HPV positive tissues as directed in the Biopsy Specimen Slide Preparation and Pretreatment procedure. If the control slides do not appear as expected, the test run is invalid.

## **INTERPRETATION OF RESULTS**

Examine the entire area of the specimen wells to assure observation of every cell on the wells. For a specimen to be identified as positive for HPV, at least one positive blue-purple nucleus must be present. Some artifactual deposit of stain may occur due to trapping of reagents under folds of tissue, giving a generalized blue stain appearance. Ensure proper interpretation of specimen slides by scoring only distinctive nuclear blue-purple staining as positive. Scan the slides using 40-100X magnification, and use 400X magnification to confirm cellular structure and signal localization.

If no positive cells are observed and if the specimen meets the criterion of adequacy, the specimen is negative for HPV DNA.

Specimens containing only stromal tissue and no positively-stained cells must be considered inadequate for determination of the presence of HPV.

## **LIMITATIONS**

- This procedure is for research use only! It is not intended for diagnostic or therapeutic use.
- Negative results do not rule out the possibility of HPV infection.
- It has been reported that there is a marked variability in the number of cells containing HPV DNA in different specimens.

## SPECIFICITY STUDIES

The specificity of the **PATHO-GENE® AP-NBT/BCIP HPV *in situ* screening assay** was tested and confirmed by examining a variety of viruses, microorganisms and cell lines for possible cross reactions. All the organisms and cell lines tested gave negative results.

## TROUBLESHOOTING GUIDE

- *Highly positive control slides display very weak signal:* Check the temperature of the heating block to ensure the surface temperature is at least 92-95°C. A smooth-surfaced block transmits heat more efficiently than a rough-surfaced block. If your block has a rough surface, increase the temperature to 94-96°C. If the 37°C block temperature is too low, the positive signals will be weaker than expected. Check the temperature of both heating blocks before starting the procedure. Protect heating blocks from drafts.
- *Tissue sections are lost from the slide during the hybridization/detection procedure:* Make sure you are using Pretreated Slides to mount biopsy sections. These slides are pretreated to enhance retention of the sections during the hybridization procedures. Use a protein-free water bath for floating sections. Avoid over-vigorous washing with wash buffer from the squeeze bottle; aim stream of buffer to the side of the well and allow the buffer to flow gently over the well.
- *Coverslips are difficult to remove:* Ensure easy removal of coverslips by using sufficient amounts of the HPV DNA Probe Mix. In addition, care should be taken whenever coverslips are removed. If a coverslip does not slide off readily, soak it off in SIGNASURE® Wash Buffer.
- *Blue stain where tissue folds or crumples:* Take special care when mounting tissue sections on the ENZO Pretreated Slides. Make sure tissue is flat and not crumpled. The stain is due to trapped reagents and improper washing. Employ a thorough washing technique which includes occasional gentle agitation of the Coplin jars during wash steps. Coplin jars of SIGNASURE® Wash Buffer must be fresh for each wash step. Avoid erroneous Interpretation of results by scoring only distinctive nuclear staining as positive.
- *Pale blue to light purple-brown background color throughout tissue section:* This may result from either improper washing or excessive thickness of the tissue section. The optimum tissue thickness is 4-6 microns. Incomplete deparaffinizing may also cause elevated background. Use fresh xylenes for

each batch of 5-8 slides and deparaffinize only a few slides at one time. Specimens that contain large amounts of burned tissue, hemosiderin or dendritic melanocytes can display patches of brown or black color. In addition, generalized blue background may result from the use of NBT/BCIP Reaction Mixture prepared with 2.5µl of NBT plus 10µl of BCIP per ml of AP Reaction Buffer. Try NBT/BCIP Reaction Mixture containing 10µl of NBT plus 10µl of BCIP per ml of AP Reaction Buffer.

- *Counterstain is very weak:* If counterstain is weaker than desired, increase incubation time in Counterstain (1X) to 4 to 6 minutes (see STEP 19).

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# Product Manual

## **GLOBAL HEADQUARTERS**

Enzo Life Sciences Inc.  
10 Executive Boulevard  
Farmingdale, NY 11735  
Toll-Free: 1.800.942.0430  
Phone: 631.694.7070  
Fax: 631.694.7501  
info-usa@enzolifesciences.com

## **EUROPE/ASIA**

Enzo Life Sciences (ELS) AG  
Industriestrasse 17  
CH-4415 Lausen  
Switzerland  
Phone: +41/0 61 926 89 89  
Fax: +41/0 61 926 89 79  
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:  
[www.enzolifesciences.com](http://www.enzolifesciences.com)