PathoGene®

HRP-AEC
Human Papillomavirus (HPV)
In Situ Typing Assay for Tissue Sections

Types 6/11, 16/18 and 31/33/51

Cat. No. ENZ-32877

For Research Use Only

PROPOSED USE

Enzo Life Sciences' ***PathoGene***® **HRP-AEC Human Papillomavirus (HPV)** ***In Situ*** **Typing Assay for Tissue Sections**, Cat. No. ENZ-32877, provides reagents and materials for (a) preparation and pretreatment and (b) hybridization/ detection and typing of HPV DNA (using Horseradish Peroxidase-AEC 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 [2-7,9-15]

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 ***PathoGene***® **HRP-AEC Human Papillomavirus (HPV)** ***In Situ*** **Typing Assay for Tissue Sections** employs separate mixtures of HPV-specific probes to identify HPV types 6/11, 16/18, or 31/33/51. HPV types 6 and 11 are primarily associated with benign lesions presenting as condylomas and mild dysplasia. HPV types 16 and 18 are more commonly associated with cervical intraepithelial neoplasia (CIN) and carcinoma *in situ* (CIS). HPV types 31, 33 or 51 can be associated with either condyloma or CIN and CIS, presumably dependent upon other, as yet undefined, contributing factors or agents. The kit can be used to detect and identify HPV DNA in tissue sections under conditions that preserve cellular morphology.

ASSAY PRINCIPLE [1,3,7,8]

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 re-form 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 ***PathoGene***® **HRP-AEC Human Papillomavirus (HPV)** ***In Situ*** **Typing Assay for Tissue Sections** is designed for use in determining the presence of HPV DNA and for determining the type of HPV DNA in infected tissue biopsy sections. Formalin-fixed, paraffin-embedded tissue sections are fixed to pre-treated slides, deparaffinized, treated with both Proteinase K (to make the specimen DNA accessible to the biotinylated DNA probes) and Quench Reagent (to eliminate endogenous peroxidase activity) and then dehydrated. Following these pretreatment procedures, the biopsy sections are stained for the *in situ* detection and identification of HPV DNA.

Three different HPV probe reagents (DNA sequences covalently labeled with biotin) are provided: one reagent that detects both HPV 6 and 11, a second that detects both HPV 16 and 18 and a third that detects HPV 31, 33 and 51. The three typing probe reagents are hybridized to three separately fixed and pretreated sections of a tissue specimen. Specific hybridization between the HPV DNA probes and DNA in the specimen is determined by the detection of biotin. Detection of biotin is accomplished in two steps. First, a streptavidin-horseradish peroxidase complex is bound to the biotin of the hybridized HPV DNA probes. In the second step the entire complex is visualized after conversion of the substrate and chromogen (hydrogen peroxide and 3-amino-9-ethylcarbazole) into a localized brick red precipitate. Following counterstaining with hematoxylin, the stained cells are observed by light microscopy. Infection with a particular type of HPV is indicated by the appearance of a brick red precipitate in the nuclei of epithelial cells of one of the three hybridized sections. Infection by more than one type of HPV can be indicated by a positive reaction with more than one of the typing probe mixtures. In such a case, separate loci of cells within the tissue sections may display positive nuclei with individual typing probe mixtures. Infection with an HPV type that is not specifically included in one of the typing mixtures may be indicated by reaction with more than one typing probe mixture in a single locus of cells within the tissue sections.

REAGENTS

HPV 6/11 Probe Reagent, 1 ml *(pink)*
Mixture of biotin-labeled HPV 6 and HPV 11-specific probes in buffered formamide and hybridization enhancers

HPV 16/18 Probe Reagent, 1 ml *(purple)*
Mixture of biotin-labeled HPV 16 and HPV 18-specific probes in buffered formamide and hybridization enhancers

HPV 31/33/51 Probe Reagent, 1 ml *(green)*
Mixture of biotin-labeled HPV 31, HPV 33 and HPV 51-specific probes in buffered formamide and hybridization enhancers

Post Hybridization Reagent, 30 ml *(white)*
Buffered formamide

Detection Reagent, 30 ml *(yellow)*
Streptavidin-biotinylated horseradish peroxidase in buffered saline and stabilizers and preservative

AEC Reagent, 1 ml *(red)*
Aminoethylcarbazole in solvent

Reaction Buffer/Substrate Reagent, 5 x 6 ml *(gray)*
Hydrogen peroxide in acetate buffer with stabilizer and preservative

Blue Counterstain, 30 ml *(blue)*
Ready-to-use aqueous-based hematoxylin

Proteinase K, 2 x 5 mg
Lyophilized powder

Quench Reagent, 30 ml *(orange)*
3% Hydrogen Peroxide in buffered saline

Wash Buffer Salts, 5 packets
Each packet yields 1 liter of buffered saline

Pretreated Specimen Slides, 40 slides
20 single-well plus 20 double-well slides for mounting formalin-fixed paraffin-embedded tissue specimens

MATERIALS REQUIRED BUT NOT PROVIDED

- Formalin-fixed biopsy specimens embedded in quality paraffin
- Equipment for making sections
- Micropipets
- One clean 500 ml 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°C ± 3°C
- One heating block (slide warmer), set at 37°C ± 1°C
- Surface thermometer
- Heating oven set at ≥ 60°C
- Coverslips
- Absorbent wipes
- Light microscope equipped for 10X to 400X magnification
- Xylenes or equivalent
- 50%, 70%, 90% and 100% Alcohol (Reagent Grade)
- Aqueous mounting medium
- Deionized or distilled water

STORAGE AND SHELF-LIFE

1. Upon receipt, remove the **AEC Reagent** and the **Blue Counterstain** from the kit. Store the **AEC Reagent** at -20°C. Store the **Blue Counterstain** at room temperature away from direct bright light. Store all other reagents at 2-8°C.
2. Store reconstituted Wash Buffer (1X) at 2-8°C when not in use.
3. Store the reconstituted 10X Proteinase K Stock Solution at or below -20°C. This solution is stable for at least one year when kept frozen. Once defrosted, **do not refreeze.**
4. 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 Wash Buffer (1X) 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.

WARNINGS

- **For RESEARCH use only. Not to be used for *in vitro* diagnostic purposes**.
- **Use a protein-free water bath for floating sections** when using ENZO pretreated specimen slides.
- Use a safety pipetting device for all pipetting. Never pipet by mouth.
- The Quench Reagent contains 3% hydrogen peroxide, a strong oxidant. Avoid direct contact with eyes and skin.
- The HPV Probe Reagents and the Post Hybridization 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.
- AEC has been classified as a possible carcinogen. This solution 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. Solutions containing AEC should be disposed of according to local regulations.

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 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. Cross contamination of samples could cause false results. Use care when preparing slides from more than one specimen. Keep specimens 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° ± 3°C) and slide warmer (37°C ± 1°C) is important. A temperature of at least 92°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 specimen slides may result in loss of sections from the slides during the pretreatment and hybridization procedures.

PREPARATION OF REAGENTS

1X Wash Buffer: As needed, dissolve a packet of ENZO **Wash Buffer Salts** in one liter of deionized or distilled water. The pH of the solution should be 7.1 ± 0.1. When not in use, the Wash Buffer should be stored refrigerated. Warm to room temperature before use. Dispense into Coplin jars as needed.

10X Proteinase K Stock Solution: Just before use, add 2.0 ml of 1X Wash Buffer to the vial containing the Proteinase K and mix gently. Pipet 0.5 ml of 10X Proteinase K Stock Solution into separate tubes and freeze all tubes that are not required for immediate use at ≤-20°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 1X 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.

Color Reaction Mixture: Prepare the **Color Reaction Mixture** fresh for use for each test run as directed in the Hybridization/Detection Procedures (STEP 16). Each kit contains enough reagent to prepare this mixture five separate times. Keep the **Color Reaction Mixture** well protected from direct bright light. When refrigerated and protected from light, this solution is stable for up to one week.

SPECIMEN COLLECTION AND STORAGE

NOTE: The PathoGene® HRP-AEC Human Papillomavirus (HPV) In Situ Typing Assay for Tissue Sections 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.

BIOPSY SPECIMEN SLIDE PREPARATION AND PRETREATMENT

The ***PathoGene®* HRP-AEC Human Papillomavirus (HPV) *In Situ* Typing Assay for Tissue Sections** 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.

NOTE: For each biopsy, prepare one double-well and one single-well slide.

STEP 1: Apply a section (4-6 microns thick) of each biopsy specimen to each well of two pretreated specimen slides (one single-well and one double-well 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, add **0.35 ml to 0.5 ml of freshly prepared 1X Proteinase K Working Solution** (see **PREPARATION OF REAGENTS**). 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 **1X Wash Buffer**. Gently agitate the Coplin jar while soaking the slides. Tap off wash buffer.

STEP 5: To inactivate any peroxidase activity, all tissue sections are treated with the Quench Reagent (see REAGENTS). Cover each well with **enough (approximately 6-8 drops, or 0.5 ml) of Quench Reagent** (orange) and incubate at 37°C for 10 minutes.

STEP 6: 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 7: Dry the slides at 37°C for 5 to 10 minutes or at room temperature. The slides can be stored desiccated at 4°C for up to one week, if necessary.

HYBRIDIZATION/DETECTION PROCEDURE

STEP 8: Remove the slides from the 37°C slide warmer. Apply **1 drop of probe** (~40 µl) from each vial of DNA Probe Reagent to the wells of the slides in the following manner:

Frosted end of the slide is to the left.

Double-Well Slide		**Single-Well Slide**
Left Well	***Right Well***	
HPV 6/11 Probe Reagent *(pink)*	HPV 16/18 Probe Reagent *(purple)*	HPV 31/33/51 Probe Reagent *(green)*

Cover each well separately being careful not to trap bubbles under the coverslips. To avoid mixing of the probe reagents, do not allow the coverslips to touch each other.

STEP 9: Place slides on a **95°C ± 3°C heating block. Incubate the biopsy specimen slides 8-10 minutes.**

STEP 10: 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 11: Removing one slide at a time from the 37°C block in the order in which it was placed on the block, remove the coverslips by tilting the slide and allowing the coverslips to slide off. If a coverslip does not slide off easily, dip the slide in **1X Wash Buffer** until the coverslip comes off. Discard coverslips and place slide in a Coplin jar containing **1X Wash Buffer**. Allow slides to stand in Wash Buffer at room temperature for **2 to 5 minutes**. **Gently** agitate the Coplin jar.

STEP 12: Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the wells, being careful not to disturb the specimen. Place slide on the **37°C heating block and apply enough (approximately 6-8 drops, or 0.5 ml) Post Hybridization Reagent** *(white)* to each well. **Incubate for 10 minutes**.

STEP 13: Removing slides from the 37°C heating block in the order and timing in which they were placed on the block, tap off **Post Hybridization Reagent** and soak slides at room temperature for **1 minute in each of three** Coplin jars filled with fresh **1X Wash Buffer**. **Gently** agitate the Coplin jars during each wash.

STEP 14: Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the wells, being careful not to disturb the specimen. Place the slide on the **37°C heating block and add enough (approximately 6-8 drops, or 0.5 ml) Detection Reagent** *(yellow)* to each well. **Incubate for 30-60 minutes.**

STEP 15: 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 slide with a **gentle** stream of **1X Wash Buffe**r from the plastic squeeze (wash) bottle for **10 seconds**. Place the slide in a Coplin jar filled with fresh room temperature **1X 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 **1X Wash Buffer**. **Gently** agitate the Coplin jars during each wash.

STEP 16: During the last soak, **STEP 15**, prepare the **Color Reaction Mixture**. To each vial of **Reaction Buffer/Substrate Reagent** *(gray)***,** add **3 drops of AEC Reagent** *(red)*. Mix thoroughly. One vial of this mixture is sufficient for 12-15 slide wells (*i.e.*, 4-5 tissue specimens). If more than 12-15 slide wells are being tested, prepare additional vials.

STEP 17: Removing one slide at a time from the Coplin jar, tap off excess wash buffer and wipe around the wells without disturbing the specimen. **enough (approximately 6-8 drops, or 0.5 ml)** freshly prepared **Color Reaction Mixture** to each well and **incubate for 15-20 minutes on the 37°C heating block.**

STEP 18: 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 **Color Reaction Mixture** and soak slides for 1 minute in each of three Coplin jars filled with fresh room temperature **1X Wash Buffer**. Then rinse with distilled or deionized water.

STEP 19: Counterstain sections by **adding enough (approximately 6-8 drops, or 0.5 ml) Blue Counterstain** *(blue)* to cover the specimen. **Incubate at room temperature for 1-2 minutes.**

STEP 20: Tap off the counterstain and then rinse slides with **1X Wash Buffer** followed by distilled or deionized water.

STEP 21: Using water as a mounting medium, view the slides using a light microscope. Brick red deposits indicate positive reactivity. If desired, slides can be permanently mounted using an aqueous histologic mounting medium compatible with AEC precipitates. **Do not use an alcohol or organic based mounting medium.**

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 brick red nucleus must be present. Some artifactual deposit of stain may occur due to trapping of reagents under folds of tissue. Ensure proper interpretation of specimen slides by scoring only distinctive nuclear brick red staining as positive. Scan the slides using 40-100X magnification, and use 400X magnification to confirm cellular structure and signal localization.

If more than one typing well contains cells with positive nuclei, it is possible that multiple HPVs are present, particularly if distinct loci of cells are positive with separate HPV DNA typing probes.

If more than one typing well contains positive nuclei in a single locus within the sections, it is possible that the specimen is HPV-infected, but that the specific type of HPV is not included in any of the typing reagents.

In strongly HPV 16/18-infected specimens, some cross reaction may be observed with the HPV 31/33/51 Probe Reagent, and *vice versa* due to the sequence homology between certain HPV DNA types.

If no positive cells are observed in any of the wells of patient specimen slides, and if the specimen meets the criterion of adequacy, the specimen is negative for HPV DNA types 6, 11, 16, 18, 31, 33 or 51.

Specimens containing only stromal tissue and no positively-stained cells must be considered inadequate for determination of the presence or the type 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.[3,8]
- It is possible for specimens to be infected with more than a single type of HPV.
- For specimens containing very few HPV-infected cells, it may not be possible to determine the HPV type.

SPECIFICITY STUDIES

The specificity of the ***PathoGene*® HRP-AEC Human Papillomavirus (HPV) *In Situ* Typing Assay for Tissue Sections** 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 specimen preparation and/or hybridization/detection procedure*s. Make sure you are using the provided Pretreated Specimen 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. Time and temperature are critical during Proteinase K treatment. Be sure not to leave the enzyme on for an extensive period of time.

- *Coverslips are difficult to remove*: Ensure easy removal of coverslips by using sufficient amounts of the HPV Probe Reagents. In addition, care should be taken whenever coverslips are removed. If a coverslip does not slide off readily, soak it off in 1X Wash Buffer.

- *Deep red stain where tissue folds or crumples*: Take special care when mounting tissue sections on the Pretreated 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 occassional gentle agitation of the Coplin jars during wash steps. Coplin jars of 1X Wash Buffer must be fresh for each wash step. Avoid erroneous interpretation of results by scoring only distinctive nuclear staining as positive.

- *Pale pink to light 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.

- *Stained slides display distinctive positive staining and good cell morphology when first observed, but on re-examination, distinctive staining patterns and/or tissue morphology are lost:* It is possible that the water evaporated drying the coverslip onto the stained slide. This will cause artifactual disruption of the staining pattern and tissue morphology. Do not allow the coverslip to dry onto the stained slide. When slides are dried and stored in the dark without a coverslip, they can be re-examined years later without loss of staining or tissue morphology. It is also possible that an organic solvent-based mounting medium was used instead of water. Avoid loss of positive signal by using water or any preferred aqueous mounting media.

- *The counterstain appears to alter the color of the precipitate*: The amount of counterstaining of the tissue will depend on the duration of the counterstaining step (STEP 19). In a heavily counterstained specimen the red precipitate may appear purplish rather than red. Use only the provided water based hematoxylin.

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