TROUBLESHOOTING

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■ Immunohistochemistry is a multi-step process that requires specialized training in the processing of tissue, the selection of appropriate reagents and interpretation of the stained tissue sections. In general, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Because of its highly complex nature, the causes of unexpected negative reactions, undesired specific staining or undesired background could be difficult to isolate. It is our hope in DakoCytomation Technical Service that the information contained in this chapter will enable you to rapidly pinpoint and resolve problems encountered during the staining procedure.

To this end, we have developed troubleshooting tools for use in the histology laboratory.

Section One is a compilation of common problems encountered when using immunohistochemical-staining reagents, the underlying causes of staining failure and recommended corrective actions. The chart is divided into sections describing little or no staining, general background staining and limited background staining.

Section Two presents a method of systematically adding one IHC reagent at a time to determine at which stage non-specific or undesired staining may be occurring in a peroxidase or alkaline phosphatase staining system.

Section Three is a simple chart used to define the type of tissue specimen, the IHC staining and ancillary reagents already in place in the laboratory, and the staining protocol used by the laboratory personnel. We encourage you to copy this chart and use it to help troubleshoot any problems you may encounter with your staining systems.

SECTION ONE

■ Troubleshooting problems commonly encountered during immunohistochemical staining.

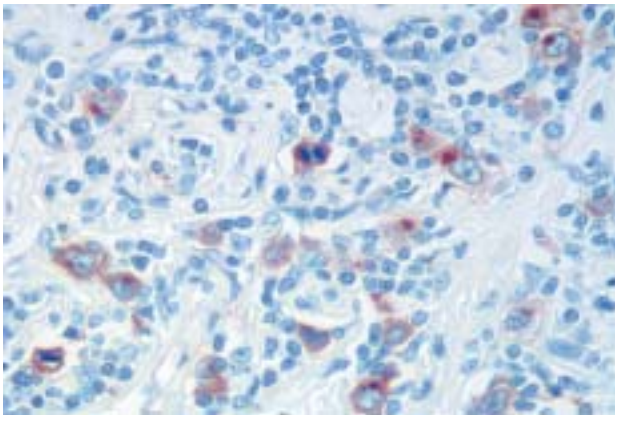

Figure 23

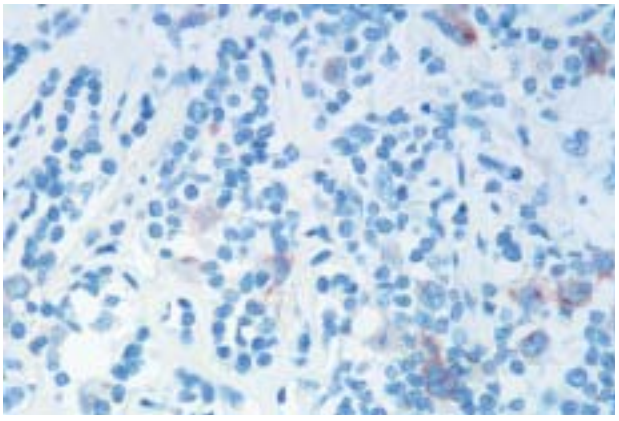

Figure 24

Ongoing studies performed in the DakoCytomation Research & Development laboratory confirm that the pH and ion content of the antibody diluent may have a significant effect on the sensitivity of monoclonal antibodies.

Hodgkin's lymphoma stained with CD30 (clone Ber-H2) antibody, using a three-stage immunoperoxidase staining system. Figure 23: anti-CD30 diluted 1:50 with Tris-HCl, pH 7.6. Figure 24: anti-CD30 diluted 1:50 with PBS, pH 7.0.

INADEQUATE STAINING

■ Little or no staining of controls or specimen tissue, except for counterstain. May show little or no background staining.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Primary antibody or labelled reagent omitted. Reagents used in the wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet or standard operating procedure reagent check-list as established by the individual laboratory.	26
Excessively diluted or excessively concentrated reagents; inappropriate incubation time and temperature.	Determine correct concentration for each reagent. Depending on the degree of staining obtained, if any, a 2- to 5- fold change in concentration may be needed. Incubation temperature and incubation time are inversely proportional and will affect results. To determine optimal incubation protocol, vary either the time or temperature for each reagent in the IHC staining system. Generally, incubation times can be extended if little or no background was detected.	12
Primary antibody diluted with inappropriate buffer. ■ Use of PBS or TBS as an antibody diluent. ■ Lack of stabilizing or carrier protein. ■ Detergent in the diluent.	Check formula and compatibility of antibody diluent. A change of ion and/or pH of the antibody diluent can cause a diminution in the sensitivity of the antibody. Addition of NaCl should be avoided. This problem is primarily seen with monoclonal antibodies.	26
Primary antibody defective; one or several secondary or ancillary reagents defective. Do NOT use product after expiration date stamped on vial.	Replace defective or expired antibody; repeat staining protocol, replacing one reagent at a time with fresh, in-date reagents. ■ Store products according to each product specific package insert. ■ If using a neat or concentrated antibody, it may be aliquoted and frozen. Avoid repeated freezing and thawing. ■ Do not freeze ready-to-use or customer diluted products. Follow manufacturer recommendations on product specification sheets, package inserts and reagent labels.	10
Dissociation of primary antibody during washing or incubation with link antibodies.	A feature of low affinity antibodies: ■ Polyclonal primary antiserum: Attempt staining at lower dilutions. ■ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity. Re-optimize incubation times for washing buffer and link antibody.	8
Use of alcohol-based counterstain and/or alcohol-based mounting media with aqueous- based chromogens.	■ Repeat staining, using water-based counterstain and mounting media. ■ Use a permanent chromogen, such as DAB, that is not affected by organic solvents.	15
Excessive counterstaining may compromise proper interpretation of results.	Use a counterstain that: ■ Will not excessively stain tissue sections. ■ Can be diluted so as not to obliterate the specific signal. ■ Reduce incubation time of the counterstain.	38

INADEQUATE STAINING (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Incorrect preparation of substrate-chromogen mixture. (See Specification Sheet)	■ Repeat substrate-chromogen treatment with correctly prepared reagent. ■ Staining intensity is decreased when excess DAB is present in the working reagent.	
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: ■ Use of PBS wash buffer with an alkaline phosphatase labelled staining system. ■ Sodium azide in reagent diluent or buffer baths for immunoperoxidase methodologies.	Check compatibility of buffer ingredients with enzyme and substrate-chromogen reagents. Repeat staining. ■ Commercial phosphate buffers may contain additives that will inhibit alkaline phosphatase activity. ■ Avoid sodium azide in diluents and buffers. A concentration of 15mM/L sodium azide, which is routinely added to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels.	26
Antigen levels are too low for detection by the employed staining method. May be due to loss of antigenic differentiation in some tumors or loss of antigenicity due to sub-optimal tissue fixation.	■ Utilize a higher sensitivity staining system. ■ Prolong incubation time of primary antibody. ■ Re-optimize incubation times and concentrations of ancillary reagents. ■ Perform antigen retrieval if applicable.	29
Steric hindrance due to high antigen level and possible prozone effect	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration may be too high.	29
Use of inappropriate fixative. ■ Use of certain fixatives may damage or destroy antigens or epitopes in the tissue specimen. ■ Use of non-cross linking fixatives may allow the elution of antigens soluble in IHC reagents. ■ Different fixatives may affect standardization of cells.	Check manufacturer's specifications regarding recommended fixative.	20
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature less than or equal to 58°C. Wax used for embedding should not exceed 60°C.	20
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature not to exceed 60°C. Note: The intensity of immunostaining may be diminished when tissue is exposed to prolonged heat. Refer to the primary antibody specification sheet for additional information.	20
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess reagent left on the tissue section will dilute the next consecutive reagent. Repeat staining, wiping away excess washing buffer and blocking serum.	12
Demasking protocol is inappropriate or has been omitted.	Some tissue antigens require proteolytic enzyme digestion or heat induced antigen retrieval performed prior to staining. Need for pretreatment depends on the type and extent of fixation, specific characteristics of the antigen and the type of antibody used. Use the pretreatment method recommended by the manufacturer. No single pretreatment is suitable for all applications.	23
Repeated reuse of antigen retrieval buffer. (See Specification Sheet)	Do not reuse buffer.	

INADEQUATE STAINING (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.	38
Failure to achieve the optimal temperature required for heat induced antigen retrieval. (See Specification Sheet)	■ Allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99°C. ■ At high altitude, the buffer will boil at less than 95°C. Utilize a closed heating system such as a pressure cooker or autoclave or a low temperature protocol if standardization of procedure is not affected.	24
Excessive or incomplete counterstaining. (See Specification Sheet)	Re-optimize concentration of counterstain and incubation time.	38
Instrument malfunction (See Manufacturer's Instrument Manual)	Ensure automated-stainer is programmed correctly and is running to manufacturer's specifications.	
■ **Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variably background staining of several tissue elements.**		
Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	23
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Re-cut tissue using sharp blade.	35
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	38
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration. Unfixed tissue tends to bind all reagents nonspecifically.	19, 38

GENERAL BACKGROUND

■ **Background seen in all control tissues and specimen tissue. May see marked background staining in several tissue elements such as connective tissue, adipose tissue and epithelium.**

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Excessive incubation with substrate-chromogen reagent. (See Specification Sheet)	Reduce incubation time.	
Substrate-chromogen reagent prepared incorrectly. (See Specification Sheet)	Repeat incubation with correctly prepared chromogen reagent.	
Link antibody cross-reacts with antigens from tissue donor.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.	27

GENERAL BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Secondary (link antibody) and/or tertiary reagents too concentrated.	Repeat staining. Determine correct concentration for each reagent. Incubation temperature and incubation time will affect results. To determine optimal incubation protocol, vary both the time and temperature for each reagent in the IHC staining protocol.	13
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for 5 minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.	8
Insufficient saline or detergent in wash buffer.	High sensitivity staining systems may require higher concentrations of saline or detergent in the wash buffer. Refer to the staining system specification sheet for optimal formulation.	35
Wrong blocking serum used.	Blocking serum and link antibody should come from the same species. Alternatively, a universal serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.	35
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.	38
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against species specimen.	27
Instrument malfunction (See Manufacturer's Instrument Manual)	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.	
■ **Specimen tissue and negative reagent control slides show background staining. Positive and negative control tissue show appropriate specific staining. May involve several tissue elements such as connective tissue, adipose tissue and epithelium.**		
Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.	19
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue. Unfixed tissue tends to bind all reagents nonspecifically.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration.	20
Sectioned portion contains crush artifact caused by grossing in tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Re-cut tissue using sharp blade.	35
Sectioned portion of specimen contains crushed, necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.	35
Excessive or unevenly applied adhesive (subbing agent) on poly-L-lysine, or silanized slides.	IHC reagents bind to these products, resulting in a light stain over the entire slide surface. Some slides may be unevenly coated, and will exhibit the above problems on only a portion of the tissue or glass.	35

GENERAL BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.	37
Tissue sections too thick.	Cut tissue sections thinner. Formalin fixed paraffin embedded tissue should be approximately 4-6µ; cryostat sections <10µ.	45
■ Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue shows expected specific staining.		
Negative control serum insufficiently diluted.	Use properly diluted negative reagent control serum. ■ For polyclonal antibodies, dilute the negative reagent control serum until the protein concentration is equal to that of the primary antibody. ■ For monoclonal antibodies, dilute the negative reagent control serum until the Ig concentration is equal to that of primary antibody.	32
Contaminating antibodies in the negative control serum are cross-reacting with proteins from the specimen tissue.	Replace the negative reagent control serum; repeat staining protocol.	32
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.	11

LIMITED BACKGROUND

■ Areas of inconsistent staining on controls, specimens and glass slides		
POSSIBLE CAUSE	**SOLUTION**	**SEE PAGE**
Protein trapped beneath the tissue during the mounting process will allow partial lifting of the section. Pooling of IHC reagents beneath the section, or partial detachment of the tissue from the slide may occur.	Avoid the use of commercial adhesives, glue, starch or gelatin in water baths when mounting tissue sections. This is particularly important when using charged slides.	38, 46
Undissolved granules of chromogen.	Insure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.	38
Incomplete removal of embedding medium.	Remove embedding medium thoroughly, using fresh reagents.	38
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.	21
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.	38

LIMITED BACKGROUND (continued)

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Partial drying of tissue prior to fixation. Unaffected areas show normal staining.	■ Immerse tissue promptly in fixative or holding reagent. ■ Keep moist during the entire staining process. ■ Use a humidity or moist chamber during incubation steps.	38
Instrument malfunction	Ensure automated-stainer is programmed correctly and is running to manufacturer's specifications.	38
■ **Adipose or connective tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Background in connective and epithelial tissue.**		
Hydrophobic and ionic interactions between immunoglobulins and lipoid substances in fatty tissue.	Nonspecific staining of fatty tissue rarely interferes with interpretation of specific staining and can usually be disregarded. Special stain with Sudan IV; fat will stain an orange to red color (normal myelin and fatty acids do not stain).	34
Primary antibody and negative reagent control serum are insufficiently diluted.	Re-optimize the dilution of the primary antibody and negative control serum.	12
■ **Epithelial tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Staining is moderate to marked, especially in epidermal epithelium. Background in epithelia accompanies background in connective tissue.**		
Both the primary antibody and negative control serum contain contaminating antibodies to epithelial elements, possibly cytokeratins.	■ Use a higher dilution of the primary antibody and negative control serum. ■ Increase the incubation time. ■ Replace the antibody.	7, 36
Excessive formalin fixation of tissues may increase protein cross-linking, resulting in tissue hydrophobicity.	Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody and/or the negative reagent control specification sheet for appropriate pretreatment.	23, 34
■ **Focal cytoplasmic staining observed in epithelium in the specimen tissue.**		
Focal cytoplasmic staining is seen, particularly in intermediate and superficial layers of the epidermis. May be caused by passive absorption of plasma proteins into degenerating epidermal cells.	This observation is rare and should not interfere with interpretation of specific staining.	37
■ **Background seen in all control and specimen tissue when using an immunoperoxidase staining system.**		
Unquenched endogenous peroxidase activity may be seen in all hemoprotein-containing specimens, including hemoglobin in erythrocytes, myoglobin in muscle cells, cytochrome in granulocytes and monocytes and catalases in liver and kidney.	■ Use alternate or prolonged peroxidase blocks or use another enzyme label such as alkaline phosphatase. ■ Eosinophils are particularly resistant to peroxidase quenching. Use special stain: eosin will stain these cells a bright red-orange.	35
■ **Background seen in all control and specimen tissue when using an alkaline phosphatase staining system.**		
Unquenched endogenous alkaline phosphatase activity may be seen in leucocytes, kidney, liver, bone, ovary, bladder, salivary glands, placenta and gastro-intestinal tissue.	Add levamisole to the alkaline phosphatase chromogen reagent or use another enzyme label such as horseradish peroxidase. Intestinal alkaline phosphatase is not quenched by the addition of levamisole. If use of an alkaline phosphatase system is necessary, the tissue may be pre-treated with 0.3N HCl.	36

LIMITED BACKGROUND (continued)

■ Background seen in all control and specimen tissue when using a biotin-streptavidin (avidin) staining system.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Endogenous protein-bound biotin (water-soluble B vitamin). High to moderate amounts may be found in adrenal , kidney, liver, spleen, gastrointestinal, lung, central nervous system, adipose and lymphoid tissue. Staining may also be seen on cells grown in culture media containing biotin as a nutrient.	Use a biotin block or another non-biotin (strept) avidin based staining system.	36

■ Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.

Cause is not understood. It is possibly due to antibodies to muscle antigens in primary and negative reagent control serum.	Should not interfere with interpretation of specific staining.	35, 36

UNDESIRED "SPECIFIC" STAINING

■ Positive staining of leukocyte membranes in specimen tissue, positive control, negative tissue control and negative reagent control.

POSSIBLE CAUSE	SOLUTION	SEE PAGE
Binding of Fc portion of Ig by Fc receptors on the cell membrane of macrophages, monocytes, granulocytes and some lymphocytes.	■ Use $F(ab')_2$ or F(ab) fragments for the primary and secondary antibodies rather than the intact antibodies. ■ Add detergent to the wash buffer.	37

■ Positive staining of histiocytes and granulocytes in the specimen tissue only, with a marker not normally reactive with these cells.

Phagocytosis of antigens may render phagocytes positive for the same.	Rare: should not interfere with interpretation of specific staining.	38

■ Positive membrane staining of specimen tissue and negative reagent control tissue when using a horseradish peroxidase staining system.

Tissue from persons infected with hepatitis B virus and expressing hepatitis B surface antigen may exhibit undesired staining.	Utilize a non-peroxidase staining system.	38

MISCELLANEOUS

■ Loss of viability of cell cultures.

POSSIBLE CAUSE	SOLUTION
DakoCytomation manufactures all antibodies for in vitro use only. These products contain preservatives, usually sodium azide. Azide is a known poison. (See Product MSDS)	Utilize an in vivo product only for application or injection into viable cells. For use on cell cultures only: Sodium azide may be dialyzed out of DakoCytomation reagents. Contact Technical Service for additional information.

SECTION TWO

Troubleshooting flow chart: Use this flow chart to determine source(s) of non-specific staining, when using an immunohistochemical protocol.

BACKGROUND STAINING ENCOUNTERED WITH HRP-PEROXIDASE REAGENTS

REAGENTS	RESULT/ACTION
SLIDE #1 Positive Control Tissue: Counterstain with hematoxylin	Brown pigment (melanin) observed: To distinguish melanin pigment from DAB chromogen, Azure B can be used as a counterstain. The melanin stains blue-green, while the DAB remains brown. An alternate method is to use AEC as the chromogen. However, if high levels of pigment exist in the tissue, the red chromogen may be partially obscured. Since bleaching protocols to remove melanin may compromise tissue antigenicity, it should be avoided if at all possible.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #2 Positive Control Tissue: DAB/AEC + Counterstain	Brown/Red color observed: Indicates endogenous peroxidase activity in the tissue sections. It is present in all hemoprotein containing tissue including erythrocytes, muscle, liver, kidney, granulocytes and monocytes. Block with 3% hydrogen peroxide, sodium azide or Peroxidase Blocking Reagent (S2001).
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #3 Positive Control Tissue: Peroxidase Block + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cell grown in culture media containing biotin (RPMI, NCTC, MEME). Block with Biotin Block (X0590) or switch to a staining system that is not dependent on the streptavidin/biotin reaction, such as the EnVision system.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #4 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: Indicates non-specific or undesired attachment of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10µL of normal serum (from the species of tissue to be stained) per 100µL of the secondary antibody.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #5 Positive Control Tissue: Peroxidase Block + Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/Red color observed: ■ May indicate non-specific attachment of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or Protein Block (X0909); add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #6 Negative Control Tissue: Perform complete staining protocol.	Brown/Red color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

BACKGROUND STAINING ENCOUNTERED WITH ALKALINE PHOSPHATASE

REAGENTS	RESULT/ACTION
SLIDE #1 Positive Control Tissue: Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: Indicates endogenous alkaline phosphatase activity in the tissue sections. It is present in liver, kidney, GI tract, bone, bladder, ovary, salivary gland, placenta, leukemic, necrotic or degenerated cells. Block with Levamisole X3021 (Intestinal alkaline phosphatase may be quenched by the addition of 0.3N HCl prior to the addition of the strept-avidin-AP).
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #2 Positive Control Tissue: Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cells grown in culture media containing biotin (RPMI, NCTC, MEME). Block with Biotin Block (X0590) or switch to a staining system that is not dependent on the streptavidin/biotin reaction, such as the EnVision system.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #3 Positive Control Tissue: Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT+ Counterstain	Red/Blue color observed: Indicates non-specific or undesired attachment of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10µL of normal serum (from the species of tissue to be stained) per 100µL of the secondary antibody.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #4 Positive Control Tissue: Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: ■ May indicate non-specific attachment of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or Protein Block (X0909); add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections.
NO STAINING SEEN. GO TO NEXT STEP. ▼	
SLIDE #5 Negative Control Tissue: Perform complete staining protocol	Red/Blue color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination. ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections.

SECTION THREE

TISSUE SPECIMEN

■ Tissue Specimen: Successful staining of tissue with an IHC marker is dependent on the type and preparation of the specimen. Record in the chart below, the species of the animal to be tested, the tissue source or organ from which it was collected, the collection method, how the specimen was fixed and tissue preparation.

Species: _____

Organ/tissue source: _____

Collection:

- ☐ Surgical specimen/biopsy
- ☐ Post-mortem specimen
- ☐ Fine needle aspirate
- ☐ Peripheral blood (include anti-coagulant)
- ☐ Brushing
- ☐ Biologic fluid
- ☐ Cell culture
- ☐ Other _____

Tissue preparation:

- ☐ Paraffin embedded
- ☐ Plastic embedded
- ☐ Cryostat section
- ☐ Cytospin
- ☐ Cell smear
- ☐ Mono-layer cultured cells
- ☐ Other _____

Tissue fixation:

Type of fixative _____

Length of time _____

Size of specimen _____

Tissue mounting:

- ☐ Slide mount
- ☐ Tissue thickness
- ☐ Gelatin, glue commercial adhesive or starch in the water bath
- ☐ Other _____

BUFFERS

■ The type of buffer selected for dilution of the primary antibody, secondary antibody and wash buffers will have a significant impact on the quality of the IHC stain. Variations include the amount of detergent present in the buffer, the pH, ion content and temperature at which it is used.

Antibody diluent:

- ☐ Tris
- ☐ TBS
- ☐ PBS
- ☐ Other _____

Wash buffer:

- ☐ Phosphate buffered saline
- ☐ Tris buffered saline
- ☐ TWEEN 20 (percentage)
- ☐ pH at 25°C
- ☐ Other _____

DEMASKING

■ Pretreatment: Tissue specimens, when fixed with a cross-linking fixative, may require pretreatment to obtain desired results. Refer to the manufacturer's primary antibody specification sheet to determine the correct protocol.

Antigen retrieval buffer:

- ☐ Target Retrieval Solution (S1700)
- ☐ High pH Target Retrieval Solution (S3308)
- ☐ EDTA
- ☐ pH 8.0
- ☐ Other _____

Heat source:

- ☐ Steamer
- ☐ Waterbath
- ☐ Autoclave/pressure cooker
- ☐ Microwave
- ☐ Other _____

Incubation time: _____

Incubation temperature: _____

Cool down time: _____

Proteolytic enzyme digestion:

- ☐ Proteinase K
- ☐ Protease XXIV
- ☐ Pepsin
- ☐ Trypsin
- ☐ Other _____

Incubation time: _____

ENDOGENOUS BLOCKS

■ Background staining is defined as unexpected or undesirable staining seen on the test or control tissue, which does not represent the target antigen. Frequent causes of background staining are endogenous enzyme activity and endogenous biotin.

Peroxidase is an enzyme of the oxido-reductase class that reacts with a substrate containing hydrogen peroxide as the electron acceptor. To block this activity, a variety of hydrogen peroxide reagents can be applied to cells producing this enzyme.

Alkaline phosphatase is an enzyme having various isoforms, which are produced in the leukocytes, liver, bone, intestine, placenta and Regan (carcinoma). Addition of levamisole to the chromogen/substrate will inhibit endogenous alkaline phosphatase activity, with the exception of the intestinal isoform. If necessary, this can be blocked with a weak acid wash, such as 0.03-0.5N HCl.

Biotin, a B vitamin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns when using a streptavidin or avidin reagent. To block this binding, a biotin/avidin block can be applied to tissue sections containing moderate to high amounts of this vitamin.

Peroxidase block:

- ☐ 3% H_2O_2
- ☐ Methanol/H_2O_2
- ☐ Sodium azide
- ☐ Peroxidase Block (S2001)
- ☐ Other _____

Alkaline Phosphatase block:

- ☐ Levamisole
- ☐ 0.03N HCl (not for use on cryostat tissue)
- ☐ Other _____

Biotin block:

- ☐ Biotin Block (X0590)
- ☐ Other _____

Protein block:

- ☐ Protein Block (X0909)
- ☐ Normal sera from host species of the secondary antibody
- ☐ Other _____

STAINING SYSTEM

A variety of routinely used immunohistochemical systems or kits are now commercially available. These include indirect methods, labelled streptavidin-biotin (LSAB), avidin-biotin complex (ABC), biotinyl tyramide amplification (CSA) and labelled polymer technology (EnVision).

To help troubleshoot, fill in the chart below

Manufacturer: _____

- ☐ Automated
- ☐ Manual

Staining kit:

- ☐ 3-stage immunoperoxidase
- ☐ LSAB
- ☐ EnVision
- ☐ Other _____

Primary antibody: _____

Dilution: (Refer to primary antibody specification sheet) _____

Incubation time and temperature: _____

Negative Reagent Control:

- ☐ Polyclonal Ig fraction of non-immunized host of the primary serum
- ☐ Isotypic Ig (Monoclonal antibody produced as cell culture supernate or ascites fluid)
- ☐ Other _____

Secondary antibody: _____

Incubation time and temperature: _____

Label: _____

Incubation time and temperature: _____

Chromogen: _____

Incubation: _____

Counterstain compatible with the chromogen:

- ☐ Harris hematoxylin
- ☐ Mayers hematoxylin
- ☐ Gill hematoxylin
- ☐ Methyl Green
- ☐ Nuclear fast red
- ☐ Other _____

Clearing Agent:

_____

Mounting Media:

_____