

# General Instructions

For Immunohistochemical Staining

November 2009

## Intended use

For In Vitro diagnostic use.

These instructions apply to Dako immunohistochemistry reagents. They may or may not apply to the product(s) contained in this shipment.

Dako antibodies are intended for laboratory use to qualitatively identify by light microscopy antigens on or in cells from either tissue or cell preparation samples. Positive and negative results aid in the classification of normal and abnormal cells and tissues and serve as an adjunct to conventional histopathology. The clinical interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper controls. Evaluations should be made within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist. Contact Dako Technical Support to report any unusual staining.

## Principles of the procedure

Dako antibodies may be used as the primary antibody with a variety of immunohistochemical (IHC) techniques including labelled streptavidin-biotin (LSAB™+ and Dako LSAB™2), labelled polymer and enhanced polymer systems EnVision™, EnVision™+, EnVision™ FLEX and FLEX+, EnVision™ G|2, EnVision™ G|2 Doublestain, ADVANCE™, Dako REAL™ and Catalyzed Signal Amplification (CSA) for the demonstration of antigens in tissue/cell samples. In general, IHC staining techniques allow for the demasking and visualization of antigens first by pretreatment with proteolytic enzymes or heat retrieval (if required), followed by the sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody (link antibody or labelled polymer), an enzyme complex, and a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. The specimen may then be counterstained and coverslipped. Results are viewed using a light microscope,

and interpreted by a qualified pathologist to aid in the diagnosis of pathophysiological processes which may or may not be associated with a particular antigen. (1-4)

## Materials required, but not supplied

Staining reagents, such as those provided in the Dako EnVision™, EnVision™+, EnVision™ G|2, EnVision™ G|2 Doublestain, EnVision™ FLEX, Dako REAL™ (Peroxidase and Alkaline Phosphatase detection systems), Dako LSAB™+, Dako LSAB™2 and Artisan Staining Systems, as well as Dako Antibody diluents, and negative control reagents. Certain antibodies may require a more sensitive detection system such as EnVision™ FLEX+, ADVANCE™, CSA, or CSA II. Check with your distributor for product availability.

Other materials required, but not supplied include equipment, chemicals and ancillary items. Equipment includes light microscope, staining jars or a Dako automated immunohistochemical staining system, timer (capable of 3-40 minute intervals) and wash bottles. Chemicals include ammonium hydroxide 15 mol/L diluted to 0.037 mol/L, counterstain such as hematoxylin, distilled water, ethanol (absolute and 95%), and xylene or xylene substitutes. Ancillary items may include absorbent wipes; control tissue (positive and negative); coverslips; aqueous mounting medium or nonaqueous permanent mounting medium; and charged, poly-L-lysine coated or silanized slides. Other ancillary components, including control slides, may be necessary. Dako products include target retrieval (demasking) solutions; antibody diluents; blocking reagents; counterstains; proteolytic enzymes; chromogenic substrates for Peroxidase and Alkaline Phosphatase; wash buffer solutions; PAP Pen; aqueous mounting media; Silanized Slides; and Control Slides. Refer to the Dako Web site, Dako catalog or contact Dako for the most current products.

## Storage

Antibodies for IHC from Dako should be stored according to the conditions detailed on their specification sheet. If using a neat (concentrated), unconjugated antibody, it may be aliquoted into convenient volumes and frozen at -20 °C. If using an antibody that was frozen, avoid repeated freezing and thawing. Frozen antibodies may be stored in small aliquots until periodic assay verification by the user detects unacceptable changes in reactivity. (See Quality Control, Assay Verification Section.) Fresh dilutions of the antibody should be made prior to use. Unused portions of antibody preparations should be discarded according to local Health and Safety regulations. Stability of diluted antibodies must be validated by the user. Each antibody is suitable for use until the expiration date printed on the product label, when stored at 2-8 °C.

There are no obvious visual signs to indicate deterioration of IHC reagents. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact Dako Technical Support.

Do not use products after the expiration date. If reagents are stored under conditions other than those specified in the package insert, they must be verified by the user. (5)

The presence of turbidity and/or a precipitate in the reagent may indicate deterioration of the reagent through loss of antibody titer or due to bacterial growth. Positive and negative tissue controls should always be run simultaneously with patient specimens in order to ascertain any loss in titer or in sensitivity. If unexpected staining is observed which cannot be explained by variations in laboratory procedures, and a problem with the antibody is suspected, contact Dako Technical Support.

## Specimen preparation

Prior to IHC staining, tissues must be fixed and processed. Fixation prevents autolysis and necrosis of excised tissues, preserves antigenicity, enhances the refractive index of tissue constituents and increases the resistance of cellular elements to tissue processing. Tissue processing includes dehydration, clearing of dehydrating agents, infiltration of embedding media, embedding and sectioning of tissues. The most common fixatives for IHC tissue preparations are discussed below.

These are guidelines only. Optimal procedures must be determined and verified by the user. For specific information regarding tissue fixation and processing, see references 1 and 6. Consult local Health and Safety regulations.

## Paraffin-embedded tissue

### *General comments*

Although 10% (v/v) neutral phosphate-buffered formalin (commonly referred to in the EU as 4% w/v buffered formalin) is the most common fixative, Dako Detection Systems (as listed in "Materials Required, Not Supplied") have been successfully used with tissues processed in a variety of fixatives. Consequently, the choice of fixative is dependent on the limitations of the primary antibody and the user's institutional or laboratory constraints.

Survival of tissue antigens for immunological staining may depend on the type and concentration of fixative, on fixation time, and on the size of the tissue specimen to be fixed. (7) It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Where possible, use of thinner specimens coupled with shorter fixation times is recommended. Prolonged exposure to fixatives may result in the masking, impairment or destruction of antigens, which contribute to reduced immunostaining. Zenker's fluid, B-5 and Bouin's have often been recommended as alternative fixatives for the preservation of tissue antigens sensitive to routine formalin fixation (10% neutral buffered formalin). (8, 9) See references 1 and 6, the

primary antibody package insert(s) and the protocol(s) supplied with the fixing reagent(s) for additional information regarding tissue fixation.

#### *Tissue fixation in formaldehyde-based solution (Neutral buffered formalin and Bouin's)*

Most formaldehyde-based fixatives contain 10% formalin, a neutral salt to maintain tonicity, and a buffered system to maintain pH. These fixatives are well tolerated by tissues and exhibit good histological penetration. However, shrinkage or distortions may occur in poorly fixed and embedded tissue specimens. Fix small blocks of tissue (10.0 x 10.0 x 3.0 mm) in 5-10 mL of neutral buffered formalin per block for up to 24 hours. Bouin's solution is an alternative formaldehyde-based fixative which contains picric acid and is suitable for use on all tissues except kidney. Specimens may be fixed from 1 to 12 hours depending on tissue thickness. Excessively fixed tissues become brittle and the appearance and quantity of lipids is adversely affected. Complete fixation with a 70% ethanol wash to precipitate soluble picrates prior to aqueous washes.

#### *Mercuric-chloride containing fixatives (B-5 and Zenker's)*

Mercuric-chloride fixatives, such as B-5 and Zenker's, frequently include a neutral salt to maintain tonicity and may be mixed with other fixatives. In general, mercuric-chloride fixatives are poor histological penetrators and are not well tolerated by tissue specimens. Consequently, small tissue blocks (7.0 x 7.0 x 2.5 mm) and short fixation periods (1 to 6 hours for B-5, and 2 to 15 hours for Zenker's) are recommended. After fixation, the tissue block(s) should be rinsed well with water and placed in 70% ethanol for wet storage or until tissue processing can be completed. Conclude fixation with tissue processing and paraffin-embedding (see Processing and Paraffin-Embedding Section). Prior to immunostaining, clear tissue sections of mercury deposits using an iodine/ethanol/sodium thiosulfate solution. (10) Exercise the necessary precautions when handling reagents containing mercury compounds.

#### *Tissue fixation in ethanol*

Ethanol is not widely employed as a fixative for routine histological techniques due to its poor penetrating ability. However, small pieces of tissue show good cytological preservation. Fix tissue blocks (5.0 x 5.0 x 2.0 mm) in absolute alcohol for 48 hours at room temperature (20-25 °C) followed by two 1-hour baths in fresh xylene and two consecutive 1-hour baths in liquid paraffin. Follow paraffin infiltration with embedding.

#### *Processing and paraffin-embedding*

After fixation, processing may be completed using an automatic tissue processor. Tissues are dehydrated using graded alcohols, cleared with xylene or xylene substitute, and infiltrated with paraffin wax. The tissue is subsequently embedded with paraffin wax in molds or cassettes which facilitate tissue sectioning. To minimize denaturing of antigens, do not expose tissues to temperatures in excess of 60 °C during processing. Tissue blocks may be stored or sectioned on completion of embedding. Properly fixed and paraffin-embedded tissues will keep indefinitely if stored in a cool place.

Slides with paraffin-embedded tissue sections can be kept for up to 3 years if stored at 2-8 °C depending on the antigen in question. (11)

#### *Adherence of paraffin-embedded tissue sections to microscope slides*

Collect sectioned tissues from paraffin-embedded blocks on clean glass slides. Dehydrate in an oven for one hour at 60 °C or less. For increased adhesion of tissue sections during IHC staining procedures, use of FLEX IHC Slides (code K8020), charged, poly-L-lysine coated, or Silanized Slides (code S3003) is suggested. When using charged, poly-L-lysine coated or silanized slides specifically omit any adhesives in the mounting water bath, such as gelatin, glue and/or commercially produced protein-containing products. Coated slides are strongly recommended for staining procedures requiring proteolytic digestion or heat-induced epitope retrieval (target retrieval).

#### *Deparaffinization and rehydration*

Prior to staining, tissue slides must be deparaffinized to remove embedding media and rehydrated. Avoid incomplete removal of paraffin. Residual embedding media will result in increased non-specific or reduced staining.

1. Place slides in a xylene bath and incubate for 5 ( $\pm$ 1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 3 ( $\pm$ 1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 3 ( $\pm$ 1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in distilled or deionized water for a minimum of 30 seconds. Unless proteolytic digestion or target retrieval is required, commence staining procedure.

Xylene and alcohol solutions should be changed weekly, or after a maximum of 200 slides. Toluene or xylene substitutes such as HistoClear may be used in place of xylene; incubation times may need to be increased and the solution may need to be changed more frequently.

If necessary, rehydrated tissues may be kept in buffer solution at 2-8 °C for up to 18 hours prior to use. Allow tissues to come to room temperature (20-25 °C) before staining.

#### *Proteolytic digestion and target retrieval*

Formaldehyde is known to induce conformational changes in the antigen molecules by forming intermolecular cross-linkages. Excessive formalin fixation can mask antigenic sites and diminish specific staining. However, these sites may be revealed with proteolytic digestion or target retrieval of tissue slides prior to immunostaining. To determine if either of these pretreatments of tissues is warranted, see the specification sheet provided with each primary antibody.

Pretreatment of tissue with proteolytic enzymes may be performed prior to staining on deparaffinized and rehydrated tissue sections. Proteolytic enzymes, such as Dako Proteinase K, RTU (code

S3202), Pepsin (code S3002), or Proteolytic Enzyme, RTU (code S3007) can be used. Use the timing recommended in the specified package insert of the enzyme. CAUTION: Overdigestion may result in nonspecific staining and/or unacceptable morphology. Rinse thoroughly with distilled water and continue with the staining procedure of the detection system instructions.

Heat-induced epitope retrieval (HIER/target retrieval) prior to IHC staining procedures results in increased staining intensity with many primary antibodies. For some antibodies, this procedure is required. Refer to the antibody specification sheet for the recommended retrieval method. Target retrieval involves immersion of tissue sections in a pre-heated buffer solution and maintaining heat, either in a PT Link, water bath, steamer (95-99 °C), pressure cooker (121 °C), or other temperature-controlled laboratory equipment. For optimal results on PT Link, use Target Retrieval Solution from EnVision™ FLEX and FLEX+ kits (codes K8000, K8002, K8010, and K8012) or optional EnVision™ FLEX Target Retrieval Solutions (codes K8004 and K8005). Other Dako target retrieval solutions include Target Retrieval Solution, pH 9 (10x), (3-in-1)<sup>a</sup> (code S2375), Target Retrieval Solution (code S1699 and S1700), and Target Retrieval Solution, Citrate pH 6 (code S2369 and S2031). Refer to individual instructions for use.

If PT Link is used together with either Target Retrieval Solution from EnVision™ FLEX and FLEX+ kits or K8004, K8005, S2375, S1699, deparaffinization, rehydration and target retrieval can be carried out in one step. The PT Link run should be followed by a Quick Dip procedure, using Wash Buffer from EnVision™ FLEX and FLEX+ kits, Optional EnVision™ FLEX Wash Buffer (20x) (code K8007), or Dako Wash Buffer 10x (code S3006).

Using the Dako EnVision™+ System, the pressure cooker method, or other temperature-controlled laboratory equipment, gives

<sup>a</sup> In the US, the Dako Target Retrieval Solution, S1699, (10x) and EnVision™ FLEX Target Retrieval Solution Low pH, K8005 are not for sale as a "3-in-1" buffer for use in the 3-in-1 procedure. Licensing under U.S. patent No. 6,649,368 B1 may be required.

stronger staining than the water bath method. Refer to instructions provided with Target Retrieval Solution or reference 12.

If the water bath method is used for retrieval, some antigens may require an additional pretreatment with proteolytic enzymes prior to heating. Tissue sections can be digested with Dako Proteinase K (code S3004) diluted 1:500 in a Tris-HCl buffer, pH 7.2-7.6 for 10 minutes at room temperature (20-25 °C).

At certain higher elevations (above 1372 m (4500 feet)), boiling of the target retrieval solution may occur prior to achieving the desired optimal temperature. In such situations, a recommended alternative procedure is to heat the slides at the maximum achievable temperature and to extend the incubation time of the slides in the target retrieval solution until the desired staining intensity is achieved. (11) An additional possible solution is to use a closed pressure system such as a pressure cooker to achieve 121 °C. However, each laboratory must determine the best method and target retrieval time for their particular circumstances.

## Frozen tissue

Frozen sections should be cut from snap-frozen tissue blocks (approximately 1.0 x 1.0 x 0.5 cm) and air-dried for 2-24 hours. Dried sections can be fixed in room temperature (20-25 °C) acetone for 10 minutes or in buffered formyl-acetone for 30 seconds. Allow sections to air-dry until completely dehydrated. Proceed with immunostaining or wrap slides in aluminum foil and store at -20 °C or lower, for up to six months. Equilibrate wrapped, frozen sections to room temperature prior to use. Post-fix sections in cold acetone (2-8 °C) for 10 minutes. Place slides in TBS bath. Gently change TBS bath several times to remove residual acetone.

If sections are too thick (greater than 4-6 µm), incorrectly fixed or unevenly dried, artifacts may result and interfere with interpretation of staining. This includes rupturing of cell membranes and chromatolysis. Nuclei may appear swollen and uniformly blue when counterstained with hematoxylin.

## Other specimens

Dako Detection Systems may also be used for staining antigens in bone sections, bone marrow, blood smears, cytopins and imprints. Smears may be air-dried for 2-24 hours and processed for immediate staining or wrapped in aluminum foil and stored at -20 °C or lower for up to six months.

Air-dried or thawed smears may be fixed for 90 seconds in acetone-methanol (1:1). Fixation in acetone or acetone-methanol-formalin (10:10:1) is also acceptable.

Osseous tissues must be decalcified prior to sectioning and processing to facilitate tissue cutting and prevent damage to microtome blades. (1) Decalcification may affect IHC staining.

For professional users in the United States, Clinical Laboratory Improvement Amendments of 1988 (42 CFR 493.1259(b)) requires that "The laboratory must retain stained slides at least 10 years from the date of examination and retain specimen blocks at least two years from the date of examination."

Consult the Dako Education Guide: Immunohistochemical Staining Methods, Fourth Edition (6) or references 1 and 2 for further details on specimen preparation.

## Precautions

1. For professional users.
2. Products may contain sodium azide ( $\text{NaN}_3$ ), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous,  $\text{NaN}_3$  may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. Biological specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and disposed of with proper precautions. (14) Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with both reagents and specimens.

If reagents come into contact with sensitive areas, wash with copious amounts of water. Wear Personal Protective Equipment when handling any human biological material and while performing the staining procedure.

4. Safety Data Sheets available for professional users on request.
5. Incubation times or temperatures other than those specified may give erroneous results. Any such changes must be validated by the user.
6. Unused solution should be disposed of according to local, State and Federal regulations.

## Staining procedure

For state-of-the-art staining performance, use FLEX Ready-to-Use antibodies. Optimized with EnVision™ FLEX and FLEX+ visualization systems, these antibodies deliver desired end results when used on PT Link and Autostainer Link or Dako Autostainer instruments.

When using concentrated (neat) antibodies, refer to specific package inserts for primary antibody dilutions and to specific Detection System Instructions for recommended procedures.

When using N-series ready-to-use antibodies, refer to the product specific package insert and the Staining Procedure section of EnVision™+, EnVision™ G2 Doublestain, LSAB™2 and LSAB™+ Detection System Instructions.

## Quality control

Differences in tissue processing and technical procedures in the user's laboratory may produce significant variability in results; regular controls need to be performed according to local guidelines for accreditation in addition to the following procedures. Professional users in the United States should consult the quality control guidelines of the College of American Pathologists (CAP) Accreditation Program for Immunohistochemistry, NCCLS Quality Assurance for Immunocytochemistry, Approved Guideline, (15) and reference 7 for additional information.

## Positive control tissue

Controls should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded in the same manner as the patient sample(s). Positive control tissues are indicative of correctly prepared tissues and proper staining techniques. One positive control tissue for each set of test conditions should be included in each staining run.

Tissues used for positive control testing should give weak positive staining in order to detect subtle changes in the primary antibody sensitivity. Commercially available tissue slides or specimens processed differently from the patient sample(s) validate reagent performance only and do not verify tissue preparation. Refer to the product specific package insert, Performance Characteristics Section for normal tissue specimens that may be used for a positive control tissue.

Known positive control tissues should only be utilized for monitoring the correct performance of processed tissues and test reagents, NOT as an aid in formulating a specific diagnosis of patient samples. If the positive control tissues fail to demonstrate positive staining, results with the test specimen should be considered invalid.

## Negative control tissue

Use a normal tissue known to be negative for the antigen being tested (refer to the product specific package insert, Performance Characteristics Section) that is fixed, processed and embedded in a manner identical to the patient sample(s) with each staining run to verify the specificity of the primary antibody and to provide an indication of background staining. The variety of different cell types present in most tissue sections offers internal negative control sites (this should be verified by the user).

If specific staining occurs in the negative control tissue, patient specimen's results should be considered invalid.

## Negative control reagent

Use a negative control reagent with each specimen to evaluate nonspecific or undesired staining and allow better interpretation of specific staining at the antigen site. Ideally, a negative control reagent contains an antibody which exhibits no specific reactivity with human tissues (non-human reactive) in the same matrix/solution as the diluted primary antibody. The non-human reactive antibody should be the same isotype and animal species as the primary antibody, diluted to the same immunoglobulin or protein concentration as the diluted primary antibody. Normal/nonimmune serum from the same species as the primary antibody, at a protein concentration equivalent to the diluted primary antibody in the same matrix/solution may be suitable for use depending on the type of primary antibody used. Refer to the package insert of each primary antibody and to Table 1 for specific recommendations. Diluent alone may be used as a less desirable alternative to the previously described negative control reagents. The incubation period for the negative control reagent should be equivalent to that of the primary antibody.

**Table 1. Examples of negative control reagents for concentrated antibodies**

<i>Primary antibody type</i>	<i>Suggested negative control reagent</i>
Monoclonal mouse FLEX Ready-to-Use antibody	Universal Negative Control for Mouse Primary Antibodies, code IR750 (for Autostainer Link instruments) and IS750 (for Dako Autostainer instruments)
Polyclonal or monoclonal rabbit FLEX Ready-to-Use antibody	Universal Negative Control for Rabbit Primary Antibodies, code IR600 (for Autostainer Link instruments) and IS600 (for Dako Autostainer instruments)
Monoclonal mouse antibody, produced in ascites	Non-human reactive monoclonal antibody produced in ascites. This negative control antibody should be of the same isotype as the primary antibody. Alternatively, normal/nonimmune mouse serum may be used, Dako code X0910
Monoclonal mouse antibody, produced in tissue culture	Non-human reactive monoclonal antibody produced in tissue culture. This negative control antibody should be of the same isotype as the primary antibody. Dako codes X0931 (IgG1), X0943 (IgG2a), X0944 (IgG2b), X0942 (IgM). Alternatively, fetal calf serum may be used.*
Polyclonal rabbit antibody, immunoglobulin fraction	Rabbit Immunoglobulin fraction (Normal), Dako code X0903
Solid-phase absorbed polyclonal rabbit antibody, immunoglobulin fraction	Rabbit Immunoglobulin Fraction (Solid-Phase Absorbed), Dako code X0936
Polyclonal rabbit antibody, whole serum	Normal/nonimmune rabbit serum, whole serum, Dako code X0902

\* Fetal calf serum is suitable for use if it is retained in the primary antibody after processing.



When panels of several antibodies are used on serial tissue sections, the negatively staining areas of one slide may serve as a negative/nonspecific binding background control for other antibodies if their dilutions are similar and they are from similar animal sources.

To differentiate endogenous enzyme activity or nonspecific binding of enzymes from specific immunoreactivity, additional patient tissues may be stained exclusively with substrate-chromogen or enzyme complexes (avidin-biotin, streptavidin, labelled polymer) and substrate-chromogen, respectively. For specific procedures, contact Dako Technical Support.

**Table 2. The purpose of daily quality control**

<i>Tissue: fixed &amp; processed similar to patient sample</i>	<i>Specific antibody &amp; detection system</i>	<i>Negative reagent control* or buffer plus same detection system as used with specific antibody</i>
Positive Control: Tissue or cells known to contain target antigen to be detected (could be located in patient tissue). Tissue which exhibits weakly positive staining is most sensitive to antibody or detection system degradation.	Controls all steps of the analysis. Validates reagent and immunostaining procedures.	Detection of non-specific background staining.
Negative Control: Tissue or cells expected to be negative (could be located in patient tissue or positive control tissue).	Detection of unintended antibody cross-reactivity to cells/cellular components.	Detection of non-specific background staining.
Patient Tissue	Detection of specific staining.	Detection of non-specific background staining.

\* Same species and isotype as the specific antibody, but not directed against the same target antigen. To detect non-specific antibody binding, e.g., binding of Fc portion of antibody by the tissue.

## Assay verification

Prior to initial use of an antibody or immunostaining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house tissues with known IHC performance characteristics representing known positive and negative tissues. Refer to the quality control procedures previously outlined in this section of the General Instructions and, for US professional users, to the quality control requirements of the

CAP Accreditation Program for Immunohistochemistry and NCCLS Quality Assurance for Immunocytochemistry, Approved Guideline. (15) These quality control procedures should be repeated for each new antibody lot, or whenever there is a change in assay parameters. Tissues listed in the product specific package insert, Performance Characteristics Section are suitable for assay verification.

## Troubleshooting

Refer to the Troubleshooting section in the Dako Education Guide: Immunohistochemical Staining Methods, Fourth Edition (6) for remedial action, or contact Dako Technical Support or the website at [www.dako.com](http://www.dako.com) to report unusual staining.

## Staining interpretation

### *Positive control tissue*

The positive control tissue should be examined first to ascertain that all reagents are functioning properly. Positive reactivity is indicated by the presence of a red (3-amino-9-ethylcarbazole, AEC), bright pink (New Fuchsin or Fast Red) or brown (3,3'-diaminobenzidine tetrahydrochloride, DAB) reaction product at the site of the target antigen. See the Staining Interpretation and Performance Characteristics sections of the product specific package insert for specific staining patterns. If the positive control tissues fail to demonstrate the expected staining pattern, all results with the test specimen should be considered invalid.

**NOTE:** The color of the reaction product may be different if substrate chromogens other than those stated are used. Refer to the substrate package insert for expected color reactions. Further, metachromasia may be observed in variations of the method of staining. (16)

Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a blue coloration of the cell nuclei. Excessive or incomplete counterstaining may compromise proper interpretation of results.

### *Negative control tissue*

The negative control tissue should be examined after the positive control tissue to verify the specificity of the labelling of the target antigen by the primary antibody. The absence of specific staining in the negative control tissue confirms the lack of antibody cross-reactivity to cells/cellular components. If specific staining, other than that described above, occurs in the negative control tissue, results with the patient specimen should be considered invalid. In negative tissues, the tissues should have a blue-purple appearance when using hematoxylin.

Nonspecific staining, if present, will be of a diffuse appearance. Sporadic staining of connective tissue may also be observed in sections from excessively formalin-fixed tissues. Use intact cells for interpretation of staining results. Necrotic or degenerated cells can exhibit nonspecific staining. (7)

False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by endogenous enzymes, such as myeloperoxidase, leucocyte alkaline phosphatase and hemoglobin pseudoperoxidase, primarily in frozen tissues and depending on the type of enzyme label used to visualize the reaction. (17)

### *Patient tissue*

Patient specimens should be examined last. Positive staining intensity should be assessed within the context of any nonspecific background staining with the negative control reagent. As with any IHC test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissue assayed. If necessary, use a panel of antibodies to identify false-negative reactions.

Refer to the product specific package insert for information regarding primary antibody immunoreactivity.

## General limitations

1. IHC is a multi-step diagnostic process that requires specialized training in the selection, fixation and processing of tissue; selection of reagents; preparation of the IHC slide; and interpretation of the staining results.
2. Tissue staining is dependent on the handling and processing of the tissue prior to staining. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. Excessive or incomplete counterstaining may compromise proper interpretation of results.
4. The clinical interpretation of any staining, or its absence must be complemented by morphological studies and proper controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the antibodies, reagents and methods used to interpret the stained preparation. Staining must be performed in a certified licensed laboratory under the supervision of a pathologist who is responsible for reviewing the stained slides and assuring the adequacy of positive and negative controls.
5. Unexpected negative reactions in poorly differentiated neoplasms may be due to loss or marked decrease of expression of antigen or loss or mutation(s) in the gene(s) coding for the antigen. Unexpected positive staining in tumors may be from expression of an antigen not usually expressed in morphologically similar normal cells, or from persistence or acquisition of an antigen in a neoplasm that develops morphologic and IHC features associated with another cell lineage (divergent differentiation). Histopathologic classification of tumors is not an exact science and some literature reports of unexpected staining may be controversial.
6. Tissues from persons infected with hepatitis B virus and containing hepatitis B surface antigen (HBsAg) may exhibit non-specific staining with horseradish peroxidase. (18)
7. Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms or other pathological tissues. (19) Contact Dako Technical Support with any documented unexpected reaction.
8. Normal/nonimmune sera from the same animal species as the secondary antisera used in blocking steps may cause false-negative or false-positive results due to autoantibodies or natural antibodies.
9. False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by endogenous biotin or enzymes, such as myeloperoxidase, leucocyte alkaline phosphatase and hemoglobin pseudoperoxidase, primarily in frozen tissues and depending on the type of immunostain used. (17)
10. Heat induced epitope retrieval (target retrieval) may result in HIER lipofuscin artifacts. Target retrieval may result in demasking of unexpected or undesired sites.
11. False-negative staining results, with or without background, may be encountered if the concentration of primary antibody is too high when used in a given staining system.
12. Ready-to-use primary antibodies are prediluted and optimized for use with specific staining systems. When used in conjunction with other Dako or manufacturer's detection systems these are no longer ready-to-use and must be re-optimized and validated according to the clinical laboratory IHC protocol.
13. Unless specifically claimed in the instructions, the performance characteristics of antibodies used for IHC have not been determined for other laboratory techniques.

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