Agilent Dako

CE

General Instructions
For Immunohistochemical Staining

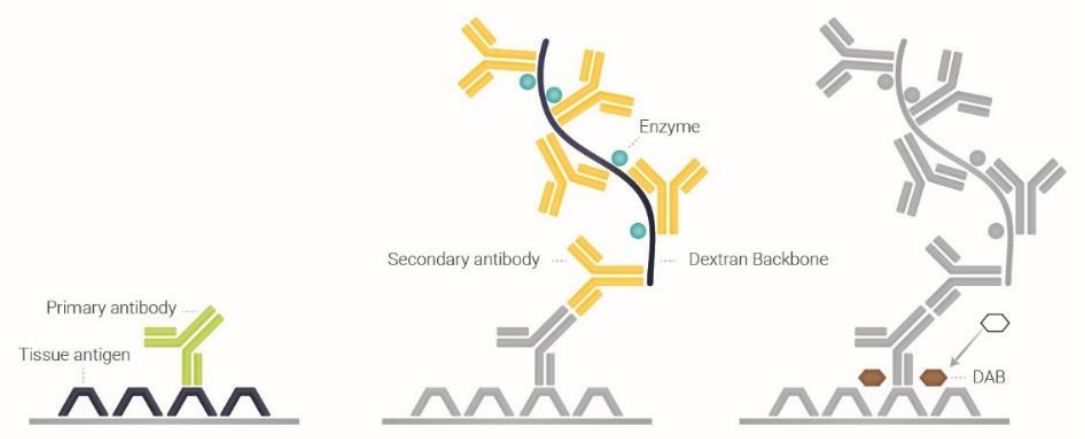
Purpose
These general instructions apply to Agilent Dako products for immunohistochemistry (IHC). Always consult the individual Instructions for Use of your product for specific instructions on the use of the product.

The information in this General Instructions for Immunohistochemical Staining are guidelines only. A validated procedure is provided in the specific Instructions for Use for each primary antibody together with the visualization system's Instructions for Use. Optimal procedures must be determined and verified by the user.

NOTE: Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Principle of Procedure
Our antibodies may be used as the primary antibody together with a variety of immunohistochemical (IHC) reagents for pre-treatment, visualization systems, and counterstaining for the demonstration of antigens in tissue/cell samples. In general, IHC staining techniques allow for the demasking and visualization of antigens in tissue sections or cell preparations first by pretreatment with heat retrieval or proteolytic enzymes (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 labeled 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 (bright field) microscope and interpreted by a qualified pathologist to aid in the assessment of pathophysiological processes, including classification of cancers, which may or may not be associated with a particular antigen.[2-5]

The user should read these General Instructions and product-specific Instructions for Use carefully and become familiar with all the components and the instrumentation prior to use.



Materials Required, but Not Supplied
See individual Instructions for Use for specific recommendation of procedure. Not all the below listed materials may be required.

Pretreatment:
Target Retrieval Solution for heat-induced epitope retrieval
Proteolytic enzyme

Primary antibody
Our concentrated antibody or FLEX Ready-to-Use antibody

Visualization:
Visualization kit with included staining reagents, such as those provided in EnVision FLEX and FLEX+, EnVision FLEX HRP Magenta, or similar

Counterstaining and mounting
Aqueous mounting medium or non-aqueous permanent mounting medium or mounting method
Hematoxylin

Agilent

Equipment and accessories
Automated immunohistochemical staining system from Agilent
PT Link pre-treatment module (not needed for Dako Omnis)
Drying oven, capable of maintaining 60 °C or less
Timer (capable of 3–40 minute intervals)
Coverslips
Light (bright field) microscope (4–40x objective magnification)
Microscope slides: FLEX IHC Microscope Slides, SuperFrost Plus slides
Absorbent wipes
Other ancillary components, including instrument-specific ancillaries and accessories, may be necessary.

Chemicals, buffers and solutions
Antibody diluents
Blocking reagents
Chromogenic substrates
Distilled or deionized water (reagent-grade quality water)*
Ethanol
Wash buffer
Xylene, toluene, or xylene substitutes

Controls
Positive and negative tissue controls
Negative control reagent

***Note: Not all sources of distilled or de-ionized water may be of sufficient quality for IHC reagent preparation. It is recommended to use reagent-grade distilled or de-ionized water corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by CLSI, or water similar in quality to be used for reagent preparation**.

Storage
Proper storage and handling of reagents and samples are essential for the performance. Do not use the product after the expiration date printed on the outside of the reagent package. Unless otherwise specified in the product's IFU, the expiry date on the label is valid for unopened as well as opened (in-use) vials when handled according to instructions. If products are stored under any conditions other than those specified, they must be validated by the user.

Return vials to specified storage conditions directly after use. Fresh dilutions of a concentrated antibody should be made immediately prior to use. Stability of diluted antibodies must be validated by the user. Unused portions of antibody preparations should be disposed of in accordance with all local, regional, national and international regulations. Each antibody is suitable for use until the expiration date printed on the product label, when stored at 2–8 ℃. After use on an automated staining platform from Agilent, store the closed vial in the dark at 2–8 °C. See individual primary antibody Instructions for Use for onboard stability on Dako Omnis staining platform.

There are no obvious visual signs to indicate incorrect product storage or handling of this product during the product's shelf life. Positive and negative control tissues as well as negative control reagent should be run simultaneously using the same protocol as the patient specimens, preferably on the same slide, to ensure the validity of the staining procedure, including reagents, tissue processing and instrument. If a problem is suspected with the product during the shelf life that cannot be explained by incorrect product storage or handling, or other variations in laboratory procedures, contact Agilent Pathology Support. Refer to the Troubleshooting and Quality Control sections for more information.

The presence of turbidity and/or a precipitate in the reagent may indicate deterioration of the reagent. Positive and negative tissue controls should always be run simultaneously with patient specimens in order to ascertain any loss in reagent quality.

Specimen Preparation
These are guidelines only. Optimal procedures must be determined and verified by the user.

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.[1] Specimens must be handled to preserve the tissue for IHC staining. Standard methods of tissue processing should be used for all specimens.[2,3] 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 and in references 2 and 6.

Paraffin-Embedded Tissue
General comments
The most common tissue fixative is 10% (v/v) neutral phosphate-buffered formalin, commonly referred to in the EU as 4% w/v buffered formalin. Most of our reagents for IHC have been successfully validated with paraffin-embedded tissues fixed in 10% formalin. This is referred to as formalin-fixed, paraffin-embedded (FFPE) tissue sections.

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. It is important to maintain optimal, standardized fixation conditions whenever possible in order to obtain reproducible staining. Formalin-fixed, paraffin-embedded tissues are generally suitable for use for Agilent Dako IHC technique. Fixation time of approximately 24 hours in 10% neutral buffered formalin (NBF) is normally recommended. Fixation times of ≤ 3 hours may result in variable biomarker detection. Prolonged exposure to fixatives may result in the masking, impairment or destruction of antigens, which contribute to

reduced immunostaining. Specimens should be blocked into a thickness of 3 or 4 mm, fixed in formalin and dehydrated and cleared in a series of alcohols and xylene, followed by infiltration with melted paraffin. The paraffin temperature should not exceed 60 °C. FFPE tissue blocks which are 5 years or older may result in a loss of immunoreactivity. Tissue specimens should be cut into sections of approximately 4 µm. After sectioning, tissues should be mounted on microscope slides. See the primary antibody Instructions for Use(s) and the protocol(s) supplied with the fixing reagent(s) for additional information regarding tissue fixation.

See individual primary antibody Instructions for Use for specific recommendation of fixative and glass slides for mounting. Alternative fixatives and use on frozen tissue are only suitable for specific products and should always be determined and verified by the user.

Tissue fixation in formaldehyde-based solution (neutral buffered formalin)
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. Optimal fixation time may vary by block size and tissue type and will typically be approximately 24 hours. Prior to immunostaining, dehydrate, clear and embed tissue sections as described below in *Processing and Paraffin-Embedding*.

Tissue fixation in Bouin's, B-5 and Zenker's fixatives
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).[7,8] 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 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.[9] 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. To preserve antigenicity, tissue sections mounted on slides should be stained within 6 months of sectioning when held in the dark at 2–8 °C (preferred), or at room temperature up to 25 °C.

Adherence of paraffin-embedded tissue sections to microscope slides
Collect sectioned tissues (~4 µm) from paraffin-embedded blocks on clean glass slides. Dry the tissue sections at 58 +/-2 °C for a maximum of one hour. For increased adhesion of tissue sections during IHC staining procedures, use of FLEX IHC Microscope Slides or Superfrost Plus slides. In a few occasions, Silanized Slides are 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 nonspecific or reduced staining.

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in absolute ethanol for 3 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 95% ethanol for 3 (±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. Consult the Instructions for Use of the used reagent.

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 equilibrate 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 heat-induced target retrieval of tissue sections prior to immunostaining. To determine if either of these pretreatments of tissues is warranted, see the Instructions for Use provided with each primary antibody.

Table 1. General pre-treatment guidelines when using Dako-Branded Primary Antibodies from Agilent

Primary Antibody	Pre-Treatment Solution	Method
Ready-to-Use for Dako Omnis	EnVision FLEX Target Retrieval Solution, High pH or EnVision FLEX Target Retrieval Solution, Low pH	Onboard Dako Omnis
Ready-to-Use for Autostainer Link 48	EnVision FLEX Target Retrieval Solution, High pH or EnVision FLEX Target Retrieval Solution, Low pH	PT Link
Concentrated Antibody	HIER, Proteolytic or no requirement	PT Link, microwave oven, water bath, enzymatic.

Always consult Instructions for Use for optimal pre-treatment method for the individual primary antibody.

Heat-induced epitope retrieval (HIER) prior to IHC staining procedures results in increased staining intensity with many primary antibodies.[10] For most of our primary antibodies, this procedure is required. Refer to the antibody Instructions for Use 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 pre-treatment module or onboard Dako Omnis. Other temperature-controlled laboratory equipment is also applicable for HIER such as water bath, microwave oven, autoclave, pressure cooker and steamer as heating sources. The optimal procedure must be determined by user. For optimal results on PT Link and Dako Omnis, use EnVision FLEX Target Retrieval Solution High pH or Low pH. Other target retrieval solutions include Target Retrieval Solution, pH 9 (10x), (3-in-1), Target Retrieval Solution Citrate pH 6.1 and Target Retrieval Solution, Citrate pH 6. Refer to individual Instructions for Uses for instructions for use.

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 and Dako Pepsin can be used. Use the timing recommended in the specified Instructions for Use of the enzyme. Overdigestion may result in nonspecific staining and/or unacceptable morphology. Rinse thoroughly with distilled water and continue with the staining

When PT Link or Dako Omnis is used together with either EnVision FLEX Target Retrieval Solution, High pH or Low pH, 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 Dako Wash Buffer.

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 appropriately diluted Proteinase K in a Tris-HCl buffer, pH 7.2–7.6 for 10 minutes at room temperature (20–25 ℃) before commencing the HIER.

Laboratories at high elevations
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. (10) An additional possible solution is to use a closed pressure system such as a pressure cooker to achieve 121 ℃. Drying of tissue sections at elevated temperatures must only be performed in a calibrated oven with uniform heat distribution. Each laboratory must determine the best method and target retrieval time for their circumstances.

NOTE: FFPE sections should not dry out during pre-treatment, during the immunohistochemical staining procedure or before mounting.

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 tris-buffered saline (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.

Only few concentrated of our antibodies are validated for use on frozen tissue. Consult the specific Instructions for Use.

Other Specimens
Immunohistochemical staining of antigens in bone sections, bone marrow, blood smears, cytospins and imprints has generally not been validated for our primary antibodies and visualization systems. Consult the specific Instructions for Use.

Consult *our Education Guide: Immunohistochemical Staining Methods, Sixth Edition*[6] or references 2 and 3 for further details on specimen preparation.

Staining Procedure
When using FLEX Ready-to-Use antibodies, use the recommended reagents and the validated protocol outlined in the Instructions for Use for optimal staining performance on the dedicated staining platform. When using concentrated antibodies, refer to product-specific Instructions for Uses for primary antibody dilutions and procedures together with recommendations outlined in the Instructions for Use for the specific visualization system.

NOTE: Any modification of the recommendations outlined in the individual Instructions for Use must be validated by the user.

Quality Control
Deviations in the recommended procedures for tissue fixation, processing and embedding in the user's laboratory may produce significant variability in results. Positive and negative control tissues as well as negative control reagent should be run simultaneously using the same protocol as the patient specimens, preferably on the same slide (see below). These quality controls are intended to ensure the validity of the staining procedure, including reagents, tissue processing and instrument. It is recommended that controls be stained on the same slide as the patient tissue. The positive control should be a tissue with positive biomarker expression fixed in the same way as the patient tissue. The negative control should be a tissue with no biomarker expression. If controls are not fixed in the same way as the test specimen, the tissue may only be used as a staining control for reagents and instrument performance.

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. The quality control guidelines of the College of American Pathologists (CAP) Accreditation Program for Immunohistochemistry, NCCLS Quality Assurance for Immunocytochemistry, Approved Guideline[11], and reference 12 contain additional information.

Positive control tissue
Controls should be 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. It is recommended that controls be stained on the same slide as the patient tissue. Internal positive elements of the test specimen may be used as positive controls.

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 Instructions for Use, Analytical and clinical performance characteristics section or Atlas of Controls 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 assessment 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 Instructions for Use, Analytical and clinical performance characteristics section or Atlas of Controls) 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
A negative control reagent can be used 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 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 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 Instructions for Use of each primary antibody and to Table 2 for specific recommendations. The incubation period for the negative control reagent should be the same as the primary antibody.

DAB precipitates may form in the tissue during the IHC staining procedure which in some cases can resemble bacteria staining. It is therefore essential to always use normal tissue known to be negative to identify unexpected DAB precipitates when staining for bacteria.

Table 2. Examples of Negative Control Reagents for Primary Antibodies

Primary Antibody Type	***Suggested Negative Control Reagent***
Monoclonal mouse FLEX Ready-to-Use antibody	Universal Negative Control for Mouse Primary Antibodies, Code IR750 (for Autostainer Link 48 instruments) and GA750 (for Dako Omnis).
Polyclonal or monoclonal rabbit FLEX Ready-to-Use antibody	Universal Negative Control for Rabbit Primary Antibodies (for Autostainer Link 48 and for Dako Omnis instruments).
Concentrated monoclonal mouse antibody, produced in tissue culture	Non-human reactive monoclonal mouse antibody produced in tissue culture. This negative control antibody should be of the same isotype as the primary antibody.
Concentrated 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.
Concentrated polyclonal rabbit antibody, immunoglobulin fraction	Rabbit immunoglobulin fraction (normal).
Solid-phase absorbed polyclonal rabbit antibody, immunoglobulin fraction	Rabbit immunoglobulin fraction (solid-phase absorbed).
Polyclonal rabbit antibody, whole serum	Normal/nonimmune rabbit serum, whole serum.

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, labeled polymer) and substrate-chromogen, respectively.

Table 3. The Purpose of Daily Quality Control

Tissue: Fixed & Processed Similar to Patient Sample	***Specific Antibody & Visualization System***	***Negative Control Reagent* or Buffer plus same Visualization System as used with Specific Antibody***
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 visualization system degradation.	Controls all steps of the analysis. Validates reagent and immunostaining procedures.	Detection of nonspecific background staining.
Negative control: Tissues 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 nonspecific background staining.
Patient tissue	Detection of specific staining.	Detection of nonspecific background staining.

* Same species and isotype as the specific antibody, but not directed against the same target antigen. To detect nonspecific 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 lab-supplied 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.[11] 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 Instructions for Use, Analytical and clinical performance characteristics section are suitable for assay verification.

Stained Slide Storage
Some fading of stained slides may occur, depending on several factors including, but not limited to, counterstaining, mounting materials and methods, and slide storage conditions. To minimize fading, store stained slides in the dark at room temperature (20-25 °C).

Staining Evaluation
The specific staining pattern is described in the individual Instructions for Use. For evaluation of immunohistochemical staining, objectives of 4x-40x magnification are appropriate. The entire tissue section should be considered, avoiding edge effects, necrotic areas, and areas with other obvious artifacts. As with any immunohistochemical test, absence of staining means that the antigen was not detected, not necessarily that the antigen was absent in the cells/tissue assayed.

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 chromogenic reaction product at the site of the target antigen. See the Staining pattern and Analytical and clinical performance characteristics sections of the product specific Instructions for Use 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.

The color of the reaction product may be different depending on the substrate chromogen. Refer to the substrate Instructions for Use for expected color reactions. Further, metachromasia may be observed in variations of the method of staining.[13]

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 labeling 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] Unless specifically mentioned in the individual Instructions for Use, there was no nonspecific staining observed in tissue types tested.

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.[14]

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 Instructions for Use for information regarding primary antibody immunoreactivity.

Interfering Substances

Endogenous interference in immunoassays is generally considered low (incidence of immunoassay interference is estimated to be less than 2%),[15] and is almost mostly limited to immunonephelometric and immunoturbidimetric methods, and not IHC, primarily due to serum indices or autoantibodies. Furthermore, the antibodies for IHC from Agilent are designed by careful selection of reagents and addition of blocking agents to minimize the effects of any interferents.

Residual embedding media resulting from incomplete removal of paraffin will result in increased nonspecific or reduced staining.

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.[14]

Not all sources of distilled or de-ionized water may be of sufficient quality for IHC reagent preparation. It is recommended to use reagent-grade distilled or de-ionized water corresponding to Clinical Laboratory Reagent Water (CLRW) standard as specified by CLSI, or water similar in quality to be used for reagent preparation.

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 nonspecific staining with horseradish peroxidase.[16]
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.[17] Contact Agilent Pathology 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.[14]
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 than the specified reagents and protocols these are no longer ready-to-use and must be re-optimized and validated according to the clinical laboratory's IHC validation protocol.
13. Unless specifically claimed in the instructions, the performance characteristics of antibodies used for IHC have not been determined for other laboratory techniques.
14. When properly stored, the reagent is stable to the date indicated on the label. Do not use reagent beyond the expiration date. Improper storage and use of reagents may lead to erroneous results.
15. The user should always ensure adherence to the maintenance schedule for the Agilent automated staining instrument. Lack of adherence to the maintenance schedule may give erroneous results.

Troubleshooting
Refer to the Troubleshooting section in the *Education Guide: Immunohistochemical Staining Methods, Sixth Edition (*6) for a comprehensive guide to troubleshooting including remedial action. This is especially useful when using concentrated primary antibodies and lab-developed tests.

Contact Agilent Pathology Support via www.agilent.com to report any unusual staining. Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the country in which the user and/or the patient is established.

Below is a list of common problems and solutions when using the FLEX RTU concept with recommended products and validated protocols.

Problem	Probable Cause	Suggested Action
1. No or weak staining of slides	1a. Wrong storage conditions used for reagents.	1a. Check that reagents have been stored correctly according to recommended storage conditions.
	1b. Reagent is used past its expiration date.	1b. Ensure reagent is not used past its expiration date.
	1c. Reagent is used past its onboard stability.	1c. Ensure reagent is not used past its onboard stability.
	1d. Inappropriate fixation method used.	1d. Ensure that patient tissue is not fixed for too short or too long a time period, and that the correct fixative was used.
	1e. Excessive heating of mounted tissue sections prior to staining may lead to loss of immunoreactivity and morphology.	1e. Dry the tissue sections at 58 ± 2 ˚C for a maximum of 1 hour, using a calibrated oven with uniform heat distribution.
	1f. Incorrect placement of slides in automated staining platform.	1f. Check placement of slides
	1g. Damaged dynamic gap lids (Dako Omnis).	1g. Check integrity of dynamic gap lids.
	1h. Correct diluent is not used to dilute concentrated reagents.	1h. Ensure that correct solution is used to dilute concentrated reagents.
	1i. Incorrect pre-treatment method is used.	1i. Ensure that correct pre-treatment specified in Instructions for Use is used.
2. Excessively strong specific staining of slides	2a. Inappropriate fixation method used.	2a. Ensure that only approved fixatives and fixation methods are used.
	2b. Distilled or de-ionized water is not used to dilute the Target Retrieval Solution concentrate.	2b. Use distilled or de-ionized water to prepare 1x Target Retrieval Solution.
	2c. Correct diluent is not used to dilute concentrated reagents.	2c. Ensure that correct diluent is used to dilute concentrated reagents.
3. Excessive non-specific staining of slides	3a. Starch additives used in mounting sections to slides.	3a. Avoid using starch additives for adhering sections to glass slides. Many additives are immunoreactive.
	3b. Sections dried after staining procedure.	3b. Avoid sections drying out during the staining procedure, including pre-treatment.
	3c. Sections dried prior to coverslipping.	3c. Avoid stained slides drying out between staining and coverslipping.
	3d. Inappropriate fixation method used.	3d. Ensure that approved fixative was used. Alternative fixative may cause excessive background staining.
	3e. Paraffin incompletely removed.	3e. Ensure that paraffin is removed completely from sections.
	3f. Non-specific binding of reagents to tissue.	3f. Ensure that correct fixation method of the specimen is used and avoid large areas of necrosis.
	3g. Correct diluent or distilled/de-ionized water is not used to dilute concentrated reagents.	3g. Ensure that correct solution is used to dilute concentrated reagents.
	3h. Incorrect pre-treatment method is used.	3h. Ensure that correct pre-treatment specified in Instructions for Use is used.
4. Tissue detaches from slides	4a. Use of incorrect glass slides.	4a. Use FLEX IHC Microscope Slides or SuperFrost Plus slides.

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Agilent Technologies Singapore (International) Pte Ltd.
No. 1 Yishun Avenue 7
Singapore, 768923
Tel. +44 161 492 7050
www.agilent.com

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